Sarcoplasmic Reticulum Function in Smooth Muscle

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Wray S, Burdyga T. Sarcoplasmic Reticulum Function in Smooth Muscle. Physiol Rev 90: 113–178, 2010; doi:10.1152/physrev.00018.2008.—The sarcoplasmic reticulum (SR) of smooth muscles presents many intriguing facets and questions concerning its roles, especially as these change with development, disease, and modulation of physiological activity. The SR’s function was originally perceived to be synthetic and then that of a Ca store for the contractile proteins, acting as a Ca amplification mechanism as it does in striated muscles. Gradually, as investigators have struggled to find a convincing role for Ca-induced Ca release in many smooth muscles, a role in controlling excitability has emerged. This is the Ca spark/spontaneous transient outward current coupling mechanism which reduces excitability and limits contraction. Release of SR Ca occurs in response to inositol 1,4,5-trisphosphate, Ca, and nicotinic acid adenine dinucleotide phosphate, and depletion of SR Ca can initiate Ca entry, the mechanism of which is being investigated but seems to involve Stim and Orai as found in nonexcitable cells. The contribution of the elemental Ca signals from the SR, sparks and puffs, to global Ca signals, i.e., Ca waves and oscillations, is becoming clearer but is far from established. The dynamics of SR Ca release and uptake mechanisms are reviewed along with the control of luminal Ca. We review the growing list of the SR’s functions that still includes Ca storage, contraction, and relaxation but has been expanded to encompass Ca homeostasis, generating local and global Ca signals, and contributing to cellular microdomains and signaling in other organelles, including mitochondria, lysosomes, and the nucleus. For an integrated approach, a review of aspects of the SR in health and disease and during development and aging are also included. While the sheer versatility of smooth muscle makes it foolish to have a “one model fits all” approach to this subject, we have tried to synthesize conclusions wherever possible.

I. INTRODUCTION AND BRIEF HISTORICAL OVERVIEW

For an excellent general historical overview of the sarcoplasmic reticulum (SR) and endoplasmic reticulum (ER), the recent review by Verkhratsky (728) can be consulted. For other general references to the SR (or ER), Ca homeostasis and SR/ER Ca-ATPase (SERCA), the following reviews are recommended: Laporte et al. (384), Pozzan et al. (573), Karaki et al. (336), Strehler and Treiman (665), Floyd and Wray (184), Rossi and Dirksen (597), Carafoli and Brini (106), Carafoli (105), and Endo (167).

An internal store of Ca in smooth muscle cells was postulated following demonstrations of Ca remaining in the cells after immersion in Ca-free solutions. This followed older observations that contractile responses, which were known to be Ca dependent, could continue for varying periods in Ca-free solutions, and that if external [Ca] had been elevated before the switch to Ca-free solution, this period was extended (278). Although we now have a more sophisticated view of Ca handling and Ca sensitivity (e.g., Refs. 13, 201, 271, 612, 769), which could also account for contraction in Ca-free solutions, the conclusion reached, i.e., there must be an internal Ca store, was correct. Electron microscopy (EM) of smooth muscle revealed a membranous system of tubules and sacs, the SR, that was both close to the periphery and caveolae as well as deep within the cell. Close apposition of the SR to mitochondria and the nucleus was also noted. This membrane system excluded extracellular markers such as ferritin and horseradish peroxidase, but was contiguous with the lumen of the nuclear envelope. Although originally described as sparse, following improvements in fixation and microscopy, these earliest accounts were replaced with terms such as “well developed” and “rich reticulum.” It is now appreciated that the SR’s distribution, amount, and shape is not only smooth muscle specific, but also changes with physiological stimuli and developmental stage and in disease.
One of the earliest suggestions that the SR in smooth muscle was the major intracellular Ca store and that it acts as in striated muscle was made by Giorgio Gabella and was based on EM studies (194–196). In the same year, Avril and Andrew Somlyo demonstrated that strontium, used as a surrogate for Ca, was accumulated in the SR of smooth muscle (aorta and pulmonary artery), and therefore, it followed that the SR could store Ca and be an activator of contraction (656). Many further important contributions were made by the Somlyos, including the demonstration using electron-probe X-ray microanalysis that [Ca] decreased in the SR following agonist stimulation (e.g., Refs. 95, 371) and that Ca cycling to and from the SR occurred during contraction and relaxation (69).

The demonstration of a rich reticular formation in smooth muscle renewed interest in examination of the role of the SR in excitation-contraction (E-C) coupling. Much of this work was looking for or assuming similarity of mechanisms to those of striated muscle. It is only in the last decade that the distinct role of the SR in smooth muscle has been elucidated. Work from Nelson’s group (70, 306, 507) and others (76, 682) showed that local Ca release from the SR can control plasma membrane excitability. We built on this and showed that there is a fundamental relationship between SR Ca release and the action potential in ureteric smooth muscle (87).

We now appreciate that the SR can have the following roles as demonstrated in Figure 1: 1) contributing to Ca homeostasis by maintaining low resting [Ca], via action of the SERCA; 2) restoration of [Ca] and relaxation following stimulation, via the action of SERCA; 3) augmenting contraction through release of Ca and producing or modifying global Ca signals in response to agonist stimulation; 4) modulating membrane excitability through activation of Ca-sensitive ion channels; 5) contributing to the efficacy of plasma membrane Ca extrusion mechanisms by vectorially releasing Ca to them; 6) contributing to the maintenance of signaling microdomains around the plasma membrane and other organelles; and 7) influencing development, aging, and the health of smooth muscle tissues.

**Fig. 1.** Functions of the SR in smooth muscle. A cartoon to demonstrate the functions of the SR. 1: Contributing to Ca homeostasis and maintenance of low resting level of intracellular [Ca] via sarco/endoplasmic reticulum Ca-ATPase (SERCA) activity. 2: Contributing to relaxation of the smooth muscle cell by taking up Ca via SERCA. 3: Contributing to Ca signals and contraction by Ca release via IP$_3$ receptor (IP$_3$R)-or ryanodine receptor (RyR)-gated Ca release channels and Ca puffs and Ca sparks. 4: Contributing to excitability via Ca-activated K and Cl channels. 5: Facilitation of Ca efflux on plasma membrane Ca-ATPase (Ca pump) or Na/Ca exchanger, via vectorial release of Ca. 6: Contributing to subcellular microdomains and organellar Ca homeostasis via Ca uptake and release between organelles. 7: Correct SR function needed for normal development and health, and SR functional change is associated with disease and aging.
This is an extensive job description for an organelle only occupying ~5% of the cell. We highlight these physiological and pathological functions inter alia.

Our approach in this review has been to synthesize a holistic view of how the SR works in smooth muscle and then discuss how these components are put together in different smooth muscles leading to tissue specificity. There is a daunting amount of literature in some areas, not all of which can be cited, and for this we apologize. There are areas of the literature where clarity is slowly emerging and others where unfortunately it remains elusive. Thus the conclusions we make to clarify, summarize, and hopefully provide insight will include a degree of speculation and author bias, and perhaps not all will stand the test of time. We offer them as discussion points in the quest of understanding SR function in smooth muscle. We have also focused wherever possible on literature obtained on intact tissues, intact muscle strips, and native smooth muscle cells, i.e., freshly dissociated and isolated. The effects of cell culturing on almost every aspect of smooth muscle E-C are so well known and long standing that investigators ignore them at their peril (116, 533).

II. STRUCTURE AND LOCATION OF SARCOPLASMIC RETICULUM IN SMOOTH MUSCLE

A. Introduction

Although it may sound out-moded to be concerned with the structure and location of the SR, recent work, for example, on membrane microdomains and SR compartmentalization, make it important that we have a clear factual understanding of SR structure and distribution, to better judge what is feasible both within the SR and in its relation to other organelles and the plasma membrane.

B. Electron Microscopic Studies

Much of our structural information concerning smooth muscle has come from EM studies. These studies addressed the size of the SR, usually compared with striated muscle and in terms of cell volume, and SR location within myocytes. By revealing these features EM made a significant contribution not just to the understanding of SR structure, but also to E-C coupling in smooth muscle. Before fluorescent probes sensitive to ion concentration were developed, electron X-ray microprobe analysis provided spatial information about the elemental composition of different areas in the myocytes, in relaxed and contracted stages. Determinations of SR Ca content are considered in section VI.

Consistent between different workers and smooth muscles is the location of the SR: close to, but not touching the plasma membrane. The SR was noted to encircle the invaginations of the surface membrane, the caveolae. Sheets of reticulum running along the longitudinal axis of the cell are a feature of all the tissues examined, e.g., vascular (152), airway (375), gastrointestinal tract and bladder (197), human blood vessels (677), developing blood vessels (693), and portal vein (69). The SR is described as peripheral (or superficial) when it is close to the plasma membrane and central (or deep) when it is located away from this membrane. The peripheral SR has been associated with Ca homeostasis, local Ca release, and interactions with plasma membrane ion channels and hence excitability, whereas the central SR has been suggested to be more directly involved in contraction by supplying Ca to the myofilaments. This is discussed further in section V.

Given the different activity and mechanisms of excitation between phasic and tonic smooth muscles, differences in the amount and localization of the SR have been sought. In vas deferens, a phasic muscle, the SR at EM level was described as being located predominantly at the periphery, whereas the tonic aorta had proportionally more SR in a central location (515). This paper also noted a central distribution for the tonic pulmonary artery and tracheals.

The smooth muscle cell's interior is packed with myofilaments and intermediate filaments. It is estimated that they, along with associated dense bodies, occupy up to 90% of the cell's volume. The intracellular organelles share the remaining volume (195). Although the earliest EM accounts described the SR of smooth muscles as being poorly developed (e.g., Refs. 194, 548, 577), this view gradually changed and is borne out by recent studies using confocal microscopy (described later). In one of the best comparative studies, Devine et al. (152) compared rabbit smooth muscles and reported that ~5% of the cell volume was accounted for by the SR in aorta and pulmonary artery and that this fell to ~2% in mesenteric vein and artery and portal vein. These findings also correlated with the varying ability of these preparations to continue contracting in zero-Ca solutions, i.e., longer in aorta than mesenteric and portal vein. However, when low values for SR volume were reported in some (vas deferens and taenia coli, 1.7%) but not all (uterus, ~7%) (506) phasic smooth muscles, the correlation with contraction time in zero Ca fell away. Somlyo's group pointed out that osmium fixation can damage smooth muscle SR and that this could have contributed to reports of being sparse (652). Thus the view gradually changed from “However, we must bear in mind that a sarcoplasmic reticulum, as we understand the structure from skeletal muscle work, is definitely not present in smooth muscles, and it seems most unlikely that the minute fraction of reticulum present in some smooth muscles is capable of modulating calcium levels” (275) to “Our major conclusion is that there is in mammalian smooth muscles a sarcoplasmic reticulum that,
while being variable in different smooth muscles, is sufficiently well developed and organised. . . . .to function as a Ca store in the process of excitation-contraction and inhibition-relaxation coupling” (152).

Notwithstanding the fact that smooth muscle SR is well developed, its volume is less than that of striated muscles (~10% of cell volume). Therefore, there are reasonable grounds to question whether the SR in smooth muscle can have the same role as in striated muscles, particularly with respect to it being the amplifier of a small Ca entry signal to provide the necessary rise of [Ca] required for contractile activation by Ca-induced Ca release (CICR). This questioning is also suggested by the generally smaller SR volume in visceral smooth muscles, where Ca entry is predominant, compared with vascular smooth muscles, where SR Ca release is more prominent.

It seems to us that the pendulum swung from initially dismissing the SR’s role in modulating Ca signals and CICR in smooth muscle to viewing the SR as playing a very similar role to that occurring in striated muscles. We are perhaps now able to readjust the pendulum and see more clearly that smooth muscle SR is important to E-C coupling, but in its own distinctive way.

C. Confocal Imaging of the SR

Confocal imaging can show the extent and three-dimensional distribution of the SR in smooth muscle and identify Ca release and uptake sites, as well as allowing changes in its Ca content to be determined. Following from the development of Ca-sensitive fluorescent indicators to monitor cytoplasmic [Ca] changes, efforts were directed at developing similar probes for intracellular organelles. One of the earliest methods was to use dicarbocyanine dye, DiOC6. This has been used in a variety of cell types as a label for SR/ER (696). Although some authors have noted its specificity for the ER (605), others considered that DiOC6 probably labels other intracellular membranes (696). Another approach to SR imaging is to target specific components associated with the SR membrane using antibodies, e.g., against ryanodine receptors (RyRs), inositol 1,4,5-trisphosphate (IP₃) receptors (IP₃Rs), or SERCA pumps. The Ca within the SR can also be targeted by fluorescent indicators. If these can be preferentially loaded into the SR lumen (as opposed to other organelles and/or cytoplasm), then these too can provide a picture of the SR, assuming Ca is not compartmentalized within the lumen. Another useful range of markers for the SR/ER has been developed from ceramide analogs bearing the fluorophore boron dipyrromethene difluoride (BODIPY) (540). To confer SR organellar specificity, this backbone is added to fluorescent ryanodine or thapsigargin (an inhibitor of SERCA) (635).

Techniques of molecular biology to specifically target the SR proteins and fuse them with fluorescent proteins such as aequorin can also be used to image the SR, although these are rarely suitable or successful in smooth muscle cells. Unlike fluorescent indicators that can be used directly in intact tissues or single cells, using genetically manufactured SR-targeted probes requires transfection of the recombinant proteins in the cells, and hence culturing. This poses two problems for smooth muscle studies. First, the well-appreciated problems of phenotypic change that occur with even short periods of culturing. Second, conventional transfection has proven difficult in most smooth muscles. However, methods based on organ-culturing and nonviral delivery, such as reversible permeabilization, get around both these difficulties. They have been shown to work well in some smooth muscles, to introduce plasmids, antisense oligonucleotides, and small interfering RNAs (siRNA), e.g., focal adhesion kinase depletion with antisense intrachalmyocyte (690), calponin depletion with antisense in aorta (311), Rho knockdown in cerebral arteries (131), and siRNA against arterial TASK-1 K channel (234). These methods have added to our understanding of the complex signaling pathways in smooth muscle, e.g., downregulation of profilin with antisense oligonucleotides was shown to inhibit force in carotid smooth muscle (691), and transfection with plasmids encoding a paxillin mutant was employed to explore its role in tension development in tracheal myocytes (529). However, these and related methods have been little used to target the SR and its regulators. There appears to be only one study, which used adenoviral vectors and targeting of apoaequorin in native tail artery cells (588). This paper should also be consulted for an informed discussion and evaluation of methods. The use of fluorescent probes to measure luminal SR content is discussed in section viB.

Several studies using fluorescent BODIPY linked to ryanodine or thapsigargin and DiOC₆ have been used in native smooth muscle: stomach (753), portal vein (221), and uterus (635, 636). Recently, the value of BODIPY ryanodine has been questioned as it may not block RyR activity, perhaps due to the presence of the fluorophore (231, 455). Using fluorescently labeled thapsigargin and ryanodine in uterine myocytes, we found that both were distributed nonhomogeneously, but in the same pattern; there was abundant labeling around the nucleus and peripherally. The close apposition of the SR to plasma membrane leads to the cell shape being clearly outlined.

Figure 2 shows examples of the SR in live smooth muscle cells, imaged using a variety of techniques.

The SR in uterine myocytes can be loaded with the low-affinity Ca indicator mag-fluo 4. With the use of this area of increased [Ca], “hot spots” can be seen. This distribution was similar to that of SERCA and RyRs.
With the use of three-dimensional image reconstruction techniques, a network of interconnecting, spirally shaped tubules, mainly running along the longitudinal axis of the cell, can be seen. This is reminiscent of earlier descriptions in fixed tissue with EM. It is notable that when uterine cells in culture were studied with antibodies to IP$_3$Rs and RyRs, a different distribution was found; they had an homogeneous (diffuse) distribution and hot spots detected by fluo 3FF, were scattered throughout the cytoplasm (792). In stomach myocytes from *Bufo marinus*, Steenbergen and Fay (661) used mag-fura 2 and found the SR to be distributed in a punctate manner, but again with a concentration peripherally and around the nucleus. Other studies describing more or less similar structures, i.e., interlacing tubules, sacs, and cisternae, and location of the SR, include cultured A7r5 (706), mesenteric artery (214), and portal vein (221). It appears that when different fluorescent indicators are used, the same pattern of SR distribution in smooth muscle myocytes is observed.

As found with EM studies, these images of the SR show it to be abundant around mitochondria and continuous with the nucleus (221, 635). There may be more SR in a peripheral location in phasic muscles (portal vein and uterus), compared with mesenteric artery and A7r5 cells, again consistent with suggestions from EM studies. Immunostains and immuno-EM of RyRs in vas deferens and urinary bladder myocytes also revealed a largely discrete, peripheral distribution for the SR, assuming that the RyRs represent the entire SR (394, 525). Ohi et al. (525) also noted colocalization with large-conductance, Ca-activated K (BK) channels, discussed further in section X.

In summary, recent data obtained by live staining of native smooth muscle cells have added confidence to earlier data on SR structure and distribution obtained with EM. There is no certainty about what the distinction between peripheral and central SR means in terms of SR components or functional consequences for any particular smooth muscle. Perhaps because of the considerable interest in local Ca signals from the SR and their relation to plasma membrane ion channels, the literature tends to emphasize the peripheral SR, and few studies have addressed the role of central SR. All phasic smooth muscles examined with confocal techniques appear to have predominantly peripheral SR. As well as the interactions between the SR and plasma membrane and caveolae, there is also interest in elucidating the functional importance for smooth muscles of having their SR enveloping mitochondria and appearing con-
tinuous with the nuclear envelope, and these aspects are discussed next.

D. SR, Caveolae, Microdomains, and Superficial Buffer Barrier

1. Introduction

An excellent review of Ca microdomains in smooth muscle was provided recently by McCarron et al. (451). This topic in other cell types has also been recently reviewed by Rizzuto and Pozzan (592).

Many fine structural studies have noted the close (15–30 nm) apposition of the SR to the plasma membrane, particularly around caveolae (565, 677). The close contact between caveolae and the SR membrane (195, 197) has led some investigators to consider if caveolae also possess Ca release mechanisms, i.e., IP$_3$R or RyRs. In mouse ileal smooth muscle, Fujimoto et al. (191) reported IP$_3$ type I receptors on caveolae. This observation has, however, never been confirmed to the best of our knowledge. It has been reported that there is a regularity in the close apposition of the two membranes (565), leading to the suggestion that there may be a structural anchor between the two (688). However, it is generally concluded from EM studies that the foot structures composed of clusters of RyR reaching to the plasma membrane and found in skeletal muscles are not a feature of smooth muscle (652), although a case for this has been made by some (490). No structural rearrangements of the plasma-SR membranes have been observed with stimulation (although this has been reported for SR-mitochondrial junctions, see sect. uE; Ref. 140).

2. Caveolae

That the membrane invaginations that are caveolae approach very close to the SR membrane remained largely a structural note of little interest to physiologists, until it became clear that caveolae were specialized regions of the plasma membrane and could facilitate cell signaling and transduction pathways (85). It is now appreciated that the protein caveolin is responsible for the invagination of the surface membrane and that caveolae are a form of membrane lipid rafts. Lipid rafts, rich in cholesterol (and sphingomyelin), are less fluid than surrounding areas (hence “raft”) and have certain proteins concentrated or excluded from them, in a dynamic fashion. For a recent review of caveolae in smooth muscle, see References 298 and 520. That these biochemical specializations of the raft/caveolar membrane are functionally important has now been shown in several smooth muscles by disrupting caveolae, usually by extracting cholesterol [rat uterus (648) and ureter (26), human myometrium (798), blood vessels (155), and bladder (273)]. A link with obesity, through elevated cholesterol and other smooth muscle pathologies, has recently been demonstrated (186, 797).

A variety of techniques have shown that BK channels localize in caveolae (26, 80, 634). As will be discussed in section xR, BK channels are targeted by local SR Ca release, Ca sparks, and their activation can cause hyperpolarization and therefore changes in excitability in smooth muscles. Thus any disruption of the close link between the SR and caveolae would be expected to have functional significance (406). The Na-K-ATPase (Na pump) is also preferentially located in caveolae (491). As this has profound effects on setting ionic gradients for Na and K and thereby also membrane potential (11), anything which modulates its activity will be expected to influence smooth muscle excitability (e.g., Refs. 56, 183, 399). Recently, it has been shown that the α2 isoform may directly regulate Ca transport and signaling, due to its spatial proximity to the Na/Ca exchanger (NCX) (184, 326, 445). In vascular smooth muscles, a link between the α2 Na pump subunit and relaxation pathways has been demonstrated (801). As with the BK channels, these components of ionic homeostasis and Ca signaling are located in caveolae and close to the SR (57). From these close appositions of SR and caveolae, and congregation of signaling components associated with (but not exclusively limited to) Ca regulation, it has been proposed that the SR in this region is part of a specialized signaling complex, sometimes referred to as a signalosome (e.g., Refs. 280, 423).

3. Microdomains

Although it is unlikely that there is a structural anchor between caveolae and SR, or that there is a Ca release function in caveolae, there is evidence that these regions in the smooth muscle myocytes form a functional unit affecting Ca signaling. This microdomain arises between the plasma membrane and SR and has the conditions and components necessary to 1) have higher [Ca] than the bulk cytoplasm, 2) vectorially release Ca, and 3) act as a barrier or buffer to the free diffusion of Ca in this domain.

The existence of Ca microdomains in smooth muscle, where the [Ca] may be several orders of magnitude greater than in the bulk cytoplasm, overcomes objections to the NCX and Ca-activated membrane channels having too low a Ca affinity to contribute to Ca homeostasis during physiological processes. If [Ca] can also change rapidly in these microdomains, then those proteins with high association constants will be activated preferentially, even when they have similar $K_a$ values (451). The relatively slow diffusion constant of Ca in smooth muscle cytoplasm ~2.2 × 10$^{-6}$ cm$^2$/s (37), compared with water, will contribute to the maintenance of the microdomain.
Many of the features that led to the postulation of a specialized space and environment between the SR and plasma membrane will also hold for regions between the SR and other organelles.

4. Superficial buffer barrier

Van Breemen and colleagues (713, 715) hypothesized in the 1980s that there is a subplasmalemmal domain that is functionally separated from the cytosolic constituents by the superficial SR and that this would be important in modulating Ca signaling and force production in smooth muscle. This peripheral SR, being so close to the sites of Ca entry, would act to scavenge and buffer extracellular Ca entry. This buffering ability would be overcome during agonist stimulation or depolarization but could act to help maintain resting [Ca] low between stimuli. Following stimulation, the superficial SR will release Ca back to the plasma membrane Ca extrusion mechanisms. Because of the difficulty of directly measuring [Ca] in microdomains, direct experimental support for them was lacking. However, with the advent of near-membrane Ca indicators (170, 809), evidence supporting or consistent with the superficial buffer barrier role of the SR in smooth muscles has been provided in a variety of preparations (vascular (587, 713), gastric (754), bladder (789), uterus (631, 793)). The sites on the SR of Ca release and uptake during these processes may be functionally and spatially separate (635). The estimates of subplasmalemmal [Ca] have been between 10 and 50 μM (gastric (170), colonic (77), arterial (809)), and the elevations of [Ca] last up to 100–200 ms (339).

Inhibition of SERCA increases bulk Ca signals produced in response to agonists, which is consistent with the superficial buffer barrier hypothesis. Bradley et al. (77) calculated that the buffer accounted for binding of ~50% of the Ca entering colonic myocytes. Recently however, this group has argued that the SR should be viewed more as a “Ca trap” and that SERCA activity cannot curtail the rise of cytosolic [Ca] but rather contributes to its decline when efflux ends (451; see also Ref. 635).

Although not generally considered in accounts of smooth muscle microdomains, a role for calmodulin in contributing to localizing Ca signals has been proposed (764). This arises from the finding that, even at low [Ca], a specific “contractile” pool of calmodulin with zero or two of its four Ca-binding sites occupied is tightly bound to the contractile machinery. Local changes of [Ca] near the myofilaments could then activate this specific calmodulin pool by saturating the four Ca-binding sites to activate myosin light-chain kinase (MLCK) and cause contraction, while more distant local [Ca] changes need not be associated with contraction (764).

E. SR and the Mitochondria

Both EM and confocal imaging studies have shown the SR and mitochondrial membranes to be in very close proximity (175, 677). The distance between them may be as small as 10 nm (515). As with caveolae, the SR may appear to engulf mitochondria, and thereby create a specialized space for Ca signaling, discussed below. Although close association between ER and mitochondria has been described in other cell types (489, 561), few have reported the almost complete enveloping, which appears to be a feature of smooth muscles. This aspect has been examined in detail at the EM level by Dai et al. (140) in pig tracheal muscle. At rest, all (99.4%) mitochondria were within 30 nm of SR membrane, and the average distance was 22 nm. The majority (82%) of mitochondria were completely encircled or enwrapped by the SR network. The regions of close contact lacked the regular spacing sometimes seen between SR and plasma membrane, but specific anchors have been suggested in other cell types (175, 241).

What makes the above data particularly interesting is that the association of mitochondria and SR changed with acetylcholine (ACh) stimulation. The number of mitochondria surrounded by SR fell to 12%, and fewer were in close contact. The authors suggest the SR unwraps from mitochondria with stimulation and extends more into the cytoplasm. These structural arrangements between SR and mitochondria have been reported in Madin-Darby canine kidney (MDCK) II cells, but it is elevated [Ca] that keeps the close association and between them and low [Ca] causes dissociation (735). Lateral movements of mitochondria relative to the SR of up to 3 μm/s have been reported in neuroblastoma cells (405).

1. Mitochondria and smooth muscle Ca signaling

There is now a large amount of literature on mitochondrial Ca signaling and the interactions between the SR and mitochondria. It is beyond the scope of this review to assess all the literature on mitochondrial Ca signaling, but we will give an account of recent data investigating SR-mitochondrial signaling in smooth muscle. The following reviews provide a good perspective on this area (296, 451, 565).

Mitochondrial Ca uptake has enjoyed a resurgence of interest (53) largely accounted for by new techniques allowing for monitoring their role in Ca signaling and the weight of accumulated evidence showing that Ca uptake occurs during physiological conditions (e.g., Refs. 49, 159, 590). Due to the low affinity of their Ca transporter (46), the capacity of mitochondria to rapidly accumulate Ca was considered only relevant during pathologically high [Ca]. That there could be microdomains of high [Ca] around mitochondria, to which the SR (or ER) would
contribute, removed the “problem” of the mitochondrial transporter having such a low affinity for Ca (591).

In smooth muscles, the following studies have shown that mitochondria can contribute to Ca signaling (114, 156, 454, 488, 515, 589). Elevations of mitochondrial [Ca] with stimulation have been demonstrated in pulmonary arterial cells and considered to curtail the cytosolic [Ca] increases (157, 488). If the mitochondrial membrane potential (usually about −180 mV with respect to cytoplasm) is dissipated to disable the mitochondria, the decay of the cytosolic Ca transient was slowed in myocytes from portal vein (225), tail artery (675), pulmonary artery (157), and cultured aortic cells (678). Although not extensively studied in smooth muscles so far, it may be that mitochondrial uptake of SR Ca is a mechanism of promoting maximal Ca release from the SR, perhaps by removing Ca-induced feedback on the SR Ca release (128, 603, 675). In their study of anococcysgeus smooth muscle, Restini et al. (589) describe cross-talk between the SR and mitochondria and show EM Figures of the close contact between the two. Interactions have also been found with smooth muscle mitochondria when SR Ca release is through RyR (124, 603). These studies found that when mitochondria were inhibited there was a slowing of the rate of Ca release and decay of the Ca transient, and a decrease in Ca sparks. The work of Chalmers and McCarron (114) suggests that in colonic myocytes mitochondrial activity promotes Ca release through IP_3Rs and that some mitochondria are situated particularly close to these receptors. They also addressed the important issue of whether the Ca entry into mitochondria during smooth muscle activity causes significant mitochondrial inner membrane depolarization, which would impair their function and ATP production. They found no change during normal Ca transients but suggest depolarization may occur during repetitive Ca oscillations or oxidative stress.

Further work is required in smooth muscles to determine if the above data can be generalized to other smooth muscles, and if cross-talk or functional outcomes differ, depending on whether Ca is released via RyR or IP_3R. More work is also needed to establish the effects of mitochondria on smooth muscle local and global Ca signals. Finally, there is emerging data indicating that mitochondria may also play a role in pacemaking in smooth muscle cells (345, 374, 741, 742), which deserves further attention (see also Refs. 160, 456, 503).

F. SR and the Nucleus

The nucleus and the SR are commonly associated in smooth muscle cells. For example in aorta, Lesh et al. (394) describe the SR forming a continuous network and being contiguous with the outer nuclear envelope. Over the last decade, the evidence has shown that the nucleus can regulate its own [Ca] and that the nuclear pores are regulated (208, 353, 436). Furthermore, identification of IP_3Rs, RyRs, and SERCA on the nuclear membrane has added to our understanding of how it may generate and regulate its Ca signals (2, 276, 415).

The SR may contribute to Ca-regulated gene transcription, via CREB or NFAT (31, 110, 217, 662). The term excitation-transcription coupling has been used to highlight that the changes in [Ca] occurring during E-C coupling will also be involved in regulating gene expression and phenotypic modulation (733).

There is only a sparse literature on interactions between the SR and nucleus in smooth muscles. In cultured aortic cells using BODIPY-RY and thapsigargin, Abrenica et al. (3) reported a discrete localization of SERCA and RyR perinuclearly and suggested that they could contribute to regulating nucleoplasmic [Ca]. In turn, a role for the nucleus as a Ca sink was also suggested by the authors. In their study of Ca sparks in portal vein myocytes, Gordienko et al. (221) reported that the majority of spark initiation sites were within 1–2 μm of the nuclear envelope (mitochondria were not particularly associated with sparks in this study). The frequency of sparks in these perinuclear regions often increased to produce a Ca wave and contraction.

In cultured aortic (A7r5) cells, Missiaen et al. (482) noted Ca release and uptake in a thapsigargin-insensitive and nonmitochondrial store, which could have been the Golgi or nucleus. However, there seems to be little or no information on such interactions in native smooth muscles, so nothing further can be said at present.

G. Lysosomes

Interest in lysosomes as Ca storage organelles was stimulated by the finding that in sea urchin eggs a recently identified new Ca mobilizer, nicotinic acid adenine dinucleotide phosphate (NAADP), was stored in organelles equivalent to lysosomes in mammalian cells (126). NAADP is discussed further in section 5. In smooth muscle, a close association between the SR and perinuclear lysosomes has been shown (350, 650), and this region is described as a “trigger zone.” Lysosomes are acidic organelles dependent on vacuolar H-ATPase activity. Drugs that inhibit the transporter, such as bafilomycin, have therefore been used to determine the importance of this Ca store to cells in which this type of Ca mobilizer has been found [myometrial (650), pulmonary (61), and coronary myocytes (800)]. In these studies a decrease in the Ca signal to agonists (endothelin-1, histamine) was found. To date, no studies in smooth muscle have tried to assess the size of the lysosomal Ca pool or looked at the dynamics of its interaction with the SR. It appears at least in some cells that the NAADP release
can trigger SR Ca release from RyRs, but not IP$_3$R, suggesting a clustering of RyRs with the release channels on the lysosomes (61).

III. SARCO/ENDOPLASMIC RETICULUM CALCIUM-ADENOSINETRIPHOSPHATASE

A. Introduction

SERCA is the major protein associated with the SR. It is a P-type ATPase responsible for transporting Ca into the SR at the expense of ATP. Calcium binding proteins enable this process to continue with an SR free ionized Ca content of ~500 µM, only ~0.5–1% of the total Ca stored in the SR (463). This sequestering of luminal Ca also means that inhibitory feedback of Ca on the ATPase is minimized. Auxiliary proteins, most notably phospholamban, as well as metabolites and pH, regulate SERCA activity. Several isoforms of SERCA exist, and expression varies between smooth muscles.

Functionally SERCA creates an internal Ca reservoir. As a by-product of this activity it will also contribute to Ca homeostasis and basal [Ca] increases within the cytoplasm when its activity is inhibited. SERCA may be considered a Ca buffer, transferring bound Ca from the cytosol to the lumen of the SR (105). SERCA also functions to expedite the removal of Ca from the cytoplasm following stimulation and thereby contributing to relaxation. Pharmacological inhibitors of SERCA allowed progress to be made in understanding its structure and roles in smooth muscle, and fluorescent indicators have permitted the monitoring of its effects on intracellular [Ca] and direct measurements of luminal [Ca]. Molecular studies have given a precise understanding of many aspects of how SERCA structure relates to function and the consequences of genetic mutations. The structure of SERCA will be described next, followed by its catalytic cycle and then isoform types and function in smooth muscle. Features of its regulation and function are shown in Figure 3.

B. Enzyme Type and Activity

Active transport is required to drive Ca into the SR. This is brought about by the P-type (ion-motive) ATPase of the SR membrane, SERCA, which is in the same enzymatic class as the Na-K-ATPase and the plasma membrane Ca-ATPase (PMCA). Amino acid sequence similarity to the Na pump is more extensive than with PMCA. Recent studies using chimeras have begun to elucidate how SERCA and PMCA are targeted to their respective membranes (36, 509); SERCA has an ER retention signal close to the NH$_2$ terminus, which is cytoplasmic facing. SERCA transports two Ca for every ATP hydrolyzed. This also distinguishes it from PMCA, which has a 1:1 stoichiometry (290). For further details on the structure of SERCA and its similarity to other type ATPases, see the following reviews (106, 676).

The demonstration that SR membrane fractions could accumulate Ca and hydrolyzed ATP was the first evidence of SERCA (161). Such investigations were helped by the abundance of SERCA in striated muscle SR, as were the subsequent structural studies. The uptake and release of Ca into the SR by SERCA occurs as a cycle of reactions and conformational changes.

C. SERCA Structure and Catalytic Cycle

The following account is taken largely from several excellent reviews (487, 573, 777, 791). The pump was purified in 1970 (428) and the crystal structure resolved in 2000 by Toyoshima (701), and the conformational changes during Ca release were elucidated 2 years later by the same group (702). The structural determination of SERCA (isoform 1a) was the first for any active transporter. The SERCAs are complex proteins with single-stranded, polypeptide chains of ~110 kDa and distinct domains (676). These domains undergo considerable movement as the enzyme performs its function, which has the effect of limiting the amount of regulation that can occur without
compromising function. Folded into the molecule are four domains: M, transmembrane domain; P, phosphorylation domain; N, nucleotide-binding domain; and A, the actuator domain. Figure 4 shows diagrammatically SERCA structure and its catalytic cycle. The Ca binding sites are in the M domain, with the two Ca ions being 5.7Å apart. There are 10 transmembrane sections in the M domain (M1-M10), and this domain forms the channel through which Ca moves. The interaction site for phospholamban, a major regulator of SERCA1 (see sect. IV) is located in the N domain, around Lys-397, although other binding sites on the M domain have been described (19, 20). Thapsigargin and cyclopiazonic acid (CPA), specific inhibitors of SERCA, bind to the M domain (Phe-256 of M3) (521) and helped with the elucidation of SERCA structure and function (102). From topological models, the structure of SERCA can also be described as having three parts: a cytoplasmic head, a stalk domain, and a transmembrane domain (442). The cytoplasmic head region constitutes around half of the total SERCA mass, and it encompasses the A, N, and P domains. The A and P domains are connected to the M domain, and the N domain is connected to the P domain.

The simplest catalytic cycle for SERCA can be described as follows: E1→2Ca·E1→2Ca·E1·ATP→2Ca·E1·P→2Ca·E2·P1→E2·P1→E2→E1 (see also Fig. 4).

The two Ca binding domains exist in either a high- or low-affinity form, known as E1 and E2 states, and allow access only from the cytosolic and luminal side, respectively. Their \( K_d \) values are \( 10^{-7} \) and \( 10^{-3} \) M, respectively. Work has suggested that two Ca ions from the cytosolic side bind, and the enzyme is subsequently phosphorylated by ATP at Asp-351. The formation of an “energy-rich” aspartyl-phosphorylated intermediate is why SERCA is classified as a P-type pump. The phosphorylation reaction is highly pH sensitive and \( H^+ \) facilitates the reaction of inorganic phosphate (P) with the Ca pump (291). Phospholamban binds preferentially to the E2 state, lowering its apparent Ca affinity. It is as this high-energy intermediate (2Ca·E1·P) that the translocation of Ca occurs. Following translocation and release of the two Ca ions into the SR lumen, the enzyme becomes a low-energy intermediary. Two or possibly three (395) protons are countertransported during the step E2·P1→E2, which aids, but does not complete, electroneutrality across the SR membrane. The protons are released into the cytoplasm following binding of the two Ca ions.

### D. SERCA Isoforms and Expression in Smooth Muscles

Three genes (ATP2A1, ATP2A2, and ATP2A3) encode the three mammalian isoforms of SERCA known as SERCA1, -2, or -3. In addition, alternative splicing occurs giving rise to SERCA1a, SERCA1b, SERCA2a, and SERCA2b. SERCA1a is found in adult fast-twitch skeletal muscle, and...
SERCA1b is in neonatal muscles. Brody’s disease is a rare inherited muscle disorder in which relaxation rates of skeletal muscle are impaired, leading to muscle stiffness. Patients have high rates of mutation in the SERCA1 gene which lower its Ca transport activity (43). SERCA3 appears to be the most diverse form, and an ever-growing number of isoforms are being discovered (15, 58, 534, 566). The first was SERCA3a, found in nonmuscle cells and which has a low apparent Ca affinity, leading to questioning of its functional role in Ca signaling (573), although it may be that it maintains high [Ca] in microdomains. Subsequently, seven additional SERCA3 gene products have been described (58, 441). SERCA3 has been found in epithelial and endothelial cells, platelets, megakaryocytes, many endocrine glands, cerebellum, pancreas, and spleen (59). It seems that SERCA3 is not found in smooth muscle cells but could appear as a contaminant from other cell types when whole smooth muscle tissues are analyzed. For example, SERCA3-deficient mice have impaired vascular and tracheal relaxation, but the effects are mediated via endothelial and epithelial cells, respectively, which express SERCA3b.

SERCA2 is the isoform found in smooth muscles, and this will be discussed in detail.

1. SERCA2

SERCA2 is 84% identical in sequence to SERCA1a. The gene for SERCA2 is located on human chromosome 12 at 12q24.11. SERCA2b has a longer (luminal) COOH-terminal tail than that of SERCA2a, which is cytoplasmic (726). SERCA2a is expressed in heart, slow-twitch skeletal muscle, and many types of smooth muscles (25). SERCA2b appears be ubiquitously expressed, leading to it being labeled as the housekeeping form of SERCA. This designation, while also consistent with it being the phylogenetically earliest isoform, is likely to be too simplistic, as different tissues express different levels of SERCA2b (777). From expression studies in COS and cardiac cells it has been suggested that, compared with SERCA2a, the 2b isoform has a lower turnover rate of ATP hydrolysis, a 10-fold lower vanadate sensitivity and Ca transport rate, but a higher apparent affinity for Ca (425, 723, 724). Removal of the last 12 amino acids from SERCA2b in mouse cardiac cells abolished these differences, suggesting the long tail in 2b is affecting SERCA activity (723). Another difference between SERCA2a and -2b is the greater stability of 2a’s mRNA, perhaps explaining its increased expression levels. Both isoforms have the same sensitivity to phospholamban and thapsigargin (426).

Although the differences between 2a and 2b are not extreme, their functional and developmental importance, at least in cardiac muscle, is great. Mice engineered to have SERCA2a replaced with SERCA2b suffer from embryonic/neonatal mortality due to malformations of their hearts (~40%), and if the switch is made in adults, they develop cardiac hypertrophy (723). Heterozygous mice developed squamous cell tumors (560). The human disease Darier-White condition, in which there are foci of separation between keratinocytes, has been associated with mutations of the SERCA2 gene, but no cardiac symptoms are apparent (608).

In smooth muscles, when comparisons have been attempted, SERCA2b expression is often greater than that of 2a. Both 2a and 2b have been described in most smooth muscles: stomach (162), aorta (15, 434, 774), uterus (94, 707), mesenteric artery (15), trachea (14, 458), ileum (162), and pulmonary (162).

Following an investigation of splice variants, a third isoform, SERCA2c, has been described in a number of cell types: epithelial, mesenchymal, hematopoietic, and cardiac (141, 207), but its expression in, or relevance to, smooth muscle is unknown.

E. Regulators of SERCA Expression and Activity

Given the importance of SERCA to SR function and hence cellular Ca homeostasis, Ca signaling, and ultimately function, it was anticipated that its function would be modulated under physiological conditions and perhaps altered in pathophysiological states. However, the structure of SERCA has evolved to translocate Ca, and this is accomplished by large conformational changes in the molecule. This requirement for substantial conformational change to function is considered to impose limitations upon how much modification, protein-protein interactions, and other coupling to effectors can occur.

1. Accessory proteins

The best known regulator of SERCA is phospholamban, a small membrane protein discussed in section IV.C, along with other protein modulators that work from the luminal side of the SR. Other cytoplasmic regulators of SERCA activity are emerging and were well reviewed by Wuytack and colleagues (719). The list includes the insulin-receptor substrate IRS1/2, the Ca binding protein S100A1, acylphosphatase, and the antiapoptotic protein Bcl-2. IRS1/2 has been found to interact with SERCA2 in an insulin-dependent manner in cultured aortic cells (9).

2. Calcium and protons

In smooth muscles (and other cell types), regulation of SERCA expression has been linked to cytoplasmic (774) and luminal [Ca] (376). This latter study noted the coordinated regulation of SERCA and the PMCA, e.g., in response to thapsigargin. It is not apparent that the linked regulation of SERCA and PMCA are functionally synergistic, i.e., increased plasmalemmal Ca extrusion on PMCA...
3. Development, hormones, and gestation

While there is considerable developmental regulation of SERCA1 and SERCA2a in striated muscle (559), and SERCA3 also shows clear ontological differences in expression, SERCA2b, which predominates in smooth muscle, appears not to be developmentally regulated.

Culturing has been shown to change SERCA isoform expression, as the cells become less differentiated (387, 534). Thus the relative increase in SERCA2a with development in aorta is reversed with culturing (387). It is worth noting here that more work is required on native smooth muscle cells if clarity concerning modulators of SERCA expression and activity is to be obtained.

Thyroxine is the best recognized hormonal modulator of SERCA expression, and the SERCA2 gene has promoter elements that bind thyroid receptors. In neonatal hypothyroidism, SERCA2a is downregulated. SERCA2a and -2b were reported to be increased in pregnant compared with non-pregnant rat myometrium (344) and in laboring compared with nonlaboring human myometrium (707). The mechanism for this upregulation is unclear, although endocrine changes might well be suspected. A recent paper by Liu et al. (401) showed that 17β-estradiol could upregulate SERCA2a in cultured cells, along with the Na-K-ATPase β subunit. Tamoxifen, the anti-estrogen drug, decreased SERCA activity. In the male urogenital system, testosterone may influence SERCA activity, given that castration in rabbits decreases its activity in corpus cavernosum (325). However, these authors also reported no change in SERCA activity in urethral myocytes.

F. Summary

There is a very large amount of literature concerning many aspects of SERCA, but it is often not obtained on smooth muscle. We have a detailed understanding of SERCA structure and catalytic mode of action. We are also aware of the different SERCA isoforms and their expression in smooth muscle. However, we can still say very little about how these differences influence the fine-tuning of smooth muscle Ca stores and hence Ca signaling. Further work in smooth muscles on how SERCA activity is affected by developmental or hormonal changes is needed.

IV. PROTEINS ASSOCIATED WITH THE SARCOPLASMIC RETICULUM

A. Introduction

SERCA, a protein associated with the SR and of pivotal importance to its functioning, has been described above. We will now discuss other proteins associated with the SR and SERCA, especially phospholamban, and those proteins whose prime role is to bind Ca within the SR lumen, i.e., calreticulin and calsequestrin. We also briefly mention other proteins that may have a role to play such as junctin. We have not considered proteins whose role is not specific to the SR. However, it is important that we know about the environment in which these proteins (and SERCA) are operating; therefore, the SR membrane and the gradients across it, a topic that has received scant attention in many discussions of the SR, will be discussed first.

B. The SR Membrane

While much attention is given to the plasma membrane surrounding the smooth muscle cell, e.g., its composition, fluidity, and microdomains, less attention has been given to that of the SR. However, it is reasonable to suggest that the activity of SR channels, transporters, and associated proteins will also be affected by the same parameters.

The SR (and ER) has a unique lipid membrane. It is the most fluid of all membranes, a direct consequence of it also being cholesterol-poor and having many unsaturated phospholipids (719). Thus SERCA has evolved to function in this specific membrane, and its optimal activity in artificial lipid bilayers, for example, occurs with a different composition than that required for maximal Na pump activity (390). The high fluidity of the SR/ER membrane presumably helps nascent proteins to fold correctly. The low cholesterol content also ensures that the SR membrane is relatively thin.

The SR membrane is notably unsaturated in nature. The major phospholipids of the SR are choline (~65%), ethanolamine (~15%), and inositol (~7%) phospholipids; together they constitute ~80% of total lipids. Of the remaining neutral lipids, cholesterol makes up 95%. Cholesterol is excluded from the phospholipid annulus around SERCA (743). The informative reviews by Tada et al. (681), Lee et al. (390), and Vangheluwe et al. (719) should be consulted for further reading on SR lipid and protein composition.
SERCA is in direct contact with several SR lipids but in a cholesterol-poor environment, and this is essential for its function, as a highly fluid lipid membrane is needed for the large conformational changes required for SERCA function. Maximal activity of SERCA is affected by the thickness and head group composition of the SR phospholipids; phosphocholine containing 18C fatty acids yielded maximal ATPase activity, while longer or shorter fatty acids lowered its activity (261, 499). Dietary fatty acid changes have been shown to change the composition of the SR membrane (674), and its cholesterol content may increase with age (373) and contribute to the decreased SERCA function found in older animals (198) as the SR membrane becomes increasingly rigid.

Variation in the lipid composition of the SR from different skeletal muscles has been described and related to differences in SERCA activity (73), but nothing is known about how composition may differ between smooth muscles. In a study of macrophage SERCA, Li et al. (397) showed that enrichment with cholesterol inhibited SERCA2b activity. This loss of fluidity may explain SERCA decreased activity, as the large conformational changes required for Ca transport are limited. In terms of macrophage cell function, the authors noted that dysfunction of SERCA could lead to protein unfolding in the ER and contribute to atherosclerotic lesions in foam cells (cholesterol-loaded macrophages).

C. Phospholamban

1. Introduction

Phospholamban is a 52-amino acid homopentameric protein, which, depending on its phosphorylation state, reversibly binds to SERCA and affects its activity (431, 639, 680). The phosphorylation of phospholamban occurs by both cAMP-dependent protein kinase (PKA) at Ser-16 and Ca/calmodulin-dependent protein kinase II (CaM kinase II) at Thr-17, and the name phospholamban was given to mean "phosphate receptor" (679); see also Katz, 1998 and associated articles in the same volume). Phospholamban inhibits SERCA by lowering its apparent affinity for Ca through direct protein-protein interactions, probably of a single phospholamban molecule associated with two SERCA molecules, and thus phospholamban may restrict the large domain movements needed for SERCA activity (21, 790). Upon phosphorylation there is a large change in phospholamban charge, and this greatly decreases its inhibitory effect on SERCA. It is clear in cardiac muscle that phosphorylation of phospholamban is a highly important part of the mechanism whereby β-agonists via cAMP increase cardiac contractility; by relieving the inhibition of phospholamban on SERCA, more Ca can enter the SR and contribute to an increased Ca release on the next heart beat, and relaxation occurs at a faster rate. In heart, chronic inhibition of SERCA, as can occur with some human phospholamban null mutations (447, 621), is associated with dilated cardiomyopathy, as contractility is diminished.

2. Distribution in smooth muscle

Phospholamban is expressed in smooth muscle, although at lower levels than occur in cardiac myocytes (378, 643). Raeymaekers and Jones (584) first reported the presence of phospholamban in smooth muscle. They found it in pig stomach and rabbit and dog aorta but not pig aorta. This group then used pig stomach to cDNA clone and sequence phospholamban (725) and concluded it was 100% sequence identical to cardiac phospholamban and was the product of the same gene. Thus any differences in effects of phospholamban between cardiac and smooth muscle cannot be explained by isoform diversity.

An immunogold EM study of phospholamban showed a patchy SR distribution of it in a variety of smooth muscles (ileum, iliac artery, and aorta), again suggesting that its density was lower than that of cardiac SR (172). Interestingly, phospholamban labeling of the outer nuclear envelope was also seen, although this observation does not appear to have been further investigated. These early studies concluded that whereas SERCA expression was approximately comparable between smooth muscles, phospholamban mRNA levels varied 12-fold, with aorta expressing low levels compared with ileum and stomach (162).

Given the importance of PKA and CaM kinase II to smooth muscle force modulation, phosphorylation of phospholamban would be predicted to be one of their targets. It has also been suggested that in smooth muscle cGMP is an important mediator of phospholamban phosphorylation (129, 583) acting at Ser-16. A recent report (363) has found that in tracheal smooth muscle phospholamban is associated with a PKA signaling complex, in addition to its expected association with SERCA. If this were also to be the case in other smooth muscles, then this may lead to facilitated IP_3-induced Ca release, rather than effects via SERCA Ca pumping, i.e., phospholamban may have multiple functions and effects in smooth muscles. As the target for several second messenger-dependent kinases, phospholamban may also play a role in Ca signaling events associated with vascular smooth muscle migration and hyperplasia, or influence endothelial Ca signals, but a discussion of this is beyond the scope of this review (117, 563, 672).

3. Functional effects

The earliest studies performed in smooth muscle to investigate phospholamban’s role involved vesicle studies of Ca uptake (582, 745). Sarcevic et al. (615), using cultured aortic smooth muscle cells, suggested that the
cGMP-mediated atrial natriuretic peptide transduction pathway involved phosphorylation of phospholamban. In 1992, Karczewski et al. (338) suggested a role for phospholamban in the nitric oxide (NO)/endothelium-derived relaxing factor (EDRF)-induced relaxation of rat aorta. This work was followed up with a paper demonstrating more directly phosphorylation of phospholamban and coronary artery relaxation (337). However, no difference in blood pressure was reported when phospholamban knockout animals were made (418), and little effect of modulators of cGMP on the kinetics of aortic force was seen (379). Thus phospholamban may play a role in the actions of endogenous vasodilators, but its effects appear to be specific to different vascular beds.

The development of the phospholamban knockout mouse has led to investigation of its role in smooth muscles. An increase in the rate of force development to phenylephrine stimulation in aorta was observed, compared with wild-type controls (378). However, these authors reported no differences when KCl was the stimulant and, importantly, no significant difference in relaxation rates. As noted above, this was also the case when cyclic nucleotides were used (379). Given the role of SERCA in facilitating Ca efflux from smooth muscle myocytes, these data do not support a prominent role for phospholamban in this process, with two caveats. First, as with all knockout studies, other compensatory mechanisms can occur, e.g., changes in plasma membrane Ca transporters or other SR proteins such as sarcolipin (see below). Second, force measurements can be an unreliable surrogate for [Ca] changes, and no simultaneous [Ca] and force measurements have been made.

The study by Lalli et al. (378) on aorta of knockout mice described above did however note that greater phenylephrine-induced force was produced, compared with controls suggesting, but not proving, that an increase in SR Ca uptake may be occurring. In subsequent studies, the same group has examined portal vein, a phasic, blood vessel, which may be considered to have its [Ca] changes controlled and regulated in a different manner from the tonic aorta (671). This study found an alteration of the contractile pattern in the knockout mice, but no difference in sensitivity to ACh, and no difference in relaxation rate to isoproteenol.

It appears then that there are some differences in blood vessels from phospholamban knockout mice compared with wild type, but there is no consistent finding and perhaps only subtle differences in the response to agonists or relaxation rates. One drawback to conclusions drawn from the studies to date is that no measurements of SR Ca uptake or release have been made on smooth muscle cells taken from these animals to directly test suggestions, such as increased SR Ca uptake or faster Ca release. Functionally no differences in blood pressure have been found in these studies.

Several visceral smooth muscles have also been used to help elucidate phospholamban’s functional contribution to Ca signaling and contractility. In a series of studies on gastric smooth muscle, Kim and colleagues (346–348) have investigated the role of phospholamban in NO-mediated relaxation. Their work suggests that CaM kinase II is responsible for the phosphorylation of phospholamban in this tissue. They conclude that the major mechanism involved in phospholamban’s effect on gastric relaxation is an increase in SERCA activity influencing Ca removal, rather than an increase of spark-spontaneous transient outward current (STOC) coupling mechanism, although neither was measured. PKA-stimulated phosphorylation of phospholamban is postulated as one of the mechanisms whereby β2-adrenergic receptors (β2-AR) act to relax airway smooth muscle (263). Stimulated by the observation that asthmatics chronically treated with β2-AR agonist develop enhanced sensitivity to bronchoconstrictors (372), transgenic mice overexpressing the β2-AR were studied (458). The authors noted that phospholamban transcripts and protein were markedly reduced in airway smooth muscle cells from these mice. These mice also had reduced constriction to methacholine. Thus they suggest that downregulation of phospholamban is a target of β2-AR agonists and protects against airway hyperactivity.

We have preliminary data on knockout mice indicating that in the uterus phospholamban can influence the pattern of contractility. Thus the pattern of spontaneous contractions compared with controls was less frequent but longer lasting, and with many of the contractions having a spiked appearance (518). This pattern was also found in portal vein from knockout mice (671).

Phospholamban is expressed in bladder smooth muscle (517). Measurements of force and [Ca] have been made in phospholamban knockout (KO) and phospholamban overexpressing (OE) mice. When stimulated by carbachol, compared with wild types, maximal increases of force and [Ca] were depressed in KOs and increased in OE mice (517). These differences were abolished when SERCA was inhibited by CPA. Resting [Ca] was also elevated in the OE mice. It was concluded that Ca uptake into the SR, rather than content, is the mechanism by which phospholamban is affecting force. Interestingly, the phospholamban OE mice also had a significant decrease in SERCA expression.

In summary, it appears to us that despite some elegant studies there are insufficient mechanistic and functional studies to date to draw many firm conclusions about the role of phospholamban in smooth muscle. Some of the expected findings of knocking out phospholamban, e.g., slowing of Ca relaxation rates, do not occur and the reported changes in force are subtle rather than obvious; certainly the profound effects on contractility seen in cardiac muscle with phospholamban alteration appear
not to occur in most smooth muscles. Few studies on smooth muscle have considered the more complex role of the SR in this tissue, e.g., providing negative feedback on Ca entry via Ca sparks, as opposed to it acting as a Ca store to augment contraction. We tentatively conclude that phospholamban is not a major physiological regulator of SERCA in most smooth muscles; its effects on SERCA, for example, are not as large as those of luminal [Ca] alteration. This difference between smooth and cardiac muscles may be due to the relative expression of SERCA isoforms between the two muscles, but we suggest it also reflects the differences in the role of SERCA between them.

D. Sarcolipin

This 31-amino acid protein appears to be a homolog of phospholamban (522); it associates with the SR membrane, and its transmembrane domains are structurally similar. Sarcolipin inhibits SERCA by lowering its apparent Ca affinity (increasing $K_m$) and $V_{max}$ (27, 205, 435, 523). It has been found in greatest abundance in fast- (199, 425, 511) and slow-twitch muscles (523) as well as cardiac muscles (720). To date, we can find no data describing sarcolipin presence in any smooth muscle.

E. Calsequestrin

1. Introduction

Calsequestrin and calreticulin (discussed next) are the two most important luminal buffers of Ca in the SR. They share many protein properties, as well as fulfilling the requirement of being a Ca buffer, i.e., binding Ca with a high capacity and low affinity. Calsequestrin was the first SR Ca binding protein to be identified (103, 429, 432). Its crystal structure has been elucidated from rabbit skeletal muscle (734). These studies suggest that three very negative thioredoxin-like domains underlie the high Ca binding capacity of calsequestrin.

2. Isoforms and expression

There is evidence for calsequestrin expression, at varying levels, in the following smooth muscles: stomach (585, 730, 778), vas deferens (729, 730), aorta (730), ileum (585), and trachea (585). It may not be present in bladder (730) or pulmonary artery (585) and is either absent or in trace amounts in uterus (472).

It is now appreciated that two genes exist for calsequestrin expression, leading to two isoforms which are 65% identical (622). These isoforms are often referred to as the fast-twitch skeletal and cardiac forms (182, 622), but slow skeletal muscles express the fast form (181) and it appears that many smooth muscles express both isoforms (730). The functional significance of smooth muscles expressing the two isoforms of calsequestrin or neither remains to be established.

Calsequestrin in skeletal muscle binds $\sim 40–50$ Ca ions with a binding constant of $\sim 1$ mM and a high off-rate (430). The cardiac isoform binds $\sim 20$ Ca ions with an affinity of 0.5 mM (51). In striated muscles calsequestrin binds to other proteins, including triadin and junctin (see sect. II D), leading to a physical association with RyRs (233, 443). It may be that this arrangement enables the Ca of the SR to be in the right place, i.e., close to the release channel (187). Regular arrays of calsequestrin with Ca attached appear as crystalline structures in the SR lumen (607). Calsequestrin has been shown to increase the RyR open probability in skeletal muscle (343).

Wuytack et al. (778) were the first to report that calsequestrin was present in SR from smooth muscle. They examined pig antrum and concluded it contained the cardiac form of calsequestrin in low amounts compared with heart, although commensurate with the lower SERCA expression in smooth muscle compared with heart. These authors also noted that calsequestrin in portal vein smooth muscles had first been proposed, on morphological grounds, by Somlyo (652) and Franzini-Armstrong (187). Vas deferens expresses both calsequestrin isoforms in about equal amounts (730). Its distribution was reported to be clustered at discrete luminal sites and on SR located superficially below the plasma membrane and rich in IP$_3$R (729). Thus a specific distribution of calsequestrin in smooth muscle as reported also for striated muscles may occur (322, 323). This contrasts with the nondiscrete distribution of calreticulin (see below).

The colocalization of IP$_3$R with calsequestrin presumably aids rapid release of messenger Ca in smooth muscles expressing both these elements. Moore et al. (491) demonstrated in toad stomach that calsequestrin was not only present in the SR in a discrete manner, but that it was associated with the superficial SR. They also demonstrated a close association between calsequestrin and the NCX and Na pump distribution on the plasma membrane, in caveolae. Thus not only is calsequestrin close to SR Ca release channels but also to exchangers important to Ca homeostasis in smooth muscle, and contributing to microdomains (as discussed in sect. II D).

Despite these several publications from a number of different laboratories, a paper appeared stating that calreticulin and not calsequestrin was the major Ca binding protein in smooth muscle SR (472). This study however only examined one type of smooth muscle, pig uterus, and actually reported that calsequestrin could be detected in uterine tissue extracts but not SR vesicles. They also used calsequestrin antibodies on cultured uterine cells and reported negligible staining, but given the culturing and a switch to a noncontractile, synthetic state, these data are difficult to interpret. This publication appears to have...
stimulated Meldolesi’s laboratory to reexamine, confirm, and extend their earlier findings of calsequestrin expression in vas deferens (730). They again demonstrated both isoforms of calsequestrin are present in vas deferens and also expressed in stomach and aorta, particularly the skeletal muscle isoform. In another study combining ultrastructure and antibody labeling, the presence of calsequestrin in guinea pig vas deferens was confirmed, as was its absence from aorta (515). Again the calsequestrin distribution was similar to that of IP₃R.

3. Function

Although calsequestrin has been demonstrated in many smooth muscles, there appears to have been no recent work investigating its function and role in Ca signaling. Thus its functions, as a binding protein for Ca, as a part of an SR complex with Ca release channels (particularly RyRs), and triadin and junctin (see sect. mG), and as a sensor of luminal Ca, can largely only be assumed by analogy to studies in striated muscles, with all the caveats that this requires.

In summary, it seems reasonable to conclude that many, but not all, smooth muscles express calsequestrin and those that do express the two different isoforms in a tissue-specific manner. It is not possible to do anything other than speculate about how these differences may relate to differences in Ca signaling or function and pathology in different smooth muscles.

F. Calreticulin

1. Introduction

Calreticulin is a 46-kDa protein, the product of a single gene, and has been the subject of recent reviews focused on its role within the ER and skeletal muscle (164, 320, 419, 468, 761). It was first described in 1974 as a high-affinity Ca binding protein of the SR binding 25 mol Ca/mol protein (531) and its cDNA code was revealed in 1989 (180, 647). Unlike calsequestrin it contains an ER retention signal. It was initially isolated from the SR and its Ca binding ability noted, which led to Ca binding being viewed as its major function. As discussed in the reviews cited above, it is now appreciated that calreticulin has many functions, including chaperoning of ER proteins, and that it is also expressed in other cellular organelles and at the cell surface. A list of its functions would now include cell adhesion (e.g., Ref. 320), antithrombotic activity, induction of NO in endothelial cells, long-term memory in *Aplysia*, and development (see Refs. 320 and 469 for review of these other functions of calreticulin). Our focus is on its role in Ca homeostasis within the SR.

2. Expression

In smooth muscle, calreticulin has been found to be expressed in the SR lumen of several different tissues. In 1992 Tharin et al. (697) reported its widespread distribution in rabbit tissues, especially smooth muscle (aorta, uterus). Calreticulin has been described as the major Ca buffer in smooth muscle (472), but as already noted, this study only examined uterine tissue, which expresses calreticulin and only trace amounts of calsequestrin. In their study of vas deferens, Villa et al. (729) found both Ca-binding proteins present and reported that calreticulin was widely distributed throughout the SR, whereas calsequestrin had a more discrete distribution and overlapped with IP₃Rs. A recent study of human esophageal smooth muscle has detected a particularly strong calreticulin signal (stronger than for cardiac muscle), as well as calsequestrin but not phospholamban (177). Both Ca binding proteins decreased in patients with reduced gastrointestinal motility and elevated esophageal pressures.

3. Function

Calreticulin may specifically affect the activity of SERCA2b, suggesting some specificity of function in smooth muscles (318). SERCA2b has an additional transmembrane segment (see sect. mG) that is thought to contain a calreticulin binding site. It has been suggested that when SR [Ca] is low, calreticulin is not bound to SERCA and thus Ca enters the SR as SERCA is maximally active. When SR [Ca] is high, calreticulin binds to the luminal tail of SERCA2b and decreases its activity (468).

In vascular smooth muscle (aorta), calreticulin increased when glucose concentration was elevated, both in vitro and in vivo (699). These effects however may be due to calreticulin’s chaperone role and a destabilization of GLUT1 mRNA, rather than direct effects on Ca signaling in the myocytes. Daniel’s group working on airway and esophageal smooth muscle (142, 143) have presented evidence that calreticulin and calsequestrin are colocalized and precipitated in the detergent-resistant membrane fraction indicative of caveolae, along with L-type Ca channels. While these data await confirmation with additional techniques to ensure that the immunoprecipitation with caveolin is not due to their loss from the SR during membrane isolation, they indicate that these Ca-binding proteins may influence caveolar function and interactions with the peripheral SR and may contribute to Ca microdomains within the smooth muscle cell. The finding of calreticulin outside the SR/ER has been reported in other cell types and explains its role in processes such as cell adhesion (320, 468).

Using a *Xenopus* oocyte expression system, calreticulin was found to suppress IP₃-induced Ca oscillations, suggestive of it acting to inhibit Ca uptake by SERCA2b (318). It has been estimated that >50% of Ca in the ER is...
bound to calreticulin (501), and if its expression is deficient, then the ER Ca storage capacity is decreased and agonist-evoked Ca release is inhibited (467, 501). Conversely, overexpression leads to increased Ca storage capacity and a decrease in store-operated Ca influx (466). Emptying of the ER Ca store stimulates expression of calreticulin (744). Thus changes in calreticulin concentration may be expected to have a direct effect on Ca signaling, as well as indirect ones, as its chaperone function is also affected (470). Cardiac development is so impaired in calreticulin knockout mice that they die at the embryonic stage (467). However, in adult heart, the level of calreticulin expression is low, and its overexpression produces arrhythmias, heart block, and death (500).

G. Triadin and Junctin

While many proteins have been identified in the SR lumen (51, 424), two that are abundant and intrinsic to the SR membrane are triadin and junctin (38, 340). They appear to anchor calsequestrin to the junctional face of the SR membrane, perhaps to keep it close to Ca release channels. The binding to calsequestrin is disrupted by low (<10 μM) and high (>10 mM) [Ca] (803). It is also possible that these two proteins are SR luminal Ca sensors and modulators of RyR open probability (51, 359, 803). To date, triadin and junctin do not appear to have been described in smooth muscle. This may be a consequence of the lack of triad and diad arrangement of the SR in smooth muscle, but it may also be due to lower expression levels, methodological difficulties (443), or that different isoforms occur. For example, recent data have identified new triadin isoforms, which colocalize with IP$_3$R (721). Thus there may be an IP$_3$R-specific complex that could be in smooth muscle. Similarly triadin and junctin by mediating interactions with calsequestrin could have function in smooth muscle if expressed there (237).

H. Summary

In summary, while it is clear that for the SR to function as a Ca store the Ca binding proteins calreticulin and calsequestrin are required, little data are available investigating the functional effects of altering their expression in smooth muscle. Nor is it clear what advantages or specificity is conferred on smooth muscles by their expressing the different proteins or their isoforms. Little recent data have been obtained in this field despite the increased interest in how luminal Ca content affects Ca signaling and functions in smooth muscle. Triadin and junctin may not be expressed in smooth muscle, but verification of this would be helpful. We concur with the words of Volpe et al. (730) written more than a decade ago on this issue that “it is therefore possible that what at present appears to be no more than a complex pleiotropism could ultimately be attributed to specific physiological characteristics of the various smooth muscles.” We have however come no closer to examining this possibility in the intervening years.

V. CALCIUM RELEASE CHANNELS

A. Introduction

Ca release from the SR in smooth muscle cells occurs through activation of two families of Ca release channels: RyR and IP$_3$R channels, which have substantial similarities in structure (113, 657). These channels are involved in control of various functions in smooth muscle cells including contraction, relaxation, proliferation, and differentiation (31). The functional role of RyR and IP$_3$R channels in these processes critically depends on molecular identity, level and proportion of expression, subcellular distribution, and the types of functional units they form with other cellular structures. Smooth muscles differ by types (vascular and visceral), location (different vascular beds or various hollow organs), size (resistance versus conduit arteries), orientation (circular versus longitudinal), and function (phasic versus tonic), and it is not surprising that the data obtained so far indicate marked differences in expression, spatial distribution, and subcellular location of different isoforms of RyRs and IP$_3$Rs and that these underlie the generation of tissue- or cell-specific Ca responses (224, 292, 493, 765, 794). NAADP is an agonist at RyRs, but there may also be a third Ca release channel, which is discussed in the section vD.

B. Ryanodine Receptors

The RyR is a homotetramer with the (~565 kDa) subunits surrounding a central Ca pore. The subunits act in a coordinated way to gate the Ca channel and are also each associated with an important 12-kDa regulatory binding protein FKBP (427, 461). Cryo-EM and three-dimensional reconstruction reveal that RyR is a symmetrical mushroom like structure with a large cytosolic assembly and a short region that traverses the SR membrane (610, 624). The ion channel-forming, membrane-spanning regions are highly conserved between different RyR isoforms and are localized to the COOH terminus. The cytosolic domain consists of >80% of the mass of the RyRs. It assumes a quatrefoil shape and is the modulatory region of the molecule, containing binding sites for Ca, adenine nucleotides, calmodulin, FKBP, as well as phosphorylation sites (624). The massive size of RyRs makes them physically the largest ion channels (almost twice as
large as the IP₃R, their closest relatives). Because of its short and wide channel region, the RyR is suitable for sudden and large release of Ca from intracellular stores.

The Ca conductance of RyR is on the order of 100 pS, and Ca is their principal endogenous effector. Studies describing the Ca dependence of channel activity in isolated preparations showed that there are sites within the protein where Ca can bind and inhibit as well as activate the channels (460). Data obtained using purified channels protein where Ca can bind and inhibit as well as activatealerted preparations showed that there are sites within the protein where Ca can bind and inhibit as well as activate the channels (460). Data obtained using purified channels protein where Ca can bind and inhibit as well as activate the channels (460).

The RyR specifically binds the plant alkaloid ryanodine, which is the reason for its name. Each of the three isoforms of RyRs are encoded by a distinct gene (670). RyR1 and RyR2 are predominantly expressed in skeletal (812) and cardiac (532, 599) muscle, respectively, while RyR3 appears to be expressed in a variety of tissues (211, 243, 389). Unlike cardiac and skeletal muscle, which express only one RyR isoform, all three RyR isoforms can be expressed in smooth muscles, with the type and the relative proportion of expression of each being tissue and species dependent. Expression of all three isoforms has been reported for smooth muscles of the pulmonary and systemic vasculatures (133, 475, 511, 787, 805) and in pregnant human and rat myometrium (439, 440). Some types of smooth muscles coexpress only two RyR isoforms: RyR1 and RyR3 are subtypes identified in airway (158), and RyR2 and RyR3 are subtypes found in rat aortic myocytes (340, 351) and duodenum (138). Expression of just the RyR2 isoform was reported for smooth muscle cells of urinary bladder (115, 490), toad stomach (781), and cerebral artery (213), and only the RyR3 isoform is expressed in rat ureteric (60) and human nonpregnant (24) and mouse pregnant uterus (358) smooth muscle cells. An alternative splicing of RyR3 subtype was recently reported for some types of smooth muscles (138, 139). In mouse, spliced short isoform (RyR3s), without channel function, are coexpressed with nonspliced functional full-length isoforms of RyR3 (RyR3L) (139). Mouse duodenum myocytes express RyR2 and spliced and nonspliced RyR3 isoforms (138). The functional significance of these nonfunctioning RyRs is discussed below.

Culturing of smooth muscle cells (132, 444, 711) or tissues (154) alters the patterns and levels of RyR expression in smooth muscle cells. Changes in RyR expression have been reported in some smooth muscle pathologies, e.g., a loss of RyR2 expression was reported for hyperactive detrusor smooth muscle (316).

2. Localization

The spatial arrangement of RyRs in smooth muscle cells is in marked contrast to the regular distribution of RyRs in sarcomeric muscles. A helical arrangement of well-developed superficial SR was reported in live smooth muscle cells double stained with fluorescently labeled indicators for the SR and RyRs (221). Immunofluorescence and immuno-EM studies also show that the subcellular distribution of RyRs closely follows that of the SR (394, 490). Both a peripheral and!cytoplasmic distribution of RyRs is found in tonic smooth muscle (394), whereas the distribution is largely peripheral in phasic smooth muscles [vas deferens (394), urinary bladder (490)]. A transition from a diffuse cytoplasmic distribution of functional, but nonsparking, RyR2 in neonatal cerebral arteries, to a distinct distribution in peripheral clusters in adult arteries, with the production of Ca sparks, indicates that Ca spark-generating units of RyRs appear late in tissue differentiation and are confined to peripheral regions of the cell (213). Distinct RyR2 staining in close proximity to the plasma membrane colocalized with voltage-gated L-type Ca channels and BK has been reported for guinea pig urinary bladder myocytes (490) and vas deferens (525). Rat pulmonary artery myocytes that express all three subtypes of RyRs have RyR2, their predominant isoform located mainly in peripheral sites, and RyR3 in the perinuclear region and RyR1s in both regions (787). In rat portal vein, the antibody-staining pattern of RyR3 is diffuse, unlike that of RyR1 and RyR2 which exhibit discrete areas of higher density staining (475). A diffuse distribution of RyR3 was also reported for smooth muscle cells of duodenum (138) and nonpregnant mouse myometrium (476).

The existing histological data would fit a model of peripherally located collections of RyR2 and RyR1 and more diffuse distribution of RyR3 in smooth muscle cells.

3. RyR function

The physiological contribution of different RyR subtypes to Ca signaling in smooth muscle cells has been recently addressed using RyR knockout mice (314, 805) and antisense oligonucleotides specifically targeting each of the subtypes (138, 139, 407, 476). In portal vein, injection of myocytes with either RyR1 or RyR2 antisense oligonucleotides abolished Ca sparks, suggesting RyR1 and RyR2 may colocalize to form Ca spark sites, while...
suppression of RyR3 did not alter Ca spark activity. In urinary bladder myocytes, RyR2s are required for generation of Ca sparks (314). Native RyR3s are either not involved (133, 314) or may even have an inhibitory effect on Ca sparks (407). However, when both RyR1 and RyR2 are inhibited with antisense oligonucleotides, and under conditions of increased SR Ca loading, RyR3 can be activated by caffeine or agonists (475, 476). RyR3 gene knockout significantly inhibits the contractile response to hypoxia but not the norepinephrine-induced Ca and contractile responses, in pulmonary artery smooth muscle cells (805).

The function of RyR3 in Ca signaling is complicated by alternative splicing of RyR3. The expression of a short isoform of RyR3 (RyR3s) in HEK293 cells can inhibit both the full-length RyR3 (RyR3L) and RyR2 subtypes (315). In native smooth muscle, the dominant negative effect of the spliced isoform of RyR3 inhibits RyR2 activation and Ca signals in duodenum (138) and the activity of RyR3 in mouse pregnant myometrium (139). These observations reveal a novel mechanism by which a splice variant of one RyR3 isoform may heteromerize with the other RyR variants and suppress the activity of these RyR isoforms via a dominant negative effect. Thus tissue-specific expression of RyR3 splice variants is likely to account for some of the pharmacological and functional heterogeneities of RyR3, for example, the lack of Ca sparks and caffeine sensitivity in rat myometrium, despite expression of all three forms of RyRs (88).

On the basis of the available information, RyR2 appears to be the most important component of Ca sparks in smooth muscle, and recent findings on RyR3 splice variants have helped unravel some of the perplexity in Ca signaling in smooth muscle. Calcium sparks are discussed in detail in section IX.

C. IP$_3$-Sensitive Ca release channels

Binding of G protein-coupled receptor agonists to their receptors in smooth muscles leads to activation of phosphatidylinositol-specific phospholipase C (PLC), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to yield IP$_3$ and diacylglycerol (DAG) (48). The IP$_3$ releases Ca from intracellular stores by binding to IP$_3$R, which are IP$_3$-gated Ca release channels, while DAG activates protein kinase C (PKC). Activation of IP$_3$Rs via activation of L-type Ca channels independent of Ca entry, so-called metabotropic Ca channel-induced Ca release, has been suggested in arterial myocytes but requires confirmation by other studies (149), and is not discussed further.

1. Expression

Three distinct IP$_3$R gene products (types 1–3) have been identified in different mammalian species (283, 546). All three isoforms of IP$_3$R are structurally and functionally related (193). The IP$_3$R isoforms differ in their sensitivities to breakdown by cellular proteases (IP$_3$R2 relatively more resistant that IP$_3$R1 and IP$_3$R3) (765). These receptors exist as tetrameric structures, with a monomeric molecular mass of ~300 kDa. Unlike RyRs, IP$_3$Rs are poorly expressed in skeletal and cardiac muscle but extensively expressed in brain and other tissues including smooth muscles (317, 471, 498, 546, 695, 760, 794). In smooth muscles, the density of IP$_3$R is ~100 times less than in brain (437, 802). In visceral smooth muscles (356, 379–381), IP$_3$Rs are expressed in greater quantity than RyRs with an overall stoichiometric ratio of IP$_3$Rs to RyRs of ~10–12:1, while in vascular smooth muscles the ratio is 3:4-1 (62).

Smooth muscle cells express multiple IP$_3$R isoforms, although as with RyRs, the level of expression of different subtypes is tissue and species dependent. Using reverse-transcriptase PCR analysis, Morel et al. (493) showed that rat portal vein expressed IP$_3$R1 and IP$_3$R2, whereas rat ureter expressed predominantly IP$_3$R1 and IP$_3$R3 (493), although in an earlier study it was reported that rat ureteric myocytes expressed all three isoforms of IP$_3$Rs (60). In the thoracic aorta and mesenteric arteries, IP$_3$R1 was the only isoform found, while IP$_3$R1 and weak expression of IP$_3$R2 were reported for smooth muscle cells of basilar artery (324). The type 1 isoform predominate in guinea pig intestinal smooth muscle cells (222) as well as in adult porcine (297) and rat aorta (693), whereas type 3 predominate in neonatal aorta (693). Subtype-specific IP$_3$R antibodies revealed that the expression of IP$_3$R1 was similar in cultured aortic cells and aorta homogenate, but expression of IP$_3$R2 and IP$_3$R3 types was increased threefold in cultured cells (693). Immunostaining and functional studies performed on isolated cells and cells in situ in rat portal vein showed two subpopulations of cells, one which predominantly expressed IP$_3$R1 and a second predominantly expressing IP$_3$R2. Distinct patterns of the Ca responses were generated by the two subpopulations of myocytes in response to agonist (493).

2. Localization

In some smooth muscles, IP$_3$R1 has been localized throughout the cell, i.e., central and peripheral SR, as visualized using EM (191, 515, 693, 729), and in the others it had a mainly subplasmalemmal location (222). IP$_3$R2 has a diffuse cytoplasmic distribution, similar to IP$_3$R1 (and IP$_3$R3), but also occurs as dense patches in the peripheral cytoplasm, a pattern that IP$_3$R1 did not exhibit (668). IP$_3$Rs were also arranged in clusters in rat ureteric myocytes, as well as throughout the cell (60). In vascular myocytes, IP$_3$R2 is distributed peripherally and associated with the nucleus in proliferating cells (668, 694). In portal vein, IP$_3$R2 was also found closely associated with
the nucleus and at the plasma membrane, whereas the IP₃R3 was distributed predominantly around the nucleus (209).

Interestingly, IP₃Rs are also localized in structures other than the ER/SR. For instance, they have been found in the nuclear envelope in many cell types, including vascular (694) and visceral (729) smooth muscles. This may be important in regulating intracellular calcium release around the nucleus when vascular smooth muscle cells switch to a more proliferating phenotype. In myocytes from basilar artery, IP₃R1 staining was detected close to both the nuclear and plasma membranes. IP₃R1 was also observed throughout the cytoplasm intermingled with the actin filaments. This observation suggests that an extensive SR network spreads from the nuclear region, throughout the cell, and comes into close association with the plasma membrane. Localization of IP₃Rs close to the cell membrane may account for the activation of Ca-induced Ca entry via nonselective cation channels (381), calcium-dependent potassium (34), or chloride channels (239).

3. Role of IP₃Rs

Interaction of IP₃ with the IP₃R is a principal mechanism for mediating the release of Ca from the ER/SR in response to agonist stimulation. The IP₃R has a single high-affinity binding site (Kᵢ value of 80 nM), and it is estimated that half-maximal release of Ca from the SR requires 40 nM InsP₃. The InsP₃ sensitivities of the isoforms vary to a limited extent and are ranked IP₃R1 > IP₃R2 > IP₃R3 (Kᵢ values of 1.5, 2.5, and 40 nM, respectively) (766). IP₃R activity not only depends on the concentration of IP₃ but also on the cytosolic concentration of Ca (283, 546). This property allows Ca release activated by the IP₃R to regulate further Ca release. IP₃R1 activity can be stimulated at [Ca] below 300 nM, while at >300 nM Ca inhibits activity. This type of biphasic Ca dependence has been reported in smooth muscle cells (52, 282) in which the IP₃R1 isoform is predominant (283). The IP₃R2 and IP₃R3 do not exhibit such marked Ca concentration-dependent inhibition (240, 586, 586). The positive regulation of the IP₃R by Ca may be largely due to a direct binding of Ca to the receptor (484). The sensitivity of IP₃R is also controlled by the SR luminal Ca content such that a reduced content also reduces IP₃-induced Ca release (481), although recent data point to the cytoplasmic aspect of the channel being important (114).

Recent studies have shown that Ca oscillations in cultured rat portal vein myocytes and rat adrenal chromaffin cells depend on IP₃R2 or an interaction between IP₃R1 and IP₃R2 (323, 326). It has been shown that coexpression of IP₃R types 1 and 2 facilitates Ca oscillations, whereas expression of IP₃R3 alone generates monophasic Ca transients (493). Since IP₃R types 1 and 2 are more sensitive to IP₃ and Ca, it was postulated that they are required for generation of spontaneous SR Ca release known as Ca puffs (i.e., local Ca release through IP₃Rs) and Ca oscillations (60, 493). In contrast, the IP₃R3 is activated by higher [Ca] and may participate in agonist-induced Ca waves (493).

The clustering of IP₃Rs provides spatially discrete release sites and enables Ca signals that arise from IP₃Rs to exist with various amplitudes and locations throughout the cell (60). These localized increases produce an increase in [Ca] throughout the cell when puffs coalesce to form transient [Ca] increases (Ca waves) that propagate through the cell in a regenerative manner (60, 72). Calcium puffs are discussed further in section IX.

A cautionary note on using pharmacological inhibitors to study SR release mechanisms was made by MacMillan et al. (433). They found that dantrolene and tetra-caine, thought to be specific RyR blockers, could affect IP₃R Ca release, i.e., they may not be as specific as previously thought, and this will have implications when interpreting such data.

4. Conclusions

The variety, distribution, and combination of IP₃Rs and RyRs in smooth muscle and their contribution to different types of signals will underlie the specificity of mechanisms, as well as the spatial and temporal parameters that are required for specificity of function in different smooth muscles. These functions include not just tissue-specific contraction and relaxation parameters, but also secretion, proliferation, and phenotypic change. In the following section we address how these SR release processes can be modulated.

D. Modulation of SR Ca Release

Recently several excellent reviews have been published on the pharmacology of the RyRs and IP₃R channels (231, 384, 670); thus here for completeness we will briefly overview the basic pharmacology of the Ca release channels in smooth muscle cells. We will then consider in a little more detail the roles of cADP ribose and NAADP, as evidence grows for their physiological role in smooth muscle.

1. RyRs

RyRs have binding sites for many agents, reflecting the huge size of these channels. Methylxanthines, imidazoles and imidazolines, perchlorates, suramin, and volatile anesthetics all activate RyR. Inhibitors of RyRs include ryanodine, ruthenium red, procaine, tetracaine, dantrolene, and octanol (for more details, see references in Ref. 384).
A) Caffeine. Caffeine, an alkaloid methylxanthine, increases the RyR Ca sensitivity by increasing its open probability \( (P_o) \) without changing its conductance, as shown in single-channel experiments on RyR purified from cardiac (601), skeletal (600), and smooth muscles (258) reconstituted into planar lipid bilayers. The threshold caffeine concentration for Ca release is 250 \( \mu \text{M} \) (404). The caffeine-induced Ca release is a two-step process: a small Ca release and then a second regenerative phenomenon, in which released Ca acts on a cluster of RyRs, triggering further Ca release. Caffeine has long been used to deplete SR Ca stores in smooth muscle; this is frequently done in a Ca-free medium containing Ca chelators such as EGTA. The transient contractile response obtained under such conditions is a qualitative estimate of the average size of the Ca stores in the SR. Caffeine, however, does not deplete the SR Ca store in those tissues expressing the RyR2 dominant negative splice variant, e.g., the uterus.

In the guinea pig ureter, which expresses effectively only RyR (91, 92), concentrations of caffeine for Ca release ranged from 0.5 to 20 \( \text{mM} \) (89). The use of caffeine in smooth muscle comes with several concerns: 1) it inhibits phosphodiesterase and therefore raises cAMP concentration (341), which at concentrations exceeding 1 \( \text{mM} \) inhibits voltage-gated Ca channels; 2) it has the potential to augment capacitative Ca entry by depletion of the SR; and 3) it inhibits the SR Ca release induced by \( IP_3 \) (480) and inositol phosphate formation (576).

B) Ryanodine. Ryanodine is a poisonous alkaloid found in the plant *Ryania speciosa* (670). The compound has an extremely high affinity for the RyR. The pharmacology of RyRs has been elegantly reviewed (231). Ryanodine has complex concentration-dependent effects on the conductance and gating of single RyR channels (670). It binds with such high affinity to the receptor that it was used as a label for the first purification of RyRs and gave its name to them. At nanomolar to low micromolar concentrations, ryanodine locks the receptors in a half-open state, whereas it closes them at micromolar concentration, irreversibly inhibiting channel opening. There is an agreement that high-affinity binding results in channel activation or subconductivity, whereas low-affinity binding leads to channel inhibition (670). Ryanodine at low concentration is frequently used to deplete the SR by causing the Ca release channels to remain in a semiconducting state. This leak from the SR has several consequences, such as preventing the SR from storing any Ca it may accumulate, loss of Ca sparks and their effects on Ca-sensitive ion channels, and loss of ability to generate IP\(_3\)R-mediated Ca waves and Ca oscillations in many types of smooth muscle. This last effect is due to ryanodine depleting common Ca stores, i.e., shared by RyR and IP\(_3\)R. The binding of ryanodine to RyRs is use-dependent, that is, the channels have to be in the activated state for it to be effective.

C) Tetracaine. Tetracaine is a potent local anesthetic and an allosteric blocker of RyR channel function. At low concentrations, tetracaine causes an initial inhibition of spontaneous Ca release events (137), while at high concentrations it blocks release completely (236).

D) Ruthenium red. Ruthenium red is an organic polycationic dye that tightly binds to tubulin dimers and RyRs. It is a potent (nM) inhibitor of RyR (783), but also inhibits mitochondrial Ca uptake (242).

2. \( IP_3 \)Rs

Pharmacological inhibitors of IP\(_3\)Rs are less abundant compared with RyRs, and unfortunately, agents used to block these receptors are nonselective. The inhibitors most commonly used include 2-aminoethoxy-diphenylborate (2-APB), heparin, and xestospongins (for a more detailed review, see Ref. 384).

A) 2-APB. 2-APB is a synthetic monomer that can form a five-membered boroxazolidine heterocyclic ring (boroxazolidone), when an internal coordinate bond is formed between the nitrogen in the ethanolamine side chain and the tricoordinated boron (664). 2-APB inhibits the IP\(_3\)R channel opening without affecting IP\(_3\) synthesis or binding. Despite earlier claims to the contrary, 2-APB is not selective for IP\(_3\)Rs as it reduces capacitative Ca entry and Ca efflux from mitochondria by inhibition of the NCX, and it may also block gap junctions (reviewed in detail in Ref. 384).

B) Heparin. Heparin is a cell-impermeant inhibitor of IP\(_3\)R and acts as a competitive inhibitor of IP\(_3\) in permeabilized smooth muscles (91). At low concentrations (up to 2 \( \text{mM} \)), heparin may be specific, but at higher concentrations (>20 \( \text{mM} \)), it chelates Ca and inhibits contraction (358).

C) Xestospongins. The xestospongins A, C, and D, araguspongine B, and demethylxestospongion B are alkaloids from the Australian marine sponge *Xestospongia sp.* (199). They are cell-permeant, potent inhibitors of IP\(_3\)Rs. Xestospongion C is the most potent and widely used in smooth muscle studies (34, 45, 169, 483). Although more studies are required, accumulated data suggest that the various isoforms of IP\(_3\)Rs differ in their sensitivities to xestospongins. Xestospongins are not selective and can inhibit voltage-gated Ca channels, which complicates their use.

3. cADPR

Cyclic ADP ribose (cADPR) is a cellular messenger for calcium signaling (235). It is derived from nicotinamide adenine dinucleotide (NAD) and ADP-ribosyl cyclase (392). It is a physiological allosteric modulator of RyRs and helps stimulate CICR at low cytosolic [Ca]. RyR
activation with high concentrations of caffeine is partly due to caffeine mimicking the binding of cADPR to RyRs. Whether the action is by direct binding to RyR or indirect (through binding to FKBP) is debated. Some reports suggest that cADPR binding makes FKBP, which normally binds RyR2, fall off RyR2. Involvement of cADPR in Ca release via RyRs has been suggested for some vascular and visceral smooth muscles (262, 757, 776), but not colonic myocytes, where Ca efflux by the Ca-ATPase was stimulated (78). Firm conclusions are difficult to draw until further studies are performed in smooth muscle and cADPR synthesis in response to agonist stimulation is demonstrated.

4. NAADP

NAADP was first suggested as a Ca-mobilizing agent after studies in sea urchin eggs (125, 391). While NAADP is similar to cADPR and may be synthesized from the same enzyme, ADP-ribosyl cyclase CD38 (but see Ref. 650), it is a distinct Ca mobilizer, using RyR to amplify its signals after releasing Ca, rather than acting to modulate RyR release as cADPR appears to do.

It has been shown that the enzymes for its synthesis and metabolism are present in smooth muscle (cultured A7r5 and mesangial cells) (795, 796). It appears to be unimportant in terms of Ca signals linked to contraction in some smooth muscles [tracheal, bronchial, and ileum (501)] but functionally important in others [coronary (799) and pulmonary (61) arterial, myometrial (650)]. Elucidation of its signaling pathway in smooth muscle has been best worked out in pulmonary myocytes. In elegant experiments where myocytes were freshly isolated and then cannulated with a micropipette, NAADP was introduced into the cytoplasm and produced Ca bursts, which on occasion produced global Ca waves and cell shortening (61). The full effects of NAADP on Ca signals in smooth muscle cells appear to need reinforcement from CICR via RyRs, recently identified as being RyR3 subtype, at least in pulmonary myocytes (350).

Recent interest has turned to identifying the cell compartment that NAADP receptors are located within. Although initially assumed to be the ER/SR and then nuclear envelope, there is growing evidence for a lysosomal, acidic organellar source in smooth muscle, and other tissues. The first evidence came from sea urchins, where lysosomal-related organelles were shown to be the store released by NAADP (149). Zhang and Li (799) have since demonstrated that the NAADP channel can be reconstituted in liver lysosomes. Kinnear et al. (350) in pulmonary arterial cells used baflomycin, an inhibitor of lysosomes, and showed that the Ca response to endothelin-1 but not PGF2α was reduced. They suggested that this lysosomal store was perinuclear and situated in close proximity (0.4 μm) to RyRs on the SR, a “trigger zone.” NAADP alone was unable to generate global Ca waves. Similar findings, i.e., baflomycin decreases NAADP-induced Ca release and contraction, were obtained in coronary arterial myocytes (800). These authors also used lipid bilayers to demonstrate that NAADP does not act directly on RyRs. In myometrial cells, the microsomal fraction releasing Ca in response to NAADP was inhibited by a drug glycyll-L-phenylalanine-β-naphthylamide (GPN) that lyses lysosomes, but SR inhibitors had no effect (650). This study also showed close apposition of fluorescent signals for lysosomes (lysotracker red) and the SR (BODIPY-TG) in myometrial cells. In peritubular myocytes, the NAADP signaling pathway has been shown to act via endothelin-1 receptor subtype B (ETB), and lipid raft integrity is needed for the endothelin-NAADP signaling pathway to operate (200), possibly due to the location of both ETB and CD38 in this compartment. Recently, Calcraft et al. (100) identified the NAADP release channel as two pore channels (TRCPs) and showed an involvement of IP3Rs in amplifying the NAADP Ca signals.

E. SR Ca Leak

As with the plasma membrane, there will be some low level leak of Ca through the SR membrane, i.e., a constitutive nonregulated process. Once luminal Ca indicators had been developed it was possible to directly monitor the decrease in SR [Ca] that occurred in the absence of stimulation. Before this the leak was assumed to be the mechanism of SR Ca depletion following inhibition of SERCA activity, and surrogates such as spark frequency were used to monitor it. That the leak of Ca from the SR or ER is not via Ca release channels (573) has been shown by, for example, blocking these channels and monitoring a decrease in luminal Ca content following SERCA inhibition, often in permeabilized cells. Indeed, it is easy to argue that this leak, or passive efflux, must be present to balance the basal SERCA activity (266). The nature of this leak is still an enigma (101).

Although the SR Ca leak will not be energy dependent, Hofer et al. (266) presented data in a fibroblast cell line that shows it to be ATP regulated; more Ca was lost at higher ATP concentration. This effect was very slow compared with Ca loss stimulated by IP3. Clearly then, there can be a link between the metabolic state of the cell and SR [Ca]. We can speculate that if ATP is compromised, e.g., during hypoxia and ischemia, then a feedback regulator such as ATP, which would reduce leak, would also therefore reduce the need for SERCA activity. It has been calculated that SERCA activity is responsible for 1–3% of a cell’s energy demand (573).

In the smooth muscle cell line A7r5, Missiaen and colleagues (478, 480) investigated the kinetics of the leak in the absence of IP3. They found a nonexponential leak
that persisted after reducing SR Ca content to 40% of its initial value. At extremely low [IP₃], Ca efflux via the leak exceeded IP₃-induced Ca efflux. These authors also speculated that there is heterogeneity of Ca stores with respect to this leak, perhaps between peripheral and central SR. Additional support for a nonrelease channel leak has come from experiments in permeabilized pancreatic acinar cells, where inhibitors of RyR, IP₃R, and Ca release by NAADP were used, and still an unchanged basal leak occurred from the ER (408). This group also found a consistent leak rate over a broad range of SR Ca loads (486). Although it may be SERCA isoform specific, there is little reason to think that the leak is due to reverse mode (486). Although it may be SERCA isoform specific, there is little reason to think that the leak is due to reverse mode.

To date, there appear to have been but a handful of papers examining the nature and extent of the SR Ca leak in smooth muscles. The amount of Ca reported upon blocking SERCA has varied, with substantial depletion of the SR seen in cells from uterus (633), stomach (753), and (cultured) aorta (478, 706) and very little depletion in bladder (218), (toad) gastric (661), and (cultured) uterus (792). These differences are consistent with estimates made in other cell types of leak rates from ~10 to 200 μmol/min (see Camello et al., Ref. 101).

For A7r5 cells the rate of loss was, expressed as a maximal fractional Ca content loss, 22%/min (478). Thus it is clear that in several smooth muscles, the SR leak could rapidly (few minutes) deplete the SR of Ca. However, it should be cautioned that none of these measurements has been made under physiological conditions, as isolated, permeabilized cells, with a pharmacopeia of inhibitors are required for these studies. Thus, in smooth muscle tissues, the contribution of the leak to setting luminal Ca levels and its modulation during stimulation to affect functional responses remains to be better investigated.

F. Summary

In this section we have reviewed SR Ca release mechanisms, their modulation by pharmacological and endogenous agents, and the process of “leak.” Molecular and pharmacological tools along with knockout animals have greatly increased our understanding of how Ca is released from the SR of smooth muscle cells. With the existence of these different Ca-releasing mechanisms, along with the different mechanisms of modulation, isoform expression, and cellular distribution, smooth muscle myocytes appear to be the most complex of all cells. Certainly striated myocytes as well as neuronal and secretory cell types are not as rich in their Ca release processes. As we have noted elsewhere, we consider this to be a reflection of the wide range of functions and phenotypic lability in smooth muscle cells.

VI. LUMINAL CALCIUM

A. Introduction

Some of the first evidence that the SR in smooth muscle is a Ca storage site was obtained histochemically after calcium had been precipitated (147, 259, 567). Better spatial resolution and the ability to look simultaneously at several ions came with the technique of electron-probe microanalysis, used to determine elemental concentrations in different regions of the myocyte. This approach led to measurements of [Ca] in the SR of 30–50 mmol/kg dry wt or 2–5 mM wet wt (69, 371), i.e., considerably higher than in the cytoplasm. These studies also detected a decrease in SR [Ca] with stimulation (655). There are, however, numerous limitations to this approach, such as the need for fixation and specialized apparatus and the small dynamic range. Determination of ⁴⁵Ca efflux can provide some indication of SR function (393, 477), but large background counts from ⁴⁵Ca bound to Ca-binding proteins, and nonspecific leaks, severely limit its usefulness. In cardiac muscle, ¹³⁵F-NMR has been used with a difluorinated version of BAPTA. The advantage of this technique is that by alternating the obtaining of ¹³⁵F data with ³¹P spectra, near-simultaneous information on ATP and phosphocreatine and pH can also be obtained.

B. Fluorescent Indicators for SR Luminal Ca Measurement

Other methods have been sought for measuring SR [Ca], of which fluorescent indicators appear the most promising. As Zou et al. (813) put it, “there is a strong need to develop Ca sensors capable of real-time quantitative Ca concentration measurements in specific subcellular environments without using natural Ca binding proteins such as calmodulin, which themselves participate as signaling molecules in cells.” In this paper they describe the development of one such set of sensors with K₄ values ranging from 0.4 to 2 mM. The [Ca] in the SR is such that K₄ values in this range are required. This along with selectivity (for Ca and the SR) and considerations of what is bound and what is free Ca, make measuring SR/ER Ca nontrivial. For a commentary on these issues around SR [Ca] determination, see Bygrave and Benedetti (96), and for a general review of [Ca] measurement, see Takahashi et al. (684).

Luminal [Ca] measurements to date in smooth muscles have only been reported for a limited number of tissues. Most of the studies have used the approach of fluorescent indicators such as Mag-fura 2 (K₄ = 49 μM; Ref. 667), which was originally developed as a probe for magnesium, and fluo 5-N, Furaptra (e.g., Ref. 667). Some indicators originally considered for cytoplasmic studies...
were found to preferentially enter organelles, and do so in a reasonably selective manner in some cell types. For example, rhod 2 will accumulate in mitochondria. In the case of the SR indicators originally developed to monitor Mg, they load relatively easily into the SR and can be used to report luminal [Ca]. These indicators include Mag-fura 5, Mag-fura 2, Mag-indo 1, and Furaptra, and it may be because of the particular efficiency of hydrolysis within the SR that they are accumulated within it (667). Indicators with less Mg dependence include fluo 3FF and fura 2FF, both of which have been used in smooth muscles (214, 792). Little is known about SR [Mg] in smooth muscle or indeed many other cell types; Sugiyama and Goldman (667) estimated it to be ~1 mM. These authors also concluded that Furaptra (another probe sensitive to Mg, with a $K_d$ of 6 mM and for Ca of ~50 $\mu$M), could be used to determine SR [Ca], without Mg impacting on its usefulness. In the aortic cell line A7r5, they estimated luminal [Ca] to be 75–130 $\mu$M.

Another approach used to investigate luminal Ca levels is to modulate its buffer capacity by using compounds that can quickly diffuse into the SR and bind Ca. As with Ca indicators for the SR, the $K_d$ for such compounds has to be in the high micromolar range, i.e., low affinity. The heavy metal chelator TPEN with a $K_d$ around 200–500 $\mu$M is such a compound (108, 571). However, TPEN may also have some side effects, such as inhibition of SERCA activity. Thus the expression of these Ca binding proteins is critical to the SR’s ability to act as a Ca store, in all cells.

Although many proteins capable of binding Ca may be expressed in the SR, the consideration here is of those which bind considerable quantities of Ca and which appear to have this as their prime role within the SR lumen. There are just two protein families that fulfil this description: calreticulin and calsequestrin. As described already in section IV, they are both high-capacity (25–50 mol/mol), low-affinity (1–4 mM) Ca binding proteins that function only or primarily to dynamically store Ca (384, 597). These two proteins have similar Ca-binding properties, molecular weights, and other protein properties, such as acidic isoelectric points (573). As noted above, specific data for either protein in smooth muscle are not abundant, or there have been controversies in the literature (472, 730) and little recent work.

C. Luminal Ca Buffers

Calcium binding proteins are present in the cytosol and intracellular organelles (703). Their role in maintaining a low cytosolic [Ca] and compartments capable of storing Ca at relatively high concentration, respectively, is crucial to Ca acting as a ubiquitous second messenger. Soluble (nonmembranous) Ca-binding proteins buffer Ca but are limited by their finite amount. Calcium pumps such as SERCA may also be viewed as membrane-bound Ca-bindings proteins, as they act to bind Ca, transport it across their constituent membrane, release it, and are then ready to complex Ca again. As such, SERCA makes a large contribution as a Ca-binding protein and a very important and efficient regulator of [Ca] inside cells (105). It has been estimated that cytoplasmic Ca buffering takes up 84 of every 85 Ca ions entering gastric smooth muscle cells (230) and 30–46:1 in bladder myocytes (145, 204).

Although cells contain millimolar [Ca], only ~0.01% is free ionized Ca. High-affinity Ca binding proteins, such as calmodulin, are present in large amounts in the cytoplasm of all mammalian cells and contribute to Ca sensitizing and exchange. Calmodulin is the best studied of the endogenous Ca sensors (proteins with high affinity and low capacity) in smooth muscle (653). It binds Ca with a $K_d$ of ~2 $\mu$M (319) and is present at ~40 $\mu$M (336). In early studies of [Ca] within smooth muscle cells (rabbit portal vein) using electron-probe microanalysis, it was concluded that there was not sufficient calmodulin to account for the [Ca] measurements made (66). In a recent study of cytosolic Ca buffering in single smooth muscle cells from guinea pig bladder, it was concluded that the Ca buffers present have a low affinity (EF-hand) for free Ca, are present at ~530 $\mu$M, and have a calculated binding ratio of ~40 (145). In this study the volume of the SR was taken to be 2%, which may be an underestimate. Other studies in smooth muscles have yielded similar values for the ratio of Ca binding: airways (178), bladder (204), larger portal vein (329), stomach (349).

Within the SR lumen, specific proteins are expressed to bind Ca. These SR luminal Ca storage proteins can be considered to act as buffers, as they keep Ca relatively loosely bound and thus available for quick release, and help prevent insoluble calcium phosphate precipitating. By reducing the amount of free or ionized Ca in the SR, they also reduce inhibition by Ca of SERCA activity. Thus it seems that the expression of these Ca binding proteins is critical to the SR’s ability to act as a Ca store, in all cells.

D. Measurements

Not withstanding caveats around indicator specificities, $K_d$ values, Mg sensitivity, and calibration methods, a few investigators have estimated/measured the SR luminal [Ca] (214, 636, 667, 755, 811). Not unexpectedly, there is a range of values, from ~60 to 150 $\mu$M, but this range is perhaps surprisingly small given the difficulties involved.

It would therefore appear that the SR free [Ca] is less in smooth muscles compared with other cell types, where values of 200–1,500 $\mu$M have been reported (33, 118, 121, 268). However, until more measurements in native smooth muscle cells are made, it is unproductive to draw further conclusions.
E. Effects of SR Ca Load

1. Introduction

How luminal SR Ca content relates to a change in cytosolic [Ca] is not easy to predict or determine. This is because there is feedback from cytosolic [Ca] onto the SR release channels (282) and a difference in buffering capacity between the SR and the cytoplasm (230). In cardiac muscles where a proper quantitative study can be made, the nonlinearity between the two can be steep (704). This has not been as well studied in smooth muscle, but the following study also points to a steep relation.

In rat uterine cells we have shown that increasing external [Ca] from 2 to 10 mM increases cytosolic [Ca], from ~120 to 270 nM, and is associated with a substantial rise (17% increase compared with steady-state value) in SR [Ca] (636). This elevation of SR [Ca], however, did not lead to any potentiation of the Ca transient elicited in response to the IP_3_‐generating agonist ATP. If the SR load was decreased (but not emptied) from its steady-state level, the ATP‐induced Ca transients fell. If the SR load was decreased below 80% of normal, no Ca transients to ATP could be elicited. Thus there is a steep dependence of IP_3_‐induced SR Ca release with SR load as it depletes, but not as it overloads, at least in uterine myocytes.

2. Relation between SR Ca load and Ca signals

The effects of altering SERCA activity on global Ca signals and function in smooth muscle cells are discussed in section VI, C and D. Here we will focus on how SERCA activity affects SR luminal [Ca] and then Ca release events. Blocking SERCA, e.g., with thapsigargin or CPA, has been shown by us and others to produce a decrease in SR Ca content, which is accelerated with agonist stimulation (16, 633, 661). In our study of uterine cells, we simultaneously measured cytosolic and luminal [Ca] changes and compared the depletion of SR Ca in response to agonist stimulation, with and without SERCA activity (633). We found that the depletion was greater when SERCA was inhibited and the intracellular Ca transients were smaller. In a follow up study we found that the rise in cytosolic [Ca] seen with increasing external [Ca] was significantly increased, from ~270 to 320 nM, when SERCA was inhibited with CPA (636). Removal of external Ca produced a fall in cytosolic [Ca] and SR [Ca]. The effects of repetitive agonist applications under conditions of low and high SR [Ca] and with and without SERCA are also described by Shmygol and Wray (636).

From these and other studies we can confidently say that agonists lower SR [Ca] and SERCA activity is required to restore, and presumably maintain, the ability of smooth muscle cells to respond to agonists. As a rundown of luminal [Ca] would be anticipated to rapidly curtail agonist‐induced Ca signals, it can be assumed that under normal physiological conditions SERCA is active during stimulation to refill the store, although as noted by Gomez‐Viquez et al. (218), the turnover rate of SERCA is slower than that of the release channels, being ~3.5 Ca/s for SERCA2b compared with ~1 × 10^6 Ca/s through RyR (462). Given the available data on SERCA and RyR expression, the number of SERCA pumps is not sufficient to compensate for the RyR Ca efflux.

In their interesting study, Gomez‐Viquez et al. (218) explored the relation between SERCA pump activity and SR Ca release channels in urinary bladder myocytes. They investigated whether SERCA activity per se rather than luminal [Ca] could influence Ca release events, or if uptake and release operate independently of each other, assuming there is sufficient [Ca] in the SR lumen. They rapidly and completely inhibited SERCA (thapsigargin) so that luminal [Ca] would be maintained (as verified from the Mag‐fura 2 signals). With SERCA inhibited, the ACh‐ or caffeine‐induced Ca release into the cytoplasm, as measured with fura 2, was reduced in both amplitude and rate of rise. Thus SERCA2b in bladder smooth muscle cells can modulate both IP_3_R and RyR Ca release events; SERCA activity is required for optimal Ca release. The authors speculate that SERCA2b pumps are modulating Ca availability in the SR. As described earlier, the SERCA2b isoform contains a carboxy tail extension that interacts with luminal Ca binding proteins, suggesting a molecular and structural mechanism for this effect.

SERCA activity can indirectly modulate Ca release events through setting the [Ca] load in the SR. Both IP_3_Rs and RyRs have been shown, in lipid bilayer experiments, to have their activity affected by luminal SR [Ca] (477, 642; see sect. V). In turn, it is SERCA activity that loads the SR with Ca (661). ZhuGe et al. (811) working on gastric myocytes noted the effects of luminal load by detecting Ca sparks and STOCs at different Ca loads. They found the frequency of both increased with load. A similar relation between the SR Ca load (altered by knocking out phospholamban) and Ca sparks had previously been reported in cardiac muscle (614). The effects of SR Ca load on STOCs was also confirmed by others (123, 452). It was found that a relatively small fall in SR [Ca] (~16%) had a profound effect on STOC frequency (decreased by 70%) (452). As mentioned earlier, in uterine myocytes, overloading the SR with Ca did not increase the simultaneously measured cytosolic Ca transient in response to ATP stimulation (636). In marked contrast, partial depletion of the SR Ca substantially reduced the amplitude of the ATP‐induced Ca release: a reduction to 80% of normal luminal SR Ca content abolished responses. Previous work had shown that uptake of Ca into the SR occurred even in the absence of external Ca, although this only replenished luminal Ca to ~50% of its resting value (633). Thapsigargin abolished both subsequent ATP‐evoked Ca release and SR refilling. Interestingly, this study found no
changes in luminal [Ca] during spontaneous activity, suggesting no role for the SR in this process, but rather supporting the view that L-type Ca entry is both necessary and sufficient for normal phasic activity in the uterus, and store-operated Ca influx is not necessary. When the SR was overloaded, spontaneous activity was abolished, consistent with a negative feedback from the SR on E-C coupling in the uterus, despite the lack of Ca sparks (88).

F. Conclusions

While it is well accepted that luminal [Ca] is a very important control regulator of both SR function and Ca signaling, the field would be well served by some additional studies in this area. For example, additional calibrated measurements of luminal [Ca] would clarify if the value in smooth muscles is ~100 μM, i.e., considerably lower than striated muscles. Will values be lower in tissues like uterus where the SR may not contribute to rhythmic activity, compared for example to blood vessels, which rely more heavily on the SR Ca store? Similarly, more simultaneous measurements of cytosolic and luminal [Ca] would add to our knowledge of how SR Ca load affects Ca signaling. Does the SR release Ca during phasic activity? Is SR Ca overload inhibitory to contractility in smooth muscles? How do changes in the expression of SR Ca buffers affect SR Ca load? Although these experiments are technically challenging, especially when combined with electrophysiological studies, the insight gained will be important and significant to the field.

VII. CALCIUM REUPTAKE MECHANISMS

A. Introduction

In section III, the structure of SERCA and its isoforms and catalytic cycle were described. The ability of SERCA to take up Ca is of course crucial to the ability of the SR to act as a Ca store. In this section we review the effect of Ca uptake by SERCA on cytosolic [Ca] levels and Ca signals, its role in plasma membrane Ca extrusion, and the effects of inhibiting its activity either pharmacologically or molecularly. (Discussions of how phospholamban and related proteins affect SERCA were dealt with in section IV.) This is then followed by a review of store-operated Ca entry in smooth muscle, exploring the relation between luminal Ca levels and Ca entry mechanisms that replenish the SR with Ca.

B. SERCA Uptake of Ca and Ca Homeostasis

For the cell to maintain low cytosolic [Ca] with respect to both the extracellular fluid and the SR, requires not just SERCA activity, but also that of the PMCA and NCX. For the cell to be in a steady state, Ca entering the cell across the plasma membrane must be removed by PMCA and NCX, and Ca released from the SR must be retaken up by SERCA. This will also be the case for Ca uptake into organelles such as the nucleus and mitochondria. There will be basal or “resting” SERCA activity, which presumably balances SR Ca leaks, as well as spontaneous Ca releases from the SR, Ca puffs, and sparks. SERCA activity is stimulated when cytosolic [Ca], from whatever source, rises and the presence of the luminal Ca buffers, calsequestrin and calreticulin, ensures that inhibition of SERCA by luminal Ca does not occur. The SR will contribute to plasma membrane Ca efflux by vectorially releasing Ca towards efflux sites. As with many other aspects of smooth muscle physiology, the exact contribution of SERCA, PMCA, and NCX will differ between them, depending on expression levels and distribution.

Perhaps the easiest way to uncover the role of SERCA in maintaining cytosolic Ca levels is to determine the effects of its inhibition. As mentioned already, there are two specific inhibitors of SERCA: thapsigargin, which is irreversible, and CPA, which is reversible. When SERCA is inhibited in smooth muscle cells there is a rise in cytoplasmic [Ca]. This rise of [Ca] can be fast or slow and then plateaus, leading to an elevated resting [Ca]. Due to the difficulties of calibrating Ca signals, many studies have simply reported a rise of [Ca] with SERCA inhibition, perhaps expressed relative to high-K depolarization or some other calibrator. Some of the first studies to explore the effect of CPA were on vascular myocytes (1, 120) and used aequorin to monitor [Ca]. With CPA there was a slow, i.e., minutes, increase in the Ca signal and basal tension. The response to norepinephrine was decreased. Rises in [Ca] in response to SERCA inhibition have also been shown in bladder myocytes (from 137 to 471 nM; Ref. 789) and uterine myocytes (from 120 to 320 nM; Ref. 636). A study of airway myocytes, however, found no significant change in resting [Ca] (~130 nM) after 25 min in 0-Ca and CPA solution (603), perhaps due to increased Ca efflux under these conditions.

C. SERCA and Ca Signaling and Contractility

Both pharmaceutical SERCA inhibition and to a more limited extent gene ablation have been used to investigate the role of SERCA in Ca signaling in smooth muscles. When SERCA2 was reduced to ~50% and aortic contractility measured, no changes from wild-type mice were found in response to KCl and phenylephrine (294). This contrasts with the significant effects found on cardiac contractility, including decreases in Ca transients and cell shortening (558). It is not clear whether the lack of effect
in the vascular preparation is due to increased SERCA reserve compared with the heart or some form of compensatory regulation of other parameters. When CPA is used to inhibit SERCA in blood vessels, clear effects are seen (504). When SERCA3-deficient mice were examined, the relaxation to ACh was reduced in aorta (402) and to substance P in trachea (334); however, as discussed earlier, these effects are not direct on the myocytes, but rather are due to endothelial- and epithelial-dependent effects. No overt phenotype is associated with the SERCA3 knockout (402). This appears to be the extent of the literature on gene targeting studies of SERCA function in smooth muscle. There is, however, a more extensive literature concerning effects obtained with CPA and thapsigargin. In most smooth muscles, a clear elevation of baseline tension is found with SERCA inhibition, and when measured, this can be seen to be accompanied by increased global Ca signals [ileal (710), aorta (417, 629), fundus (562), esophageal (640), uterus (377, 446, 682)].

Before the link between SR Ca release and BK channels and membrane hyperpolarizations were elucidated, many authors interpreted their data, i.e., SERCA inhibition increases [Ca] and contraction, in terms of the SR blocking or buffering the flow of Ca to the myofilaments. The spark-STOC coupling mechanisms, however, can directly account for the effect of SERCA inhibition in many, but importantly, not all smooth muscles. The effects of SERCA inhibition on Ca signaling and contractility in phasic smooth muscles can be very pronounced. For example, in the neonatal uterus (519) or term myometrium (707), the normal phasic activity becomes tonic-like.

D. SERCA Ca Efflux and Relaxation

Although SERCA takes up Ca and aids restoration of [Ca] to resting levels following smooth muscle stimulation, there is evidence from uterine smooth muscle to show that acting on its own, it does not significantly alter the decay of the Ca transient, but rather, it enhances efflux mechanisms. In isolated uterine myocytes (631) when PMCA and NCX were blocked, intracellular [Ca] did not fall after stimulation (644). However, if SERCA was blocked, but not PMCA and NCX, then the decay of the Ca transient was slowed. This was the case whether Ca efflux was via PMCA or NCX or both. We concluded that when SERCA is active, it takes up a portion of Ca entering the cell, and the SR then releases Ca in a directed manner towards the plasma membrane extruders, thereby maximizing their efficiency (see also Refs. 184, 445). A study in airway myocytes also concluded that the decay of the Ca transient was not altered by SERCA, although it is active (603). These authors suggested that mitochondria take up Ca and contribute to the decrease in [Ca]. In an earlier study in isolated tracheal myocytes, SERCA had been shown to contribute to the decay of agonist-induced Ca transients as the decay was slower when SERCA was inhibited (640).

E. Store-Operated Ca Entry

1. Introduction

The Ca content of the SR acts as a powerful factor controlling the open probabilities of both RyR and IP$_3$R channels (see sects. IV and VII). Depletion of Ca stores in smooth muscle cells by SERCA pump inhibitors results in an inhibition of Ca sparks and STOCs in those tissues producing them. This in turn can lead to depolarization of the cell membrane and activation of Ca entry via L-type Ca channels, as was reported for rat cerebral arteries (507). However, in addition to activation of L-type Ca channels, depletion of SR Ca can also activate nifedipine-resistant Ca entry, through opening of Ca release-activated Ca channels (CRAC). In nonexcitable cells, ER Ca depletion opens voltage-independent plasma membrane Ca channels through which extracellular Ca enters the cell to refill the store. These are termed “capacitative calcium entry” (CCE) channels (542, 579, 580). The role of CCE, also known as store-operated Ca entry (SOCE) and Ca entry mechanism across the sarcolemma of the smooth muscles, is still being determined.

Recent work has identified the specific molecular components, Stim and Orai, which constitute the Ca sensor in the ER/SR and the Ca channel, respectively, and their discovery has helped to clarify the mechanisms of CCE. The process and mechanisms of SOCE have been best studied in nonexcitable cells, where the process was first discovered, and studies in smooth muscle are relatively sparse. Therefore, an overview of current knowledge taken from the literature on nonexcitable cells will be given to set the scene for smooth muscle.

2. Overview of store depletion, Orai, STIM1, and TRPCs

This account is based on information in several recent reviews that have appeared since the discovery of Stim (stromal interaction molecule 1) proteins (99, 173, 189, 260, 524, 569, 581, 649).

Agonist binding to cell membranes and subsequent generation of IP$_3$ leads not only to ER Ca release, but also to activation of Ca channels in the plasma membrane. This connected depletion and loading of the ER Ca store was termed capacitative Ca entry by Putney in 1986 (578) and is synonymous with the term store-operated Ca entry. This form of Ca entry is the major route of Ca entry in nonexcitable cells, which lack functional voltage-gated Ca channels. It was subsequently demonstrated that the Ca
entry could be stimulated without agonist or IP3, if the ER Ca was depleted, e.g., using SERCA inhibitors, particularly thapsigargin (545, 686). Detecting the rise of cytoplasmic [Ca] and the associated small Ca release-activated Ca current \(I_{\text{crac}}\) (272) resulting from this store depletion-activated Ca entry was difficult. This led to the suggestion from experiments on arterial myocytes that the refilling of the SR occurred by-passing the cytoplasm, i.e., direct coupling between the plasma membrane and SR, or that the Ca was in a restricted/protected space between the two, and not accessible by the myofilaments, as no contraction occurred (111). However, given the existence of \(I_{\text{crac}}\), in both excitable and nonexcitable cells, Ca clearly is entering the cytoplasm, and the failure to detect a rise in [Ca] in most cell types is now attributed to the close proximity between ER/SR and the plasma membrane and the rapid buffering of the Ca entry by the ER/SR.

Much experimental effort was put into elucidating the molecular events between the ER and plasma membrane, which could activate the capacitative store-operated Ca entry. Until a few years ago, the discussion would be focused around canonical TRPCs, which were discovered in Drosophila and shown to be activated after an increase in PLC activity (245). These channels, expressed in a large variety of isoforms in mammalian cells, almost became accepted as “the” store-operated channels responsible for capacitative Ca entry. However, as discussed by Putney (581), these reports were often from overexpressing or constitutively active preparations and the TRPC channels did not reproduce the known properties of \(I_{\text{crac}}\) and were usually cationic rather than Ca-selective channels.

While it is too early to be categorical, the evidence that TRPC channels are responsible for capacitative Ca entry is not strong. It is also the case that since 2006 new putative channels in the form of Orai proteins have been discovered, which appear to be much better candidates. Orai is a plasma membrane protein which when mutated results in abrogation of \(I_{\text{crac}}\) and severe combined immunodeficiency syndrome (174). It appears to be unlike other previously described Ca channels. Mammalian cells can express three types of Orai, 1–3, and all are capable of forming Ca channels, probably as homotetramers. A recent paper suggests that Orai is primarily a dimer in the plasma membrane under resting conditions, and upon activation by the COOH terminus of Stim, the dimers dimerize, forming tetramers that constitute the Ca-selective channel (553). These and other data make it hard to propose that TRPC channels may contribute to capacitative Ca entry via inclusion in an Orai/TRPC Ca channel (153, 398). Orai1 forms the most conductive Ca channel function (575, 788). When Orai1 is expressed with another protein, Stim1, large \(I_{\text{crac}}\) currents are observed (552, 804).

Stim1 was discovered as a cell surface protein (530) and was related to the ER in 2005 (400, 595) as its Ca sensor, responsible for activating capacitative Ca entry. Thus Stim1 knockout reduces \(I_{\text{crac}}\) and SOCE. Stim1 is a phosphoprotein with a single transmembrane domain. The NH2 terminus of Stim has an EF hand domain in the ER lumen that is considered to be the sensor of luminal [Ca]. It has been calculated that the EC50 for Stim1 redistribution is between 200 and 400 μM luminal Ca (82, 651, 660) and that oligomerization of about four Stim1 proteins occurs with the conformational change in the NH2 terminus as Ca dissociates. This oligomerization is necessary for activation of \(I_{\text{crac}}\) (575, 788), and mutation of this region produces constitutive activation of Ca entry (464). Both Stim and Orai are required to produce \(I_{\text{crac}}\). Although not completely clear, the mechanism of activation appears to involve a redistribution of Stim1 in the ER when Ca dissociates from it, i.e., as the ER depletes. This redistribution brings Stim closer to the plasma membrane (within 10–25 μm) in a punctate pattern on the ER, with some workers (541) but not all (148) suggesting that it takes up a lipid raft distribution (541). This redistribution that precedes \(I_{\text{crac}}\) by ~6–10 s (775) presumably enables Stim1 to directly interact with Orai channels, and Ca enters in these discrete regions of the plasma membrane (416). Evidence for this comes from a variety of biophysical and imaging techniques as well as coimmunoprecipitation. It may be, however, that there is no direct coupling between Orai and Stim and another factor, such as “Ca-influx factor” (CIF), is stimulated by Stim redistribution and CIF (or another messenger) is needed for Orai activation, perhaps involving activation of phospholipase A2 by CIF displacing calmodulin (63). Another protein identified in the Stim family, Stim2, is structurally ~60% identical to Stim1, but does not appear to play any major role in capacitative Ca entry (595), but may contribute to regulating basal [Ca] via ER Ca sensing (82).

Most of the studies mentioned above were performed on immune or exocrine cells. How much evidence is there for these mechanisms and proteins in smooth muscle? Although only a few years from their discovery, the excitement around Stim and Orai has already led to studies investigating their expression and role in smooth muscle tissues.

### 3. Orai, Stim, and TRPCs in smooth muscle

The study of capacitative/store-operated Ca entry in smooth muscles is more complicated than it is in nonexcitable cells, due to the greater variety of Ca entry mechanisms, i.e., voltage-gated and receptor-operated channels, in addition to channels that are opened in response...
to a depletion of SR Ca. Previous work had investigated TRPC channels as the putative Ca entry mechanism opened by store Ca depletion, but the growing consensus is that they are not responsible for $I_{\text{crac}}$. Therefore, it may be argued that TRPCs are unlikely to be responsible for SOCE in smooth muscles. This has already been suggested by some (153, 722, 738) but contested by others (e.g., Ref. 7). TRPC1, the TRP most thought to be involved in SOCE and highly expressed in smooth muscles, had no effect on vascular function or SOCE when knocked out (12). In TRPC6 knockout mice, enhanced DAG and agonist-induced current in smooth muscle cells was reported, i.e., the opposite result expected if TRPC6 is involved in SOCE (188). RNA interference (RNAi)-based high-throughput screens also revealed no effect on SOCE activation of TRP channel knockdown, in contrast to Stim (400). As with studies on nonexcitable cells, caution must be applied to studies where genes have been knocked out or overexpressed, and in smooth muscle, when cells have been cultured, the physiological relevance of such studies is even harder to interpret (274). Nevertheless, while it is premature to rule TRPCs out of involvement in SOCE in smooth muscle cells, especially as much of the Orai work has been conducted on nonexcitable cells, it nevertheless does not appear profitable to cover in great depth previous studies related to a role in SOCE of TRPC, especially as this has recently been reviewed (7). A recent report by Dehaven et al. (148) demonstrated that TRPC functions independently of Orai1 in vascular myocytes (A7r5 and A10 cell lines) and suggests that TRPCs are activated by PLC and do not involve Stim1 as the Ca sensor and TRPCs open nonselective cation channels when stimulated by arginine vasopressin. Roles for TRPCs other than in store-operated Ca entry in smooth muscle have been well demonstrated for receptor-operated channels, where they constitute nonspecific cation channels (39, 457) and stretch channels (658), but these are outside the scope of this review.

There is already evidence to support the Stim-Orai model for SOCE in some smooth muscles: airway, Stim1 (549), Orai (550); vascular, Stim (153, 413, 685), Stim and Orai (47, 210); and uterus, Stim and Orai (611). The list of smooth muscles expressing Stim and Orai is expected to grow rapidly as more studies are performed. To date however, there is already evidence that capacitative Ca entry/SOCE in some smooth muscles at least occurs via the Stim-Orai mechanism. Dietrich et al. (153) inhibited SOCE using siRNA against Stim1, whereas TRCP1 knock-out did not affect it. Further support for a physiological function for these proteins in smooth muscles comes from data showing that their expression levels are altered at different sites and in disease. Thus Orai and Stim were upregulated in proliferating arterial myocytes, leading the authors to suggest that they play a critical role in the altered Ca handling that occurs during vascular growth and remodeling (47). Stim1 expression was greater in the distal than proximal part of the pulmonary artery, where SOCE is also greatest (413). Stim1 and Orai1 were also found to be upregulated in aortas from hypertensive rats (210). Knockdown of Orai1 reduced thapsigargin-induced Ca influx and reduced $I_{\text{crac}}$ (210).

Thus, in summary, Orai and Stim appear to constitute the molecular mechanism underlying capacitative Ca entry and $I_{\text{crac}}$ in nonexcitable cells, and there is mounting evidence for this also to be the case in smooth muscle. To us, the most urgent questions revolve around whether there is only one form of SOCE in smooth muscle and if this involves only the Stim-Orai mechanism. The relative simplicity of Ca entry in nonexcitable cells falls away in studies of smooth muscle, as SOCE is a variable feature between myocytes, and several other Ca entry pathways exist. In addition, there are only relatively few studies that have gone beyond demonstrating a putative SOCE, i.e., mechanistic details, measurement of $I_{\text{crac}}$, and biophysical and imaging studies are sparse, leading to interpretation based on only a few myocyte preparations. The interaction of TRPCs and Orai channels, if any, also requires resolution.

4. Evidence for store-operated Ca entry in smooth muscle

As one might anticipate from knowing that the contribution of voltage-gated Ca entry varies between smooth muscles, being high for example in uterine (771) and intestinal (64) and low in many large blood vessels and tracheal myocytes (7), so the contribution of Ca entry through SOCE and/or other voltage-independent routes such as nonspecific receptor-operated cation channels (ROCs) shows considerable variation between smooth muscles (44). Although ROC activity is a feature of agonist binding, e.g., norepinephrine, and is important for their physiological effects (39), it does not involve SR Ca depletion, and therefore, these channels are not discussed in detail further.

In smooth muscles, SOCE has been associated with a second, delayed Ca response to agonist applications; the IP$_3$ formed causes an initial fast and transient release of Ca from the SR, followed by the slower process of SOCE which produces a more or less sustained Ca signal, often of lower amplitude than the initial transient rise. If Ca sensitization mechanisms are also stimulated by the agonist, then the force responses will not be directly related to the Ca signal and force may continue long after [Ca$^2+$] has fallen.

A sustained increase in [Ca$^2+$] due to extracellular Ca entry following agonist application has been taken as evidence for SOCE by many investigators, sometimes augmented by data from putative blockers of SOCE such as SKF96365 and La$^{3+}$. It is generally accepted that there are
no specific blockers of SOCE, so pharmacological approaches are helpful but not definitive. Separating SOCE from voltage-gated Ca channel and ROC-mediated Ca entry must also be accomplished. This is often done by not using agonists, depleting the SR of Ca using SERCA inhibitors, and preventing L-type Ca entry, and interpretation is further simplified by the use of single cells. Such conditions make it hard to relate the findings to physiological conditions. With the use of these approaches, rises in [Ca] considered to be due to SOCE have been reported in many smooth muscles, e.g., colonic (370), portal vein (538), cultured A10 cells (784), inferior vena cava (120), aorta (265), coronary arteries (731), ileal myocytes (526), pulmonary (216), esophagus (740), and gallbladder myocytes (492).

There have been few studies in smooth muscle of the membrane currents or conductance changes associated with SOCE. Recordings of what would be called \( I_{\text{crac}} \) in nonexcitable cells are often denoted as \( I_{\text{soc}} \) in smooth muscle, underlining the uncertainty around whether SOCE in smooth muscle uses the same entry mechanism as in nonexcitable cells. An added complexity to the study of \( I_{\text{crac}}/I_{\text{soc}} \) in smooth muscle is that the plethora of ion channels means that any changes in cytoplasmic [Ca] due to SOCE could evoke changes in other currents, e.g., opening of Ca-activated Cl channels, giving rise to inward current and therefore complicating interpretation. Studies of \( I_{\text{crac}} \) should ideally therefore be performed with heavy buffering of Ca, e.g., 10 mM EGTA or BAPTA. From experiments using whole cell and single-channel recordings, evidence for SOCE has been provided in a variety of smooth muscles. The first recording of \( I_{\text{crac}} \) was made in mouse anococcygeous myocytes in 1996 (746). Subsequent recordings of a conductance elicited by store Ca depletion were made in portal vein (5, 403), mesenteric (609) and pulmonary arteries (216, 705), and airway (549). Thus, as expected, most evidence for significant SOCE comes from blood vessels, where reliance on L-type Ca entry is less than in many phasic muscles.

Work particularly from Large’s group (7) has questioned whether SOCE is the same process in smooth muscle as it is in nonexcitable cells and if different forms of SOCE occur in smooth muscle cells. As discussed above, there is mounting evidence for Orai and Stim in smooth muscles, including vascular and airway, although this does not prove that \( I_{\text{crac}} \) and SOCE are identical to those described in nonexcitable cells. In a careful review of the biophysical properties and activation mechanism for SOCE in a small number of different smooth muscles, Albert et al. (7) concluded from measurements of unitary conductance (not current) that the Ca permeability of SOCs in smooth muscle myocytes was much smaller than measured for \( I_{\text{crac}} \) in nonexcitable cells and that therefore in myocytes SOCE is via nonspecific cation channels. Such channels will contribute to SR Ca refilling but also to depolarization of the myocytes. Albert et al. (7) argue that such channels may also have TRPCs associated with them and be activated by factors other than Stim; mention has been made of Ca-independent phospholipase A2 (iPLA2) activating SOCE (644), and there is also evidence for a role for PKC (6, 609). Finally, store-independent activation of SOCE involving PKC has been described by this group in portal vein (6), with the suggestion that there is a constitutively active driver of SOCE.

What the relation of these channels is to classical SOCE/capacitative Ca entry remains to be established, but clearly such channels fall outside the conventional definition of SOCE. The use of single-channel recording in isolated patches of membrane clearly provides experimental clarity, but it is difficult to relate these events to physiological activity in intact cells or tissue, where [Ca] would not be clamped and Stim-Orai activity would be present.

There are data which indicate that refilling of the store occurs directly from the myoplasm (267, 486). It was shown that when intracellular [Ca] was clamped around resting levels, using Ca chelators, store refilling occurred in the absence of external Ca, suggesting that mechanisms directly linking Ca influx to store refilling might not be necessary (54, 267, 486). Certainly, SR Ca uptake when cytoplasmic [Ca] rises (in the absence of external Ca) has been directly demonstrated (636).

If the plasma membrane Ca leak is small and SERCA activity large, then many cycles of SR Ca release and uptake could occur without the need for Ca entry. In those smooth muscles where L-type Ca entry is large and agonists produce depolarization, then there is little need for a separate SOCE/CCE mechanism. For smooth muscles such as the uterus, SOCE may be a mechanism that is only important during the strong agonist-driven contractions of labor, and that is absent/unused at other times (518, 707). The heterogeneous nature of the stores (see sect. vii) may also contribute to differences in SOCE between smooth muscles. For example, in arterial myocytes, the ryanodine/caffeine-sensitive store was not sensitive to either thapsigargin or CPA, whereas the IP\(_3\)-sensitive Ca store was depleted by both of them (307). From the results obtained on colonic myocytes, which have two functionally distinct SR Ca stores, it was suggested that the common store (\( S_\alpha \)) expressing both IP\(_3\)R and RyR was dependent on an external Ca source for replenishment, and the second store containing only RyR (\( S_\beta \)) refills directly from the myoplasm (185). Also in colonic myocytes, the mechanisms that control Ca entry into the cytoplasm involve two types of channels, one of which is voltage sensitive and the other is voltage insensitive (453). It is also suggested that the influx of Ca via these two channels produces a high subplasmalemmal [Ca] accessed by the common store (453). However, other authors have found that the magnitude of SOCE in colonic...
myocytes was the same irrespective of whether just IP$_3$R or IP$_3$.R and RyR stores were depleted (370). The refilling mechanisms may depend on the location of the SR. Peripherally located stores may be functionally more closely linked to Ca entry via SOCE channels (215), while those located centrally could be refilled from the myoplasm. Another consideration is the type of SERCA associated with each of the stores. The Ca binding affinity ($K_{m}$) for SERCA2a is 0.31 $\mu$M and for SERCA2b is 0.17 $\mu$M (479). SERCA2b activity can be modulated by calmodulin (229) and phospholamban (162), and again this could affect cross-talk between the store and plasma membrane. If different isoforms of SERCA are associated with different stores, this could explain differences in their ability to refill the SR in the presence of different [Ca].

6. Summary

It seems to us, based on the current state of knowledge, that SOCE has been demonstrated in some but not all smooth muscles and that this will occur through a Stim-Orai mechanism when the channel involved gives rise to $I_{\text{calc}}$. Variations on this mechanism may occur in some smooth muscles, but it remains to be established if these are true variations on SOCE, or different channels such as ROCs. TRPCs may contribute to variation of SOCE in smooth muscle, leading to decreased Ca selectivity, but this is far from certain. More studies, both molecular and physiological, are clearly called for, and it is important to have more information from nonvascular myocytes. This remains a controversial and complex area (396) but one of some importance. The role of SOCE pathways may change with disease states, e.g., hypertension and bronchoconstriction, as well as phenotype, e.g., changes in arterial myocytes with proliferation, migration, or contraction (396), and thus an increased understanding could lead to new therapeutic targets.

There is little doubt that TRP channels are expressed in many smooth muscles, particularly, but not exclusively, tonic ones. As discussed, this expression does not necessarily equate to SOCE function. One recent interesting paper has provided evidence that TRPC channels become converted to SOCE channels as Stim1 leads to their movement from non-raft (TRCP) to raft (SOCE) membrane locations (10), whereas another recent study had TRPC channels in raft domains not Orai1 (148). There is evidence from only a limited number of smooth muscles of $I_{\text{calc}}$; whether this scarcity is due to the difficulties of recording these tiny currents or their absence from most smooth muscles is impossible to determine at the moment. Studies on cultured cells are limited models for native smooth muscle cells, as the downregulation of the normal components of E-C coupling and changes in SOCE will distort findings (512). The recent discoveries of Orai and Stim should help better study store-operated Ca entry in all cell types. Firm conclusions must await the ability to demonstrate store-operated entry under physiological conditions, e.g., agonist depletion, along with direct measures of luminal SR Ca content. It is worth noting that in cardiac muscle where the SR plays a dominant role in E-C coupling, no such SOCE mechanism exists. Perhaps in those smooth muscles where Ca entry is the dominant feature of force production, SOCE will also not be important. In contrast, in those smooth muscles that are more reliant on SR Ca contributions to contraction and have significant neurohormonal stimulation, then store-operated Ca entry may have an important role.

VIII. COMMON OR SEPARATE CALCIUM POOLS

A. Introduction

The presence of two types (at least) of Ca release channels on the SR, which can interact with each other to control local and global Ca signals, adds another level of complexity to the regulation of Ca signaling by the SR in smooth muscles. The scope for such interactions in any particular smooth muscle will depend on the morphological arrangement of the store(s), isoform expression, receptor distribution, and the cytoplasmic and luminal [Ca] (Fig. 5). There is reasonable evidence to support the view that the SR has heterogeneous compartments of releasable Ca in smooth muscle cells. In view of this, it is helpful to frame the discussion that follows according to the type of Ca store. These have been classified into three subtypes: $S_a$ (containing both IP$_3$ and RyRs), $S_p$ (containing only IP$_3$ receptors) (281, 285), and $S_y$ (containing only RyRs) (307). The relative distribution of each of these stores can vary considerably, not only among different types of smooth muscles but also among different species and with development. In the studies described below, a couple of general points should be borne in mind. First, a variety of different agonists, appropriate to particular smooth muscles, have been used as a mechanism to generate IP$_3$, and any differences in their other actions are often ignored. Second, when conclusions are based on pharmacological data, the specificity or otherwise of the drugs must not be overlooked. Mention has already been made of the problems associated with this approach to IP$_3$ receptor blockade, and similar concerns over some agents used to inhibit RyRs have also been raised (433).

B. One Store With One Type of Receptor

One of the clearest examples of cells having just one type of Ca release channel is provided by the ureter. In rat
ureteric myocytes, SR Ca release was shown to be agonist sensitive but not caffeine sensitive. In the guinea pig ureter, Ca release was caffeine sensitive but agonist insensitive. These data suggest that IP₃R plays the key role in control of Ca release in rat and RyRs in the guinea pig ureteric cells (91, 92). Within the above classification, rat has an Sᵦ store and guinea pig ureter Sᵧ.

It was subsequently shown that rat ureteric myocytes generated ryanodine-resistant, heparin-sensitive Ca puffs and Ca waves when stimulated by IP₃ or ACh (60, 91), while guinea pig ureteric cells generated ryanodine-sensitive Ca sparks enhanced by low concentrations of caffeine (76, 87). The functional data obtained on rat ureter were also supported by the data obtained using molecular biology and immunocytochemistry techniques (60, 493). Thus rat myocytes expressed predominantly IP₃Rs (all three isoforms) and only a trace of one isoform of RyR (RyR3) (60).

In single myometrial cells from pregnant rats (17), oxytocin and ACh evoked an initial peak in [Ca] followed by sustained elevations. These data support the idea that oxytocin and ACh activate IP₃R to generate Ca sparks, resulting in the propagation of Ca waves.

**Fig. 5.** Diagram of proposed SR arrangements and possible function in smooth muscle cells. A, a–e: schematic diagram showing proposed arrangements of the SR Ca stores reported for different types of smooth muscles. The Ca store in many types of smooth muscle cells can be compartmentalized into smaller SR elements that might contain either RyR (green) or IP₃R (red) Ca release channels or a mixture of these channels (blue). RyRs can be clustered to form specialized Ca release units located in close proximity to the cell membrane where they generate Ca sparks. IP₃Rs in most types of smooth muscle are homogeneously distributed in the cell and are mainly involved in generation of Ca waves and Ca oscillations, with or without involvement of the RyR channels. B: schematic diagram illustrating Ca spark/spontaneous transient outward current (STOC) coupling mechanism discovered by Nelson et al. (507), which acts as a powerful negative-feedback mechanism controlling excitability and electrical activity and has been reported in the majority of smooth muscles. Ca sparks can be activated by Ca entering the cell via L-type Ca channels (Ca-induced Ca release; CICR) or an increase in the luminal level of Ca by SERCa pump. BKCa, Ca-activated K channels; VOC, voltage-operated Ca channels. C: possible mechanisms for the generation of global Ca waves in smooth muscle cells. In a, the Ca wave is produced by activation of RyR channels via CICR triggered by Ca. In b, the Ca wave is triggered by IP₃, but propagated and amplified by RyR channels (cooperative action between two types of Ca release channels). In c, the Ca wave is triggered, amplified, and propagated by IP₃, RyR channels.
by a smaller sustained rise. The transient increase in \([\text{Ca}]\) was abolished by heparin, an inhibitor of IP_3-induced Ca release (ICR) and thapsigargin. In contrast, the transient \([\text{Ca}]\) response induced by oxytocin was unaffected by ryanodine. In permeabilized fibers of pregnant rat myometrium, caffeine did not produce contraction, whereas both IP_3 and the ionophore A23187 evoked contractile responses (620). These data show that myometrial cells possess an \(S_\beta\) but not \(S_\alpha\) store.

C. One Store With Two Types of Receptors

The majority of smooth muscle cells are both agonist sensitive and caffeine sensitive and have one common store (\(S_\alpha\)) shared by both IP_3R and RyR (62, 449, 537, 755). In addition, these cells may have additional, i.e., separate, agonist- or caffeine-sensitive Ca stores (673, 687). For example, in rat mesenteric artery myocytes (32), norepinephrine and caffeine produced a transient increase in \([\text{Ca}]\) in Ca-free solution. In the presence of norepinephrine, caffeine or thapsigargin elevated \([\text{Ca}]\). However, if thapsigargin or caffeine were added first, the subsequent application of norepinephrine did not increase \([\text{Ca}]\). These results suggest the existence of two types of Ca stores: one store sensitive to both caffeine and agonist (\(S_\alpha\)) and one sensitive to caffeine and thapsigargin but not to agonists (\(S_\alpha\)). Evidence that in some smooth muscles there is just one functional store but that it possesses both receptor types comes from direct imaging of the store (755). One difficulty in demonstrating either one or multiple Ca stores comes from the reciprocal actions of both the release channels on luminal Ca, i.e., if the Ca store is shared, as both IP_3R and RyR are sensitive to cytosolic and luminal \([\text{Ca}]\), the release of Ca from one population will affect the \(P_\alpha\) of the other. There are many examples described below of reciprocal and synergistic effects on Ca signaling arising from having both IP_3R and RyRs in the same smooth muscle cells. This does not, however, by itself prove that they access entirely the same Ca store. Identical patterns of store depletion upon exposure to caffeine or agonist, consistent with the idea that IP_3R and RyRs release Ca from the same store, have been demonstrated, highly suggestive of a single Ca store (755).

D. Two Stores, Two Types of Receptors

Early experiments based on the ability of caffeine and IP_3 to release Ca from permeabilized strips of taenia caeci, portal vein, and pulmonary artery demonstrated the existence of two separate Ca stores, with one gated by both RyRs and IP_3Rs (\(S_\alpha\)) and the other by IP_3Rs alone (\(S_\beta\)) (281, 282). In taenia caecum and pulmonary artery, IP_3 alone or in combination with caffeine-induced Ca release, which was about twofold larger than that induced by caffeine alone. Inhibition of \(S_\alpha\) store via its depletion produced by caffeine and ryanodine did not affect ICR from the \(S_\beta\) store (281). The contribution of the \(S_\beta\) store to total Ca release induced by IP_3 was 60% in taenia caecum, 40% in pulmonary artery, and only 6% in portal vein (281). In experiments performed on intact pulmonary artery myocytes, depletion of Ca stores with ryanodine and caffeine eliminated subsequent caffeine-induced intracellular Ca transients, but had little or no effect on the IP_3-mediated intracellular Ca transient induced by agonists (307). Experiments performed on intact terminal arterioles also revealed the existence of two separate caffeine-sensitive and agonist-sensitive Ca stores. In these blood vessels, Ca oscillations induced by agonists were resistant to the combined action of caffeine and ryanodine (74, 75). Recent work from McCarron and Olson (455) has highlighted the difficulty of using functional data to deduce information about the existence of two stores. They note that the appearance of two stores may arise because of partial depletion of SR Ca and luminal Ca regulation of the receptors terminating release from one receptor only.

E. Role of Ca Release Channels and Store Type in Ca Signaling

There are two key questions to be addressed here. First, can the complete set of Ca signals associated with contraction arise in smooth cells using just IP_3Rs or just RyRs? Second, how are IP_3-initiated Ca signals influenced by RyR Ca release and vice versa? There is general agreement that the initiation of Ca signaling is a response to agonists caused by the release of Ca from the SR via IP_3Rs (212). However, whether Ca released from the IP_3R then activates RyR to generate further release by CICR to amplify the signal and support propagation of Ca waves in a regenerative way, is still a controversial issue. As mentioned above, rat ureteric myocytes do not express RyR1 or RyR2, the subtypes which play the major role in CICR in smooth muscle (133). The ability of these myocytes to generate Ca puffs and Ca waves in response to agonists or IP_3s, which are resistant to ryanodine and antibodies to RyR (60), demonstrates that the entire Ca release and signaling process in smooth muscles can arise from IP_3R activity alone, i.e., without RyR involvement (60, 91, 92). Thus the answer to the first question posed above is yes; normal agonist-induced Ca signals and contractions can be produced by IP_3s alone.

There are examples of interactions between IP_3Rs and RyRs with the most noted being an amplification of IP_3Rs responses by RyRs. With the use of a variety of approaches, including blocking of the RyR with ryanodine (or ruthenium red, which may have additional actions) or
using antibodies to RyRs (62), effects on IP₃ Ca signals have been demonstrated in a wide variety of smooth muscles. These effects include abolition of agonist-dependent Ca oscillations (333, 535, 638) and inhibition of Ca waves (34, 62, 303, 331). There are also many examples of IP₃, modulating RyR events, and affecting both local and global Ca signals (299). Receptor activation leading to the generation of IP₃, increased spark discharge from frequent discharge sites (FDS) in vascular (18, 62, 219) and visceral (755) smooth muscle cells. It has been postulated in portal vein that IP₃ produced by the basal activity of PLC activates IP₃Rs to recruit neighboring clusters of RyRs within the FDS to generate large Ca sparks (219). Inhibition of IP₃Rs results in a decrease in the frequency of Ca sparks, Ca oscillations, and waves in response to agonists in visceral and vascular smooth muscles (18, 62, 299, 333, 535, 638).

A physiological consequence of interaction between IP₃- and RyR-sensitive Ca channels will be positive feedback amplifying Ca release. In porcine tracheal smooth muscle cells, ryanodine and ruthenium red inhibited ACh-induced Ca oscillations (333, 574). Similarly, the P2Y receptor agonists UTP and 2-methylthio-ATP each increased the occurrence of Ca waves in rat cerebral artery and murine colonic myocytes, effects which were blocked by ryanodine (34, 303). In rabbit cerebral artery (332) and renal resistance arteries (307), agonist-induced Ca oscillations were blocked by ryanodine, suggesting that RyR-dependent CICR contributed to the [Ca] rise.

Agonist-induced propagating Ca oscillations that originate from FDSs have been demonstrated in several smooth muscles. For example, ACh-induced Ca oscillations in tracheal smooth muscle originate from areas of the cell displaying the highest frequency of Ca sparks but require SR Ca release through IP₃ receptor channels for initiation (333, 535, 638). In gastric myocytes, Ca waves evoked by carbachol were initiated from the same sites as those evoked by caffeine, and these sites also colocalized with spontaneous spark sites. These observations suggest that RyRs were involved in both cases, and the carbachol responses were inhibited by both IP₃R and RyR blockers (755). Inhibition of RyRs with antibodies or with ryanodine abolished agonist-dependent Ca transients and waves, in both visceral and vascular smooth muscles with one functional store shared by both types of receptor channels (62, 223, 300, 755, 756).

These findings suggest that agonists can stimulate Ca release in smooth muscle via mechanisms that are both IP₃R and RyR dependent and support a model in which activation of IP₃Rs acts as a trigger leading to secondary activation of RyRs. Does this mean that all IP₃R-activated responses are amplified by RyRs when they are expressed? Recent observations strongly suggest the scope for such interactions varies among different types of smooth muscles and depends on receptor distribution and isoform expression.

There is much evidence that in some smooth muscles in which IP₃R and RyR share the same store, there is a noncooperative relationship between the two types of channels (29, 185, 449). For example, adrenergic activation decreased the frequency of Ca sparks in intact rat resistance arteries; this was suggested to be due to a decrease in the SR Ca content (449). Blockade of IP₃Rs in smooth muscle cells from both the gastric antrum and vas deferens did not decrease, but in fact increased, both caffeine-releasable store content and spontaneous spark activity (755, 756). However, in rat resistance artery myocytes, IP₃R blockade did not affect the temporal or spatial characteristics of Ca sparks (380).

There is, therefore, clear evidence that increases and decreases in IP₃R activity can lead to reciprocal changes in the activity of RyRs through changes in luminal SR [Ca]. However, some inhibition of Ca sparks by agonists could be explained by activation of PKC, which also decreases Ca spark frequency, by directly inhibiting RyRs (70).

In equine tracheal myocytes, the Ca release evoked by the IP₃-generating muscarinic agonist methacholine was unaffected, while that evoked by caffeine was blocked by ruthenium red (739). In colonic myocytes, ryanodine alone did not inhibit IP₃-evoked [Ca] increases, but when applied with caffeine allowing ryanodine to lock RyR channels in a subconducting state, IP₃-mediated [Ca] increases were blocked. These results were attributed to the drug’s indirect inhibition of the IP₃-mediated response by store depletion of Ca, rather than to involvement of RyR in Ca release (185). Similar data have been reported for the smooth muscle cells of interpulmonary bronchioles and arterioles, in which under normal conditions the RyR was not essential for generating agonist-induced Ca oscillations (29, 556, 557). These results were consistent with the observation that the release of caged Ca was able to initiate CICR via RyR in the smooth muscle of the interpulmonary bronchioles only when the cells were previously depolarized with KCl to overload the Ca stores and sensitize the RyR.

Acetylcholine, norepinephrine, and nerve stimulation each released Ca in porcine coronary, rabbit mesenteric, and rat tail arteries, respectively (284, 299, 342, 449). The release of Ca by the neurotransmitters was inhibited by ryanodine in each study and attributed to the drug’s ability to open RyRs and deplete the Ca store. Norepinephrine-evoked Ca waves persisted in tail artery segments maintained in organ culture for 3 days with ryanodine, while Ca sparks and Ca release evoked by caffeine were lost, suggesting that RyR does not directly contribute to Ca waves in this preparation (154). Ruthenium red and tetracaine (each of which abolished the [Ca] increase evoked by caffeine without depleting the store) did not affect the Ca oscillations induced by agonists in pulmo-...
nary arterial myocytes (279). In guinea pig colonic myocytes in the absence of RyR activity, repetitive Ca release events induced by photolysis of caged IP$_3$, were not inhibited by tetracaine or ryanodine (185). This suggests that IP$_3$ receptor activity alone accounted for the Ca waves and loss of the ability of agonists to induce SR Ca release after caffeine results from Ca depletion in a common store (185).

F. Summary

From the above we propose that the functional implications of RyRs in neurotransmitter-induced Ca waves or oscillations depend on their density relative to IP$_3$R and that they share the same store. In smooth muscles displaying a higher density of IP$_3$R than of RyRs (e.g., colonic smooth muscles, Ref. 758), the Ca responses to neurotransmitters will mainly depend on activation of IP$_3$ receptors alone (e.g., Ref. 366). In smooth muscles displaying a higher density of RyRs than IP$_3$R (e.g., rat portal vein), the Ca waves and oscillations induced by neurotransmitters will depend on activation of both IP$_3$Rs and RyRs. However, comparative functional experiments correlated with expression and distribution of IP$_3$R and RyR channels in other types of smooth muscles are needed to expand this conclusion to all types of smooth muscles.

To summarize these data, we can conclude that the functional studies that employed blockers of SERCAs in conjunction with activators of IP$_3$Rs and RyRs shed some light on the morphological organization of intracellular Ca stores. In some types of smooth muscles, morphological studies employing immunofluorescence and molecular biology techniques provided information that could explain the functional data. However, to fully understand the functional role of the SR in smooth muscles, confocal microscopy and immuno-EM are needed to examine the relationship between IP$_3$R and RyR isoforms and SERCA, to substantiate conclusions drawn from these functional studies.

IX. ELEMENTAL CALCIUM SIGNALS FROM SMOOTH MUSCLE SARCOPLASMIC RETICULUM

A. Ca Sparks

1. Introduction

Calcium sparks were first identified in cardiac (122, 544) and skeletal (354, 708) muscles, where their fundamental role in the generation of global Ca signals underlying phasic contractions has been well established. In smooth muscle cells Ca sparks were first observed in cerebral artery and showed biophysical and pharmacological characteristics similar to those of cardiac muscle cells (507). Since then, similar events have been described in a wide variety of smooth muscle types including different types of arteries and arterioles (70, 90, 192, 305, 473, 568, 749, 751), portal vein (219, 221, 474), urinary bladder (127, 254–256, 288, 312), gastrointestinal tract (34, 35, 220, 223, 351, 808, 809, 811), airways (369, 810), gallbladder (572), and guinea pig ureter (76, 87). A lack of Ca sparks has been reported for smooth muscle cells of neonatal cerebral arteries (213), nonpregnant mouse (476), and pregnant and nonpregnant rat myometrium (88).

Calcium sparks in smooth muscle can occur spontaneously (76, 87, 220, 507) or after activation by one of the following factors: 1) low concentrations (1 mM) of caffeine (76, 87, 213, 307, 807), 2) elevation of global [Ca] caused by Ca entering through L-type Ca channels (312, 508), 3) increase in the SR Ca content (87, 811), and 4) stretch (312).

2. Frequent discharge sites

Irrespective of the mechanism of activation, Ca sparks repeatedly arise from a few specialized regions adjacent to the superficially located SR within the myocytes; these have been termed FDS (65, 87, 220, 221, 288, 312, 508). These areas are enriched with clusters of RyR2 channels (133, 213, 525). It should, however, also be noted that frequent discharge events away from the cell membrane have also been reported in smooth muscle cells from rat portal vein (18, 65) and gastrointestinal tract (220, 807). The number of FDS varies not only among different types of smooth muscle cells (65, 87, 219, 220, 807), but also among different populations of the same type of smooth muscle (65). The number of FDS is increased in the presence of low concentrations of caffeine (76, 307, 807) or after increasing the SR Ca content (87, 811). Such a non-uniform distribution of FDS in smooth muscle may arise from either the irregular clustering of RyR Ca release channels, or nonuniform distribution of the SERCA pump on the SR. Clustering of RyR2 in peripheral zones of SR was an absolute requirement for the formation of sites generating spontaneous Ca sparks coupled to STOCs in arteriolar myocytes (213).

The properties of Ca sparks in isolated cells appear to be relatively similar in different types of smooth muscle cells (for details, see reviews in Refs. 536, 750) and do not differ from those observed in intact preparations (70, 87, 305, 507, 568). Thus, although variations in their biophysical parameters have been noted, Ca sparks appear to be more stereotypic in different types of smooth muscles than the global Ca signaling controlling contractility.
3. Functional role of Ca sparks

The physiological role of Ca sparks has been a subject of intense investigation in a variety of smooth muscles. Calcium sparks are the fundamental units of SR Ca release during E-C coupling in cardiac muscle, synchronized in time by the action potential. Cardiac sparks occur through the CICR process, and the physical and functional coupling between Ca influx and RyR channels is tight and intimate (50, 122, 127). RyR channels are positioned in junctional SR elements within short distances (~20 nm) of voltage-dependent Ca channels in the transverse tubules (50, 759). Pharmacologically CICR in cardiac muscle can be easily tested using ryanodine, or a low concentration of caffeine. In cardiac muscle, ryanodine produces a strong negative inotropic effect associated with marked inhibition of the global Ca transient, but with no effect on the action potential. Caffeine produces a transient positive inotropic effect associated with an increase in systolic [Ca] with no change of the Ca current.

Cardiac myocytes express RyR2. The majority of smooth muscles also express RyR2 and a1C L-type Ca channels (511), which colocalize with them (490, 525). It is perhaps not surprising then that a cardiac-like mechanism of CICR was expected to be present, acting as an amplifying system for the global Ca transient, especially in phasic smooth muscle cells. However, it should be recalled that the highly organized junctional arrangement between plasma membrane and SR seen in striated muscles does not occur in smooth muscles, and junctional proteins such as junctin and triadin may be absent (see sect. iv).

Even more importantly, there are significant differences in the activation by Ca between cardiac and smooth muscles. In cardiac muscle, global Ca is the determinant of contraction under normal physiological conditions. However, in smooth muscle cells, myosin light chain phosphorylation catalyzed by MLCK and activated by the Ca-calmodulin complex is a key factor in the control of mechanical activity. Data obtained in some types of smooth muscle cells show that MLCP phosphorylation can achieve its maximal level at submaximal levels of Ca (654). Thus, unlike cardiac muscle where high cytoplasmic [Ca] is required to achieve a high level of force, in smooth muscle cells a high level of force can be achieved at moderate levels of Ca, especially during agonist-induced stimulation, when sensitizing pathways involving PKC or Rho-associated kinase inhibition of myosin light-chain phosphatase (MLCP) can also be activated (625, 692).

Finally, Ca-activated ion channels are conspicuously expressed in smooth muscle cells but are largely absent from the heart. Thus, as will be described below, a role for Ca sparks in affecting excitability, as well as modulating Ca signals, has emerged as their prime function in many smooth muscles.

The first work in which smooth muscle Ca sparks were observed (507), in myocytes of cerebral arteries, showed that sparks did not contribute to contraction. Rather, the authors demonstrated that sparks were involved in relaxation of tone via activation of Ca-activated large-conductance K channels (BK). The discovery of Ca sparks provided the missing link in explaining the origin and functional role of STOCs, which had been observed earlier in a number of smooth muscle cells (41). The role of Ca sparks/STOCs coupling mechanism and its functional role in tonic smooth muscles has now been thoroughly investigated (507), and well reviewed (304, 750).

In contrast, the role of Ca sparks in phasic smooth muscles, particularly their possible role in amplification of the global Ca signal via CICR, is still under investigation, and there is no consensus on this issue. It appears to us that different types of smooth muscles utilize Ca sparks in a variety of ways to match their physiological function. Unlike cardiac muscle, smooth muscle cells express Ca-activated K and Cl channels, which can be targeted by Ca sparks, and in this way control excitability.

4. Ca sparks in phasic smooth muscles

In cardiac myocytes, each action potential results in a contraction that derives from RyR-mediated calcium release, triggered by an increase in [Ca] due to influx via L-type Ca channels. The signal gain is quite high, since each channel opening results in a Ca spark (activation of several RyRs), the duration of which is longer than the L-type Ca channel opening (104). Phasic smooth muscles are also activated by Ca transients controlled by the action potential. However, information on the Ca signal in the cytosol following the action potential is limited, even though it is crucial to the understanding of the coupling of membrane excitation to Ca-sensitive cellular functions. Thus, despite the broad expression of L-type Ca channels and RyR in many phasic smooth muscles, the existence and nature of CICR in these nonsarcomeric cells, in which the distribution of L-type Ca channels and RyR differs substantially from an orderly dyadic pattern in sarcomeric muscles, is not well established.

Simultaneous recording of electrical activity and intracellular [Ca] have been performed in some intact phasic smooth muscles, e.g., guinea pig ureter (87), pregnant rat myometrium (88), and urinary bladder (288) and isolated cells from guinea pig ileum (247, 248, 254), guinea pig vas deferens (361), and mouse urinary bladder (494). In urinary bladder, vas deferens, uterus, and ileum, the action potential usually appears as brief (20–40 ms) spikes, while in ureter it consists of a spike followed by a long-lasting (400–800 ms) plateau component. In all these four smooth muscles, the Ca transient is triggered by the
action potential, and both are inhibited by L-type Ca channel blockers, indicating that Ca influx is a key factor in the generation of the global Ca transients. Is CICR contributing to the global Ca transients associated with the action potential in smooth muscles?

In the guinea pig ileum where Ca sparks have been observed (220), ryanodine and thapsigargin produced stimulant rather than inhibitory effects on the Ca transients associated with the action potential by increasing the amplitude and duration of the spike (203, 288, 312, 494). Voltage-clamp experiments performed on ileum (361), cerebral arteries (330), portal vein (133), vas deferens and bladder (288), and pregnant myometrium (630) demonstrated that Ca entry via L-type Ca channels can trigger some Ca release via RyRs. At the same time, other workers have failed to detect CICR in voltage-clamped smooth muscle cells isolated from other or even the same type of smooth muscles. For example, CICR was not detected in voltage-clamped smooth muscle cells of portal vein (329), gastrointestinal tract (79, 781), and airways (178).

A discussion of the physiological role of CICR in urinary bladder smooth muscle cells illustrates the complexity in this area and the data which are hard to unify. Elegant work performed on voltage-clamped guinea pig urinary bladder myocytes by Ganitkevich and Isenberg (203) showed that Ca transients induced by long depolarizing voltage steps were inhibited ~70% by ryanodine. This suggested that a relatively high gain “cardiac type” of CICR operates in these myocytes, at least under these experimental conditions. The importance of a functional contribution of CICR to E-C coupling and contraction in urinary bladder smooth muscles has been assessed by several research groups, using different species and experimental models (127, 247, 254, 490, 494, 525). Results from these studies addressing the susceptibility to contraction in bladder myocytes treated with ryanodine are not in agreement. For example, Hashitani and Brading (247) showed the intracellular [Ca] rise induced by spontaneous action potentials in the guinea pig was reduced to ~60% by ryanodine. In a later publication (494), the stimulant action of ryanodine on the mechanical activity of the guinea pig bladder was reported. Reduction of spontaneous or electrically evoked contractions by ryanodine have been reported for mouse bladder myocytes (494). However, ryanodine has no significant effect on the [Ca] and spontaneous contractions in rat bladder myocytes (252). These data suggest that the contribution of RyRs and CICR to E-C coupling in the bladder differs between species and experimental conditions.

Imaizumi et al. (288) in guinea pig urinary bladder myocytes using brief (50 ms) depolarizing voltage steps, obtained the first direct evidence that Ca “hot spots” (equivalent to Ca sparks) could be transiently activated in cells depolarized to −20 mV. These events lasted for >0.5 s and spread as Ca waves when depolarization was to 0 mV. The increase in global [Ca] elicited by 50 ms depolarization to 0 mV reached a peak 60–100 ms after the start of depolarization, indicating that the spread of Ca hot spots continued even after most voltage-dependent Ca channels had closed. Recently, Kotlikoff and co-workers (127) have also investigated the temporal relationship between the Ca current and local and global Ca signaling in rabbit urinary bladder myocytes, under voltage- and current-clamp conditions with physiologically relevant protocols of stimulation. Localized photolysis of caged Ca initiated CICR, but multiple excitation pulses were needed and IP₃R channels were also activated (313). Calcium channels were shown to activate RyRs to produce CICR in the form of Ca sparks and propagated Ca waves. Both the initial Ca spark and the subsequent Ca waves occurred through the opening of RyRs, since both were eliminated by ryanodine, and neither was affected by dialysis with heparin (127). They concluded that 1) L-type Ca channels could open without triggering Ca sparks, 2) triggered Ca sparks could be observed after channel closure, 3) the trigger stimulus for the Ca release process is not a local but a global rise in intracellular [Ca], and 4) this results in a functional uncoupling of a single action potential from Ca release.

5. Loose coupling

The small number of Ca spark sites evoked by the Ca current, the delay between Ca channel and RyR channel openings, as well as the inhibitory effect of BAPTA on Ca sparks in urinary bladder smooth muscle cells suggested that compared with cardiac myocytes, a fundamentally different coupling process operates in smooth muscle, which was termed “loose coupling” (127, 368). Features of the loose coupling system are low gain (multiple Ca channels must open to produce sparks), discriminated responses (release takes the form of local Ca sparks or globally propagated Ca waves), and a marked lengthening of signal duration (Ca waves last far longer than the action potential). The delayed activation of CICR and the subsequent propagation of a Ca wave, which could last up to 1 s, suggests that CICR in contrast to cardiac muscle (122, 127) cannot be controlled by a brief (40–60 ms) single action potential, and its functional role is yet to be established (368). Thus, in urinary bladder myocytes paradoxically, global rises of [Ca] are required to activate Ca sparks. The concept of loose coupling can also be explained by an increase in the SR [Ca] and activation of [Ca] sparks. An increase in the luminal [Ca] produces profound effects on both the frequency and the amplitude of Ca sparks in smooth muscle (811).

Both cytosolic and SR Ca levels would be expected to increase with enhanced Ca entry. The proximity of the SR to the plasma membrane and the existence of the uptake
mechanisms by the SERCA pump provide the structure for what has been termed by van Breemen the "superficial buffer barrier" (714), discussed also in section II. Since inhibition of SERCA has little effect on the global rise of [Ca] induced by depolarizing voltage steps (e.g., Refs. 77, 507), it can be suggested that only a small portion of the SR is involved in active ATP-dependent accumulation of Ca, which does not affect the global rise of [Ca]. Similarly, many myocytes have FDS which, when releasing Ca in the form of Ca sparks, have little effect on the global rise of [Ca] (330). However, this suggestion should be experimentally tested by studying the spatial distribution of SERCA pumps in smooth muscle cells. Since the effects of ryanodine on the guinea pig urinary bladder were mimicked by CPA, the BK channel inhibitor iberiotoxin, and BAPTA (247), it was suggested that CICR targets BK channels via Ca sparks and in this manner controls excitability of this smooth muscle.

Another well-studied smooth muscle with respect to CICR and the role of Ca sparks is the guinea pig ureter. In contrast to bladder smooth muscles, Ca transients and phasic contraction in the guinea pig ureter are controlled by a single long-lasting (400–800 ms) plateau-type action potential of myogenic origin, which gives rise to a long-lasting Ca transient (76, 87). Parameters of the Ca transients are tightly coupled to the parameters of the action potentials (76, 87, 93) and thus meet the criteria of both tight and loose coupling. Early contractile studies in intact guinea pig ureteric smooth muscle preparations identified ryanodine-sensitive calcium release by caffeine (93, 287) but not agonists (89, 91, 92), and thus these myocytes serve as an excellent experimental model to investigate the role of CICR in phasic smooth muscles. The STOCS, associated with spontaneous transient hyperpolarizations and sensitive to caffeine, were recorded in myocytes under voltage- and current-clamp conditions (91, 92). We observed Ca sparks arising from several FDS in isolated cells and intact preparations (87). In guinea pig ureteric smooth muscle cells, low caffeine concentration increased the frequency of Ca sparks and inhibited the action potential, Ca transients, and phasic contractions. These components of E-C coupling were restored by ryanodine, CPA or TEA, and iberiotoxin (blockers of BK channels) (76). Under voltage-clamp conditions, caffeine at low concentrations had no inhibitory effect on the Ca current and slightly potentiated global rises of [Ca], suggesting some contribution of CICR to the generation of depolarization-evoked increases in global [Ca]. However, caffeine markedly increased the frequency and amplitude of STOCs and increased the overlap between Ca and BK channel currents when voltage-clamped ureteric myocytes were depolarized to 0 mV (76). Caffeine had no effect on the action potential and Ca transients in the presence of TEA, again suggesting that CICR had little contribution to global rises of [Ca] controlled by the action potential. The lack of effect of ryanodine and CPA on the Ca transients and the reversal of the inhibitory effects of caffeine by these agents suggest that the contribution of CICR to global Ca signaling is minimal, and the major source of Ca is action potential-stimulated Ca entry.

In the guinea pig ureter, the duration of the plateau component of the action potential is affected by inward Ca current ($I_{\text{Ca}}$) and outward BK ($I_{\text{BK}}$) currents (287, 382). Normally there is a substantial delay between $I_{\text{Ca}}$ and STOCs in ureteric myocytes (287, 382), which enables the generation of the long-lasting plateau. However, if this delay is shortened or eliminated, as is the case with caffeine, the action potential is blocked or markedly reduced in duration leading to the inhibition or reduction in the duration of the Ca transient. Thus these data fit the hypothesis of loose coupling between $I_{\text{Ca}}$ and CICR. This mechanism is an important factor in controlling the duration of the plateau component of the action potential, which in turn plays a key role in controlling the amplitude of phasic contractions (93). A decrease or elimination of the delay between $I_{\text{Ca}}$ and STOCs, and a slight increase in $I_{\text{Ca}}$-independent amplitude of the global Ca transient occur under voltage-clamp conditions in the presence of caffeine, suggesting that $I_{\text{Ca}}$ can be tightly coupled to Ca sparks. The mechanism of loose coupling between $I_{\text{Ca}}$ and STOCs (Ca sparks) in guinea pig ureteric cells can be explained by a prominent role of luminal Ca in the activation of Ca sparks/STOCs coupling mechanism. Indeed, we and others have recently shown that the Ca content of the SR following a global Ca signal is substantially increased (77, 633), and this was well correlated with a long-lasting discharge of Ca sparks after L-type Ca channels were closed (87, 93).

The Ca content of the SR was also shown to play a critical role in the generation of spontaneous Ca sparks and STOCs in smooth muscle of the gastrointestinal tract (811). We have established that the transient eruption of Ca sparks plays a key role in the control of the long-lasting period of refractoriness, which serves as an important pacing and protecting mechanism in the ureter (87). In the guinea pig bladder, the repolarizing phase of the action potential, as well as the frequency of spike discharge in one burst, are also potentiated by inhibitors of the SR or BK channels (247, 248, 254, 256). These data would also fit a loose coupling between $I_{\text{Ca}}$ and STOCs, acting as an important mechanism controlling the parameters of the action potential and excitability of these types of smooth muscles, as observed by Collier et al. (127).

To summarize these data, we conclude that the large contribution of BK channels activated by superficial CICR to the negative feedback of membrane excitability and/or the regulation of action potential shape, is well-established and a characteristic of many types of smooth muscles. The question of a possible role of CICR as a contrib-
utor to the global Ca signal is still a matter for future experiments. That inhibition of SR function by SERCA inhibitors results in >70% inhibition of the [Ca] rises in the area of discharge sites in urinary bladder and vas deferens strongly suggests that CICR is present in these tissues and can produce a significant contribution to the cytoplasmic [Ca] rise. However, as under the same conditions, that global rises of [Ca] were not inhibited, but in fact were slightly potentiated, as also reported for a number of intact phasic smooth muscles, suggests that CICR is confined to microdomains. Studies using other types of smooth muscles and different species should be carried out. High temporal and spatial resolution and analyses of the Ca release from the SR should be correlated with the isoform expression and spatial distribution of different types of RyRs.

6. Ca sparks and myogenic tone

Many resistance arteries possess an intrinsic “myogenic” tone, activated in response to elevation of intravascular pressure by a graded membrane depolarization, opening Ca channels, and leading to elevation in [Ca] and constriction (507). It has been proposed that Ca sparks, by targeting BK channels, generate STOCs, which cause a tonic hyperpolarizing influence on the membrane potential of small pressurized arteries with myogenic tone (507). Inhibition of Ca sparks or BK channels causes membrane depolarization, leading to further elevation of [Ca], and additional increases in muscle tone in pressurized cerebral arteries (330, 356, 507). Thus, in resistance arteries, a Ca sparks/STOCs coupling mechanism can act as a negative-feedback mechanism to oppose myogenic Ca entry via Ca channels and promote relaxation (507). However, under voltage-clamp conditions, global rises of [Ca] induced by depolarizing voltage step in myocytes isolated from these arteries were significantly inhibited by ryanodine. This suggested that CICR is also present in these cells and can act as an amplifying mechanism (507). However, under normal physiological conditions, this mechanism may contribute little, as blocking Ca sparks in the presence of blockers of BK channels has little effect on global [Ca] and arterial tone (330). Myogenic tone is associated with moderate depolarization (356, 357, 507), and it was suggested that L-type Ca channels operate in a “low-activity mode,” i.e., the myocytes never depolarize sufficiently to achieve a high channel P_{o}.

We conclude that the Ca sparks/STOCs coupling mechanism acts as a powerful vasodilator in vascular beds where membrane potential depolarization acts as an activating mechanism to generate tone. If muscle tone is activated via stimulation of Ca entry by SOC or ROC channels, this mechanism will be ineffective, as it will be in controlling tone produced by the Ca oscillations associated with activation of IP_{3}Rs. However, this mechanism can play an important role in disabling the mechanisms of generation of the action potential, which is a feature of most of the arteriolar smooth muscle cells and enables them to be regulated by local factors.

B. Ca Puffs

The term Ca puffs refers to the small local increases in [Ca] that occur when IP_{3}Rs open spontaneously or in response to agonists. Unlike sparks, Ca puffs have not been detected in vascular myocytes despite the obvious presence of IP_{3}Rs. So far, Ca puffs have been detected in only two smooth muscles: rat ureter (60) in which agonist-induced Ca release plays a dominant role in modulating contraction (34, 86) and murine colonic myocytes. In rat ureteric myocytes, Ca puffs were observed during release of low concentrations of IP_{3} from a caged precursor, or by low concentrations of ACh. They were also observed spontaneously in Ca-overloaded myocytes (91). Spontaneous Ca puffs were observed in intact rat ureteric preparations (60).

Calcium puffs in tissues appear to have a wide range of amplitudes, time courses, and spatial spread, suggesting that the IP_{3}Rs exist in clusters of variable numbers of channels and that within these clusters a variable number of channels can be recruited (86). Calcium puffs in the ureteric myocytes were blocked selectively by intracellular applications of heparin and an antibody to IP_{3}R, but were unaffected by ryanodine and intracellular application of an antibody to RyR (60). When stimulated by agonists, propagated, ryanodine-resistant Ca waves appeared to result from the spatial recruitment of Ca-release sites by diffusion. This is consistent with data from multicellular preparations, where ryanodine-resistant agonist-induced Ca release was reported (34). Both Ca signals provide an integrated mechanism to regulate contractility in smooth muscle cells, where RyRs are absent, or poorly expressed.

The possible role of Ca puffs in the control of excitability of rat ureteric smooth muscle cells has not been investigated, although we have observed spontaneous transient outward/inward currents (STOICs) (Burdyga and Wray, unpublished data), which could result from activation of the BK and Cl/Ca channels (91, 92).

Localized Ca transients and puffs, resistant to ryanodine and inhibited by xestospongin, U-73122 (an inhibitor of PLC), occurred spontaneously or during P2Y receptor stimulation in murine colonic myocytes (34). The puffs were coupled to the activation of both BK and small-conductance Ca-activated K (SK) channels. Thus the release of Ca by G protein-mediated activation of PLC can be linked to inhibitory responses via Ca puffs targeting SK channels. This is in marked contrast to the usual finding that IP_{3}-dependent mechanisms are used by excitatory agonists in smooth muscles.
A role for IP₃Rs in the generation of Ca oscillations and waves seems to be a common feature of smooth muscles. The absence of local Ca signals, i.e., Ca puffs, is therefore surprising. The paucity of Ca puffs might be explained by there being little or no clustering of the receptors in most smooth muscles. It may also be that Ca puffs have not been sought. Whether puffs are present in other smooth muscles and whether they can contribute to the control of excitability seems to us to be an important question that should be urgently addressed.

X. SARCOPLOMASCIC RETICULUM AND PLASMALEMMAAL ION CHANNELS

A. Overview of Ca-Activated Ion Channels

In contrast to skeletal and cardiac muscle, smooth muscle cells express several types of Ca activated ion channels on the plasmalemma that can be differentially activated by SR Ca release events, such as Ca sparks, Ca puffs, and Ca waves. There are two major populations of Ca-activated channels that can be activated by SR Ca release in different types of smooth muscles: Ca-activated K (K_{Ca}) and Ca-activated Cl (Cl_{Ca}) channels. The K_{Ca} channels are composed of at least three different members, which are separate molecular entities. Their different K conductances have led to their being known as big (BK), intermediate (IK), or small (SK) K channels. These channels also exhibit different inhibition profiles to various toxins. (The BK channel is also referred to as the maxi K_{Ca}.) Coupling between SR-mediated Ca events and these Ca activated ion channels can lead to changes in membrane potential and/or the action potential and thus serve as powerful negative- or positive-feedback mechanisms, controlling excitability and contractility of smooth muscles. Ca sparks can activate either or both of these Ca-activated channels producing STOCs (507), spontaneous transient inward currents (STICs), or STOCs (811). Spontaneous transient membrane hyperpolarizations have also been reported in coronary artery (202) and guinea pig ureter (87) and spontaneous transient membrane depolarizations in urethral (249, 287) smooth muscles, confirming that STOCs and STICs can affect the membrane potential of smooth muscles. A Ca spark/STOC coupling mechanism can also directly affect the parameters of the action potential by increasing the repolarizing current and thus decreasing the amplitude and duration of the action potential, as was shown for urinary bladder (247, 248, 254, 256, 288) and ureter (474). Calcium waves may also indirectly influence excitability and contractility of smooth muscles through activation of Ca-dependent ion channels. In rat portal vein myocytes, Ca sparks were demonstrated to activate STOCs, while Ca waves selectively activated Cl_{Ca} channels (474). Interestingly, STICs but not STOCs were selectively blocked by inhibition of IP₃Rs (623), suggesting that the variability in Ca release events can also modulate the cell membrane potential through differential targeting of K_{Ca} and Cl_{Ca} channels.

B. BK Channels

Transient outward K currents were first described in smooth muscle cells by Benham and Bolton (1986), who used the term STOC to describe these events, which were later identified in a wide variety of smooth muscle cells (41, 70, 87, 150, 264, 277, 287, 308, 332, 382, 495, 507, 516, 528, 568, 604, 617, 806, 810, 811). The native BK channels consist of four identical α subunits that create a functional BK channel and four modulatory β subunits which combine with the channel forming α subunits and affect their sensitivity to Ca (83, 93, 459, 514, 712). Immunocytochemical observations using antibodies against the BK channel α subunit, Ca channel α₁C subunit and RyR, showed that the BK and RyR but not the Ca channel form clusters in myocytes (525). The BK channels have an estimated single-channel conductance of ~80 pS at ~40 mV with a physiological K concentration gradient and occur at a density of ~1–4 channels/μm² (41, 641, 689). Direct evidence for the causal relationship between Ca sparks and transient BK currents has been supplied by studies combining whole cell patch clamping with simultaneous confocal imaging of Ca in voltage-clamped myocytes (83, 150, 302, 352, 474, 555, 749, 810). These experiments revealed good correlations between the temporal characteristics of Ca sparks and STOCs, although BK currents showed a faster decay relative to the Ca spark. These data are in good agreement with immunohistochemical observations that showed the close proximity of Ca spark-generating sites and BK channels (525).

A Ca spark/STOC coupling mechanism is present in many visceral and vascular smooth muscles and acts as a powerful negative-feedback mechanism, protecting smooth muscle cells from overexcitability. Inhibition of Ca sparks with ryndone or SERCA pump inhibitors or their target BK channels (by low concentration of TEA or iberiotoxin) results in augmentation of vascular tone (507) and an increase in the excitability and termination of the refractory period in phasic smooth muscles (87, 247). The importance of BK channels has been demonstrated with transgenic mice. Targeted deletion of regulatory β1-subunits of BK channels resulted in animals that developed an increase in vascular tone and hypertension (618) as well as other pathologies such as ataxia (619), urinary incontinence (465), and erectile dysfunction (752). BK channels have a relatively low affinity for Ca. For example, at a physiological membrane potential of ~40 mV, the Kₘ of BK channels for Ca is ~20 μM (554). Calcium reaches high levels (10–100 μM) close...
to the SR release site (505, 555), and the plasma membrane is in close proximity; thus BK channels are activated despite their low affinity for Ca. A single Ca spark can produce ~20 mV hyperpolarization in an isolated myocyte through activation of BK channels (202, 507). Vascular myocytes have a relatively high density of BK channels and do not require clustering of BK channels above a spark site. However, in visceral smooth muscle cells, where the density of BK channels is low, clustering of BK channels in the patches of the plasma membrane at sites frequently discharging sparks is required (525, 809).

C. SK and IK Channels

There are four different isoforms of the SK channel, SK1-4 (480), with SK4 also known as IK1. The cloning of SK channels revealed the existence of at least three members of a family (SK1-3) (362), which were first identified in cultured rat skeletal muscle (55). These channels are highly sensitive to Ca (EC_{50} = 0.5–0.7 μM) and have a small single-channel conductance (4–14 pS). In addition, they are selectively blocked by the bee venom toxin apamin (135, 190, 247, 250, 254, 256). Of the three cloned SK channel subtypes (SK1-3), SK1 and SK3 channels are least sensitive to apamin (nanomolar apamin was required for their inhibition), and SK2 channels were more sensitive and inhibited in the subnanomolar range (67, 362, 626, 727).

Since their initial discovery, SK channels have been described in a number of cell types, including rat pituitary cells (381), T lymphocytes (227), and adrenal chromaffin cells (543). They have also been identified in some types of smooth muscles, e.g., urinary bladder (698), stomach (673), colon (364), uterus (360, 450), renal artery (206), portal vein (293), and the pancreatic arterioles (666). Their distribution and functional importance are currently being established (247, 248, 256, 663), but there is evidence that SK3 is particularly relevant to contractility and excitability in smooth muscle. Functional studies performed on the intact bladder revealed SK channels contribute to the afterhyperpolarization (AHP) following trains of action potentials (255), as apamin blocked the AHP and increased excitability and contractility without affecting other parameters of the action potential.

There is evidence that both SR Ca release and plasmalemmal Ca entry can activate SK channels. Hererra and Nelson (256) reported that SK channels in the bladder are activated by the rise in [Ca] which occurs upon Ca entry through L-type Ca channels during the action potential but not by Ca sparks (256). The inability of Ca sparks to activate SK channels was explained by the small density of the SK channels near the discharging sites. Indeed, the density of SK channels in bladder myocytes was estimated to be ~200 times lower than that of BK or L-type Ca channels (257). The activation of SK channels occurs through the binding of Ca to calmodulin, which is constitutively bound to the COOH terminus of the channel (503). Thus SK channels are well suited to respond to changes in global [Ca] (0.1–1 μM), since calmodulin is saturated by [Ca] below 1 μM. However, Kong et al. (368) showed that IP_{3}-induced Ca release in colonic myocytes activated SK. Their activation has been suggested to underlie the hyperpolarization and relaxation associated with inhibitory purinergic input on these cells. The SK channels are also responsible for the hyperpolarization in response to release of ATP from enteric inhibitory motor-neurons (360). More studies are required before it is known if this is common to other smooth muscles.

When mice were produced in which SK3 was absent, no aberrant phenotype was observed (68). However, overexpression of SK3 unexpectedly led to difficulties in breathing and parturition. Labor was prolonged and described as dystocic. These data suggest SK channel down-regulation may be required for parturition and labor. Modzelewsk et al. (485) subsequently showed that apamin inhibits NO-induced relaxation of the myometrium, but did not affect spontaneous contractions. In bladder, inhibition of SK3 channels has been associated with instability (257).

The IK channel, also known as KCNN4, K3.1, and formerly SK4 (527, 747), is the product of different genes from SK1-3 and exhibits only 40% amino acid sequence identity (40). The IK have conductances of 50–70 pS (718). These channels are blocked by TRAM-34 and clotrimazole, are sensitive to charybdotoxin but insensitive to apamin, and are not time or voltage dependent (506). It is now considered that IK channels are the Gardos channels of erythrocytes, responsible for their high K permeability (269).

In the endothelium, IK channels contribute to endothelial-derived hyperpolarizing factor (EDHF)-mediated responses. Deletion of IK channels attenuates ACh-induced hyperpolarization of endothelial cells and vascular myocytes (637; see also Ref. 179). Blood pressure was elevated in the IK knockout mice in agreement with a role for EDHF in its regulation (81).

The IK channel may be associated with proliferating arterial and airway smooth muscle (510, 628). The latter authors showed IK expression in human airway and suggest that their upregulation plays a role in disease. The mRNA for IK channels has also been found in human trachea (628). It is likely that IK channels will contribute to setting the membrane potential relatively negative in the cells that express them. As mentioned already, IK channels in endothelial cells can lead to hyperpolarization of vascular myocytes. In human pulmonary artery, IK expression and current have been shown, but these are likely to come from endothelial cells not myocytes (301, 564). Thus, although there is a physiological importance
of IK channels to smooth muscle, most of this appears to be due to indirect effects.

In summary, although there is now good evidence for SK channels playing an important role in control of excitability and contraction in some types of smooth muscles, the functional role of these channels in other types of smooth muscles is still not clear. The Ca signals that activate SK and IK channels in different types of smooth muscles are not well established. The situation is even more complex for blood vessels, which contain both endothelial and smooth muscle, both of which can express different sets of BK, SK, and IK channels. However, one can speculate that SK channels are expressed and functional in smooth muscle cells that are controlled by a burst of action potentials. Data obtained on intact urinary bladder myocytes suggest that SK channels are involved in control of the AHP which sets a "mini refractory period" between action potentials. During this period, voltage inactivation of Ca channels is prevented; thus the channels are available for generation of the next action potential. SK channels are well suited for this function, i.e., controlling AHP as they are more sensitive to [Ca] than BK and thus can be activated by a relatively small rise in cytoplasmic [Ca] produced by a brief action potential. To achieve a graded rise of force in bladder, uterus, and intestinal muscles, a burst of action potentials is required which produces Ca oscillations. This in turn will result in a gradual summation of force and the generation of the large synchronized contractions needed for voiding or parturition. This mechanism of generation of muscle "tone" in phasic action potential-generating smooth muscles has many advantages over muscle tone produced by sustained membrane potential depolarization. One can also speculate that Ca oscillations are well suited to the generation of gradually rising force induced by changing the frequency of action potentials. Smooth muscles controlled by single action potentials, such as ureter, appear not to express SK channels. Instead, a Ca sparks/STOCs coupling mechanism operates, setting the refractory period to ensure that there is no summation of the action potential, which in the case of the ureter would impair the flow of urine and lead to kidney damage.

D. Ca-Activated Cl Channels

Chloride channels sensitive to Ca are a second population of Ca-sensitive plasma membrane ion channels that respond to both local (Ca sparks or puffs) or global (Ca spikes, waves, and oscillations) SR Ca release events. They are encoded by a family of genes and found in many smooth muscles (84, 163, 226). In the majority of smooth muscle cells, electrochemical driving force for Cl ($E_{Cl}$) is in the range of −20 to −30 mV, i.e., more positive than the resting membrane potential, which is in the range of −50 to −60 mV. Thus the $E_{Cl}$ is outwardly directed, and activation of Cl currents will result in diffusion of Cl out of the cell, causing membrane depolarization, i.e., an increase in the excitability of smooth muscle cells (321). Ca-activated Cl channel currents activated by agonist-induced global Ca signals from IP$_3$-sensitive Ca stores have been identified in a number of smooth muscles including coronary artery (355), anococcygeus muscle (97, 98), portal vein (539), pulmonary artery (737), esophagus (4), trachea (310), intestine (717), myometrium (17), and rat ureter (625). In some types of smooth muscle, Ca sparks were shown to activate Cl channels to promote membrane depolarization and contraction (369, 810). STICs (810) were first observed in myocytes from trachea (309) and portal vein (386, 736) and have subsequently been reported in mesenteric vein (716), trachea (251, 308, 810), and pulmonary artery (270, 369), suggesting that a Ca sparks/STIC coupling mechanism serves a specific functional role in these types of smooth muscles. The properties of STICs are consistent with Ca sparks causing the transient activation of Cl channels in the plasma membrane, as they are abolished by chloride channel blockers and are inhibited by the depletion of SR Ca stores (251, 386, 736, 810).

The coexistence of Cl and BK channels in the sarcolemmal membrane adjacent to Ca spark sites suggests another level of fine control of membrane excitability. In tracheal smooth muscle cells, STICs have been demonstrated to be activated by Ca sparks in conjunction with STOCs, leading to generation of biphasic STOCs (369, 810). There are some notable differences in the properties of STICs and STOCs. The decay of STICs ($t_{1/2} = 65–182$ ms, Ref. 810) is similar to the decay of Ca sparks (810) and is much slower than the decay of STOCs ($t_{1/2} = 9–43$ ms, Ref. 810). The amplitudes of STICs and STOCs are similar with similar driving forces for Cl and K even though the unitary conductance of the BK channel (80 pS) is ~30-fold greater than the Cl channel (2.6 pS) (369)). Therefore, a STIC appears to represent the activation by a Ca spark of at least 600 clustered Cl channels. The activation characteristics and Ca sensitivities of BK channels and Cl channels are also different. BK channels are both Ca and voltage sensitive, and depolarization increases their sensitivity to [Ca] (107, 134, 136, 438, 508). The Cl channels appear to have a higher affinity for Ca (half-maximal activation at ~370 nM, Ref. 538) than BK channels and are less voltage sensitive.

The precise effect on membrane potential of the simultaneous activation of Cl and BK channels by a Ca spark will depend on a number of factors. These include the poor voltage dependence of Cl channels and high voltage dependence of BK channels whose $P_{0}$ changes about e-fold for a 12- to 14-mV change in potential (42, 383) and their conductances in the cell membrane. Thus, at negative potentials (−70 to −60 mV), the predominant effect
of Ca spark discharge will be to trigger STICs (810), since at these potentials the electrochemical driving force for Cl will be greater than that for K. The discharge of STICs will cause membrane depolarization and thus serve as a positive-feedback mechanism to trigger the activation of voltage-gated Ca channels and excitation of the myocytes. As depolarization increases the frequency of Ca sparks and STOCs, sensitivity of BK channels to Ca and the electrochemical driving force for K will also be increased. This will make BK more active and result in the generation of high-amplitude STOCs and hyperpolarization. This would lead to closure of voltage-gated Ca channels and relaxation of smooth muscle, as was reported for pressurized cerebral arteries (507). High sensitivity of ClCa to [Ca] and low sensitivity to voltage make them ideal ion channels to be targeted by agonists to elicit excitatory effects on smooth muscle cells. Unlike STICs, the whole cell ClCa currents decay much faster than global Ca transients as the inactivation of ClCa current is determined not by intracellular [Ca], but by phosphorylation of the channel by CAM kinase II (739). Therefore, the decay of a STIC appears to be determined by the decline of Ca sparks, whereas the decay of the whole cell ClCa currents is controlled by CAM kinase II activity.

To summarize these data, we conclude that the expression of a range of different ion channels enables muscles to perform diverse functions in the human body. Recent data clearly indicate a role of the SR in the Ca signaling that controls smooth muscle function, via coupling Ca release from the SR to activation of ion channels. Discovery of Ca sparks, waves, and oscillations that can target several Ca-regulated channels (BK, SK, and IK) can produce positive or negative feedback controlling contractility. For example, some tonic contractions, which can be achieved via sustained membrane depolarization or discharge of bursts of action potentials, can be effectively decreased via activation of a Ca sparks/STOCs coupling mechanism. BK channels coupled to Ca sparks may produce long refractory periods, whereas SK channels targeted by Ca entry may be associated with short refractory periods, as discussed above. Still other mechanisms may be present in different smooth muscles, and a better understanding of them would aid understanding of the control of their contractility under normal and pathological conditions.

XI. SARCOPLASMIC RETICULUM AND DEVELOPMENT AND AGING

In the nervous system and heart there is a relatively large amount of literature on the role of Ca homeostasis and the SR, and a growing understanding of their importance to aging, development, and pathology. Less is known for smooth muscle, but there are some data specific to fetal and/or neonatal smooth muscles and aging tissues and their SR, and this will be briefly presented. Perhaps given the diversity of expression in Ca release channels between different adult smooth muscles, it should not be expected that a uniform thesis can be presented. As some papers have examined both development and aging, these data will be discussed together as appropriate. As changes in [Ca] are considered to be important for gene expression and pathological conditions in very many tissues, their role in development and aging in smooth muscles would seem to be worthy of study. It should also be acknowledged that changes involving the SR may be direct, e.g., SERCA expression, or indirect, e.g., due to changes in Ca buffering or to protein-coupled receptor sensitivity and IP₃ production. Other changes with development, e.g., development of force (502, 613), or Ca sensitivity (682), are not considered here.

Although a complete data set is not available for any smooth muscle, it would seem reasonable to conclude that a change in the role of the SR occurs with the transition from fetal and neonatal life to adulthood. There is evidence for this at both structural and biochemical levels, as well as from physiological studies. Care has to be taken in the interpretation of these data as it is not always possible to distinguish SR from ER, and thus to establish if the greater ER protein production in the neonates contributes to these differences. In addition, not all studies have been able to report directly on either intracellular or luminal [Ca]. Consequently, although changes in SR Ca uptake and release and Ca pool size can explain the data, effects on processes such as Ca sensitization or excitability cannot always be excluded. Nevertheless, a greater contribution of SR to smooth muscle function in the neonate has been identified in a number of instances, including uterus, bladder, and some blood vessels.

Following examples in other tissues, where aging has been linked to dysfunctions in Ca homeostasis and the SR (409, 570, 616), studies in smooth muscles have also pointed to alterations in Ca signaling (448, 593, 779). From this review of the data, it is apparent that cytoplasmatic [Ca] can rise with aging in smooth muscles. This in turn may be attributed to a decline in SERCA activity, although more studies on this point are required. This rise in resting [Ca] will produce overcontraction and contribute to dysfunctions associated with aging such as hypertension and incontinence urge.

XII. GENDER

The effects of female hormones on the vasculature and other smooth muscles are well recognized. While a discussion of these interesting differences is beyond the...
scope of this review, a little can be said about the SR and gender.

The earliest and still best documented effects of gender on SR function come from studies on cardiac muscle, including human myocardium (119, 144, 365, 388, 547). SERCA levels are depressed in failing hearts, as is phospholamban phosphorylation. The latter characteristic, however, was only found in male hearts. Men have a greater incidence of coronary heart disease and have augmented myocardial ischemic reperfusion injury, which has been related to increased SR Ca load (“overload”) (119). In smooth muscle, the few studies we have located have been on vascular smooth muscle. The findings of lower blood pressure in women than men, and the demonstration of male rats in hypertensive strains developing a greater elevation of blood pressure and with earlier onset than females, can be partly attributed to estrogen. There is an estrogen-dependent, protective enhanced release of NO in female endothelial cells (112). The cardiac studies mentioned above would also point to mechanisms directly present in the vascular myocytes and involving the SR, as well as other aspects of Ca homeostasis. In addition, female hypertensive rats still exhibit an enhanced vasodilatory response in the absence of endothelium, compared with males (328).

Aortic cells isolated from male hypertensive rats proliferated more rapidly than those from female rats (28). There were no differences in resting [Ca] between the cells, but a more pronounced increase in [Ca] to angiotensin II occurred in males (411). As the authors noted, this could indicate a greater ability of agonist to mobilize Ca from the SR or release threshold in males. In a follow up study, intracellular [Ca] was measured and the augmented Ca response to angiotensin II in males confirmed. However, SERCA inhibition with thapsigargin produced a much larger [Ca] rise in females (328). There were no differences in resting [Ca] between male and female hypertensive rats (328). Thus a role for increased Ca entry rather than SR release, in males, would appear more likely (412). Gender differences in Ca entry pathways have previously been demonstrated (496). This study also found no evidence for gender-based differences in SR Ca release. Effects of estrogens on SERCA expression in smooth muscles have been suggested from a few studies (see sect. mE).

It seems to us that there is little direct evidence thus far for effects of gender on Ca handling and SR function in smooth muscle cells. It is, however, premature to decide whether this is due to a lack of significant effects or the small amount of data currently available. Given the high incidence and morbidity of cardiovascular disease, it would be fruitful to have more information from smooth muscles on these issues.

**XIII. pH, METABOLISM, AND SARCOPLASMIC RETICULUM FUNCTION**

**A. Introduction**

That changes in pH can affect smooth muscle function has long been known (645). These changes can be intracellular (pHi), occurring for example with changes of metabolism and activity, or extracellular (pHo), consequent to changes in perfusion or systemic pH disturbance, for example (768). The mechanisms by which a change in pHi or pHo can affect E-C coupling in smooth muscle are numerous. Our focus is on SR Ca release processes and SERCA activity. For details on pH and smooth muscles, see the following reviews (22, 732, 772). Changes in pH are closely associated with metabolic activity and accompanied by changes in ATP and Pi concentrations, which as we discuss will also influence SR function.

**B. pH**

Release through RyR has been shown to be pH sensitive, with alkalization increasing and acidification decreasing the sensitivity of the release channels to Ca (151, 513, 763). In a skinned portal vein preparation (286), reduction in pH increased CICR, suggesting in this simplified system, where bathing [Ca] is maintained constant, that the RyR are either open for longer or have an increased P_o as pH falls. In intact preparations, this effect may be masked, as other processes involved in Ca homeostasis are affected. In a recent study in intact rat aortic preparations, it was concluded that acidic pH induces Ca release from RyRs and contributes to the rate of rise of contraction (594). Studies on cardiac SR microsomes and lipid bilayers have shown that a fall in pH lowers the P_o of the RyR (e.g., Refs. 602, 782). In isolated cells, Ca sparks are reduced, which is also consistent with a fall in RyR P_o (30). In pressurized cerebral arteries, Heppner et al. (253) showed that a small alkalinization, from pH 7.4 to 7.5, increased the frequency of Ca sparks and, upon raising it further to pH 7.6, caused a shift from sparks to Ca waves in the cells and vasoconstriction. These effects were blocked by ryanodine, but not inhibitors of IP_3R. The IP_3R, however, is also pH sensitive (71, 146, 232, 709); alkalization increases the sensitivity of IIcR. These effects may be due to changes of pH affecting IP_3 binding to the receptor (497), or IP_3 production (8). Alkalization will also increase the P_o of IP_3R (324, 767). Changes of pH were also reported to be more potent when acting at IP_3R3 rather than IP_3R1 (146). Further data are required to confirm isoform pH sensitivity.

As noted in section m, protons are translocated during SERCA activity from lumen to cytoplasm (395, 785) and may contribute to stabilization of SERCA when its Ca
binding sites are vacant (700). The proton countertransport has been reported to stop around luminal pH 8.0 (551). Thus it may be anticipated that pH will affect SERCA Ca pumping. Grover’s group have studied the effects of pH alteration on SERCA activity in a variety of smooth muscle preparations and concluded that alkalization decreased its activity and acidification increased it (165, 166, 228). As a decrease in pH inhibits kinase activity, then phosphorylation of phospholamban will be reduced, which in turn will produce a decrease in SERCA activity (606). Stimulation of SOCE, by SR Ca depletion, produced, which in turn will produce a decrease in SERCA activity in smooth muscles and concluded that alkalinization decreased its activity and acidification increased it (165, 166, 228).

Thus, although sometimes overlooked as investigators focus on ion channels and changes in myofilament Ca sensitivity, effects of pH alterations on Ca release and SERCA activity in smooth muscles will be significant factors in functional changes.

C. Metabolism

Here we address how metabolic changes such as ATP, phosphocreatine (PCr), and Pi levels affect SERCA activity. Most methods of determining SERCA activity couple its cycling to ATP hydrolysis via measurements of NADH, with the rate of NADH disappearance being proportional to the rate of ATP hydrolysis by SERCA (414). Other approaches target the production of Pi by SERCA activity in colorimetric-based assays (e.g., Ref. 109). In a recent paper, Williams et al. (762) described an HPLC-based method that also measured ATP, ADP, and PCr. The SERCA activity is calculated from the changes in ATP and ADP. The authors were also able to confirm that the PCr is used locally via creatine kinase to synthesize local ATP for SERCA (244, 366). Thus functional coupling and localization between PCr, creatine kinase, and SERCA occurred. This specific binding of creatine kinase to SERCA had previously been reported (e.g., Refs. 367, 598). As PCr levels are much lower in smooth compared with striated muscles (385, 659, 773), this positioning of creatine kinase by the SERCA membrane may be particularly useful in smooth muscles. Local metabolic control of SERCA may be advantageous during conditions of metabolic stress, in maintaining SERCA function and preventing cytoplasmic [Ca] from becoming too high.

Investigators have suggested, based on the location of glycolytic enzymes, that there is also a coupling between glycolysis and SERCA activity (168, 780). In smooth muscle, a large body of work, particularly from Lorenz and Paul (410), has led to the theory of metabolic compartmentation. Although beyond the scope of this review, there is good evidence that ATP, provided by oxidative phosphorylation, supports contraction, whereas that from glycolysis supports membrane pumps in smooth muscle (246, 295, 420, 421). There is also a much greater lactate production and use of glycolysis under normoxic conditions in smooth muscle compared with many other cell types (422, 770). During hypoxia or ischemia, effects of pH and changes in metabolites on SERCA activity will be expected to contribute to contractile dysfunction (683).

XIV. MATHEMATICAL MODELING

Given the accepted complexities of SR Ca uptake and release events, and their reciprocal effect on cytoplasmic [Ca], plasmalemmal ion channels, and other organelles such as the mitochondria, there is merit in developing models of E-C coupling in smooth muscle cells. One considerable obstacle to the use of such models is the lack of quantitative data for many of the essential parameters, e.g., SR [Ca]. Even setting aside experimental differences such as temperature, species, and stimulation mode, for some smooth muscles, e.g., urethra and anococcygeus, many necessary measurements are simply not available; others such as vascular and uterine smooth muscles have been better studied.

There are several mathematical models of various aspects of E-C coupling in smooth muscle now available [e.g., airway, (376), mesenteric artery (335), cerebral artery (786)]. Other models have addressed more general questions, such as superficial buffer barrier (37), slow waves (289), protein interactions (171), and IP3 changes (176). The use of such models could provide significant insight into the workings of the SR by taking an integrated approach. As such models become more detailed and SR focussed, and the “missing” parameters are experimentally determined, then a better overall description and understanding of the SR in different smooth muscles should emerge.

XV. CONCLUSIONS AND FUTURE DIRECTIONS

The SR is vital to the modulation and regulation of force in smooth muscles. Pathophysologies are associated with perturbation of SERCA, Ca release mechanisms, and regulatory proteins, but our appreciation of this lags behind studies on striated muscles. This can largely be attributed to the lack of clarity around the regulation of normal SR function in smooth muscle, which in turn is affected by genuine differences between tissues. It is uncommon for investigators to address more than one smooth muscle in their studies, leading to pockets of detailed information in specific areas, but a lack of breadth. There are areas where real progress has been made, such as unraveling the Ca spark-STOC mechanism of regulating excitability and function. Other areas remain controversial, such as the role of TRPCs in store-operated
Ca entry. A key question remaining to be answered is whether in smooth muscle SR Ca depletion leads to $I_{\text{crac}}$ only through the Stim-Orai pathway. Another key question is how important SOCE is to those smooth muscles whose main source of Ca for contraction is L-type Ca entry; need such mechanisms be required if SERCA can refill from the plasmalemmal Ca entry? More measurements of SR luminal Ca are also required, as they could provide important mechanistic insight.

Another area of unfinished business is the role of the SR in shaping Ca signals in smooth muscles. With Ca-activated ion channels that can increase or decrease membrane excitability being expressed in the same myocyte, elucidation of the role of local SR Ca release to global Ca signals is difficult. We have suggested that consideration of the pattern of excitation, i.e., single short-lasting or plateau-type action potential or trains of action potentials, may dictate the involvement or otherwise of the SR with BK, IK, and SK and Cl$_{\text{ca}}$ channels playing a role. Imaging and molecular biology techniques have supported the views expressed over 30 years ago that there will be microdomains of Ca and signaling moieties within the myocyte. The SR Ca release channels and Ca-activated ion channels form part of the underlying mechanism, with both the plasma membrane and SR membrane having signaling microdomains.

The existence of different SR Ca stores, which may or may not completely intercommunicate, also leads to specialization within smooth muscles. Figure 5 illustrates some features of the store. This complexity of Ca signaling in smooth muscle (and other tissues) is added to by the growing evidence that other organelles, including mitochondria, lysosomes, Golgi, and nucleus, interact with the SR (ER). Furthermore, along with Ca and IP$_3$ as Ca release agents, newer messenger/modulators, including NAADP, cADPR, and possibly the L-type Ca channel, need to be brought into the mix. Presumably the right mix of messenger(s) and Ca store(s), along with the right place and time, are how the myocyte decodes the Ca stimulus.

With time, the SR will give up more of its secrets. This requires laboratories applying a range of techniques, including functional, electrophysiological, pharmacological, and molecular biological studies. We are encouraged that the increased use of intact tissues rather than cultured cells for molecular biology, along with the success of nonviral transfection methods, will result in much new data which are physiologically relevant and not dogged by concerns about the phenotypic changes occurring with culturing. Ultimately, an improved understanding of the SR of smooth muscles will contribute to the development of new therapeutic approaches to tackle diseases. Given the pivotal role of smooth muscle to all the major systems of the body, this is a worthwhile goal.

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