

Maintenance of intracellular Ca^{2+} basal concentration in airway smooth muscle (Review)

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Abstract. In airway smooth muscle, the intracellular basal Ca^{2+} concentration $[\text{Ca}^{2+}]_i$ must be tightly regulated by several mechanisms in order to maintain a proper airway patency. The $[\text{Ca}^{2+}]_i$ is efficiently regulated by sarcoplasmic reticulum Ca^{2+} -ATPase 2b, plasma membrane Ca^{2+} -ATPase 1 or 4 and by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Membranal Ca^{2+} channels, including the L-type voltage dependent Ca^{2+} channel (L-VDCC), T-type voltage dependent Ca^{2+} channel (T-VDCC) and transient receptor potential canonical 3 (TRPC3), appear to be constitutively active under basal conditions via the action of different signaling pathways, and are responsible for Ca^{2+} influx to maintain $[\text{Ca}^{2+}]_i$. The two types of voltage-dependent Ca^{2+} channels (L- and T-type) are modulated by phosphorylation processes mediated by mitogen-activated protein kinase kinase (MEK) and extracellular-signal-regulated kinase 1 and 2 (ERK1/2). The MEK/ERK signaling pathway can be activated by G-protein-coupled receptors through the α_q subunit when the endogenous ligand (i.e., acetylcholine, histamine, leukotrienes, etc.) is present under basal conditions. It may also be stimulated when receptor tyrosine kinases are occupied by the appropriate ligand (cytokines, growth factors, etc.). ERK1/2 phosphorylates L-VDCC on Ser⁴⁹⁶ of the β_2 subunit and Ser¹⁹²⁸ of the α_1 subunit, decreasing or increasing the channel activity, respectively, and enabling it to switch between an open and closed state. T-VDCC is also probably phosphorylated by

ERK1/2, although further research is required to identify the phosphorylation sites. TRPC3 is directly activated by diacylglycerol produced by phospholipase C (PLC_β or γ). Constitutive inositol 1,4,5-trisphosphate production induces the release of Ca^{2+} from the sarcoplasmic reticulum through inositol triphosphate receptor 1. This ion induces Ca^{2+} -induced Ca^{2+} release through the ryanodine receptor 2 (designated as Ca^{2+} 'sparks'). Therefore, several Ca^{2+} handling mechanisms are finely tuned to regulate basal intracellular Ca^{2+} concentrations. It is conceivable that alterations in any of these processes may render airway smooth muscle susceptible to develop hyperresponsiveness that is observed in ailments such as asthma.

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1. Introduction

In unstimulated tissues, numerous cellular mechanisms contribute to the influx and efflux of Ca^{2+} to and from the cytoplasm in order to maintain homeostasis of intracellular basal Ca^{2+} concentrations $[\text{Ca}^{2+}]_i$, a phenomenon that occurs in almost all cells (1-7). In smooth muscle at rest, $[\text{Ca}^{2+}]_i$ must be kept tightly within the range of 100 and 150 nM (8-15) to maintain an equilibrium between contraction and relaxation. In these cells, the processes of Ca^{2+} influx and efflux preserve the myogenic tone, resting membrane potential and sarcoplasmic reticulum (SR) Ca^{2+} refilling (1,10,16-18). It has been proposed that the influx process involves entry of extracellular Ca^{2+} through L-type voltage dependent Ca^{2+} channels (L-VDCCs) (10,19-22), receptor-operated

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Ca^{2+} channels (ROCCs) activated by agonists (23-28) and store-operated Ca^{2+} channels (SOCCs, capacitative Ca^{2+} entry) activated by SR- Ca^{2+} depletion (10,29-33). An additional cytosolic Ca^{2+} source is the SR, that is the main intracellular Ca^{2+} store, activated via inositol 1,4,5-trisphosphate (IP_3) receptor channels (30,34-36) and ryanodine-receptor (RyR) channels (35,37-40). Ca^{2+} extrusion from the cytoplasm is accomplished via the action of membrane and sarcoplasmic Ca^{2+} ATPases and $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) in its forward mode (41-49).

Pivotal work on basal Ca^{2+} influx performed in aortic vascular smooth muscle cells using a pharmacological approach, demonstrated two predominant mechanisms of basal Ca^{2+} entry: One associated with L-VDCCs, accounting for ~23-43% of the total Ca^{2+} entry, and another associated with SOCCs, which contributed ~30% of the total (50).

In a recent study on airway smooth muscle (ASM), the present authors observed that the basal Ca^{2+} entry was mediated by L-VDCCs and probably also a constitutively active transient receptor potential canonical 3 (TRPC3) channel (18), which is described below. However, the mechanisms that maintain their permeability to Ca^{2+} have yet to be elucidated.

In the present review, current knowledge regarding different structures that maintain the $[\text{Ca}^{2+}]_i$ in ASM, including those involving L- and T-VDCCs, TRPC3, membrane and sarcoplasmic Ca^{2+} -ATPases, NCX in its forward mode, IP_3 and RyRs, is discussed, including the most recent findings associated with the phosphorylation of L- and T-VDCCs and the dependence of TRPC3 on diacylglycerol (DAG).

For a better understanding of the participation of each of these proteins in the $[\text{Ca}^{2+}]_i$ regulation of ASM, novel unpublished data from studies by our group have been included. Firstly, Fig. 1A shows the maximal reduction of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) produced under Ca^{2+} free medium. This maneuver allowed determination of the proportional effect of each protein in the handling of $[\text{Ca}^{2+}]_i$.

2. VDCCs

L- and T-VDCCs have been described in different types of smooth muscle (19,51,52); in particular, L-VDCC expression has been abundantly reported in the ASM of different species, including human (20,21,53-56). Opening of both types of channel is dependent on membrane depolarization, allowing the entry of Ca^{2+} , which subsequently contributes to contraction and SR Ca^{2+} refilling (9,10,19,20,57).

Several subunits for L-VDCC have been described: $\text{Ca}_v1.1$, $\text{Ca}_v1.2$, $\text{Ca}_v1.3$ and $\text{Ca}_v1.4$ (58). In ASM, L-VDCC had generally been characterized by pharmacological and electrophysiological methods (19). However, the presence of all the subunits of this channel was recently reported in rat bronchial smooth muscle (59). Nevertheless, in bovine and guinea-pig tracheal myocytes, only $\text{Ca}_v1.2$ and $\text{Ca}_v1.2$ - $\text{Ca}_v1.3$, respectively, were observed (21,60). As identified recently by the present authors and shown in Fig. 1B and E, in guinea-pig ASM, D-600 (methoxyverapamil hydrochloride), a blocker of L-VDCC, significantly decreased the $[\text{Ca}^{2+}]_i$, corroborating that this channel is constitutively active and contributes towards maintaining the $[\text{Ca}^{2+}]_i$ (18). It is well known that this channel is greatly dependent on the membrane voltage, and in canine

ASM our group observed that its membrane potential at rest is approximately -59 mV, and is held steady. Furthermore, when the tissue was stimulated with carbachol, a cholinergic agonist, its membrane was depolarized, and when the depolarization reached -45 mV, it started oscillating (20). These oscillations are nifedipine-sensitive, and therefore corresponded to the opening and closing of the L-VDCC (61). Since the membrane potential at rest is unchanging, it was highly improbable that the voltage was influencing its opening at this stage.

Recently, a study in rat cardiomyocytes demonstrated that extracellular signal-regulated kinases 1 and 2 (ERK1/2), the mitogen-activated protein kinases (MAPKs), are able to phosphorylate L-VDCC at two sites: On Ser⁴⁹⁶ of the β_2 subunit and Ser¹⁹²⁸ of the α_1 subunit. Phosphorylation on the β_2 subunit or the α_1 subunit decreased or increased the L-VDCC activity, respectively (62). Thus, it may be hypothesized that in ASM, MAPK kinase (MEK)-ERK1/2 signaling may be involved in the continual opening and closing of the channel under basal conditions. This pathway may be associated with receptor tyrosine kinases (RTKs), which are activated by basal cytokines or growth factors. Our group previously demonstrated that ERK1/2 are present in the phosphorylated state in unstimulated bovine ASM (9). Fig. 1D and E show that the addition of U-0126, an inhibitor of ERK1/2, to guinea-pig tracheal myocytes significantly diminished the $[\text{Ca}^{2+}]_i$ until reaching a plateau. The addition of D-600 did not further modify the $[\text{Ca}^{2+}]_i$, confirming that phosphorylation of the L-VDCC through the MEK-ERK1/2 pathway is possibly involved in its constitutive active mode. Therefore, the ERK1/2 signaling pathway may be responsible for phosphorylating the β_2 Ser⁴⁹⁶ and α_1 Ser¹⁹²⁸ sites, serving to switch the L-VDCC between an open and closed state (Fig. 1F).

Treatment with mibefradil, a T-VDCC blocker, also significantly lowered $[\text{Ca}^{2+}]_i$ in the guinea-pig tracheal myocytes, implying the participation of this channel in sustaining $[\text{Ca}^{2+}]_i$ (Fig. 1C and E). The presence of T-VDCC has been reported in this tissue (19), and the expression of $\text{Ca}_v3.1$, $\text{Ca}_v3.2$ and $\text{Ca}_v3.3$ subunits has been detected in ASM by immunohistochemistry (63). In this context, unexpectedly our group found that the addition of mibefradil following U-0126 did not further diminish $[\text{Ca}^{2+}]_i$ (Fig. 1D). This finding suggested that T-VDCC could also be regulated by the ERK1/2 signaling pathway. Recent studies have shown that T-VDCC may be modified by several serine/threonine protein kinase pathways, suggesting that this channel is susceptible to undergo phosphorylation (64); however, further research is required in this regard to determine the functional impact that ERK1/2 signaling has on the T-VDCC. Notably, in sensitized guinea-pigs that developed an airway inflammatory state, the expression level of L-VDCC was not modified (60). This finding indicated that these channels appear not to participate in the modification of $[\text{Ca}^{2+}]_i$ that is observed in inflammatory ailments, such as asthma (65).

3. TRPC channels

In smooth muscle, TRPC channel genes code for ROCC and SOCC, which have an important role in intracellular Ca^{2+} homeostasis, while recently transient receptor potential vanilloid 1 (TRPV1) was revealed to be involved in the modulation of

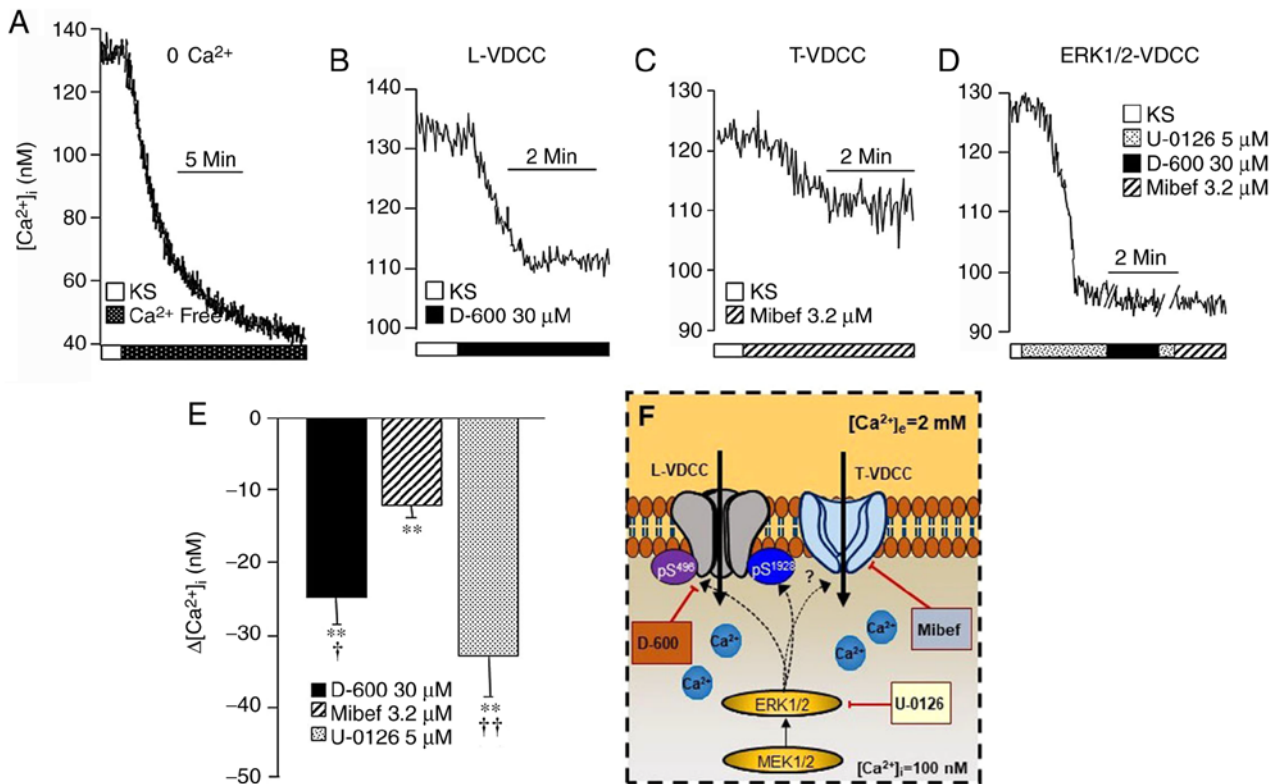


Figure 1. In guinea-pig airway myocytes at rest, L-VDCC and T-VDCC contribute towards maintaining the $[Ca^{2+}]_i$, and apparently are phosphorylated through the MEK-ERK1/2 pathway. Upper traces are representative of the intracellular Ca^{2+} measurements through fura-2AM in the different experimental protocols. (A) Representative trace showing the amplitude of the reduction in the $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} . The addition of (B) D-600 (an L-VDCC blocker; n=12) or (C) Mibef (a T-VDCC blocker; n=13) significantly lowered the $[Ca^{2+}]_i$ to differing extents. (D) Blockade of MEK-ERK1/2 kinase with U-0126 (n=12) markedly diminished the $[Ca^{2+}]_i$, and the administration of D-600 or Mibef did not lead to any further decreases in the altered $[Ca^{2+}]_i$ (n=6). (E) Bar graph depicting the statistical analysis of the different experimental protocols. Each bar represents the mean \pm standard error of the mean. **P<0.01 when compared with their respective $[Ca^{2+}]_i$ values; †P<0.05, ††P<0.01 with respect to the Mibef group (according to the Student-Newman-Keuls multiple comparison test). (F) Schematic representation of regulation of the basal activity of the VDCCs. The MEK signaling pathway through ERK1/2 phosphorylates the β_2 Ser⁴⁹⁶ (pS⁴⁹⁶) and α_1 Ser¹⁹²⁸ (pS¹⁹²⁸) sites, switching the L-VDCC and probably also the T-VDCC between an open and closed state. D-600, Mibef or U-0126 diminished the $[Ca^{2+}]_i$, (for further details, see the 'VDCCs' section). These results suggest that, under basal conditions, the two types of VDCC are continuously phosphorylated through the MEK pathway, which is responsible for their constitutive activity. L-VDCC, L-type voltage-dependent channel; T-VDCC, T-type voltage dependent Ca^{2+} channel; $[Ca^{2+}]_i$, intracellular basal Ca^{2+} concentration; MEK, mitogen-activated protein kinase kinase; ERK1/2, extracellular-signal-regulated kinase 1/2; Mibef, mibefradil; KS, Krebs' solution.

ASM tone and Ca^{2+} handling during agonist-induced contraction (66). In general, due to their ionic permeability, all TRPC channels are considered to be non-selective cation channels (NSCCs) (67,68). Thus far, all known TRPC channel activity has been shown to be associated with a phospholipase C (PLC) signaling pathway (69,70). In this context, it has been proposed that certain TRPC channels, including TRPC1, -2 and -3, are dependent on SR- Ca^{2+} depletion due to IP₃ production [a process termed store-operated Ca^{2+} entry (SOCE)] (36,71-75). On the other hand, ROCCs also include TRPC channels (TRPC3, -4, -5, -6 and 7), although these are activated by DAG, the other metabolite of PLC activity, and are independent of SR- Ca^{2+} depletion (69,70,76). In this context, only TRPCs 3, 6 and 7 are directly activated by DAG not involving protein kinase C (69,76), whereas TRPCs 4 and 5 are inhibited by protein kinase C, since their activity may be observed when this kinase is blocked (70).

In ASM, previous studies have reported the presence of almost all TRPC channel subtypes (TRPC1, -2, -3, -4, -5 and -6), with the exception of TRPC7 (67,68). Several TRPC channels have been shown to be constitutively active in different

types of tissue. For example, TRPC1 and -4 were proposed to be continuously active in C57 mice skeletal myocytes (77); likewise, TRPC7 in human embryonic kidney cells (76), while TRPC3 was also observed to be constitutively active in rabbit ear artery and mouse airway myocytes (78,79). In this regard, our recent study demonstrated that, in guinea-pig ASM, this channel was also involved in maintaining the $[Ca^{2+}]_i$ and preserving smooth muscle basal tone (18). The role of this channel in $[Ca^{2+}]_i$ is illustrated in Fig. 2, where the addition of 2-aminoethoxydiphenyl borate (2-APB), a blocker of the TRPC3 channel (80), markedly diminished the $[Ca^{2+}]_i$ (Fig. 2A and E). Furthermore, Pyr3, another specific TRPC3 channel blocker (81), also lowered $[Ca^{2+}]_i$ by a similar extent (Fig. 2B and E). These results suggested that TRPC3 is constitutively active in guinea-pig ASM, even though the mechanism underlying this phenomenon has yet to be fully elucidated.

Since almost all TRPC channel subtypes are expressed in ASM, in this review the DAG analog, 1-oleoyl-2-acetyl-sn-glycerol (OAG), was used to investigate the possible functional role of the channels present in this tissue. Fig. 2C shows that the addition of OAG to tracheal myocytes induced a transient

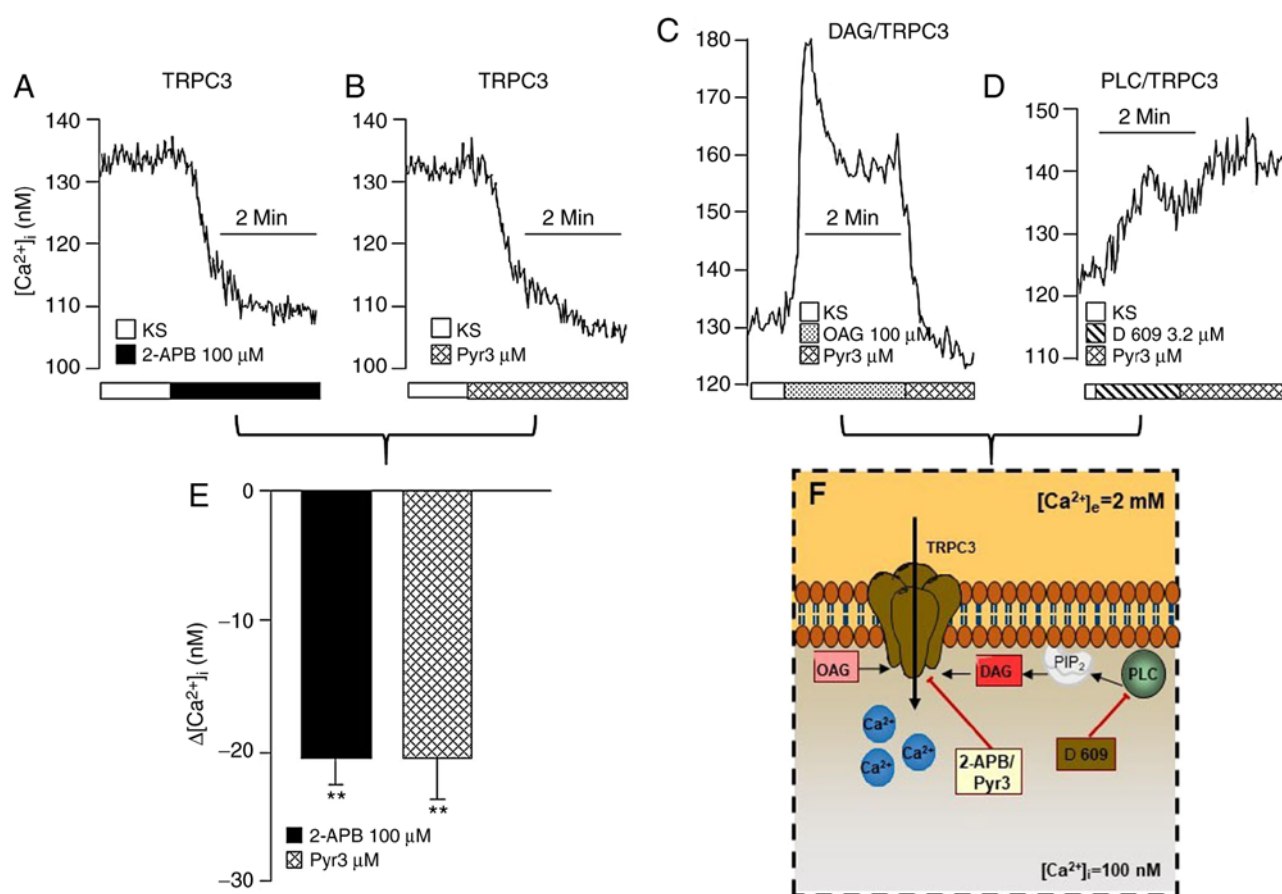


Figure 2. Membrane TRPC3 channel also contributes to $[\text{Ca}^{2+}]_i$ in guinea-pig airway smooth muscle. The upper traces shown are representative of the different experimental protocols. The addition of (A) 2-APB (a blocker of TRPC3; $n=5$) or (B) Pyr3 (a specific TRPC3 blocker; $n=5$) lowered the $[\text{Ca}^{2+}]_i$. (C) The addition of OAG, a DAG analog, induced a transient peak of the $[\text{Ca}^{2+}]_i$, followed by a plateau. The application of Pyr3 to the Ca^{2+} plateau returned Ca^{2+} to its basal level, indicating that the main TRPC channel functionally active in airway smooth muscle at rest is TRPC3. (D) Incubation with D-609, an inhibitor of PLC, produced a small incremental increase in the $[\text{Ca}^{2+}]_i$, and the addition of Pyr3 no longer diminished the $[\text{Ca}^{2+}]_i$. (E) Bar graph illustrating that the effects elicited by 2-APB and Pyr3 on $[\text{Ca}^{2+}]_i$ are similar. Each bar represents the mean \pm standard error of the mean. $**P < 0.01$ compared with the respective $[\text{Ca}^{2+}]_i$ value. (F) Schematic representation of the basal activity regulation of the TRPC3 channel. The results suggest that, under basal conditions, TRPC3 may oscillate between an open and closed state in the plasma membrane, i.e., these channels are constitutively active in this tissue, and are regulated by PLC through DAG. See the 'Transient receptor potential canonical channels' section for further details. PIP₂, phosphatidylinositol 4,5-bisphosphate; TRPC3, transient receptor potential canonical-3; 2-APB, 2-aminoethoxydiphenyl borate; OAG, 1-oleoyl-2-acetyl-sn-glycerol; DAG, diacylglycerol; PLC, phospholipase C.

peak in the $[\text{Ca}^{2+}]_i$ followed by a plateau. This response could have been developed through TRPC3 and/or TRPC6 channels, since these are both directly activated by DAG (69). However, after having reached the Ca^{2+} plateau induced by OAG, the addition of Pyr3 led to a return of $[\text{Ca}^{2+}]_i$ to its basal level. This finding indicated that the predominant TRPC channel that is functionally active in guinea-pig ASM, is TRPC3. Our group has postulated that TRPC3 is one of the channels involved in the maintenance of $[\text{Ca}^{2+}]_i$ (18), probably in a DAG-dependent manner. This lipid molecule is produced via the PLC or phospholipase D (PLD) pathways. It has been reported in rabbit ear artery myocytes that the PLD pathway produces DAG to sustain the constitutive activity of TRPC3 that contributes to the resting membrane potential (78,82). In ASM, protein kinase A was reported to regulate PLD activity, and it has been postulated that this phospholipase may be involved in the molecular mechanism underlying cyclic adenosine 5'-phosphate (c-AMP)-mediated relaxation in this tissue (83). By contrast, PLC has been shown to be predominantly involved in the IP_3 - Ca^{2+} signaling pathway and in contraction (35). Therefore, in this review, we investigated if PLC

may participate in DAG production in ASM at rest by using tricyclodecan-9-yl xanthogenate (D-609, a relatively specific inhibitor of PLC) (84) to inhibit this enzyme activity. It was observed that the addition of Pyr3 following D-609 to tracheal myocytes did not result in any further notable perturbations of the $[\text{Ca}^{2+}]_i$ (Fig. 2D). Thus, these results suggested that PLC generates DAG, which subsequently leads to the activation of TRPC3 under basal conditions in order to maintain $[\text{Ca}^{2+}]_i$ in ASM (Fig. 2F). Conceivably, the activity of PLC may be regulated by endogenous ligands of RTKs, or by G-protein-coupled receptors.

It has been demonstrated that the expression levels and activity of the TRPC3 channel are greatly augmented in ASM cells obtained from sensitized mice (79). This may lead to an increase in the $[\text{Ca}^{2+}]_i$, which could contribute to airway hyperresponsiveness in asthma.

The TRPV receptors, which are other members of the TRP family, have been implicated in mechanical stretch-induced Ca^{2+} influx in human ASM (85). In this context, TRPV1 is expressed in these cells, and was shown to be involved in Ca^{2+} oscillations and the maintenance of contraction by cholinergic

agonists (66). However, any role in terms of maintaining the ${}_b[\text{Ca}^{2+}]_i$ has not yet been elucidated, and this requires further research.

4. Capacitative Ca^{2+} entry

SR- Ca^{2+} depletion mediated by IP_3 induces the established mechanism of capacitative Ca^{2+} entry. The first studies on this were performed by Putney (31) in non-excitabile cells. Capacitative Ca^{2+} entry also occurs in smooth muscle via Ca^{2+} influx through diverse membrane channels (32,86). One of these Ca^{2+} influx mechanisms involves two types of protein associated with the SOCE pathway: Stromal interaction molecules (STIMs) and Orai proteins (87,88), both of which have been characterized in vascular smooth muscle and ASM (89,90). Orai are plasma membrane proteins, and three isoforms from different genes have been characterized: Orai1, -2 and -3 (91). On the other hand, two homologs of STIM have been identified: STIM1 and STIM2, both of which are located in the SR membrane (88,92,93). Regarding the two protein groups, Orai1 and STIM1 are the proteins that are chiefly expressed in ASM, and are responsible for the capacitative Ca^{2+} entry (89,94). Briefly, STIM1 on the SR functions as a Ca^{2+} sensor, monitoring the organelle's Ca^{2+} content (95). When the SR- Ca^{2+} store is depleted, STIM1 forms an aggregate with other STIM1 molecules, thereby forming structures designated as 'puncta', which interact with Orai1 plasma membrane proteins to promote capacitative Ca^{2+} entry (89). Additionally, in several cell types it has been postulated that STIM/Orai may interact with TRPC channels, thereby establishing an alternative mechanism for capacitative Ca^{2+} entry (89,96). It is noteworthy that, in ASM, IP_3 has been demonstrated to directly open membranal TRPC3 channels. This recent finding implies that IP_3 mediates SR- Ca^{2+} depletion (i.e., capacitative Ca^{2+} entry) and also a direct, independent Ca^{2+} influx by TRPC channels (36). In this context, in one of our previous studies, we demonstrated that, in unstimulated airway myocytes, capacitative Ca^{2+} entry was not activated unless the SR Ca^{2+} content fell below 50% (8). However, it is well known that capacitative Ca^{2+} entry is activated by contractile agonists that act through the PLC_β - IP_3 signaling cascade (32), therefore providing no certainty that it does contribute to the maintenance of ${}_b[\text{Ca}^{2+}]_i$.

5. $\text{Na}^+/\text{Ca}^{2+}$ exchanger

The $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) is a membrane Ca^{2+} -handling protein that introduces three Na^+ ions to the cytoplasm, while extruding one Ca^{2+} when in its forward mode. By contrast, in its reverse mode, it introduces Ca^{2+} and extrudes Na^+ (42). To activate the reverse mode (NCX_{REV}), the entry of Na^+ through an NSCC, and probably L-VDCC in proximity to the NCX, is required (21,41,48,97). The NCX is encoded by three gene isoforms, which generate NCX1, -2 and -3 (98-100). NCX1, extensively distributed in mammalian cells, has 17 different splicing variants that are tissue-specific and define the exchanger's ionic sensitivity and regulation (101). NCX2 has no splicing variants and is located predominantly in the brain, spinal cord, gastrointestinal and kidney tissues, whereas NCX3 has five splice variants expressed in brain and skeletal

muscle (101). In ASM, the NCX1.3 splicing variant is the main isoform present (102).

In airway myocytes, it has been proposed that NCX participates in the physiology of $[\text{Ca}^{2+}]_i$, including SR- Ca^{2+} refilling (10,57), although it has been given a minor role in Ca^{2+} homeostasis (43). In this context, we have observed that NCX blockade with amiloride, a blocker of both the forward and reverse NCX modes, or KB-R7943, a blocker of NCX_{REV} , had no noticeable effect on ${}_b[\text{Ca}^{2+}]_i$, indicating a minor role of this protein in terms of ${}_b[\text{Ca}^{2+}]_i$ regulation (unpublished data). Nevertheless, its participation in Ca^{2+} regulation, accomplished mainly through NCX_{REV} , becomes evident when ${}_b[\text{Ca}^{2+}]_i$ is increased and acquires a new steady-state (Fig. 3A). In this context, in a murine chronic model of allergen-induced airway hyperresponsiveness, it was shown that the levels of NCX1 were significantly augmented, and that NCX_{REV} activity was increased (103). Furthermore, in human myocytes, the addition of pro-inflammatory cytokines, including tumor necrosis factor- α (TNF α) and interleukin (IL)-13, also increased the expression of NCX1 and favored NCX_{REV} activity (104). These findings suggested that, during inflammation, NCX_{REV} could significantly contribute to an increase in the ${}_b[\text{Ca}^{2+}]_i$, which would predispose airway smooth muscle to hyperresponsiveness.

6. Ca^{2+} -ATPases in ASM

Ca^{2+} -ATPases form part of a large family of membrane proteins defined as P-type ATPases, including the plasmalemmal Ca^{2+} -ATPase (PMCA) and the SR Ca^{2+} -ATPase (SERCA, or sarco/endoplasmic reticulum Ca^{2+} -ATPase) (105).

The PMCA extrudes Ca^{2+} against a high concentration gradient to contribute to ${}_b[\text{Ca}^{2+}]_i$. It exists in a 1:1 relationship with ATP, is electroneutral via $\text{H}^+/\text{Ca}^{2+}$ exchange, and its affinity for Ca^{2+} and transport efficiency is increased by calmodulin. PMCA1-4 are the products of four different genes with several splice variants (105). PMCA1 and -4 are ubiquitous, and have lower affinity for calmodulin, whereas PMCA2 and PMCA3 have high calmodulin affinity (105,106).

In ASM, the primordial function of PMCA in Ca^{2+} homeostasis was demonstrated late in the 20th century (43). Shortly afterwards, the expression of this pump in canine ASM was reported (107). More recently, in rat bronchial myocytes, the presence of PMCA1 and PMCA4 was confirmed, and the participation of these two isoforms in Ca^{2+} homeostasis was demonstrated (108).

On the other hand, SERCA is, in part, electrogenic, since it introduces two Ca^{2+} ions to the SR, at the same time releasing at least four H^+ ions to the cytoplasm (105). Additionally, it has been demonstrated that SERCA transports two Ca^{2+} ions for each hydrolyzed ATP molecule, and it appears to be the main system for controlling $[\text{Ca}^{2+}]_i$ in muscular cells (105).

SERCA pumps are produced by three genes: SERCA1, -2 and -3. They are subjected to alternative splicing, resulting in the isoforms, SERCA1a-b, SERCA2a-c and SERCA3a-f (105,109). In smooth muscle cells, the SERCA isoforms predominantly present are 2a and 2b (109), whereas in ASM, SERCA2b is the predominant isoform (110).

By measuring $[\text{Ca}^{2+}]_i$ in the absence of extracellular Ca^{2+} , the addition of thapsigargin, a SERCA blocker, to rat bronchial

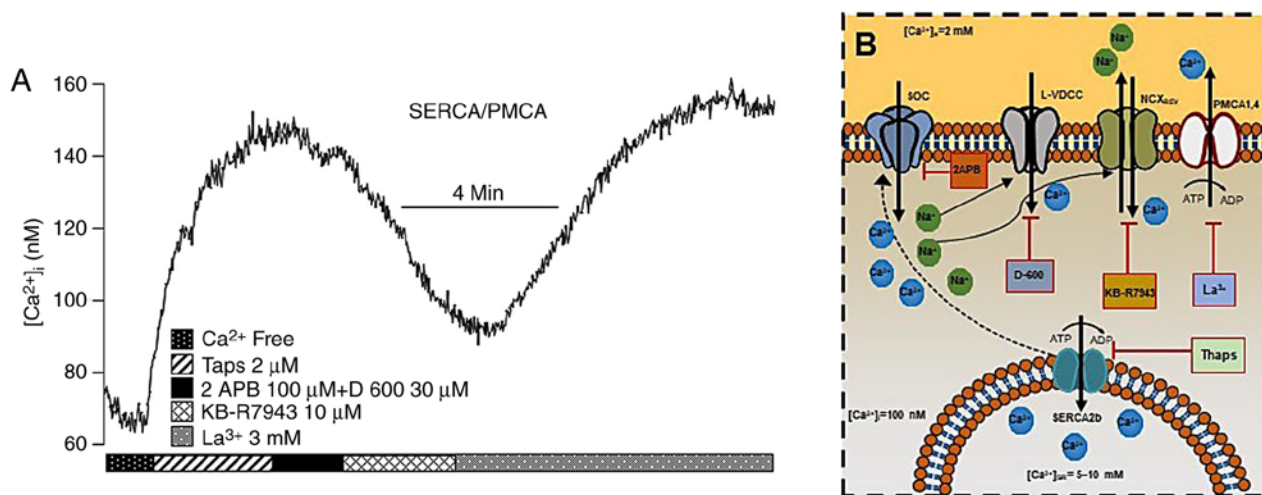


Figure 3. In guinea-pig airway smooth muscle, SERCA and PMCA actively participate in maintaining the $[\text{Ca}^{2+}]_i$. (A) The blockade of SERCA with Thaps ($n=6$) increased the $[\text{Ca}^{2+}]_i$ until a new basal steady state was reached due to capacitative Ca^{2+} entry involving L-VDCC and SOCC. At this point, the NCX changes to its reverse mode, probably due to the entry of Na^+ through SOCC and L-VDCC, and thereby becomes the main contributor to sustaining the Ca^{2+} plateau since KB-R7943 brought $[\text{Ca}^{2+}]_i$ to a new basal steady state. The addition of lanthanum (La^{3+}), a non-specific PMCA blocker, led to large increase in $[\text{Ca}^{2+}]_i$, thus indicating that the former new Ca^{2+} basal state was maintained by PMCA activity. Note that all experimental protocols were performed in Ca^{2+} -containing Krebs solution, with the exception of the first 1.5 min at the beginning of the experiment. (B) Schematic representation of the roles of SERCA, PMCA, NCX and NCX_{REV} in maintaining $[\text{Ca}^{2+}]_i$. For further details, see the 'Na $^+$ /Ca $^{2+}$ exchanger' and the 'Ca $^{2+}$ -ATPases in ASM' sections. NCX, Na $^+$ /Ca $^{2+}$ exchanger; SERCA, sarcoplasmic reticulum Ca $^{2+}$ -ATPase; PMCA, plasmalemmal Ca $^{2+}$ -ATPase; Thaps, thapsigargin; L-VDCC, L-type voltage-dependent channel; SOCC, store-operated Ca $^{2+}$ channel.

myocytes produced a transient Ca^{2+} peak that returned to its basal value. At this point, lanthanum, a PMCA blocker, induced a sustained $[\text{Ca}^{2+}]_i$ increment that promoted apoptosis (108), demonstrating the central functional role of the two pumps in Ca^{2+} handling in ASM. In this regard, it has been proposed that there is a functional coupling between PMCA and SERCA to maintain Ca^{2+} homeostasis (49). Under physiological conditions (i.e., in the presence of extracellular Ca^{2+}), we found in guinea-pig tracheal myocytes that thapsigargin increased $[\text{Ca}^{2+}]_i$ until a plateau was reached (Fig. 3A). It is well known that, in ASM, this Ca^{2+} increment is due to capacitative Ca^{2+} entry (i.e., SOCE) predominantly via the TRPC3 channel, a process that also produces membrane depolarization due to the entry of Na^+ (79,111), consequently leading to L-VDCC opening and further Ca^{2+} and Na^+ entry (10,18,21,36,79,112). At this stage, the NCX may change to its reverse mode (i.e., NCX_{REV}) due to the Na^+ entry, thereby becoming the main contributor towards sustaining the Ca^{2+} plateau due to SERCA blockade. This proposition was corroborated using an NCX_{REV} -mode blocker, KB-R7943, which brought $[\text{Ca}^{2+}]_i$ to a new basal Ca^{2+} steady state (Fig. 3A) that was maintained by the PMCA activity. At this point, the addition of lanthanum, a non-specific PMCA blocker, led to a marked increase in $[\text{Ca}^{2+}]_i$, probably inducing cellular apoptosis, as was suggested by a previous study (108). Taken together, these results corroborated that, under physiological conditions, SERCA and PMCA exert a primordial role in regulating $[\text{Ca}^{2+}]_i$ homeostasis, whereas NCX_{REV} only participates when $[\text{Ca}^{2+}]_i$ is modified and acquires a new steady state (Fig. 3A and B).

Studies associated with the effects of pro-inflammatory cytokines on the ASM SERCA have demonstrated that overnight exposure of human airway myocytes to TNF α or IL-13 decreases the expression of SERCA that, in turn, diminishes the reuptake of SR- Ca^{2+} (113). Notably, these authors also revealed

that, unlike other species, e.g., in porcine airways (114), human ASM SERCA does not express phospholamban, but is directly phosphorylated by Ca^{2+} /calmodulin-dependent protein kinase II (113). Thus, it is possible that in an inflammatory process such as asthma, SR-ATPase activity is decreased, which may lead to an increase in the $[\text{Ca}^{2+}]_i$ to a new steady state, favoring an augmented response to bronchoconstrictor agonists. The same phenomenon may also be occurring as far as the PMCA is concerned; however, further research is required in this field.

7. Ryanodine and IP $_3$ receptors

RyR is a non-selective cation channel that releases Ca^{2+} from the SR and, in mammals, its three isoforms, RyR1, -2 and -3, are the products of different genes (115). All three isoforms are expressed in smooth muscle, including ASM (115,116). Cyclic ADP-ribose (cADPR) is considered to be their endogenous ligand in airway myocytes, which is regulated by the membrane-bound protein, CD38 (117). This protein has ADP-ribosyl cyclase and hydrolase activity, and is involved in the synthesis or degradation of cADPR, respectively (118,119).

The IP $_3$ receptor (ITPR) is another non-selective cation channel that releases Ca^{2+} from the SR via IP $_3$ generated by the G $_q$ α signaling pathway (35). It has three isoforms (ITPR1, -2 and -3) derived from different genes, which share ~60-80% amino acid homology (120,121). These receptors have also been identified in different smooth muscles types, including ASM (36,122-124).

In 1993, Ca^{2+} 'sparks' were described in heart muscle (125), and these were associated with the Ca^{2+} -induced Ca^{2+} release from RyRs (126). In guinea-pig tracheal myocytes, the presence of spontaneous Ca^{2+} sparks was observed for the first time in

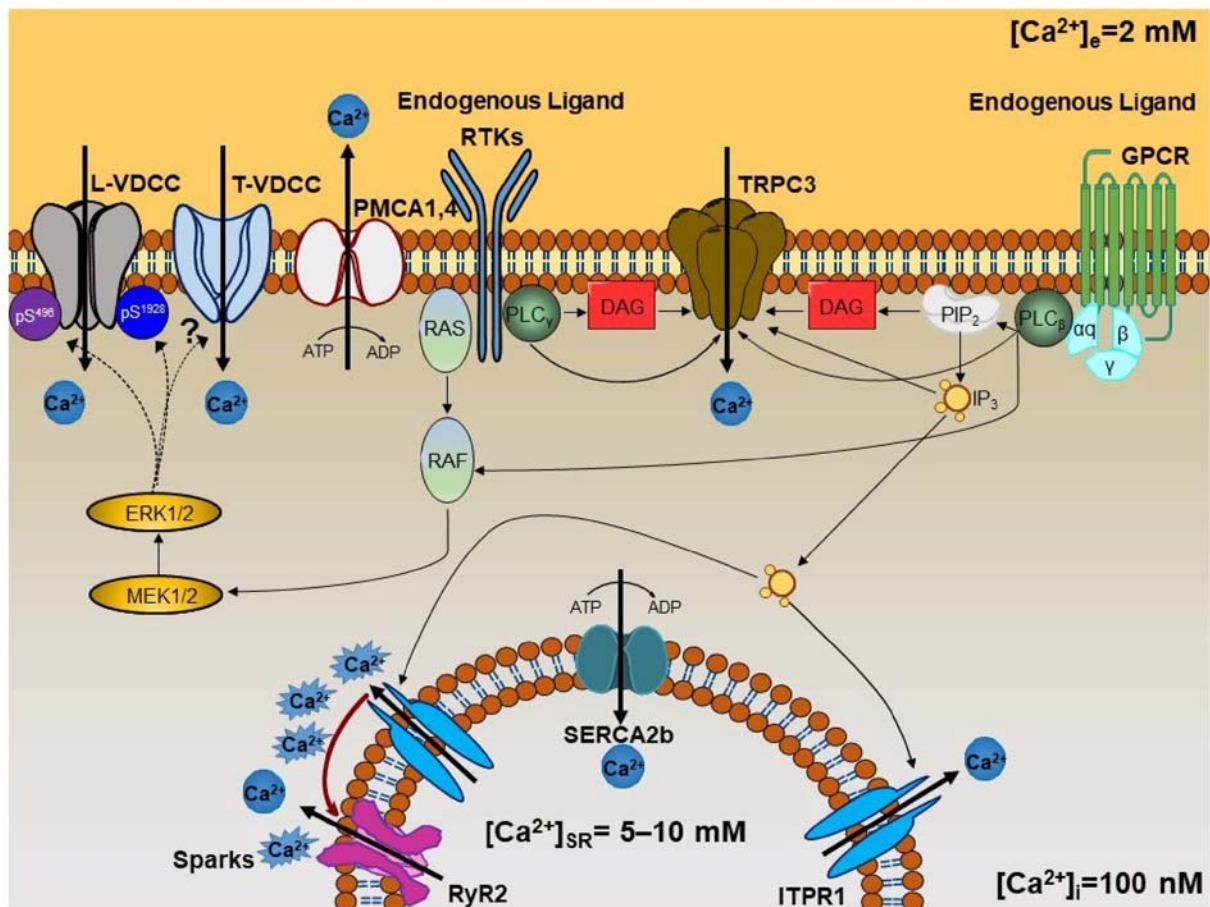


Figure 4. Schematic representation of the mechanisms involved in the maintenance of ${}_i[Ca^{2+}]_i$. Membranal Ca^{2+} channels, such as L-VDCC, T-VDCC and TRPC3, appear to be constitutively active under basal conditions through different signaling pathways. The two types of voltage-dependent Ca^{2+} channel may be modulated by phosphorylation processes mediated by mitogen-activated protein kinase ERK1/2 signaling. This signaling pathway can be activated by GPCRs through the α_q subunit when the endogenous ligand is present under basal conditions (i.e., acetylcholine, histamine, leukotrienes, etc.). It may also be stimulated when RTKs are occupied by the appropriate ligand (cytokines, growth factors, etc.). ERK1/2 phosphorylates L-VDCC on Ser⁴⁹⁶ of the β_2 subunit and Ser¹⁹²⁸ of the α_1 subunit, decreasing or increasing the channel activity, respectively, enabling it to switch between an open and closed state. T-VDCC is probably also phosphorylated by ERK1/2, but further research is needed to identify the phosphorylation sites (see Fig. 1D). TRPC3 is directly activated by DAG and IP₃ arising from PLC β or PLC γ , the first coupled to the α_q subunit of GPCR, and the second to RTKs. Constitutive IP₃ production induces SR- Ca^{2+} release through ITPR1. This Ca^{2+} induces Ca^{2+} -induced Ca^{2+} release through the RyR2 (designated as Ca^{2+} 'sparks'). Finally, $[Ca^{2+}]_i$ is efficiently regulated by the SERCA2b and PMCA1 or PMCA4. L-VDCC, L-type voltage-dependent channel; T-VDCC, T-type voltage dependent Ca^{2+} channel; TRPC3, transient receptor potential canonical-3; ERK1/2, extracellular-signal-regulated kinase 1/2; GPCR, G-protein-coupled receptor; RTK, receptor tyrosine kinase; DAG, diacylglycerol; IP₃, inositol 1,4,5-trisphosphate; PLC, phospholipase C; SR, sarcoplasmic reticulum; ITPR, IP₃ receptor; RyR, ryanodine receptor; SERCA, sarcoplasmic reticulum Ca^{2+} -ATPase; PMCA, plasmalemmal Ca^{2+} -ATPase.

1998 (127). Subsequently, in urinary bladder smooth muscle, these Ca^{2+} sparks were characterized as the elementary release of Ca^{2+} from RyRs (128), and this finding was later corroborated in mouse ASM, occurring predominantly through RyR2 (116,129). In this context, studies on the pulmonary artery revealed that Ca^{2+} sparks are activated by Ca^{2+} released via ITPR (130), as well as in ASM (129). The physiological role of these Ca^{2+} sparks in guinea-pig tracheal myocytes was well established. Essentially, they produce spontaneous transient outward currents caused by large-conductance Ca^{2+} -activated K^+ channels; they also induce spontaneous transient inward currents accomplished through Ca^{2+} -activated Cl-channels (127). Therefore, all these components may serve an important role in the basal state regulation of the ASM by stabilizing the membrane potential, the ${}_i[Ca^{2+}]_i$ and the basal contractile tone.

Interestingly, further lines of research have demonstrated that pro-inflammatory cytokines (predominantly TNF α),

promote the augmentation of CD38-cADPR signaling and increase Ca^{2+} responses to agonists (117,131), a phenomenon that is probably mediated by an augmentation of ${}_i[Ca^{2+}]_i$. Furthermore, TNF α also enhances G $_q\alpha$ protein expression, thereby increasing the ASM response to carbachol (132). However, upregulation of the IP₃- Ca^{2+} signaling pathway and any consequent modification of the ${}_i[Ca^{2+}]_i$ in an inflammatory context, such as in asthma, has not readily been identified, and this requires further research.

8. Conclusion

The current review has discussed how several Ca^{2+} handling mechanisms are finely tuned to regulate the ${}_i[Ca^{2+}]_i$, summarized in Fig. 4. It is conceivable that alterations in any of these processes could render ASM susceptible to developing the type of hyperresponsiveness that is commonly observed in ailments such as asthma, and this warrants further study.

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Availability of data and materials

The datasets presented in the current review are available from the corresponding author on reasonable request.

Authors' contributions

With particular regard to the previously unpublished work presented herein, the contribution of each author was as follows. JRG and ACG performed the assays of intracellular Ca²⁺ levels. EFS performed enzymatic isolation of tracheal myocytes, participated in the assays of intracellular Ca²⁺ levels and data analysis, and provided critical ideas during the writing of the manuscript. BS contributed to the data analysis and writing of the manuscript. LMM contributed to the design and global supervision of the study, data analysis and writing of the manuscript, and was responsible for submitting the paper for publication. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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