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Calcium and Signal Transduction

Edited by John N. Buchholz and Erik J. Behringer

CALCIUM AND SIGNAL TRANSDUCTION

Edited by **John N. Buchholz**
and **Erik J. Behringer**

Calcium and Signal Transduction
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Physiology

Volume 1

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Scope of the Series

Modern physiology requires a comprehensive understanding of the integration of tissues and organs throughout the mammalian body, including the expression, structure, and function of molecular and cellular components. While a daunting task, learning is facilitated by our identification of common, effective signaling pathways employed by nature to sustain life. As a main example, the cellular interplay between intracellular Ca^{2+} increases and changes in plasma membrane potential is integral to coordinating blood flow, governing the exocytosis of neurotransmitters and modulating genetic expression. Further, in this manner, understanding the systemic interplay between the cardiovascular and nervous systems has now become more important than ever as human populations age and mechanisms of cellular oxidative signaling are utilized for sustaining life. Altogether, physiological research enables our identification of clear and precise points of transition from health to development of multi-morbidity during the inevitable aging process (e.g., diabetes, hypertension, chronic kidney disease, heart failure, age-related macular degeneration; cancer). With consideration of all organ systems (e.g., brain, heart, lung, liver; gut, kidney, eye) and the interactions thereof, this Physiology Series will address aims of resolve (1) Aging physiology and progress of chronic diseases (2) Examination of key cellular pathways as they relate to calcium, oxidative stress, and electrical signaling & (3) how changes in plasma membrane produced by lipid peroxidation products affects aging physiology.

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Preface

Since the development of microelectronic clamping methodology and fluorescent indicators for direct measurement of dynamic intracellular calcium transients, our understanding of biological signal transduction has progressed dramatically since the 1980s. Calcium is a universal signal in biology that modulates gene expression, transmitter and hormone release, muscular movement, and even "programmed" cell death. This book contains 8 carefully reviewed chapters from a diverse set of expert biologists throughout the world who have conducted research in the general area of calcium signaling in organisms ranging from bacteria to humans. In accord with priorities of resolving human disease, the reader will also benefit from learning calcium's role in cellular signaling pathology relating to acute or chronic conditions such as vomiting, sepsis, obesity, hypertension, and cancer.

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Section 1

Calcium Signaling in Multiple Cellular Models

Chapter 1

Regulation of Calcium Signaling by STIM1 and ORAI1

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Additional information is available at the end of the chapter

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Abstract

STIM1 and ORAI1 proteins are regulators of intracellular Ca^{2+} mobilization. This Ca^{2+} mobilization is essential to shape Ca^{2+} signaling in eukaryotic cells. STIM1 is a transmembrane protein located at the endoplasmic reticulum, where it acts as an intraluminal Ca^{2+} sensor. The transient drop of intraluminal Ca^{2+} concentration triggers STIM1 activation, which relocates to plasma membrane-endoplasmic reticulum junctions to bind and activate ORAI1, a plasma membrane Ca^{2+} channel. Thus, the Ca^{2+} influx pathway mediated by STIM1/ORAI1 is termed store-operated Ca^{2+} entry (SOCE). STIM and ORAI proteins are also involved in non-SOCE Ca^{2+} influx pathways, as we discuss here. In this chapter, we review the current knowledge regarding the role of SOCE, STIM1, and ORAI1 in cell signaling, with special focus on the modulation of the activity of kinases, phosphatases, and transcription factors that are strongly influenced by the extracellular Ca^{2+} influx mediated by these regulators.

Keywords: calcium, signaling, SOCE, STIM, ORAI

1. Introduction

Cell signaling is the network of reactions and interaction of molecules that allow cells to react to a wide range of stimuli. In this response, many pathways are involved, so cells are able to adapt to changing conditions. One of the mechanisms to respond to external stimuli is mediated by receptors, that is, proteins located at the plasma membrane that communicate the extracellular and the intracellular medium. A significant strategy that cells acquired early in their evolution was the modification of the composition of the intracellular milieu, so the ionic

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composition is different across the plasma membrane. This strategy is expensive in terms of the consumption of energy, since the ionic composition of the intracellular medium is modified by pumping out some ions from the cytosol. However, this is cost-efficient because it provided the possibility to proliferate and to gain cellular specialization. In this regard, free calcium (Ca^{2+}) concentration in the cytosol of cells is much lower than that observed in the external medium, so there are mechanisms to remove the excess of free Ca^{2+} from the cytosol, such as extruding Ca^{2+} to the extracellular medium or to intracellular Ca^{2+} stores. This pumping is carried out by plasma membrane Ca^{2+} pumps and by endoplasmic reticulum Ca^{2+} pumps, respectively. Also, buffering of Ca^{2+} with Ca^{2+}-binding proteins is another strategy to keep cytosolic free Ca^{2+} concentration ($[Ca^{2+}]i$) within the low nanomolar range (~100 nM). The reason why the $[Ca^{2+}]i$ is tightly controlled is because this level is a second messenger in cell signaling, that is, transient variations of $[Ca^{2+}]i$ communicate a signal to be transmitted. For instance, during fertilization of mammalian oocytes, a series of short-term cytosolic increases of $[Ca^{2+}]i$ occurs in the oocyte for ~20 h after the fusion with sperm. These transient and short spikes are required to release the arrest of the cell cycle and to stimulate the transition from the fertilized oocyte to 1-cell embryo (zygote). The level of $[Ca^{2+}]i$ is also involved in many other cellular events, like the control of gene expression, vesicular trafficking, neurotransmitter release, cytoskeletal dynamics, and so on.

Cytosolic Ca^{2+} spikes and Ca^{2+} waves are generated by the opening of Ca^{2+}-specific ion channels located at the plasma membrane and subcellular organelles. When they become activated, plasma membrane Ca^{2+} channels let the influx of extracellular Ca^{2+} so the $[Ca^{2+}]i$ rapidly increases, triggering the activation of Ca^{2+}-sensitive effectors. As the main intracellular Ca^{2+} store, the endoplasmic reticulum (ER) also contains Ca^{2+} channels that become activated upon certain stimuli to let the transient release of Ca^{2+} to the cytosol. Then, elevated $[Ca^{2+}]i$ activates Ca^{2+} pumps to reduce the level of free Ca^{2+} in the cytosol, making possible the temporal increase of $[Ca^{2+}]i$ which is essential for its role as a messenger. The speed of the Ca^{2+} rise, as well as the Ca^{2+} removal, together with the time that this elevation lasts, define the temporal Ca^{2+} signaling, or Ca^{2+} signature, a critical point in the activation of subsequent events. Similarly, the specific distribution of Ca^{2+} channels and pumps define the spatial Ca^{2+} signature. The spatiotemporal control of the Ca^{2+} signaling is relevant for determining the regulation of different signaling pathways that finally lead to diverse actions. In summary, it is not only important to know how Ca^{2+} levels are altered upon specific stimuli, but also their specific duration, shape, and subcellular localization.

In this chapter, we summarize the current knowledge regarding the role of the STIM and ORAI proteins family. Because of their role as ER intraluminal Ca^{2+} sensors, STIM proteins have been recently involved in the modulation of several Ca^{2+}-dependent signaling pathways. ORAI proteins are Ca^{2+} channels located at the plasma membrane that regulate the influx of Ca^{2+}, in some cases under the control of STIM proteins. Thus, cooperation of both proteins is critical for Ca^{2+} influx, Ca^{2+} signaling, and cell physiology.

2. General overview of STIM and ORAI proteins

In humans, there are two different genes coding for STIM proteins: STIM1 and STIM2. STIM1 gene shows three known transcriptional variants that generate the proteins STIM1 (canonical),

Gene	Transcript(s)	Protein	Protein official name
ENSG00000167323	NM_001277961.1	NP_001264890.1	STIM1 isoform 1, or STIM1L
	NM_003156.3	NP_003147.2	STIM1 isoform 2 (canonical)
	NM_001277962.1	NP_001264891.1	STIM1 isoform 3, or STIMS
ENSG00000109689	NM_001169117.1	NP_001162588.1	STIM2 isoform 3
	NM_001169118.1	NP_001162589.1	STIM2.1, STIM2β
	NM_020860.3	NP_065911.3	STIM2.2, STIM2α
ENSG00000276045	NM_032790.3	NP_116179.2	ORAI1
ENSG00000160991	NM_001126340.2	NP_001119812.1	ORAI2 isoform a
	NM_001271818.1	NP_001258747.1	ORAI2 isoform a
	NM_001271819.1	NP_001258748.1	ORAI2 isoform b
	NM_032831.3	NP_116220.1	ORAI2 isoform a
ENSG00000175938	NM_152288.2	NP_689501.1	ORAI3

Table 1. Accession number for genes and reference sequences (RefSeq) of transcriptional variants and proteins.

STIM1L (the longest isoform), and STIM1S (the shortest isoform). For STIM2 gene, also three transcriptional variants have been described coding for proteins STIM2, STIM2.1 (or STIM2 beta), and STIM2.2 (or STIM2 alpha) (see **Table 1**).

Also in humans, three different genes code for ORAI proteins: ORAI1, ORAI2, and ORAI3. ORAI1 gene yields a single product (ORAI1 protein, also known as calcium release-activated calcium channel protein 1), whereas ORAI2 gene produces two variants (isoforms 1 and 2), and ORAI3 gene generates a single transcriptional variant and a single protein isoform (**Table 1**).

STIM1 protein is a positive regulator of store-operated Ca^{2+} entry (SOCE) [1, 2], a Ca^{2+} influx pathway regulated by the filling status of intracellular Ca^{2+} stores, mainly the ER. Although there is a significant pool of STIM1 at the plasma membrane, most STIM1 is ER-resident. When located at the ER, STIM1 shows a single transmembrane domain (TM) with the N-terminus toward the intraluminal space of this organelle. The Ca^{2+}-sensitive EF-hand domain, together with a sterile-α-motif (SAM), constitute an intraluminal Ca^{2+} sensor, with an apparent dissociation constant for Ca^{2+} of 250 μM [3]. When the intraluminal Ca^{2+} concentration drops below this Kd, the dissociation of Ca^{2+} from the EF-hand domain is transmitted to the SAM domain, and to the cytosolic domain of the protein leading to its activation [4]. The cytosolic domain shows a well-studied calcium release-activated calcium (CRAC) activation domain (CAD), with a series of short coiled-coil (CC) domains that bind to ORAI1 plasma membrane channels to activate Ca^{2+} influx [5]. STIM1 protein also shows a Ser/Pro rich domain, close to a short sequence of four amino acids that binds to the microtubule plus-end binding protein EB1 [6], and finally a terminal Lys-rich domain which is critical for the activation of non-ORAI1 Ca^{2+} channels, such as TRPCs [7].

STIM2 and STIM1 share >60% sequence identity, and STIM2 also senses intraluminal Ca^{2+} concentration although with different sensitivity, since the dissociation constant for Ca^{2+}

(~500 μM) is twofold higher than that of STIM1 [8], suggesting that STIM2 becomes activated with smaller changes in intraluminal Ca^{2+} levels, whereas STIM1 activates Ca^{2+} entry upon more severe conditions [9].

ORAI1 is a plasma membrane protein with four transmembrane domains with the N- and C-termini oriented to the cytosol. The Ca^{2+} channel is formed by a hexamer of ORAI1 monomers, with the Ca^{2+} pore in the center of the hexamer [10, 11]. Both the N- and C-terminal domains are involved in the binding to STIM1 [12]. The paralogues ORAI2 and ORAI3 share 63% and 58% sequence identity with ORAI1, being the extracellular loop 3 that connects TM domain 3 and 4, significantly larger in ORAI3.

3. STIM1-ORAI1-mediated Ca^{2+} influx

The mechanism of activation has been well documented for the complex STIM1-ORAI1. At resting state, STIM1 is distributed on the surface of the ER, where its cytosolic domain is folded in a tight state due to an intramolecular clamp between domains CC1a1 and CC3 [13]. The inactive and resting STIM1 is a dimer [14], and the activation of Ca^{2+}-release from intracellular stores due to the activation of the phosphoinositide pathway, leads to the transient depletion of Ca^{2+} levels within the ER. The consequent Ca^{2+} dissociation from the intraluminal domain of STIM1 triggers the activation of the protein in a more extended state that lets STIM1 to form oligomers [15]. In contrast to what it has been observed for inactive STIM1, which shows a high mobility on the ER surface while it is bound to EB1 and microtubules, STIM1 oligomers are quite immobile when they reach ER-PM junctions. This oligomerization has been extensively documented when STIM1 is targeted with fluorescent tags (**Figure 1**).

The binding of STIM1 to the microtubule plus-end tracking protein EB1, ensures the targeting of STIM1 to ER-PM junctions [16]. However, this binding to EB1 is not required for the activation of ORAI1. STIM1 dissociates from EB1 by a mechanism regulated by the phosphorylation of a set of serine residues (Ser575, Ser608, and Ser621) adjacent to the EB1-binding site [17]. This STIM1 phosphorylation is mediated by the kinases ERK1/2, which become activated in the absence of Ca^{2+}-influx by the activation of tyrosine kinase receptors at the plasma membrane [18–20]. Thus, STIM1 bound to EB1 travels to ER-PM junctions [6], but it dissociates from EB1 to bind to ORAI1 [17]. The physical interaction between STIM1 and ORAI1 is fully required for gating the Ca^{2+} channel. This interaction is mediated by the aforementioned CRAC activation domain (CAD) of STIM1 and both the cytosolic C-terminus and the N-terminus of ORAI1 [21], although the binding to the N-terminus is slightly weaker [22]. The stoichiometry of the complex STIM1-ORAI1 is also in the center of debate, but the current accepted proposal supports a 1:1 to 2:1 ratio in order to activate the channel [23, 24].

The high selectivity of ORAI1 for Ca^{2+} is due to the pore design, with a selectivity filter mediated by the acidic glutamate residue E106 at the first transmembrane domain [26]. Once Ca^{2+} influx is activated, a negative feedback controls the excessive Ca^{2+} entry, and Ca^{2+}/calmodulin mediates this mechanism of inactivation. A short domain (residues 470–491) with seven acidic amino acids, close to the CAD binding domain, is directly involved in the Ca^{2+}-dependent inactivation [27]. Also in this report, Mullins et al. identified a membrane-proximal N-terminal domain of ORAI1 (residues 68–91) that binds calmodulin (CaM) in a Ca^{2+}-dependent manner

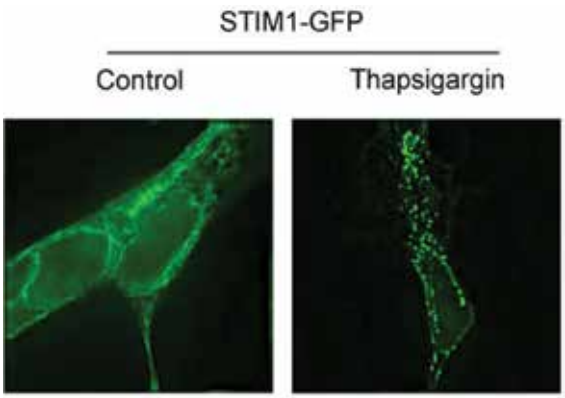

Figure 1. HEK293 cells stably expressing STIM1-GFP were incubated in Hank's balanced salt solution (HBSS) (left) or in Ca^{2+}-free HBSS with 1 μM thapsigargin (right) to trigger Ca^{2+} store depletion. After 10-min incubation cells were fixed and visualized under wide-field fluorescence microscopy. In control cells (left panel), STIM1-GFP showed a localization that matched with endoplasmic reticulum. Thapsigargin induced aggregation of STIM1-GFP revealed by the clustering of GFP fluorescence (right panel), a result that demonstrated that the recombinant protein STIM1-GFP was sensitive to store depletion (reprinted from reference [25]).

[27], supporting a model in which Ca^{2+}/CaM binds to the N-terminus of ORAI1 to trigger channel inactivation.

The large Rab GTPase CRACR2A mediates another mechanism that controls and prevents excessive Ca^{2+} entry. At low intracellular Ca^{2+} levels, CRACR2A enhances the binding of STIM1 to ORAI1, but at higher $[Ca^{2+}]i$, that is, after ORAI1 activation, CRACR2A dissociates from ORAI1, inhibiting SOCE [28]. ORAI1 residues involved in the binding to CRACR2A are the same as those that bind Ca^{2+}/CaM, so Ca^{2+}/CaM and Ca^{2+}-free CRACR2A are competitors for ORAI1.

STIMATE, a protein encoded by TMEM110 gene, is an ER-resident protein and a modulator for the activity of the STIM1-ORAI1 complex [29]. When Ca^{2+} dissociates from STIM1, the conformational change to a more extended structure of STIM1 facilitates the binding between STIMATE and STIM1-CC1 domain, avoiding the inhibition of CAD domain. This is the reason why STIMATE promotes the full extended conformation and the formation of STIM1 clustering at ER-PM junctions. In addition, the protein SARAF has been described as a negative regulator of SOCE [30]. As an ER membrane-resident protein, SARAF associates with STIM1 to promote Ca^{2+}-dependent inactivation of SOCE. In this regard, a conserved STIM1 (448–530) C-terminal inhibitory domain (CTID) has been reported to regulate Ca^{2+}-dependent inhibition [31]. CTID shows the capability to promote access of SARAF to the STIM1-ORAI1 activation region (SOAR or CAD), thus promoting inactivation of SOCE.

Additional regulators of the complex STIM1-ORAI1 have been reported, including septins [32] and RASSF4 [33]. Septin filaments and phosphatidylinositol-4,5-bisphosphate (PIP_2) polarize in ER-PM junctions before store-depletion and facilitate STIM1 targeting to these junctions, where STIM1 recruits ORAI1. On the other hand, RASSF4 (RAS association domain family 4) also regulates SOCE by affecting the translocation of STIM1 to ER-PM junctions. Finally, a recent report has shown that ORAI1, as well as STIM1 phosphorylated at ERK1/2-target sites, are recruited at the leading edge of migrating cells, where ORAI1 binds cortactin, a regulator

of plasma membrane ruffling [34]. This membrane ruffling is the reorganization of the cortical cytoskeleton required for the formation of filopodia and lamellipodia, and STIM1-KO (knockout) and ORAI1-KO cells, engineered by CRISPR/Cas9 genome editing, showed defective membrane ruffling and largely diminished cell migration [34], demonstrating that Ca^{2+} influx through STIM1-activated ORAI1 is essential for these events.

4. The role of STIM1 and ORAI1 on cell signaling

Given the importance of Ca^{2+} in many signaling pathways, the impact of STIM1, ORAI, and SOCE on cell signaling is also remarkable. Increasing evidence prove the significant role of this Ca^{2+} entry pathway in cell physiology and tissue homeostasis, and we focus here on the role of STIM and ORAI proteins on modulators of signaling pathways, such as kinases, phosphatases, and transcription factors. We also describe recent findings that unravel how STIM1 and ORAI1 are modulated by posttranslational modifications.

4.1. MAPK pathway

The close relationship between SOCE and mitogen-activated protein kinases (MAPKs) was revealed by Machaca and Haun [35], when they investigated the inactivation of SOCE in Xenopus oocyte maturation. SOCE is an active pathway in almost all eukaryotic cells, but during M-phase of cell cycle it becomes inactivated [36–38]. Machaca and Haun demonstrated that SOCE inactivation at germinal vesicle breakdown of Xenopus oocytes coincided with an increase in levels of MAPK and maturation-promoting factor (MPF), but they also demonstrated that MPF triggered SOCE inactivation by inhibiting the coupling between store depletion and SOCE activation, and not by blocking Ca^{2+} influx through SOCE channels [35].

In cells at interphase, some evidence supports a role for SOCE on ERK1/2 activation. In this regard, it has been proposed that SOCE activates extracellular signal-regulated kinases 1/2 (ERK1/2) in parotid acinar cells [39] and melanoma cells [40]. This proposal fits well with SOCE as an upstream regulator of ERK1/2. However, this proposal does not seem to be applicable to all cell lines, since ERK1/2 can be activated in the absence of extracellular Ca^{2+} and therefore in the absence of Ca^{2+} influx in HEK293 cells [20], the Ishikawa adenocarcinoma cell line [18], osteosarcoma U2OS cells [34], and prostate PC3 cells [19]. In addition, STIM1 knockdown did not modify phosphorylation of MEK1/2-ERK1/2 in gastric cancer cells [41], and STIM1 and ORAI1 knockdown did not inhibit the activation of ERK1/2 in response to EGF [42]. Moreover, ERK1/2 is fully activatable in STIM1-KO PC3 cells [19], with no active SOCE, demonstrating that SOCE is dispensable for ERK1/2 activation.

On the contrary, increasing evidence demonstrates that SOCE is a target for ERK1/2 activity, and that ERK1/2 is an upstream regulator of STIM1 and SOCE (reviewed in [43]). Pozo-Guisado et al. reported that STIM1 is phosphorylated by ERK1/2 at residues Ser575, Ser608, and Ser621 [25]. This phosphorylation is required for the full activation of STIM1 and for triggering the dissociation of STIM1 from microtubules [17]. Accordingly, phospho-STIM1 is enriched at the leading edge of migrating cells, that is, in the vicinity of receptor tyrosine

kinases [34], where phospho-STIM1 acts in cooperation with ORAI1 to regulate the Ca^{2+} influx that rules cell migration. Consequently, phospho-STIM1 is an effector of ERK1/2 and an essential mediator for the activation of Ca^{2+} influx upon stimulation of cells with IGF-1 [20], or EGF [18, 19, 34].

Other MAPKs, such as p38 MAPK, have been shown to regulate SOCE, although by an indirect mechanism. Transforming growth factor beta (TGFβ) regulates megakaryocyte maturation and platelet formation by upregulating the expression of the serum-glucocorticoid inducible kinase SGK1 [44, 45], which is p38 MAPK-dependent [46]. SGK1 stimulates nuclear translocation of transcription factor NF-κB, which upregulates ORAI1 expression, increasing SOCE. This increase was demonstrated to be sensitive to p38 MAPK inhibition, SGK1 inhibition, and NF-κB inhibition, demonstrating the role of p38 MAPK in the upregulation of SOCE [47]. Another proposal was reported by Sundivakkam et al. [48], who reported that p38 MAPK directly phosphorylates STIM1. In this report, it was shown that pharmacological inhibition of p38 MAPK increased SOCE and that p38β knockdown prevented STIM1 phosphorylation and potentiated SOCE. However, this report did not identify the phosphorylated Ser/Thr residue(s), since the findings were based on the use of a phospho-Ser antibody [48], but not a site-specific phospho-specific antibody.

4.2. cAMP and PKA

The crosstalk between SOCE and cAMP-activated pathways has been investigated thoroughly since the molecular description of STIM1 and ORAI1. For instance, the Ca^{2+}/CaM-stimulated adenylyl cyclase 8 (AC8) was found to be activated by SOCE and co-localized with STIM1 and ORAI1 in lipid rafts [49]. Interestingly, other authors found that lowering the concentration of free Ca^{2+} within the ER led to recruitment of adenylyl cyclases, enhancing the production of cAMP with the subsequent PKA activation, being this action independent of the $[Ca^{2+}]i$ [50]. Because activation of STIM1 and translocation to ER-PM junctions were required for coupling ER-Ca^{2+} depletion and adenylyl cyclase activity, without altering $[Ca^{2+}]i$, those authors proposed the occurrence of a pathway termed store-operated cAMP signaling (SOcAMPS), a pathway that was later confirmed for other AC isoforms, such as AC3 [51]. More recently, it was confirmed that other Ca^{2+} channels, including TRPC1, were also involved in the activation of ACs [52]. It has been reported that STIM1 interacts with the plasma membrane adenylyl cyclase 6 to regulate melanogenesis [53], and this interaction is mediated by the Ser/Pro-rich C-terminal region of STIM1. These reports, together with the finding that ORAI1- and SOCE-deficient fibroblasts showed impaired cAMP production and cAMP-dependent signaling [54], strongly support the direct relationship between Ca^{2+} depletion at ER stores, STIM1 activation, and enhanced production of cAMP.

As for other pathways, the regulation between STIM1 and PKA seems to be reciprocal. In addition to the CRAC channel ORAI1, the plasma membrane-resident STIM1 activates store-independent arachidonic acid regulated Ca^{2+} (ARC) channels, and this activation depends on the phosphorylation of STIM1 at Thr389 by PKA, which requires the scaffold protein AKAP79 [55]. This phosphorylation triggers a structural change in the SOAR region of STIM1 (also known as CAD) being essential for the selective activation of ARC channels [56].

4.3. Other kinases and pathways

It is known that Ca^{2+}-influx is upregulated by phosphoinositide 3-kinase (PI3K) signaling in platelets [57, 58]. Because PI3K signaling involves activation of SGK1, and this kinase has been shown to be a stimulator of ORAI1 expression [59], it is accepted that PI3K modulates SOCE by upregulation of the CRAC channel. In B16B6 melanoma cells, constitutive activation of Src and PKB/Akt was revealed to be due to the activation of SOCE in lipid rafts, which promoted Ca^{2+}-dependence of the Src activity to trigger tumor signaling events [60], as it reported for lung metastasis of melanoma cells in a xenograft mouse model [61]. However, this is not shared by other cancer cells, as in prostate PC3 cells, with low levels of active ERK1/2 due to constitutive activation of PKB/Akt, Src is fully activatable in a Ca^{2+}-independent manner by epidermal growth factor (EGF) [19].

Protein kinase C (PKC) phosphorylates ORAI1 at residues Ser27 and Ser30 [62]. More precisely, Kawasaki et al. demonstrated that the knockdown of the isoform PKCβ led to an increase of Ca^{2+} influx, and that recombinant PKC phosphorylated ORAI1 in vitro and in vivo at these two amino acids, an effect that inhibited the Ca^{2+} transport through ORAI1 [62]. No other phospho-residues have been characterized in detail in ORAI1. In airway smooth muscle cells, rottlerin, a PKCδ-selective inhibitor, reduced phorbol esters-triggered SOCE, without affecting total levels of STIM1 and ORAI1 [63]. However, the mechanism of this inhibition remains to be elucidated. PKC also inhibited SOCE in hepatocytes treated with amiodarone, an experimental design to mimic the accumulation of lipids during steatosis [64]. Because selective inhibition of PKC reversed SOCE to normal values, it was concluded that lipid accumulation triggers PKC-dependent SOCE impairment. Also, accumulation of palmitate is cytotoxic in kidney cells, and high levels of palmitate triggered Ca^{2+} depletion in the ER, in addition to mitochondrial stress. This depletion is antagonized by the inhibition of fatty acid transporters, inhibition of phospholipase C (PLC), and inhibition of PKC [65]. Once again, the mechanism that links PKC and the regulation of STIM1/ORAI1 remains elusive.

In 2011, Mungai et al. reported that hypoxia augmented cellular reactive oxygen species (ROS), without a significant alteration of energy charge values [66]. Hypoxia triggered an increase in $[Ca^{2+}]$i, relocalization of STIM1 to ER-PM junctions, and phosphorylation of AMP-activated protein kinase (AMPK), in the absence of its upstream regulator LKB1 (liver kinase B1). These events were due to the ROS-dependent activation of ORAI1, which led to an increase in $[Ca^{2+}]$i and activation of CaMKK2 (or CaMKKβ), an upstream activator of AMPK [66, 67]. Finally, a recent report from Yang et al. described how STIM1-ORAI1 mediated autophagy in endothelial progenitor cells exposed to oxidized low-density lipoprotein to mimic hypercholesterolemia. This treatment caused stimulation of Ca^{2+} influx mediated by STIM1-ORAI1, activation of CAMKK2 and decrease of mTOR activity with the subsequent activation of autophagy [68]. Similarly, in hepatocarcinoma cells, mitochondrial fission increased cytosolic Ca^{2+} levels that activated the NF-κB pathway, upregulating STIM1 expression and the subsequent SOCE [69]. The relative increase of $[Ca^{2+}]$i also activated NFAT-dependent upregulation of Drp1, promoting a positive loop to rise levels of mitochondrial fission.

4.4. NF-κB, NFAT, CREB, and other transcription factors

As we mentioned above, SOCE is essential for platelet activation, and it is known the key role of ORAI1 in response to thrombin [70, 71]. ORAI1 transcripts were found significantly reduced

in platelets and megakaryocytes from SGK1-KO mice, and transfection of megakaryocyte with constitutively active SGK1 increased phosphorylation of the IκB kinase (IKKα/β), which phosphorylates the inhibitor protein IκBα, promoting nuclear translocation of NF-κB subunit p65 [59]. In addition, Eylenstein et al. defined, by chromatin immunoprecipitation (ChIP) experiments, the promoter regions accounting for NF-κB-sensitive genomic regulation of STIM1 and ORAI1 [72], supporting further the conclusion that upregulation of ORAI1 and STIM1 by SGK1-dependent NF-κB signaling leads to the upregulation of SOCE, which in turn upregulates expression of other transcription factors like fibroblast growth factor 23 (FGF23) [73]. Other transcription factors are known to regulate STIM1 expression, such as Wilms tumor suppressor 1 (WT1) and early growth response 1 (EGR1), which were found by analyzing the STIM1 promoter with the TESS search system (University of Pennsylvania) [74]. Finally, NEUROD2, a neurogenic transcription factor, has been described as a negatively regulator of STIM1 expression, an activity that limits the level of STIM1 in cortical neurons [75].

Regarding NF-κB, reciprocal regulation seems to link this transcription factor and SOCE. In addition to the activation of SOCE by NF-κB described above, Liu et al. reported that T-cell activation triggered by the binding of antigen to T-cell receptor stimulated SOCE and that this Ca^{2+} entry activated a PKCα-mediated phosphorylation of p65 NF-κB at Ser536, an event that controls nuclear localization and transcriptional activity of NF-κB [76].

STIM1 and ORAI1 are also well-known activators of the protein phosphatase calcineurin, which activates the nuclear factor of activated T cells (NFAT) [77]. Once NFAT becomes dephosphorylated by calcineurin the transcription factor is internalized into the nucleus. Indeed, the activation and nuclear translocation of NFAT was the reporter used by Feske et al. when they searched for regulators of SOCE using a Drosophila RNA interference screening, a report that led to the description of ORAI1 as the channel that mediates the Ca^{2+} release-activated Ca^{2+} current, or CRAC [78]. Because NFAT modulates the expression of a wide range of genes, it is involved in many pathways, and also in the regulation of the expression of other regulators of transcription, including IRF4, BATF, and Bcl-6 [79]. NFAT is not the only transcription factor activated by the axis STIM1-ORAI1-Ca^{2+}/CaM-calcineurin because Ca^{2+} influx through ORAI1 stimulates the transcription factor EB (TFEB), promoting the activation of chemokines genes [80]. SOCE also activates the Ca^{2+}/cAMP response element binding protein (CREB), a transcription factor that regulates expression of many genes, at least in cultured smooth muscle cells and intact arteries [81]. In this regard, it was observed that mitochondrial Ca^{2+} uptake was reduced in lymphocytes lacking STIM1 or ORAI1, an effect that was due to reduced mitochondrial Ca^{2+} uniporter (MCU) expression [82]. ChIP and promoter analyses revealed that CREB directly binds the MCU promoter, revealing that SOCE regulates the Ca^{2+} uptake capability of mitochondria by regulating Ca^{2+}-dependent activation of CREB [82].

Ca^{2+} influx regulates myoblasts differentiation, and shortly after the molecular description of STIM and ORAI proteins, it was reported that silencing STIM1, Orai1, or Orai3 reduced SOCE and myoblast differentiation [83]. This positive effect on myoblasts correlated with the expression of MEF2 and myogenin, two transcription factors involved in skeletal muscle development, although it is still unclear the molecular pathway that links STIM1/ORAI1 and the activation of the transcription factors. In cerebellar granule neurons cultured in low concentration of extracellular potassium, mimicking resting conditions, SOCE promoted the degradation of transcription factor Sp4, a regulator of neuronal morphogenesis and function [84].

Another important molecular interactor of STIM1 is the hypoxia-inducible factor-1 alpha (HIF-1α), which is upregulated during hepatocarcinoma growth [85]. Li et al. found that HIF-1α directly controls STIM1 transcription, but also that STIM1-mediated SOCE is required for HIF-1α accumulation in hepatocarcinoma cells via activation of Ca^{2+}/CaM-dependent protein kinase II, revealing a mutual dependence of STIM1 and HIF-1α in the regulation of Ca^{2+} transport and tumor growth [85].

High levels of ORAI1 and STIM1 are found in many types of cancer cells. In gastric cancer tumor progression, this higher expression is associated with a negative impact on survival rates of patients, an effect that was partially due to targeting expression of metastasis-associated in colon cancer-1 (MACC1) [86], an essential regulator of the transcription for the gene coding for the hepatocyte growth factor receptor, MET. Similarly, a recent report described that STIM1 promotes cell migration and the epithelial-to-mesenchymal transition (EMT) by activating TGF-β, Snail and Wnt/β-catenin pathways in prostate cancer cells [87].

Finally, an excellent report from Stephan Feske laboratory [54], described how SOCE is crucial for mitochondrial fatty acid oxidation, and that Ca^{2+} entry through ORAI1 was essential to activate adenylyl cyclase, cyclic AMP production, the transcriptional regulator peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) and peroxisome proliferator-activated receptor alpha (PPARα), which is mediated by the activation of CREB.

5. ORAI3 and calcium signaling

Shortly after the molecular description of ORAI1 as the CRAC channel, it was revealed the essential role of other ORAI proteins in cell signaling. The involvement of ORAI3, together with ORAI1, in arachidonic acid-regulated Ca^{2+} (ARC) channels was early proposed [88, 89]. In contrast to SOC channels, the activation of ARC channels depends on the pool of STIM1 resident in the plasma membrane [90]. More interestingly, ORAI1 and ORAI3 show a differential sensitivity to reactive oxygen species, due to the extracellularly located Cys195 residue which is found in ORAI1, but not in ORAI3. The differential redox sensitivity underlies the differential responses between naïve and T helper lymphocytes, an event that lets T(H) cells proliferate and secrete cytokines in oxidative environments [91].

ORAI3, but not ORAI1, was also involved in the activation of PLCδ in response to arachidonic acid, an activation that controls oscillation frequency of Ca^{2+} spikes triggered by carbachol [92]. ORAI3 channels are overexpressed in estrogen receptor-positive breast cancer cells [93], and it was later demonstrated, using the MCF-7 cancer cell line, that silencing ORAI3 slows down cell cycle and triggers arrest at G1 phase [94]. EGF triggers Ca^{2+} entry through ORAI3, and the channel is transcriptionally upregulated by the estrogen receptor alpha (ERα) [95]. It is now accepted that cancer cells show a remodeling of ORAI proteins, with an enhanced participation of ORAI3 compared to noncancerous cells, suggesting that heteromerization of ORAI3 and ORAI1 is a common feature in malignant transformation [96].

6. Future directions

During the past decade, a significant progress was made regarding the molecular description of the proteins involved in store-operated Ca^{2+} entry. Although some details remain unclear,

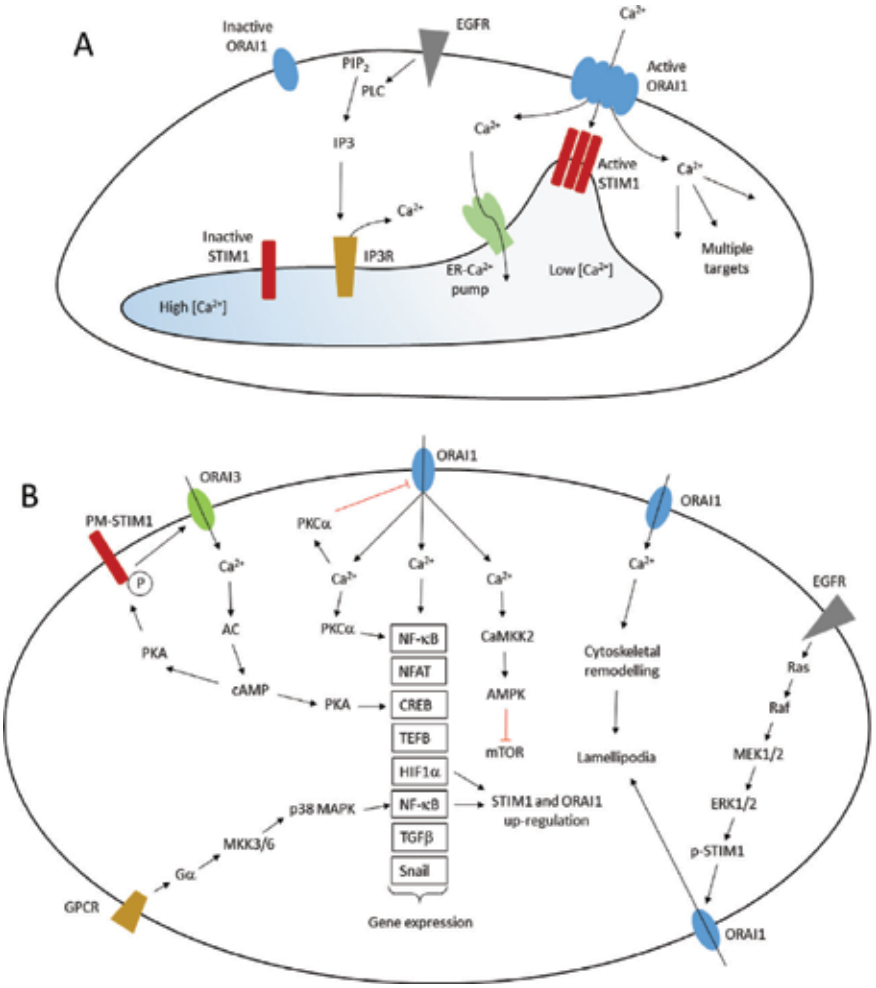

Figure 2. Activation of SOCE by STIM1/ORAI1 and pathways involved in SOCE-dependent signaling. Panel A: diverse stimuli that triggers the activation of the phospholipase C pathway, such as activation of EGF receptor (EGFR), stimulate the production of inositol 1,4,5-trisphosphate (IP3) which binds and activates IP3 receptor (IP3R) at the endoplasmic reticulum (ER). This activation leads to the release of Ca^{2+} from the ER, with the subsequent transient depletion of intraluminal [Ca^{2+}] and the activation of STIM1. Ca^{2+}-unbound STIM1 aggregates in oligomers and translocates to plasma membrane (PM)-ER junctions where it binds and activates ORAI1. Extracellular Ca^{2+} entry through ORAI1 activates multiple Ca^{2+}-dependent targets, as shown in panel B, but also provides a Ca^{2+} source to replenish intraluminal Ca^{2+} levels. This replenishment is accomplished by the ER-Ca^{2+}-ATPase which pumps Ca^{2+} into the ER lumen. Panel B: schematic illustration of the most important pathways regulated by STIM1/ORAI1. AC, adenylyl cyclase; GPCR, G protein-coupled receptor; PM-STIM1, plasma membrane-resident STIM1; p-STIM1, phosphorylated STIM1.

a topology of STIM1-ORAI1 contact sites, selectivity filters in ORAI1, and the role of post-translational modifications have been reported for both proteins. The involvement of STIM and ORAI proteins in different pathways is now much clearer, and they are now considered master regulators of Ca^{2+}-dependent signaling pathways. However, in many cases pathways were studied in cancer cell lines in vitro, so physiological models are required to evaluate the importance of STIM1 and ORAI1 in the pathophysiology of cells in vivo. Nevertheless, primary cell cultures and established cell lines constitute a widely accepted experimental approach for basic studies in cell signaling and understanding the role of STIM1/ORAI1 in cell biology and cell signaling. With these tools, we have reached the conclusion that STIM1 and ORAI1 are involved in the control of Ca^{2+} refilling within the ER. More important, STIM1 and ORAI1 directly modulate Ca^{2+} signaling in a wide variety of pathways, with a significant role in gene expression, cell migration, and tumor cell metastasis (**Figure 2**). Because the expression of STIM1/ORAI1 is deregulated in cancer cells, it is required to evaluate the relative importance of STIM1/ORAI1 as pharmacological targets for the treatment of disease, not only with the use of in vitro cell cultures, but also in animal models for the study of human disease.

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Conflict of interest

The authors declare no conflict of interests.

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Chapter 2

Calcium and Cell Response to Heavy Metals: Can Yeast Provide an Answer?

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Abstract

Despite constant efforts to maintain a clean environment, heavy metal pollution continues to raise challenges to the industrialized world. Exposure to heavy metals is detrimental to living organisms, and it is of utmost importance that cells find rapid and efficient ways to respond to and eventually adapt to surplus metals for survival under severe stress. This chapter focuses on the attempts done so far to elucidate the calcium-mediated response to heavy metal stress using the model organism *Saccharomyces cerevisiae*. The possibilities to record the transient elevations of calcium within yeast cells concomitantly with the heavy metal exposure are presented, and the limitations imposed by interference between calcium and heavy metals are discussed.

Keywords: heavy metal, calcium, stress adaptation, *Saccharomyces cerevisiae*, aequorin

1. Introduction

Responding to environmental stimuli is a prerequisite for cell adaptation to the ever-changing conditions in the cell surroundings. Stress conditions such as sudden changes of temperature, pH, irradiation, or elevations in various chemicals concentration need to be sensed by the cell in order to respond and adapt to these changes. Calcium ions are one of the most widespread second messengers in the eukaryotic cell, being responsible for triggering many responses to external stress conditions [1]. Various biotic and abiotic stresses induce an increase in cytosolic calcium ions ($[Ca^{2+}]_{cyt}$), which in turn activate many proteins involved in signaling pathways, from yeast to humans [2]. Thanks to easy manipulation, rapid growth, genetic

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amenability and with many genes bearing resemblance with higher eukaryotic genes, the yeast *Saccharomyces cerevisiae* is one of the widely used model organisms which helped in elucidating a wide variety of molecular mechanisms conserved along evolution, related to cell cycle and cell proliferation, homeostasis, adaptation and survival [3]. Among many others studies, *S. cerevisiae* was used as a model to investigate the Ca^{2+}-mediated responses to a variety of stimuli: hypotonic stress [4–6], hypertonic and salt stress [7], cold stress [8], high ethanol [9], β-phenylethylamine [10], glucose [11, 12], high pH [13–15], amidarone and antifungal drugs [16, 17], oxidative stress [18], eugenol [19, 20], essential oils [21, 22], or heavy metals [23, 24]. This chapter focuses on the studies made on *S. cerevisiae* cells in the effort to understand the role of calcium in cell response to heavy metal exposure.

Heavy metals represent a constant threat to clean environments as they are constantly released in the course of various anthropogenic activities (**Figure 1**), both industrial (mining, electroplating, smelting, metallurgical processes, nanoparticles, unsafe agricultural practices) and domestic (sewage and waste, metal corrosion), all in the context of rapid industrialization and urbanization [25]. Heavy metals as contaminants are included in the category of persistent pollutants, because they cannot be destroyed or degraded. Being natural components of the earth crust, the environmental contamination becomes serious when heavy metals have the possibility to leach into surface or underground water, or undergo atmospheric deposition and metal evaporation from the water resources [26–28]. The ultimate threat imposed by the spread of heavy metals into the environment is their accumulation in the living organisms (**Figure 1**) via the food chain [29], inducing serious illnesses in animals and humans [30–34].

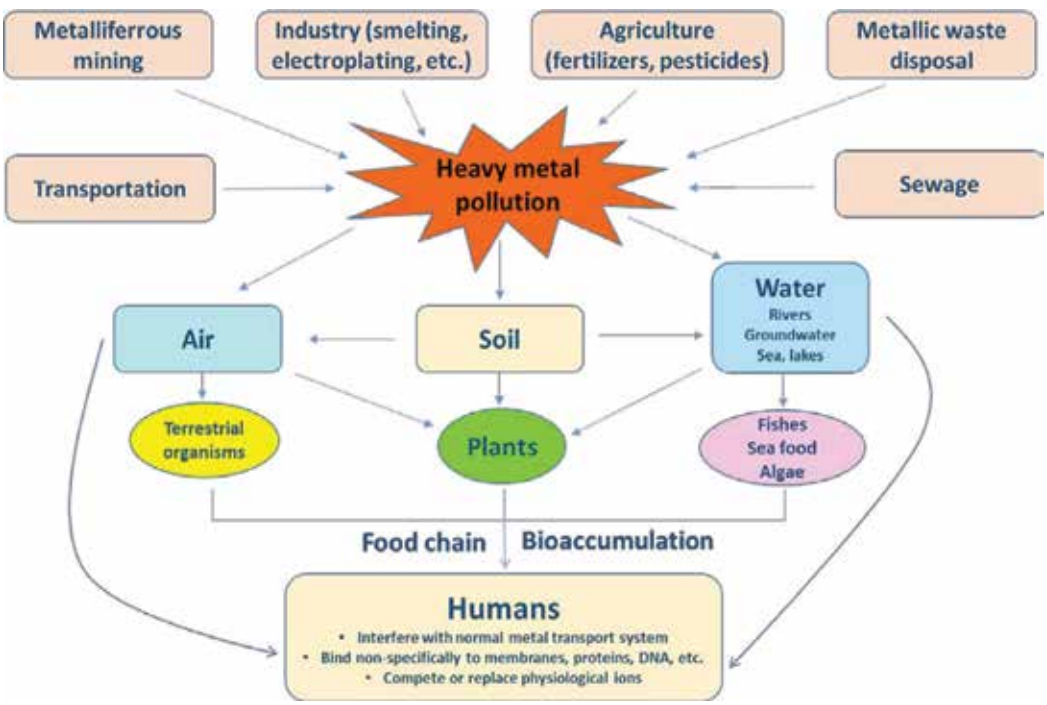

Figure 1. Schematic representation depicting the sources of heavy metal pollution and the impact on the environment and organisms.

Some heavy metals (Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, Mo^{2+}, and Zn^{2+}) are essential for life, contributing to various biochemical and physiological functions in the living organisms. The nutritional requirements of these elements are generally low and they must be present in food in trace concentrations [35]. However, excessive exposure to higher concentrations is deleterious, representing a threat to living organisms [36]. Other heavy metals (Ag^{+}, Cd^{2+}, Pb^{2+}, Hg^{2+}) are not essential for life and have no established biological roles, but they are highly toxic because they compete with the essential metals for their biological targets or they simply bind nonspecifically to biomolecules; these metals are able to induce toxicity at low doses [37]. Essential or not, the hazardous heavy metals such as Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+} are known to be major threats to the environment [38]. The molecular mechanisms involved in heavy metal transport and homeostasis have been intensively studied in *S. cerevisiae* [3], along with many aspects regarding their toxicity, tolerance, accumulation, or extrusion [38–47]. Some of the relevant studies performed in *S. cerevisiae* correlating heavy metal exposure to calcium-related mechanisms are presented in the following section.

2. Calcium transport and homeostasis in *S. cerevisiae*

Intracellular calcium ions are important second messengers in all organisms, including yeast. The mechanisms involved in calcium transport and homeostasis in *S. cerevisiae* cells have been extensively studied [48–50]. Under normal conditions, the $[Ca^{2+}]_{cyt}$ is maintained very low (50–200 nM) at external Ca^{2+} concentrations ranging from <1 μM to >100 mM [51, 52]. Abrupt changes in the environment can be transduced inside the yeast cells by sudden elevations in $[Ca^{2+}]_{cyt}$ which can be the result of Ca^{2+} influx from outside the cell, Ca^{2+} release from internal stores (usually vacuole), or both (**Figure 2**). The yeast plasma membranes contain at least two different Ca^{2+} influx systems, the high-affinity Ca^{2+} influx system (HACS) and the low-affinity Ca^{2+} influx system (LACS), the former being responsible for Ca^{2+} influx under stress conditions [50]. The HACS consists of two proteins, Cch1p and Mid1p, which are expressed and colocalize to the plasma membrane. These two subunits form a stable complex that is activated in response to sudden stimulation, boosting the influx of Ca^{2+} from the extracellular space. In *S. cerevisiae*, Cch1p is similar to the pore-forming α1 subunit of mammalian L-type voltage-gated Ca^{2+} channels (VGCCs) [53], while Mid1p is as a stretch-activated Ca^{2+}–permeable cation channel homologous to $\alpha 2\delta$ subunit of animal VGCCs [54]. HACS is regulated by Ecm7p, a member of the PMP-22/EMP/MP20/Claudin superfamily of transmembrane proteins that includes the λ subunits of VGCCs. Ecm7p is stabilized by Mid1p, and Mid1p is stabilized by Cch1p under non-signaling conditions [55].

Changes in the cell environment are signaled by a sudden increase in $[Ca^{2+}]_{cyt}$ which can be a consequence of either external Ca^{2+} influx via the Cch1p/Mid1p channel on the plasma membrane [4–14, 56], release of vacuolar Ca^{2+} into the cytosol through the vacuole-located Ca^{2+} channel Yvc1p [18, 57], or both (**Figure 2**). After delivering the message, the level of $[Ca^{2+}]_{cyt}$ is restored to the normal very low levels through the action of Ca^{2+} pumps and exchangers. Thus, the Ca^{2+}-ATPase Pmc1p [58, 59] and a vacuolar Ca^{2+}/H^{+} exchanger Vcx1p [60, 61] independently transport $[Ca^{2+}]_{cyt}$ into the vacuole, while Pmr1p, the secretory Ca^{2+}-ATPase, pumps $[Ca^{2+}]_{cyt}$ into endoplasmic reticulum (ER) and Golgi along with Ca^{2+} extrusion from the cell [62, 63]. These responses are mediated by the universal Ca^{2+} sensor protein calmodulin that

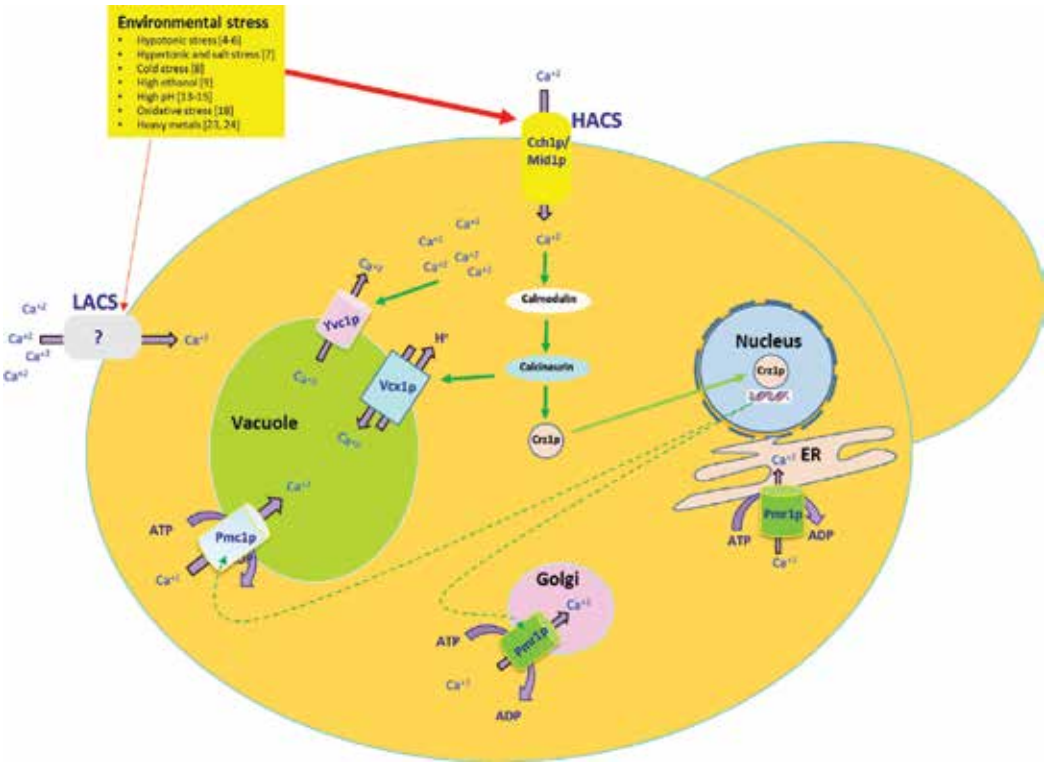

Figure 2. The mechanisms by which yeast cell regulate cell calcium. Under external stresses, the plasma membrane Ca^{2+} influx systems HACS (high-affinity Ca^{2+} influx system) and to a lesser extent LACS (low-affinity Ca^{2+} influx system) are activated, resulting in a rapid influx of Ca^{2+} into the cytosol. Transient increases in intracellular Ca^{2+} concentrations may also be due to release from internal compartments, mainly the vacuole, via Yvc1p. Unlike mammalian cells, where the main Ca^{2+} stores reside in the endoplasmic reticulum (ER), in yeast the intracellular stores are situated in the vacuole compartment. The increased cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) are sensed by calmodulin, activating calcineurin. Activated calcineurin acts on its downstream target Crz1p, inducing its translocation from cytoplasm to nucleus to further induces the expression of a set of Ca^{2+}/calcineurin-dependent target genes, including *PMC1* and *PMR1*. Calcineurin also regulates Vcx1p at post-transcriptional level. Subsequently, the $[Ca^{2+}]_{cyt}$ concentration is reduced to basal levels via uptake by organelles, especially vacuole (by means of Pmc1p and Vcx1p) and Golgi (by means of Pmr1p).

can bind and activate calcineurin, which inhibits at the post-transcriptional level the function of Vcx1p [60, 64, 65] and induces the expression of *PMC1* and *PMR1* genes via activation of the Crz1p transcription factor [64, 65]. The release of Ca^{2+} from intracellular stores stimulates the extracellular Ca^{2+} influx, a process known as capacitative calcium entry [66]. Inversely, the release of vacuolar Ca^{2+} via Yvc1p can be further stimulated by the Ca^{2+} from outside the cell as well as that released from the vacuole by Yvc1p itself in a positive feedback called Ca^{2+}-induced Ca^{2+} release (CICR) [67–70].

3. Aequorin, a transgenic molecular tool for detecting $[Ca^{2+}]_{cyt}$ changes in *S. cerevisiae*

As a second messenger, Ca^{2+} triggers a variety of cascade responses by temporarily activating Ca^{2+}-binding components of signaling pathways which can lead either to adaptation to

the environmental changes or to cell death [71]. To determine the $[Ca^{2+}]_{cyt}$ fluctuations during cell exposure to environmental changes, it is necessary to have an system capable to detect the sudden and transient elevations in $[Ca^{2+}]_{cyt}$. This was made possible by the isolation of aequorin, a Ca^{2+}-binding photoprotein, isolated from the luminescent jellyfish, *Aequorea victoria*. Aequorin consists of two distinct units, the apoprotein apoaequorin (22 kDa) and the prosthetic group, coelenterazine, which reconstitute spontaneously in the presence of molecular oxygen, forming the functional protein [72–74]. Aequorin has become a useful instrument for the measurement of intracellular Ca^{2+} levels, since it has binding sites for Ca^{2+} ions responsible for protein conformational changes that convert through oxidation its prosthetic group, coelenterazine, into excited coelenteramide and CO_2 (**Figure 3A**). As the excited coelenteramide relaxes to the ground state, blue light (λ_{max} 469 nm) is emitted and can be easily detected with a luminometer [75].

The expression of cDNA for apoaequorin in yeast cells and subsequent regeneration of apoaequorin into aequorin provide a noninvasive, nontoxic and effective method to detect the transient variations in yeast $[Ca^{2+}]_{cyt}$ [76]. The yeast strains to be analysed must express the *A. victoria* apoaequorin, and they need to be reconstituted into fully active aequorin by association with coelenterazine (**Figure 3B**). The latter cannot be synthesized by yeast itself; therefore, the way to achieve reconstitution is to incubate the apoaequorin-expressing cells with coelenterazine, prior to Ca^{2+} determination. Coelenterazine is a hydrophobic molecule, and therefore, it is easily taken up across yeast cell wall and membrane, making aequorin suitable as a Ca^{2+} reporter [52, 77]. Aequorin has a number of advantages over other Ca^{2+} indicators as follows: because the protein is large, it has a low leakage rate from cells compared to lipophilic dyes and it does not undergo intracellular compartmentalization or sequestration. Also, it does not disrupt cell functions, and the light emitted by the oxidation of coelenterazine does not depend on any optical excitation, so problems with auto-fluorescence are eliminated [78]. The primary limitation of aequorin is that the prosthetic group coelenterazine is irreversibly consumed to produce light. Such issues led to developments of other genetically encoded calcium sensors including the calmodulin-based sensor cameleon, which were less successful in yeast, due to their size [79].

In *S. cerevisiae*, the reconstituted aequorin is used primarily to detect the Ca^{2+} fluctuations in the cytosol [76]; there have been few attempts to obtain apoaequorins targeted to various cell compartment in yeast. One notable example was the construction of a recombinant apoaequorin cDNA whose product localizes in the ER lumen; using this product, a steady state of 10 μM Ca^{2+} was detected in the ER lumen of wild type cells, and it was possible to demonstrate that the Golgi pump Pmr1p also controls, at least in part, the ER luminal concentration of Ca^{2+} [63]. Nevertheless, no reports on Ca^{2+} fluctuation in the ER in response to environmental stress are available in yeast. Surprisingly, no vacuole-targeted aequorin has been reported in yeast, in spite of the fact that the vacuole is the main storage compartment for Ca^{2+} in yeast; instead, the vacuolar Ca^{2+} traffic was determined indirectly, using genetic approaches (knockout mutants of various Ca^{2+} pumps and transporters) [61, 80] or blockers of the Ca^{2+} influx across the plasma membrane. This latter approach makes use of cell-impermeant Ca^{2+} chelators such as 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) [18] or of lanthanide (Ln^{3+}) ions, which are efficient blockers if ion channels due to size similarity between Ca^{2+} and Ln^{3+} [80]. Of all Ln^{3+}, Gd^{3+} is the most widely used as Ca^{2+}-channel blocker. It was shown that at 1 mM concentration in the medium all the cations from the Ln^{3+} series block Ca^{2+} entry into

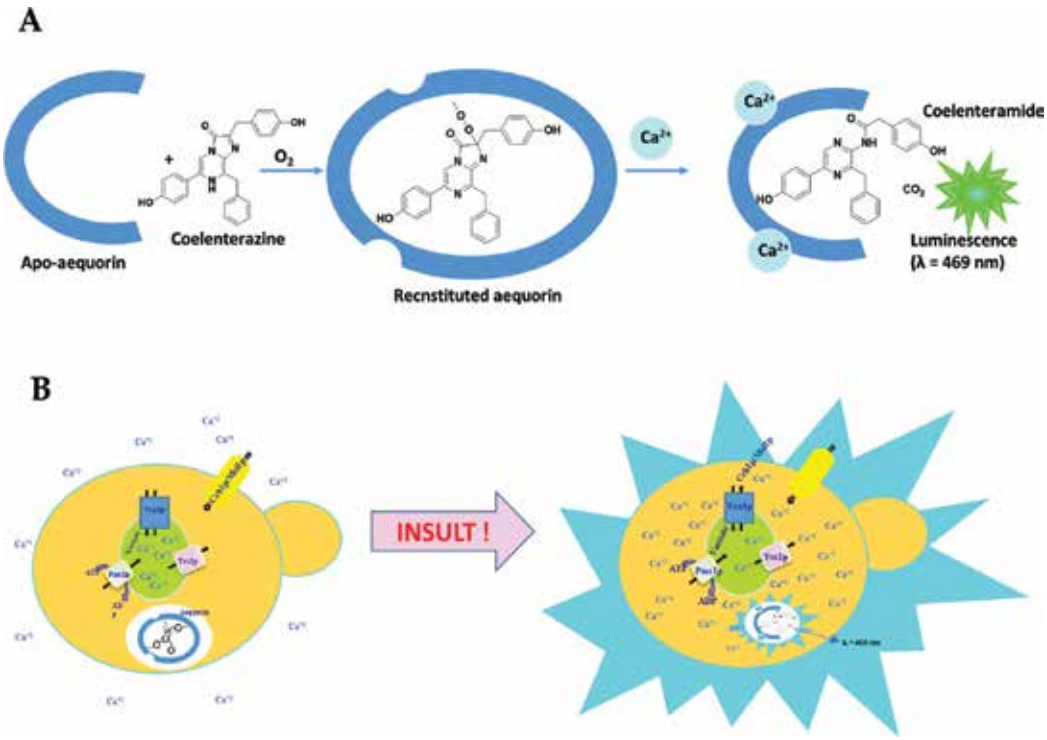

Figure 3. Transgenic aequorin as a tool for measuring intracellular Ca^{2+}. A. Schematic representation of aequorin bioluminescence [72–74]. Cells expressing apo-aequorin are first incubated with the cell-permeant coelenterazine to produce functional aequorin. When Ca^{2+} binds to aequorin, the protein undergoes a conformational change leading to the destabilization of the peroxide group (-O-O-), linking apoaequorin to coelenterazine, decomposing it to to coelenteramide and CO^2; the coelenteramide, which is in an excited state, generates blue light (λmax = 469 nm). B. Schematic representation of Ca^{2+}-induced bioluminescence of yeast cells expressing reconstituted aequorin in the cytosol. When cells are exposed to an insult (e. g., environmental stress) the secondary messenger Ca^{2+} ions enter the cytosol and bind to aequorin, rendering the cell luminescent. Luminescence traces indicate the intensity and the duration of the $[Ca^{2+}]_{cyt}$ wave [75, 76].

cytosol with the exception of La^{3+} (lanthanum) and to a lesser extent, Pr^{3+} and Nd^{3+} [81]. Care must be taken when using Ln^{3+} as channel blockers, as it was shown that at low concentrations Ln^{3+} may leak into the cytosol via the Cch1p/Mid1p system [82].

4. Correlations between calcium and heavy metal exposure as seen in *S. cerevisiae* cells

When grown in media contaminated with heavy metals, the yeast cell wall is the first to get in contact with the surplus cations present in the cell surroundings. If the contamination is not excessive, the cations would probably get stuck at this level, due to the mannoproteins that compose the outer layer of the cell wall (alongside of β-glucans and chitin) which are heavily phosphorylated and carboxylated, decorating the cell façade with a negatively charged shield prone to bind to positively charged species, such as the metal cations [83]. Excess metal ions which escape the negatively charged groups on the cell wall surface penetrate the porous cell

wall and reach the membrane to exert their toxic effect by disrupting the lipid bilayer or by assaulting the membrane transporters.

Several heavy metals (Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+}) are essential for life in their ionic forms, acting mainly as cofactors for a variety of enzymes. They are necessary only in minute amounts inside the cell (hence their denomination as "trace" elements); if their concentration goes beyond the physiological threshold they become toxic by nonspecifically binding to any biomolecule bearing a negative charge or a metal-chelator fragment. The bipolar nature of trace metals determined the development of intricate cellular systems dedicated to their uptake, buffering, sequestration, intracellular trafficking, compartmentalization and excretion. As in many other directions of study, *S. cerevisiae* brought a considerable contribution to the understanding of the molecular mechanism involved in trace metal transport and homeostasis [3, 38–47]. Several heavy metal transporters were identified at the plasma membrane level (**Figure 4A**), with both high and low affinity. For example, Ctr1p, Smf1p and Zrt1p are involved in the high-affinity uptake of Cu^{+}, Mn^{2+} and Zn^{2+}, respectively [84–86]. Low-affinity plasma membrane transporters are more numerous and less specific: Fet4p for Fe^{2+}, but also for Cu^{2+}, Cd^{2+}, Mn^{2+}, and Zn^{2+}; Zrt2p for Zn^{2+}, but also for Fe^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, Mn^{2+} [87, 88]. Transporters for phosphate or amino acids were also shown to participate in the low-affinity transport of Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, and Ni^{2+} [89, 90]. All these transporters are likely to be assaulted by surplus metals (**Figure 4B**) when cells are exposed to contaminated environments [91].

To have any chance of survival under heavy metal stress, the cell needs to be one step ahead of the "villain" ions and to get prepared for defense by using various strategies. The attempts

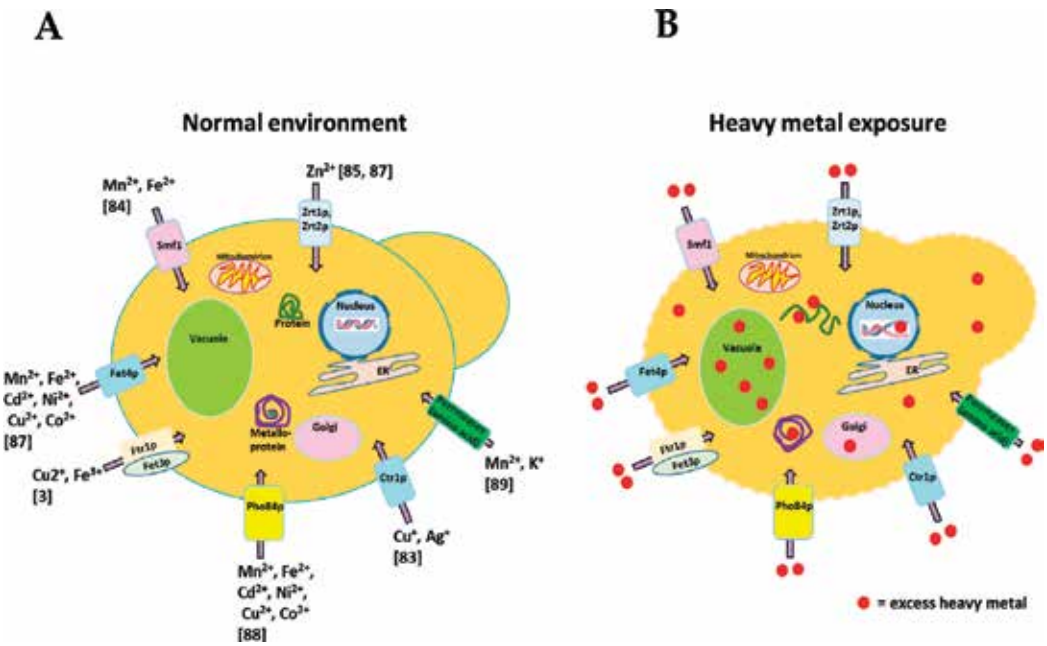

Figure 4. Toxicity of heavy metal exposure. A. Schematic representation of transporters involved in the uptake of essential metals under normal conditions. B. Under high surplus of heavy metals, the transporters will carry the excess cations into the cell, where they bind non-specifically to biomolecules, altering their structure and functionality [91].

to understand the role of calcium in preparing the yeast cell to resist the heavy metal attack are summarized in the following sections.

4.1. Cd^{2+}

Cd^{2+} is one of the most studied non-essential heavy metals as it is a global environmental pollutant present in soil, air, water, and food, representing a major hazard to human health [92]. External Cd^{2+} was shown to unequivocally induce the $[Ca^{2+}]_{cyt}$ elevations in *S. cerevisiae*, as recorded in aequorin-expressing cells, which responded through a sharp increase in the $[Ca^{2+}]_{cyt}$, just a few seconds after being exposed to high Cd^{2+} [23]. Interestingly, the chemically similar Zn^{2+} and Hg^{2+} failed to elicit $[Ca^{2+}]_{cyt}$ elevations under the same conditions [23]. The response to high Cd^{2+} depended mainly on external Ca^{2+} (transported through the Cch1p/Mid1p channel) and to a lesser extent on the vacuolar Ca^{2+} (released into the cytosol through the Yvc1p channel). The adaptation to high Cd^{2+} was influenced by perturbations in Ca^{2+} homeostasis in that the tolerance to Cd^{2+} often correlated with sharp Cd^{2+}-induced $[Ca^{2+}]_{cyt}$ pulses (**Figure 5A**, **B**), while the Cd^{2+} sensitivity was accompanied by the incapacity to rapidly restore the low levels of $[Ca^{2+}]_{cyt}$ [23] (**Figure 5C**).

It had been suggested that Cd^{2+} toxicity was a direct consequence of Cd^{2+} accumulation in the ER and that Cd^{2+} does not inhibit disulphide bond formation (which could account for the lack of response in the case of Zn^{2+} and Hg^{2+}) but perturbs calcium metabolism. Cd^{2+} activates the calcium channel Cch1/Mid1 under low external Ca^{2+}, which also contributes to Cd^{2+} entry into the cell [93]; the protective effect of Ca^{2+} may be the result of competitive uptake between the two cations at the plasma membrane. In this line of evidence, it was shown that excess concentration of extracellular Ca^{2+} attenuates the Cd^{2+}-induced ER stress [94]. It was

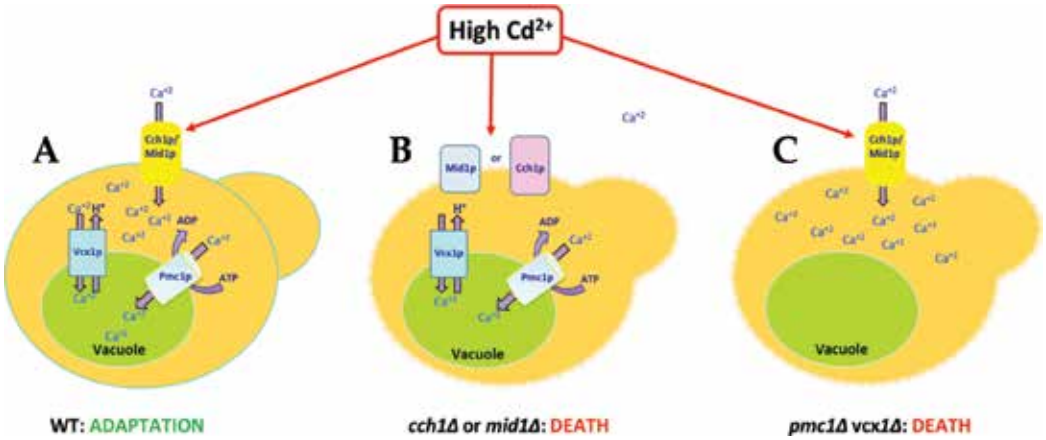

Figure 5. Cd^{2+}-induced $[Ca^{2+}]_{cyt}$ elevations mediate cell adaptation or cell death under Cd^{2+} stress. A. In normal (WT, wild type) cells, surplus Cd^{2+} induces Ca^{2+} entry via Cch1p/Mid1p channel, then $[Ca^{2+}]_{cyt}$ is rapidly restored to low levels by the action of vacuolar Pmc1p and Vcx1p, allowing adaptation to high Cd^{2+}. B. Cells lacking Cch1p or Mid1p (knock-out mutants *cch1Δ* or *mid1Δ*) die under Cd^{2+} stress, as Ca^{2+} does not enter the cell in sufficient quantity to signal the Cd^{2+} excess. C. Cells lacking both Pmr1p and Vcx1p (double knock-out mutant *pmr1Δ vcx1Δ*) die under Cd^{2+} stress, as $[Ca^{2+}]_{cyt}$ cannot be rapidly restored to the low physiological levels [23].

determined that divalent Cd^{2+} and Ca^{2+} have very similar physical properties, with ionic radii of Ca^{2+} (0.97 Å) and Cd^{2+} (0.99 Å) giving similar charge/radius ratios, meaning that these ions are able to exert strong electrostatic forces on biological macromolecules [95]. Under such circumstances, the Cd^{2+}-induced aequorin luminescence observed could also be the result of aequorin binding to Cd^{2+} instead of Ca^{2+}. This was not the case though: when measuring the Cd^{2+} accumulation in yeast cells, it was revealed that the Cd^{2+}-induced aequorin luminescence occurred significantly faster than the Cd^{2+} uptake, indicating that the luminescence produced was the result of increase in $[Ca^{2+}]_{cyt}$ [23].

4.2. Cu^{2+}

Cu^{2+} is one of the most important essential metals: a variety of enzymes require copper as a cofactor for electron transfer reactions [96]. Nevertheless, when in excess, Cu^{2+} is very toxic in the free form because of its ability to produce free radicals when cycling between oxidized Cu^{2+} and reduced Cu^{+}. Studies correlating Ca^{2+} with Cu^{2+} toxicity in yeast are scarce, but it had been known that the inhibitory effect of Cu^{2+} on glucose-dependent H^{+} efflux from *S. cerevisiae* could be alleviated by Ca^{2+} [97]. The role of Ca^{2+} in mediating the cell response to high concentrations of Cu^{2+} was investigated in parallel with Cd^{2+}, and it was noted that exposure to high Cu^{2+} determined broad and prolonged $[Ca^{2+}]_{cyt}$ waves which showed a different pattern from the $[Ca^{2+}]_{cyt}$ pulses induced by high Cd^{2+} [23]. In contrast to Cd^{2+}, Ca^{2+} – mediated responses to high Cu^{2+} depend predominantly on internal Ca^{2+} stores [24] (**Figure 6A**).

It was found that the cell exposure to high Cu^{2+}-induced broad Ca^{2+} waves into the cytosol which were accompanied by elevations in cytosolic Ca^{2+} with patterns that were influenced by the Cu^{2+} concentration but also by the oxidative state of the cell [18, 24]. When Ca^{2+} channel deletion mutants were used, it was revealed that the main contributor to the cytosolic Ca^{2+} pool under Cu^{2+} stress was the vacuolar Ca^{2+} channel, Yvc1p, also activated by the Cch1p-mediated Ca^{2+} influx (**Figure 6**). Using yeast mutants defective in the Cu^{2+} transport across the plasma membrane, it was found that the Cu^{2+}-dependent Ca^{2+} elevation could correlate with the accumulated metal, but also with the Cu^{2+} – induced oxidative stress and the overall oxidative status. Moreover, it was revealed that Cu^{2+} and H_2O_2 acted in synergy to induce Ca^{2+}-mediated responses to external stress [24]. Interestingly, other redox active metals such as Mn^{2+} or Fe^{2+} were inactive in inducing $[Ca^{2+}]_{cyt}$ waves ([23], unpublished observations), probably because these metals are less redox-reactive than the Cu^{2+}/Cu^{+} couple (**Figure 6D**) under aerobic conditions [98].

4.3. Mn^{2+}

High manganese failed to elicit Ca^{2+} elevations irrespective of the magnitude of the insult applied ([23]; unpublished observations). The response was monitored over a wide range of concentrations (from the quasi-physiological 0.5 mM to the super lethal 50 mM) and times (up to 60 min of exposure). Of all the cations, Mn^{2+} is the closest to Ca^{2+} in terms of ionic radius and charge. This similarity is so relevant that Mn^{2+} effectively supports yeast cell-cycle progression in place of Ca^{2+} [99]. This similarity probably renders the cell irresponsive to high concentrations of an otherwise toxic metal. A more subtle Mn^{2+}-Ca^{2+} interplay exists though, being

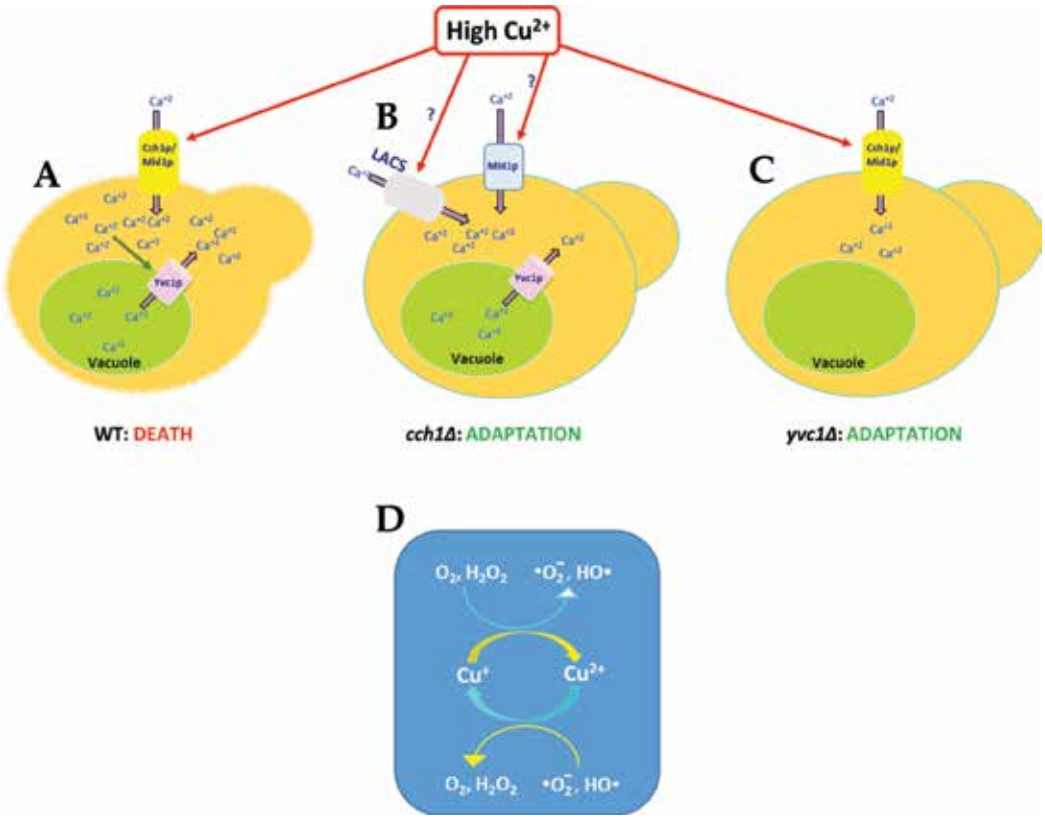

Figure 6. Cu^{2+}-induced $[Ca^{2+}]_{cyt}$ elevations mediate cell adaptation or cell death under Cu^{2+} stress. A. In normal (WT, wild type) cells, surplus Cu^{2+} induces $[Ca^{2+}]_{cyt}$ elevations as Ca^{2+} enters via Cch1p/Mid1p channel or is released from the vacuole via Yvc1p, in a positive feed-back. The normal low levels of $[Ca^{2+}]_{cyt}$ are not rapidly restored as in the case of Cd^{2+}-exposure, and the cells die. B. Cells lacking Cch1p (but not Mid1p) exhibit lower elevations in Cu^{2+}-induced $[Ca^{2+}]_{cyt}$ and are more tolerant to Cu^{2+} stress. C. Cells lacking Yvc1p (knock-out mutant *yvc1Δ*) exhibit very low elevations in Cu^{2+}-induced $[Ca^{2+}]_{cyt}$ and adapt easily to Cu^{2+} stress [24]. The cell behavior described in A-C is similar to the Ca^{2+}-mediated response to oxidative stress [18], suggesting that the Cu^{2+}-induced $[Ca^{2+}]_{cyt}$ changes may be indirectly mediated by the formation of reactive oxygen species during copper shuffling between oxidative states Cu^{2+}-Cu^{+} (D).

manifested at several levels [41]. For example, high Mn^{2+} is controlled by calcineurin/Crz1p-regulated Pmr1p and Pun1p [100]. Importantly, the tolerance of yeast cells to Mn^{2+} is related to both Pmr1p and Vcx1p [41, 64, 65, 101] two determinants of maintaining low $[Ca^{2+}]_{cyt}$ by transporting the ions to the vacuole and Golgi/ER, respectively [60–63]. The Ca^{2+}-dependent response to Mn^{2+} surplus seems to be induced not by external Mn^{2+}, but by the cations accumulated inside the cell. For example, it was found that internal Mn^{2+} can be redistributed by calcium-stimulated vesicle trafficking [102].

4.4. Fe^{2+}

Fe^{2+} toxicity can be the result of direct ionic effect, but the indirect effect of catalyzing Fenton reactions, in which highly reactive oxygen species arise, represents the main concern raised

by Fe^{2+} surplus. As in the case of Mn^{2+}, excess Fe^{2+} did not elicit sudden elevations in $[Ca^{2+}]_{cyt}$ upon exposure [23]. It had been reported that yeast strains lacking the components of the Cch1p/Mid1p plasma membrane channel were hypersensitive to Fe^{2+}. When measuring the relative Ca^{2+} accumulation, it was noted that iron stress also increased the residual Ca^{2+} uptake in the *cch1Δ mid1Δ* double knockout mutant [8]. As the Ca^{2+} measurements in this study were done radiometrically, there must have been a considerable lag between application of the stimulus and Ca^{2+} measurement (unlike aequorin determinations, which allow Ca^{2+} detection simultaneously with stimulus application), and the mutant's sensitivity towards Fe^{2+} might have been caused by Ca^{2+} lingering in the cytosol, as in the case of Cd^{2+}-sensitive mutants [23].

4.5. Other metals

The surplus of heavy metals such as Ni^{2+}, Co^{2+}, Pb^{2+}, Hg^{2+}, and Ag^{+} did not have the ability to rapidly induce elevations in $[Ca^{2+}]_{cyt}$. In some cases, (Ni^{2+} and Co^{2+}) exogenous Ca^{2+} alleviated the toxicity of the metal ions, but this effect was rather related to the inhibition of Co^{2+} or Ni^{2+} uptake by Ca^{2+} [103].

5. Concluding remarks

In this chapter, we attempted to highlight the studies made in *S. cerevisiae* which correlate the exposure to high concentrations of heavy metals with the Ca^{2+}-mediated cellular responses. *S. cerevisiae* is a very good model to study the cell response to sudden changes of metal concentration in the environment; such studies were greatly facilitated by the ease of obtaining yeast cells expressing aequorin in the cytosol, thus allowing the real-time detection of $[Ca^{2+}]_{cyt}$ fluctuations. By combining Ca^{2+} monitoring under metal stress with the genetic approaches that make use of mutants with perturbed heavy metal or Ca^{2+} homeostasis, important aspects related to cell adaptation or cell death under heavy metal stress have been elucidated. Using yeast cells expressing aequorin in the cytosol provides answers regarding the immediate Ca^{2+}-mediated responses, which are crucial for deciding the cell fate. Nevertheless, to understand the Ca^{2+}-mediated cell responses which occur at later phases, developing sensitive Ca^{2+} sensors targeted to specific compartments is still a desiderate for future studies.

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Chapter 3

The Endothelium: The Vascular Information Exchange

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Additional information is available at the end of the chapter

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Abstract

Maintenance of adequate blood flow to tissues and organs requires that endothelial cells dynamically respond in a stimulus-specific manner to elicit appropriate changes in smooth muscle contractility and thus, arterial diameter. Endothelial cells can be stimulated directly by increases in blood flow and by humoral factors acting on surface receptors, as well as through flux of second messengers from smooth muscle cells activated by release of neurotransmitters from perivascular nerves. The ability of endothelial cells to generate stimulus-specific responses to these diverse inputs is facilitated by organization of ion channels and signaling proteins into microdomains that permit finely-tuned, spatially-restricted Ca^{2+} events to differentially activate key effectors such as nitric oxide (NO) synthase and Ca^{2+}-activated K^+ (K_{Ca}) channels. NO is a diffusible mediator which acts locally to cause vasodilation. Opening of K_{Ca} channels causes hyperpolarization of the endothelial membrane potential which spreads to surrounding smooth muscle cells to also cause local vasodilation. However, once initiated, hyperpolarization also spreads longitudinally through the endothelium to effect coordinated changes in blood flow within multiple arterial segments. Thus, the signaling pathways activated by a particular stimulus determine whether it's effects on arterial diameter are localized or can impact blood flow at the level of the vascular bed.

Keywords: endothelium, calcium, nitric oxide, microdomain, potassium channels

1. Introduction

Appropriate local control of blood flow through resistance arteries is critical to the functioning of tissues and organs, and to regulation of blood pressure. Lying at the interface between the blood and smooth muscle cells of the vessel wall, the endothelium plays a vital role in this dynamic process by transducing diverse chemical and mechanical stimuli into

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coordinated changes in arterial diameter. Endothelial cells respond to vasodilatory stimuli by releasing diffusible mediators such as nitric oxide (NO) and prostacyclin (PGI_2) and by initiating membrane hyperpolarization that spreads to surrounding smooth muscle cells via myoendothelial gap junctions (MEGJs) to inhibit contractility, a mechanism termed endothelium-dependent hyperpolarization (EDH) [1–3]. NO and PGI_2 are local mediators that diffuse to surrounding smooth muscle cells to cause relaxation. Once initiated, EDH spreads to surrounding smooth muscle cells to affect relaxation but conduction of hyperpolarization longitudinally through the endothelial layer means that EDH also contributes to coordination of changes in blood flow in multiple arterial segments within a vascular bed [4]. Thus, the ability of a stimulus to engage diffusible mediators versus EDH determines whether it's effects on arterial diameter and thus blood flow, are restricted to the local area or can impact blood flow at the level of the vascular bed.

Increases in endothelial intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) drive these vasodilator pathways; NO is synthesized from L-arginine by the Ca^{2+}-calmodulin-dependent enzyme NO synthase (NOS) [3], PGI_2 is generated by the actions of cyclooxygenase on arachidonic acid released by the actions of Ca^{2+}-dependent phospholipase A_2 on membrane phospholipids [5, 6], and opening of Ca^{2+}-activated K^+ (K_{Ca}) channels causes hyperpolarization [3, 7]. Global changes in endothelial $[Ca^{2+}]_i$ have been widely studied [8, 9], but development of new technologies such as high-speed, high-resolution confocal Ca^{2+} imaging and generation of transgenic mice expressing genetically encoded Ca^{2+} indicators has allowed resolution of a wide range of transient Ca^{2+} events within endothelial cells of intact arteries to provide a growing body of support for the concept of stimulus-specific engagement of effectors underpinned by spatially and temporally discrete Ca^{2+} signaling patterns that occur independently of changes in bulk endothelial $[Ca^{2+}]_i$ [10–13].

Pulsars [10] and wavelets [11] are spontaneous, short-lived, (<0.03 s duration) spatially fixed Ca^{2+} events originating from distinct clusters of inositol 1,4,5-trisphosphate ($InsP_3$) receptors on the membrane of endoplasmic reticulum (ER). First identified in mouse mesenteric artery and hamster skeletal muscle arteriolar endothelial cells, these events predominantly occur close to endothelial projections that abut or form MEGJs with smooth muscle cells [10, 11] and exert a basal vasodilator influence through activation of intermediate conductance (IK_{Ca}) Ca^{2+}-activated K^+ channels. Their dependence on $InsP_3$ provides a mechanism by which pulsars are linked to and regulated by G protein-coupled receptor (GPCR) signaling. Elevation of $InsP_3$ by endothelium-dependent vasodilators [10] or by flux of $InsP_3$ from smooth muscle cells following stimulation of α_1-adrenoceptors [11] increases pulsar size and/or frequency through recruitment of new sites and a reduction in the interval between pulsars at a given site. In porcine coronary arteries, $InsP_3$-dependent Ca^{2+} events similar to pulsars propagate into longer lasting Ca^{2+} waves (>8 s duration) facilitated by the longitudinal arrangement of ER/$InsP_3$ receptors to promote directional Ca^{2+}-induced Ca^{2+} release along the endothelial cell axis and are associated with activation of both NOS and K_{Ca} channels [12].

Sparklets are generated by spatially restricted Ca^{2+} influx through members of the transient receptor potential (TRP) ion channel family [14, 15]. Sparklets were first identified in mouse mesenteric arteries under experimental conditions in which $InsP_3$-mediated pulsars were

eliminated [14]. Exposure of the endothelium to TRPV4 agonists and/or acetylcholine increased the activity of these discrete Ca^{2+} signals which were linked to activation of both IK_{Ca} and small conductance (SK_{Ca}) Ca^{2+}-activated K^+ channels, effects which were absent in arteries from mice lacking TRPV4 [14]. In rat cremaster arterioles, clustering of TRPV4-mediated sparklets in endothelial projections was linked exclusively to activation of IK_{Ca} channels [16] and in mouse small pulmonary arteries, shear stress-stimulated TRPV4 activity was linked to NO production [17]. Larger endothelial sparklets mediated by simultaneous opening of two TRPA1 and leading to activation of IK_{Ca} channels were shown to underlie dilation to reactive oxygen species in rat cerebral arteries [18]. We will now discuss how grouping of Ca^{2+} signaling and effector proteins into microdomains allows dynamic, stimulus-specific Ca^{2+} events which determine the recruitment of effectors thus the degree to which blood flow is impacted.

2. Stimulus-specific endothelial Ca^{2+} signaling

2.1. Shear stress

In vivo, endothelial sensing of laminar shear stress, the tangential frictional force exerted by blood flowing across the cell surface, plays a dominant role in acute modulation of vascular tone and therefore, tissue perfusion [19–21]. In the majority of resistance arteries, increases in blood flow stimulate endothelium-dependent relaxation of surrounding smooth muscle cells and increase arterial diameter, a response termed flow-mediated dilatation. Flow also influences gene expression and structural remodeling with areas of disturbed flow and reduced shear stress is associated with development of atherosclerotic plaques [22]. Measurement of acute responses to increases in shear stress is the most widely used clinical index of endothelial function and vascular health with attenuation of flow-induced dilation associated with increased risk of cardiovascular diseases [23, 24]. Indeed, reductions in shear stress are a likely mechanism by which endothelial function is altered with inactivity, an effect which can be overcome by exercise interventions [25, 26].

In animals and humans, acute shear stress-induced vasodilation can be mediated by both NO and EDH [27–31]. Although endothelial cells express both SK_{Ca} and IK_{Ca} channels, data from isolated arteries indicate that it is SK_{Ca} channels that play a predominate role in mediating the EDH component of this response. Deletion of SK_{Ca} but not IK_{Ca} channels impaired both NO and EDH-mediated dilation to shear stress stimulation in mouse isolated carotid arteries [32]. In rat isolated perfused mesenteric beds, shear stress-induced modulation of sympathetic vasoconstriction was prevented by both the NOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME) and apamin, a selective blocker of SK_{Ca} channels, but not by the IK_{Ca} channel inhibitor TRAM-34 [33]. Similarly, shear stress-evoked dilation of mouse isolated coronary arteries was inhibited by apamin [34] and L-NAME [35].

Mechanotransduction, the conversion of increases in shear stress into changes in arterial diameter, is reliant upon rises in endothelial $[Ca^{2+}]_i$ mediated by Ca^{2+} entry. In vitro studies have identified multiple candidates as potential endothelial mechanosensors including

integrins [36], tyrosine kinase receptors [37], intercellular junction proteins [38], and P2X4 receptors which are cation channels activated by adenosine triphosphate (ATP) [39]. Work with transgenic mice has implicated GPR68, a proton-sensing rhodopsin-like GPCR [40], inwardly rectifying K^+ channels [41] and PIEZO1, a Ca^{2+}-permeable, non-selective cation channel [42, 43]. However, recently, it is the role of TRPV4 in endothelial responses to increases in shear stress that has received particular attention. This Ca^{2+} permeable channel can be directly activated by shear stress [34, 44] via membrane deformation or through a lever-like action involving cytoskeletal linkages to molecules embedded in the glycocalyx [45, 46], the layer of proteoglycans and glycoproteins that covers the luminal surface of the endothelium, or indirectly through upstream production of arachidonic acid metabolites [47]. Genetic deletion of TRPV4 results in blunted flow-mediated dilation of mouse carotid and mesenteric arteries [31, 47, 48] and pharmacological inhibition of these channels blocked flow-evoked increases in endothelial $[Ca^{2+}]_i$ in isolated mouse mesenteric, human coronary, and rat carotid and gracilis arteries [31, 49–51]. In bovine coronary endothelial cells block of TRPV4 inhibited both shear stress-evoked increases in $[Ca^{2+}]_i$ and activation of SK_{Ca} channels [34], indicating that there may be a direct link between TRPV4-mediated Ca^{2+} influx and SK_{Ca} channel activity. This idea is supported by the demonstration that in rat pulmonary arteries, vasodilation to the TRPV4 agonist GSK1016790A was mediated by activation of SK_{Ca} channels [52]. In the same vessels, and in mouse small pulmonary arteries, shear stress-stimulated TRPV4 activity was also linked to NO production [17] suggesting a further link between TRPV4 and NOS.

Building on these findings, several lines of evidence now support the notion that acute increases in shear stress cause Ca^{2+} influx through TRPV4 channels to selectively activate both SK_{Ca} channels and NOS, and that this pathway is enabled by organization of TRPV4, NOS, SK_{Ca} channels and the caveolae scaffold protein caveolin-1 into microdomains within caveolae, flask-shaped structures on the endothelial cell surface rich in signaling proteins [34, 53, 54]. SK_{Ca} channels are localized to the luminal membrane of endothelial cells in rat mesenteric arteries [55] and SK_{Ca} channel protein was co-immunoprecipitated with caveolin-1 from endothelial cells of the same arteries and from porcine coronary arteries [56]. It is well established that endothelial NOS is localized to caveolae where it is negatively regulated through its interaction with caveolin-1 [57]. Increases in $[Ca^{2+}]_i$ promote recruitment of Ca^{2+}-calmodulin to displace caveolin-1 from NOS thereby activating it [53]. In bovine coronary and human microvascular endothelial cells, SK_{Ca} channels were co-localized with caveolin-1, NOS and TRPV4 channels within microdomains at the luminal endothelial cell surface [34, 57, 58]. Furthermore, in mesenteric arteries from mice lacking caveolin-1, endothelial TRPV4 channel activity was impaired indicating that a direct interaction between TRPV4 and caveolin-1 may be functionally important for Ca^{2+} entry in response to shear stress [57]. Caveolin-1 has been shown to initiate downstream signaling in response to increases in shear stress [59] leading to the suggestion that caveolae act as mechanosensors to elicit a cascade of events that promote vasodilation. In line with this proposal, shear stress-induced dilation is defective and endothelial SK_{Ca} current reduced in coronary and carotid arteries of mice lacking caveolin-1, an effect rescued by re-introduction of endothelial specific caveolin-1 [58, 60].

Together these findings, gathered using a range of approaches and from a number of different arteries, support an elegant model in which shear stress-evoked Ca^{2+} influx through TRPV4 channels on the luminal surface of endothelial cells leads to spatially-restricted Ca^{2+} sparklets

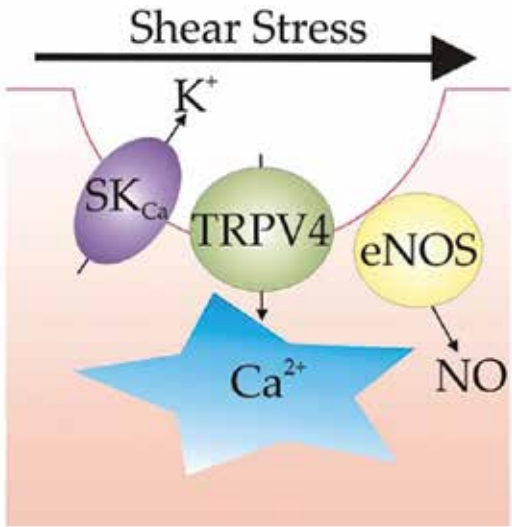

Figure 1. Model of localized endothelial Ca^{2+} signaling evoked by increases in shear stress. Shear stress-evoked Ca^{2+} influx through TRPV4 channels on the luminal surface of endothelial cells leads to spatially-restricted Ca^{2+} sparklets within a signaling microdomain to selectively activate SK_{ca} channels and endothelial NOS (eNOS).

within a signaling microdomain to selectively activate SK_{Ca} channels and NOS to elicit vasodilation (**Figure 1**). However, a number of questions remain to be addressed. Shear stress increases PGI_2 production in bovine coronary and human umbilical vein endothelial cells [34, 61] and rabbit isolated femoral arteries [62], and hydrogen peroxide (H_2O_2) contributes to flow-mediated dilation in coronary arterioles from patients with heart disease [63] but the functional role of these factors in acute flow-mediated vasodilation has not fully been explored. A significant component of flow-induced dilation remained in isolated mesenteric arteries of mice lacking TRPV4 [31] which could indicate that, as suggested in earlier reports, Ca^{2+}-independent processes may also contribute to this response [64] or the involvement of another route for Ca^{2+} influx. The possibility that flow-induced increases in endothelial cell $[Ca^{2+}]_i$ are stimulated by localized release endothelium-derived paracrine mediators such as ATP, substance P or acetylcholine, first proposed over 30 years ago [65, 66], has recently received renewed support with the demonstration that endothelial organic cation transporters release acetylcholine in response to increases in shear stress in rat isolated carotid arteries [67]. The same study suggests that $InsP_3$-mediated Ca^{2+} release from ER stores and Ca^{2+} entry through TRPC but not TRPV4 contributes to flow-induced endothelial Ca^{2+} signaling in these vessels. This finding highlights the fact that further work is required to elucidate the differential signaling networks underlying endothelial responses to acute increases in shear stress in different arteries.

2.2. Agonists at endothelial GPCRs

Many endogenous and exogenous chemicals bind to GPCRs leading to stimulation of EDH and production of NO, PGI_2 and other diffusible mediators such as epoxyeicosatrienoic acids and H_2O_2, to cause vasodilation [1, 2, 68]. Measurements of bulk endothelial $[Ca^{2+}]_i$ established

the role of $InsP_3$-mediated Ca^{2+} release and subsequently store-operated Ca^{2+} entry (SOCE) in this process [9]. The mechanism underlying endothelial SOCE has been controversial but recent evidence supports a model in which Ca^{2+} store depletion allows spatial reorganization of Ca^{2+} sensor protein stromal interaction molecules (STIMs) so that they can aggregate into clusters that physically interact with Ca^{2+}-selective Orai channels at the ER-plasma membrane junction [69–72]. TRPC and TRPV4 can also interact with STIMs [73] and studies of knock-out mice have provided evidence for a role for TRPC4 in acetylcholine-evoked SOCE in aortae [74] and for TRPV4 in SOCE in mesenteric [75] and carotid arteries [76]. A receptor-operated Ca^{2+} entry mechanism can also be mediated by DAG-induced activation of TRPC and TRPV channels. For example, in human umbilical vein endothelial cells, bradykinin stimulated both translocation of DAG-sensitive TRPC6 to the cell membrane and Ca^{2+} influx [77, 78]. Expression of mRNA for another ER Ca^{2+} release channel, the ryanodine receptor (RyR), has been detected in endothelial cells of human mesenteric arteries [79], and RyRs have been suggested to mediate Ca^{2+} oscillations in cultured bovine aortic and human umbilical vein endothelial cells [80] but to date, ryanodine has been shown to have no effect on endothelial Ca^{2+} signaling or vasodilation [12, 81]. There is significant variation in the reported contribution of EDH, NO and other mediators to agonist-evoked dilation, both in terms of differences between agonists and arteries. Thus, for the purposes of this chapter we will limit our discussion to three agents commonly used to stimulate endothelium-dependent vasodilation in experimental studies, acetylcholine, ATP and substance P.

The first evidence that differential endothelial Ca^{2+} signaling underlies agonist-evoked EDH and NO came from a study of rat isolated middle cerebral arteries in which EDH-dependent vasodilation to purinergic agonists required a larger increase in $[Ca^{2+}]_i$ than for NO [82]. Measurements of global $[Ca^{2+}]_i$ indicated that different sources of Ca^{2+} contributed to agonist-stimulated production of NO and EDH; NO production is associated with SOCE [83] whereas EDH is linked to both $InsP_3$-mediated Ca^{2+} release and SOCE [84]. Similarly, both agonist evoked SOCE and NO production are suppressed in isolated aortae from mice lacking STIM1, the primary endothelial STIM [85]. Building on these findings, data accrued over the past 15 years from functional, histological, Ca^{2+} imaging and immunohistochemical studies of intact arteries and endothelial-smooth muscle co-cultures support agonist-evoked EDH and NO release being mediated by distinct Ca^{2+} signaling within specialized domains.

2.2.1. EDH

EDH is mediated by opening of both IK_{Ca} and SK_{Ca} channels but their relative contribution to agonist-evoked vasodilation, based on the effects of selective pharmacological inhibitors, displays significant variation between agonists and arteries. Simultaneous block of both IK_{Ca} and SK_{Ca} channels is required to inhibit acetylcholine-evoked EDH in mesenteric arteries from rats and mice, and guinea-pig coronary arteries [86–88] whereas in rat cerebral and human mesenteric arteries the same response is largely reliant on IK_{Ca} channels [89, 90].

IK_{Ca} and SK_{Ca} channels display a differential spatial distribution within endothelial cells and their contribution to agonist-evoked EDH appears to be mediated by different signaling pathways. In mesenteric arteries from rats, mice and humans, IK_{Ca} channels are localized within

regions associated with MEGJs in which ER membrane, $InsP_3$ receptors, gap junction connexins, TRPC3 and TRPV4 have also been identified [10, 11, 14, 55, 90–93]. In mouse and rat mesenteric arteries, acetylcholine increased the frequency and number of $InsP_3$-dependent Ca^{2+} pulsars in this region which were linked to activation of IK_{Ca} channels to evoke EDH [10, 93] (**Figure 2**). TRPC3 may support this process by providing Ca^{2+} entry for refilling of $InsP_3$-sensitive ER stores, and/or direct activation of both SK_{Ca} and IK_{Ca} channels [91, 94]. However, in other reports acetylcholine exclusively stimulates TRPV4 in the vicinity of MEGJs to generate Ca^{2+} sparklets and which in turn activate IK_{Ca} channels in mouse mesenteric arteries [14, 95]. This occurs via a mechanism dependent on the anchoring protein AKAP, and is consistent with deletion of TRPV4 resulting in blunting of acetylcholine-evoked increases in endothelial $[Ca^{2+}]_i$ and loss of EDH in mouse mesenteric arteries [75, 76]. TRPV3 [96] and TRPA1 [97] are also expressed in endothelial cells and activators of these channels can certainly initiate increases in Ca^{2+} signaling and EDH in cerebral arteries, but a role for these channels in agonist-stimulated EDH has yet to be demonstrated.

In contrast to IK_{Ca} channels, SK_{Ca} channels are associated with caveolae and are diffusely distributed across the cell membrane with a higher level expression at endothelial-endothelial cell borders [12, 55, 90, 93]. Also, unlike IK_{Ca} channels, evidence is lacking for a direct link between agonist-evoked, $InsP_3$-mediated Ca^{2+} events and SK_{Ca} channel activity. Instead, it

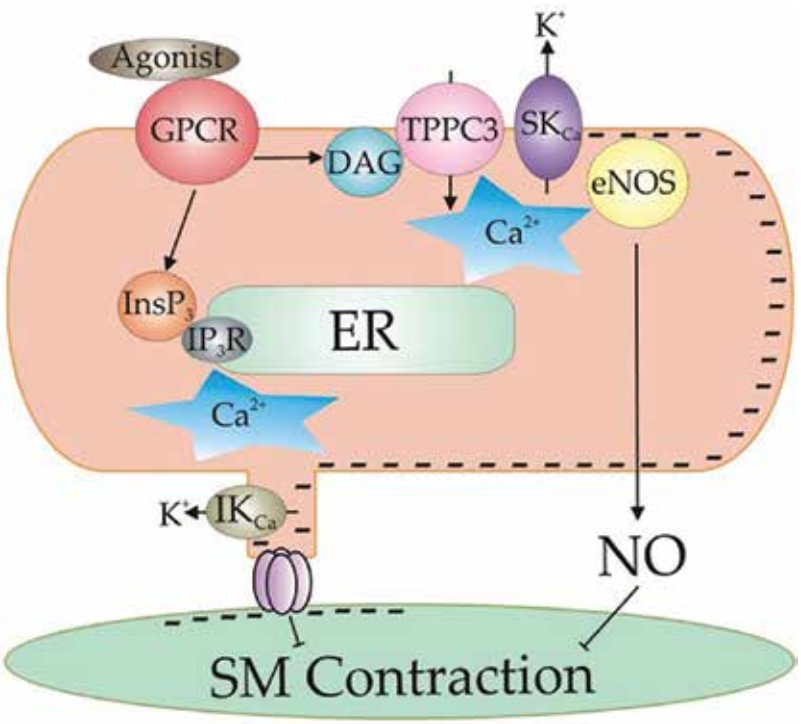

Figure 2. Schematic showing discrete Ca^{2+} signaling events elicited by agonists at endothelial GPCRs. $InsP_3$-dependent Ca^{2+} pulsars are linked to activation of IK_{Ca} channels to evoke EDH whereas Ca^{2+} influx through DAG-activated TRPC channels is the primary source of Ca^{2+} for agonist stimulation of endothelial SK_{Ca} channels and eNOS.

appears that Ca^{2+} influx through TRP channels is the primary source of Ca^{2+} for agonist stimulation of endothelial SK_{Ca} channels [94, 98]. In mouse cerebral artery, ATP caused rapid trafficking of TRPC3 to the plasma membrane to provide Ca^{2+} influx to selectively activate SK_{Ca} channels to cause EDH [98] (**Figure 2**). As described earlier, TRPV4 are also associated with caveolae and are a source of Ca^{2+} for SK_{Ca} channel activation in response to increases in shear stress but whether this relationship accounts for engagement of SK_{Ca} channels by agonists has not been explored.

2.2.2. NO

In contrast to EDH, the role of localized Ca^{2+} signaling in agonist-evoked NO release has received little attention. NOS, TRPV4 and TRPC3 are located in caveolae microdomains, and deletion of either channel blunts acetylcholine-evoked NO release and NO-mediated relaxation in mouse mesenteric and carotid arteries [75, 99] suggesting they may provide a source of Ca^{2+} for agonist-driven NOS activation. Heteromultimers of TRPV4-TRPC1 channels mediate vasorelaxation of rabbit mesenteric arteries in response to stimulation of the Ca^{2+}-sensing receptor through NO production [100] but the underlying Ca^{2+} dynamics were not assessed. A recent study has shown that TRPV4-mediated sparklets underlie ATP driven activation of endothelial NOS in mouse small pulmonary arteries. The resulting NO initiates vasodilation and also guanylyl cyclase-protein kinase G signaling in the endothelium that limits TRPV4 channel cooperativity and serves as a negative feedback signal to regulate TRPV4 channel function [17]. This description of ATP-evoked, spatially distinct TRPV4 sparklets and localized TRPV4-NOS signaling support a novel paradigm that NOS can be activated by spatially restricted Ca^{2+} signals, and identifies TRPV4 channels as a key regulator of NOS activity in the pulmonary microcirculation.

In contrast, in porcine isolated coronary arteries, substance P increased the occurrence of discrete $InsP_3$-dependent endothelial Ca^{2+} events in a concentration-dependent manner; low concentrations primarily increased the number of Ca^{2+} events and at higher concentrations the number of Ca^{2+} events saturated while the magnitude of individual events increased [12]. This pattern correlated with a greater role for NO in vasorelaxation at lower concentrations suggesting subtle Ca^{2+} signal expansion at low stimulation levels may preferentially target NOS. A key finding of this study was that idiosyncratic Ca^{2+} signal expansion corresponded with coronary artery vasorelaxation whereas global changes in $[Ca^{2+}]_i$ did not highlighting that frequency modulation of discrete Ca^{2+} signals is the primary driver of this functional response and that measurement of changes in bulk $[Ca^{2+}]_i$ do not adequately describe the Ca^{2+} signaling pathways that underlie endothelium-dependent vasodilation.

2.2.3. Membrane potential and Ca^{2+} microdomain signaling

Production of NO and stimulation of EDH have long been regarded as separate mechanisms for agonist-evoked vasodilation but several lines of evidence indicate that there may be a facilitatory relationship between endothelial SK_{Ca} and IK_{Ca} channel activity and NO. SK_{Ca} channel activity has been linked to NO-mediated vasodilation to agonists with deletion of these channels causing impaired NO-mediated dilation to acetylcholine in mouse carotid arteries and increased expression enhancing NO-mediated dilation of cremaster arterioles

[32]. In rat mesenteric arteries, block of SK_{Ca} and IK_{Ca} channels reduces agonist-evoked, NO-mediated vasorelaxation and NO release [101]. Conversely, activators of endothelial K_{Ca} channels can enhance NO release from cultured endothelial cells, enhance ATP-induced increases in cytosolic Ca^{2+} concentration and NO synthesis in rat cremaster arterioles, and elicit NO-mediated relaxation in mesenteric arteries [102–104].

Lacking voltage-operated Ca^{2+} channels, endothelial Ca^{2+} influx is mediated by TRP channels and so membrane hyperpolarization may be required to maintain an appropriate electrochemical driving force for agonist-induced Ca^{2+} influx and also to prevent channel inactivation and/or reduction in unitary conductance [105, 106]. Membrane depolarization does inhibit both agonist-induced increases in $[Ca^{2+}]_i$ and NO release in cultured endothelial cells [107, 108], and in rat isolated basilar arteries, endothelial depolarization was associated with a reduction in NO-mediated relaxation to acetylcholine [109]. Nonetheless, the ability of hyperpolarization to regulate Ca^{2+} entry by increasing the electrical driving force is controversial. The large concentration gradient (~20,000-fold for extracellular versus intracellular) [110] and driving force for Ca^{2+} entry raising the question of whether a small amplitude hyperpolarization will be insufficient to modulate Ca^{2+} entry. In rat mesenteric and cerebral arteries, that certainly appeared to be the case as changes in global endothelial $[Ca^{2+}]_i$ were independent of changes membrane potential [89, 111]. However, more recent work with endothelial cell tubes isolated from resistance arteries has provided renewed support for hyperpolarization enhancing acetylcholine-evoked Ca^{2+} influx through TRPV4 [112] and indicate that pharmacological activation of SK_{Ca} and IK_{Ca} channel may not only enhance Ca^{2+} entry to further amplify K_{Ca} channel activity, but also boost NO production [113]. In mouse mesenteric arteries, acetylcholine-evoked TRPV4-dependent Ca^{2+} signaling was inhibited in arteries from mice lacking IK_{Ca} channels indicating that in these arteries, endothelial stimulation drives sufficient IK_{Ca}-dependent Ca^{2+} entry through TRPV4 to enhance dynamics [13]. IK_{Ca} channel activity modestly augmented Ca^{2+} event amplitude but the most notable impact was in recruiting new Ca^{2+} firing sites as well as increasing firing frequencies at pre-existing sites. In the same study, increasing or decreasing SK_{Ca} expression had little additional effect on the occurrence of Ca^{2+} events but did promote increased amplitudes and durations indicating that SK_{Ca} channels may play a role in positive feedback Ca^{2+} regulation by shaping the size and time course of individual events. In porcine coronary arteries stimulation of NOS by $InsP_3$-dependent, large amplitude-low frequency Ca^{2+} waves [12], exactly the types of events which were lost in mesenteric arteries from mice with an endothelial specific knockout of SK_{Ca} channels [114], suggests that SK_{Ca} channels are required for their development. As mentioned above, deletion of SK_{Ca} channels impaired NO-mediated dilation to acetylcholine [32] and together, these findings support the notion that their role in protraction of Ca^{2+} events may be important in stimulation NOS.

2.3. Myoendothelial feedback

The sympathetic nervous system regulates total peripheral resistance and is a key modulator of resistance artery diameter through release of noradrenaline and co-transmitters such as ATP and neuropeptide Y [115]. Noradrenaline causes vasoconstriction through activating α_1-adrenoceptors on vascular smooth muscle cells, a process which is limited by engagement of

endothelial mechanisms through myoendothelial feedback. The current model of myoendothelial feedback involves flux of $InsP_3$ from smooth muscle to endothelial cells to elicit localized increases in Ca^{2+}, activation of IK_{Ca} channels and possibly NOS, to limit smooth muscle contractility [11, 91, 116]. This model is supported by ultrastructural and histochemical studies showing that in rat mesenteric and basilar, and hamster retractor feed arteries, MEGJ connexins and IK_{Ca} channels are in close spatial association with ER and $InsP_3$ receptors within endothelial projections that extend through the internal elastic lamina to make contact with smooth muscle cells [11, 55, 91, 94]. In hamster retractor feed arteries, myoendothelial feedback is fully accounted for by EDH. The α_1-adrenoceptor agonist phenylephrine induced localized, $InsP_3$-mediated Ca^{2+} signaling events within endothelial projections and block of endothelial IK_{Ca} channels enhanced smooth muscle depolarization and vasoconstriction [11]. In rat basilar arteries in which NO makes a major contribution to myoendothelial feedback, smooth muscle depolarization to 5-HT was accompanied by IK_{Ca} channel-mediated endothelial hyperpolarization. Inhibition of IK_{Ca} channels, gap junctional communication, TRPC3 or NOS potentiated smooth muscle depolarization to 5-HT in a non-additive manner indicating that rather being distinct pathways, NO and endothelial IK_{Ca} channel activity are part of an integrated mechanism for the regulation of agonist-induced vasoconstriction [91]. In the latter study, Ca^{2+} signaling was not investigated and the link between IK_{Ca} channel activity and NO production was not defined. However, NOS has now been localized close to MEGJs [117] and in co-cultures stimulation of smooth muscle cells with phenylephrine leads to MEGJ specific NOS phosphorylation within endothelial cells to increase NO [118]. Also, in mouse mesenteric vessels, phenylephrine stimulated endothelial TRPV4 sparklets in an $InsP_3$-dependent manner, to engage SK_{Ca} and IK_{Ca} channels as well as, to a lesser extent, NOS [17]. Thus, given the ability of IK_{Ca} channels to modulate endothelial Ca^{2+} dynamics [12, 113, 114], it may be proposed that activation of IK_{Ca} channels at MEGJs following stimulation of smooth muscle cells by GPCR agonists, may amplify dynamic Ca^{2+} signals to enhance NO production.

3. Local versus conducted responses

The majority of studies described in this chapter have been conducted on isolated resistance arteries which in in vivo would be part of branching network of resistance vessels supplied by feed arteries in which effective control of blood flow requires coordinated behaviour amongst arterial segments [119]. As described above, diffusible mediators such as NO act locally to increase arterial diameter. In contrast, K_{Ca} channel-mediated hyperpolarization leads to both local dilation and conduction of the response through the endothelium for distances of several millimeters. This conduction allows for coordination of changes in arterial diameter in multiple vessel segments and so optimizes blood flow [4, 119, 120]. That is not to say that diffusible mediators do not play a role in global blood flow regulation within vascular beds. A recent study of the vascular bed of the mouse gluteus maximus muscle revealed that NO and EDH provide complementary endothelial pathways for ascending vasodilatation to optimize oxygen delivery to the muscle. EDH of downstream arterioles conducts along the endothelium into proximal feed arteries to cause dilation, and NO is released in response to luminal shear stress which increases secondary to downstream dilatation [120].

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4. Conclusion

It has become apparent over the past 15 years that endothelial Ca^{2+} signaling patterns underlie the engagement of effectors such as NOS and/or K_{Ca} channels. The physiological significance of these stimulus-specific signaling pathways is not just that they determine the mediator of vasodilation, but also the scope of the impact of each stimulus on blood flow. Stimuli which predominantly elicit release of diffusible mediators will elicit local vasodilation whereas those that initiate EDH have the potential to dilate multiple arterial segments and so affect tissue perfusion. Further work is required to determine if the patterns of Ca^{2+} signaling described here have widespread applicability, and how they are impacted by age, sex and cardiovascular risk factors. Investigation of how changes in the components of signaling microdomains contribute to the etiology of endothelial dysfunction in conditions such as diabetes and hypertension may lead to the identification of new therapeutic targets.

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Conflict of interest

The authors have declared no conflict of interest.

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Chapter 4

Mineralocorticoid Receptor in Calcium Handling of Vascular Smooth Muscle Cells

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Abstract

For decades, the mineralocorticoid receptor (MR) antagonists have been used for the management of cardiovascular diseases; however, the molecular mechanisms involved in their beneficial effects are not fully understood. Recent publications point to the fundamental role of aldosterone and vascular MR in the regulation of arterial tone, vascular contractility, and cell proliferation. However, the intricate transduction machinery activated by vascular MRs has begun to be revealed with the help of transgenic rodent models and novel transcriptional analysis approaches. Specifically, in this chapter, we review and discuss the most recent contributions about the fine-tuning that the MR exerts on the expression and function of ion channels that participate in calcium handling of vascular cells and the therapeutic implications for hypertension and cardiovascular diseases.

Keywords: calcium channels, calcium handling, vascular smooth muscle, aldosterone, mineralocorticoid receptor

1. Introduction

Recent research efforts on the mineralocorticoid receptor (MR) signaling have revealed a cluster of new pathophysiological mechanism mediated by vascular MR in which aldosterone (Aldo) plays a pivotal role; however, the molecular pathways are not completely elucidated. In this chapter, we review and discuss novel contributions about the structure, ligand activation, and additional mechanisms that confer selectivity for Aldo of the MR in vascular tissues. In addition, we review the fine-tuning that the MR exerts on the expression and function of

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ion channels that participate in Ca^{2+} handling of vascular smooth muscle cells and the therapeutic implications for hypertension and cardiovascular diseases.

In 1972, Crabbé demonstrated that Aldo was interacting with cytoplasmic receptors and that the steroid-protein complex acted as activator triggering the synthesis of mRNA and proteins [1]; thus, Aldo was the first identified mammalian steroid hormone that exerted transcriptional actions via some kind of cytoplasmic/nuclear receptors [2]. Several classes of mineralocorticoid receptors were identified in both epithelial and surprisingly in non-epithelial tissues such as cardiomyocytes, endothelial cells (ECs), and vascular smooth muscle cells (VSMCs) [2], auguring the future actions of Aldo in the cardiovascular system.

2. Mineralocorticoid receptor structure and expression in vascular tissues

The MR is a ligand-activated transcription factor that belongs to the nuclear receptor superfamily [3]. MR was originated by a process of gene duplication from a common ancestor that diverged into the glucocorticoid receptor (GR) and the MR [4]. In 1987 the group of Arriza et al. cloned the MR from human placenta [5]. The human MR (hMR) is coded by a unique gene (*Nr3C2*, due to its belonging to the subfamily 3 of nuclear receptors, group C, member 2) located in chromosome 4, locus q31.1, and with a length of about 75Kb. The gene encodes a polypeptide chain of 984 aa (~107 KDa) [5]. The orthologous gene in rat encodes for a protein of 981 aa (**Figure 1**) and share an identity of 90.1% with the hMR. *Nr3C2* contains 10 exons; the first 2 of them (1α and 1β) comprise the 5′-noncoding sequences, whereas the following exons (2–9) are harboring the functional domains of the protein. It has been reported that at

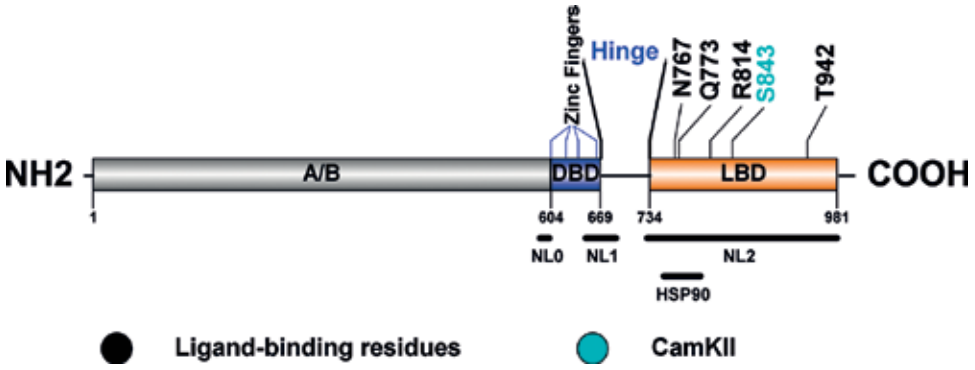

Figure 1. Mineralocorticoid receptor structure. Linear representation of rat MR sequence with respective protein domains. The MR contains an N-terminal transactivation domain of variable lengths (A/B) and a DNA binding domain (DBD) with two zinc fingers involved in the recognition of specific DNA sequences within the promoters of target genes and named hormone response elements (HRE); a flexible hinge is connecting the DBD to the ligand-binding domain (LBD) in the C-terminal region. The residues N767, Q773, R814, and T942 are part of the ligand-binding pocket. The MR also contains three nuclear localization signals (NL0, NL1, and NL2) and multiple phosphorylation sites, and between them is Ser843 that is a target of calcium/calmodulin-dependent protein kinase type II (CamKII).

least three variants of MR mRNA (α, β, and γ) are encoded in a tissue-specific manner under the control of different gene promoters [6].

The MR receptor has the same protein structure as other members of the nuclear receptor superfamily. The MR is composed of an A/B domain (1–604 aa) with a transactivation function and several serine and threonine phosphorylation sites [7] and a DNA binding domain (DBD, 604–699 aa) with two zinc finger motifs that recognize DNA-specific sequences named hormone response elements (HRE), normally found in the promoters of target genes [8]. After the DBD, the hinge D region (670–733 aa) is found and, finally, the C-terminal region (734–981 aa) that is harboring the ligand-binding domain (LBD) with a pocket (which comprises Asp767, Gln773, Arg814, and Thr942) involved in the recognition of agonist and antagonist [9] (**Figure 1**).

2.1. Mineralocorticoid receptor expression in different tissues

The direct and specific actions of Aldo require MR expression in target tissues. For a long time, it was thought that MR was expressed exclusively in kidney epithelial cells and that Aldo was secreted only by the adrenal gland. However, a cumulative evidence has showed the presence of MR in non-epithelial tissues, such as the colon, salivary glands, trachea, heart [10], adipocytes [11], brain [5], skeletal muscle [12], leucocytes, macrophages [13], and vessels [14–19] (**Table 1**).

Tissue or cell type	Detection method	Reference
Kidney	NB	[5]
Gut (gastrointestinal tract)	NB	[20]
Brain	NB	[5]
Hippocampus	NB	[5]
Heart	Im	[10]
Skeletal muscle	WB	[12]
Endothelial cells	RT-PCR	[17]
Vascular tissues		
Aorta	Im, WB, [^{3}H]Aldo bindings	[10, 16, 19, 21, 22]
Carotid artery	Im	[10]
Cerebral artery	WB	[18]
Coronary artery	RT-PCR, Im, WB	[10, 16, 19]
Humeral artery	Im	[10]
Mesenteric artery	Im, RT-PCR, WB	[10, 15, 19]
Pulmonary artery	Im, NB	[10, 14]
Renal artery	Im	[10]
Saphenous vein	RT-PCR, WB, WB	[23]

Tissue or cell type	Detection method	Reference
Umbilical vein		[23]
Adipocytes	Im	[11]
Macrophages	PCR, Im	[13]
Lymphocytes	PCR	[13]

Im, immunohistochemistry; NB, Northern blot; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; WB, Western blot technique.

Table 1. Cell types and tissues expressing MR.

Specifically in blood vessels, the group of Marc Lombès showed for the first time the expression of MR in ECs and SMCs of the aorta and pulmonary artery by immunostainings and [^{3}H]Aldo binding [10]. Although with a low level of immunostaining signal, the MR was found in small arteries such as the carotid, humeral, mesenteric, coronary, and renal arteries. Interestingly, neither immunostaining nor [^{3}H]Aldo binding allowed the detection of MR in the vena cava and portal vein [10]. Hatakeyama et al. detected MR mRNA in primary cultures of ECs and SMCs from human pulmonary arteries [14]. Later, Takeda et al. demonstrated the presence of MR mRNA in mesenteric arteries of stroke-prone spontaneously hypertensive rats (SHRSP) [15]. Using RT-PCR the MR mRNA was detected in a human aorta [21]. Jaffe and Mendelsohn also demonstrated that MR was expressed in VSMCs from aorta and heart vasculature, specifically in coronary arteries [16]. In the case of cerebral arteries (CA), MR expression has been showed by Western blots, where MR levels were higher in CA from females than males [18]. All of these data established the foundation for understanding MR action in vascular tissues. Nowadays we know that MR is indeed expressed in the cardiovascular system supporting its direct role in vascular pathophysiology.

3. Mineralocorticoid receptor: mechanism of activation and regulation in vascular tissues

In basal conditions, the MR is located at the cytoplasm forming a complex with heat-shock proteins, Hsp90 and Hsp70, that stabilize its structure in a conformation where the ligand-binding site is ready to interact with the hormone [24, 25]. Once the ligand is bound, the MR is subjected to a series of conformational changes (**Figure 2**). The interaction between N-terminal and C-terminal domains of MR induces the dissociation of heat-shock proteins and corepressors, allowing MR translocation to the nucleus. However, it has been reported also that Hsp90 follows MR into the nucleus [26]. Inside the nucleus the MR dimerizes, binds to HRE, and recruits a co-regulator complex to induce the transcription of target genes [27, 28]. Interestingly, in some animal models of kidney and cardiac injury that showed normal plasma Aldo concentrations, the MR is activated through a ligand-independent pathway, involving a direct interaction between a small GTPase, Ras-related C3 botulinum toxin substrate 1 (Rac1) and the MR [29], though evidence of this pathway is still lacking in vessels.

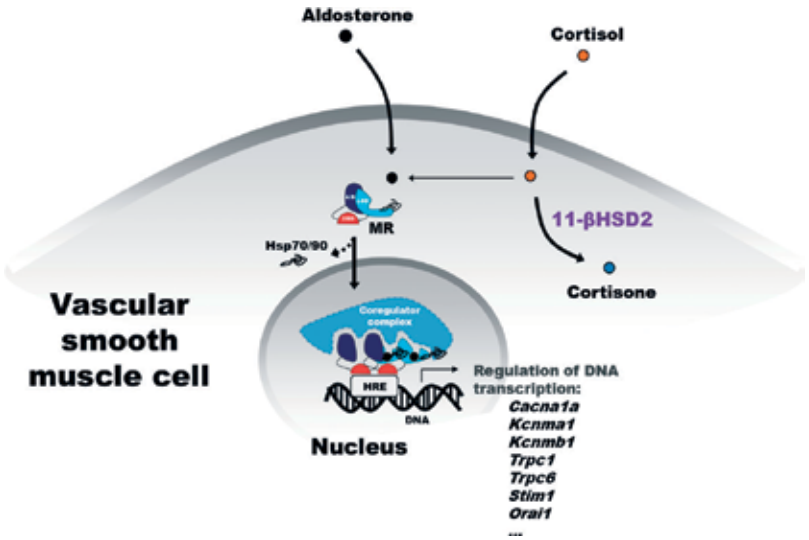

Figure 2. Schematic representation of the MR activation mechanism by Aldo in vascular smooth muscle cells. Once MR binds Aldo in the cytoplasm, it is subjected to conformational changes that allow the dissociation of heat-shock proteins (Hsp70/90), the unmasking of nuclear localization signal, and finally the MR translocation to the nucleus where the MR dimerizes (homodimer complex) and binds to hormone response elements (HRE). The MR dimer recruits a co-regulator complex for regulating the transcription of target genes. Both Aldo and cortisol bind to MR with similar affinity. The mechanism that confers MR selectivity for Aldo depends on the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11 βHSD2), which converts cortisol to cortisone, the latter has a low affinity for MR. Some of the MR target genes in SMC from different vascular beds are listed [19, 30–32]. Abbreviations: *Cacna1a*, VDCC subunit alpha1 A; *Kcnma1*, BK_{Ca} channel (alpha subunit); *Kcnmb1*, BK_{Ca} channel (beta subunit 1); *Trpc1*, transient receptor potential cation channel subfamily c member 1; *Trpc6*, transient receptor potential cation channel subfamily c member 6; *Stim1*, stromal interaction molecule 1; *Orai1*, ORAI calcium release-activated calcium modulator 1.

The high homology between the LBD structure of MR and GR receptors helps to explain why the MR binds cortisol and corticosterone and glucocorticoids with similar affinity with Aldo [33]. Cortisol and Aldo bind to hMR with similar affinity [5, 9]. Because the serum levels of cortisol are higher (from 100 to 1000 times more) than Aldo, then it is expected that the occupancy of MR by cortisol predominates. However, a mechanism that confers MR selectivity for Aldo depends on the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), which converts cortisol to cortisone, the latter has a low affinity for MR [34]. Several papers have demonstrated the co-expression of MR with a functional 11βHSD2 in different vascular beds including mesenteric [15] and coronary arteries [16, 35], though with an indirect determination in CA [36]. These studies support that vascular tissues are Aldo-specific targets and that the action of 11βHSD2 is the physiological mechanism that excludes the activation of MR by cortisol. Furthermore, these data support that the MR-Ado complex has greater stability and higher transcriptional efficiency than the MR-cortisol complex [9].

3.1. Importation of MR into the nucleus

Immunostainings of MR in SMC from coronary arteries have showed the presence of MR in both cytoplasm and nucleus, even in the absence of a ligand. After exposure to Aldo, MR

is located mainly in the nucleus [16]. Like other nuclear receptors, the MR is a protein of a considerable molecular mass (~107 KDa) that exceeds the calculated size for its passive diffusion through the nucleus; thus, it requires specific signals for its nuclear transport. Specifically the importation of MR into the nucleus is controlled through three nuclear localization signals (NLS): the first NLS (NL0) is a serine-/threonine-rich sequence located at the N-terminal region, the second is a NLS (NL1) located at the DBD, and the third is a NLS (NL2) within the LBD. The presence of several NLS in different regions of the MR structure suggests a redundant mechanism to assure its mobilization toward the nucleus as part of the essential mechanism of its transcriptional activity [37].

3.2. MR posttranslational modifications

The MR is subjected to several posttranslational modifications (PTM) such as phosphorylation, ubiquitination, sumoylation, and acetylation that regulate its localization, activity, and stability. Phosphorylation is the most common PTM since the MR contains more than 30 (putative and experimentally assessed) phosphorylation sites that allow to consider it as a phosphoprotein (**Figure 2**). The multiple phosphorylation sites of the MR generate a double band in SDS-PAGE due to a shift of approximately 30 kDa in its apparent molecular mass. The physiological function of all these phosphorylation sites is still under study or completely unknown as in the case of vascular MR. A report has shown that the phosphorylation of Ser843 (in the LBD) prevents MR ligand binding and activation [38]. In contrast, Amazit et al. reported an increase in the MR phosphorylation after Aldo binding, suggesting a ligand-dependent process [39]. Walther et al. reported that phosphorylation of residues inside the NL0 might modulate the MR transport into the nucleus [37]. Finally, Faresse et al. found that the MR is monoubiquitinylated at its basal state and that the Aldo-stimulated MR phosphorylation induces its polyubiquitinylation, destabilization, and degradation [40]. The physiological role of MR-PTM has not been determined in VSMCs.

3.3. Genomic effects of aldosterone in vessels

Aldo exerts its effects in vascular tissues via non-genomic (which are not subjects of this chapter) and genomic MR-dependent pathways. In the case of MR-dependent genomic actions in VSMCs, Jaffe and Mendelsohn investigated the MR-mediated gene transcription activity in SMC of human coronary arteries (HCSMCs) by microarray and quantitative RT-PCR assays. These researchers showed that Aldo modulated the expression of VSMC genes that contribute to vascular inflammation and fibrosis. Additionally, by using a MR response element (MRE) reporter driving the expression of the luciferase gene, they demonstrated that Aldo activated MR in HCSMCs. The Aldo transcriptional effects were regulated in a dose-dependent manner, starting at 1 nM, which is consistent with the Kd for Aldo-MR interaction of ~1–2 nM [16]. Similarly, Newfell et al. evaluated the gene expression profile in Aldo-treated aorta ex vivo, identifying 72 genes that were regulated by Aldo, some of them in a concentration-dependent fashion (1, 10, 30, and 100 nM). Between the Aldo-regulated genes, several of them are involved in oxidative stress, nitric oxide (NO) signaling, vascular proliferation, and fibrosis. Moreover, it has been showed that the MR transcriptional activity is blunted by MR antagonists, and

actinomycin D (a transcriptional inhibitor) supporting an MR-dependent effect of Aldo in vessels [16, 19, 30, 41]. Furthermore, an increasing body of evidence has underlined the ability of MR to modulate the expression of ion channels in several vascular beds, unveiling the role of MR in vascular physiology and pathology [19, 30, 42, 43].

4. Pathological role of vascular mineralocorticoid receptor in blood vessels

It has been confirmed that MR presents extrarenal actions [42, 44, 45] as Na^+ handling alone cannot fully explain the development of hypertension and associated cardiovascular mortality; but these actions are still poorly understood. In fact, there are multiple clinical studies in which mineralocorticoid receptor antagonists (MRA) reduce the incidence of heart attacks and cardiovascular mortality [46, 47].

4.1. Role of vascular MR in oxidative stress

In vitro and in vivo data suggest a vascular MR activation in stimulating oxidative stress, inhibiting vascular relaxation, and contributing to vessel inflammation, fibrosis, and remodeling. MR activation may promote vascular aging and atherosclerosis contributing to the pathophysiology of heart attack, stroke, and possibly hypertension [42]. The balance between damaging reactive oxygen species (ROS) and protective NO determinates the vascular oxidative stress. ROS interact with NO decreasing the NO bioavailability. In vivo experiments in rats support that activation of MR signaling contributes to the vascular dysfunction induced by βAR overstimulation associated with endothelial NO synthase uncoupling reducing NO production and bioavailability [48]. Meanwhile, in the presence of endothelial dysfunction, vascular injury, or high vascular oxidative stress (for instance, in patients with cardiovascular risk factors), ROS production increases via VSMC-MR-mediated activation of NADPH oxidase (a ROS generator) [23, 49, 50] promoting impaired EC-dependent vasorelaxation and consequently increasing vasoconstriction and blood pressure (BP) [51].

4.2. Role of MR in vascular remodeling

Vessel injury induces a pathological response termed vascular remodeling which contributes to human ischemic vascular disease. Adverse vascular remodeling limits vessel lumen diameter and increases vascular stiffness associated with fibrosis, thereby contributing to organ ischemia and hypertension. MR activation contributes to vascular remodeling by acting synergistically with endothelial damage, angiotensin II (Ang II), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) [52–55]. These processes involve both genomic (upregulation of genes involved in cell migration, proliferation, and matrix modulation) and non-genomic mechanisms (via MAPK and the c-Src/Rho) [56]. In the pulmonary artery, MR activation induced the proliferation of VSMC, an effect prevented by spironolactone [57]. Moreover, in a VSMC-MR knockout mouse model, carotid injury-induced and

aldosterone-enhanced vascular fibroses were attenuated; thus, VSMC-MR is necessary for aldosterone-induced vascular remodeling [58]. In aged VSMC-MR-deficient mice (18-month-old), a decrease in aortic collagen content was found [42], suggesting that VSMC-MR play a role in vascular fibrosis. Unlike in an aldosterone/salt hypertension model, the specific VSMC-MR inactivation also leads to the attenuation of arterial stiffening preventing the cell-matrix attachment proteins but without significant modification in vascular collagen/elastin ratio [22]. Other studies support that, after injury, aldosterone-infused animal developed vascular remodeling and MR antagonist reversed those effects [59–61]. Pharmacological inhibition of MR has also demonstrated beneficial effects such as increased lumen and outer diameters of the middle cerebral artery of spontaneously hypertensive stroke-prone rats [62]. Moreover, in a clinical study, treatment with the MR antagonist eplerenone improves the degree of arterial stiffness in hypertensive patients [63]. In conclusion, these studies support that VSMC-MR plays a direct role in vascular remodeling.

4.3. Participation of MR in vascular inflammation

MR plays a key role in the pathogenesis of vascular disease including atherosclerosis and hypertensive vasculopathy, where the role of inflammation has been studied in the last years. In patients with atherosclerosis, high levels of aldosterone in serum predict a substantial increase in subsequent myocardial infarction or death. In vitro studies with human VSMC and ECs have shown that MR activation directly promotes the expression of inflammatory genes [64]. Interestingly, in an in vivo model, spironolactone reduced the number of inflammatory cells in the grafted vein without changing total SMC content, suggesting that MR signaling may contribute to graft remodeling through inflammatory processes rather than SMC hypertrophy [65]. Moreover, in experimental models of atherosclerosis, it was confirmed that the plaque progression was enhanced by Aldo and prevented by MR antagonists [66–68]. The pro-atherogenic genes (*CTGF*, *MT1*, and *PGF*) are also vascular MR-regulated genes [41]. MR signaling also contributes to vascular inflammation in animal models of hypertension. In experimental models of hypertension, MR inhibition reduced the vascular inflammation even without changes in BP, supporting that MR activation participates in vascular inflammation and damage through a BP-independent process [69]. Vascular calcification is a late stage found in atherosclerosis, particularly in the elderly and in patients with renal failure [70]. In VSMCs from human coronary artery, MR activation by Aldo upregulated the expression of genes implicated in vascular calcification, including bone morphogenetic protein 2 (*BMP-2*), alkaline phosphatase (*ALP*), and osteoprotegerin [16]. Also, in an in vitro model, MR activation by Aldo or cortisol stimulated vascular *ALP* [71]. MR is also involved in vascular calcification by regulating the expression of the phosphate transporter *Pit1*, which has an osteogenic function in the smooth muscle ameliorated by spironolactone [72]. However, in a different in vitro study using VSMC isolated from the aorta, it was showed that pro-calcification effects of corticosterone and 11-DHC are mediated directly by MR, but the expression of *Osterix*, *BMP-2*, and *Pit-1* was unaltered [73]. These in vitro studies support that MR is involved in the late stage of atherosclerosis: vascular calcification. All these studies support that vascular MR activation participates in the inflammatory response and contributes to the complications associated with atherosclerotic vascular disease.

4.4. Vascular MR and hypertension

Hypertension represents an aging-associated cardiovascular risk factor. It is known that renal MR regulates the BP and the MR has been considered an antihypertensive target for decades. The association between high levels of Aldo and hypertension was proposed when some forms of hypertension were found associated to primary hyperaldosteronism; also, the positive correlation of high levels of Aldo with high MR expression and hypertension has pointed out to a key role of MR in the establishment of the hypertensive phenotype. Moreover, about 50 years ago, the MR antagonist spironolactone decreased BP in hypertensive patients [74] and in patients with other types of hypertension [75]. The antihypertensive effects of MRA have been analyzed in clinical trials demonstrating a BP reduction in hypertensive patients with primary aldosteronism [76–78] with no changes in plasma K^+ concentration, a marker of renal MR activation [44, 79]. The meta-analysis by Dahal et al. showed that spironolactone reduced systolic BP and this effect was not associated with an increased risk of hyperkalemia compared to placebo [80]. Antihypertensive effects of MRA were analyzed also in resistant hypertension that affects at least 10–15% of all patients. Several studies support that low-dose spironolactone provides significant additive BP reduction in subjects with resistant hypertension [75, 81–85]. PATHWAY-2 was the first randomized and controlled trial to compare spironolactone with other BP-lowering drugs in a well-characterized population of patients with resistant hypertension. In this study it was demonstrated that MRA reduced systolic BP with no hyperkalemia risk [86]. In addition, a role of MR in pulmonary hypertension has been recently identified. MRA treatment initiated at the time of the pulmonary arterial hypertension stimulus prevents the pulmonary vascular hyperplasia and reduces systemic BP [57, 87]. Thus, MR activation may be equally important in patients with and without an established diagnosis of primary aldosteronism. The pathogenesis of MR-associated hypertension in the presence of physiological levels of Aldo in plasma might be mediated by MR activation by other pathways, for instance, MR overexpression, sensitivity, and/or overstimulation by other factors.

The effect of MR blockade in the development of hypertension has been also assessed in experimental models. In the Dahl salt-sensitive model, MRA attenuated the progressive rise in systolic BP in rats fed with a high-salt diet [88, 89]. Whole body disruption of MR in mice results in neonatal lethality from dehydration by renal Na^+ and water loss; thus, transgenic mouse models allowing cell-specific targeting of MR expression have been used to understand the role of MR in vascular tissues and its potential implication in BP regulation. The participation of vascular MR in BP regulation has been analyzed by using two different mouse models: a tamoxifen-inducible VSMC-specific MR inactivation model (via the smooth muscle actin promoter [42]) and a constitutive model of VSMC-specific MR inactivation (via the SM22 promoter; [22]). No transgenic models with targeted MR overexpression in the VSMCs have been reported yet.

The genetic inactivation of MR in adult (2 months of age) mice prevented the increase in BP induced by aging. SMC-MR-deficient mice developed reduced spontaneous myogenic tone. However, the vascular structure and stiffness of resistance arteries from aged SMC-MR-deficient were similar to those from control mice, supporting the notion that SMC-MR contributes to vascular tone and BP regulation independently of structural changes in the

vasculature [42]. The constitutive model of VSMC-specific MR inactivation reported a similar basal BP decrease in 5-month-old MR-KO mice [22]. The BP phenotype in both inactivated VSMC-MR model mice is independent of Na^+ intake and renal MR function supporting a role for VSMC-MR in BP regulation. Interestingly, tamoxifen-inducible VSMC-MR inactivation prevented the in vivo increase in BP induced by Ang II infusion but not by aldosterone-salt challenge [22, 42]. Inactivation of VSMC-MR was also shown to decrease the contractile response to KCl and extracellular Ca^{2+} [62]. The role of the vascular MR could also depend on the vascular bed that is considered. In the future, the use of transgenic models will allow us to decipher the contribution of endothelial MR and VSMC-MR in the different vascular beds and the possible implication in BP regulation [90].

5. Calcium handling proteins are targets of vascular MR receptors

5.1. L-Type Ca^{2+} channel

Recognized as a universal second messenger in various cellular processes and cell types, Ca^{2+} signal plays a critical role in many cellular processes, including, but not limited to, gene transcription and excitation-contraction (EC) coupling [91, 92]. Although almost all biological responses are mediated by Ca^{2+}-dependent and Ca^{2+}-controlled processes, Ca^{2+} signals need to be finely coordinated and precisely regulated. Ubiquitously expressed in the whole body, $Ca_v1.2$ is the main route of Ca^{2+} entry in VSMCs, essential for vascular EC coupling and control of myogenic tone [93]. As a heteromultimeric channel, L-type Ca^{2+} channel (LTTC) is formed by four associated subunits: $Ca_v1.2\alpha1C$, $Ca_v1.2\beta$, $Ca_v1.2\alpha2\delta$, and $Ca_v1.2\gamma$. Undebatable the main subunit, the pore-forming $Ca_v1.2\alpha1C$ region, has also been the target of drugs with antihypertensive properties [94–96], although its effectiveness has been achieved only in a subset of hypertensive patients [97]. Importantly $Ca_v1.2\alpha1C$ channels are expressed as two distinct tissue-specific transcripts of *Cacna1c* driven by two alternative promoters P1 and P2, encoding, respectively, for a long "cardiac" ($Ca_v1.2$-LNT) and for a short "vascular/brain" ($Ca_v1.2$-SNT) N-terminal region [98]. In VSMCs, LTTC is activated in response to the membrane depolarization, allowing a small fraction of Ca^{2+} influx, which is sufficient to trigger VSMC contraction. Thus, sustained voltage-dependent Ca^{2+} influx through the LTCCs maintains a tonic level of vasoconstriction and provides an excitatory template upon which endogenous vasoactive substances may act to modulate arterial diameter and BP.

Although previous studies have demonstrated that aldosterone modulates VSMC Ca^{2+} currents [99–101], the mechanisms remain to be determined. A landmark study showed that VSMC-specific MR knockout mice (VSMC-MR-KO) are protected against the age-associated rise of BP [42]. Importantly, aged VSMC-MR-KO mice showed decreased myogenic tone and attenuated vascular contraction in mesenteric arteries in response to a LTCC opener. Moreover, mRNA level of $Ca_v1.2\alpha1C$ subunit was dramatically downregulated in aortas from aged VSMC-MR-KO mice, suggesting that MR may regulate VSMC $Ca_v1.2$ expression. However, this phenomenon seems indeed to be an age-dependent effect, since a latter study did not validate, at protein level, the downregulation of $Ca_v1.2$ in aortas from young

VSMC-MR-KO mice [102]. Furthermore, during the aging process, MR expression increases in resistance vessels along with a decline in the microRNA (miR)-155 abundance, suggesting that $Ca_v1.2$ is a downstream target of miR-155 regulation [103].

Adding more pieces to the puzzle, we recently showed in cardiomyocytes that aldosterone regulates $Ca_v1.2$-LNT by recruiting MR onto targeted genomic regions in "cardiac" *Cacna1c* P1-promoter [19]. Importantly, we deciphered that aldosterone, through MR-dependent mechanism, dramatically activates the "cardiac"-specific *Cacna1c* P1-promoter, even in blood vessels, conferring a new molecular signature to $Ca_v1.2\alpha1C$ in this tissue that minimizes Ca^{2+} channel blocker actions, a mechanism that might participate to treatment-resistant hypertension, as recently proposed [86]. These findings were further validated using a hypertensive rat aldosterone-salt model, as previously described [104]. Although our data showed that aldosterone/MR impairs 1,4-dihydropyridine sensitivity in VSMC through alternative splicing of $Ca_v1.2\alpha1C$, further studies are needed to validate whether this mechanism participates in the resistant hypertension.

5.2. Ca^{2+}-activated potassium channels

Ca^{2+}-activated potassium channels (KCa), mainly the large conductance KCa channels (BKCa), have been recognized as another important target of MR in blood vessels [105]. BKCa plays a critical role in limiting arterial contraction by producing VSMC hyperpolarization through transient outward K^+ current in response to increased intracellular Ca^{2+} concentration [106]. However, three subtypes of KCa have been identified in blood vessels and categorized according to their conductance: small (SKCa), intermediate, and BKCa. Small- and intermediate-conductance channels are mainly expressed in the ECs, while BKCa channels are predominately expressed in VSMCs.

Previous studies have shown that increased plasma aldosterone concentration enhances vascular KCa function [105]. Oppositely, it was demonstrated that mice lacking the pore-forming BKCaα subunit led to an elevation of BP resulting from hyperaldosteronism, which was accompanied by decreased serum K^+ levels, as well as increased vascular tone in small arteries [107]. Accordingly, impaired acetylcholine-mediated relaxation in isolated coronary arteries has been shown in mice model with cardiac-specific overexpression of aldosterone synthase (MAS mice) [30]. These findings correlate with decreased mRNA and protein expression of BKCa α and $\beta1$ subunits in the heart and coronary artery of MAS mice. Moreover, in vitro treatment of rat aortic VSMCs with increasing concentrations of aldosterone led to a reduced BKCa subunit expression in a concentration-dependent manner. Thus, these findings suggest that augmented local aldosterone production likely acts in a paracrine fashion way suppressing BKCa expression in the surrounding coronary VSMC, thereby contributing to the impaired endothelium-dependent VSMC relaxation. Intriguingly, despite aged VSMC-MR-KO mice displaying lower BP than age-matched WT mice, no significant changes were observed in aortic mRNA expression and function of BKCa in mesenteric VSMC [42]. Furthermore, aldosterone-treated aorta for 24 h with 10^{-8} M of aldosterone did not modify mRNA expression of BKCa α and $\beta1$ subunits [19]; thus, further studies are needed to clarify the effect of MR activation in the expression and activity of BKCa channels.

As mentioned above, SKCa is predominantly expressed in ECs, where it contributes to endothelium-derived hyperpolarization (EDH) of VSMC resulting in vasorelaxation of resistance arteries [108]. In a previous study, circulating aldosterone level was significantly higher in mice fed with high-fat diet (HFD) compared to lean mice; however, despite the restoration of endothelium-dependent vasodilation, eplerenone treatment further increased plasma aldosterone levels of HFD-fed obese mice [109]. Recently, using similar obese model, plasmatic aldosterone concentration was also augmented in male and female mice, whereas no change was found in endothelial cell-specific MR knockout mice (EC-MR-KO) subjected to HFD [110]. In males, obesity impaired NO-dependent vasodilation of resistance arteries, which was compensated by enhancement of EDH of VSMC along with an increase in mesenteric protein expression of SKCa3, while any change was observed in EC-MR-KO. On the other hand, in females, EDH component of VSMC relaxation was impaired, whereas the expression of SKCa3 remained unchanged in control and EC-MR-KO underwent to HFD [110]. Altogether, these results uncover distinct sex-specific mechanisms driving vascular dysfunction, suggesting personalized therapies to prevent vascular disorders.

5.3. Transient receptor potential channels

In VSMC, Ca^{2+} entry from the extracellular space involves a variety of plasmalemmal Ca^{2+} channels, which also involve the superfamily of transient receptor potential (TRP) channels, such as TRPC (canonical), TRPM (melastatin), TRPV (vanilloid), and TRPP (polycystin) [111]. Widely expressed in visceral and vascular SMC, changes in the expression and activity of these channels are implicated in a variety of physiological and pathophysiological consequences [112]. Although TRPM subfamily contains eight isoforms (TRPM1–8), which exhibit a variety of cation permeability, only TRPM6 and M7 seems to be Ca^{2+} and Mg^{2+} permeable. Interesting, aldosterone (100 nM) transiently upregulates mRNA TRPM7 expression in rat VSMC from 2 to 6 h after the onset of treatment, restoring to control level after 24 h of treatment [113]. However, up to date, no studies have been done to evaluate whether aldosterone modulates the expression of TRPV and TRPP channels.

Originally thought to contribute solely to restoring Ca^{2+} concentration under store depletion, creating a capacitative Ca^{2+} entry, commonly termed as store-operated Ca^{2+} entry (SOCE), the role of SOCE is much more diverse than just refilling Ca^{2+} stores [114] but also contributing to vascular contractility, VSMC proliferation, and differentiation [112]. In addition, Ca^{2+} entry from the extracellular space may also occur through Ca^{2+}-permeable store-independent channels, named as receptor-operated channels (ROC), which their activity depends on second messengers produced by downstream effectors from a vast array of G protein-coupled receptors [114]. TRPC subfamily comprises seven members (TRPC1–TRPC7), with the TRPC2 gene being a pseudogene in humans [114].

Although TRPC1 seems to be the most abundant isoform expressed in rat mesenteric arteries, only the expression of TRPC6 was increased in deoxycorticosterone acetate (DOCA)-salt hypertensive rats [31]. Moreover, A7r5 cells treated with aldosterone (1 μM for 24 h) also displayed increased mRNA and protein levels of TRPC6 [31]. Accordingly, it was shown that coronary rings cultured for 7 days with aldosterone (100 nM), without fetal bovine

serum to preserve the contractile phenotype, displayed higher coronary contractility in both endothelium-denuded and endothelium-intact rings, while co-treatment with spironolactone prevented this effect [115]. Recently, we demonstrated that rat aorta treated with aldosterone (10 nM for 24 h) did not reveal changes in the expression of TRPC1, C3, C4, C5, and C6 [19]. Altogether, these studies suggest a concentration-dependent increase of TRPC channels, since we have previously demonstrated an upregulation of TRPC1, C4, and C5 in cardiomyocytes upon aldosterone concentrations higher than 100 nM [43]. Moreover, one of the features of metabolic syndrome is the elevated plasma aldosterone level [116], which has been associated with increased TRPC1 and TRPC6 expression in coronary arteries compared to lean pigs [32].

Another critical component of SOCE is the protein Orai (comprising Orai1, Orai2, and Orai3), which forms a family of highly Ca^{2+}-selective channels that are regulated by stromal-interacting molecules (STIM1 and STIM2) [111, 112]. In neonatal cardiomyocytes, Orai1 was significantly increased by 100 nM and 1 μM aldosterone treatment, whereas lower concentrations (1 and 10 nM) had no effect [43]. However, Stim1 expression remained unchanged even at the highest concentration tested (1 μM) [43]. Similarly, we recently observed, in blood vessels, that treatment with 10 nM of aldosterone for 24 h does not affect the expression of either Orai1 or Stim1 [19].

6. Perspectives

Hypertension is a substantial public health problem, affecting 25% of the adult population in industrialized societies. This disorder is a major risk factor for many common causes of morbidity and mortality including stroke, myocardial infarction, congestive heart failure, and end-stage renal disease. Thus, substantial effort has been devoted to defining the pathogenesis of BP variation. Aldo, through the activation of MR in tubular epithelial cells, has a well-known function on water balance and BP homeostasis. The renal hemodynamic consequences of excess mineralocorticoids—Na^+ and water retention and K^+ secretion—ultimately lead to hypertension. However, the kidney is no longer regarded as the primary site for mineralocorticoid modulation of BP. MR is consistently expressed in both ECs and VSMCs of blood vessels, and its activation by Aldo at pathological concentrations (10 nM) is associated with several types of vascular dysfunction, including atherosclerosis and hypertension. However, despite the recent understanding about the mechanisms involved in the activation of MR mainly in pathological conditions, further research is still required to determine the physiological role of MR-VSMCs in blood vessels.

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Conflict of interest

The authors declare no conflict of interest.

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Section 2

Calcium Signaling in Prokaryotic Cell Models

Chapter 5

Calcium Signaling in Prokaryotes

Delfina C. Domínguez

Additional information is available at the end of the chapter

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Abstract

Calcium (Ca^{2+}) functions as a universal messenger in eukaryotes and regulates many intracellular processes such as cell division and gene expression. However, the physiological role of Ca^{2+} in prokaryotic cells remains unclear. Indirect evidence suggests that Ca^{2+} is involved in a wide variety of bacterial cellular processes including membrane transport mechanisms (channels, primary and secondary transporters), chemotaxis, cell division and cell differentiation processes such as sporulation and heterocyst formation. In addition, Ca^{2+} signaling has been implicated in various stages of bacterial infections and host-pathogen interactions. The most significant discovery is that similar to eukaryotic cells, bacteria always maintain very low cytosolic free Ca^{2+}, even in the presence of millimolar extracellular Ca^{2+}. Furthermore, Ca^{2+} transients are produced in response to stimuli by several agents. Transport systems, which may be involved in Ca^{2+} homeostasis are present in bacteria but none of these have been examined critically. Ca^{2+}-binding proteins have also been identified, including proteins with EF motifs but their role as intracellular Ca^{2+} targets is elusive. Genomic studies indicate that changes in intracellular Ca^{2+} up and downregulate hundreds of genes and proteins suggesting a physiological role. This chapter presents an overview of the role of Ca^{2+} in prokaryotes summarizing recent developments.

Keywords: Ca^{2+} signaling in bacteria, calcium binding proteins, Ca^{2+} homeostasis in bacteria, prokaryotic Ca^{2+} transporters

1. Introduction

Intracellular free Ca^{2+} serves as a universal messenger in all eukaryotic cells [1–4]. Cells respond to environmental stimuli by transient changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), which are utilized by cells to transmit information. Physiological responses also depend on the speed, magnitude and spatiotemporal patterns of the Ca^{2+} signal [5]. Basal

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levels of free cytosolic calcium are regulated by Ca^{2+}-binding proteins, primary and secondary transporters and cytosolic Ca^{2+} stores preventing calcium phosphate toxicity [1, 3].

Although the role of Ca^{2+} in prokaryotes is still unclear, there is increased evidence favoring a role for ($[Ca^{2+}]_i$) in signal transduction in bacteria. Indirect evidence shows that Ca^{2+} affects several bacterial physiological processes including: chemotaxis, cell differentiation such as spore development and heterocyst formation, membrane transport (channels, primary and secondary transporters), virulence and host pathogen interactions [4, 6–10]. Similar to eukaryotes, bacteria maintain cytosolic free Ca^{2+} within the nM range even in the presence of mM extracellular Ca^{2+} [11–15]. Ca^{2+}-stimulus-response has been documented during environmental stress, toxicants [16–18] carbohydrate metabolites [19, 20], iron acquisition, quinolone signaling and type III secretion, which are secretory systems comprised of proteins found in pathogenic Gram negative bacteria that are used to infect eukaryotic cells [21, 22]), suggesting that Ca^{2+} signals are relevant to microbial physiology. Primary and secondary transporters including channels (Ca^{2+}, K^+, Na^+) have been identified in various genera of bacteria. Data show that the level of similarity with eukaryotic counterparts is striking. For example sodium channels show high degree of conservation but their structure is simpler [23]. The ATPase found in *B. subtilis* is analogous to the typical eukaryotic type IIA family of P-type ion-motive ATPases [24]. However, direct evidence that these transporters regulate the concentration of cytosolic free Ca^{2+} is limited. There is evidence of calcium binding proteins (CaBP) in several genera of bacteria, including proteins with EF-hand domains [25, 26], and other calcium motifs such as β-rolls motif, Greek key motif, repeats in toxin and Big Ca^{2+} domain [27–30] but their functional role needs to be investigated. Proteomic and transcriptomic studies in *E. coli*,

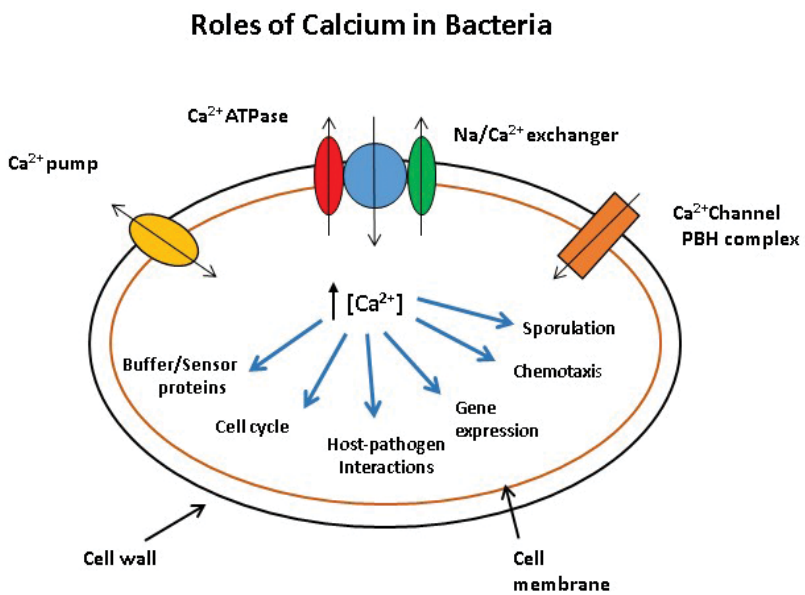

Figure 1. Possible roles of calcium in bacteria.

P. aeruginosa and *B. subtilis* showed that hundreds of genes and proteins are up/downregulated by changes in ($[Ca^{2+}]_i$) but the physiological role needs to be elucidated [20, 21, 23, 31, 32]. **Figure 1** illustrates the potential roles of Ca^{2+} in prokaryotes.

Despite the progress made in recent years, the role of Ca^{2+} in prokaryotes remains intriguing and unclear. Disappointingly many studies have not been followed up and the understanding of the role of Ca^{2+} in prokaryotes lags behind. Questions that need to be answered are: why bacteria maintain a very low cytosolic free Ca^{2+}? Do bacteria utilize the high Ca^{2+} gradient to trigger cell events? What are the molecular mechanisms for Ca^{2+} regulation in bacteria? Does intracellular Ca^{2+} play a role in provoking and regulating cell events? This chapter reviews the work done in this field and will present recent developments.

2. Ca^{2+} homeostasis in bacteria

Initial measurements of $[Ca^{2+}]_i$ in bacteria were a challenge because of the unique physical characteristics of bacterial cells (tiny size, cell walls and membrane), the difficulty in manipulating live cells and the toxicity of reagents [13, 33]. Other concerns included those associated with Ca^{2+} research such as contamination and lack of selectivity of Ca^{2+} chelators [34–36]. With the introduction of molecular technology, the photoprotein aequorin gene was expressed in bacterial cells to measure cytosolic free Ca^{2+} in live cells. In this way, several investigators were able to continuously monitor cytosolic free-Ca^{2+} in several genera of bacteria [12–14]. A crucial discovery was that all bacteria tested maintained very low levels of cytosolic free Ca^{2+}, even in the presence of 1–10 mM extracellular Ca^{2+} (**Figure 2**). Cytosolic free Ca^{2+} in bacterial cells ranges from 100 to 300 nM, very similar values to those observed in eukaryotic cells [11, 13, 14]. These findings suggest that microbial cells must have transport systems (influx and efflux), proteins or other structures that may serve as intracellular free Ca^{2+} targets that may play a role in the maintenance of Ca^{2+} homeostasis.

The role of channels, ATPases and exchangers in Ca^{2+} homeostasis has not been investigated critically and none of these have been experimentally proven to transport Ca^{2+} specifically. The contribution of bacterial CaBP to Ca^{2+} homeostasis remains undetermined [26, 37]. However, recent work shows that the disruption of particular ATPases (PA2435, PA3920), the exchanger (PA2092) and a putative EF-hand protein, is evidence that these transporters are necessary to maintain low intracellular Ca^{2+} levels in *P. aeruginosa* [15, 38]. A proteomic analysis in *B. subtilis* showed that several cytosolic proteins appear to bind Ca^{2+}, as determined by Ca^{2+} autoradiography [32]. Some of these proteins, identified by liquid chromatography/mass spectrometry include: a potential cation transport ATPase, fructose biposhate aldolase, DnaK 70 and adenylate kinase. These proteins were induced when cells were treated with extracellular divalent cation chelator ethylene glycol tetraacetic acid (EGTA) and reduced when treated with high extracellular Ca^{2+}. None of these proteins however had Ca^{2+} binding domains [32]. Notably genes encoding fructose biposhate aldolase, DnaK 70 and adenylate

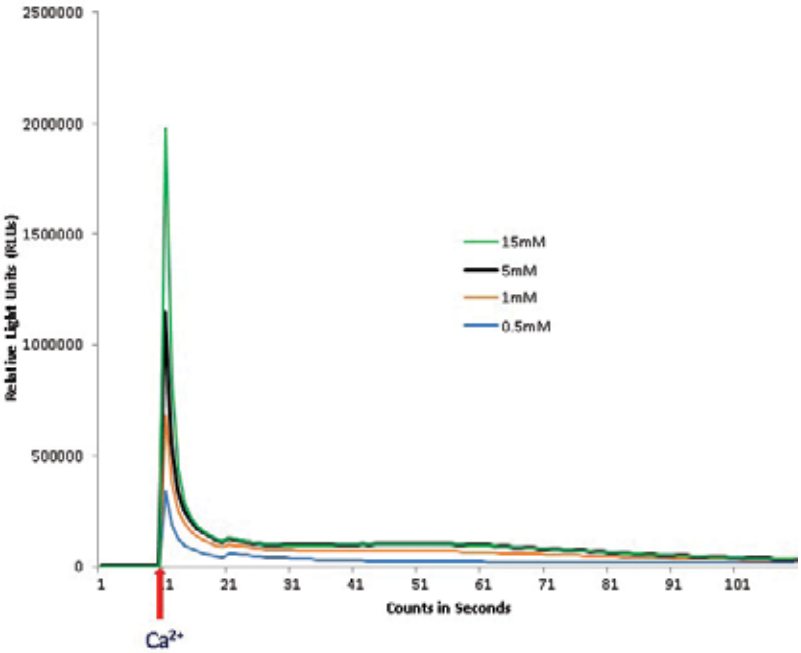

Figure 2. Cytosolic free Ca^{2+} in Bacillus subtilis cells. *B. subtilis* Cells were transformed with a plasmid containing the gene for the photoprotein aequorin. Light emission was recorded in a luminometer after challenging the cells with different $CaCl_2$ concentrations: 0.5, 1, 5, and 15 mM. J Anal Bioanal Tech reproduced with permission.

kinase were found to be modulated by Ca^{2+} in *E. coli* [20]. These findings suggest that perhaps other proteins and anionic protein groups yet to be characterized may be involved in buffering intracellular free Ca^{2+}.

Bacterial cells lack organelles such as endoplasmic reticulum and mitochondria, which function as Ca^{2+} sinks in eukaryotes. However, some bacteria contain membrane-bound vesicles (acidocalcisomes) and polyphosphate granules that accumulate and store Ca^{2+} [39–42]. Other structures that bind Ca^{2+} in significant amounts are DNA and the complex poly-(R)-3 hydroxybutyrate (PHB)-polyphosphate (PP) [43–45]. Moreover, the periplasmic space, which is a region between the inner cytoplasmic membrane and the bacterial outer membrane and that has been found in both Gram negative and Gram positive bacteria [46–48], is another structure that has been reported that may play a role in storing and buffering Ca^{2+} [49]. Intracellular free Ca^{2+} measurements within the periplasmic space in live *E. coli* cells revealed that this structure can store 3–6-fold Ca^{2+} with respect to the external medium [49]. Chang and co-workers [50] also demonstrated high concentrations of Ca^{2+} associated with the cellular envelope in *E. coli* cells as determined by X-ray mapping and electron loss spectroscopy.

Altogether, the aforementioned data suggest that bacterial cells may have different mechanisms to maintain cytosolic Ca^{2+} homeostasis. Further work should be performed to elucidate how and why bacterial cells maintain low levels of intracellular free Ca^{2+}.

3. Influx and efflux transport systems in bacteria

3.1. Influx

The existence of cation (Na^+ and K^+) and anion (Cl^-) channels, ATPases and exchangers have been documented in several genera of bacteria [4, 51]. Despite high resolution structure of some bacterial channels the physiological function reminds unknown [7]. Several bacteria have mechanosensitive ion channels that have large conductances (nanosiemens range) thus it would be expected to allow Ca^{2+} into cells. However, gene knockouts of major mechanosensitive channels in *E. coli* (MscL and MscS) still showed large Ca^{2+} influx [2, 52] and the Ca^{2+}-dependent K^+ channels of the archaea *Methanobacterium thermoautotrophicum* and *Thermoplasma volcanium* are activated at millimolar Ca^{2+} concentrations questioning the physiological relevance since Ca^{2+} signals occur within micromolar range. On the other hand, deletion of the SynCaK, a Ca^{2+}-dependent K^+ channel in cyanobacteria resulted in increased resistance to heavy metals suggesting a physiological role for Ca^{2+}-mediated channels [53].

So far the best evidence of a Ca^{2+} influx channel in bacteria is the nonproteinaceous complex polyhydroxybutyrate-polyphosphate (PHB-PP). The channel is highly selective for Ca^{2+} at a physiological pH [54]. This preference has been attributed to a high density negative charge along the polyphosphate backbone. The complexes are abundant in stationary phase and correlate with high rise in cytosolic Ca^{2+}. These complexes have many characteristics of protein Ca^{2+} channels: voltage-activated, conduct Ca^{2+}, Sr^{2+} and Ba^{2+} and are blocked in a concentration-dependent manner by La^{3+}, Co^{2+} and Cd^{2+} [44, 45, 55]. However, the genes encoding the synthesis of PHB complex remain to be properly identified and characterized. A figure of the putative channel is shown in **Figure 3**.

More recently, Bruni et al. [52] employing a sensor that simultaneously reports voltage and Ca^{2+} showed that Ca^{2+} influx is induced by voltage depolarization in *E. coli*. These exciting findings support the idea that bacteria may sense their environment through voltage-induced Ca^{2+} fluxes, similar to eukaryotic cells.

3.2. Efflux

In most bacteria, Ca^{2+} is apparently exported by Ca^{2+} exchangers, Ca^{2+}/H^+ or Ca^{2+}/Na^+ antiporters. These are low-affinity Ca^{2+} transport systems that use the energy stored in the electrochemical gradient of ions. Ca^{2+} exchangers differ in ion specificity and have been identified in a number of bacterial genera [11, 56]. In *E. coli*, the proteins ChaA, YrbG and PitB were reported as potential Ca^{2+}/H^+ [57, 58], Ca^{2+}/Na^+ antiporters [59] and Ca^{2+}/PO_4^{3+} symporter respectively. Knockout of corresponding genes showed no effect on either Ca^{2+} influx or efflux [19, 20] raising questions about the role of these proteins. Potential redundancy is not ruled out. More recently, the multidrug transporter LmrP from *Bacillus lactis* has a predicted EF-hand motif with a Kd = 7.2 μM and two acidic residues (Asp-235 and Glu-327) binding Ca^{2+}. LmrP was shown to selectively bind Ca^{2+} and Ba^{2+} and mediates selective Ca^{2+} efflux via electrogenic exchange [60]. A predicted transporter PA2092 from *P. aeruginosa* might be involved in Ca^{2+} efflux since intracellular Ca^{2+} accumulates after disruption of the corresponding mutant [15].

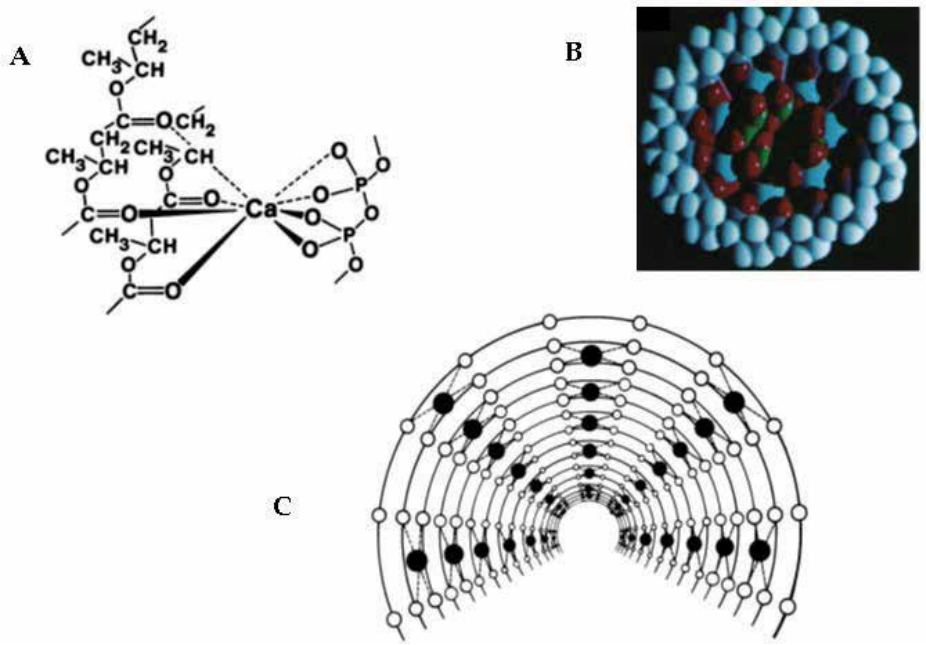

Figure 3. Coordination geometry of Ca^{2+} in the PHB-PP helix. (A) Calcium forms ionic bonds with four phosphoryl oxygens of poly-P and ion-dipole bonds with four ester carbonyl oxygens of poly-hydroxybutyrate (PHB) to form a neutral complex with distorted cubic geometry. (B) Computer model horizontal cross section showing the poly-P helix with the poly (HE) helix with Ca^{2+} surrounded by the oxygen moieties of both polymers. The seven Ca^{2+} displayed are from two turns of the poly-P helix. Light blue, hydrogen; dark blue, carbon; red, oxygen; green, phosphorous; aqua, Ca^{2+}. (C) View down the poly(HB) cylinder. Ca^{2+} (closed circles) bound to carbonyl ester oxygens (open circles) in a pattern that links each turn of the helix alternatively to the proximal turns above and below. Reusch and Sadoff [45]. Courtesy of Reusch RN.

P and F-type Ca^{2+} ATPases have been described in bacteria. ATPases that were purified and shown to translocate or have Ca^{2+}-dependent phosphorylation include:

the P-type ATPase from *Synechocystis sp.* showed vanadate sensitivity, which appears to be homologous to eukaryotic SERCA [61, 62], the F-type ATPase from *Flavobacterium odoratum* also vanadate-sensitive, phosphorylated only in the presence of Ca^{2+} [63] and the *Listeria monocytogenes* ATPase, which has low Ca^{2+} affinity, and it is induced at alkaline pH [64]. The *in vivo* function of these proteins remains to be characterized. Other ATPases that have been identified by bioinformatics include: CaxP from *Streptococcus pneumoniae* [65], YloB from *B. subtilis* [24], PacL from *Synechococcus* sp. [66] and PA2435 and PA3920 from *P. aeruginosa* [15].

Work by Naseem et al. [20] demonstrated that ATP is essential for Ca^{2+} efflux, and there is a possibility that ATP may regulate Ca^{2+} efflux through an ATPase. It was shown that the gene atpD, which encodes a component of an F-type ATPase is required for a normal Ca^{2+} efflux function. Although no specific transporter was shown here, the result is important, indicating that ATP is surely necessary for transport of Ca^{2+} by a still unknown ATPase.

Bacterial transporters have not been studied systematically and knowledge about these proteins is limited. It appears that prokaryotes have multiple transporters with some redundancy.

Besides protecting from toxic effects the question arises is Ca^{2+} transport in bacteria linked to signaling? What is the contribution of these transport systems in Ca^{2+} homeostasis?

4. Bacterial Ca^{2+} binding proteins (CaBP)

If a change in cytosolic free Ca^{2+} is to have any effect on bacterial physiology, bacterial cells must have intracellular Ca^{2+} targets in addition to influx and efflux mechanisms. Identification of such intracellular Ca^{2+} targets remains elusive. Nevertheless, a number of prokaryotic CaBP have been discovered by a combination of approaches: molecular technology and bioinformatics. According to Zhou et al. [26], sequence analyses of prokaryotic genomes showed the presence of 397 putative EF-hand proteins. However, most of these proteins with a few exceptions (Calerythrin from *Saccharopolyspora erythrea*, Calsymin from *Rhizobium etli*, the *Brucella abortus* Asp24, *Streptomyces coelicolor* CabA, CabD and Ccbp from *Anabaena* sp.) are hypothetical proteins [37, 67, 68]. Few proteins have been studied biochemically and none of these have been characterized functionally.

Five classes of EF-hand motifs have been reported in bacteria. The typical helix-loop helix EF-hand structure seen in Calerythrin and Calsymin, the *ex*tracellular *Ca*$^{2+}$-*b*inding *r*egion (Excalibur), which has a shorter loop containing 10 residue motif DxDxDGxxCE found in various bacteria, the longer 15 residue Ca^{2+}-binding loop seen in the *E. coli* lytic transglycosylase B, and the fourth and fifth classes lacking the first or second helix as described in the *C. thermocellum* dockerin and the *Sphingomona* ssp. alginate-binding protein, respectively [25, 26]. **Table 1** presents the five classes of bacterial EF-hand and EF-hand-like motifs proteins with known structures. The presence of the Ca^{2+} binding motifs must be tested for functional necessity or for viability of the organism.

Other Ca^{2+} motifs found in various bacteria include the Ca^{2+}-binding β-roll motif, which includes proteins containing a region referred as repeats-in-toxin (RTX) [27, 69, 70] and a family of proteins with a signature sequence Proline P-Glutamate E Polymorphic GC-rich Repetitive Sequence (PE_PGRS) [71, 72], the Greek key motif present in the βγ-crystallin superfamily containing Ca^{2+}-binding proteins in Eubacteria and Archaea [29, 73–76] and finally the Big domain motif comprising proteins with an immunoglobulin-like domains [30, 77]. Most of these proteins however, are extracellular proteins and some require Ca^{2+} within the μM to mM range to bind compared to eukaryotic cells that have high Ca^{2+} binding affinity within lower μM to nM range. Nevertheless, reports have shown that cytosolic free Ca^{2+} in *E. coli* can increase to tens of micromolar without any loss of viability, suggesting that bacterial Ca^{2+} targets may have lower affinity for Ca^{2+}.

Prokaryotic CaBP encompass a diverse group of proteins that exhibit great structural variety. Binding of Ca^{2+} may provoke folding to a functional state or may lead to protein stabilization. Structural characteristics of these proteins suggest they may act as buffers, may play a structural role and/or may function as sensors/signal transducers. Much more research is needed to characterize biochemically and genetically bacterial Ca^{2+}-binding proteins offering exciting possibilities and a challenge for the future.

Organism	Protein name	Accession number	a.a. number	EF-hand/ EF-hand-like motif	Potential role of Ca^{2+}	Refs.
Saccharopolyspora erythrea	Calerythrin	P06495	177	Helix-loop-helix	Buffer	[4, 24]
Rhizobium etli	Calsymin	Q9F6V9	293	Helix-loop-helix	Transducer	[4, 36]
Thermotoga maritime	4-α-Glucano-transferase	P80099	441	Helix-loop-helix	Unknown	[4, 24]
Escherichia coli	B Slt35	P41052	361	Helix-loop-helix	Structural	[4, 24]
Bacillus anthracis	Protective antigen	P13423	764	Helix-loop-helix	Structural	[4, 23]
Clostridium thermocellum	Dockerin	A3DCJ4	350	Helix-loop-helix	Structural	[4, 23]
Salmonella typhimurium	Periplasmic galactose binding protein	P23905	332	Helix-loop-strand	Structural	[4, 23]
Sphingomonas sp	Periplasmic alginate binding protein	Q9KWT6	526	Helix-loop-loop	Regulatory	[4, 23]
Pseudomonas aeruginosa	Alkaline protease	Q03023	479	Strand-loop-strand	Unknown	[4, 24]
Halothermothrix	α-Amylase A	Q8GPL8	515	Strand-loop-helix	Structural	[4]

Protein accession numbers in UniProtKB database. Reproduced with permission from Elsevier. Dominguez et al. [4].

Table 1. Examples of bacterial proteins containing EF-hand and EF-hand-like motifs with known structure.

5. Ca^{2+} signaling

The hypothesis that Ca^{2+} acts as a messenger in bacterial cells is based on the observation that environmental signals induce changes in the level of cytosolic free Ca^{2+}. Microorganisms must quickly adapt to changes in the environment in order to survive. Therefore, bacteria must have evolved sophisticated regulatory networks to constantly monitor signals that are critical for their continued existence. How bacterial cells sense the external signal has not been determined yet but experimental observations suggest that may occur through different mechanisms including: cytosolic-free Ca^{2+} transients, membrane sensors, two component systems and its regulatory proteins, and Ca^{2+} sensors transducing the signal.

Over the years, evidence of a Ca^{2+}-mediated stimulus response in bacteria has been documented. Since 1977, Ordal reported that cytosolic Ca^{2+} controlled the rotation of the flagella in

B. subtilis cells. Later work corroborated that cytosolic Ca^{2+} transients affect bacterial motility in *E. coli*, possibly through the phosphorylation of the Che proteins [78–80]. The involvement of Ca^{2+} as a signal transducer in a variety of environmental conditions, where cytosolic free Ca^{2+} is elevated as a result of the stimulus, has been shown in various organisms including: oxidative stress in *B. subtilis* [81], heat/cold shock, and salt and osmotic stress in *Anabaena* strain PCC7120 [14, 82], carbohydrate fermentation products in *E. coli* [19], organic solvents, pharmaceuticals and antibiotics in cyanobacteria [16, 17].

Evidence that membrane-bound proteins may be able to transduce Ca^{2+} signal was shown *in vitro* using the chimeric protein Taz1. Under low concentrations of Ca^{2+}, Taz was phosphorylated leading to the activation of porin genes in *E. coli* [83, 84]. No *in vivo* studies have been followed up. A more recent report in *Vibrio cholera*, showed that Ca^{2+} greatly enhances the transmembrane virulence regulator (TcpP) activity by increasing protein-protein interaction in the presence of bile salts, leading to the activation of downstream virulence factors [10].

Two component regulatory systems, consisting of a sensor kinase and a transcriptional activator, are commonly used by bacteria to sense and respond to environmental signals. Several of these systems have been shown to respond to extracellular Ca^{2+}. In the PhoPQ system in *Salmonella typhimurium* and *P. aeruginosa*, PhoQ is a Mg^{2+}, Ca^{2+} sensor that modulates transcription in response to cation levels. The binding of PhoQ to Ca^{2+}, Mg^{2+} or Mn^{2+} keeps the protein in a repressed state inhibiting the transcription of many virulent genes [85, 86]. In *V. cholera*, the *ca*lcium *r*egulated *s*ensor (carS) and regulator (carR) were shown to be decreased when bacterial cells grew in Ca^{2+} supplemented medium. Further analysis demonstrated that expression of vps (*Vibrio* polysaccharide) genes and biofilm formation are negatively regulated by the CarRS two-component regulatory system [87]. In *V. parahemolyticus*, Ca^{2+} influences gene expression for type III secretion systems ($T3SS_1$) and swarming. A transcription factor called CalR was shown to repress T3SS1 and swarming, which in turn were linked to a σ^{54}-dependent regulator [22]. Another two-component system AtoS-AtoC, which mediates the regulation of PHB complexes in *E. coli* is induced by Ca^{2+}. It was shown that the highest accumulation of PHB complexes occurred in AtoS-AtoC expressing *E. coli* cells compared to deletion mutants AtoSC at high Ca^{2+} concentration in cytosolic and membrane fractions [88, 89]. More recently, in *P. aeruginosa*, the two-component regulator PA2656-PA2657 genes were induced by $CaCl_2$. Deletion mutations and transcriptome analysis revealed that this two-component system may be responsible for regulating the expression of periplasmic proteins and affecting Ca^{2+} homeostasis [90].

Bacterial CaBP that may be involved in in signal transduction include CabC, which may be regulating spore germination and aerial hyphae formation in *Streptomyces coelicolor* [91]. The recently reported EfhP from *P. aeruginosa* that is required for Ca^{2+} homeostasis [38] and other two EF-hand proteins from *S. coelicolor* and *S. ambofaciens* whose function remains to be discovered [92, 93].

Despite all the information accumulated over the past few years, Ca^{2+} signaling in bacterial physiology remains to be elucidated. Further work is needed to uncover the specific nature of the Ca^{2+} signal transduction, its components and their specific regulation and function.

6. Ca^{2+} signals during host-pathogen interactions

Pathogenic bacteria have evolved various strategies to successfully colonize and cause infection in their hosts. Intracellular Ca^{2+} mobilization has been implicated as an important signaling event during bacterial adhesion, invasion and intracellular replication during infection [6]. Interestingly, some pathogens induce Ca^{2+} increases while others interfere with the Ca^{2+} signal to promote invasion [9, 94, 95]. However, despite the significant role of Ca^{2+} signaling during pathogenesis, the mechanisms underlying how bacterial cells and their virulent factors manipulate Ca^{2+} mobilization in host cells remains to be elucidated. This section will present some examples of the role of Ca^{2+} in host-pathogen interactions.

Neisseria meningitidis (meningococci) is the causative agent of bacterial meningitis. Pili are one of the major virulent factors of meningococci. Pili are bacterial structures that play an important role in adhesion to host cells. Analyzing the role of Ca^{2+} during *N. meningitides* infection, Asmat et al. [9] found that the meningococcal protein PilC1 triggered a significant increase of cytosolic Ca^{2+} in human brain microvascular endothelial cells (HBMEC), which was critical for adherence and subsequent internalization into host endothelial cells. Use of the Ca^{2+} chelator, BAPTA-AM, significantly reduced PilC1-mediated meningococcal adherence. Mutants deficient in PilC1 were not able to increase cytosolic Ca^{2+} in endothelial host cells. Pretreatment of host cells with the phospholipase inhibitor, U73122, indicated that the Ca^{2+} increase in endothelial cells was mediated by phospholipase C (PLC). Similar findings where Ca^{2+} mediated adherence to host cells occur through pili were reported in *P. aeruginosa* [96] and efficient internalization via PLC was reported in *Campylobacter jejuni* [97] and *Borrelia burgdorferi* [98].

Shigella is another pathogen that utilizes Ca^{2+} signaling during infection of epithelial cells. *Shigella* is the etiologic agent of bacillary dysentery. This pathogen invades the intestinal mucosa producing massive destruction of the colonic epithelium by eliciting a strong inflammatory response [6]. As early as 5 min after bacterial contact with epithelial cells *Shigella* induces local Ca^{2+} signals in the host cell, which remodel the cytoskeleton allowing bacterial entrance to the cells. Global Ca^{2+} signals are involved in later stages of infection promoting slow cell death as a result of plasma membrane permeabilization and increased cytosolic Ca^{2+}. *Shigella* also manipulates the Ca^{2+} signal to interfere with immune responses and inflammation [99]. Global Ca^{2+} signals have also been associated with an induced decrease of sumoylation by *Shigella*. Sumoylation is a posttranslational modification by *S*mall *U*biquitin *Mo*difier (SUMO) proteins, which is an essential regulatory mechanism involved in several processes including protein stability, cell cycle, cell communication and gene expression [100]. At late time of postinfection, *Shigella* induces inhibition of sumoylation through activation of calpain proteases, which degrade SUMO proteins [101]. Inhibition of Ca^{2+} influx or calpain activity prevented shigella-induced loss of sumoylation. On the other hand Ca^{2+} treatment and inomycin resulted in sumoylation inhibition [101]. Knowledge of how pathogens interfere with SUMO enzymatic machinery is limited and remains to be characterized.

Several bacterial pathogens secrete potent virulence factors such as pore-forming toxins. These toxins perforate host cell membranes in order to deliver virulence factors, escape from

phagosmes or disrupt cell-cell junctions (Tran Van Nhieu [6]; Reboud et al. [102]). Interestingly, some pore-forming toxins such as *Listeria* listeriolysin O (LLO) induce Ca^{2+} oscillations as a result of a direct Ca^{2+} influx via the pore-forming toxin. An interesting feature of LLO is that pores open and close in a synchronized fashion leading to long lasting Ca^{2+} oscillating signaling provoking a broad spectrum of cellular responses during infection [6, 103]. Two other pore-forming toxins produced by the opportunistic pathogens *P. aeruginosa* (ExlA) and *Serratia marcescens* (ShlA), which share similar structural and functional aspects, have the capacity to trigger Ca^{2+} influx leading to disruption of cell-cell junctions of epithelial and endothelial cells. This influx of Ca^{2+} activates a metalloproteinase called 10(ADAM 10), which cleaves cadherin inducing cell-cell-junction breakdown and loss of tissue integrity [102].

There is a great diversity of Ca^{2+}-dependent processes that pathogens utilize to cause infection. However, studies on bacterial induced Ca^{2+} signaling are limited. More research is needed in this filed to understand the mechanisms of how bacterial virulence factors regulate second messengers such as Ca^{2+} and Ca^{2+}-dependent events during the infectious processes.

7. Conclusion

The role of Ca^{2+} in bacteria is a fascinating field that still remains unexplored. It is clear that evidence supporting the role of calcium as a regulator in prokaryotes is accumulating. However, the extent and significance remains unclear. A systematic assessment and careful analysis of the processes involving calcium warrants further analysis.

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Conflict of interest

The author has no conflict of interest.

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Section 3

Calcium Signaling and Physiologic Consequences in Chemotherapeutic and Cancer Pathology

Chapter 6

Role of Calcium in Vomiting

Weixia Zhong and Nissar A. Darmani

Additional information is available at the end of the chapter

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Abstract

Cisplatin-like chemotherapeutics cause vomiting via calcium (Ca^{2+})-dependent release of multiple neurotransmitters/mediators (dopamine, serotonin, substance P, prostaglandins and leukotrienes) from the gastrointestinal enterochromaffin cells and/or the brainstem. Intracellular Ca^{2+} signaling is triggered by activation of diverse emetic receptors (including neurokininergic NK_1, serotonergic 5-HT_3, dopaminergic D_2, cholinergic M_1, or histaminergic H_1), whose stimulation in vomit-competent species evokes emesis. Other emetogens such as cisplatin, rotavirus NSP4 protein, and bacterial toxins can also induce intracellular Ca^{2+} elevation. Our findings demonstrate that application of the L-type Ca^{2+} channel (LTCC) agonist FPL 64176 and the intracellular Ca^{2+} mobilizing agent thapsigargin (a sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor) cause vomiting in the least shrew. On the other hand, blockade of LTCCs by corresponding antagonists (nifedipine or amlodipine) not only provide broad-spectrum antiemetic efficacy against diverse agents that specifically activate emetogenic receptors such as 5-HT_3, NK_1, D_2, and M_1 receptors, but can also potentiate the antiemetic efficacy of palonosetron against the nonspecific emetogen, cisplatin. In this review, we will provide an overview of Ca^{2+} involvement in the emetic process; discuss the relationship between Ca^{2+} signaling and the prevailing therapeutics in control of vomiting; highlight the current evidence for Ca^{2+}-signaling blockers/inhibitors in suppressing emetic behavior and also draw attention to the clinical benefits of Ca^{2+}-signaling blockers/inhibitors for the treatment of nausea and vomiting.

Keywords: cisplatin, vomiting, antiemesis, Ca^{2+}, L-type Ca^{2+} channel

1. Introduction

Acute (≤24 h) and delayed (>24 h) phases of chemotherapy-induced nausea and vomiting cause distressing side-effects which affect the well-being and quality of life of cancer patients

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receiving chemotherapy, especially cisplatin [1]. Major neurotransmitter mechanisms underlying chemotherapy-induced nausea and vomiting have been subject of considerable research over the past 45 years. As presented in brief in **Figure 1**, cancer chemotherapeutics such as cisplatin evoke vomiting via local release of a variety of emetic neurotransmitters/mediators (including dopamine, serotonin (5-HT), substance P, prostaglandins and leukotrienes) both from the enterochromaffin cells of the gastrointestinal tract and the brainstem emetic loci in the dorsal vagal complex containing the nucleus tractus solitarius, the dorsal motor nucleus of the vagus and the area postrema [2–4]. The area postrema and the nucleus tractus solitarius contain large numbers of fenestrated capillaries which lack blood-brain barrier and permit neurons in both areas access to blood-borne circulating factors including emetogens [5]. The chemoreceptor trigger zone, in the area postrema has high concentrations of emetic receptors for serotonin (5-HT_3), dopamine ($D_{2/3}$), neurokinin (NK_1), and opioids (μ), among others [2]. Direct stimulation of these receptors in the chemoreceptor trigger zone by emetogens is one important mechanism by which vomiting can occur [6]. The nucleus tractus solitarius receives emesis-related information from the area postrema as well as the gastrointestinal tract conveyed by vagal afferents. The dorsal motor nucleus of the vagus receives axonal projections from nucleus tractus solitarius [7] and sends emetic signals via motor efferent pathways to the gastrointestinal tract and modulates vomiting behaviors [2, 5, 8, 9] (**Figure 1**). In addition, chemotherapeutic drugs may evoke release of emetic neurotransmitters/mediators from the gastrointestinal tract into the blood to be directly delivered to the area postrema via a

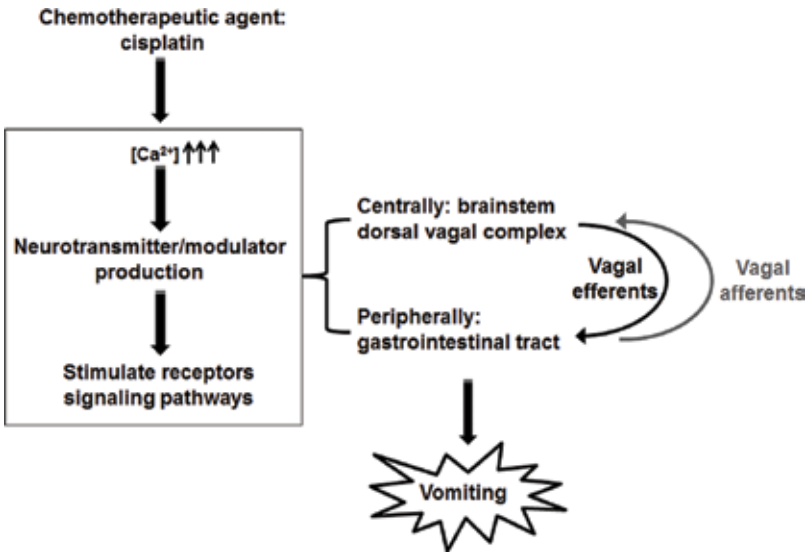

Figure 1. Brief illustration of the mechanisms underlying vomiting induced by chemotherapeutic agent cisplatin. Mechanisms underlying cisplatin-induced vomiting can be simplified as: (1) cisplatin can increase cytoplasmic Ca^{2+} level to evoke Ca^{2+}-dependent release of emetic neurotransmitters/mediators at the brainstem emetic loci, the dorsal vagal complex, and subsequently activates diverse receptors and their corresponding signaling pathways. These emetic signals are output to the gastrointestinal tract via efferents to trigger vomiting [2, 4–9]; (2) cisplatin-induced peripheral release of neurotransmitters/mediators from the gastrointestinal tract into the blood can directly stimulate the dorsal vagal complex, activate receptors signaling pathways and trigger vomiting [2, 10]; and (3) the peripherally-released emetic neurotransmitters/mediators stimulate their corresponding receptors present on vagal afferents in the gastrointestinal tract which indirectly activate brainstem emetic nuclei and trigger vomiting [6].

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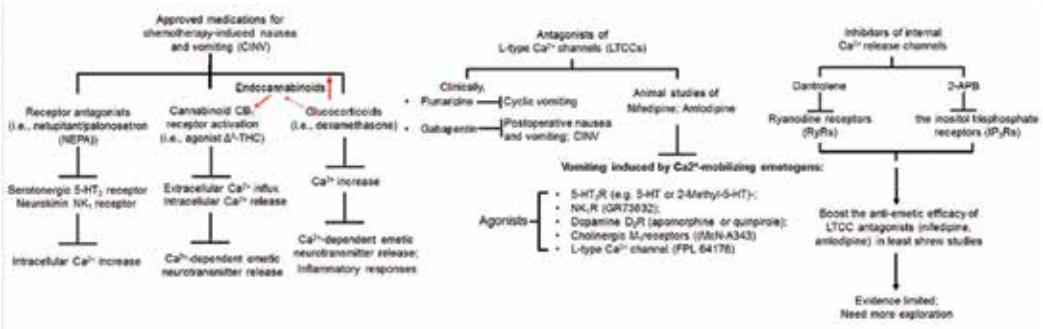

Figure 2. Overview of evidence for suppression of Ca^{2+} signaling involved in anti-vomiting actions of antiemetic agents. (1) Netupitant and palonosetron are highly selective respective antagonists of NK_1Rs and $5\text{-}HT_3Rs$ are approved to treat the acute- and delayed- phases of chemotherapy-induced nausea and vomiting (CINV) in cancer patients [79–82]. Our studies [83–86] indicate that suppression of Ca^{2+} signaling is involved in antiemetic efficacy of both palonosetron and netupitant. (2) Cannabinoids such as delta-9-tetrahydrocannabinol exert their antiemetic efficacy via direct activation of CB_1 receptors (CB_1R) [92, 94, 98–100]. The ability of CB_1R agonists to suppress both extracellular Ca^{2+} influx [111–115] and intracellular Ca^{2+} release from the sarco/endoplasmic reticulum stores [15, 117], result in inhibition of Ca^{2+}-dependent neurotransmitter release [108] and is probably the fundamental mechanisms underlying the antiemetic efficacy of CB_1R cannabinoid agonists against CINV [95–97]. (3) Glucocorticoids such as dexamethasone reduce both acute and delayed CINV [6]. Glucocorticoids' ability to decrease the abnormal elevation of cytosolic Ca^{2+} concentration [122], and subsequently control Ca^{2+}-dependent neurotransmitter release [6, 121, 126] and inflammatory responses [6]. Increased release of endocannabinoids and subsequent CB_1R activation may also be involved in antiemetic actions of glucocorticoids [123–125]. (4) The L-type Ca^{2+} channel (LTCC) antagonist flunarizine can reduce cyclic vomiting in patients [151, 152]. Gabapentin binds to the alpha-2/delta auxiliary subunits of LTCCs, and exerts inhibitory actions on trafficking and activation kinetics of LTCCs [153]. Gabapentin can be used as an anti-nausea and antiemetic agent in postoperative nausea and vomiting [154, 155] and in CINV [156, 157]. (5) LTCC antagonists (nifedipine and amlodipine) are broad-spectrum antiemetics when delivered systemically against diverse specific and nonselective emetogens. (6) Suppression of intracellular Ca^{2+} release from the sarco/endoplasmic reticulum through the inositol trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) may be additional targets for the prevention of nausea and vomiting, since functional and physical linkages between Ca^{2+} channels on cell membrane and IP_3Rs/RyRs play a role in Ca^{2+} signaling [160–166]. In the least shrew emesis model, the RyRs antagonist dantrolene can potentiate the antiemetic efficacy of amlodipine against $5\text{-}HT_3R$ agonist 2-Methyl-5-HT-induced vomiting [25]; and dantrolene together with the IP_3R antagonist 2-APB can potentiate the antiemetic efficacy of nifedipine against thapsigargin-induced vomiting [70].

blood-borne pathway which then triggers vomiting [2, 10], and/or the released emetic neurotransmitters/mediators stimulate their corresponding receptors present on vagal afferents in the gastrointestinal tract which indirectly activate brainstem emetic loci primarily in the nucleus tractus solitaries to trigger vomiting [6].

Ca^{2+} is not only one of the most universal and versatile signaling molecules, it is also an extremely important factor in both the physiology and pathology of living organisms. At rest, diverse cells have strict and well-regulated mechanisms to maintain low nM cytosolic Ca^{2+} levels [11]. Cytoplasmic Ca^{2+} concentration is a dominant factor in determining the amount of transmitter released from nerve terminals [12]. Thus, Ca^{2+} mobilization can be an important aspect of vomit induction since it is involved in both triggering the quantity of neurotransmitter released coupled with receptor activation, as well as post-receptor excitation-transcription coupling mechanisms [13]. Studies using Ca^{2+} imaging performed in vitro in the brainstem slice preparation suggest that emetic agents evoke direct excitatory effects on cytosolic Ca^{2+} signals in vagal afferent terminals in the nucleus tractus solitarius which potentiate local neurotransmitter release [5, 14, 15]. Therefore, chemotherapeutics including cisplatin seem to activate emetic circuits through a number of neurotransmitters released in a Ca^{2+}-dependent

manner in specific vomit-associated neuroanatomical structures. In both the periphery and the brainstem, emetic neurotransmitters/mediators—such as acetylcholine, dopamine, 5-HT, substance P, prostaglandins, leukotrienes, and/or histamine—may act independently or in combination to evoke vomiting after cisplatin administration [16] (**Figure 1**). In this review, we focus on the current evidence supporting the significance of Ca^{2+} signaling in emesis generation and its relationship to antiemetic efficacy, as well as the corresponding development of potential novel antiemetic medications, as shown in brief in **Figure 2**.

2. Emerging roles of Ca^{2+} in emesis

2.1. Emetic receptor stimulation increases intracellular Ca^{2+} concentration

Excitatory receptor activation by corresponding agonists can increase cytosolic Ca^{2+} levels via both mobilization of intracellular Ca^{2+} stores (e.g., endoplasmic reticulum = ER) and influx from extracellular fluid [17]. The evoked cytoplasmic Ca^{2+} increase may result from direct activation of ion channels, or indirectly via signal transduction pathways following G protein-coupled receptor activation. The neurokinin NK1 receptor (NK_1R) is a member of the tachykinin family of G-protein-coupled receptors. NK_1R stimulation by substance P or corresponding selective agonists such as GR73632, can increase cytosolic Ca^{2+} concentration. In fact GR73632-induced activation of NK_1Rs can evoke intracellular Ca^{2+} release from the sarco/endoplasmic reticulum stores via Gα/q-mediated phospholipase C pathway, which subsequently evokes extracellular Ca^{2+} influx through L-type Ca^{2+} channels (LTCCs) [17–19]. The serotonergic 5-HT_3 receptor (5-HT_3R) is a Ca^{2+}-permeable ligand-gated ion channel [20]. Cell lines studies have demonstrated that activation of 5-HT_3Rs by 5-HT or its analogs can evoke extracellular Ca^{2+} influx into cells in a manner sensitive to both 5-HT_3R antagonists (tropisetron, MDL7222, metoclopramide) and LTCC blockers (verapamil, nimodipine, nitrendipine) [20–24]. These studies suggest that both L-type- and 5-HT_3-receptor Ca^{2+}-permeable ion channels are involved in extracellular Ca^{2+} influx evoked by 5-HT_3R agonists. Moreover, 5-HT_3R activation indirectly causes release of Ca^{2+} from ryanodine-sensitive intracellular Ca^{2+} stores subsequent to the evoked extracellular Ca^{2+} influx which greatly amplifies the cytoplasmic concentration of Ca^{2+} [23]. In fact, our findings from behavioral studies in the least shrew emesis model [25] further support the notion of Ca^{2+}-induced Ca^{2+} release following 5-HT_3R stimulation, which will be discussed in more detail in Section 3.4. Other emetogens such as agonists of dopamine D_2- [26, 27], cholinergic M_1- [28, 29], histaminergic H_1- [30, 31], and opiate μ- [32, 33] receptors, as well as cisplatin [34], prostaglandins [35, 36], rotavirus NSP4 protein [37, 38] and bacterial toxins [39, 40] also possess the potential to mobilize Ca^{2+} which involve extracellular Ca^{2+} influx and/or Ca^{2+} release from intracellular Ca^{2+} pools. Much of the discussed evidence has been acquired from isolated cells.

The least shrew is an emesis-competent mammal whose reactions to common emetogens are well-defined and correlate closely with human responses [2]. 2-Methyl-5-HT is a well-known selective emetic agonist targeting the emesis-prone 5-HT_3Rs [4]. This vomit-competent species is an excellent animal model for studying the emetic activity of diverse agents [2]. In fact least shrews exhibit dose-dependent full emetic responses to intraperitoneal administration of

both the peripherally-acting 5-HT, as well as to its central nervous system-penetrating analog, 2-Methyl-5-HT [4, 41, 42]. In our studies, incubation of least shrew brainstem slices containing the dorsal vagal complex emetic loci with 2-Methyl-5-HT, results in a rapid increase in intracellular Ca^{2+} concentration as reflected by an increase in fluo-4 AM fluorescence intensity in a palonosetron (a 5-HT_3R antagonist)/nifedipine-sensitive manner [22, 25].

2.2. Emetic potential of Ca^{2+} channel activators: behavioral and immunohistochemical evidence

A variety of Ca^{2+}-permeable ion-channels mediating extracellular Ca^{2+} influx are present in the plasma membrane. Among them are voltage-gated LTCCs, which can be activated by membrane depolarization, and serve as the principal route of Ca^{2+} entry in electrically excitable cells such as neurons and muscle [43, 44]. Recently we have acquired direct evidence for the proposal that Ca^{2+} mobilization is an important facet in the mediation of emesis. In fact we have identified the novel emetogen FPL64176 (**Figure 2**), a selective agonist of LTCCs, which causes vomiting in the least shrew in a dose-dependent manner [45, 46]. All tested shrews vomited at a 10 mg/kg dose of FPL64176 administered intraperitoneally (i.p.). LTCCs have been shown to be present in enterochromaffin cells of guinea pig and human small intestinal crypts [47]. Furthermore, in these cells FPL64176 not only can enhance cytosolic Ca^{2+} concentration, but also increases 5-HT release from enterochromaffin cells [47]. The latter findings may have underpinnings for the mechanisms underlying FPL64176-evoked vomiting observed in least shrew model of emesis. FPL64176 (10 mg/kg., i.p.) can cause Ca^{2+}-dependent 5-HT release from shrew intestinal enterochromaffin cells which in turn could increase vagal afferent activity via stimulation of 5-HT_3 receptors, thereby indirectly triggering emetic signals in the brainstem [2, 48].

Our most recent work has focused on the Ca^{2+}-mobilizing agent thapsigargin (**Figure 3**), a specific and potent inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump which transports the free cytosolic Ca^{2+} into the lumen of the sarco/endoplasmic reticulum to

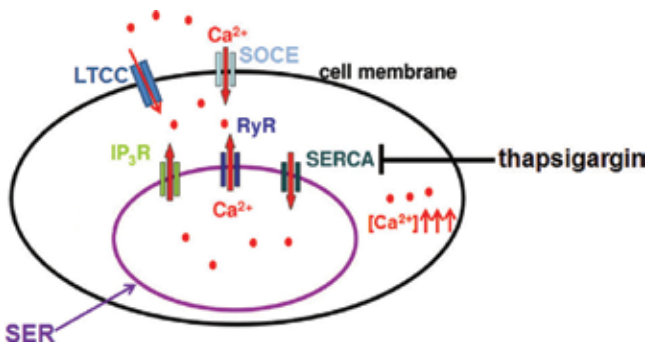

Figure 3. A schematic representation of extracellular Ca^{2+} influx and intracellular Ca^{2+} release contributing to thapsigargin-elicited Ca^{2+} mobilization. Intracellular Ca^{2+} release from the sarco/endoplasmic reticulum (SER) Ca^{2+} stores through the inositol triphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) is counter-balanced by continuous Ca^{2+} uptake from the cytoplasm into SER stores by the SER Ca^{2+}-ATPase pump (SERCA). Thapsigargin is a specific inhibitor of SERCA and thus enhances cytosolic levels of Ca^{2+}, a process involving SER Ca^{2+} release via IP_3Rs and RyRs as well as extracellular Ca^{2+} entry through Ca^{2+} channels located in the plasma membrane including store-operated Ca^{2+} channels (SOCE) and L-type Ca^{2+} channels (LTCCs) [49–60].

counter-balance the cytosolic intracellular Ca^{2+} release from the sarco/endoplasmic reticulum into the cytoplasm via the inositol trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) [49–51]. Thapsigargin also causes intracellular release of stored Ca^{2+} from the sarco/endoplasmic reticulum into the cytosol which subsequently evokes extracellular Ca^{2+} influx predominantly through store-operated Ca^{2+} entry (SOCE) in non-excitable cells [52–54]. In total, these events lead to a significant rise in the free concentration of cytosolic Ca^{2+} [55–57]. In addition, a partial involvement of LTCCs in thapsigargin-evoked contraction has also been demonstrated in rat stomach smooth muscle cells [58], rat gastric smooth muscle [59], and cat gastric smooth muscle [60]. On the other hand, the potential of thapsigargin as a Ca^{2+}-modulating cancer chemotherapeutic agent has been evaluated in both cells and animal models [61]. Thapsigargin-evoked increases in cytosolic Ca^{2+} concentration can lead to cell apoptosis, which can result in eradication of cancer cells of the breast [62], prostate [63], colon [64] and kidneys [65]. Clinically, a prodrug form of thapsigargin, mipsagargin, is currently under clinical trial as a targeted cancer chemotherapeutic agent with selective toxicity against cancer cells in tumor sites with minimal side-effects to the host [66–69]. In our studies, intraperitoneal administration of thapsigargin (0.1–10 mg/kg) caused vomiting in least shrews in a dose-dependent, but bell-shaped manner, with maximal efficacy at 0.5 mg/kg. An important consideration for the emetic potential of thapsigargin is that it augments the cytosolic levels of free Ca^{2+} in emetic loci as a result of SERCA inhibition as indicated in our latest discussed finding [70], which is the first study to reflect emesis as a major side-effect of thapsigargin when delivered systemically.

c-Fos induction has been used to evaluate differential neuronal activation [71]. Our lab has applied immunostaining and detected c-Fos induction in the brainstem emetic nuclei to demonstrate central responsiveness to peripheral administration of a variety of emetogens [4, 70, 72, 73]. The participation of the central emetic neurons in FPL64176-induced vomiting is further indicated by evoked c-Fos expression in brainstem emetic nuclei, the nucleus tractus solitarius and the dorsal motor nucleus of the vagus (unpublished data). Thus, the blood-brain barrier permeable agent FPL64176 [74–76] could excite emetic neurons directly in the nucleus tractus solitarius and the dorsal motor nucleus of the vagus. Thapsigargin (0.5 mg/kg) also causes increases in c-Fos immunoreactivity in the brainstem emetic nuclei including the area postrema, the nucleus tractus solitarius and the dorsal motor nucleus of the vagus [70].

3. Ca^{2+} intervention mechanisms relevant to antiemetic approaches

3.1. Receptor antagonist antiemetic regimens such as netupitant/palonosetron (NEPA)

The ultimate aim of prophylactic management of chemotherapy-induced nausea and vomiting is to abolish both the acute- and delayed phases of vomiting which will help to improve the well-being and quality of life of cancer patients receiving chemotherapy. Cisplatin-like chemotherapeutics cause release of multiple emetogenic neurotransmitters in both the central nervous system and the gastrointestinal tract and no available single antiemetic administered alone can provide complete efficacy. Significant initial work had suggested that while activation of $5\text{-}HT_3Rs$ by serotonin in the gastrointestinal tract is involved in the mediation of acute phase of chemotherapy-induced nausea and vomiting, the delayed phase is due to stimulation

of NK_1Rs subsequent to release of substance P in the brainstem [77, 78]. However, our more recent findings suggest that 5-HT and substance P are concomitantly involved in both emetic phases in the gastrointestinal tract as well as in the brainstem [2, 16]. While netupitant is a highly selective and a longer-acting second generation NK_1R antagonist, palonosetron is considered as a second generation 5-HT_3R antagonist with a unique antiemetic profile in both humans [79, 80] and the least shrew model of emesis [45]. A successful regimen of an oral fixed combined dose of netupitant/palonosetron (NEPA) (**Figure 2**) has been formulated with over 85% clinical efficacy, good tolerability, and high central nervous system penetrance for the prophylactic treatment of acute and delayed chemotherapy-induced nausea and vomiting in cancer patients receiving chemotherapy [9, 81, 82].

Recent evidence accumulated from HEK293 cells stably transfected with 5-HT_3Rs suggest that suppression of Ca^{2+} signaling is involved in antiemetic efficacy of both palonosetron and netupitant. Indeed, Rojas et al. [83, 84] have shown that palonosetron causes a persistent inhibition of 5-HT_3R function as reflected by a near complete suppression of 5-HT-evoked extracellular Ca^{2+} influx. They have further demonstrated that palonosetron can prevent enhancement of substance P-induced intracellular Ca^{2+} release in response to serotonin in NG108–15 cells expressing both 5-HT_3Rs and NK_1Rs [85]. Our Ca^{2+} monitoring studies performed on acutely-prepared least shrew brainstem slices also demonstrate that palonosetron can abolish enhancement of intracellular Ca^{2+} levels in brainstem slices evoked by the selective 5-HT_3R agonist 2-Methyl-5-HT [25]. The latter finding provides more relevant ex-vivo evidence for the Ca^{2+}-modulating antiemetic effect of palonosetron in a vomit-competent species. The role of netupitant in suppression of substance P-evoked enhancement of intracellular Ca^{2+} levels has also been demonstrated via Ca^{2+} mobilization assays in vitro in CHO cells expressing the human NK_1Rs. Moreover, pronetupitant, an intravenous alternative to the oral netupitant, appears to be more potent than netupitant in both in vitro Ca^{2+} measurement studies and in vivo animal behavioral evaluations of substance P in rats [86]. In addition, another clinically approved NK_1R antagonist antiemetic rolapitant, has been shown to suppress the ability of the selective NK_1R agonist GR73632 to evoke intracellular Ca^{2+} release [9, 87–89]. The discussed findings clearly suggest that Ca^{2+} is a major player in the initiation of vomiting evoked by diverse emetogens.

3.2. Cannabinoid CB_1 receptor agonists

Before the introduction of first generation 5-HT_3R antagonists, several phyto- and synthetic cannabinoids including dronabinol (delta-9-tetrahydrocannabinol, Δ^9-THC (**Figure 2**)), levonantradol and nabilone, were evaluated in cancer patients for suppression of chemotherapy-induced nausea and vomiting that were not effectively controlled by other available antiemetics [2, 90]. Cannabinoids are increasingly being tested as antiemetics against cisplatin-induced emesis in animal experiments using house musk shrews [91], ferrets [92], or least shrews [73, 93]; nausea-related behavior in rats [91]; radiation-induced emesis in the least shrew [94]; as well as both phases of chemotherapy-induced nausea and vomiting in the clinic [95–97]. Cannabinoid agonists exert their antiemetic efficacy via direct activation of CB_1 receptors (CB_1R) since their antiemetic effects were reversed by CB_1R antagonists [92, 94, 98–100]. Significant evidence for a role for CB_2Rs in emesis is currently lacking [101]. The presence of CB_1Rs in the brainstem nuclei involved in emesis has been confirmed, with a high density of CB_1R immunoreactivity in the dorsal motor nucleus of the vagus and the medial subnucleus

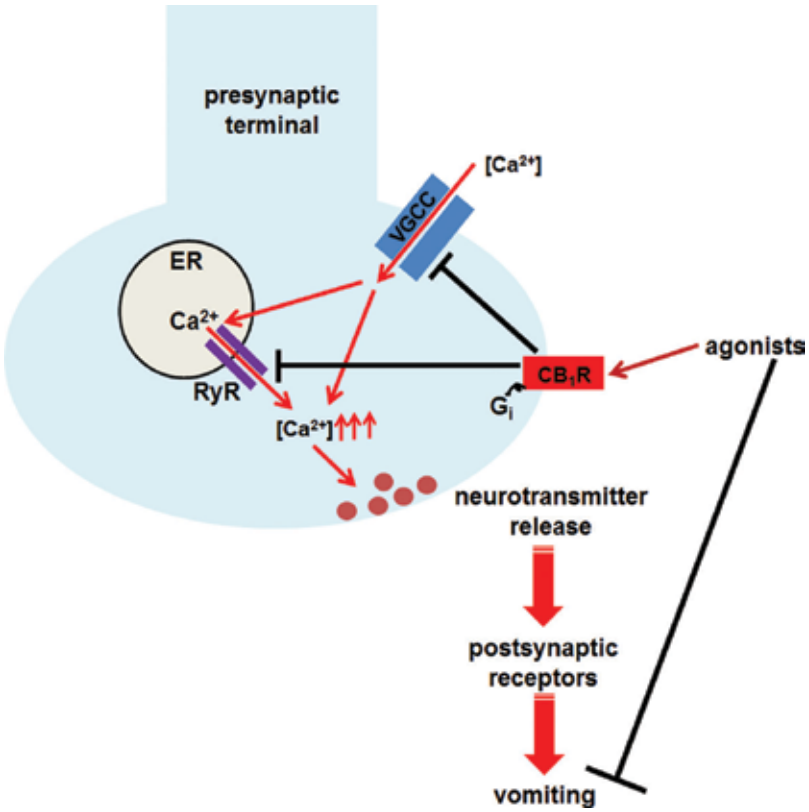

Figure 4. A schematic explanation of the antiemetic action of cannabinoid CB_1R agonists from the perspective of Ca^{2+} signaling. Activation of CB_1R initiates a $G_{i/o}$ mechanism leading to the downregulation of extracellular Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs) as well as endoplasmic reticulum (ER) Ca^{2+} release via ryanodine receptors (RyRs) which has the potential to be activated by extracellular Ca^{2+} entry through VGCCs. The reduction in cytosolic Ca^{2+} attenuates Ca^{2+}-dependent emetic neurotransmitter release, which further results in a reduction in postsynaptic neuronal activation, and ultimately suppression of the vomiting behavior [93, 103, 117].

of the nucleus tractus solitarius, a moderate density in the commissural subnucleus of the nucleus tractus solitarius, and lower densities in the area postrema and dorsal subnucleus of the nucleus tractus solitarius [73, 92]. CB_1R distribution has been also observed in the myenteric plexus of the stomach and duodenum [92]. Furthermore, CB_1Rs have been localized in the myenteric plexus of the rat and guinea pig intestine in nearly all cholinergic neuron terminals [102, 103]. These as well as behavioral evidence [42] suggest that the antiemetic action of cannabinoids involve both the central dorsal vagal complex and intestinal emetic loci. In addition, primary cultures of guinea-pig myenteric neurons express CB_1Rs and exogenously added cannabinoids suppress their neuronal activity, synaptic transmission and mitochondrial transport along axons [104]. Moreover, the $CB_{1/2}R$ agonist WIN55212-2 can suppress intestinal activity since it can attenuate the electrically-evoked contractions of the myenteric plexus-longitudinal muscle preparation of the guinea-pig small intestine in a Ca^{2+}-dependent and CB_1R-specific manner [105]. Thus, CB_1R agonists in the in vivo setting can also suppress the gastrointestinal tract motility [104]. Using whole-cell patch-clamp recordings in brainstem slices, Derbenev et al. [106, 107] have shown that activation of presynaptic CB_1Rs in the dorsal

vagal complex inhibits synaptic transmission to the dorsal motor nucleus of the vagus neurons, which may explain suppression of visceral motor responses caused by cannabinoids.

Furthermore, in the central nervous system CB_1R stimulation can result in inhibition of Ca^{2+}-dependent neurotransmitter release from presynaptic nerve terminals which consequently leads to inhibition of neurotransmission [108]. In chemotherapy-induced nausea and vomiting, the CB_1R-mediated antiemetic action of cannabinoids appears to be directly related to presynaptic inhibition of release of emetic neurotransmitters from nerve terminals. **Figure 4** may help to explain the antiemetic action of cannabinoid CB_1R agonists from the Ca^{2+} perspective. Indeed, the adenylyl cyclase/cyclic AMP (cAMP)/protein kinase A (PKA) signal transduction system is a well-established emetic signaling pathway [109]. PKA activation is known to phosphorylate both Ca^{2+} ion channels on plasma membrane and intracellular endoplasmic IP_3Rs, which respectively increase extracellular Ca^{2+} influx and internal Ca^{2+} release from the sarco/endoplasmic reticulum stores [110]. CB_1Rs are known to be Gi/o-protein coupled receptors which mediates inhibition of adenylate cyclase. This inhibition has been proposed to be the fundamental reason for CB_1R agonists attenuating Ca^{2+}-dependent emetic neurotransmitter release which would ultimately reduce postsynaptic neuronal activation in both dorsal vagal complex and gastrointestinal tract [93, 103]. Moreover, dose-dependent inhibitory action of cannabinoid CB_1R agonists on extracellular Ca^{2+} influx via a number of voltage-gated Ca^{2+} channels residing in the cell membrane including N-type, P/Q type and L-type have been demonstrated in multiple experimental systems [111–115]. Additionally, cannabinoid CB_1R agonists also block $5\text{-}HT_3Rs$ in a non-competitive manner and thus prevent extracellular Ca^{2+} influx [115, 116].

Furthermore, CB_1R agonists appear to inhibit the intracellular Ca^{2+} release channels located on the sarco/endoplasmic reticulum membrane, RyRs. Ca^{2+}-induced Ca^{2+} release is a well-established feature of Ca^{2+} signal amplification. During neuronal activation, Ca^{2+}-induced Ca^{2+} release Ca^{2+} signaling involves increased concentration of cytoplasmic Ca^{2+} via extracellular Ca^{2+} influx through voltage-gated Ca^{2+} channels (e.g., LTCCs) present on the cell membrane, which then causes release of stored intracellular Ca^{2+} from the sarco/endoplasmic reticulum into the cytosol through RyRs [117]. In fact RyRs have a wide distribution in the central nervous system including the brainstem [118]. RyRs not only can regulate Ca^{2+} homeostasis, but also other critical brain functions including neurotransmitter release [117]. Increased serum levels of the pro-inflammatory cytokine, tumor necrosis factor alpha (TNF-α), is associated with chemotherapy-evoked vomiting [119]. TNF-α can excite vagal afferent terminals by augmenting Ca^{2+} release from sarco/endoplasmic reticulum stores via sensitization of RyRs which subsequently amplifies neurotransmission in the brainstem [15]. Cannabinoid CB_1R agonists prevent the TNF-α-evoked sensitization of RyRs and therefore attenuate intracellular Ca^{2+} release from the sarco/endoplasmic reticulum stores [15]. Peripheral RyRs also play a critical role in agonist-evoked Ca^{2+} oscillations in gut epithelial cells [120]. Therefore, the ability of CB_1R agonists in preventing both extracellular Ca^{2+} influx as well as intracellular Ca^{2+} release from the sarco/endoplasmic reticulum stores may be the fundamental mechanisms underlying the broad-spectrum antiemetic efficacy of CB_1R cannabinoid agonists.

3.3. Glucocorticoids

Glucocorticoids, used primarily as anti-allergic and anti-inflammatory drugs. They are also effective, either alone or in combination with other antiemetics, for the suppression of nausea

and vomiting. Indeed, dexamethasone (**Figure 2**), one of the clinically used glucocorticoids, is effective in reducing both acute and delayed chemotherapy-induced nausea and vomiting, and when combined with 5-HT_3 or neurokinin NK_1 antagonists, it is utilized in patients receiving high emetogenic chemotherapy [6]. Glucocorticoids' antiemetic effect has been related to its inhibitory effects in the following facets: (i) glucocorticoids control the inflammatory response involved in mediating chemotherapy-induced nausea and vomiting by reducing the production of inflammatory mediators such as cytokines, chemokines, inducible nitric oxide synthase, and increasing the gene transcription of anti-inflammatory proteins [6]; (ii) glucocorticoids can inhibit 5-HT and substance P release, both of which can evoke emesis [6, 121], (iii) glucocorticoids can cross the blood-brain barrier and can exert direct central inhibitory effects on the nucleus tractus solitarius [6], which may be due to a decrease in abnormal elevation of cytosolic Ca^{2+} concentration as well as downstream Ca^{2+} signals and the maintenance of Ca^{2+} homeostasis within the cell [122], (iv) inhibitory actions of glucocorticoid could also be due to increased release of endocannabinoids, anandamide and 2-arachidonoylglycerol, evoked by glucocorticoid administration which will then be followed by subsequent CB_1R activation as well as glucocorticoid facilitation of synaptic γ-aminobutyric acid (GABA) release and suppression of glutamate release [123, 124]. The endocannabinoid system is composed of CBRs, endocannabinoids and the enzymes involved in their synthesis. Anandamide and 2-arachidonoylglycerol are among the well-studied endocannabinoids and endogenous activators of CBRs [125]. The role of CB_1R agonists as antiemetics was discussed in Section 2.2. It has been suggested that dexamethasone may decrease motion sickness through modulation of the endocannabinoid/CB_1 receptor system on the terminals of the nucleus tractus solitarius neurons that project to the output neurons of the DMNV as well as by endocannabinoid/CB_1 receptor system-mediated inhibition of transmitter release from interneurons of the nucleus tractus solitarius [99, 126]. Selective elevation of 2-arachidonoylglycerol by inhibition of its major metabolic enzyme monoacylglycerol lipase, have been shown to suppress lithium chloride evoked vomiting in the house musk shrew (*Suncus murinus*) [127]. However, intraperitoneal administration of the endocannabinoid 2-arachidonoylglycerol can evoke vomiting in the least shrew in a dose-dependent manner probably via its rapid metabolism to arachidonic acid which is also a potent emetogen in this species [128]. Moreover, the cancer chemotherapeutic agent cisplatin can increase 2-arachidonoylglycerol but not anandamide levels in the least shrew brain [129].

4. Perspective in developing new antiemetic candidates

4.1. Antiemetic efficacy of LTCC blockers in the least shrew model of emesis

Nifedipine along with amlodipine, are among the most studied of Ca^{2+} channels blockers, and both belong to the dihydropyridine subgroup of LTCC antagonists. Relative to nifedipine, a fast and short-acting LTCC antagonist with a plasma half-life of 1.2 h, amlodipine is slow and longer acting, more extensively bound to plasma protein, with a larger volume of distribution, more gradual elimination, with a half-life of over 30 h [130–134]. We have evaluated the antiemetic efficacy of both nifedipine and amlodipine (**Figure 2**) by assessing mean emesis frequency and the percentage of shrews vomiting, and demonstrated that both LTCC blockers [45, 46] behave as broad-spectrum antiemetics when delivered systemically against diverse specific emetogens,

including FPL 64176 (10 mg/kg, i.p.), the peripherally-acting and non-selective 5-HT_3R agonist 5-HT (5 mg/kg, i.p.), the peripherally/centrally-acting and more selective 5-HT_3R agonist 2-Methyl-5-HT (5 mg/kg, i.p.), the dopamine D_2R-preferring agonist quinpirole (2 mg/kg, i.p.), the non-selective dopamine D_2R agonist apomorphine (2 mg/kg, i.p.), the nonselective cholinergic agonist pilocarpine (2 mg/kg, i.p.), the M_1-preferring cholinergic agonist McN-A343 (2 mg/kg, i.p.), and the selective neurokinin NK_1R agonist GR73632 (5 mg/kg, i.p.). The vomiting behavior was recorded for 30 min. Our results suggest that both amlodipine and nifedipine act by suppressing the influx of extracellular Ca^{2+}, thereby delay the onset as well as protecting least shrews from vomiting, further supporting our proposed Ca^{2+} hypothesis of emesis. Nifedipine appears to be more potent than amlodipine against vomiting caused by FPL64176, 5-HT, 2-Methyl-5-HT, GR73632, quinpirole and McN-A343. These potency disparities could be explained in terms of their pharmacokinetic and pharmacodynamic differences [130–139].

Unlike the above tested emetogens which can evoke vomiting within minutes of administration, cisplatin (10 mg, i.p.) requires more exposure time (30–45 min) to begin to induce emesis since only its metabolites are emetogenic. The relative efficacy of amlodipine (5 mg/kg., i.p.) in reducing the frequency of cisplatin-evoked early vomiting by 80% compared with the observed lack of antiemetic action of nifedipine up to 20 mg/kg [45, 46], could be explained in terms of positively charged amlodipine associating more slowly with LTCCs, requiring more exposure time not only to reach its sites of action, but also to compensate for its slower receptor binding kinetics, which can lead to a more gradual onset of antagonism [140]. In addition, intracerebroventricular microinjection of another LTCC antagonist, nitrendipine, has been shown to attenuate nicotine-induced vomiting in the cat [141], which further supports the discussed broad-spectrum antiemetic efficacy of nifedipine and amlodipine as observed in the least shrew model. Cisplatin-based chemotherapeutics induce both immediate and delayed vomiting in humans and in vomit-competent animals [16, 142, 143]. In the least shrew, cisplatin (10 mg/kg, i.p.) causes emesis over 40 h with respective peak early- and delayed-phases occurring at 1–2 and 32–34 h post-injection [144]. Amlodipine, due to its unique pharmacokinetics, may offer practical advantages over other calcium antagonists in cisplatin-evoked delayed emesis.

4.2. Potentiation of antiemetic efficacy of 5-HT_3R antagonists when combined with LTCC blockers

In 1996 Hargreaves and co-workers [20] demonstrated that members of all three major classes of LTCC antagonists can prevent the ability of the 5-HT_3 receptor-selective agonist 1-(m-chlorophenyl)-biguanide to increase intracellular Ca^{2+} concentration in cell lines that possess either one or both of these two different Ca^{2+}-ion channels. The latter interaction is not competitive since the binding site for the different classes of LTCC antagonists appear not to be the same as the serotonin 5-HT_3R binding site itself (i.e., the orthosteric site) but instead, is an allosteric site in the 5-HT_3 receptor channel complex. Furthermore, 5-HT release from enterochromaffin cells can be prevented by antagonists of both 5-HT_3Rs and LTCCs [145, 146]. These findings provide possible mechanisms via which antagonists of both LTCCs and 5-HT_3Rs can mutually prevent the biochemical and behavioral effects of their corresponding selective agonists, including the vomiting behavior induced by their corresponding selective agonists FPL64176 and 2-Methyl-5-HT as we reported previously [45]. We have further demonstrated that when non-effective antiemetic doses of their selective antagonists (nifedipine

and palonosetron, respectively) are combined [45], the combination significantly and in an additive manner attenuate both the frequency and the percentage of shrews vomiting in response to either FPL 64176 or 2-Methyl-5-HT. Furthermore, although nifedipine alone up to 20 mg/kg dose failed to protect shrews from acute cisplatin-induced vomiting, its 0.5 mg/kg dose, significantly potentiated the antiemetic efficacy of a non-effective (0.025 mg/kg) as well as a semi-effective (0.5 mg/kg) dose of palonosetron. In another study we also utilized a combination of non-effective doses of amlodipine (0.5 mg/kg or 1 mg/kg) with a non- or semi-effective dose of the 5-HT_3R antagonist palonosetron (0.05 or 0.5 mg/kg) [46]. The combined antiemetic doses produced a similar additive efficacy against vomiting induced by either FPL 64176 or cisplatin. In fact relative to each antagonist alone, the combination was at least 4 times more potent in reducing the vomit frequency and provided more protection against FPL 64176-induced vomiting. The observed additive antiemetic efficacy of a combination of 5-HT_3- (and/or possibly NK_1-) with LTCC-antagonists in the least shrew suggests that such a combination should provide greater emesis protection in cancer patients receiving chemotherapy in a manner similar to that reported between 5-HT_3- and NK_1-receptor antagonists both in the laboratory [144, 147] and in the clinic [148]. Although in our investigation, the mechanism underlying the additive antiemetic efficacy of combined low doses of LTCC antagonists with 5-HT_3R antagonists was not directly studied, the published literature points to their interaction at the signal transduction level involving Ca^{2+} [20, 149, 150].

4.3. Clinical use of LTCC blockers as anti-nausea/antiemetic medication

There are several published clinical case reports that demonstrate Ca^{2+} channel blockers may provide protection against several causes of nausea and vomiting. The LTCC antagonist flunarizine (**Figure 2**) was shown to reduce cyclic vomiting on acute basis in one patient [151] and prophylactically in 8 other patients [152]. Gabapentin is a gamma-aminobutyric acid (GABA) analog and is predominantly used in the clinic for the management of pain [3]. Gabapentin binds to the alpha-2/delta auxiliary subunits of voltage-gated Ca^{2+} channels (VGCCs) (i.e., LTCCs), and exerts inhibitory actions on trafficking and activation kinetics of VGCCs [153] (**Figure 2**). Moreover, several other reports indicate that gabapentin can also be used as a well-tolerated, less-expensive and promising anti-nausea and antiemetic agent in diverse conditions including: postoperative nausea and vomiting [154, 155], moderately or highly emetogenic chemotherapy-induced nausea and vomiting, particularly effective against delayed chemotherapy-induced nausea and vomiting [156], and both acute and delayed nausea induced by chemotherapy [157], as well as hyperemesis gravidarum [158]. When combined with dexamethasone, gabapentin can also significantly reduce the 24-h incidence of postoperative nausea and vomiting [159]. Alpha-2/delta subunits of VGCCs control transmitter release and further facilitate excitatory transmission [153]. Gabapentin's interaction with neuronal alpha-2/delta subunits of VGCCs and subsequent downregulation of neuronal Ca^{2+} signaling in emesis relevant sites, such as the dorsal vagal complex, is postulated to play a critical role in its anti-nausea and anti-vomiting effects [3].

4.4. Intracellular Ca^{2+} release channels: possible targets for suppression of emesis

A functional and physical linkage between LTCCs and RyRs appears to exist and plays an important role in intracellular Ca^{2+} release following voltage-dependent Ca^{2+} entry through LTCCs

during neuronal depolarization to generate a transient increase in cytosolic Ca^{2+} [160–162]. Physical attachment of IP_3Rs to plasma membrane Ca^{2+} influx channels through conformational coupling has also been proposed as one of the mechanisms connecting depletion of internal Ca^{2+} stores with stimulation of extracellular Ca^{2+} influx [163]. For example, Ca^{2+} release from IP_3Rs was shown to couple with extracellular Ca^{2+} influx through LTCCs in non-excitable cells such as Jurkat human T lymphocytes [164] and drosophila S2 cells [165], as well as in excitable cells such as submucosal neurons in the rat distal colon [166]. We have found that $5\text{-}HT_3R$-mediated vomiting triggered by 5 mg/kg 2-Methyl-5-HT is insensitive to the intracellular Ca^{2+} release channel IP_3R antagonist 2-APB, but in contrast, was dose-dependently suppressed by the RyR antagonist, dantrolene [25]. Furthermore, a combination of the semi-effective doses of amlodipine and dantrolene was more potent than each antagonist being tested alone [25]. Significant reductions (70–85%) in the frequency of Ca^{2+} mobilizer thapsigargin-evoked vomiting (see Section 1.2) were observed when shrews were pretreated with antagonists of either IP_3Rs (2-APB at 1 and 2.5 mg/kg, i.p.)- or RyRs (dantrolene at 2.5 and 5 mg/kg, i.p.)-ER luminal Ca^{2+} release channels. Moreover, while a mixture of 2-APB (1 mg/kg) and dantrolene (2.5 mg/kg) did not offer additional protection than what was afforded when each drug administered alone, a combination of the latter doses of 2-APB plus dantrolene with a partially effective dose of nifedipine (2.5 mg/kg), led to a complete elimination of thapsigargin-evoked vomiting [70]. In another set of experiments [167], we found that pretreatment with the IP_3R inhibitor 2-APB causes a significant reduction in NK_1R agonist GR73632-induced emesis, however the RyR inhibitor dantrolene did not. Thus, RyRs and IP_3Rs can be differentially modulated by various emetogens and their antagonists provide further efficacy when combined with LTCC antagonists (**Figure 2**). Suppression of Ca^{2+} release from the sarco/endoplasmic reticulum stores through IP_3Rs and RyRs may be additional targets for the prevention of nausea and vomiting.

4.5. Ca^{2+}-related signaling pathways in emesis

4.5.1. The role of cAMP-PKA in vomiting

In mammals, cyclic AMP (cAMP) is synthesized by 10 adenylate cyclase isoforms [168]. One of the best-studied second messenger molecules downstream of selected G-protein coupled receptors is cAMP. It is an example of a transient and diffusible second messenger involved in signal propagation by integrating multiple intracellular signaling pathways [169]. cAMP activates protein kinase A (PKA) which results in phosphorylation of downstream intracellular signals. The adenylyl cyclase/cAMP/PKA signaling pathway can phosphorylate Ca^{2+} ion-channels found on the plasma membrane and intracellular IP_3Rs [110]. These Ca^{2+} channels respectively increase extracellular Ca^{2+}-influx and intracellular Ca^{2+}-release [110]. The emetic role of cAMP has been well established (**Figure 5**), since microinjection of cAMP analogs (e.g., 8-bromocAMP) or forskolin (to enhance endogenous levels of cAMP) in the brainstem dorsal vagal complex emetic locus area postrema, not only can increase electrical activity of local neurons, but also induces vomiting in dogs [170]. Moreover, administration of 8-chlorocAMP as a potential chemotherapeutic in cancer patients can evoke nausea and vomiting [171]. Furthermore, phosphodiesterase inhibitors (PDEI) such as rolipram prevent cAMP metabolism and consequently increase cAMP tissue levels, which leads to excessive nausea and vomiting in humans [172]. In fact, one major side-effect of older PDEIs is excessive nausea and vomiting which often precludes their use in the clinical setting [173]. In addition, we have

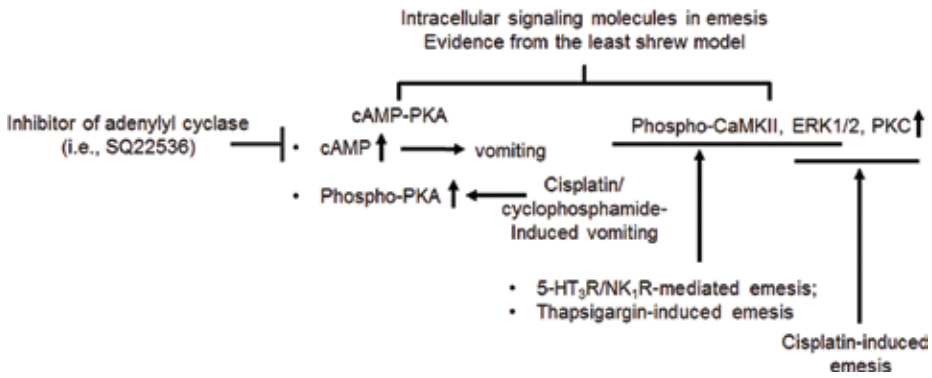

Figure 5. Summarized behavioral and biochemical evidence for intracellular signaling molecules (cAMP, PKA, CaMKII, ERK1/2, PKC) related to emesis based on the least shrew emesis model. First, cyclic AMP (cAMP) is synthesized by adenylate cyclase and cAMP activates protein kinase A (PKA) [110, 168]. The adenylyl cyclase/cAMP/PKA signaling pathway can mediate vomiting. Indeed, increased levels of endogenous cAMP can evoke vomiting in animal models [109, 170] as well as humans [171–173], which can be prevented by adenylate cyclase inhibitor SQ22536 [109]. Evoked PKA-phosphorylation is associated with peak vomit frequency during both immediate- and delayed-phases of vomiting caused by cancer chemotherapeutics including cisplatin and cyclophosphamide in the least shrew [109, 144, 149]. In addition, Ca^{2+}/calmodulin kinase IIα (CaMKIIα) and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) phosphorylation in the least shrew brainstem were elevated in vomiting evoked by the 5-HT_3R agonist 2-Methyl-5-HT [46], thapsigargin [70], or the selective NK_1R agonist GR73632 [167]. Phosphorylation of protein kinase Cα/βII (PKCα/βII) and ERK1/2 in least shrew brainstem were also upregulated in the vomiting induced by cisplatin [144, 149].

demonstrated that increased brain cAMP levels evoke vomiting which can be prevented by SQ22536 (**Figure 5**), an inhibitor of adenylyl cyclase [109]. Moreover, PKA-phosphorylation is associated with peak vomit frequency during both immediate- and delayed-phases of vomiting caused by either cisplatin or cyclophosphamide in the least shrew [109, 144, 149] (**Figure 5**).

4.5.2. Activation and inhibition of CaMKII, ERK1/2, PKC, and Akt are correspondingly linked to emesis induction and prevention

Vomit-associated Ca^{2+} mobilization as well as time-dependent Ca^{2+}/calmodulin kinase IIα (CaMKIIα) and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) phosphorylation in the least shrew brainstem occurs: (i) following 5-HT_3R-evoked vomiting caused by its selective agonist 2-Methyl-5-HT [46], (ii) thapsigargin-induced emesis in the least shrew [70], as well as (iii) NK_1R-mediated vomiting evoked by the selective NK_1R agonist GR73632 in the least shrew [167] (**Figure 5**). Our additional behavioral evidence that inhibitors of CaMKII or ERK1/2 attenuate the evoked emesis provides further credence for involvement of CaMKII and ERK1/2 downstream of the discussed emetic receptors/effectors. Furthermore, other published evidence support phosphorylation of protein kinase Cα/βII (PKCα/βII) and ERK1/2 in least shrew brainstem are associated with cisplatin-induced emesis [144, 149] (**Figure 5**). In fact significant upregulation of ERK1/2 phosphorylation occurs with peak vomit frequency during both the immediate and delayed phases of emesis caused by cisplatin in the least shrew [144, 149].

It has been suggested that glucocorticoids' antiemetic efficacy could be due to their anti-inflammatory effects [174] probably via a reduction in the synthesis of prostaglandins and leukotrienes [175], both of which can be increased during chemotherapy [6]. Although not all, but several prostaglandins (e.g., PGE2 and PGF2a) and cysteinyl leukotrienes (e.g., LTC_4

and LTD_4), appear to be potent emetogens [72, 149, 176, 177]. Our findings demonstrate that unlike other leukotrienes (e.g., LTA_4, LTB_4 and LTF_4), the above discussed leukotrienes are effective emetogens with the following potency order: $LTC_4 = LTD_4 > LTE_4$. Regarding LTC4, the evoked vomiting was shown to be suppressed in a dose-dependent manner in the least shrew by the antiasthmatic drug pranlukast, the corresponding cysteinyl leukotrienes receptor 1 (CysLT1R) antagonist [72]. Although not available in the USA, the cost of other members of this class of drugs (montelukast and Zafirlukast) that are sold in the USA is less than one dollar per pill. Based on pranlukast's efficacy against LTC_4-induced vomiting [72], we envisaged it may have potential utility against cisplatin-evoked emesis. Our most recent publication [178] shows the potential of pranlukast (currently used for the treatment of various respiratory disorders including asthma), as a new class of antiemetic for the suppression of the acute- and delayed- phases of cisplatin-evoked vomiting in the least shrew. An intraperitoneal (i.p.) dose of 10 mg/kg pranlukast by itself significantly reduced the mean frequency of vomits by 70% and fully protected 46% of least shrews during the delayed-phase of cisplatin (10 mg/kg, i.p.)-evoked vomiting. Although pranlukast tended to substantially reduce both the mean frequency of vomits and the number of shrews vomiting during the early-phase, these reductions failed to attain significance. When pranlukast was combined with a first (tropisetron)- or a second (palonosetron)-generation 5-HT_3R antagonist, it potentiated their antiemetic efficacy during both acute- and delayed-phases of cisplatin-evoked vomiting. Moreover, pranlukast potentiated the antiemetic efficacy of serotonin 5-HT_3 receptor antagonists, tropisetron and palonosetron, against chemotherapy-induced nausea and vomiting. In fact per hour efficacy antiemetic profile of pranlukast combined with palonosetron or tropisetron during both phases of chemotherapy-induced nausea and vomiting in the least shrew resembles those of: (i) the NK_1 receptor antagonist netupitant (5 mg/kg) plus palonosetron (0.1 mg/kg) in the same species [144]; (ii) netupitant plus ondansetron in ferrets [179]; and (iii) ondansetron plus aprepitant in combination with dexamethasone in ferrets [179]; and (iv) palonosetron plus netupitant in combination with dexamethasone in ferrets [179]. If analogs of pranlukast such as montelukast and zafirlukast can also provide similar antiemetic potential, then clinical trials should be initiated since this class of drugs are relatively inexpensive than available effective antiemetic regimens against chemotherapy-induced nausea and vomiting. Our related biochemical data indicates the mechanisms of antiemetic action of pranlukast are linked to suppression of cisplatin-elicited PKCα/βII, ERK1/2 and PKA activation (phosphorylation) in the least shrew brainstem [178]. Moreover, suppression of these signaling molecules may be shared in the anti-inflammatory signaling pathway of pranlukast.

When antiemetic mechanism of action of pranlukast against LTC4-induced vomiting or cisplatin-induced responses is discussed, Ca^{2+} is also an essential element. Montelukast and pranlukast were found to inhibit nucleotide-induced Ca^{2+} mobilization in a human monocyte-macrophage-like cell line, DMSO-differentiated U937 [180]. CysLT1 receptors belonging to the rhodopsin family of the G protein-coupled receptor genes respond to LTD4 with a strong increase in cytosolic Ca^{2+} concentration partially sensitive to pertussis toxin, and with the activation of the Ras-MAPK cascade totally dependent upon $G_{i/o}$ [144]. These signaling effects were totally inhibited by various specific CysLT1-receptor antagonists, and CysLT1 antagonists inhibit both the P2Y agonist-induced activation of phospholipase C and intracellular Ca^{2+} mobilization [144].

5. Conclusion

Chemotherapy-induced nausea and vomiting is a particularly distressing side-effect of chemotherapeutics for oncology patients both physically and psychologically. The use of 5-HT_3R antagonists combined with NK_1R antagonists, has enhanced physician's ability to further suppress nausea, the rates of acute- and delayed-vomiting in cancer patients receiving chemotherapy. In addition to the commonly reported adverse effects of these agents (including headache, diarrhea, constipation, hiccups, and fatigue), many patients still experience nausea and delayed vomiting [181–183]. Furthermore, the use of second generation 5-HT_3R and NK_1R antagonists for the prevention of chemotherapy-induced nausea and vomiting is currently cost-prohibitive for most patients in the world. Mechanisms that cause nausea are only partially understood and probably in part overlap with those of vomiting. There are still unmet needs for newer and less expensive therapeutic options to improve the treatment across the entire spectrum of chemotherapy-induced nausea and vomiting. Additional studies should involve combinations of agents that inhibit other neurotransmitter systems involved in nausea and vomiting.

As concluded in **Figure 2**, this systematic review shows clear evidence that Ca^{2+} modulation is an important contributor to antiemetic and probably anti-nausea signaling pathways. LTCC blockers, antagonists of intracellular IP_3Rs and RyRs Ca^{2+} release channels as well as CysLT1R antagonists have the potential to provide less expensive (e.g., nifedipine, amlodipine, dantrolene, and pranlukast) broad-spectrum antiemetic agents for the clinic against diverse causes of nausea and vomiting. The discussed findings from the least shrew should help to open new avenues of research in other established animal models of emesis as well as in patients, targeting not only the already discussed Ca^{2+} channels, but also other Ca^{2+} channels that exist on both the plasma membrane and the membranes of intracellular organs such as the sarco/endoplasmic reticulum and mitochondria.

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Chapter 7

Calcium Signaling Initiated by Agonists in Mesenchymal Stromal Cells from the Human Adipose Tissue

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Abstract

Mesenchymal stromal cells (MSCs) from different sources represent a heterogeneous population of proliferating non-differentiated cells that contain multipotent stem cells capable of originating a variety of mesenchymal cell lineages. By using Ca^{2+} imaging and the Ca^{2+} dye $Fluo^{-4}$, we studied MSCs from the human adipose tissue and examined Ca^{2+} signaling initiated by a variety of GPCR ligands, focusing primarily on adrenergic and purinergic agonists. Being characterized by a relative change of $Fluo^{-4}$ fluorescence, agonist-induced Ca^{2+} responses were generated in an "all-or-nothing" fashion. Specifically, at relatively low doses, agonists elicited undetectable responses but initiated quite similar Ca^{2+} transients at all concentrations above the threshold. The inhibitory analysis and Ca^{2+}/IP^3 uncaging pointed at the phosphoinositide cascade as a pivotal pathway responsible for agonist transduction and implicated Ca^{2+}-induced Ca^{2+} release (CICR) in shaping agonists-dependent Ca^{2+} signals. Altogether, our data suggest that agonist transduction in MSCs includes two fundamentally different stages: an agonist initially triggers a local, gradual, and relatively small Ca^{2+} signal, which next stimulates CICR to accomplish transduction with a large and global Ca^{2+} transient. By involving the trigger-like mechanism CICR, a cell is capable of generating Ca^{2+} responses of virtually universal shape and magnitude at different agonist concentrations above the threshold.

Keywords: Ca^{2+} signaling, G-protein coupled receptors, calcium-induced calcium release, IP_3 receptors, mesenchymal stromal cells, adipose tissue

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1. Introduction

Mesenchymal stromal cells (MSCs) are described as a heterogeneous cellular pool that includes immature cells responsible for the replenishment of supportive and connective tissues due to their capability of maintaining self-renewal and multipotent differentiation [1–3]. By unique biologic properties, cultured MSCs from different sources attract sufficient interest in the fields of regenerative medicine and immunotherapy [4–6]. Despite evident progress in MSC biology spurred by the therapeutic potential of these cells, current knowledge on their receptor and signaling systems remains scarce. Evidence exists that MSCs are capable of sensing complex extracellular cues, including hormones, cytokines, and nucleotides [7, 8]. This implies that MSCs employ multiple surface receptors and signaling pathways to adjust their physiological functions to specific tissue microenvironment.

Here, we studied MSCs derived from the human adipose tissue and examined Ca^{2+} signaling initiated by a variety of agonists of G-protein coupled receptors (GPCRs). We specifically focused on adrenergic and purinergic signaling systems that attracted us for the following reasons. It has been known for a long time that noradrenaline released by sympathetic nerves regulates distinct physiological processes in the adipose tissue such as lipid and glucose metabolism and secretion of distinct signaling molecules, including adipocytokines and cytokines [9]. Hence, MSCs that reside in the adipose tissue can be subjected to the action of noradrenaline and factors released by adipocytes on adrenergic stimulation. Purinergic agonists have been documented as an important factor determining MSC fate [7, 8, 10–12]. Reportedly, ATP serves both as an adipogenic regulator and an osteogenic factor, while its downstream product adenosine switches off adipogenic differentiation and promotes osteogenesis [13, 14]. Damaged tissues are an abundant source of extracellular ATP that may be converted by extracellular nucleotidases to ADP and eventually to adenosine [15]. It therefore might be expected that MSCs are exposed to and regulated by nucleotides and adenosine when these cells migrate *in vivo* or are transplanted *ex vivo* into an injured tissue.

The responsiveness to purines and pyrimidines is widespread among eukaryotic cells, which express numerous purinoreceptors from the P1 and P2 families. The P1 subgroup includes four G-protein-coupled receptors (A_1, A_{2A}, A_{2B}, A_3) recognizing adenosine as an endogenous agonist [16]. The more diverse P2 family is composed of ionotropic P2X and metabotropic P2Y receptors. P2X receptors are cationic channels specifically gated by ATP, while P2Y receptors are activated by multiple purine and pyrimidine nucleotides or by sugar nucleotides and couple to intracellular second messenger pathways by heteromeric G proteins [17, 18]. In mammals, seven genes encode P2X subunits ($P2X_{1–7}$) that can form homo- and heterotrimeric cation channels with noticeable Ca^{2+} permeability [19, 20]. The P2Y subfamily includes eight members ($P2Y_{1,2,4,6,11,12,13,14}$), which are distinct by ligand specificity and coupling to downstream signaling pathways, including the ubiquitous phosphoinositide cascade [17, 18].

Nine genes encode human adrenoreceptors, which all belong to the GPCR superfamily and compose three distinctive subgroups, including three α_1 (α_{1A}, α_{1B}, α_{1D}), three α_2 (α_{2A}, α_{2B}, α_{2C}), and three β (β_1, β_2, β_3) receptor subtypes. Canonically, α_1-adrenoreceptors couple to G_q and

are ubiquitously involved in Ca^{2+} signaling [21]. Although α_2 isoforms widely regulate adenylyl cyclase via G_i, their coupling to phospholipase C (PLC) and Ca^{2+} mobilization has also been documented [22]. All three β-subtypes are linked to adenylyl cyclase by G_s, although β_2 and β_3 also couple to G_i, and directly do not control intracellular Ca^{2+} [23]. Given that certain isoforms of adrenergic and purinergic receptors are coupled to Ca^{2+} mobilization in diverse cell types, we considered Ca^{2+} imaging as an adequate approach to detail purinergic and adrenergic transduction in MSCs.

2. Materials and methods

2.1. Cell isolation and culturing

MSCs of the first passage were obtained from the Faculty of Basic Medicine at Lomonosov Moscow State University. All procedures that involved human participants were performed in accordance with the ethical standards approved by the Bioethical Committee of the Faculty based on the 1964 Helsinki declaration and its later amendments. The study involved 21 healthy (not suffered from infectious or systemic diseases and malignancies) individuals from 21 to 55 years old, and informed consent was obtained from each participant.

Cells were isolated from subcutaneous fat tissue of healthy donors using enzymatic digestion as previously described [24]. Briefly, the adipose tissue was extensively washed with two volumes of Hank's Balanced Salt Solution (HBSS) containing 5% antibiotic/antimycotic solution (10,000 units of penicillin, 10,000 μg of streptomycin, and 25 μg of Amphotericin B per mL; HyClone), fragmented, and then digested at 37°C for 1 h in the presence of collagenase (200 U/ml, Sigma-Aldrich) and dispase (10 U/ml, BD Biosciences). Enzymatic activity was neutralized by adding an equal volume of culture medium (HyClone™ AdvanceSTEM™ Mesenchymal Stem Cell Basal Medium for human undifferentiated mesenchymal stem cells containing 10% of HyClone™ AdvanceSTEM™ Mesenchymal Stem Cell Growth Supplement (CGS), 1% antibiotic/antimycotic solution (HyClone) and centrifuged at 200 g for 10 min. This led to the sedimentation of diverse cells, including MSCs, macrophages, lymphocytes, and erythrocytes, unlike adipocytes that remained floating. After removal of supernatant, a lysis solution (154 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA) was added to a cell pellet to lyse erythrocytes, and cell suspension was centrifuged at 200 g for 10 min. Sedimented cells were resuspended in the MSC culture medium and filtered through a 100-μm nylon cell strainer (BD Biosciences). As indicated by flow [24], after isolation and overnight preplating, the obtained cell population contained not only MSC cells that basically represented the most abundant subgroup but also admixed macrophages and lymphocytes. The two last cell subgroups were dramatically depleted by culturing for a week in the MSC culture medium and humidified atmosphere (5% CO_2) at 37°C. The obtained MSC population was maintained at a subconfluent level (~80% confluency) and passaged using HyQTase (HyClone). By using the methodology described previously [25], cultured cells were demonstrated to differentiate into the osteogenic, chondrogenic, and adipogenic directions, the finding confirming their multipotency. In experiments, MSCs of the second to fourth passages were usually used.

2.2. Preparation of cells for Ca^{2+} imaging

Before assaying with Ca^{2+} imaging, cells were maintained in a 12-socket plate for 12 h in the medium described above but without antibiotics. For isolation, cells cultured in a 1-ml socket were rinsed twice with the Versene solution (Sigma-Aldrich) that was then substituted for 200 μl HyQTase solution (HyClone) for 3–5 min. The enzymatic treatment was terminated by the addition of a 0.8 ml culture medium to a socket. Next, cells were resuspended, put into a tube, and centrifuged at 50 g for 45 s for moderate sedimentation. Isolated cells were collected by a plastic pipette and plated onto a photometric chamber of nearly 150 μl volume. The last was a disposable coverslip (Menzel-Glaser) with attached ellipsoidal resin wall. The chamber bottom was coated with Cell-Tak (BD Biosciences), enabling strong cell adhesion. Attached cells were then loaded with dyes for 20 min at room temperature (23–25°C) by adding Fluo-4 AM (4 μM) and Pluronic (0.02%; all from Molecular Probes) to a bath solution. Loaded cells were rinsed with the bath solution for several times and kept at 4°C for 1 h prior to recordings. Generally, incubation of MSCs at low temperature stabilized intracellular Ca^{2+} and decreased a fraction of spontaneously oscillating cells.

2.3. Ca^{2+} imaging and uncaging

Experiments were carried out using an inverted fluorescent microscope Axiovert 135 equipped with an objective Plan NeoFluar 20x/0.75 (Zeiss) and a digital EMCCD camera LucaR (Andor Technology). Apart from a transparent light illuminator, the microscope was equipped with a handmade system for epi-illumination via an objective. The epi-illumination was performed using a bifurcational glass fiber. One channel was used for Fluo-4 excitation and transmitted irradiation of a computer-controllable light-emitting diode (LED) LZ1-00B700H (LED Engin). LED emission was filtered with an optical filter ET480/20x (Chroma Technology). Fluo-4 emission was collected at 535 ± 25 nm by using an emission filter ET535/50 m (Chroma Technology). Serial fluorescent images were usually captured every second and analyzed using Imaging Workbench 6 software (INDEC). Within the 1-s acquisition period, the 480 nm LED was switched on for only 200 ms, during which cell fluorescence was collected. This protocol allowed for minimizing photobleaching of Fluo-4 at a sufficiently high signal-to-noise ratio achievable by adjusting LED emission. This enabled us to reliably assay cell responsiveness to different compounds applied serially for up to 60 min. Deviations of cytosolic Ca^{2+} from the resting level were quantified by a relative change in the intensity of Fluo-4 fluorescence ($\Delta F/F_0$) recorded from an individual cell.

Another channel was connected to a pulsed solid laser TECH-351 Advanced (680 mW) (Laser-Export, Moscow). This unit operated in a two harmonic mode and generated not only 351 nM UV light used for Ca^{2+} uncaging but also visible light at 527 nm. The last could penetrate into an emission channel through nonideal optical filters and elicit optical artifacts during uncaging. For Ca^{2+} or IP_3 uncaging, cells were loaded with 4 μM Fluo-4-AM (Invitrogen) and 4 μM NP-EGTA-AM (Invitrogen) or 4 μM caged-Ins(145)P3/PM (SiChem) + 0.02% Pluronic (Invitrogen) for 30 min at 23°C. The basic bath solution contained (mM): 110 NaCl, 5.5 KCl, 2 $CaCl_2$, 0.8 $MgSO_4$, 10 glucose, 10 HEPES-NaOH, and pH 7.4 (≈270 Osm). When necessary, 2 mM $CaCl_2$ in the bath was replaced with 0.5 mM EGTA + 0.4 mM $CaCl_2$, thus reducing free Ca^{2+} to nearly 260 nM at 23°C as calculated with the Maxchelator program (http://maxchelator.

stanford.edu). In this low Ca^{2+} bath solution, the glucose concentration was increased to 13 mM to keep osmolarity. All chemicals used in experiments described below were applied by the complete replacement of the bath solution in a 150-μl photometric chamber for nearly 2 s using a perfusion system driven by gravity. The used salts and buffers were from Sigma-Aldrich, and agonists and inhibitors were from Tocris.

3. Results

In a typical experiment, nearly a hundred of MSCs loaded with Fluo-4 resided in a photometric camera, and their responsiveness to different ligands was assayed with Ca^{2+} imaging. Consistently with observations of others [3], functional heterogeneity was characteristic of a MSC population derived from each particular donor. Although a variety of GPCR agonists were found to stimulate Ca^{2+} signaling in MSCs, including ATP, ADP, noradrenaline or adrenaline, acetylcholine or its analog carbachol, GABA, glutamate, serotonin, and UTP, only a relatively small group of cells in a given MSC population was specifically responsive to a particular agonist (**Figure 1**). Overall, nearly 10^3 MSCs were sequentially stimulated by multiple agonists applied at different combinations, and a particular cell was either irresponsive to all stimuli or responded to one, rarely two, particular compound (**Figure 1A–C**). ATP-sensitive cells composed the most abundant subgroup of 9–15% (12% on average), depending on MSC preparation (**Figure 1B**). The percentage of cells responsive to other agonists was on average: ADP—7.1, adenosine—8.7, carbachol—3.4, GABA—5, glutamate—1.2, noradrenaline—6.7, serotonin—6.6, and UTP—6 (**Figure 1B**). The more or less accurate analysis of distribution of MSC responsivity was performed for nucleotides. In designated experiments, wherein cells were sequentially stimulated by ATP, ADP, and UTP, 125 purinergic MSCs were assayed overall, and only 13 cells (10%) were found to respond to all three agonists at the indicated concentrations (**Figure 1C**). Both ATP and ADP stimulated Ca^{2+} signaling in 40 cells (32%) that did not respond to UTP; 33 cells (26%) preferred the ATP-UTP pair. In addition, 20, 9, and 7 cells (16, 7, and 6%) responded exclusively to ATP, ADP, or UTP, respectively (**Figure 1C**).

Thus, the results presented above (**Figure 1**) clearly demonstrated that responsiveness to a given agonist varied from cell to cell. Note that GPCRs from most subfamilies, e.g. P2Y receptors, can couple to several signaling pathways, depending on cellular context [26–29]. Hence, in cells nonresponsive in terms of Ca^{2+} signaling to a particular agonist, appropriate GPCRs might be either not expressed or not coupled to Ca^{2+} mobilization.

3.1. Dose dependence of MSC responses to adrenergic and purinergic agonists

In the present study, we focused on transduction of adrenergic and purinergic agonists capable of stimulating Ca^{2+} signaling in the MSC cytoplasm. We first aimed at evaluating dose dependencies of cellular responses to tested agonists. The analysis, which initially involved adrenergic transduction, revealed that Ca^{2+} responses varied with noradrenaline concentration in an "all-or-nothing" fashion. In other words, noradrenaline never caused detectable effects, when applied below 100 nM, but above the threshold of 100–200 nM, it elicited marked Ca^{2+} transients that were similarly shaped irrespective of agonist concentration (**Figure 2A**).

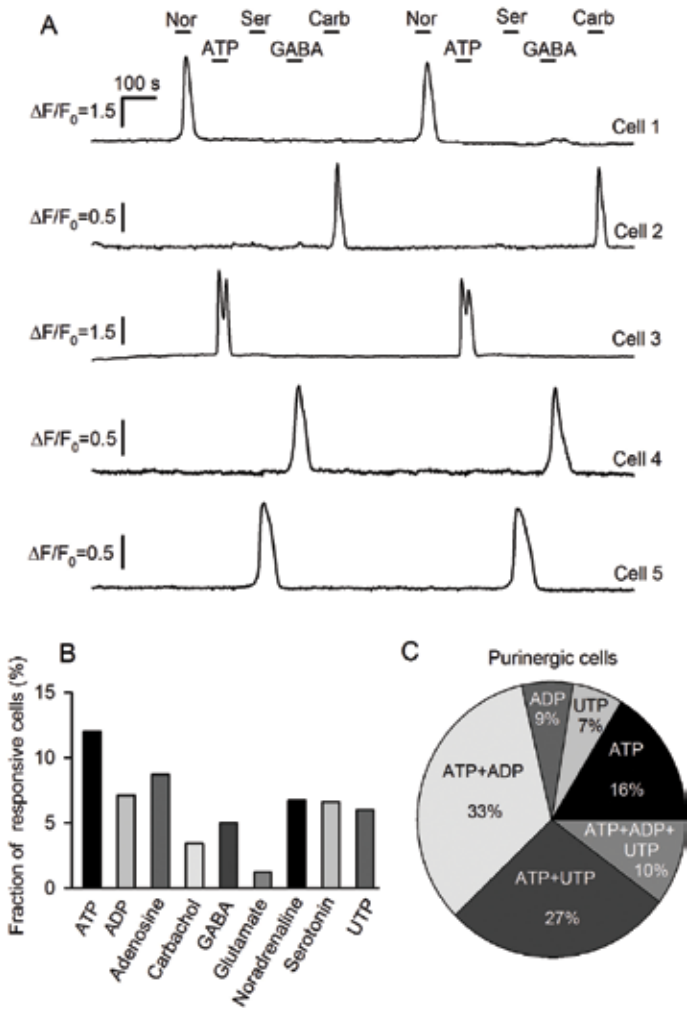

Figure 1. Functional heterogeneity of MSCs from the human adipose tissue. (A) Concurrent monitoring of intracellular Ca^{2+} in five different cells loaded with Fluo-4. The selected agonists were applied as indicated by the horizontal lines above the upper trace. (B) Fractional distribution of 426 MSCs that responded to at least one from the following serially applied agonists, including 10 μM ATP (adenosine triphosphate), 3 μM ADP (adenosine diphosphate), 10 μM adenosine (Adeno), 20 μM carbachol (carb), 20 μM GABA (gamma-aminobutyric acid), 10 μM glutamic acid (Glut), 0.5 μM noradrenaline (Nor), 10 μM serotonin (Ser), and 10 μM UTP (uridine triphosphate). (C) Distribution of MSC responsiveness to sequentially applied ATP (3 μM), ADP (3 μM), and UTP (10 μM) among a population of 125 cells, each being sensitive to at least one nucleotide.

Since we expected to obtain a somewhat gradual dose dependence, we considered the possibility that at concentrations used, noradrenaline might elicit too high Ca^{2+} transients, which all saturated Fluo-4 fluorescence, thus appearing alike. However, the permeabilizing agent saponin (0.1 mg/mL) evoked marked Ca^{2+} signals that exceeded noradrenaline responses by the factor of 1.5–2 (17 cells; **Figure 2A**). These observations indicated conclusively that MSC responses to varied noradrenaline could not be equalized due to saturation of the Ca^{2+} dye. The further analysis of MSC responsivity pointed out that the "all-or-nothing" phenomenon

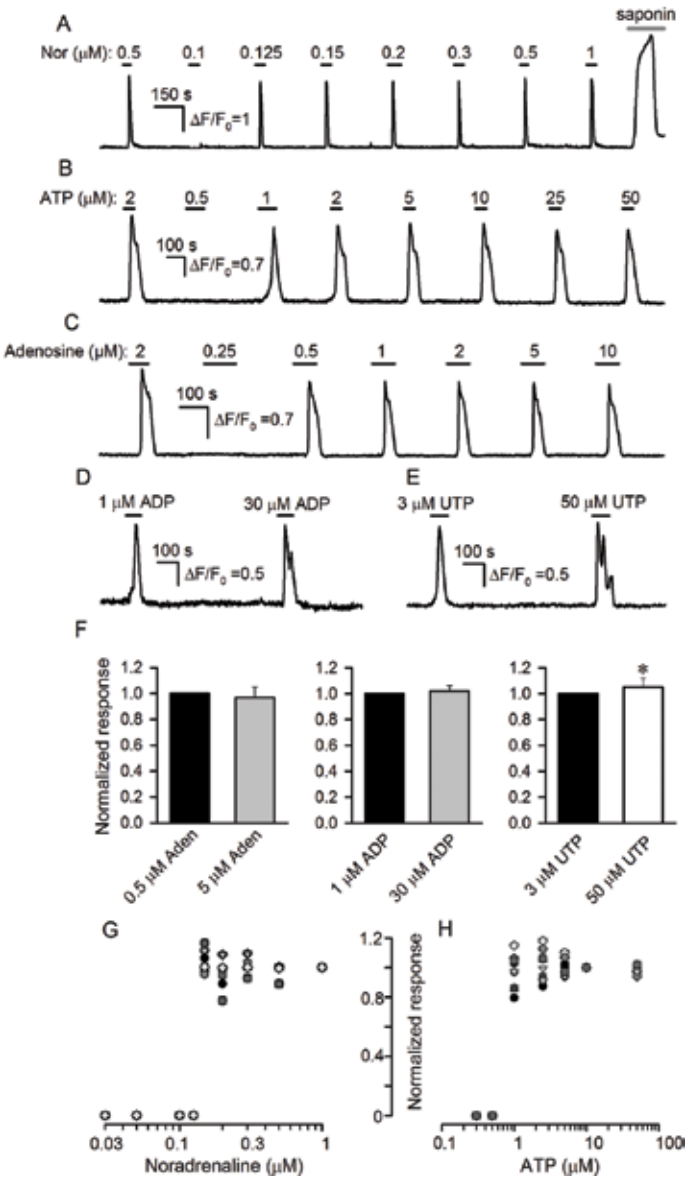

Figure 2. Agonists evoke Ca^{2+} responses in an "all-or-nothing" manner. (A–E) Monitoring of intracellular Ca^{2+} in five different MSCs serially stimulated by noradrenaline (A), ATP (B), ADP (C), UTP (D), and adenosine (E) at varied concentrations as indicated. In A, 0.1 mg/ml saponin was applied (arrow) in the end of the recording to demonstrate that Fluo-4 fluorescence was not saturated by Ca^{2+} bursts elicited by noradrenaline. (F) Summary of MSC responses to adenosine (n = 21; left panel), ADP (n = 16; middle panel) cells, and UTP (n = 11; right panel). For each assayed cell, a response to a particular agonist at low concentration was taken equal 1. The data are presented as mean ± S.D. The difference between responses to adenosine at 0.5 and 5 μM and to ADP at 1 and 30 μM ADP is statistically insignificant (student t-test, $p < 0.05$). The asterisk indicates significant difference ($p < 0.05$) of UTP responses at 3 and 50 μM. (G) Superimposed dose dependences of noradrenaline responses recorded from 10 cells that exhibited the threshold of 150 nM. For each cell, serial noradrenaline responses were normalized to a response to 1 μM noradrenaline. (H) Superimposed dose dependences of ATP responses recorded from eight cells that exhibited the threshold of 1 μM. In each case, ATP responses were normalized to a response to 10 μM ATP. In (G) and (H), each particular symbol corresponds to an individual cell.

was intrinsic for the agonist-dependent Ca^{2+} signaling in general, including purinergic transduction. In particular, submicromolar ATP was ineffective, while the nucleotide elicited Ca^{2+} transients in the MSC cytoplasm at 1–2 μM and higher (**Figure 2B**). The adenosine responses were characterized by the threshold of 0.2–0.3 μM and were similarly shaped at higher concentrations (9 cells; **Figure 2C**). For ADP- and UTP-responses, the threshold concentrations ranged within 0.5–2 and 3–6 μM, respectively. Although we did not carefully characterize MSC responses to adenosine, ADP, and UTP at widely and gradually varied concentrations, it appeared that dose-response curves for these agonists were also step-like. For example, Ca^{2+} transients of close magnitudes were usually elicited by adenosine at 0.5 and 5 μM (21 cells), ADP at 1 and 30 μM (16 cells), and UTP at 3 and 50 μM (11 cells) (**Figure 2C–F**).

In the case of noradrenaline and ATP, the dose dependence of MSC responses was carefully evaluated in designated experiments, wherein an agonist dose was gradually varied in a wide range of concentrations (**Figure 2A**, **B**). During this prolonged assay, responsiveness of many cells was liable to rundown, thus impeding the quantitative analysis. Overall, we identified 21 cells that generated sufficiently robust responses to noradrenaline at 30 nM–10 μM with the threshold of 100–200 nM. Among them, 10 cells, which exhibited the same threshold of 150 nM, were taken for the analysis. To compare different experiments, responses of each particular cell recorded at variable agonist concentrations were normalized to a response to 1 μM noradrenaline and superimposed as shown in **Figure 2G**, where different symbols correspond to individual cells. Despite some data scattering, normalized cellular responses were localized in the narrow range of 0.8–1.2 (**Figure 2G**), clearly demonstrating that in all cases, the dose dependence was a step-like rather than gradual. Similar inference came from the analysis of 32 ATP-sensitive cells that showed quite robust responses to the nucleotide gradually applied at 0.5–50 μM. Of them, nine MSCs generated rather similar Ca^{2+} signals at gradually increasing ATP doses with the threshold of 1 μM (**Figure 2B**, **H**).

One more notable feature of MSC responses was that Ca^{2+} transients were markedly postponed relative to a moment of agonist application. The characteristic time of response delay (τ_d, **Figure 3A**) gradually decreased with noradrenaline and ATP concentration (**Figure 3B**, **C**). For instance, Ca^{2+} transients triggered by noradrenaline were retarded by 38–55 s at the threshold stimulation (**Figure 3A**, left response), whereas the delay was reduced to 17–26 s at the concentration of 1 μM and higher (**Figure 3A**, right response). The detailed assay of the dose-delay dependence was not carried out for the other agonists. Nevertheless, the comparison of MSCs responses obtained at low and saturated concentrations of adenosine, ADP, or UTP revealed a marked decrease in response delay as the agonist dose raised (**Figure 3D**). As discussed below, two distinct mechanisms are presumably responsible for specific dependencies of the magnitude and delay of MSC responses on agonist concentration.

3.2. Agonist transduction involves the phosphoinositide cascade and Ca^{2+}-induced Ca^{2+} release

In certain experiments, we analyzed coupling of adreno- and purinoreceptors to Ca^{2+} mobilization in the MSC cytoplasm. When MSCs were pretreated with U73122 (2–5 μM), a poorly reversible inhibitor of PLC, all assayed cells became completely nonresponsive to tested agonists, including noradrenaline (17 cells), ATP (39 cells), adenosine (11 cells), UTP (7 cells), and

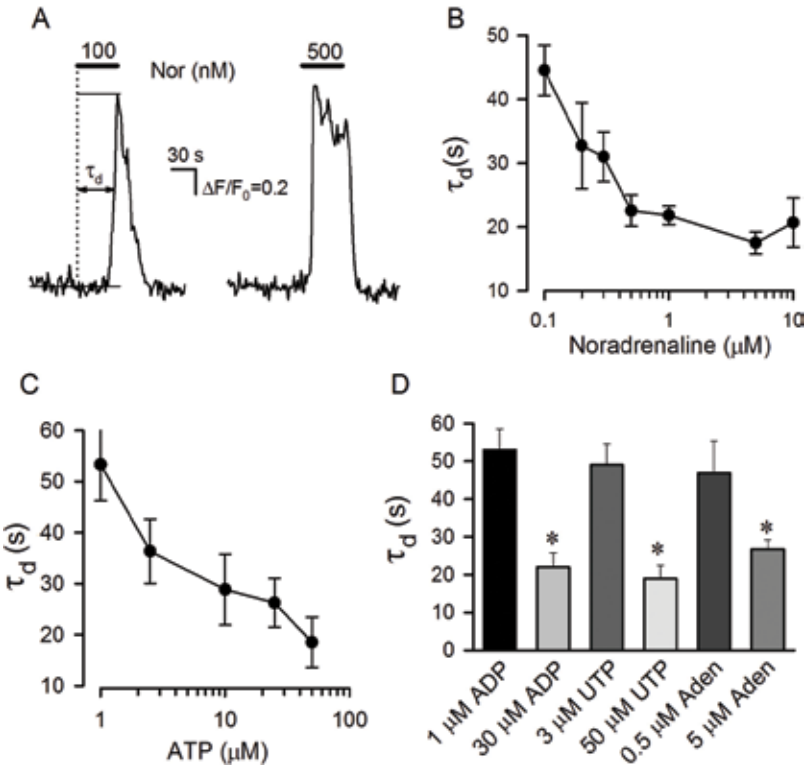

Figure 3. Dose dependence of agonist response delay. (A) Representative Ca^{2+} transients elicited by noradrenaline at 100 nM (threshold concentration) and 500 nM in the same cell. These noradrenaline responses were delayed relative to the moment of agonist application by 55 and 16 s, respectively. The characteristic time of the response delay (τ_d) was calculated as a time interval necessary for a Ca^{2+} transient to reach the half-magnitude. (B, C) Response lag versus noradrenaline (B) and ATP (C) concentration. The data were obtained from 10 adrenergic (**Figure 2A**, **G**) and 8 purinergic (**Figure 2B**, **H**) MSCs. (D) Delay of MSC responses to ADP (n = 16), UTP (n = 11), and adenosine (n = 21) at indicated concentrations. In (B–D), the data are presented as mean ± S.D.

ADP (5 cells) (**Figure 4A–C**, **G–I**). The inhibitory effect of U73122 on MSC responsiveness was apparently specific as the much less effective analog U73343 (2–5 μM) never canceled MSC responses to the nucleotides (**Figure 4A–C**, **G**, **H**). Moreover, the decrease of external Ca^{2+} from 2 mM to 260 nM weakly or negligibly affected Ca^{2+} transients elicited by ATP (26 cells), noradrenaline (31 cells), adenosine (7 cells), UTP (14 cells), and ADP (13 cells) (**Figure 4C**, **D**, **G–I**). Thus, the agonist-stimulated Ca^{2+} signaling in MSCs involved GPCRs that were basically coupled by the phosphoinositide cascade to Ca^{2+} release rather than to Ca^{2+} entry. Note also that the step-like dose dependence of ATP responses (**Figure 2B**, **H**) and their insignificant sensitivity to external Ca^{2+} (**Figure 4G**) indicated that P2X receptors could provide only a weak, if any, contribution to Ca^{2+} signaling triggered by ATP in the MSC cytoplasm.

Given the aforementioned effects of U73122 on MSC responses, there might be little doubt that the IP_3 receptor, a common effector downstream of PLC [30], was involved in transduction of assayed agonist. Expectedly, the IP_3 receptor blocker 2-APB (50 μM) suppressed Ca^{2+} signaling initiated by ATP (21 cells), noradrenaline (19 cells), adenosine (5 cells), ADP (9 cells), and UTP (10 cells) (**Figure 4D–I**)). In contrast, 50 μM ryanodine, a ryanodine receptor

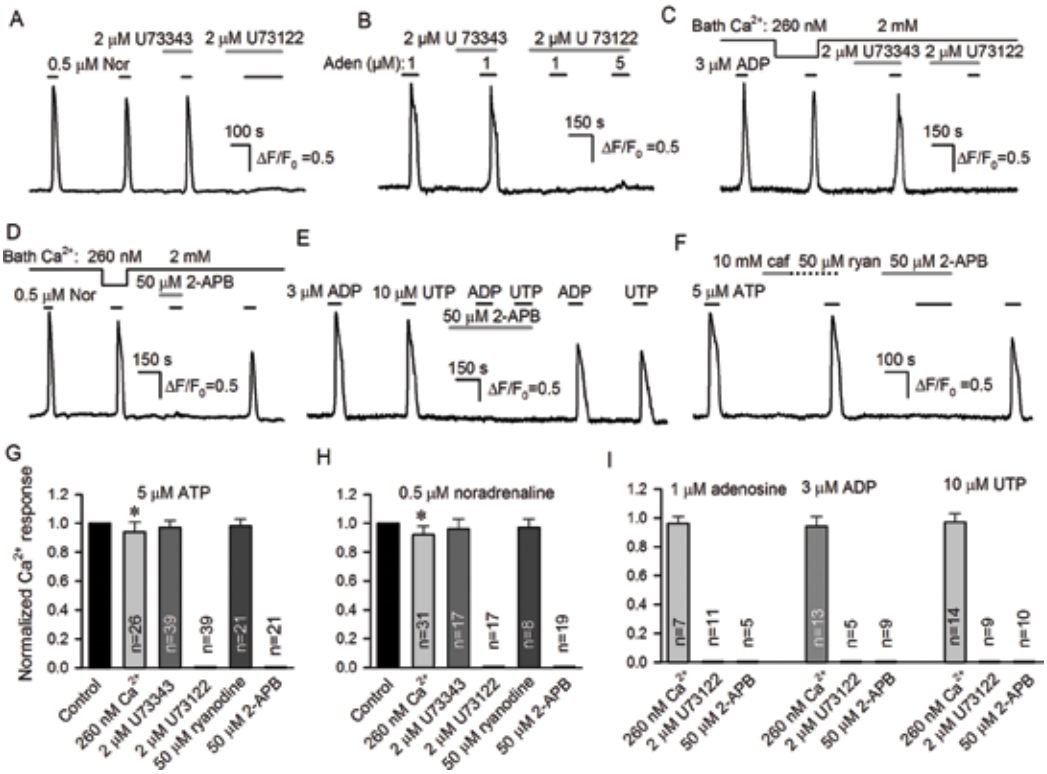

Figure 4. Involvement of the phosphoinositide cascade in agonist transduction. (A–C) PLC inhibitor U73122 (2 μM) suppressed MSC responsivity to different agonists, including 0.5 μM noradrenaline (A), 1 μM adenosine (B), and 3 μM ADP (C), while its much less effective analogue U73343 (2 μM) was ineffective in all cases. (C, D) Reduction of external Ca^{2+} from 2 mM to 260 nM weakly or negligibly affected Ca^{2+} responses to agonists, including 3 μM ADP and 0.5 μM noradrenaline. Extracellular Ca^{2+} was not completely removed because MSCs poorly tolerated prolonged exposure to a Ca^{2+}-free solution. (E, F) IP_3 receptor blocker 2-APB (50 μM) reversibly suppressed MSC responses, particularly, to 3 μM ADP, 10 μM UTP, and 5 μM ATP. (F) Caffeine and ryanodine, an agonist and antagonist of ryanodine receptors, respectively, negligibly affected cytosolic Ca^{2+} and ATP responsivity. (G–I) Summary of effects of indicated compounds and low Ca^{2+} on MSC responses to the tested agonists; n means the numbers of cells assayed in the particular case. The data are presented as mean ± S.D.; the asterisk indicates statistically significant difference (student t-test, $p < 0.05$).

antagonist, was ineffective in all cases (**Figure 4F–I**). These findings suggested a negligible role for ryanodine receptors in agonist transduction. Consistently, their agonist caffeine (10 mM) insignificantly affected cytosolic Ca^{2+} in ATP-responsive MSCs (7 cells; **Figure 4F**). It should be noted that 2-APB blocks not only IP_3 receptors but also a variety of Ca^{2+}-entry channels [31–33]. Given however that MSC responsiveness to P2Y agonists insignificantly depended on external Ca^{2+} and therefore on Ca^{2+} influx (**Figure 4G–I**), we inferred that 2-APB exerted the inhibitory action mainly by targeting IP_3 receptors.

The monotonic and gradual dependence of cellular responses on agonist concentration has been reported for a variety of cellular systems, including those that employ GPCRs coupling to Ca^{2+} mobilization [34–36]. In contrast, Ca^{2+} responses were generated by MSCs in an "all-or-nothing" manner (**Figure 2**). This step-like dose dependence of response magnitude is poorly explicable and apparently inconsistent with the gradual relation between response delay and agonist concentration (**Figure 3**) if agonist transduction involves solely PLC-dependent

production of an IP_3 burst and proportional Ca^{2+} release via IP_3 receptors. To address this problem, we assumed that the agonist transduction occurred in two separated consecutive steps. Initially, an agonist produced a Ca^{2+} signal most likely being small, local, and gradually dependent on stimulus intensity. When exceeding the threshold, this local and poorly resolved Ca^{2+} signal pushed massive Ca^{2+}-induced Ca^{2+} release (CICR) [37–40] to accomplish transduction with a large and global Ca^{2+} signal. By involving the trigger-like mechanism CICR, a cell generates Ca^{2+} responses of virtually universal shape and magnitude at different agonist concentrations above the threshold (**Figure 2**). Rising with agonist proportionally, the initial gradual Ca^{2+} signal reached a CICR threshold for the time that should have shortened with agonist concentration, thus underlying the gradual dose-delay dependence observed (**Figure 3B**, **C**).

To clarify functionality of the CICR mechanism in MSCs and its contribution to agonist responses, we used Ca^{2+} uncaging that allowed for generating as fast and intensive cytosolic Ca^{2+} bursts as necessary for initiating the CICR process. In designated experiments, MSCs were loaded with both Fluo-4 and NP-EGTA. The last is photolabile Ca^{2+} chelator with high affinity to Ca^{2+} (Kd ~ 10^{-7} M), so that in a resting cell (~100 nm free Ca^{2+}), nearly half NP-EGTA molecules are bound to Ca^{2+} ions. The absorption of ultraviolet (UV) light by NP-EGTA disrupts the coordination sphere responsible for Ca^{2+} binding, thus liberating Ca^{2+}ions and producing a step-like increase in cytosolic Ca^{2+} [41]. Because a UV laser we employed for uncaging was in fact a biharmonic light source emitting at 351 and 527 nm, a light stimulus caused an optical artifact that was seen as a marked overshoot in a recording trace of cell fluorescence acquired at 535 ± 25 nm.

In this series, caged Ca^{2+} was released by moderate UV pulses during several seconds to somehow simulate the suggested Ca^{2+} signal initially produced by agonists in the MSC cytoplasm. As illustrated in **Figure 5A**, light stimuli triggered in adrenergic MSCs (n = 33) two fundamentally different types of Ca^{2+} responses. The relatively short, 2-s in the given case, UV pulse produced an optical artifact that was followed by a small Ca^{2+} jump without evident delay (**Figure 5A**, left panel, response 1 and right panel, thick line). This Ca^{2+} signal exhibited exponential relaxation presumably mediated by Ca^{2+} pumps. The sequential 4-s and 6-s UV flashes elicited biphasic Ca^{2+} transients of nonproportional magnitudes (**Figure 5A**, left panel). Indeed, compared to a 2-s UV pulse, one could expect 4- and 6-s light stimuli to liberate nearly twice and three times more Ca^{2+} ions, respectively. Meanwhile, 4-, 6-, and 8-s flashes usually triggered the similar Ca^{2+} transients that exceeded a response to a 2-s pulse by an order of magnitude (**Figure 5A**, left panel). None of the known Ca^{2+}-dependent mechanisms but CICR could amplify and shape an initial Ca^{2+} signal produced by NP-EGTA photolysis in such a way (**Figure 5A**, right panel, response 1 vs. response 2). In addition, the representative cell (**Figure 5A**, left panel) was insensitive to 50 nM noradrenaline but similarly responded to the agonist at 0.5 and 1 μM concentrations. Similar results were obtained with other eight MSCs that tolerated prolonged serial stimulation with both UV and noradrenaline. Note that biphasic cell responses to light and noradrenaline were quite similar by shape and magnitude (**Figure 5A**, right panel, thin line 2 and circled line 3). Interestingly, light responses exhibited the delay that shortened with UV pulse duration (**Figure 5**, left panel). Similar experiments were performed with purinergic MSCs (n = 23) and basically identical results were obtained (**Figure 5B**). These findings support the idea that the delay of agonist responses (**Figure 3**) could be determined by the initial gradual Ca^{2+} signal.

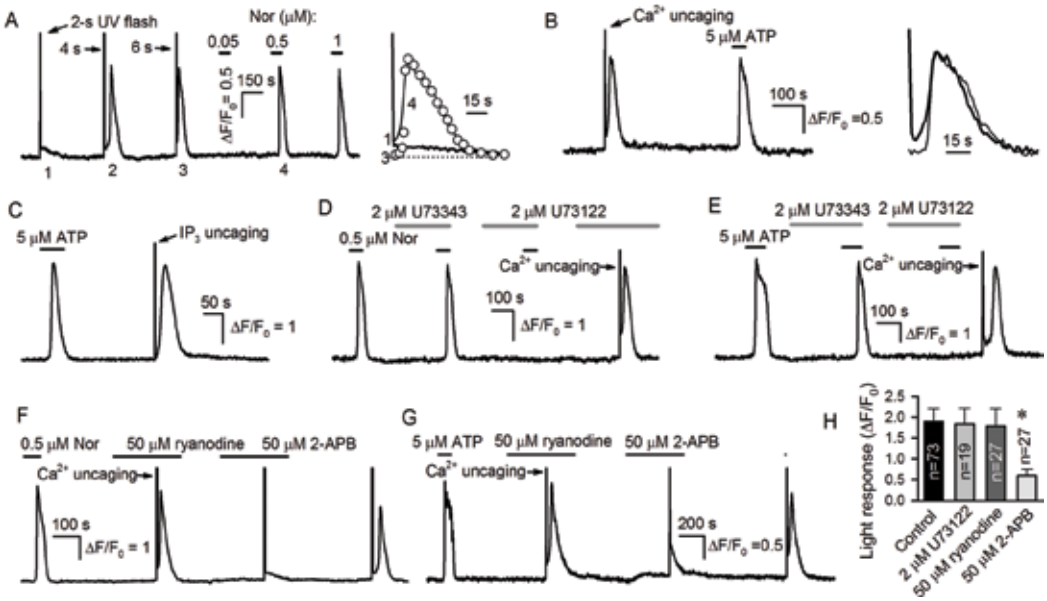

Figure 5. Evidence for Ca^{2+}-induced Ca^{2+} release in MSCs. (A) Left panel—Ca^{2+} transients resulted from Ca^{2+} uncaging in a NP-EGTA loaded cell by UV flashes of varied durations and Ca^{2+} responses to noradrenaline at the indicated concentrations. Right panel—The superimposition of the responses numbered in (A) as 1 (thick line), 3 (circles), and 4 (thin line). (B) Left panel—Cellular responses to Ca^{2+} uncaging produced by a 4-s UV flash and to 5 μM ATP. Right panel—The superimposition of the light (thick line) and ATP (thin line) responses shown in the left panel. (C) ATP (5 μM) and uncaging of IP_3 by a 2-s UV flash elicited similar responses in a cell loaded with caged-Ins(145)P3/PM. (D, E) PLC inhibitor U73122 (2 μM) dumped MSC responsiveness to 0.5 μM noradrenaline (D) and 5 μM ATP (E) but did not prevent agonist response-like Ca^{2+} transients resulted from Ca^{2+} uncaging by 4-s UV flashes. (F, G) 2-APB (50 μM) completely abolished biphasic agonist-like responses to Ca^{2+} uncaging by 4-s UV flashes, while 50 μM ryanodine was ineffective. In the experiments presented in (A–G), emission of a UV laser was weakened by the factor 10, so that Ca^{2+} uncaging should have lasted for 4 s to liberate as many Ca^{2+} ions as necessary for stimulating CICR. This gradual release of caged Ca^{2+} somewhat slowed the rising phase of a biphasic Ca^{2+} transient produced by CICR, thereby making a lag between a UV flash and a light response clearly visible. (H) Summary of effects of 2 μM U73122, 50 μM ryanodine, or 50 μM 2-APB on Ca^{2+} transients elicited by 4-s UV flashes. The data are presented as mean ± SD; the asterisk indicates statistically significant difference (student t-test, $p < 0.05$).

Similar to Ca^{2+} uncaging (**Figure 5A**), uncaging of IP_3 produced agonist-like responses in purinergic (n = 14) and adrenergic (n = 6) MSCs (**Figure 5C**). It was therefore possible that Ca^{2+} uncaging could simulate agonist-like responses by stimulating Ca^{2+}-dependent PLC [42–44], which quickly generated a sufficient IP_3 burst, thereby enhancing activity of IP_3 receptors and triggering CICR. To verify this possibility, several adrenergic (n = 12) and purinergic (n = 7) MSCs loaded with NP-EGTA were subjected to Ca^{2+} uncaging in the presence of U73122. Although this PLC inhibitor expectedly rendered MSCs nonresponsive to the agonists, the cells normally responded to UV flashes (**Figure 5D**, **E**). The ineffectiveness of U73122 (**Figure 5D**, **E**, **H**) provided strong evidence that PLC activation was not obligatory for generating light responses, thereby demonstrating that CICR initiated by UV flashes was directly stimulated by Ca^{2+} ions liberated from NP-EGTA.

Reportedly, ryanodine and inositol 1,4,5-trisphosphate (IP_3) receptors, Ca^{2+}-gated Ca^{2+} release channels operating in the endo/sarcoplasmic reticulum, are exclusively responsible for CICR

in apparently all cells [39, 42, 44]. To evaluate a relative contribution of IP_3 and ryanodine receptors to CICR in MSCs, we examined effects of their antagonists on Ca^{2+} signals associated with Ca^{2+} uncaging. While 50 μM ryanodine was ineffective, 50 μM 2-APB dramatically and reversibly changed a shape and magnitude of UV responses in adrenergic (n = 16) and purinergic (n = 11) MSCs (**Figure 5F–H**). In the presence of 50 μM ryanodine, Ca^{2+} uncaging elicited agonist-like biphasic Ca^{2+} responses that were delayed relative to stimulatory UV flashes (**Figure 5F**, **G**, 2nd responses). Thus, despite the inhibition of ryanodine receptors, Ca^{2+} uncaging was still capable of stimulating robust CICR in MSCs responsive to the agonists. With 50 μM 2-APB in the bath, a UV pulse entailed a brief Ca^{2+} jump that relaxed monotonically and was smaller by the factor 3–4 (**Figure 5F**, **G**, 3rd responses; **Figure 5H**). This indicated that Ca^{2+} uncaging failed to initiate CICR with inhibited IP_3 receptors. Moreover, when 2-APB was removed to restore activity of IP_3 receptors, a UV flash triggered a biphasic Ca^{2+} transient again (**Figure 5F**, **G**, 4th responses). These observations indicated that basically IP_3 receptors were responsible for CICR in adrenergic and purinergic MSCs.

3.3. Adrenoreceptor subtypes involved in Ca^{2+} signaling

Nine human genes encode adrenoreceptors, including α_{1A}, α_{1B}, α_{1D}, α_{2A}, α_{2B}, α_{2C}, β_1, β_2, and β_3 isoforms [45]. Previously, we demonstrated that transcripts for α_{1B}-, α_{2A}-, and β_2-adrenoreceptors were invariably present in total MSC preparations [24]. Given that both α_1- and α_2-adrenoreceptors are routinely coupled to PLC and Ca^{2+} mobilization in different cells [21, 22], either or both of these isoforms might be responsible for Ca^{2+} transients generated by MSCs in response to noradrenaline (**Figure 2A**). In contrast, β_2-adrenoreceptors, which generally involve adenylyl cyclase as a downstream effector [23], could not be an essential contributor to Ca^{2+} signaling in adrenergic MSCs.

To uncover a role of the particular isoform, we performed recordings using agonists and antagonists specific for α_1- or α_2-adrenoreceptors. Overall, 35 noradrenaline-responsive cells were treated with phenylephrine/cirazoline and prazosin (α_1-agonists and antagonist, respectively) as well as with guanabenz/B-HT 933 and yohimbine (α_2-agonists and antagonist, respectively). Most of them (29 cells, 83%) were irresponsive to phenylephrine (1–10 μM), and their noradrenaline responses were not inhibited by 10 μM prazosin. In contrast, guanabenz (10–50 μM) and B-HT 933 (10 μM) were quite effective (**Figure 6A**). In particular, 50 μM guanabenz stimulated Ca^{2+} signaling in all noradrenaline-responsive MSCs (**Figure 6A–C**). Consistently, 2 μM yohimbine dumped cellular responses to noradrenaline and guanabenz (**Figure 6A**). Six cells (17%) were sensitive to both 10 μM phenylephrine and 50 μM guanabenz (**Figure 6B**, **C**). These findings indicate that the α_2-subtype, evidently α_{2A}, predominantly mediates Ca^{2+} signaling initiated by noradrenaline in MSCs, although in a minor MSC subpopulation, both α_1- and α_2-isoforms could be involved in adrenergic transduction.

3.4. Effects of isoform-specific agonists and antagonists of P2Y receptors

In mammalians, the P2Y subgroup includes eight GPCRs ($P2Y_{1,2,4,6,11–14}$) that exhibit certain specificities to nucleotides, depending on species [18, 46]. The expression of purinoreceptors in MSCs was analyzed previously, and transcripts for multiple P2Y receptors were detected,

namely, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{13}$, and $P2Y_{14}$, while $P2Y_{12}$ transcripts were not detected in total MSC preparations [47]. Although this P2Y array is sufficient to account for MSC capability to detect ATP, ADP, and UTP, it was impossible to evaluate a contribution of a particular P2Y isoform based on MSC responses to these natural P2Y agonists (**Figure 2B**, **E**, **F**). To address this issue, we used isoform-specific P2Y agonists and antagonists.

The human P2Y family contains two ATP receptors, including specialized $P2Y_{11}$ and also $P2Y_2$ that recognizes both UTP and ATP as full equipotent agonists [18]. Although also known as a partial $P2Y_1$ agonist, ATP was hardly capable of stimulating $P2Y_1$-signaling in MSCs at low micromolar concentrations due to much lower efficacy than ADP [48]. We tried to evaluate a contribution of $P2Y_{11}$ and $P2Y_2$ to MSC responsiveness to ATP. Among 181 MSCs assayed in this series, 169 cells (93%) became nonresponsive to ATP (3 μM) in the presence of 30 μM NF 340, a specific $P2Y_{11}$ antagonist. These NF 340-sensitive cells did not respond to the $P2Y_2$

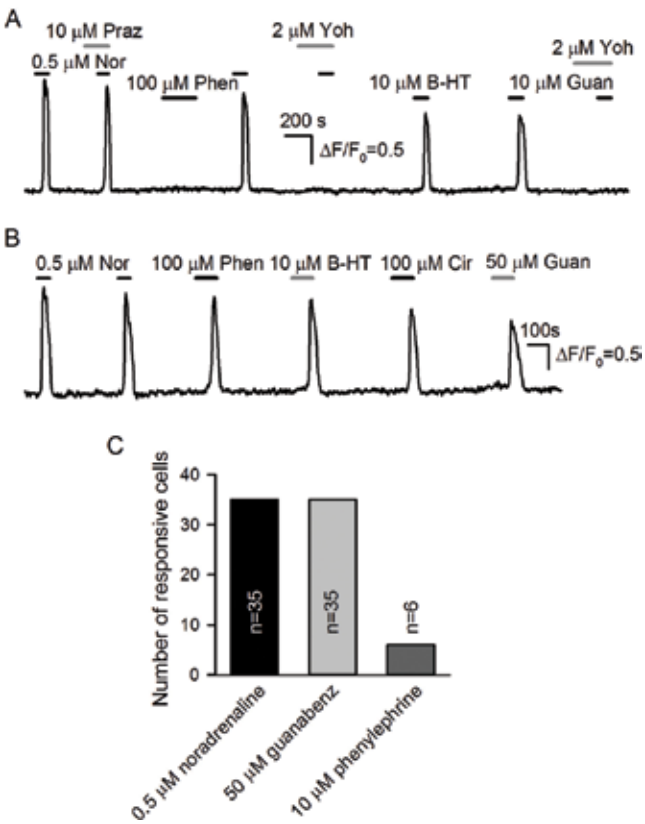

Figure 6. Sensitivity of MSCs to adrenergic agonists and antagonists. (A) In most (83%) of noradrenaline-sensitive MSCs, α2-receptor agonists B-HT 933 and guanabenz stimulated Ca^{2+} signaling in contrast to the α1-receptor agonists phenylephrine and cirazoline that were ineffective. Consistently, Ca^{2+} signaling stimulated in such cells by noradrenaline and guanabenz was canceled in the presence of the α2 antagonist yohimbine, while the α1 antagonist prazosin was ineffective. (B) Small subpopulation (17%) of noradrenaline-sensitive cells responded to both α2 and α1 agonists. (C) Responsiveness of 35 MSCs sequentially stimulated by 0.5 μM noradrenaline, 50 μM guanabenz, and 10 μM phenylephrine.

agonist MRS 2768 (10 μM) (**Figure 7A**, cell 1 and **Figure 7B**). In a subpopulation of rare MSCs (12 cells) that were capable of generating Ca^{2+} transients on 3 μM ATP in the presence of NF 340, 11 cells also responded to 10 μM MRS 2768 (**Figure 7A**, cell 2 and **Figure 7B**). Thus, MSCs that were insensitive to NF 340 presumably employed $P2Y_2$ or both $P2Y_2$ and $P2Y_{11}$ to detect ATP.

While the $P2Y_{11}$ antagonist was highly effective (**Figure 7A**, **B**), most ATP-sensitive MSCs were surprisingly nonresponsive to NF 546 (10 μM), the specific $P2Y_{11}$ agonist reported to be even more effective than ATP [49]. Among 127 cells that responded to 3 μM ATP, 10 μM NF 546 stimulated Ca^{2+} signaling solely in 9 cells (7%; **Figure 7C**, **D**). At the moment, we cannot provide any valid explanation for very low efficacy of NF-546 relative to ATP (**Figure 7D**). Perhaps, this synthetic ligand is a biased agonist that enables coupling of $P2Y_{11}$ to the phosphoinositide cascade by involving only a certain G-protein type, which is absent or relatively less abundant in most of the MSCs.

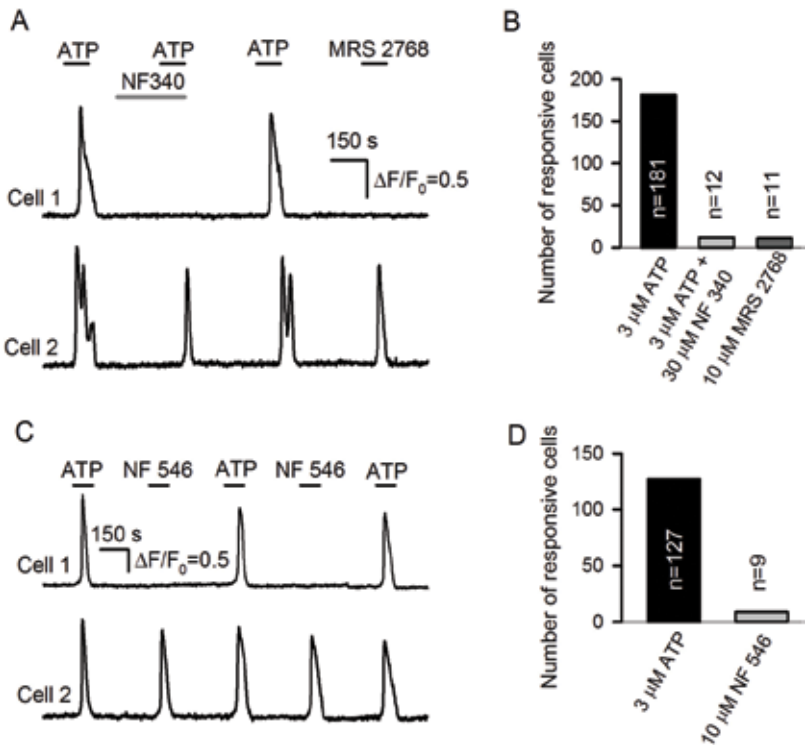

Figure 7. Sensitivity of MSCs to agonists and antagonists of $P2Y_2$ and $P2Y_{11}$ receptors. (A) Representative responses of two concurrently assayed cells to ATP (3 μM) and to the $P2Y_2$ agonist MRS 2768 (10 μM). The great majority (93%) of ATP-sensitive MSCs were rendered nonresponsive by 30 μM NF 340, a $P2Y_{11}$ antagonist, and such cells never responded to 10 μM MRS 2768 (Cell 1). Uncommon cells that remained sensitive to ATP in the presence of 30 μM NF 340 responded to 10 μM MRS 2768 as well (Cell 2). (B) Responsiveness of 181 MSCs to 3 μM ATP and 10 μM MRS 2768 assayed in control and in the presence of NF 340. (C) Representative concurrent recordings from an ordinary cell insensitive to 10 μM NF 546 (Cell 1) and from an occasional cell responsive to this specific $P2Y_{11}$ agonist (n = 127; Cell 2). (D) Responsiveness of 127 MSCs to 3 μM ATP and 10 μM NF 546.

UTP is a full agonist for $P2Y_2$ and $P2Y_4$ that were identified in MSCs at the population level [47]. It therefore was unclear whether a particular cell employs either or both of these P2Y receptors for monitoring extracellular UTP. We analyzed the sensitivity of 95 UTP-responsive MSCs to MRS 2768 and MRS 4062, specific agonists of $P2Y_2$ and $P2Y_4$ receptors, respectively. Consistently with the analysis of ATP-responsive cells (**Figure 7B**), we found only 9 (9.5%) of 95 UTP-sensitive cells to react to 10 μM MRS 2768 (**Figure 8A**, cell 3 and **Figure 8B**).

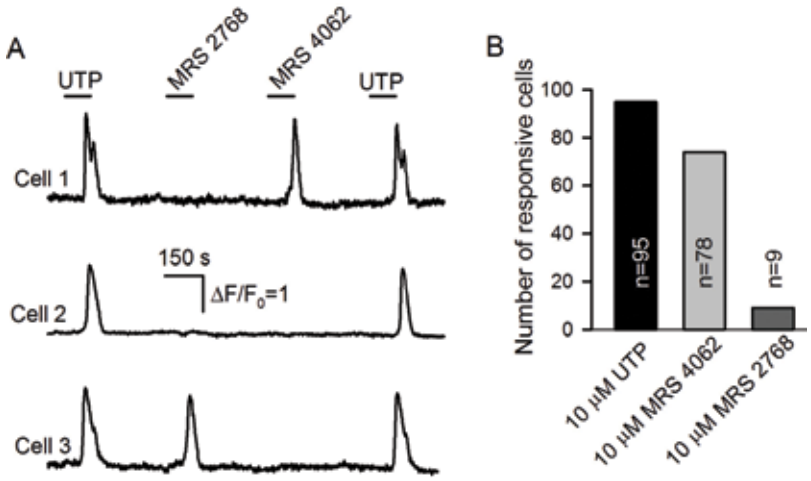

Figure 8. Sensitivity of UTP-responsive MSCs to $P2Y_2$ and $P2Y_4$ agonists. (A) Representative recordings from purinergic MSCs stimulated by UTP (10 μM), MRS 2768 (10 μM), and the agonists of $P2Y_4$ receptor MRS 4062 (10 μM), in series. (B) Responsiveness of UTP-sensitive MSCs (n = 95) to MRS 2768 and MRS 4062.

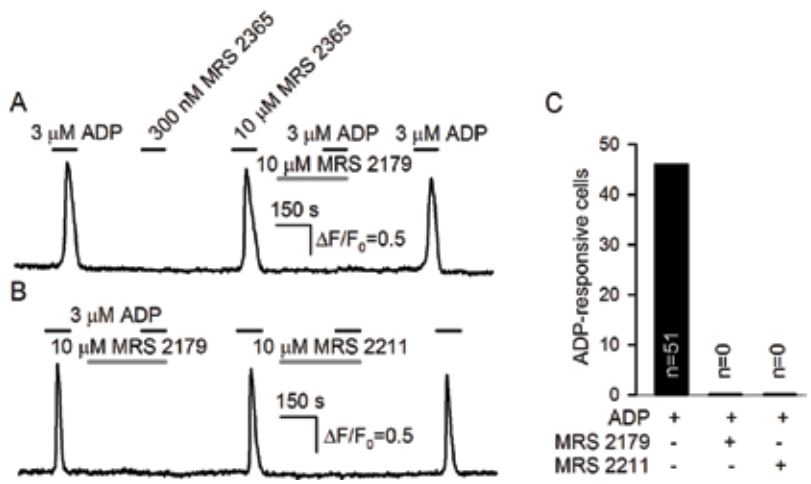

Figure 9. Contribution of $P2Y_1$ and $P2Y_{13}$ to ADP responsiveness. (A) Representative MSC responses to 3 μM ADP and to the $P2Y_1$ agonist MRS 2365 applied at 300 nM and 10 μM. All cells treated with 10 μM MRS 2179 (n = 65) became nonresponsive to 3 μM ADP. (B) When applied alone at 10 μM, antagonists of $P2Y_1$ (MRS 2179) and $P2Y_{13}$ (MRS 2211) inhibited responses of MSCs to 3 μM ADP (46 cells). (C) Summary of responses of 51 MSCs to 3 μM ADP in control and in the presence of MRS 2179 or MRS 2211.

In contrast, 78 cells (82%) responded to 10 μM MRS 4062 (**Figure 7A**, cell 1 and **Figure 7B**). These findings suggested that predominantly $P2Y_4$ was responsible for Ca^{2+} signaling evoked in MSCs by UTP, while $P2Y_2$ was either expressed in a very small subpopulation of $P2Y_4$-negative cells or not coupled to Ca^{2+} mobilization in a great majority of $P2Y_4$-positive cells.

Extracellular ADP is detected by cells with $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$. The analysis of ADP responsiveness was performed on 102 MSCs sensitive to 3 μM ADP (**Figure 8A**) that might be recognized by $P2Y_1$ and/or $P2Y_{13}$ receptors, given that $P2Y_{12}$ transcripts were not found in MSCs. To evaluate a role of the $P2Y_1$, 65 of 103 ADP-sensitive MSCs were treated with MRS 2365, a highly potent and selective $P2Y_1$ agonist that displays no activity at $P2Y_{12}$ and $P2Y_{13}$ at submicromolar concentrations [50]. MRS 2365 was ineffective at 100–300 nM but triggered Ca^{2+} signaling in 16 (25%) of 65 MSCs at 10 μM (**Figure 9A**). Because MRS 2365 specifically stimulates $P2Y_1$ with EC_{50} ~ 1 nM [50], this agonist might bring about a nonspecific action at 10 μM. On the other hand, MRS 2179 (10 μM), a $P2Y_1$ antagonist with IC_{50} = 0.15 μM [49], inhibited ADP responses in all MRS 2365-treated MSCs (65 cells; **Figure 9A**). Given that other P2Y receptors were hardly inhibited by 10 μM MRS 2179 [49], the observed effects of the specific agonist and antagonist of the $P2Y_1$ receptor were rather inconsistent. To reconcile these contradictory findings, we considered the possibility that both $P2Y_1$ and $P2Y_{13}$ should have been activated by ADP concurrently to mobilize Ca^{2+} in MSCs. If so, nanomolar MRS 2365 was ineffective, activating solely $P2Y_1$, while 10 μM MRS 2365 stimulated activity of both $P2Y_1$ and $P2Y_{13}$ [50], thus triggering Ca^{2+} signaling in MSCs. This concept predicted that MSCs would be unable to respond to ADP if either $P2Y_1$ or $P2Y_{13}$ was inhibited. In line with this idea, we assayed sensitivity of 51 ADP-responsive MSCs to both MRS 2179 (10 μM) and MRS 2211 (10 μM), a $P2Y_{13}$ antagonist. It turned out that either of these compounds rendered each of 51 assayed cells nonresponsive to ADP (**Figure 9B**, **C**). Altogether, our findings (**Figure 9A–C**) indicated that only those MSCs, which functionally expressed both $P2Y_1$ and $P2Y_{13}$ receptors, were capable of generating robust Ca^{2+} responses to ADP.

4. Discussion

Virtually in all cell types, extracellular cues can mobilize intracellular Ca^{2+} to regulate a variety of diverse cellular functions, such as fertilization, proliferation, secretion, metabolism, gene expression, mobility, and muscle contraction. How can the Ca^{2+} ion, a chemically simple substance, control so many different physiological processes? The plausible explanation comes from the versatility of Ca^{2+} signaling mechanisms that can mediate Ca^{2+} signals with variable kinetics, amplitude, duration, and spatial patterning, depending on cellular context and stimulation [30, 37, 42].

Transduction of multiple agonists involves GPCRs coupled to $PLC\beta_{1-4}$ isoforms that hydrolyze the precursor lipid phosphatidylinositol 4,5-bisphosphate to produce two second messengers, IP_3 and diacylglycerol. The primary mode of action of IP_3 is to bind to IP_3 receptors and release Ca^{2+} from the endoplasmic reticulum (ER) [30, 51, 52]. Three different isoforms of the IP_3 receptor have been identified (IP_3R1, IP_3R2, and IP_3R3) and shown to serve as a tetrameric IP_3-gated Ca^{2+} channel [30, 51–53]. IP_3R1, IP_3R2, and IP_3R3 are distinct by physiological

properties, thus allowing cells to generate specific Ca^{2+} signals with different spatial and temporal characteristics to control diverse cellular functions [30, 52]. In addition to IP_3, Ca^{2+} is the primary coregulator of IP_3 receptors [30, 51, 52, 54]. The full activation of the IP_3 receptor occurs when IP_3 has occupied the IP_3-binding domains on all four subunits [55]. This is associated with a conformational change, which sensitizes the Ca^{2+}-binding site. The binding of cytosolic Ca^{2+} to this site markedly increases the open probability of the IP_3 receptor channel [54], so that Ca^{2+} ions released from the ER can additionally stimulate activity of IP_3 receptors. This positive feedback mediates CICR. Meanwhile, the action of cytosolic Ca^{2+} is bimodal: stimulating IP_3 receptors at low levels, Ca^{2+} becomes inhibitory above 300 nm [54]. This multimodal control of the IP_3 receptor by IP_3 and Ca^{2+} is central to various aspects of intracellular Ca^{2+} signaling [30, 52].

In the present work, we studied MSCs from the human adipose tissue and examined intracellular Ca^{2+} signaling initiated by certain GPCR agonists, including adenosine, ATP, noradrenaline, and some others. Although all first messengers tested here were effective, only a relatively small MSC group responded to a particular agonist. These specifically responsive cell subpopulations overlapped weakly or negligibly, depending on agonists (**Figure 1**). This finding is hardly surprising in light of a widely accepted idea that a MSC population from different sources represents a heterogeneous mixture of diverse cells, including multipotent and more committed progenitor cells [1, 3, 56, 57]. Yet, cultured MSCs are not synchronized and dwell in different phases of the cell cycle. It therefore might be expected that divergent intracellular signaling is inherent in a MSC population containing both proliferating and quiescent cells. The aforementioned factors could underlie intrinsic heterogeneity of a MSC population discussed previously [56, 57]. It also should be mentioned that most of assayed MSCs were found by us nonresponsive to a particular agonist solely in terms of Ca^{2+} signaling that necessitated coupling of appropriate GPCRs to Ca^{2+} mobilization. Meanwhile, many GPCR isoforms are in fact promiscuous in that they may be coupled to a variety of downstream signaling pathways, depending on G-proteins involved. For instance, the $P2Y_{1,2,4,6,11}$ subtypes of purinoreceptors are canonically coupled by G_q/G_{11} to the phosphoinositide cascade and Ca^{2+} mobilization, whereas $P2Y_{12,13,14}$ control cAMP production by inhibiting adenylyl cyclase through G_i/G_o. The unique capability of $P2Y_{11}$ is to stimulate G_s [18]. In addition, apart from ubiquitous coupling to PLC and adenylyl cyclase, P2Y receptors can also engage effectors such as MAP, PI3, Akt, and PKC kinases; small G-proteins; NO synthase; transactivation of growth factor receptors; and some others [26–29]. Hence, a fraction of MSCs sensitive to a given agonist might be in fact much more abundant than that evaluated by Ca^{2+} imaging (**Figure 1B**, **C**), because the tested compounds could stimulate not only Ca^{2+} mobilization but also other signaling events.

The agonist-dependent Ca^{2+} signaling in MSCs was mostly detailed by us for noradrenaline and certain nucleotides. By using subtype-specific agonists and antagonists, it was shown that mainly a_2-adrenoreceptors mediated Ca^{2+} mobilization triggered by noradrenaline in adrenergic MSCs (**Figure 6**). In purinergic MSCs, presumably $P2Y_{11}$ serves as a primary ATP receptor (**Figure 7**), UTP responsiveness is largely mediated by $P2Y_4$ (**Figure 8**), while both $P2Y_1$ and $P2Y_{13}$ are involved in detecting ADP (**Figure 9**). The responsivity of MSCs to noradrenaline and ATP and apparently to adenosine, ADP, and UTP exhibited a peculiar dose dependence:

undetectably affecting intracellular Ca^{2+} below the cut-off concentration, a particular agonist initiated Ca^{2+} transients that were large and quite similarly shaped at all doses above the threshold (**Figure 2**). In contrast to this step-like dose-response curve, the dependence of response delay on agonist concentration was gradual (**Figure 3**). The inhibitory analysis and Ca^{2+} uncaging approach showed that agonist transduction universally involved the classical phosphoinositide cascade and CICR mechanism (**Figures 4** and **5**) that employed IP_3 receptors rather than ryanodine receptors (**Figures 4E**, **F** and **5F**, **G**).

To reconcile the "all-or-nothing" dose-response curve and gradual dose-delay dependence, we surmised that agonist-evoked Ca^{2+} signaling in MSCs includes two different but coupled stages. Primarily, agonists stimulate IP_3 production, activation of IP_3 receptors, and generation of an initial, presumably local and gradual Ca^{2+} signal. Next, this local Ca^{2+} signal stimulates CICR that produces a global Ca^{2+} signal. Some evidence suggests that the Ca^{2+} store responsible for the initial Ca^{2+} signal may be physically separated from the Ca^{2+} store that provides CICR. Indeed, when cells were overloaded with NP-EGTA due to the twofold excess of NP-EGTA-AM concentration compared to the standard loading protocol (see Methods), a MSC population became poorly sensitive to ATP. However, several UV flashes usually rendered MSC responsive (**Figure 10**). Presumably, overloading with NP-EGTA excessively increased the Ca^{2+}-buffering capacity of the cell cytoplasm, thereby significantly diminishing the initial agonist-induced Ca^{2+} signal and its speed. The photodistraction of NP-EGTA decreased exogenous Ca^{2+} buffer to a physiologically more relevant level, thus recovering MSC responsiveness to ATP. Note that in line with multiple reports, relatively slow Ca^{2+} buffer EGTA is unable to cancel Ca^{2+}-dependent processes mediated by local intracellular Ca^{2+} signals. For instance, 1 mM EGTA does not prevent activation of Ca^{2+}-gated BK channels by Ca^{2+} transients originated by both Ca^{2+} influx via voltage-gated Ca^{2+} channels and Ca^{2+} release stimulated by muscarine [58]. By analogy and based on the observation that Ca^{2+} uncaging was still capable of triggering CICR in MSCs overloaded with NP-EGTA (**Figure 10**), we suggested that NP-EGTA, slow Ca^{2+} buffer [59], could hardly repeal stimulation of IP_3 receptors by Ca^{2+} ions released through this IP_3-gated conduit. If so, the Ca^{2+} store and IP_3 receptor pool mediating CICR should be spatially separated from agonist-dependent machinery that

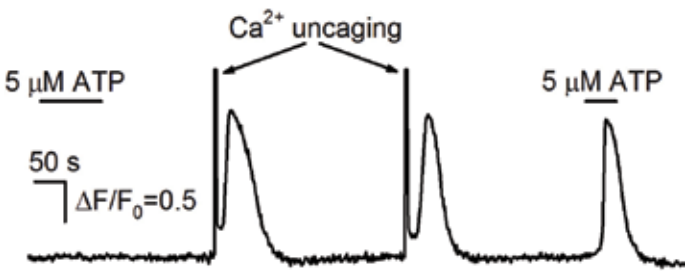

Figure 10. MSCs overloaded with NP-EGTA became nonresponsive to agonists. In this particular experiment, 76 cells, which have been pre-incubated with 4 μM Fluo-4-AM and 8 μM instead of 4 μM NP-EGTA-AM, were simultaneously assayed. None of these cells generated Ca^{2+} responses to the first application of 5 μM ATP. As exemplified by the presented fluorescence trace, two sequential Ca^{2+} uncaging by 4-s UV flashes rendered 7 of 76 cells sensitive to 5 μM ATP. The same phenomenon was observed in two more similar experiments.

generates an initial, local, and gradual Ca^{2+} signal. Otherwise, it is difficult to explain why in cells overloaded with NP-EGTA, agonist responses disappeared contrary to light responses associated with Ca^{2+} uncaging (**Figure 10**).

5. Conclusion

Note in conclusion that the specific features of agonist responses, including kinetics and magnitude, all-or-nothing behavior and gradual dose-response delay were correctly reproduced by Ca^{2+} signals elicited by Ca^{2+} uncaging (**Figure 5**). This supports the idea that agonist-evoked Ca^{2+} signaling in MSCs includes two different but coupled stages. Initially, agonists stimulate coupling of suitable GPCRs via appropriate G-proteins to PLC, thus triggering IP_3 production, activation of IP_3 receptors (IP_3R_{grad}) followed by the release of Ca^{2+} ions from Ca^{2+} store. This machinery generates an initial, presumably local and gradual Ca^{2+} signal (**Figure 11**). When exceeding the threshold, this local Ca^{2+} signal stimulates CICR that is mediated by IP_3 receptors (IP_3R_{CICR}) presumably located in another, spatially separated Ca^{2+} store. By involving the trigger-like mechanism CICR, a cell generates Ca^{2+} responses of virtually universal shape and magnitude at different agonist concentrations above the

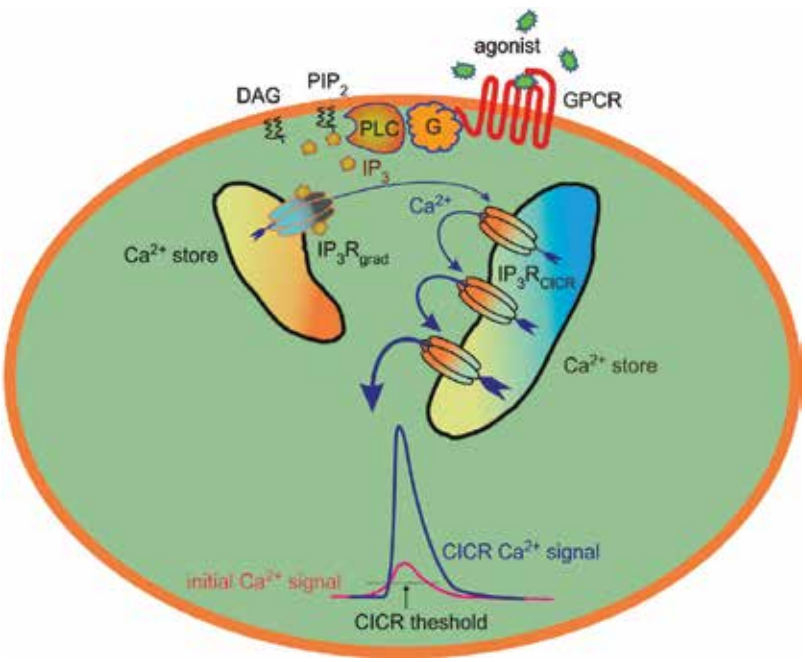

Figure 11. Working model of agonist transduction in MSCs. The binding of agonists to GPCRs stimulates PLC-dependent hydrolysis of PIP_2 to DAG and IP_3. The consequent activation of IP_3 receptors (IP_3R_{grad}) mediates Ca^{2+} release from related Ca^{2+} store, producing an initial Ca^{2+} signal that gradually rises with agonist concentration (red curve). As soon as this signal reaches the threshold level (dotted line), the process determining agonist-dependent delay of a cellular response, one stimulates IP_3 receptors of another type (IP_3R_{CICR}) in separated Ca^{2+} store and triggers CICR. This provides a significant amplification mechanism that finalizes transduction with a large and global Ca^{2+} signal (blue curve).

cut-off dose. Of course, the presented model is a simplification of the actual transduction process, and roles for other common contributors to intracellular Ca^{2+} signaling, including Ca^{2+} pumps, mitochondria, Ca^{2+} buffer as well as Ca^{2+}-dependent enzymes and ion channels, remain to be elucidated.

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Chapter 8

Alterations in Calcium Signaling Pathways in Breast Cancer

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Additional information is available at the end of the chapter

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Abstract

Breast cancer is the second most common cancer in women and the fifth cause contributing to death due to the cancer condition. It is essential to deeply understand the complex cellular mechanisms leading to this disease. There are multiple connections between calcium homeostasis alterations and breast cancer in the literature, but no consensus links the mechanism to the disease prognosis. Among the cells contributing to the breast cancer are the breast telocytes, which connect through gap junctions to other cells, including cancer cells and myoepithelial cells. Multiple proteins (i.e., voltage-gated calcium channels, transient receptor potential channels, STIM and Orai proteins, ether à go-go potassium channels, calcium-activated potassium channels, calcium-activated chloride channels, muscarinic acetylcholine receptors, etc.) coupled with calcium signaling pathways undergo functional and/or expression changes associated with breast cancer development and progression, and might represent promising pharmacological targets. Unraveling the mechanisms of altered calcium homeostasis in various breast cells due to the cancer condition might contribute to personalized therapeutic approaches.

Keywords: breast cancer, human breast stem cells, human breast epithelial cells, human breast myoepithelial cells, human breast adipocytes, human breast telocytes, calcium homeostasis alterations

1. Introduction

Breast cancer is the most common and the most frequently diagnosed cancer and is one of the most lethal malignant lesions in women worldwide, being the second leading cause of cancer death in women, after lung cancer [1].

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1.1. Cell subtypes in human breast cancer and the alterations in calcium homeostasis

The mammary gland is an exocrine, compound tubuloalveolar gland [2]. Each mammary gland has 15–20 glandular lobes in its structure, each lobe being a separate gland with its excretory channel (galactophore channel) that opens at the level of the nipple via the galactophore pore [3]. The glandular lobe consists of glandular lobules delimited by fibrous connective tissue and fat. Lobules have a radiant arrangement, each opening through a lactiferous duct in the nipple, presenting a dilation called lactiferous sinus before opening. Lobules are composed of parenchyma and stroma, and made of loose connective tissue [4]. Each lobule consists of alveoli, lined by cuboidal epithelial cells, which secrete milk, and lactiferous ducts, lined by columnar epithelium, both epithelia surrounded by an outer layer of myoepithelial cells. The stromal cell population is composed of mesenchymal cells, including adipocytes, fibroblasts, and immune cells.

1.1.1. Human breast stem cells

The mammary gland structures are capable of self-renewal due to the presence of mammary stem cells (MSCs) and multipotent adult stem cells. Mammary stem cells lead to the growth of the mammary gland during puberty and are responsible for its further development during pregnancy. Besides their intrinsic self-renewal capacity, normal stem cells call for the adoption of other additional mechanisms to protect them from the microenvironmental pressure that may overdrive the stem cell pool [5]. MSCs are able to reconstitute a completely functional mammary gland upon orthotopic transplantation [6]. MSCs can differentiate into mature epithelial cells of either myoepithelial or luminal lineage through a series of lineage-restricted progenitor intermediate cells. Of major importance is also the long-term survival and expansion of MSCs [7] and the additional accumulation of genetic and/or epigenetic alterations in those cells that may increase the susceptibility of neoplastic transformation [8]. Van Keymeulen et al. demonstrated that the mammary gland contains different types of long-lived stem cells [9].

In breast cancer, both stem cells and progenitor cells are a potential candidate for tumorigenesis, and this could underpin the extraordinary phenotypic heterogeneity of malignant breast tumors. Also, different breast tumor subtypes might be linked to distinctive mammary stem cells and progenitor cells within the mammary epithelia as suggested by different studies [10]. Understanding how these cell subpopulations influence the normal epithelial differentiation hierarchy and the development of mammary tumors could have a significant impact on the proper taxonomy of these lesions.

Unlike the mouse counterparts, the detection of human breast stem cells may be difficult due to the lack of reliable surface makers. Also, the profile of MSCs is less predictable compared to mouse models [11].

To identify MSCs, several studies have employed multiple methods, including: 5-bromo-2-deoxy-uridine (BrdU) label-retention studies, nonadherent mammosphere cultures, cell-surface markers, such as Sca1 and CD49f, labeling MSCs with lipophilic fluorescent dye PKH26 during mammosphere growth and Hoechst dye efflux [12, 13]. These methods were

very helpful for the further detection and characterization of signal transduction pathways such as the Notch, Wnt, and Hedgehog pathways that may be crucial for the self-renewal and fate determination of MSCs.

Identification of breast cancer stem cells is strictly dependent on cell-surface markers. Several markers have been proposed such as hyaluronan receptor (CD44), signal transducer CD24 (CD24), CD133 (Prominin-1), integrins CD29 (β1) and CD49f (α6), aldehyde dehydrogenase-1 (ALDH1), as tumor-initiating cells in breast cancer progression with high metastatic potential and in high-grade tumors resistant to therapeutic treatments [14–17]. However, currently, there is no agreement regarding the phenotypic characterization of breast cancer stem cells. In addition to this discord, the great heterogeneity of breast tumors reflected by a myriad of histological subtypes with variable clinical presentations and diverse molecular signatures also contributes to this major shortcoming. The intrinsic molecular taxonomy describes five major subtypes of breast cancer (luminal-A, luminal-B, basal-like, HER2, and normal-like) which overlap with various clinicopathological classification systems and correlate with clinical behavior being vital for patient's management. In addition, different breast cancer stem cells phenotypes have been described contributing to the proper characterization and nomenclature of breast malignant lesions. Several immunohistochemical markers have been characterized showing that the prevalence of stem cell-like markers varies according to tumor histological subtype [18].

In the light of these facts, some studies have proposed ALDH1 as an independent prognostic marker in breast cancer. Ginestier et al. showed a prevalence of 30% for ALDH1 positivity in a cohort comprising 577 breast tumors from two independent tumor sets. They also showed that ALDH1 expression correlates with a high histological grade, human epidermal growth factor receptor type 2 (HER2) overexpression, and absence of estrogen receptor and progesterone receptor expression [19]. A similar study [20] highlighted the worst prognosis of breast cancer patients with ALDH1 expression. There is no agreement in this matter, as other studies fail to find these correlations [21] even in more aggressive breast tumor subtypes such as inflammatory breast cancer.

With all these conflicting results, the reliability of ALDH1 expression as a clinical prognostic factor is doubtful, thus increasing the need for a standard protocol and more rigorous evaluation criteria, as well as consideration of the dissimilarity between whole-tissue staining versus tissue microarray staining [22].

Alterations in calcium homeostasis frequently occur in some pathological conditions such as malignant proliferation and could have a key role to play in the near future of some targeted therapeutic approaches. Some recent studies have shown that exposure of breast cancer cells to chemotherapy (i.e., carboplatin) induces Ca^{2+} release and leads to an enrichment of breast cancer stem cells [23]. Lu et al. have documented that chemotherapy induces the expression of glutathione S-transferase omega 1 (GSTO1), a factor which is dependent on hypoxia-inducible factor 1 (HIF-1) and HIF-2. In turn, low level of GSTO1 revokes carboplatin-induced breast cancer stem cell enrichment, decreasing tumor initiation and metastatic potential and delaying tumor recurrence after chemotherapy. The authors also found that GSTO1 interacts with the ryanodine receptor (RYR1) and increases calcium release from the endoplasmic reticulum. In this manner, high levels of cytosolic calcium activate proline-rich tyrosine kinase 2 (PYK2)/tyrosine-protein kinase (SRC)/signal transducers and activators of transcription factors 3

(STAT3) signaling pathways, leading to an increased expression of pluripotency factors and breast cancer stem cell enrichment. Concurrent HIF inhibition blocks chemotherapy-induced GSTO1 expression and breast cancer enrichment [23]. The authors have concluded that these combining effects may improve clinical outcome in breast cancer patients.

Not just chemotherapeutic agents are responsible for the release of free intracellular calcium. Petrou et al. investigated the effect of several ion channel modulators such as amiodarone, dofetilide, furosemide, minoxidil, loxapine, and nicorandil in prostate and breast cancer cell lines, PC3 and MCF7, respectively and found that in all investigated cases, calcium levels were increased by modulator concentrations comparable to those used clinically [24]. However, the way these modulators act on breast cancer stem cells remains unknown.

Calcium-and integrin-binding protein (CIB1) depletion impairs cell survival and tumor growth in triple-negative breast cancer by inducing genetic programs that reduce proliferation and survival and mediate differentiation and cancer stem cell function and epithelial to mesenchymal transition [25]. The authors also observed an almost complete cell death in MDA-468 cells after extended CIB1 depletion, suggesting that CIB1-depleted cells do not become stem cells, but rather gain some stem-like features as they are dying.

1.1.2. Human breast epithelial cells

The lactiferous ducts spread into the breast through a series of branches decreasing in caliber from the nipple to the terminal ductal-lobular units (TDLU) that are surrounded in specialized, hormonally responsive stroma. Extralobular ducts are lined by columnar epithelium that is supported by myoepithelial cells and a basement membrane composed of elastic fibers. The epithelium of the luminal duct can give rise to ductules, and fully formed lobules can originate directly from these anatomic structures in the nipple or at deeper levels of the mammary ductal system [26]. The lobules that consist of groups of alveolar glands encompassed by specialized vascularized stroma are connected by intralobular ductules that combine to form a single TDLU that drains into the extralobular ductal system.

The normal microscopic appearance of the lobules is not constant because the structure and histological appearance of the lobule in the mature breast are subject to individual changes associated with the menstrual cycle, pregnancy, lactation, exogenous hormone administration, therapy, aging, and menopause. The inactive lobular glands are lined by a single layer of cuboidal epithelial cells supported by underlying, loosely connected myoepithelial cells. The intralobular stroma contains more capillaries and is less densely collagenized than the interlobular surrounding stroma. Immunoreactivity for hormone receptors (ER and PR) is also variably expressed in lobules in a checkerboard staining pattern: the intensity and frequency of staining varies considerably from patient to patient and in many individual slides from one area to another. ER PR are sporadically positive in normal luminal epithelial cells, but some areas are, however, completely negative for those receptors. ER, PR, and AR are almost always negative in myoepithelial cells.

The 3D cultures of human breast epithelial cells have been designed in order to mimic the normal and pathological tissue architecture [27].

The regulation of signaling pathways and homeostasis of free intracellular Ca^{2+} can entail many cellular and physiological consequences, which may lead to changes in Ca^{2+} levels during lactation [28]. The Ca^{2+} influx has a decisive part in determining the concentration of Ca^{2+} in breast epithelial cells. Breast glandular proliferation, differentiation, and lactation are regulated by several local and systemic hormones, of which estrogen is one of the most important hormones. The regulators of estrogen and its receptor are modulators of proliferation and differentiation of breast epithelial cells [29]. The effect of estrogen on epithelial breast cells is done mainly through genomicpathways, but nongenomic mechanisms are particularly dependent on Ca^{2+} signaling [30].

Some studies on the MCF-7 breast cell line concluded that breast epithelium proliferation is influenced by Ca^{2+} through activation of mitogen-activated protein kinase (MAPK) by 17β-estradiol [31]. It is known that several Ca^{2+}-related proteins can cause changes in cellular functions, leading to many breast lesions, including cancer and hypercalcemia-related malignancy, which have a poorer prognosis and have often a more aggressive nature been associated with metastasis [32]. The way calcium is involved in the differentiation of breast epithelial cells is closely dependent on vitamin D3. By modulating Ca^{2+} metabolism, vitamin D3 plays a crucial role in the regulation of cell proliferation and differentiation [33].

Various epidemiological studies [34, 35] suggest that vitamin D3 deficiency might increase cancer incidence, but no spontaneous tumors have been reported in mice models lacking 1,25(OH)2D3 or deficient in its receptor until recently [36]. The authors observed, for the first time, diverse types of spontaneous tumors in vitamin-D3-deficient mice for more than 1 year of age. The authors concluded that the tumors developed due to increased oxidative stress, cellular senescence, and senescence-associated secretory phenotype molecules, such as hepatocyte growth factor, mediated via its receptor c-Met. As such, vitamin D3 prevents tumorigenesis by inhibiting oxidative stress and inducing tumor cellular senescence in mice, and the study provides direct evidence supporting the role of vitamin D deficiency in increasing cancer incidence.

Calcium levels play an important role in mitochondria-induced apoptosis and epithelial breast cell necrosis [37], and reduction of Ca^{2+} content in the endoplasmic reticulum lumen is associated with resistance to apoptosis [38]. The release of calcium from the endoplasmic reticulum can be triggered by different molecules, even natural ones like resveratrol, a product commonly found in grapes. Resveratrol triggers the release of calcium from the endoplasmic reticulum, which in turn activates the calpain protease that ultimately leads to degradation of the plasma membrane by calcium-dependent ATPase isoform 1 [39].

Human breast epithelial cells with stem-like phenotype have been also demonstrated to be sensitive to the pathophysiological changes in calcium metabolism. To date, Wang et al. showed that how antioxidant medium is superior in terms of prolonged growth for normal breast epithelial cells that expressed stem cell phenotypes. The characteristics of these mammary stem cells include the deficiency in gap junctional intercellular communication, expression of Oct-4, and the ability to differentiate into basal epithelial cells and to form organoid showing mammary ductal and terminal end bud-like structures [40]. Their study concluded that using this new method of growing breast cancer epithelial cell with stem cell phenotype

in a medium with low calcium levels and antioxidants will be of real use in the future studies of mammary development, breast carcinogenesis, chemoprevention, and cancer therapy.

1.1.3. Human breast myoepithelial cells

Myoepithelial cells lie between the epithelial cell layer and the basal lamina, where they establish a network of slender processes covering the overlying epithelial cells. The branching cytoplasmic network of the myoepithelial cell processes can be seen especially in scanning electron micrographs [41]. Spindle-shaped ductal myoepithelial cells lie parallel to the long axis of the duct and form a continuous layer. Contraction of myoepithelial cells in lobules and around ducts contributes to the flow of milk during lactation. The location of the myoepithelial cells between luminal epithelial cells and the basal lamina is an ideal location for them favoring communication with both compartments.

The histologic appearance and immunoreactivity of myoepithelial cells are highly variable, especially in pathologic conditions, and depend on the degree to which the myoid or epithelial phenotype is accentuated in a particular situation. Myoepithelial cells usually display nuclear reactivity for p63, which is the most useful marker for detecting these cells in normal and lesional tissues. Epithelioid myoepithelial cells can have reduced p63 reactivity. Other useful myoepithelial markers are α-smooth muscle actin, calponin, CD10, CKS/6, myosin, p75, and S100 (**Figure 1**, *left*). The presence or absence of myoepithelial cells, at least as demonstrated by routinely used immunostains and is very valuable in discriminating against neoplastic in situ lesions (**Figure 1**, *right*) versus malignant, infiltrative ones.

While the luminal epithelial cell has received plenty of attention as the most functionally active milk-producing cells and as the most probable target cell for tumorigenesis, attention on myoepithelial cells has begun to grow with the acknowledgment that these cells play an active role in branching morphogenesis and tumor suppression.

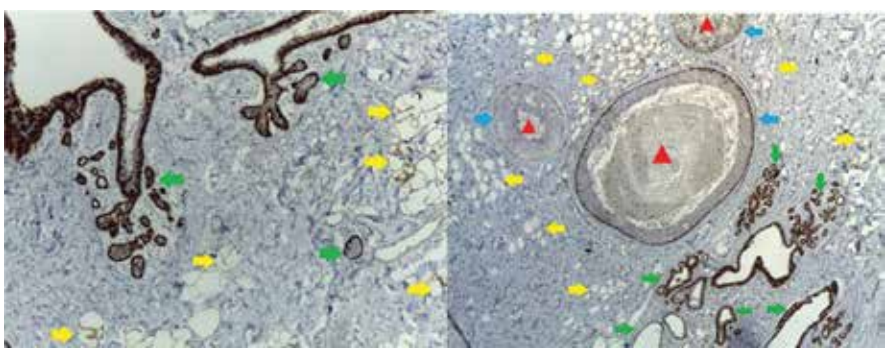

Figure 1. (Left) Immunostain for S100 highlighting the myoepithelial cells layer in a normal terminal ductal lobular unit (TDLU)—green arrow and some adjacent adipocytes—yellow arrow showing strong nuclear and cytoplasmic positivity (immunostain S100 with DAB chromogen, 100× magnification). (Right) Immunostain for S100 highlighting the intact myoepithelial cell layer (blue arrow) at the periphery of some foci of high-grade ductal carcinoma in situ (DCIS) with central comedo-type necrosis (red triangle). Also, note the adjacent normal TDLU (green arrow) and adipocytes (yellow arrow). Immunostain S100 with DAB chromogen, 40× objective.

The function of myoepithelial cells is strongly dependent on regulation of intracellular calcium. These cells contract in response to oxytocin secretion during lactation to generate the contractile force required for milk ejection. It is difficult to understand whether the alteration of calcium metabolism of myoepithelial cells plays a role, if any, in carcinogenesis. Even if it has been demonstrated that store-operated Ca^{2+} entry was mediated by a functional Orai3 in estrogen receptor-expressing (ER^{+}) breast cancer cells [42], the tumorigenesis impact of these findings on myoepithelial cell remains largely unknown, because it is known that most breast carcinomas originate in the epithelial cells and the spectrum of myoepithelial proliferative breast lesions in scarce.

However, disruption in calcium metabolism may alter the functionality of myoepithelial cells. On this issue, some studies have shown the important role of Orai1 store-operated calcium channels in lactation [43]. Davis et al., using genetically modified mouse models, observed that the store-operated Ca^{2+} channel Orai1 delivers over 50% of the calcium ions present in the secreted milk. They also demonstrated the role of Orai1 as a principal regulator of oxytocin-mediated alveolar unit contractility, milk ejection, general myoepithelial function, and survival.

S100 protein is expressed in myoepithelial cells. The S100 gene family is a Ca^{2+}-binding protein with low molecular weight [44]. The members of the S100 family have a myriad of cell functions such as cell proliferation, apoptosis, differentiation, cancer invasion, and metastasis. S100A2 is involved in breast tumorigenesis being downregulated in some cases, which led to the invasion of breast cancer cells [45]. The S100A4 expression is associated with tumor progression and metastatic potential [46]. In a similar manner, S100A7 is not only overexpressed in high-grade ductal breast carcinoma but also in in situ high-grade lesion (DCIS), and some studies suggested that the concomitant expression of S100A7, S100A8, and S100A9 in a class of breast cancers was associated with poor prognosis [47].

1.1.4. Human breast adipocytes

Apart from the epigenetic and genetic changes that occur within epithelial cells leading to breast proliferative lesions, it has shown that tumor initiation and progression also depend on the intricate intercellular dialog between tumor epithelial cells and the surrounding stromal cells [48]. Among the different cell types comprised in the breast stroma, the most abundant are those of adipose origin, mainly mature adipocytes, preadipocytes, and adipose-derived stromal/stem cells (ASCs). Besides the structural role that breast tissue has, it also has an important bioactive function [49, 50]. In pathologic condition, it is of great importance the interaction that is established between tumor cells and stromal adipocytes within the invasive front characteristic of breast cancers [51, 52]. Similar to breast myoepithelial cells, breast adipocytes also express s100 protein—**Figure 1** [53].

The role of calcium metabolism in such events is poorly understood, but some studies have found that some proteins such as calpains [54] and calpastatin [55] may be altered in adipose-derived stromal cells being responsible for their enhanced invasion potential.

Vitamin D3 triggers apoptosis in breast cancer cells and adipocytes by inducing an apoptotic signal by increasing the concentration of intracellular Ca^{2+}. This signal acts as an apoptotic

initiator that bluntly recruits calcium-dependent apoptotic effectors such as calpain and caspase 12, in both breast cancer cells and adipocytes. Some studies suggested that inducing apoptosis with vitamin D3, particularly in the tumor-surrounding adipose tissue involved in tumor progression, can contribute to the antitumoral effects of this hormone and may be of real therapeutic interest to include calcium-dependent apoptotic proteases as molecular targets for new therapeutic and preventive agents in breast cancer and obese patients [56].

1.1.5. Human breast telocytes

Recently, several papers were describing the presence of a new cellular type—the telocytes (TCs)—in the stroma of the mammary gland [57–59]. TCs are characterized by a small cellular body and extremely long telopodes with alternative regions of dilations called podoms and veil-like cytoplasmic extensions called podomeres [60–62]. Although there are numerous attempts to differentiate these cells from other cellular types such as fibroblasts, endothelial cells, mesenchymal stem cells, immune cells, a specific immunohistochemical marker was still not found [63]. The most specific markers, which are nowadays used for their identification, are CD34 and PDGFR alpha or beta [64, 65]. Genomic and proteomic approaches were also used to determine their uniqueness and have shown that telocytes are distinct from the other types listed above [66–70].

Mou et al. investigated the immunohistochemical characteristics and potential functions of TCs in reconstituted breast cancer tissue and found that they express c-kit/CD117, CD34, and vimentin. A very interesting observation is that TCs communicate with breast cancer cells as well as with other stromal cells [58, 71, 72]. Together with other stromal cells, TCs inhibited the breast cancer cell apoptosis and facilitated their proliferation and the formation of typical nest structure assembly in breast cancer, in vitro [58].

Rusu et al. described in an immunohistochemical study some CD34+/CD10±/c-kit-/vimentin- cells found in the inter- and intralobular stroma, which they considered to be TCs and suggested a stem cell-like features based on the expressed markers and changing phenotype [59]. Although a lot of studies are needed to talk about a certain function of TCs, a possible contribution to the mechanisms of carcinogenesis is not negligible, by the modification of the tumor microenvironment. As our team previously showed, TCs are not pacemakers but modulate the activity of the surrounding cells using calcium signaling [73]. Our results showed that uterine TCs express T-type calcium channels that might play a role in the generation of endogenous bioelectric signals responsible for the regulation of the surrounding cell behavior [74, 75].

2. Calcium signaling alterations in breast cancer

The most important proteins coupled with calcium signaling pathways and that have been described as key players in breast cancer cells are summarized in **Figure 2**.

2.1. Resting potential and calcium oscillations in breast cancer

The resting potential of human breast adenocarcinoma cells (estrogen receptor-positive MCF7 and triple-negative MDA-MB-231) is more positive (with approximately 27–30 mV) than normal

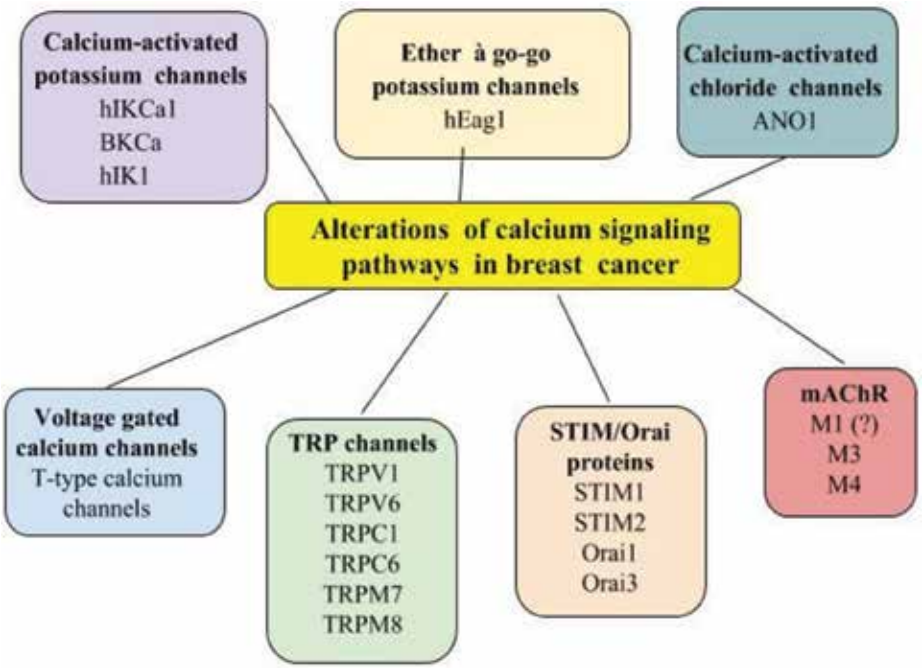

Figure 2. Proteins associated with calcium signaling pathways that have been described to undergo alterations in breast cancer cells.

human mammary epithelial cells [76]. Additionally, breast cancer cells react distinctly with respect to normal mammary epithelial cells in response to the changes in the extracellular ions (e.g., K^+ or Ca^{2+}) [76]. The differences in resting potential between breast cancer cells and normal cells may be useful in the development of anticancer-targeted therapies based on charged liposomes, which are considered among the promising liposome-based therapeutical approaches [77].

MDA-MB-231 breast cancer cells with high-metastatic potential also exhibit spontaneous Ca^{2+} oscillations in comparison with MCF7 breast cancer cells with a low-metastatic potential [78]. Interestingly, these spontaneous oscillations were absent in breast cancer cells with a low-metastatic potential, even in increased extracellular K^+ concentration conditions that determined the augmentation of their basal Ca^{2+} level [78]. This feature of presenting spontaneous calcium oscillations in metastatic cells might be further exploited in understanding the cellular mechanisms standing behind and in finding adequate therapies.

2.2. Voltage-gated calcium channels in breast cancer

Patch-clamp studies on human mammary epithelial cells (HMEC) indicated the absence of voltage-gated calcium currents [79].

The contribution of T-type calcium channels to breast cancer was investigated in several studies. To date, the expression of the α1H subunit, but not the α1C or α1G subunits, of the voltage-gated calcium channels was demonstrated in MCF-7 breast cancer cells [80]. Among T-type calcium channels, Cav3.1, but not Cav3.2, was demonstrated to play an important

role in the inhibition of proliferation and apoptosis in MCF-7 human breast cancer cells [81]. Patch-clamp recordings demonstrated the presence of T-type voltage-gated calcium currents (ICaT) in MCF7 breast cancer cells [79].

A meta-analysis of public microarray datasets in clinical cancer tissue samples identified the upregulation of several VGCCs transcripts (e.g., *CACNA1C*, *CACNA1D*, *CACNA1B*, *CACNA1G*, and *CACNA1I*) in breast cancer and their involvement in the development and cancer progression [82]. Oppositely, another meta-analysis indicated the downregulation of the same VGCCs transcripts in breast cancer [83].

2.3. Transient receptor potential channels in breast cancer

Transient receptor potential (TRP) channels have been documented to play an important role in the development and progression of cancer. TRPC1, TRPC6, TRPM7, TRPM8, and TRPV6 channels were described to be upregulated in human breast ductal adenocarcinoma in comparison with the adjacent nontumoral tissue, and correlations with the proliferative parameters or the invasiveness cell capacity have been evidenced [84]. Moreover, several studies documented the role of TRP channels in different breast cancer cell lines. In detail, TRPM7 was demonstrated by silencing experiments to contribute to the migration and invasiveness of MDA-MB-435 breast cancer cells by signaling through the MAPK, but not through Akt, pathway [85]. TRPM8 was detected in BT-474 and MDA-MB-231, but not in MCF7, breast carcinoma cells, while menthol-evoked Ca^{2+}-oscillations were recorded in all three breast cancer cell lines [86]. TRPV6 had a high level of expression in breast adenocarcinoma tissue [87]. TRPV1 is functionally expressed in SUM149PT breast cancer cells, a model for a very aggressive form of breast cancer (i.e., triple-negative breast cancer) [88], and in MCF7 breast cancer cells [89, 90].

2.4. STIM and Orai proteins in breast cancer

In nonexcitable cells, such is the case of breast cells, both store-operated calcium entry (SOCE) and store-independent calcium pathways have been described, which involve the activation of stromal interaction molecules (STIM) and Orai proteins.

STIM/Orai proteins play an important role in the breast physiology. To date, in lactation, Orai1 is upregulated, while Stim1, but not Stim2, is downregulated [91]. Moreover, calcium-influx through Orai1 was described to be necessary for mammary epithelial cells for concentrating milk with Ca^{2+} and for milk expulsion through alveolar unit contraction [91].

In cancer metastasis, STIM/Orai proteins have been described to be involved by two major mechanisms: (i) upregulation or increased functional activation and (ii) molecular switching [92]. In estrogen-receptor-positive breast cancer cells (e.g., MCF7, BT474, ZR751, T47D, and HCC1500 cancerous cell lines) but not in estrogen-receptor-negative breast cancer cells (e.g., MCF10A and 184A1 normal breast epithelial cells, and MDA-MB231, BT20, and HCC1937 cancerous cell lines), Orai 3 mediates the STIM1/2 and Orai3 pathway and the Ca^{2+}-selective Ca^{2+} release-activated Ca^{2+} current (I(CRAC)), while Orai1 does not mediate the STIM1/Orai1 pathway [42]. Further on, estrogen-receptor-α knockdown downregulates Orai3 without any effect on Orai1, decreases Orai3-mediated SOCE, and diminishes the I(CRAC) current in estrogen-receptor-positive MCF7 cells [93].

The expression of Orai3 was identified to be higher in MCF-7 breast cancer cells versus normal MCF-10A mammary epithelial cells, while its silencing inhibits the MCF-7 cell proliferation, arrests the cell cycle in the G1 phase, downregulates cyclin-dependent kinases 4/2, cyclins E and D1, and determines the accumulation of p21(Waf1/Cip1) (a cyclin-dependent kinase inhibitor) and p53 (a tumor-suppressing protein) [94].

In STIM1 siRNA- or Orai1 siRNA-treated MDA-MB-231 human breast tumor cells, a reduction in the migration process was identified [95]. Additionally, overexpression of STIM1 and Orai1 in MCF-10A epithelial cells increased their invasiveness [95]. Experiments on immunodeficient NOD/SCID mice injected with MDA-MB-231 human breast tumor cells were stably transfected with STIM1 siRNA, or Orai1 siRNA, but not with control siRNA, which demonstrated the inhibition of metastasis [95].

Interestingly, a signaling pathway independent of endoplasmic reticulum calcium stores or STIM1 and STIM2 protein activation was identified in MCF-7 breast cancer cells (the secretory pathway calcium ATPase 2 (SPCA2)/Orai1 signaling) [96], where SPCA2 is located in the Golgi apparatus.

2.5. Ether à go-go (hEag1) K^+ channels in breast cancer

Several pieces of evidence indicate the role played by Ether à go-go (hEag1) K^+ channels in breast cancer cells invasiveness. To date, blocking or silencing hEag1 channels in MDA-MB-231 breast cancer cells induces membrane depolarization and subsequent diminishment of Ca^{2+} influx through Orai1, which affects cell migration and proliferation [97].

2.6. Calcium-activated potassium channels in breast cancer

Prolactin increases the current density of the human Ca^{2+}-activated K^+ channels (hIKCa1) in MCF-7 breast cancer cells, involved in the cell proliferation in a dose-dependent manner and activating the Janus kinase (JAK2)-coupled cytokine receptor pathway [98].

Large conductance Ca^{2+}-activated K^+ channels (BKCa) were described to be expressed in several breast cancer cell lines (e.g., UACC893, SK-BR-3, and MDA-MB-231) [99]. Intermediate-conductance, Ca^{2+}-activated K^+ channels (hIK1) are also functionally expressed in MCF7 breast cancer cells, and their current density and basal cytosolic Ca^{2+} concentration being augmented in cells synchronized at the end of the G1 or S phase with respect to the cells in the early G1 phase [100]. Caveolin-1 was demonstrated to colocalize with BKCa in MCF-7 breast cancer cells, and silencing caveolin-1 induces increased activation and upregulation of BKCa [101].

2.7. Calcium-activated chloride channels in breast cancer

Calcium-activated chloride channel anoctamin 1 (ANO1) was demonstrated to be highly expressed in breast cancer cell lines and primary tumors and was considered to be a predictive factor for the disease degree and poor prognosis [102]. ANO1 activation was demonstrated to be done via the EGF receptor and calmodulin-dependent protein kinase II signaling, and its expression was associated with tumor cell survival [102].

An extensive clinical study including 431 patients with invasive ductal breast carcinoma and 46 patients with fibroadenoma analyzed the expression of anoctamin 1 (Ano1, TMEM16A), one of the members in the Ano family [103]. The study identified a correlation between Ano1 overexpression and the good prognosis in patients with lower clinical stage (stage I or II) of the breast cancer or in patients with triple-negative breast cancers [103].

Integrating these results, it is still premature to evaluate if Ano1 is or is not a predictive factor in good/poor prognosis in breast cancer patients, and if it is a suitable pharmacological target. It would be very useful to have more insights into the cellular mechanisms related to Ano1 activation. In our opinion, a possible scenario would be that the opening of calcium-activated chloride channels induces chloride efflux, membrane depolarization followed by the calcium influx through VGCCs. The high level of expression of calcium-activated chloride channels in breast cancer cells might be correlated with the tendency of these cells to be more depolarized with respect to the normal surrounding cells. However, this scenario would explain only the clinical data showing the association between Ano1 overexpression and the poor prognosis in patients with breast cancer.

2.8. Muscarinic acetylcholine receptors in breast cancer

Muscarinic receptors have been described to be expressed in several non-neuronal cell types, including endothelial cells, smooth muscle cells, or bladder and gastrointestinal tract [104–107]. In multiple malignancies, including breast, prostate, lung, ovary, pancreas, prostate, skin, stomach, uterus and colon cancer, muscarinic receptors have been demonstrated to contribute to cell proliferation and cancer progression [108–110].

In particular, muscarinic receptors have also been described in normal murine mammary (NMuMG) cells, normal human-breast-derived MCF-10A cells, and homogenates of surgical samples derived from normal human mammary tissue by Western blot and radioligand binding assays [111–113]. The expression of muscarinic acetylcholine receptors, M_3 and M_4, was detected in MCF-7 breast cancer cells [114]. Additionally, the expression of muscarinic acetylcholine receptors was evidenced in LM2, LM3, and LMM3 cell lines derived from spontaneous mammary adenocarcinomas developed in Balb/C mice [115–117]. These studies demonstrated the involvement of muscarinic receptors in the tumor cells proliferation, progression, and angiogenesis [115–117], by a mechanism involving the stimulation of the nitric oxide synthase activity [114].

Muscarinic receptors, M_1, M_3 and M_5, are coupled with $G_{q/11}$ proteins that activate phospholipase C (PLC) and determine the release of calcium from intracellular reservoirs. In MCF7, human breast cancer cells evidenced the activation of MAP by muscarinic receptors [118]. Considering that acetylcholine exerts neurocrine, paracrine, and autocrine regulation of cancer cell proliferation, migration, etc., we might consider muscarinic receptors among the master-players in breast cancer.

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3. Calcium signaling pathways as pharmacological targets in breast cancer

3.1. Calcium oscillations as pharmacological targets in breast cancer

Tetrodotoxin (TTX), a blocker of voltage-gated sodium channels (VGSCs), was demonstrated to diminish the number of oscillating MDA-MB-231 breast cancer cells and to reduce the amplitude and the frequency of the Ca^{2+} oscillations (i.e., spontaneous calcium transients) in the same cells [78]. While TTX had no effect on the basal calcium level in nonoscillating MDA-MB-231 breast cells, when applied in high extracellular K^+ conditions augmented the intracellular calcium concentration in both oscillating and nonoscillating MDA-MB-231 cells [78].

Previous studies have demonstrated that the blockade of VGSCs inhibits the invasion of endocrine-resistant breast cancer cells [119]. It is very interesting the correlation between the blockade of VGSCs and the reduction of spontaneous calcium oscillations. Therefore, considering that only metastatic cells are characterized by spontaneous calcium transients in comparison with low-metastatic or normal cells, a possible therapeutic strategy would be to diminish calcium transients by applying targeted pharmacological agents against VGSCs. An interesting approach would be to encapsulate voltage-gated sodium channels antagonists in charged liposomes and to target only highly metastatic breast cancer cells.

3.2. Voltage-gated calcium channels as pharmacological targets in breast cancer

1 mM Mn^{2+} and 0.1 mM Ni^{2+} ions blocked the fast activation and inactivation of the T-type calcium currents in MCF7 breast cancer cells [79].

Low doses (10–20 μM) of verapamil, an antagonist of voltage-gated calcium channels, blocked the growth of triple-negative MDA-MB-231 breast cancer cells, while high doses (100 μM) reduced by 90% the triple-negative MDA-MB-231 and MCF7 breast cancer cells [76].

Cav3.2 channels were demonstrated to play an important role in the mechanisms involved in chemoresistance. To date, trastuzumab resistance was demonstrated to be correlated with high mRNA Cav3.2 levels in SKBR3 breast cancer cells [120]. Patients with estrogen receptor-positive breast cancer that had a poor clinical outcome presented a significant Cav3.2 upregulation [120]. Moreover, patients with HER2-positive breast cancer presented a positive correlation between the Cav3.2 expression and patient survival upon chemotherapy [120].

3.3. TRP channels as pharmacological targets in breast cancer

TRP channels have been demonstrated to be actively involved in the development and progression of breast cancer. Therefore, finding efficient strategies for blocking-/silencing-specific TRPs in breast cancer cells might represent a good strategy to diminish the breast cancer progression.

Among the active compounds that act on TRP channels, polyunsaturated fatty acids (PUFA) have been evidenced to act on TRPC3, to reduce MCF-7 breast cancer cell proliferation and migration and to inhibit the TRPC-mediated calcium entry [80]. These compounds are particularly interesting as they are common in the diet, and previous studies have already shown their positive effects in cardiovascular diseases, including atherosclerosis, arrhythmias, etc. [121, 122]. The increased tumor vascularization is among the typical alterations and some PUFA have been demonstrated to exert effects against angiogenesis, inflammation, and cancer [123]. In this context, the action of PUFA on TRP channels is explaining their effects against breast tumors, either by diminishing angiogenesis in tumors or by reducing the proliferation/migration of tumor cells.

The Rho-associated kinases, ROCK1 and ROCK2, are considered as important therapeutic targets in breast cancer, being already demonstrated their critical role in cancer cell migration and invasion [124]. Identifying efficient Rho-kinase inhibitors is of particular interest in several subtypes of cancer, including breast cancer. Some Rho-kinase inhibitors have been identified to inhibit TRP channels. To date, fasudil, a Rho-kinase inhibitor, upregulates the expression of TRPC1 and TRPV2 in breast cancer cell lines (e.g., ZR-75-1, MCF7, and MDA-MB-231) [125]. Additionally, another Rho-kinase inhibitor, Y-27632, was demonstrated to downregulate TRPM6 and to upregulate TRPC7 in the same breast cancer cells [125].

TRPV1 seems to be one of the most promising TRP channels as a pharmacological target in breast cancer. To date, capsaicin inhibits cancer growth in SUM149PT breast cancer cells, by triggering apoptotic/necrotic mechanisms, while capsazepine diminishes these effects [88]. MRS1477, a positive allosteric modulator of TRPV1, when co-applied with capsaicin, diminished the fraction of MCF7 breast cancer apoptotic cells but was ineffective against tumor growth in MCF7 tumor-bearing immunodeficient mice [89]. Another study demonstrated that doxorubicin and melatonin exert synergistic effects against apoptosis and mitochondrial oxidative stress in MCF-7 breast cancer cells by activating TRPV1 [126]. The chemotherapy agent, 5-fluorouracil, also exerts its apoptotic effect in MCF7 breast cancer cells via TRPV1 channels [127]. The anticancer effects of cisplatin mediated by TRPV1 have to be potentiated by co-application of selenium or alpha-lipoic acid on MCF-7 breast cancer cells [128, 129]. Interestingly, selenium was shown to diminish the electromagnetic radiation (900 MHz) effects in MDA-MB-231 breast cancer cell line mediated by TRPV1 activation [130].

Although, tamoxifen is commonly known as a selective estrogen receptor modulator and used in estrogen receptor-positive breast cancer cells, it was also shown to be effective in estrogen-receptors negative tumors. Recent data indicate that it exerts antiproliferative effects in MCF7 breast cancer cells by an estrogen receptor-independent pathway that involves TRPV6 channels [87].

N-(3-aminopropyl)-2-([(3-methylphenyl)methyl]oxy)(20)-N-(2-thienylmethyl)benzamide (AMTB), an inhibitor of TRPM8, was also described to diminish the proliferation and migration of MDA-MB-231 and SK-BR-3 breast cancer cells via a TRPM8-independent mechanism involving voltage-gated sodium channels [131].

3.4. STIM and Orai as pharmacological targets in breast cancer

Blocking Ca^{2+} influx with EGTA, Ni^{2+}, or SKF96365 in STIM1 siRNA-, or Orai1 siRNA-treated MDA-MB-231 human breast tumor cells decreased the number of invasive tumor cells [95].

BALB/c mice with 4 T1 tumor cells implanted in the mammary glands were injected with the store-operated channel blocker SKF96365 (10 mg/kg, daily, 20 days treatment), and the lung metastasis was diminished up to 20% [95]. Moreover, intraperitoneal administration of SKF96365 (10 mg/kg, daily, 4 weeks treatment) in NOD/SCID mice with MDA-MB-231 human breast tumor cells determined a significant reduction of lung metastasis after 1 week of treatment, and no metastasis recurrence was observed in 2 weeks after drug withdrawal [95].

3.5. hEAG1 channels as pharmacological targets in breast cancer

Astemizole, an antihistaminic drug, was shown to block hEAG1 channels in MDA-MB-231 breast cancer cells [97]. The authors have demonstrated that blocking hEag1 with astemizole or silencing induces the breast cancer depolarization and consequently reduces the calcium influx and the cell migration without any influence on the cell proliferation [97]. These data are clinically valuable as hEAG1 are overexpressed in invasive ductal carcinoma breast cancer or metastatic lymph nodes [97, 132] and their co-expression with HIF-1α is correlated with tumor size, lymph node status, and tumor stage [132], and the possibility of pharmacologically blocking these channels might represent a promising therapy. Moreover, astemizole may be used to pharmacologically discriminate hEAG from the related hERG potassium channels in MCF-7 breast cancer cells [133].

Insulin-like growth factor-1 (IGF1) is known to ubiquitary stimulate the growth of various cells in the human body, and also to strongly inhibit the programmed cell death [134]. IGF1 was shown to activate hEAG1 channels in breast cancer cells via an Akt-dependent signaling pathway [135]. Corroborating the antiapoptotic activity of IGF1 with its activatory effect exerted on hEAG1, we can conclude that hEAG1 plays an important role in breast cancer proliferation and its blocking is of particular interest in finding an efficient anticancer therapy.

3.6. Calcium-activated potassium channels as pharmacological targets in breast cancer

The hIKCa1 blockers, TRAM-34, and clotrimazole, or siRNA-hIKCa1 inhibit the prolactin-induced proliferation of the MCF7 breast cancer cells [98].

Iberiotoxin inhibits large conductance of Ca^{2+}-activated K^+ channels (BKCa) in three types of breast cancer cell lines (e.g., UACC893, SK-BR-3, and MDA-MB-231), eliciting cellular depolarization, attenuating the anchorage-independent growth [99]. Oppositely, HER-2/neu-overexpressing SK-BR-3 cells were insensitive to iberiotoxin [99].

3.7. Calcium-activated chloride channels as pharmacological targets in breast cancer

CaCCinh-A01, an inhibitor of calcium-activated chloride channels ANO1, diminishes breast cancer cell viability and colony formation [102]. Recent clinical studies showed that ANO1 is upregulated in breast cancer in comparison with fibroadenoma [103]. Moreover, patients with progesterone receptor-positive or HER2-negative breast cancer, or breast cancer patients treated with tamoxifen, have an upregulation of ANO1, which can be considered as a predictive factor for longer overall survival [103].

3.8. Muscarinic acetylcholine receptors as pharmacological targets in breast cancer

IgG purified from the serum of breast cancer patients mimics the effect of carbachol by activating muscarinic acetylcholine receptors in MCF-7 breast cancer cells [136]. Moreover, these autoantibodies purified from the serum of breast cancer patients regulate the MCF7 breast cancer cell migration and the MMP-9 activity, and these effects are reduced by atropine, 4-DAMP (M_3 receptor antagonist), and tropicamide (M_4 receptor antagonist) [137].

Carbachol, an agonist of muscarinic acetylcholine receptors, acts on M_1 and M_3 receptors in the MCF7 breast tumor cells and potentiates tumor progression, by activating nitric oxide synthase via phospholipase C and protein kinase C signaling pathways [114]. Carbachol also elicits the mobilization of intracellular-free Ca^{2+} and induces the phosphorylation of MAPK/ERK in MCF-7 human breast cancer cells, while pretreatment with wortmannin or LY294002 (selective inhibitors of phosphoinositide 3-kinase), with genistein (nonselective inhibitor of tyrosine kinases) or with PP2 (specific Src tyrosine kinase inhibitor), diminished the carbachol-induced MAPK/ERK phosphorylation [118].

Moreover, carbachol upregulates the vascular endothelial growth factor-A in MCF7 tumor cells and determines angiogenesis, while atropine reverts its effects [136]. Carbachol treatment (20 hours) increased the tumor cell death and its administration in subthreshold concentrations in conjunction with paclitaxel potentiates cell death [138, 139], while atropine reverts these combined effects [138]. Interestingly, the combined treatment with carbachol (low doses) and paclitaxel induced the death of breast tumor MCF-7 cells, via the increased activity of nitric oxide synthase 1 and 3, and the reduced activity of arginase II, but the drug combination was ineffective against the nontumorigenic epithelial MCF-10A cell line, due to the absence of muscarinic acetylcholine receptors [140].

Although several preclinical studies indicated the pharmacological potential of M_3 antagonists in inhibiting tumor growth (e.g., melanoma, pancreatic, breast, ovarian, prostate, and brain cancers), no clinical trials have been done [109].

4. Conclusion

In conclusion, calcium signaling alterations occur in multiple cellular components, human breast stem cells, human breast epithelial cells, human breast myoepithelial cells, human breast adipocytes, human breast telocytes, etc., including those which contribute to the development and progression of breast cancer. Moreover, several molecular actors (e.g., voltage-gated calcium channels, TRP channels, STIM/Orai proteins, hEag1 K^+ channels, calcium-activated potassium channels, calcium-activated chloride channels, muscarinic acetylcholine receptors, etc.) are playing an important role in calcium-altered homeostasis associated with breast cancer that might be considered as potential pharmacological targets. Considering the interplay between the above-described calcium signaling pathways, the most efficient strategy against breast cancer would simultaneously target several molecular players.

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Since the development of microelectronic clamping methodology and fluorescent indicators for direct measurement of dynamic intracellular calcium transients, our understanding of biological signal transduction has progressed dramatically since the 1980s. Calcium is a universal signal in biology that modulates gene expression, transmitter and hormone release, muscular movement, and even "programmed" cell death. This book represents a compilation of chapters from a diverse set of expert biologists throughout the world who have conducted research in the general area of calcium signaling in organisms ranging from bacteria to humans. In accord with priorities of resolving human disease, the reader will also benefit from learning calcium's role in cellular signaling pathology relating to acute or chronic conditions such as vomiting, sepsis, obesity, hypertension, and cancer.

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