Invited Review: Mechanisms of calcium handling in smooth muscles

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highlighted topics

Signal Transduction in Smooth Muscle
Invited Review: Mechanisms of calcium handling in smooth muscles

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Sanders, Kenton M. Invited Review: Mechanisms of calcium handling in smooth muscles. *J Appl Physiol* 91: 1438–1449, 2001.—The concentration of cytoplasmic Ca^{2+} regulates the contractile state of smooth muscle cells and tissues. Elevations in global cytoplasmic Ca^{2+} resulting in contraction are accomplished by Ca^{2+} entry and release from intracellular stores. Pathways for Ca^{2+} entry include dihydropyridine-sensitive and -insensitive Ca^{2+} channels and receptor and store-operated nonselective channels permeable to Ca^{2+}. Intracellular release from the sarcoplasmic reticulum (SR) is accomplished by ryanodine and inositol trisphosphate receptors. The impact of Ca^{2+} entry and release on cytoplasmic concentration is modulated by Ca^{2+} reuptake into the SR, uptake into mitochondria, and extrusion into the extracellular solution. Highly localized Ca^{2+} transients (i.e., sparks and puffs) regulate ionic conductances in the plasma membrane, which can provide feedback to cell excitability and affect Ca^{2+} entry. This short review describes the major transport mechanisms and compartments that are utilized for Ca^{2+} handling in smooth muscles.

calcium channel; ryanodine receptor; inositol trisphosphate receptor; calcium sparks; capacitative calcium entry

CALCIUM IS A FUNDAMENTAL second messenger in smooth muscle cells. Increasing cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), and binding to calmodulin and activation of myosin light chain kinase, is the primary stimulus for contraction. To activate the contractile apparatus, Ca^{2+} must increase globally throughout the cytoplasm. The Ca^{2+} utilized for activation of the contractile apparatus enters the cytoplasmic compartment during periods of membrane depolarization, mechanical distortion, or stimulation by agonists. Release of Ca^{2+} from intracellular stores is a second means of increasing $[Ca^{2+}]_i$. After an excitatory event, relaxation and Ca^{2+} homeostasis are achieved by reuptake of Ca^{2+} into stores and extrusion into the extracellular space. These events are accomplished by at least a dozen specialized Ca^{2+} transporters and ion channels, which are arranged in membranes separating at least five distinct compartments and capable of facilitating Ca^{2+} movements up and down significant electrochemical gradients. This brief review provides a general overview of Ca^{2+} entry mechanisms, factors that regulate uptake and release from intracellular stores, and extrusion mechanisms. Further discussion will be provided about integrated Ca^{2+} handling mechanisms such as localized Ca^{2+} transients, which can provide either positive or negative feedback in regulating the excitability of smooth muscle cells. Additional recent reviews on this general subject are also available from other authors (cf. Refs. 26, 57, 63, 75, 87).

CA^{2+} ENTRY MECHANISMS

Dihydropyridine-sensitive Ca^{2+} channels. Much of the Ca^{2+} that activates the contractile apparatus in smooth muscles enters cells during periods of depolarization via dihydropyridine (DHP)-sensitive Ca^{2+} channels (Fig. 1). These channels are composed of pore-forming α-subunits and several accessory subunits that may regulate pore formation, gating, and

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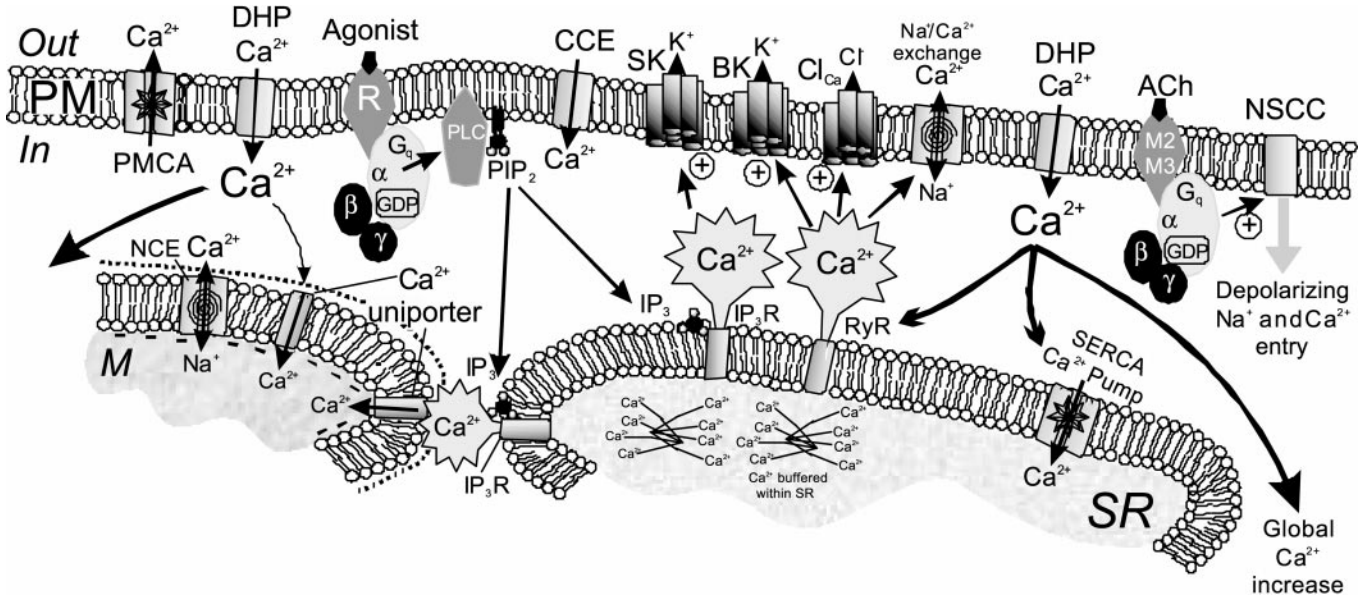

Fig. 1. Main essentials of Ca^{2+} handling. At least 5 compartments are relevant to Ca^{2+} signaling in smooth muscle: *1*) extracellular solution, *2*) subsarcolemmal region between sarcoplasmic reticulum (SR) and plasma membrane (PM), *3*) SR, *4*) mitochondria (M), and *5*) general cytoplasm. As discussed in the text, many transport proteins are involved in Ca^{2+} handling. Depolarization activates dihydropyridine-sensitive Ca^{2+} channels (DHP Ca^{2+}). Other Ca^{2+} entry mechanisms include agonist-activated nonselective cation channels (NSCC, activated by muscarinic stimulation featured in figure) and capacitative Ca^{2+} entry (CCE) channels. The amount of Ca^{2+} entry through NSCC is controversial, but these channels yield depolarization that activates DHP Ca^{2+} channels. Ca^{2+} entering cells can increase global cytoplasmic Ca^{2+} and cause contraction. Part of the Ca^{2+} entering cells may be taken up ("buffered") by superficial Ca^{2+} stores, such as the SR and mitochondria. Sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps provide the mechanism to sequester Ca^{2+} into the SR, and this requires energy to pump Ca^{2+} up a steep concentration gradient. Ca^{2+} is highly buffered within SR. The Ca^{2+} uniporter in the inner membrane of mitochondria (outer membrane depicted schematically by dotted line) provides an uptake mechanism, and this occurs down a large electrochemical gradient for Ca^{2+} (mitochondria inside very negative) generated by proton pumping by the electron transport chain. Ca^{2+} homeostasis in mitochondria is maintained by Na^+/Ca^{2+} exchange (NCE). Many excitatory agonists bind to receptors coupled to G proteins (G_q/G_{11}) and activate phospholipase C to generate inositol trisphosphate (IP_3). IP_3 binds to receptors in the SR membrane and causes Ca^{2+} release. This can sum with Ca^{2+} entry mechanisms and contribute to global Ca^{2+} transients. IP_3-dependent Ca^{2+} release can also stimulate Ca^{2+} uptake into mitochondria and localized release through IP_3 receptors (IP_3R; Ca^{2+} puffs). Localized Ca^{2+} transients can also originate from ryanodine receptors (RyR; Ca^{2+} sparks). Local Ca^{2+} transients result in high concentrations of Ca^{2+} in the subsarcolemmal region and can stimulate Ca^{2+}-activated conductances in the plasma membrane, such as small-conductance Ca^{2+}-activated K^+ channels (SK), large-conductance Ca^{2+}-activated K^+ channels (BK), and Ca^{2+}-activated Cl^- channels (Cl_{Ca}). The response to Ca^{2+} sparks and puffs depends on the spatial proximity of RyR and IP_3R to specific types of Ca^{2+}-activated conductances and may vary between smooth muscle cells. Cellular Ca^{2+} homeostasis is maintained by 2 transporters that extrude Ca^{2+} into the extracellular medium: plasma membrane Ca^{2+} pump (PMCA) and NCE proteins. Many forms of intracellular regulation exist that affect the performance of the transporters shown in the figure. See text for details regarding regulatory mechanisms.

kinetics of the channels. At least six genes encode Ca^{2+} channel α-subunits, and a splice variant, $\alpha_{1C\text{-}b}$, forms channels in smooth muscles (46). The $\alpha_{1C\text{-}b}$-subunit carries Ca^{2+} current and provides the voltage and DHP sensitivity of these channels. The α-subunits are large proteins with four repeating segments, each with six membrane-spanning domains (S_1–S_6). The pore selectivity for Ca^{2+} is thought to be due to the region between S_5 and S_6 (44).

DHP-sensitive Ca^{2+} channels are activated by depolarization of the plasma membrane, and there is a presumed voltage sensor in the S_4 domain of the α-subunit, as in other voltage-gated channels (21). In some smooth muscles, depolarization from extracellular stimuli, such as neurotransmitters, activates DHP-sensitive Ca^{2+} channels; if threshold is reached, a Ca^{2+} action potential is generated. An action potential brings substantial Ca^{2+} into cells and elicits strong contractions. In many cases, however, activation of delayed rectifier K^+ channels, which have activation kinetics similar to the Ca^{2+} channels, impedes the generation of action potentials, and Ca^{2+} channels are activated in a more sustained manner (i.e., the channels maintain a low, but significant, open probability as long as the depolarization is maintained). Depolarization also results in inactivation that slightly lags the activation phase. Inactivation is both voltage and Ca^{2+} dependent (37, 107). The latter is conveyed by intracellular Ca^{2+} (probably to a large extent by the Ca^{2+} that enters cells through the channel). The voltage dependence of activation and inactivation is such that inactivation is incomplete through a range of potentials in which significant activation occurs (i.e., approximately −60 to −20 mV). Thus, at some voltages,

DHP-sensitive Ca^{2+} channels are capable of sustained openings and sustained inward current. The voltage range in which this occurs is known as "window current" (24).

The magnitude of sustained Ca^{2+} current in the range of window current is small, but the amount of Ca^{2+} influx relative to cell volume is significant. DHP-sensitive Ca^{2+} channels have a high rate of Ca^{2+} permeation (38, 94). Integration of the inward current during step depolarization within the window current range showed that depolarization in the range of -40 to -20 mV increased cytoplasmic Ca^{2+} in colonic muscle cells by tens of micromolars (31, 106). With the assumption of 100-fold buffering (62), the increase in $[Ca^{2+}]_i$ is sufficient to elicit contraction (6, 106). Tonic smooth muscles with membrane potentials within the window current range have constant influx in Ca^{2+} by this pathway, and small voltage changes are capable of significantly altering $[Ca^{2+}]_i$ (31). Ca^{2+} influx through DHP-sensitive Ca^{2+} channels explains to a significant degree the coupling between changes in membrane potential and contraction and explains the steep relationship between voltage and force in smooth muscles (79, 85).

Regulating Ca^{2+} influx through DHP-sensitive Ca^{2+} channels is an important means of controlling the contractile state of smooth muscles. Some vasodilators, such as nitric oxide, working through cGMP and protein kinase G, directly regulate the open-probability DHP-sensitive Ca^{2+} channels (cf. Ref. 23). However, in many smooth muscles, these channels are not the primary target for regulation by agonists or second messengers. In many cases, alterations in Ca^{2+} influx are regulated by voltage, and changes are mediated by activation of subsidiary conductances. For example, K^+ channels are activated to produce outward current, hyperpolarize membrane potential, and reduce Ca^{2+} influx or nonselective cation channels or Cl^- channels are opened to generate inward current, depolarize membrane potential, and increase Ca^{2+} influx.

Other voltage-dependent Ca^{2+} channels. Ca^{2+} channels insensitive to DHP have been found in some smooth muscles. A recent example is the DHP-insensitive, rapidly inactivating, voltage-dependent Ca^{2+} channels in the terminal branches of guinea pig mesenteric artery (80). The fraction of these channels increased in lower branches of mesenteric arterial tree, and the conductance contributed significantly to Ca^{2+} entry. The DHP-insensitive channels had unique biophysical and pharmacological properties, but the molecular entity responsible for this conductance has not been identified. Others have reported that T-type or low-voltage-activated channels are expressed and contribute to Ca^{2+} entry in smooth muscles (45, 102). Mibefradil (Ro-40-5967) has been suggested as an antagonist of T-type channels in vascular muscles, but the selectivity of this compound has been questioned (cf. Refs. 10, 69).

Nonselective cation channels. Endogenous agonists activate nonselective cation currents and Ca^{2+}-dependent Cl^- currents in smooth muscles. Both inward currents can contribute to Ca^{2+} entry via depolarization and activation of voltage-dependent Ca^{2+} channels. Although Cl^- current is important in this process, this review will not discuss this family of conductances because it is not a direct source of Ca^{2+}. The reader is directed to other reviews (such as Ref. 66).

ACh, acting via muscarinic receptors, activates a nonselective cation current (I_{ACh}) in vascular and visceral smooth muscles (e.g., Refs. 8, 32, 51–54, 60, 73; Fig. 1). At the negative potentials of smooth muscle cells, most of the current through this conductance is carried by Na^+, and the inward Na^+ current is responsible for a significant part of the depolarization caused by muscarinic stimulation. I_{ACh} is voltage dependent in many cells, and the current reverses near 0 mV, demonstrating its nonselectivity. I_{ACh} is not directly activated but facilitated by intracellular Ca^{2+} (52, 86, 97, 110). Activation of I_{ACh} is blocked by pertussis toxin, and the current can be directly activated by dialysis of guanosine 5′-*O*-(3-thiotriphosphate) (53), demonstrating the role of a G-protein-dependent mechanism. Antibodies to the α-subunit of G_i or G_o were also shown to block activation by ACh (110). I_{ACh} may be opened by ACh binding of M_2 receptors working through G_i/G_o and facilitated via M_3 receptors that are coupled to phospholipase C (PLC), D-myo-inositol 1,4,5-trisphosphate (IP_3) production, and Ca^{2+} release (14). The single-channel conductance of I_{ACh} appears to be 20–30 pS (55, 64, 108). Several ions and drugs block I_{ACh} (including Gd^{3+}, Ni^{2+}, Cd^{2+}, quinine and fenamates), but specific blockers have not been identified.

I_{ACh} is permeable to Ca^{2+}, but there is controversy over whether this conductance is a significant direct source for Ca^{2+} entry (see Ref. 86). I_{ACh} conducts Ca^{2+}, and Inoue and Isenberg (51) showed that the current was of equal magnitude when external Na^+ was replaced with Ca^{2+}. The question remains, however, as to what extent the channels conduct Ca^{2+} in physiological ionic gradients. Some investigators argue that I_{ACh} provides enough Ca^{2+} influx to affect $[Ca^{2+}]_i$, (cf. Refs. 32, 64). Such a conclusion is supported by the following observation: a rapid reduction in extracellular Ca^{2+} while I_{ACh} is activated immediately decreases $[Ca^{2+}]_i$ and a rapid increase in extracellular Ca^{2+} increases $[Ca^{2+}]_i$. In addition, rapid application of a blocker of I_{ACh}, such as Ni^{2+}, also immediately decreases $[Ca^{2+}]_i$. Fleischmann and co-workers (32) calculated that up to 14% of I_{ACh} is carried by Ca^{2+} in airway smooth muscle cells.

Other agonists, such as adrenergic agents and peptides, also activate nonselective cation conductances in smooth muscle cells (cf. Refs. 70, 81, 111). These currents are similar but not identical to I_{ACh}. A major difference is that these conductances are not, in general, facilitated by intracellular Ca^{2+}, suggesting they are due to species of ion channels different from I_{ACh}.

The molecular entities responsible for nonselective cation conductances in smooth muscles have not yet been identified; however, a recent study offers possible insights into the molecular nature of these channels. Inoue and co-workers (56) showed that expression of a

transient receptor potential protein (TRP6) in HEK293 cells resulted in a current with biophysical and pharmacological properties similar to the nonselective cation current activated by adrenergic stimuli in portal vein cells. Treatment of cultured portal vein myocytes with TRP6 antisense oligonucleotides inhibited immunoreactivity to TRP6 antibodies and reduced the nonselective cation conductance activated by adrenergic stimulation.

P2X receptors. ATP is released as a neurotransmitter from autonomic neurons and affects the activity of many smooth muscles (18). ATP can function as either an excitatory or inhibitory neurotransmitter. As an excitatory transmitter, ATP typically activates P2X receptors ($P2X_1$, $P2X_2$, and $P2X_4$), which are receptor-operated cation channels expressed by smooth muscle cells (16, 83, 103). In a variety of native smooth muscle cells, ATP activates a cation current (9) that is similar in characteristics to heterologously expressed P2X receptors (99). Activation of P2X receptors, such as those of human saphenous vein myocytes, is associated with a transient, nonselective cation current and increased $[Ca^{2+}]_i$ (72). These authors concluded that the rise in $[Ca^{2+}]_i$ due to ATP was partly due to Ca^{2+} entry through P2X channels.

Stretch-sensitive nonselective cation channels. Mechanical stretch can also activate Ca^{2+}-permeable ion channels in smooth muscles. For example, in voltage-clamped urinary bladder cells, longitudinal stretch activated an inward current due to a Gd^{3+}-sensitive, nonselective cation conductance (115). In cells from mesenteric resistance arteries, cell inflation generated an inwardly rectifying, nonselective cation conductance (95) that was permeable to Ca^{2+} and blocked by Gd^{3+}.

Capacitative Ca^{2+} entry. In many cells, depletion of internal Ca^{2+} stores is coupled to activation of a Ca^{2+} entry pathway (cf. Ref. 91). This is known as store-operated Ca^{2+} entry or capacitative Ca^{2+} entry (CCE). Drugs that deplete stores without activating G-protein-coupled receptors have been used in investigations of CCE because this technique makes it easier to distinguish CCE from receptor-operated Ca^{2+} influx. In the presence of L-type Ca^{2+} channel blockers, depletion of Ca^{2+} stores with thapsigargin activated a sustained Ca^{2+} influx independent of IP_3-dependent Ca^{2+} release (119). Other studies have shown that depletion of stores with sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pump inhibitors caused DHP-insensitive enhanced tone or increased $[Ca^{2+}]_i$, and these effects were due to Ca^{2+} influx. How store depletion activates a conductance in the plasma membrane is unclear, but this process may involve a diffusible factor or some direct interaction between proteins in the sarcoplasmic reticulum (SR) and plasma membranes.

Most data suggesting the existence of CCE are from studies in which cells or tissues were loaded with fluorescent Ca^{2+} indicators to assay the end result of CCE-increased cytoplasmic Ca^{2+}. If Ca^{2+} enters smooth muscle cells during this process, it should generate an inward current (Fig. 1). It has been far more difficult to measure this current; however, there are reports of inward currents resulting from pharmacological depletion of Ca^{2+} stores.

Freshly dispersed cells from the mouse anococcygeus were studied with the whole cell configuration of the patch-clamp technique, and membrane currents induced by cyclopiazonic acid (CPA) were characterized (113). After voltage-dependent Ca^{2+} currents and K^+ currents were blocked, CPA activated two components of inward current. The first component, which was transient, was a Ca^{2+}-activated Cl^- current. The second, sustained component had a nearly linear current-voltage relationship with a reversal potential of +31 mV. When extracellular Ca^{2+} was removed, the reversal potential shifted to +18 mV. The authors determined that this current was due to a nonselective cation conductance. Treating cells with caffeine generated a similar current. The CPA-induced nonselective cation was blocked by Cd^{2+} (100 μM) and SKF-96365 (10 μM) but not by La^{3+}. In similar experiments, currents were measured while changes in cytosolic Ca^{2+} were monitored with fura 2 (114). The sustained current noted previously was associated with increased $[Ca^{2+}]_i$. Both the current and the change in $[Ca^{2+}]_i$ were blocked by Cd^{2+} and SKF-96365, suggesting that the nonselective cation current was responsible for CCE in mouse anococcygeus cells.

Other studies have reported a conductance in vascular smooth muscle cells that is activated by a diffusible factor (Ca^{2+} influx factor) produced by yeast and human platelets (100). Application of thapsigargin activated 3-pS cation channels in cell-attached membrane patches (101). The same channels were activated when cells were loaded with 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid to deplete stores without raising intracellular Ca^{2+}. The 3-pS channels were shown to be cation channels and nonselective for Ca^{2+}, Sr^{2+}, Ba^{2+}, Na^+, K^+, and Cs^+. The authors concluded that this conductance might be responsible for CCE in vascular smooth muscle cells.

A recent report has proposed that the molecular entity responsible for CCE may be encoded by *trp* genes (118). Transcripts of *trp1* were expressed in smooth muscle cells of resistance arterioles, arteries, and veins. Antibodies specific for TrpC1, a gene product of *trp1*, showed expression of TrpC1 protein in vascular smooth muscle cells and found the protein localized in the plasma membrane. Peptide-specific binding of the antibody blocked store-operated Ca^{2+} channel activity.

INTRACELLULAR CA^{2+} UPTAKE MECHANISMS

Sarcoplasmic reticulum. Storage of Ca^{2+} in cellular organelles also provides important physiological regulation and the potential for release of Ca^{2+} during physiological signaling (Fig. 1). The main storage compartment is the SR, and this organelle has a major role in maintaining low $[Ca^{2+}]_i$. The volume of SR appears to vary between smooth muscles, but, in general, the SR forms an extensive intracellular network that is

capable of Ca^{2+} uptake, storage, and specialized release. SR volume is estimated to be 1.5–7.5% of smooth muscle cell volume. SR is typically more abundant in tonic (e.g., aorta) than phasic (e.g., portal vein) smooth muscle. Much of the surface of SR in smooth muscles is closely associated with the plasma membrane (27), such that release of Ca^{2+} can greatly influence the concentration of Ca^{2+} near the inner surface of the plasma membrane. This organization has profound consequences for Ca^{2+} signaling (see below in *Ca^{2+} sparks*).

The SR is surrounded by a membrane that is not freely permeable to Ca^{2+}. Specialized, active Ca^{2+}-ATPases, known as SERCA pumps, exist in the SR membrane; these pumps generate and maintain about a 10,000-fold Ca^{2+} gradient between the SR lumen and the cytoplasm. Three genes encode SERCA pumps, and two subgroups of SERCA2 (SERCA2a and SERCA2b) have been identified. Most smooth muscles express SERCA2b (115 kDa) and SERCA 3 (105 kDa) (116). SERCA pumps utilize the energy from ATP hydrolysis to translocate Ca^{2+} from the cytoplasm to the lumen of the SR. After Ca^{2+} is pumped into the SR, it is buffered by proteins, such as calreticulin and calsequestrin. These proteins can bind large amounts of Ca^{2+}. As a result of high-affinity Ca^{2+} uptake and intraluminal SR buffering, the actual Ca^{2+} store is estimated to reach Ca^{2+} concentrations of 10–15 mM (105).

SERCA pumps are regulated by phospholamban, a small transmembrane protein (52 amino acids) that assembles as a 6-kDa homopentamer (2). Regulation of SERCA pumps occurs through an inhibitory association between phospholamban and the Ca^{2+}-ATPase that can be relieved by phosphorylation with either protein kinase A or G (92). Enhancing Ca^{2+} uptake tends to reduce basal levels of Ca^{2+} and shorten Ca^{2+} transients initiated by depolarization and/or agonist stimulation. Thus phosphorylation of phospholamban may be one of the ways in which agonists that enhance production of cAMP and cGMP produce net inhibitory effects.

Studies of the function of SERCA pumps have been strongly aided by specific SERCA pump inhibitors, such as thapsigargin and CPA (see review, Ref. 68). When SERCA pumps are inhibited, a major source of Ca^{2+} regulation is lost, Ca^{2+} leaks into the cytoplasm, and cells are unable to maintain typically low cytoplasmic concentrations. Uptake of Ca^{2+} after Ca^{2+} transients is also compromised, extending periods of contraction. For example, in guinea pig urinary bladder smooth muscle, CPA slowed recovery of basal Ca^{2+} levels after a depolarization-induced Ca^{2+} transient by a factor of four (35), thus demonstrating the importance of SERCA pumps in the process of relaxation.

Mitochondria in Ca^{2+} uptake. Evidence from a variety of cell types suggests that mitochondria play an important role in Ca^{2+} homeostasis. Mitochondria develop quite negative membrane potentials by extrusion of protons via the electron transport chain. This creates a strong electrochemical gradient for Ca^{2+} entry, and a Ca^{2+} conductance in the inner membrane of mitochondria, the Ca^{2+} uniporter, facilitates the uptake of Ca^{2+}. In voltage-clamped gastric smooth muscle cells, the rate of Ca^{2+} extrusion after Ca^{2+} loading by voltage-dependent mechanisms was reduced by 50% after treatment with inhibitors of mitochondrial Ca^{2+} uptake (28). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a mitochondrial protonophore that collapses the electrochemical gradient for Ca^{2+} uptake, prolonged the decay of Ca^{2+}-activated Cl^- currents in portal vein myocytes that were activated by Ca^{2+} entry through voltage-dependent Ca^{2+} channels (41). Decay of Ca^{2+} transients was also prolonged in rat femoral artery cells by CCCP (61). These authors suggested that mitochondrial Ca^{2+} uptake may be most important when $[Ca^{2+}]_i$ levels are high and SERCA pumps may be more important when $[Ca^{2+}]_i$ levels are in a low range. Thus the duration of Ca^{2+} transients initiated by voltage-dependent mechanisms in smooth muscles appears to be reduced by mitochondrial Ca^{2+} uptake. Part of the effects of protonophores may be mediated by cell acidification. When pH buffering was increased in guinea pig urinary bladder cells, the effects of CCCP on slowing the decay of Ca^{2+} transients was greatly reduced (35). Thus intracellular pH may also be an important factor in regulating Ca^{2+} handling in smooth muscle cells.

High-resolution imaging of HeLa cells with specifically targeted green fluorescent proteins have shown very close associations between endoplasmic reticulum and mitochondria (93). Thus, when IP_3-dependent release channels are opened, mitochondria are exposed to much higher local Ca^{2+} concentrations than reached during global Ca^{2+} transients. It is possible that a similar close relationship between SR and mitochondria exists in smooth muscle cells. Release of Ca^{2+} from the SR with caffeine also stimulated Ca^{2+} uptake into mitochondria, as shown by changes in rhod 2 fluorescence (a mitochondrial Ca^{2+} indicator) in toad gastric muscle cells (29). A close functional relationship between SR and mitochondria has also been suggested in experiments on aortic smooth muscle cells by showing that mitochondrial Ca^{2+} increased along with cytoplasmic Ca^{2+} when cells were stimulated with either phenylephrine (release from IP_3 receptors) or caffeine [release from ryanodine receptors (RyRs)] (43). However, mitochondrial Ca^{2+} transients were delayed and prolonged compared with cytoplasmic Ca^{2+} transients. Others have found that mitochondrial Ca^{2+} uptake affects Ca^{2+} transients initiated by IP_3-dependent (i.e., receptor-mediated) Ca^{2+} release (77). These authors suggested that, after IP_3-dependent release of Ca^{2+}, mitochondrial Ca^{2+} uptake may regulate the Ca^{2+} concentration near IP_3 receptors and thus preserve the sensitivity of IP_3 receptors for subsequent Ca^{2+} release. A recent study has also suggested that mitochondrial Ca^{2+} uptake following Ca^{2+} release from IP_3 receptors is essential for pacemaker activity in interstitial cells of Cajal, the cells that provide electrical pacemaker activity in gastrointestinal muscles (112). More investigation is needed to fully appreciate

the role of mitochondria in modulating Ca^{2+} transients in smooth muscle cells.

INTRACELLULAR CA^{2+} RELEASE MECHANISMS

Ryanodine receptors. One of the channels that release Ca^{2+} from the SR binds the plant alkaloid, ryanodine, and is most commonly referred to as the RyR. Cytoplasmic Ca^{2+} activates RyR channels, and thus they are also referred to in the literature as Ca^{2+}-induced Ca^{2+} release (CICR) channels. This term is less specific because the second type of Ca^{2+} release channel, IP_3 receptors (see below), can also produce CICR, but only in the presence of IP_3. At least three isoforms of RyRs have been cloned (RyR1–RyR3). RyR2 and RyR3 are the primary isoforms in smooth muscle cells. RyR2 channels are formed by four monomers, each of nearly 5,000 amino acids and weighing ~565 kDa (84). These channels are activated by caffeine and locked into a subconductance state by ryanodine (47). This explains the effectiveness of these compounds in emptying Ca^{2+} stores. Ruthenium red blocks RyRs.

Micromolar concentrations of cytoplasmic Ca^{2+} are the primary activator of RyR channels in smooth muscles (30, 48). The amount of Ca^{2+} necessary to initiate CICR in smooth muscles (>1 μM) may be much higher than experienced by smooth muscle cells during peak excitability. Therefore, the physiological significance of CICR was questioned. Voltage-clamp experiments on urinary bladder smooth muscle (36) and portal vein (42) demonstrated that Ca^{2+} entry can trigger Ca^{2+} release via RyRs. However, others have reached opposite conclusions about the importance of CICR in smooth muscles. For example, Kamishima and McCarron (62) were unable to demonstrate CICR in portal vein myocytes; similar findings were obtained in studies of tracheal myocytes (33). Recent studies have shown that Ca^{2+} entry through DHP-sensitive Ca^{2+} channels can activate CICR in smooth muscle cells of urinary bladder and couple to the occurrence of Ca^{2+} sparks and Ca^{2+} waves, but the coupling is loose (25). DHP-sensitive Ca^{2+} channels can open without initiating Ca^{2+} release, and Ca^{2+} sparks were observed after DHP-sensitive Ca^{2+} channels closed. Thus the amount of Ca^{2+} entering through DHP-sensitive Ca^{2+} channels was typically insufficient to initiate CICR, or the spatial organization between RyR and DHP-sensitive Ca^{2+} channels was such that Ca^{2+} entry did not necessarily achieve CICR. The physiological importance of this mechanism is likely to be limited to specific smooth muscles that have high-current densities through DHP-sensitive Ca^{2+} channels and appropriate spatial associations with RyR channels.

IP_3 receptors. Stimulation by a variety of agonists binding to G-protein-coupled receptors in smooth muscles results in activation of phospholipase C and metabolism of phosphatidylinositol phosphate to IP_3. IP_3 activates Ca^{2+} release via a second class of Ca^{2+} release channels, known as IP_3 receptors. Three genes encode IP_3 receptors, and each channel is made of up of four subunits of ~300 kDa that form homotetrameric or heterotetrameric channels (87).

Activation of IP_3 receptors by its ligand is regulated by cytoplasmic Ca^{2+}, and there is a biphasic relationship between the open probability of IP_3 channels and Ca^{2+} release (11, 49, 74). A rise in $[Ca^{2+}]_i$ from basal levels to ~300 nM increases the potency of IP_3 in activating channel openings, but higher concentrations reduce the effectiveness of IP_3. Thus high levels of $[Ca^{2+}]_i$ provide negative feedback for the release of more Ca^{2+}. The potentiating effects of <300 nM Ca^{2+} on open probability of IP_3 channels provides a mechanism for CICR via IP_3 receptors. Potentiation of openings of both IP_3 receptor channels and RyR channels provides the possibility of interactions between Ca^{2+} release mechanisms. If these channels are located close to each other in the SR membrane, then it is possible for release of Ca^{2+} from one to stimulate release from the other. This type of interaction tends to be amplified by agonists that enhance IP_3 levels, and under some conditions can lead to regenerative Ca^{2+} waves (see Ref. 50).

Studies of the role of IP_3 receptors in smooth muscle were complicated for many years by the lack of specific, cell-permeable antagonists. Heparin, a nonpermeable and relatively nonselective antagonist, was the main agent used, but it had to be introduced into cells with patch pipettes or through cell permeabilization. Others have used IP_3-receptor antibodies that specifically block channel activation (67, 98); however, these also proved to be impermeable. Membrane-permeable compounds, such as xestospongin C (34) and 2-aminoethoxydiphenyl borate (2-APB; Ref. 76), have been shown to block IP_3-dependent Ca^{2+} release. These agents are potentially useful for investigations of IP_3-receptor-dependent Ca^{2+} signaling. Xestospongins have some efficacy in blocking RyRs; however, these compounds are 30% less potent in this action than they are in blocking IP_3 receptors. 2-APB has no known effects on RyRs; however, at concentrations >90 μM, it causes Ca^{2+} release and elevation in $[Ca^{2+}]_i$.

CA^{2+} EXTRUSION MECHANISMS

Plasma membrane Ca^{2+}-ATPase. To offset the influx of Ca^{2+} during excitable events, cells need mechanisms to remove Ca^{2+} to restore Ca^{2+} homeostasis. A major mechanism for Ca^{2+} extrusion is the plasma membrane Ca^{2+}-ATPase (PMCA), which uses energy from ATP to pump Ca^{2+} up the steep electrochemical gradient from cytosol to extracellular space. This pump is thought to be electron neutral because the Ca^{2+} pumped to the extracellular space is exchanged for two protons. Thus Ca^{2+} extrusion results in uptake of H^+, and this has to be compensated for by transporters such as Na^+/H^+ exchange. There are no known specific inhibitors of PMCA, but nonspecific P-type transporter inhibitors, such as lantanides and vanadate, can inhibit PMCAs (19).

PMCAs are the products of at least four genes, and the isoforms 1 and 4 are widely expressed (20).

PMCA1b is the most common and has a molecular mass of ~140 kDa. PMCAs are activated by binding of calmodulin to the COOH-terminal end. This removes autoinhibition and increases the affinity for Ca^{2+} and the transport rate (75). PMCAs are also regulated by protein kinases, and phosphorylation of sites near the calmodulin binding site by protein kinases A and G or by Ca^{2+}/calmodulin kinase reduces autoinhibition and facilitates Ca^{2+} transport (117). For example, stimulation of cultured vascular smooth muscle cells with nitroglycerin caused enhanced Ca^{2+} extrusion (65).

Na^+/Ca^+ exchange. In addition to active Ca^{2+} extrusion, some smooth muscles may rely on Na^+/Ca^{2+} exchange as a means of rapid Ca^{2+} extrusion. Ca^{2+} extrusion by this mechanism utilizes energy from the electrochemical gradient for Na^{2+} and transports three Na^{2+} into the cell while removing one Ca^{2+}. There is some controversy about the relative contribution of Na^+/Ca^{2+} exchange in smooth muscles (see Ref. 63). Generally, the test for Na^+/Ca^{2+} exchange is to determine whether smooth muscle accumulates Ca^{2+} in the presence of a reduced Na^+ gradient; however, there are problems with this approach, such as the ability of the SR to capture much of the accumulated Ca^{2+}. Recovery from elevated $[Ca^{2+}]_i$ in voltage-clamped myocytes from the guinea pig ureter was not seriously affected when the Na^+ gradient was decreased by 25–50%, and these authors concluded that Na^+-independent Ca^{2+} extrusion is mainly responsible for regulating $[Ca^{2+}]_i$ under the conditions of their experiments (1). In contrast, when toad gastric muscles were voltage clamped with a protocol designed to cause Ca^{2+} accumulation, clear evidence was obtained for Na^+-dependent extrusion of Ca^{2+} (78). This became the dominant means of extrusion when $[Ca^{2+}]_i$ exceeded 400 nM. Recently, mice deficient in Na^+/Ca^{2+} exchanger (NCX1) were shown to have markedly impaired tension development in aortic muscles in response to Na^+-free solutions, suggesting a role for Na^+/Ca^{2+} exchangers in Ca^{2+} handling in the aorta (109). In reviewing the literature, it is reasonable to conclude that the relative contribution of Na^+/Ca^{2+} exchange to Ca^{2+} extrusion varies between preparations, and very careful experiments may be necessary to observe the contributions from this mechanism in some smooth muscle cells.

INTEGRATED CA^{2+} SIGNALING

Superficial buffer barrier. The proximity of the SR to the plasma membrane and the existence of Ca^{2+} entry mechanisms in the plasma membrane and uptake mechanisms in the SR provide the structure for what has been termed the superficial buffer barrier. This concept suggests that a significant portion of the Ca^{2+} entering cells may be taken up into the SR to "buffer" transmembrane Ca^{2+} signals (Fig. 1). Unloading of Ca^{2+} may also be preferentially directed at the plasma membrane to ensure efficient extrusion. One of the initial observations suggesting a superficial buffer barrier was the finding that, in some smooth muscles, the rate, more than the magnitude, of Ca^{2+} entry was important in determining contractile force (104). In accordance with this idea, it was found that preloading the SR increased the transduction of Ca^{2+} entry to contraction, and unloading the SR delayed the development of force when Ca^{2+} entry was initiated. Recent studies of canine airway smooth muscle cells confirmed these observations and showed that contractions induced by KCl were enhanced when the SR store was inactivated with CPA or with ryanodine or by overfilling (59). When the filled state of the SR was reduced, KCl contractions were reduced. Another feature of the superficial buffer barrier is that agonists that tend to increase Ca^{2+} release from IP_3 receptors effectively short-circuit the uptake in the SR and enhance contraction in this manner (104).

Ca^{2+} sparks. Release of Ca^{2+} from RyR can result in highly localized, transient increases in Ca^{2+} concentration (Fig. 1). These events have been referred to as Ca^{2+} sparks (58, 82). Ca^{2+} sparks can result in very high local increases in Ca^{2+} (i.e., estimated to be at least 10 μM close to the site of release; see Ref. 88), and the proximity of RyR in the SR to the plasma membrane creates significant transient elevations of Ca^{2+} near the plasma membrane where numerous important Ca^{2+}-dependent proteins, including Ca^{2+}-dependent ion conductances, are located. Coupling of Ca^{2+} sparks to activation of Ca^{2+}-dependent conductances leads to transient changes in transmembrane ionic currents, but, in an intact tissues, where cells are electrically coupled, periodic Ca^{2+} sparks and transient currents may sum to affect the global conductance of the tissue. If there is a predominance of coupling between Ca^{2+} sparks to K^+ currents (e.g., via large-conductance Ca^{2+}-activated K^+ channels or "BK" channels), then the syncytial effect of Ca^{2+} sparks will be net outward current and hyperpolarization. With this design, mechanisms that enhance Ca^{2+} spark frequency or amplitude will tend to increase outward current and provide negative feedback to depolarization. It is also possible for Ca^{2+} sparks to couple to channels that generate inward currents (e.g., Ca^{2+}-activated Cl^- channels) and produce depolarization (120). Different smooth muscles utilize these mechanisms in a variety of ways, and other papers within this highlighted topic series of short reviews will discuss these specific mechanisms in more detail.

The first evidence for the role of Ca^{2+} sparks in regulating plasma membrane ionic conductances came from the observation that voltage-clamped smooth muscle cells held at depolarized potentials (i.e., −40 to −10 mV) generated large spontaneous transients outward currents (STOCs; Refs. 7, 13). Benham and Bolton (7) found that STOCs were due to the periodic activation of many BK channels and found that, when Ca^{2+} stores were depleted by caffeine or agonists, STOCs ceased until the stores were reloaded. At the time, microfluorometry techniques were not sensitive enough to detect the localized Ca^{2+} transients that underlie STOCs. Application of confocal microscopy and the use of fluorescent Ca^{2+} binding molecules with high quantum yield (e.g., fluo 3) during the 1990s

provided the resolution needed to detect Ca^{2+} sparks in smooth muscle. Utilization of these techniques have demonstrated Ca^{2+} sparks in a variety of smooth muscle cells (e.g., Refs. 40, 82, 88, 96, 120) and intact tissues (e.g., pressurized cerebral arteries; Ref. 58). Ca^{2+} sparks appear to be the result of a cluster of RyRs releasing Ca^{2+} at nearly the same time. Ryanodine (by blocking Ca^{2+} release) and thapsigargin (by unloading Ca^{2+} stores) inhibit Ca^{2+} sparks and the openings of BK channels that result from sparks. BK channels activated by Ca^{2+} sparks cause hyperpolarization and dilate pressurized arteries. Ryanodine and thapsigargin depolarize and constrict arteries, similar to blockers of BK channels. Thus, in vascular tissues that utilize this mechanism, Ca^{2+} sparks indirectly produce vasodilation via openings of BK channels.

The actual release of Ca^{2+} from a given spark site is significant and has been estimated to be due to a Ca^{2+} current of 4 pA of ~10-ms duration (22). This exceeds the amount of current due to a single RyR channel and suggests cooperativity between RyRs, possibly due to CICR. It is possible that Ca^{2+} from a single channel stimulates release from other closely clustered channels. In arterial muscle cells, Ca^{2+} sparks have a rise time of ~20 ms and a decay half-time of 50–60 ms. These events are highly localized and have a spatial spread of only ~2.4 μm at the point of half amplitude. Because RyRs are spatially close to the plasma membrane, relatively large changes in local Ca^{2+} result. The amplitude of sparks and the coupling between sparks and Ca^{2+}-dependent proteins are of critical importance to the physiological consequence of this phenomenon. Regulation of the frequency and amplitude of Ca^{2+} sparks may be an important means of coupling receptor activation to electrical responses. Studies have shown that second-messenger-coupled mechanisms regulate Ca^{2+} sparks. For example, Ca^{2+} sparks recorded from rat coronary and cerebral arteriole myocytes were increased in frequency by cAMP-dependent mechanisms (90) and reduced by protein kinase C-dependent mechanisms (15). The changes in the frequency of sparks may have been modulated by altering Ca^{2+} uptake into the SR or by affecting the Ca^{2+} sensitivity of RyR. Ca^{2+} sparks in smooth muscles may regulate many cellular processes in addition to membrane conductance. Future studies will greatly expand our knowledge of this aspect of Ca^{2+} handling and additional cellular events, such as cell differentiation, proliferation, and gene expression (39).

Some authors have suggested that triggering of Ca^{2+} sparks is coupled to specific targeting of activator Ca^{2+} through DHP-sensitive Ca^{2+} channels to RyR (3, 71). This requires alignment of Ca^{2+} channels in the plasma membrane with RyR in the SR. Caveolae contain DHP-sensitive Ca^{2+} channels, and it was found that disruption of caveolae with methyl-*ss*-cyclodextrin (dextrin) reduced the amplitude, frequency, and spatial spread of Ca^{2+} sparks (71). These data suggest that Ca^{2+} sparks may be generated in a microdomain containing both caveolae and SR, and Ca^{2+} entry through the plasma membrane L-type Ca^{2+} channels may initiate Ca^{2+} release from a cluster of RyR.

The importance of coupling between Ca^{2+} sparks and BK channels has been demonstrated with transgenic mice. BK channels are composed of pore-forming α- and regulatory β_1-subunits. The β_1-subunit increases the Ca^{2+} and voltage sensitivity of BK channels. Targeted deletion of β_1-subunits resulted in animals with elevated arterial blood pressure (17, 89). Studies on dispersed cells showed that the frequency and amplitude of Ca^{2+} sparks were unaffected in these animals; however, the coupling between Ca^{2+} sparks and BK channels was shown to be greatly diminished. Relative absence of STOCs resulting from the breakdown in coupling between Ca^{2+} sparks and BK coupling would tend to produce more depolarized cells and greater basal activation of voltage-dependent Ca^{2+} entry. Thus the defect in Ca^{2+} spark to BK coupling predisposed these animals to a greater degree of vasoconstriction and hypertension.

Ca^{2+} puffs. Localized Ca^{2+} transients in some smooth muscle cells are not blocked by ryanodine. In a study of murine colonic myocytes, Ca^{2+} transients were reduced in magnitude and frequency by xestospongin C, a blocker of IP_3 receptors (4). Thus it is more appropriate to refer to these events as "Ca^{2+} puffs" (Fig. 1). Ca^{2+} release via IP_3 receptors may be an important means of coupling between G-protein-regulated receptors and Ca^{2+}-dependent ionic conductances in the plasma membrane. In support of this idea, it was shown that stimulating cells with the P2Y receptor agonist 2-methylthio-ATP (2-MeS-ATP) increased the incidence of Ca^{2+} puffs in colonic myocytes. Secondary support of the idea that Ca^{2+} transients were due to IP_3-dependent release came from the observation that spontaneous Ca^{2+} transients and the effects of 2-MeS-ATP were blocked by U-73122, an inhibitor of PLC.

It was also shown that, when Ca^{2+} release from IP_3 receptors was stimulated with 2-MeS-ATP, the localized Ca^{2+} puffs had a tendency to develop into Ca^{2+} waves, which spread locally or in some cases throughout the cells. The development of waves depended on recruitment of Ca^{2+} release from RyR, suggesting cooperation between these two release mechanisms for agonist responses. As discussed previously, because both IP_3 receptors and RyR are sensitive to cytoplasmic Ca^{2+}, release of Ca^{2+} from one type of channel might increase the open probabilities of other channels nearby. Similar hypotheses have been put forward for stimulation of portal vein cells with norepinephrine (12) and of rat cerebral artery smooth muscle cells via UTP (57). Ca^{2+} waves in colonic myocytes may be restricted to a compartment near the plasma membrane because despite transcellular spread of waves contractions were not elicited. It was also found that IP_3-receptor-mediated puffs were coupled to both BK channels and small-conductance Ca^{2+}-activated K^+ channels (SK) in colonic myocytes. SK channels are known to be responsible for the hyperpolarization response due to release of ATP from enteric inhibitory

motoneurons. Thus release of Ca^{2+} by G-protein-mediated activation of PLC can be linked to an inhibitory response in colonic cells via localized Ca^{2+} release and activation of Ca^{2+}-activated K^+ channels.

The finding that G-protein-dependent activation of PLC and subsequent activation of Ca^{2+} release is coupled to K^+ channels seems contradictory to the well-described IP_3-dependent mechanism used by many excitatory agonists in smooth muscles. Thus the effects of ACh on Ca^{2+} transients were also examined because Ca^{2+} transients coupled to STOCs and hyperpolarization would tend to override the excitatory nature of cholinergic responses. In murine colonic smooth muscle cells, ACh reduced localized Ca^{2+} transients and STOCs (5). These effects were accompanied by a rise in $[Ca^{2+}]_i$. The inhibitory effects of ACh on Ca^{2+} puffs were mimicked by nonreceptor-mediated increases in basal Ca^{2+} and blocked by inhibitors of nonselective cation conductances (e.g., Gd^{3+} and SKF-96365). When the rise in basal Ca^{2+} was blocked, ACh profoundly increased Ca^{2+} transients and promoted the generation of Ca^{2+} waves. These events were coupled to enhancement in STOCs. The results showed that the rise in $[Ca^{2+}]_i$ that accompanies muscarinic stimulation of colonic muscles inhibits localized Ca^{2+} transients that could undermine the excitatory effects of ACh by activating Ca^{2+}-activated K^+ channels. The inhibition of Ca^{2+} transients by increased $[Ca^{2+}]_i$ might be explained by the bell-shaped relationship between $[Ca^{2+}]_i$ and sensitivity of IP_3 receptors to IP_3 (Refs. 11, 49, 74; and see *IP$_3$ receptors* above).

SUMMARY AND CONCLUSIONS

In summary, Ca^{2+} homeostasis in smooth muscles is complicated and dependent on many cellular proteins and specialized compartments (Fig. 1). So important are these mechanisms in regulating $[Ca^{2+}]_i$ and the contractile state of muscles that minor defects in function can greatly affect the mechanical activity of smooth muscle organs. With what is already known about basic mechanisms that regulate Ca^{2+} transport proteins, we are beginning to understand how defects in these mechanisms contribute to pathophysiological conditions. In the near future with genetic analyses and experiments on transgenic animals, it should be possible to determine the defects in Ca^{2+} homeostasis mechanisms in a wider variety of pathphysiological conditions.

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