Pharmacological Modulation of Sarcoplasmic Reticulum Function in Smooth Muscle

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Abstract—The sarcoplasmic reticulum (SR/ER) is the primary storage and release site of intracellular calcium (Ca\(^{2+}\)) in many excitable cells. The SR is a tubular network, which in smooth muscle (SM) cells distributes close to cellular periphery (superficial SR) and in deeper aspects of the cell (deep SR). Recent attention has focused on the regulation of cell function by the superficial SR, which can act as a buffer and also as a regulator of membrane channels and transporters. Ca\(^{2+}\) is released from the SR via two types of ionic channels [ryanodine- and inositol 1,4,5-trisphosphate-gated], whereas accumulation from the
cytoplasm occurs exclusively by an energy-dependent sarco-endoplasmic reticulum Ca\textsuperscript{2+}-ATPase pump (SERCA). Within the SR, Ca\textsuperscript{2+} is bound to various storage proteins. Emerging evidence also suggests that the perinuclear portion of the SR may play an important role in nuclear transcription. In this review, we detail the pharmacology of agents that alter the functions of Ca\textsuperscript{2+} release channels and of SERCA. We describe their use and selectivity and indicate the concentrations used in investigating various SM preparations.

I. Smooth Muscle Cell Ca\textsuperscript{2+} Handling and Role of the Sarccoplasmic Reticulum

A. Ca\textsuperscript{2+} Handling

The most abundant cation in the vertebrate body is calcium (Ca\textsuperscript{2+}), where in humans it amounts to 20 to 30 g/kg body weight. There are large reservoirs of Ca\textsuperscript{2+} in the form of depots in bone, which are available to the body for cellular processes. Despite the availability of such a large, extracellular source of Ca\textsuperscript{2+}, cells have an organized internal store of Ca\textsuperscript{2+} that is readily available for rapid release as needed upon membrane excitation. The identification and universal acceptance of the SR as a Ca\textsuperscript{2+} store and sink is a relatively late event in smooth muscle research and begs the question of its utility in establishing a maintained cellular response in the face of a very large, inwardly facing gradient of accessible Ca\textsuperscript{2+} that is of infinite abundance relative to enzymatic requirements.

1Abbreviations: SR, sarcoplasmic reticulum; [Ca\textsuperscript{2+}]\text{c}yt, concentration of cytoplasmic free Ca\textsuperscript{2+}; ER, endoplasmic reticulum; SERCA, sarco/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase(s); InsP\textsubscript{3}, inositol 1,4,5-trisphosphate; InsP\textsubscript{4}, InsP\textsubscript{3}-gated Ca\textsuperscript{2+} channel/InsP\textsubscript{3} receptor; ER, endoplasmic reticulum; PM, plasma membrane; RyR, Ca\textsuperscript{2+}-gated channel/ryanodine receptor(s); PMCA, plasma membrane Ca\textsuperscript{2+}-ATPase pump(s); HEK, human embryonic kidney; CICR, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release; SBB, superficial buffer barrier; IICR, inositol 1,4,5-trisphosphate-induced Ca\textsuperscript{2+} release; InsP\textsubscript{3}R, InsP\textsubscript{3}-gated Ca\textsuperscript{2+} channel/InsP\textsubscript{3} receptor; CCE, capacitative calcium entry; SK&F, 1-[(3-(4-methoxy-phenyl)propoxy)-4-methoxyphenethyl]-1-diethylami-

As Pozzan et al. (1994) and others have argued, the diffusion of Ca\textsuperscript{2+} within the cell is not an unimpeded process, so the presence of various immobile binding sites for Ca\textsuperscript{2+} imposes severe constraints on its ability to reach a rapid and useful cellular concentration at critical sites within the cell, especially in the case of cells with relatively large volumes. The intracellular diffusion coefficient for Ca\textsuperscript{2+} in water is 7 × 10\textsuperscript{-6} cm\textsuperscript{2}/s, which is more than 10 times that in cytoplasmic extracts containing intracellular Ca\textsuperscript{2+} buffers (Allbritton et al., 1992). This impediment to the movement of Ca\textsuperscript{2+}, coupled with the presence of intracellular structures close to the plasma membrane, ensures local areas of high Ca\textsuperscript{2+} concentrations, which in turn may lead to a regenerative release of Ca\textsuperscript{2+} from the SR. In careful mathematical modeling of Ca\textsuperscript{2+} movement in cells using physiological constraints, Kargacin (1994) calculated that the concentration of Ca\textsuperscript{2+} below the plasma membrane reaches ~8 μM during the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) process.

The release of Ca\textsuperscript{2+} from internal stores ensures a more diffuse and timely increase in cytoplasmic Ca\textsuperscript{2+} that is coordinated with the ensuing entry of extracellular Ca\textsuperscript{2+}. Through such an intricate dependence of the timely movement of Ca\textsuperscript{2+} from within the cell and from extracellular sources, the cell is able to initiate Ca\textsuperscript{2+} oscillators that act as regional “switches” and “relays” within cytoplasmic domains (Bootman et al., 2002). For instance, in the smooth muscle cell, Ca\textsuperscript{2+}-mediated contractile regulation by extracellular Ca\textsuperscript{2+} influx and/or SR Ca\textsuperscript{2+} release could take place principally by two mechanisms. The first is by direct activation of the contractile apparatus through global cytoplasmic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\text{cyt}) increase throughout the cytoplasm (Sanders, 2001). A second pathway is via indirect regulation of plasma membrane excitability by an increase in [Ca\textsuperscript{2+}]\text{cyt} spatially localized to a narrow gap (20–40 nm in depth) between the plasma membrane and the superficially located SR, termed the plasma membrane-SR junctional space (Lee et al., 2002a) (see Section I.A.) (Fig. 1). [Ca\textsuperscript{2+}]\text{cyt} increase in this junctional space may also be the source of cell-wide [Ca\textsuperscript{2+}]\text{cyt} oscillations and waves coupled either to contractile activity or relaxation (Pabelick et al., 2001b; Lee et al., 2002a).
The SR has many structural features of the endoplasmic reticulum (ER), sharing many intracellular chaperone proteins and other histological features. The existence of a SERCA and of Ca\(^{2+}\) release channels reveals that the SR is, in fact, a region of the ER that specializes in Ca\(^{2+}\) homeostasis, predominantly Ca\(^{2+}\) release and uptake. Thus, the SR is in reality an intracellular networking system whereby different cytoplasmic domains are connected in an intercommunicating and independent manner. In reality, the SR and ER are constantly multitasking—some portions of the structures are involved in protein trafficking, while other portions are being earmarked for Ca\(^{2+}\) signaling (Berridge, 2002). These portions of the SR/ER are constantly remodeling, with this process being driven by stability of the proteins and the Ca\(^{2+}\) load (Berridge, 2002). The SR is also contiguous with the nuclear envelope and thus may play a role in Ca\(^{2+}\)-dependent gene regulation via Ca\(^{2+}\)-dependent transcription factors such as cAMP responsive element-binding proteins (CREB) and nuclear factor for activated T cells (NFAT) (Cartin et al., 2000; Gomez et al., 2003; Hill-Eubanks et al., 2003). InsP\(_3\)R are also found in the SR membrane and may regulate the opening of the nuclear pores (Stehno-Bittel et al., 1995).

The release and accumulation of intracellular Ca\(^{2+}\) regulates many aspects of cellular functions, including hormone and neurotransmitter release, endothelial secretion, muscle contractile activity, cell division, growth and migration, and apoptosis. It is, in fact, difficult to imagine an aspect of mammalian cell function that is not in some way regulated by the availability of Ca\(^{2+}\). With this in mind, it becomes apparent why there has been a burgeoning global interest in understanding how cells regulate all aspects of Ca\(^{2+}\) availability. Our interest in cellular Ca\(^{2+}\) handling owes much to the seminal observations of Ringer in 1883, who first demonstrated the absolute need for Ca\(^{2+}\) in muscle excitation-contraction activity coupling. The impetus for the search for drugs that modify the cellular responses to Ca\(^{2+}\) comes largely from the need to treat a variety of diseases thought to involve abnormal handling of Ca\(^{2+}\). Thus, an extensive array of pharmacological tools altering Ca\(^{2+}\) entry, release, sensitization, and extrusion has been created. In this review, we summarize the pharmacology of drugs frequently used to modulate intracellular Ca\(^{2+}\) sequestration and release in smooth muscle.

Smooth muscle cells are spindle-shaped, with the widest part being \(\sim 2 \text{ to } 5 \mu m\), and the length ranging up to 500 \(\mu m\) in visceral muscle and to \(\sim 150 \mu m\) in vascular tissues. The plasma membrane and SR come into close contact with the SR membrane running parallel with the plasma membrane for distances of 1 \(\mu m\) or more (Devine et al., 1972; Gabella, 1983). The geometrical shape of smooth muscle cells ensures a disproportionate ratio of membrane (PM, sarcoplasmic membrane) surface/cytoplasm (cytosol, sarcomoplasm) ratio. For example, visceral smooth muscle cells have a volume of 2500 to 3000 \(\mu m^3\) and a cell surface area of 5000 \(\mu m^2\) (not allowing for caveolae), producing an approximated cell surface/volume ratio of 1.5 \(\mu m^{-1}\), which is equivalent to that of erythrocytes. For a comprehensive review of the ultrastructural features of smooth muscle, see the overview by Gabella (1983).

In vascular smooth muscle cells, there are \(2.7 \mu m^2\) of cell surface for every cubic micrometer of cell volume. This large cell surface/cell volume ratio in smooth muscle favors exquisite regulation of cell surface processes by instantaneous changes in intracellular composition, such as the regulation by (presumably) spontaneously released Ca\(^{2+}\) from the SR, known as “Ca\(^{2+}\) sparks”, of Ca\(^{2+}\)-activated K\(^+\) (K\(_{Ca}\)) (see reviews by Nelson et al., 1995; Jaggar et al., 2000; Wellman and Nelson, 2003) and chloride (Cl\(_{Ca}\)) channels (Kotlikoff and Wang, 1998). Earlier studies by Benham and Bolton (1986) and by Stehno-Bittel and Sturek (1992) lead to the suggestion that the frequently observed spontaneous K\(^+\) currents recorded in smooth muscle (Benham and Bolton, 1986; Ohyya et al., 1987; Desilets et al., 1989; Hume and Leblanc, 1989) occur in regions of the cell where the SR and the PM are closely apposed. This Ca\(^{2+}\) release from the SR, presumed to occur spontaneously, occurs in close proximity to K\(_{Ca}\) channels and reaches \([Ca^{2+}]_{cyt}\) of 10 to 100 \(\mu M\) and an average size of 13 \(\mu m^2\), which covers \(\sim 1\%\) of the 1300 \(\mu m^2\) of the smooth muscle membrane (Perez et al., 1999). This release of Ca\(^{2+}\) occurs via Ca\(^{2+}\)-gated channel/ryanodine receptor (RyR) channels, likely the RyR2 subtype with an ancillary role for RyR3 (Lohn et al., 2001). However, the global change in cytoplasmic Ca\(^{2+}\) due to the spontaneous release of Ca\(^{2+}\) sparks from the SR is less than 2 nM (Jaggar et al., 2000). Ca\(^{2+}\) sparks originating from the SR occur with a relatively low frequency of 1 Hz (allowing for a tonic
hyperpolarization throughout the electrically coupled smooth muscle), and the spreading distance in smooth muscle is ~1.0 to 2.5 μm (Jaggar et al., 2000). Thus, there is the appearance of a specialized subsarcolemmal signaling space where high local concentrations of Ca\textsuperscript{2+} (~10 μM) exist in microdomains without significant impact on global cytosolic Ca\textsuperscript{2+}.

B. Sarcoplasmic Reticulum

Unlike other membrane systems, such as the mitochondrial inner membrane, there is no potential difference across the SR membrane. The concentration of Ca\textsuperscript{2+} in the extracellular space is between 1 and 10 mM, whereas the [Ca\textsuperscript{2+}]\textsubscript{cyt} in the cytoplasm is in the order of 0.1 μM, thus creating a large inwardly directed electrochemical gradient forcing Ca\textsuperscript{2+} entry across the plasma membrane. The cell has several mechanisms for maintaining a low [Ca\textsuperscript{2+}]\textsubscript{cyt}, which at the same time also ensures that the appropriate transient peak levels of Ca\textsuperscript{2+} are reached during activation. Notable among these are active processes such as Ca\textsuperscript{2+} extrusion across the plasma membrane into the extracellular reservoir by the plasma membrane Ca\textsuperscript{2+}-ATPase pump (PMCA) and also accumulation of the ion into the SR by the SERCA. Although these two pumps essentially accomplish the same effect of rapidly reducing [Ca\textsuperscript{2+}]\textsubscript{cyt} levels, they have different physicochemical properties and regulatory mechanisms (Grover and Khan, 1992; Raeymaekers and Wuytack, 1993). Using indirect methods in a large vein, Nazer and van Breemen (1998) concluded that nearly half the cytoplasmic free Ca\textsuperscript{2+} load is extruded via the PMCA, with an equal role for the SERCA and the plasma membrane Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in removal of the remainder. Much is known about the molecular identity of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in smooth muscle (Nakasaki et al., 1993; Juhaszova et al., 1996), although details of its isoform distribution and functionality awaits further characterization.

Some investigators have suggested that the plasma membrane pathways (PMCA and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger) account for only 20 to 40% of Ca\textsuperscript{2+} removal (Cooney et al., 1991), whereas others have concluded that the PMCA removes only ~10 to 20% of Ca\textsuperscript{2+} from the cell (Kargacin and Fay, 1991). However, in a resistance artery from the brain, Kamishima and McCarron (1998) proposed roles only for the Ca-ATPase pumps (SERCA and PMCA), and not the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, in removal of free Ca\textsuperscript{2+} from the cytoplasm. In keeping with this, Kargacin and Kargacin (1995) proposed that the SERCA pumps are likely to make the largest single contribution to Ca\textsuperscript{2+} removal and can reduce Ca\textsuperscript{2+} at a rate of ~60 to 80% the rate of Ca\textsuperscript{2+} removal seen in cells during a Ca\textsuperscript{2+} transient. Thus, there exists some uncertainty about the precise roles and relative importance of the extrusion mechanisms for cytoplasmic Ca\textsuperscript{2+}. In part, this is related to the lack of specific inhibitors of the various processes that govern Ca\textsuperscript{2+} homeostasis. It is likely that many of the quantitative estimates described in the literature regarding Ca\textsuperscript{2+} pool sizes, diffusion rates, and other physicochemical parameters exhibit variations in estimates due largely to differences in methods, species, and tissues.

An important initial step in elucidating the contribution and role of the various modalities of Ca\textsuperscript{2+} extrusion mechanisms is the description of caloxin, a peptide that inhibits Ca\textsuperscript{2+} extrusion by the PMCA (Chaudhary et al., 2001; Holmes et al., 2003). The search for a selective and specific inhibitor for the PMCA is considered by many to be enigmatic; the limitation of caloxin [IC\textsubscript{50} value ~0.4–1 mM in red cell leaky ghosts, which mainly express PMCA4 (Holmes et al., 2003)] is that it is a peptide and thus not amenable to routine use. However, it produces an endothelium-dependent relaxation in intact rings of rat aorta (0.34 mM) (Chaudhary et al., 2001). Caloxin does not inhibit Mg\textsuperscript{2+}-ATPase or Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (Holmes et al., 2003) and has been shown to also inhibit PMCA activity in human mesenchymal stem cells (Kawano et al., 2003) and human embryonic kidney (HEK) cells (De Luisi and Hofer, 2003). A third and relatively novel family of Ca\textsuperscript{2+} pumps is the Ca\textsuperscript{2+}/Mn\textsuperscript{2+}-ATPases, which occur predominantly in the Golgi compartment of eukaryotic cells (reviewed by Wuytack et al., 2002, 2003).

The rise of intracellular Ca\textsuperscript{2+} is vital to cell function, but Ca\textsuperscript{2+} availability must occur rapidly and in sufficient concentration at required intracellular targets. This is accomplished by Ca\textsuperscript{2+} release from the SR either as a regenerative release of Ca\textsuperscript{2+} via CICR occurring through activation of RyR, or as InsP\textsubscript{3}-induced Ca\textsuperscript{2+} release (ICCR, occurring through activation of InsP\textsubscript{3}/Ca\textsuperscript{2+} release channel) (see Section I.G.2.). The RyR was originally shown to bind [\textsuperscript{3}H]ryanodine (see Section II.B.3.a.), an agent that was then known to alter SR Ca\textsuperscript{2+} release events in skeletal muscle. Within the SR, a fraction of Ca\textsuperscript{2+} is bound to various Ca\textsuperscript{2+} storage proteins such as calsequestrin. (see Section I.E.). Once released, the diffusion rate of Ca\textsuperscript{2+} in the cytoplasm is limited by the presence of various high-affinity binding proteins, so that the rate of Ca\textsuperscript{2+} diffusion in the cytoplasm (10–100 μm/s) is ~3 to 30% the rate in free solution, which occurs at 320 μm/s (Tsien and Tsien, 1990).

The description of an intracellular network of membrane systems that was later to be named SR was first described in skeletal muscle more than 100 years ago (Veratti, 1902). It was appreciated that muscle had an intrinsic “relaxing activity”, which was later ascribed to the ability of SR membranes to accumulate Ca\textsuperscript{2+} at the expense of ATP hydrolysis (Ebashi and Lipmann, 1962). The SR is a system of anastomosing intracellular membranes organized into tubules that occupy between 1.5 to 3% of the cell volume, with greater volumes being present for instance in smooth muscle cells from large, conduit type arteries (Devine et al., 1972). This distri-
bution of SR and its implications in arteries of varying sizes has not been revisited since the initial description by Devine et al.; it is likely that these estimates may be revised with the use of more modern techniques and the patterns of SR distribution better defined with regard to cell structure and colocalization with cellular elements. This represents a daunting task since we now know that the complex architecture of the SR is made functionally complicated by the presence of regions that specialize in Ca$^{2+}$ uptake and release, with other adjoining parts involved in the assembly or degradation of proteins. The variable volume of SR in smooth muscle cells may also be a reflection of cell synthetic activity, so that large arteries, which synthesize more extracellular proteins than smaller diameter arteries, have a correspondingly larger SR volume (Somlyo, 1980), although this supposition has not been rigorously examined. Also, the SR volume of smooth muscle cells, at least in the uterus, is increased by estrogen and during pregnancy (Shoenberg, 1958; Ross and Klebanoff, 1971). Thus, proliferative, developing smooth muscle cells tend to have more SR (Campbell et al., 1971), as is the case for some injured and hypertensive vascular smooth muscle cells (Raeymaekers and Wuytack, 1993).

The SR contains several ionic species and of note is the following: 1) the Na$^+$ and Cl$^-$ concentrations are similar to those present in the cytoplasm, indicating that the SR is not effectively in contact with the extracellular space, 2) the Ca$^{2+}$ concentration in the SR measured by electron probe analysis is \(\sim 30\) to 50 mmol/kg dry weight, and 3) the Ca$^{2+}$ concentration near the plasma membrane is not uniformly distributed, with areas of low (\(\sim 1\) mmol/kg dry weight) and high ("hot spots") concentrations. There is a concordance of junctional elements of the SR (those portions close to the plasma membrane) and areas of Ca$^{2+}$ hot spots. It is estimated that the average number of SR elements indicated by hot spots that lie within 50 nm from the plasma membrane is between 3 and 5 per cell (Bond et al., 1984b). One consequence of this is that sufficiently high local concentrations (in mM) of Ca$^{2+}$ are reached near the plasma membrane, allowing for local, intracellular regulation of Ca$^{2+}$ entry (Huang et al., 1989) in smooth muscle.

That the SR acts as a sink for Ca$^{2+}$, i.e., can actively accumulate Ca$^{2+}$, was initially demonstrated by Somlyo and Somlyo (1971). These investigators took advantage of the fact that the SR can accumulate Sr$^{2+}$ and Ca$^{2+}$ by the same transport mechanisms so that the SR became loaded to a greater extent with Sr$^{2+}$ after incubation in a depolarizing solution. Based on the use of electron probe analysis, Somlyo and Somlyo (1971) estimated that the Ca$^{2+}$ content of the central SR increases 3- to 4-fold following depolarization of smooth muscle. When Casteels and Droogmans (1981) used $^{44}$Ca$^{2+}$ to determine the content of the SR (junctional, central), they estimated it to be 60 \(\mu\)M/kg wet weight. Using the same technique, they also showed that 1) the maximal rate of filling of Ca$^{2+}$ under normal conditions of external ionic composition (1.5 mM Ca$^{2+}$) is nearly 70 \(\mu\)mol/kg, and 2) the permeability of Ca$^{2+}$ across the plasma membrane is regulated by the extent of filling of the agonist-sensitive pool of SR Ca$^{2+}$ (Casteels and Droogmans, 1981). This was the forerunner of what would later be described as "capacitative Ca$^{2+}$ entry", as described by Putney (1986).

C. Capacitative Ca$^{2+}$ Entry

In addition to functioning as a store and a sink for Ca$^{2+}$, the SR is also at the origin of two cell signaling processes: 1) the SR generates Ca$^{2+}$ sparks that, in smooth muscle cells, regulate the plasma membrane electrical potential through modulation of K$_{Ca}$ channels (Nelson et al., 1995; Jaggar et al., 2000), and 2) the content of the SR determines entry of extracellular Ca$^{2+}$ through "store-operated calcium entry" (Casteels and Droogmans, 1981) or "capacitative calcium entry (CCE)" (Putney, 1986; Putney et al., 2001). Thus, both Ca$^{2+}$ sparks and CCE act to regulate Ca$^{2+}$ entry, albeit through separate but related mechanisms.

The designation of CCE is by analogy with an electrical capacitor; a charged or full intracellular store prevents Ca$^{2+}$ entry through this pathway, whereas a discharged or empty store facilitates Ca$^{2+}$ entry and refilling of the store. The CCE model thus proposes that when the SR store is stimulated to discharge Ca$^{2+}$, either by InsP$_3$-generating signal such as stimulation of G-protein coupled (G$_{i}/$G$_{11}$-) receptors or by receptor/InsP$_3$-independent means, such as through SERCA inhibition, there is a fall in the Ca$^{2+}$ content of the SR, which then signals a novel Ca$^{2+}$ pathway on the plasma membrane so that refilling of the store can occur. The refilling is rapid and allows for a constant internal store of Ca$^{2+}$ to be available for smooth muscle oscillations and maintained tone. The pharmacology of CCE is in its infancy, with agents of generally low specificity being used (Putney et al., 2001). Notable among these are \([\text{Gd}^{3+}, <1; \text{econazole, 2–10; miconazole, 1.0; flufenamic acid, 33; eicosatetraynoic acid, 4.0; SK&F 96365, 3–50; 2-aminoethoxydiphenylborate (2-APB), 30; LU52396, 2.0; and L-651,582, 1.2 (see Putney et al., 2001 for complete list). The lack of a specific inhibitor stems from a paucity of information regarding two key aspects of the model for CCE: 1) the molecular nature of the signaling between a depleted SR and the plasma membrane, and 2) the specific details of the membrane events (ion channels, receptor mechanisms) activated during the process. Notwithstanding these limitations, there has been progress made in unraveling details regarding CCE, and a clearer picture is starting to emerge, at least in some cell types.

In isolated portal vein smooth muscle cells, norepinephrine (NE) causes a transient increase in Ca$^{2+}$ due to SR activation; this is rapidly followed by a more
sustained increase in Ca\(^{2+}\) due to entry from extracellular sources (Pacaud et al., 1993). In rat aorta, inhibition of SERCA with low concentrations (1 \(\mu\)M) of cyclopiazonic acid (CPA) increases [Ca\(^{2+}\)]\(_{\text{cyt}}\) without causing contractile activity, suggesting that increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\) in noncontractile compartments are being affected by CPA (Tosun et al., 1998). Low concentrations of CPA induce increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\) that are similar to those produced by KCl, but unlike the latter, fail to induce contraction. On the other hand, higher concentrations of CPA (10–20 \(\mu\)M) appear to increase [Ca\(^{2+}\)]\(_{\text{cyt}}\) beyond the noncontractile compartment. Of interest is that the increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\) produced by low concentrations of CPA is insensitive to L-type voltage-gated Ca\(^{2+}\) channel (Ca\(_{\text{L}}\)) blockers, such as verapamil, but was inhibited by Ni\(^{2+}\) (Tosun et al., 1998). Vasodilation in rabbit and mouse aorta by nitric oxide (NO) has been linked to inhibition of CCE, possibly by the effect of NO in rapidly filling the SR store via stimulation of SERCA (Cohen et al., 1999).

A unique but poorly characterized current, Ca\(^{2+}\) release-activated Ca\(^{2+}\) current (\(\text{I}_{\text{CRAC}}\)), characterizes the depletion-activated entry of Ca\(^{2+}\). Hoth and Penner (1993) first identified a Ca\(^{2+}\) current activated by store depletion (in mast cells) and several aspects of this current were noted: It had a very low conductance (~0.02 pS), high Ca\(^{2+}\) selectivity, inward rectification, inhibition by intracellular Ca\(^{2+}\), blockade by Ni\(^{2+}\) and Cd\(^{2+}\), and lack of voltage-dependent gating (Hoth and Penner, 1993). A current with similar characteristics has also been reported in the mouse anococcygeus muscle, where it is termed \(I_{\text{DOC}}\) and has a unitary conductance of ~10 pS (Wayman et al., 1998). CCE has been described in a number of smooth muscles (Ito et al., 2000; Weirich et al., 2001; Young et al., 2001); and these findings have been reviewed recently (Gibson et al., 1998; Albert and Davis, 1996). There is some evidence to support the notion that store depletion activates tyrosine kinases to signal Ca\(^{2+}\) entry in a number of cell types including smooth muscle (Pacaud and Bolton, 1991a; Wijetunge et al., 1992; Doi et al., 2000) and endothelial cells (Jacob, 1990; Sharma and Davis, 1996). In rat aortic smooth muscle cells, depletion of the SR with thapsigargin or bradykinin stimulates phospholipase D to generate phosphatidic acid, which enhances sustained Ca\(^{2+}\) entry (Walter et al., 2000). Pharmacological evidence in support of CCE in maintaining the basal tone in resistance arteries has been documented in the rat cremaster arterioles (Potocnik and Hill, 2001). Whereas the CCE was not altered by disruption of the cytoskeleton in cremaster arterioles (Potocnik and Hill, 2001), there is marked inhibition of CCE when actin filaments are disrupted in endothelial cells (Bishara et al., 2002). Although the molecular identity of the channel responsible for CCE remains elusive, there is persuasive evidence that it has considerable homology with Drosophila transient receptor potential channel (TRP) proteins, which are involved in phototransduction in the fruit fly where they mediate CCE. TRP channels are a large family (at least 20 genes) of plasma membrane, nonselective cationic channels. This latter feature of being nonselective makes them depolarizing agents, and their Ca\(^{2+}\) permeability suggests a role in intracellular Ca\(^{2+}\) signaling. The TRP channels have six transmembrane segments and are activated by products of G-protein coupled receptor (GPCR) stimulation. The properties of TRP have recently been reviewed (Zhu and Birnbaumer, 1998; Nilius, 2003). TRP shares sequence homology with voltage- and second messenger-gated Ca\(^{2+}\) channels, and this homology has a long evolutionary history, being also present in Caenorhabditis elegans. However, a concern is that TRP channels are not highly selective for Ca\(^{2+}\), and the available evidence indicates that none of the TRP proteins induce a conductance with the known properties of CCE (Vennekens et al., 2002). The TRP(C3) channel may be directly linked to InsP\(_3\)R activation, whereby InsP\(_3\)R activation leads to entry of extracellular Ca\(^{2+}\) (Boulay et al., 1999). It is likely then that CCE is not mediated by a single TRP protein but is possibly occurring through a channel formed by a multimeric structure containing various combinations of TRP and TRP-like proteins (Montell, 1997). The vagaries of the CCE have found detractors who present evidence that key elements of store-operated Ca\(^{2+}\) entry are incompatible with generating oscillatory [Ca\(^{2+}\)]\(_{\text{cyt}}\) signals (Shuttleworth, 1999) or that depletion of the internal stores for Ca\(^{2+}\) does not always lead to CCE (Haller et al., 1996).

D. Sarcoplasmic Reticulum Fractions and Interaction with Mitochondria

Popescu and Diculescu (1975) segregated smooth muscle SR into three regions: peripheral, deep, and central. In general, the peripheral SR is located close to the plasma membrane and sometimes in apposition with the caveolae (equivalent to junctional SR described above). This SR element is in contact with the deep SR positioned near the myofilaments and is in continuity with the central SR deeper within the cell and associated with the nuclear membrane (Forbes et al., 1977, 1979). Notable details of the analogy of smooth muscle SR to that of striated muscle have been painstakingly pointed out by Forbes et al. (1979) who described as “peripheral SR” the collection of sacules, tubules, and cisternae lying in close apposition (gap of 10–20 nm) to the inner plasmalemmal surface. One functional implication of the peripheral SR is that it dampens the impact of the basal Ca\(^{2+}\) entry by acting as a “superficial buffer barrier” (SBB), which then reinforces this buffering capacity by causing a vectorial extrusion of Ca\(^{2+}\) to the extracellular space (van Breemen et al., 1995) (Fig. 2). Another implication of the close apposition of the SR and plasma membrane is that sufficiently high concentra-
tions of local are reached to activate the Na⁺-Ca²⁺ exchanger and also to activate spontaneous outward K⁺ currents. This was recorded by Tomita and Bulbring (1969) and later visualized and described in detail by Nelson et al. (1995).

The concept of the SBB as proposed by van Breemen (see van Breemen et al., 1995) is being challenged. Using more direct approaches, McCarron’s data suggests a different model of SR-plasma membrane arrangement that essentially argues against the SBB model. In elegant experiments, Bradley et al. (2002) made two important findings: first, that Ca²⁺ accumulation by the SR proceeds even when Ca²⁺ influx ceases, implying that for this to be possible, the SR and plasma membrane need not necessarily be closely apposed; and second, close apposition of the SR and plasma membrane is instead essential for Ca²⁺ removal by the Na⁺-Ca²⁺ exchanger. Using an array of biophysical constants related to Ca²⁺ diffusion characteristics and microdomain constraints, Kargacin’s group present intriguing data suggesting that the rate of Ca²⁺ uptake by the SR is insufficient to significantly alter the dynamics of a Ca²⁺ transient, either in its magnitude or spread (Bazzazi et al., 2003). Immunofluorescence imaging reveals no obvious differences in the density of Ca²⁺ pumps or phospholamban between the peripheral (superficial) SR and deep (central) SR.

In addition to the distinct distribution patterns of SR within the cell, there is also some evidence that SR fractions represent heterogeneous compartments of releasable Ca²⁺. In this regard, Golovina and Blaustein (1997) reported that, in mesenteric arteries, there are two or possibly three functionally distinct compartments: a region (~55%) that empties and refills in a InsP₃- and RyR-independent manner (e.g., not affected by CPA and thapsigargin) and a second region (~22%) that empties and fills only in response to caffeine (RyR pool), with other regions (~16%) being responsive to CPA and caffeine. Consistent with this, there is a portion of the SR that appears to be more richly endowed with InsP₃R than RyR (Wibo and Godfraind, 1994). Other studies propose two stores of Ca²⁺ that are distinguished by their Ca²⁺ refilling sources: the store that expresses only RyR is filled by cytoplasmic Ca²⁺, whereas the store that expresses both RyR and InsP₃R is filled by extracellular Ca²⁺ (Flynn et al., 2001). In contrast to these studies, Itoh et al. (1983) provided compelling evidence that the Ca²⁺ stores associated with IICR (NE-induced) and CICR (caffeine-induced) are identical; additional support for this also comes from

![Fig. 2. Preferential sequestration by the peripheral SR of the extracellular Ca²⁺ entering the cell in the restricted subsarcolemmal space as proposed in the superficial buffer barrier model. 1, extracellular Ca²⁺ enters the vascular smooth muscle cells through voltage-dependent or -independent Ca²⁺ channels. 2, it accumulates in the restricted subsarcolemmal space. 3, it is taken up by the peripheral SR through its SERCA. Sarcolemma = plasma membrane, subsarcolemmal = subplasmalemmal, myoplasmic = cytoplasmic.](image-url)
the findings of Leijten and van Breemen (1984) and more recently from Bradley et al. (2002), who depleted caffeine-sensitive Ca\(^{2+}\) stores through release with flash photolysis of caged InsP\(_3\).

It has been known for some time that mitochondria frequently envelop the central and peripheral portions of the SR (Forbes et al., 1979). Furthermore, mitochondria isolated from smooth muscle cells actively sequester Ca\(^{2+}\), as demonstrated by Somlyo and Somlyo (1971). The mitochondria are also, in many instances, located near the caveolae (Somlyo, 1975; Forbes et al., 1979), and this raises the possibility of transfer of ions between these three structures. Some direct evidence for an important regulatory role of mitochondria on intracellular Ca\(^{2+}\) homeostasis were provided by Loew et al. (1994), Drummond and Tuft (1999), and McCarron and Muir (1999). Keeping with this role for mitochondrial interaction with the SR and plasma membrane with regard to Ca\(^{2+}\) cycling are the recent findings of Kamishima and Quayle (2002) and Szado et al. (2003). Evidence in freshly dissociated vascular (Drummond and Tuft, 1999; Gurney et al., 2000) and visceral (McCarron and Muir, 1999) smooth muscle cells also suggest a functional integration between SR Ca\(^{2+}\) release and mitochondrial Ca\(^{2+}\) uptake under physiological conditions. For instance, membrane potential, and thus Ca\(^{2+}\) entry, can be regulated by mitochondrial Ca\(^{2+}\) accumulation, since mitochondrial Ca\(^{2+}\) uptake has recently been shown to modulate Ca\(^{2+}\) spark activity in isolated rat cerebral artery smooth muscle cells (Cherenov and Jaggar, 2004). Furthermore, new evidence from rat ventricular cardiac muscle cells (permeabilized cells, microsomes, and RyR2 reconstituted into planar lipid bilayers) demonstrated that physiological concentrations of NADH inhibit CICR and that NADH oxidation, likely right at the mitochondrial level, is tightly linked to and essential for this effect. This suggests that it is an important physiological negative feedback mechanism, coupling SR Ca\(^{2+}\) fluxes and mitochondrial energy production (Cherednichenko et al., 2004). In contrast, there appears to be no role for mitochondria in Ca\(^{2+}\) decay following agonist activation, as shown in rat myometrial smooth muscle cells (Shmigol et al., 1999). The dependence of Ca\(^{2+}\) entry from the extracellular space on the status of ER Ca\(^{2+}\) content and the influence of the mitochondria on this interaction (Parekh, 2003), as well as the transmission of InsP\(_3\)-generated Ca\(^{2+}\) signals to the mitochondria, have recently been reviewed (Hajnoczky et al., 2000a,b, 2002; Pacher et al., 2000; Szalai et al., 2000; Csordas and Hajnoczky, 2001, 2003).

**E. Ca\(^{2+}\) Storage by the Sarcoplasmic Reticulum**

Once the SR accumulates Ca\(^{2+}\), it is loosely bound and available for release; however, the divalent cation does not exist in an ionized form, since this would lead to inhibition of the SERCA, as mentioned above. Hence, there exist within the SR storage proteins capable of binding large quantities of Ca\(^{2+}\) in a complex that is readily available for dissociation when Ca\(^{2+}\) release is triggered. The total concentration of intracellular Ca\(^{2+}\) buffers in smooth muscle cells is estimated to be \(\sim\)200 to 300 \(\mu\)M (Bond et al., 1984a; Carafoli, 1987; Allbritton et al., 1992) or 500 \(\mu\)M (Daub and Gantiukевич, 2000). The storage proteins that are expressed at the highest levels in the SR are calsequestrin and calreticulin. These proteins have high capacity (25–50 mol/mol) and low affinity (1–4 mM) for Ca\(^{2+}\). Calsequestrin is the product of two different genes that have a 65% homology; smooth muscle is thought to express both isoforms but in much reduced quantities compared with other muscle types (Pozzan et al., 1994). On the other hand, calreticulin exists in multiple isoforms. There may be some structural similarity between these two Ca\(^{2+}\)-binding proteins (calsequestrin and calreticulin), since there is antibody cross-reactivity. It is of note that once the SR accumulates Ca\(^{2+}\), there is preferential binding to proteins, such as calsequestrin, that are strategically located close to the Ca\(^{2+}\) release channels. In particular, calsequestrin is retained within the SR lumen by the presence of discrete string-like molecular anchors (Pozzan et al., 1994). In a detailed study of the vas deferens, Villa et al. (1993) provided intriguing evidence that the Ca\(^{2+}\) proteins are not uniformly distributed in the SR. They reported that the peripheral portion of SR is rich in calsequestrin (which, incidentally, is also enriched with InsP\(_3\)R), whereas calreticulin was more evenly distributed in the cell. This selective distribution of binding proteins is in agreement with the hot spots for Ca\(^{2+}\) stores reported earlier by Bond et al. (1984a). However, by combining immunogold labeling and immunohistochemical studies, Nixon et al. (1994) concluded that calsequestrin is absent in tonic muscles (aorta), although it was located in the superficial regions of cells from tonic smooth muscle (vas deferens), but the InsP\(_3\)R distribution was largely in the peripheral SR in both tissue types. There is some evidence that protein kinase C (PKC) can directly reduce the Ca\(^{2+}\) storage by thapsigargin-sensitive [i.e., involving SERCA (see Section II.A.1.b.)] mechanisms in cells from the rat aorta. This unloading of the SR content by PKC is suggested to be defective in hypertension (Neusser et al., 1993).

**F. Estimates of Ca\(^{2+}\) Content in the Sarcoplasmic Reticulum**

There is a large variation in the estimate of Ca\(^{2+}\) concentration within mammalian intracellular stores, ranging from 1 \(\mu\)M to 5 mM, with these values being subject to the limitations of the method used [e.g., aequorin measures free Ca\(^{2+}\) in the SR, whereas electron probe X-ray microanalysis measures total Ca\(^{2+}\)] and the cell type studied (Meldolesi and Pozzan, 1998). With the advent of more precise technology, other more direct measurements of the Ca\(^{2+}\) content of the SR have been made. For example, Gantiukевич and Hirche (1996) de-
terminated that the quantity of Ca\(^{2+}\) released by acetylcholine (Ach) is 680 attomoles in smooth muscle; this translates to \(\sim 230\) \(\mu\)moles of total Ca\(^{2+}\) per liter of cytoplasmic volume. Assuming that this released Ca\(^{2+}\) is derived only from the SR, then the SR content would be 7.5 mM per liter SR. In permeabilized smooth muscle cells where uptake of the SR was monitored with fura-2 acid, it is estimated that the SERCA pumps can remove Ca\(^{2+}\) at a rate that is 45 to 75% the rate at which Ca\(^{2+}\) is removed from the cytoplasm of intact cells during transient Ca\(^{2+}\) signals (Kargacin and Kargacin, 1995). Kargacin and Kargacin (1995) calculated that the SR of a single smooth muscle cell could store more than 10 times the amount of Ca\(^{2+}\) required to generate a single transient contractile response.

The use of fluorescent indicators has revolutionized the study and understanding of intracellular Ca\(^{2+}\) regulation and has also allowed a more accurate quantification of intraluminal free Ca\(^{2+}\) in smooth muscle SR. However, an important limitation of this technique is that frequently these dyes, particularly the acetoxymethoxy ester derivative-type dyes, are prone to compartmentalization in various other intracellular organelles as well (Takahashi et al., 1999). Thus, Hofer and Schulz (1996) have determined that, at least in fibroblasts, only 88% of the loaded low-affinity Ca\(^{2+}\) indicator mag-fura-2 (furaptra) (Martinez-Zaguilan et al., 1998) is restricted to the thapsigargin-sensitive ER. In fact, potential contribution of a confounding signal stemming from its accumulation in mitochondria must be kept in mind (Gurney et al., 2000) (see Section I.D. for discussion of functional significance). Despite this limitation, the indicator has been used to monitor SR free Ca\(^{2+}\) changes in several intact smooth muscle preparations (Hiromi and Iino, 1994; Sugiyama and Goldman, 1995; Steenbergen and Fay, 1996; Golovina and Blaustein, 1997; Gomez-Viquez et al., 2003), as has been mag-indo-1, another low-affinity Ca\(^{2+}\) indicator (Pesco et al., 2001). Indeed, although it is possible to use high-affinity Ca\(^{2+}\) indicators such as fluo-3 or fura-2, the use of these lower affinity Ca\(^{2+}\) indicators may be more suitable to monitor dynamic changes in Ca\(^{2+}\) stores. The use of aquorin targeted to the ER, as initially described Kendall et al. (1992), has been a significant but technically limiting advance (Alvarez and Montero, 2002) and has been used only in limited number of smooth muscle preparations (Szado et al., 2003).

**G. Ca\(^{2+}\) Uptake and Release by the Sarcoplasmic Reticulum**

It has been known for some time that there is a basal leak of Ca\(^{2+}\) from the cell: it is likely that under resting physiological conditions, all cells are exposed to some endogenous low-level stimulation by Ca\(^{2+}\)-releasing agents, of which there are many. This will undoubtedly cause a basal leak of Ca\(^{2+}\) from the SR, which has been confirmed in a number of tissues by tracking increases in cytoplasmic Ca\(^{2+}\) or decreases in SR Ca\(^{2+}\) content after maximal inhibition of SERCA with pharmacological tools. This basal leak in various cell types is in the range of 20 to 200 \(\mu\)M/min (Camello et al., 2002; Lomax et al., 2002), and it was shown to be 22% per min in cultured smooth muscle cells (Missiaen et al., 1996). It is thus clear that, in the majority of cell types, this leak is of sufficient magnitude to deplete the SR in a few minutes and forms the largest source of Ca\(^{2+}\) efflux from the SR (Missiaen et al., 1996). To offset it, SERCA act to increase SR luminal Ca\(^{2+}\) by removing Ca\(^{2+}\) from the cytoplasm. Interestingly, pumping activity does not lead to the buildup of a membrane potential across the SR membrane during Ca\(^{2+}\) uptake (neither does it occur during Ca\(^{2+}\) release) because of the coupling of an efflux of H\(^{+}\) to this uptake (Inesi and Hill, 1983). There is also evidence for an inward movement Cl\(^{-}\) (Pollock et al., 1998) helping to maintain SR electrical neutrality during Ca\(^{2+}\) uptake.

Thus, the SR performs several functions that are directly aided by the presence of SERCA: 1) it acts as a reservoir of releasable Ca\(^{2+}\) (the concentration of Ca\(^{2+}\) in the SR lumen is nearly three times greater than in the cytoplasm), 2) it buffers the Ca\(^{2+}\) leak into the cell that is driven by the steep electrochemical gradient according to the superficial buffer barrier hypothesis proposed by van Breemen and colleagues (1995), 3) it sequesters Ca\(^{2+}\) to facilitate smooth muscle relaxation, 4) it provides a pool of Ca\(^{2+}\) that is ideally located to activate hyperpolarizing currents via stimulation of K\(_{\text{Ca}}\) channels, although the spontaneous release of discreet amounts of Ca\(^{2+}\) termed Ca\(^{2+}\) sparks (Nelson et al., 1995), and 5) it provides Ca\(^{2+}\) from InsP\(_{3}\)-sensitive stores to activate Cl\(_{\text{Ca}}\) currents and therefore sustain regenerative changes in membrane potential in gastric muscle (Hirst, 1999; Hirst and Edwards, 2001; Hirst et al., 2002).

1. **Ca\(^{2+}\) Pump (Sarco/Endoplasmic Reticulum Ca\(^{2+}\)-ATPase)**. The pumping of Ca\(^{2+}\) by the SERCA, which belongs to the P-type ion pumps family, is a cycle of chemical reactions leading to a series of conformational states divided into two main groups, termed E\(_{1}\) and E\(_{2}\), which are based on a general model describing P-type ATPases activity (the E\(_{1}\)/E\(_{2}\) model) (Martonosi, 1996; Moller et al., 1996; Adebanjo et al., 1999; Lee, 2000). The E\(_{1}\) conformations have a high affinity for Ca\(^{2+}\) (\(K_{D} = 10^{-7}\) M) and can be phosphorylated by MgATP to form a high-energy phosphorylated intermediate, E\(_{1}\)P. Furthermore, their Ca\(^{2+}\)-binding sites are only accessible from the cytoplasm and not from the SR lumen. By contrast, the E\(_{2}\) conformations have a lower affinity for Ca\(^{2+}\) (\(K_{D} = 10^{-3}\) M) and can be phosphorylated by inorganic phosphate (P\(_{i}\)), in the absence of Ca\(^{2+}\), to form a low energy phosphorylated intermediate (E\(_{2}\)P), and their Ca\(^{2+}\)-binding sites are only accessible from the SR lumen and not from the cytoplasm.
The pumping cycle (Fig. 3) is thus initiated by the binding of two Ca\(^{2+}\) ions to high-affinity binding sites on the cytoplasmic surface of the SERCA that is in an E\(_1\) conformation with high-affinity Ca\(^{2+}\) binding sites (E\(_1\)[Ca\(_2\)]), which is followed by the binding of a molecule of ATP (MgATP-E\(_1\)-[Ca\(_2\)]. This leads to autophosphorylation of the enzyme and release of MgADP resulting in an E\(_1\)P-[Ca\(_2\)] intermediate. The energy released from the high-energy phosphate bond leads to major conformational changes, through hinge-type or sliding motions, affecting the Ca\(^{2+}\) binding sites: the conversion from the high-energy E\(_1\)P to the low-energy E\(_2\)P (E\(_2\)P-[Ca\(_2\)]. The Ca\(^{2+}\) ions are then released to the SR lumen from the now low-affinity Ca\(^{2+}\) binding sites (E\(_2\)P). The cycle is then terminated by hydrolysis of the bound phosphate (E\(_2\)), countertransport of H\(^+\) ions to maintain electroneutrality of the SR membrane (although this countertransport of H\(^+\) does not completely balance the charge carried by Ca\(^{2+}\), making the Ca\(^{2+}\)-ATPase electrogenic), and the conversion of E\(_2\) into E\(_1\) to reset the Ca\(^{2+}\) pumping cycle. Variants of this model have been proposed (Martonosi, 1996; Moller et al., 1996; Adelbanjo et al., 1999; Lee, 2000) as well as an alternative model not based on the E\(_1\)/E\(_2\) dichotomy (Jencks, 1992). As Ca\(^{2+}\) accumulates within the lumen, the rate of SERCA activity would be expected to slow down due to a negative feedback. To overcome this limitation, the SR is endowed with Ca\(^{2+}\) buffering proteins (see above). The stoichiometry of 1ATP:2Ca has been proven only for SERCA1; in tissues such as smooth muscle, there is only indirect evidence from Hill coefficients (Mg ATPase being 1 and 2 for Ca\(^{2+}\)) for SERCA pump activation (Grover and Samson, 1986).

The SERCA are 110-kDa proteins and are encoded by three genes: SERCA1 is largely expressed in fast-twitch skeletal muscle, with 1a in adult and 1b in neonatal muscle, SERCA2a is mainly in cardiac and slow-twitch muscle, and SERCA2b is mainly in smooth muscle and most nonmuscle cells, whereas SERCA3 is present on a more widespread basis (East, 2000; Sorrentino and Rizzuto, 2001). Smooth muscle mainly expresses the SERCA2b isoform (>70%), with the SERCA2a and SERCA3 isoforms forming the remainder of the SERCA population (Lytton et al., 1989; Wuytack et al., 1989; Eggermont et al., 1990; Amrani et al., 1995; Trepkova et al., 2000; Wu et al., 2001). Both isoforms are splice variants transcribed from the SERCA2 gene (Wu and Lytton, 1993). The SERCA2b has a higher affinity for Ca\(^{2+}\) than SERCA2a (Verboomen et al., 1992) and a lower turnover rate for both Ca\(^{2+}\) transport and ATP hydrolysis (Lytton et al., 1992). The maximal rate of Ca\(^{2+}\) uptake by smooth muscle SR (loaded with oxalate to create linear kinetics) is ~100 nmol/kg/min, which is lower (by two times) than in striated muscle (Raeymaekers, 1982; Raeymaekers and Jones, 1986). This reduced rate of Ca\(^{2+}\) uptake is most likely due to the reduced density of SERCA in smooth muscle (Wuytack et al., 1989).

The activity of SERCA is largely regulated by phospholamban, a small protein of 52 amino acids that forms a homopentamer and is present in the smooth muscle SR membranes. Although it was shown to be expressed in the porcine gastric smooth muscle (Raeymaekers and Jones, 1986) and rabbit aorta (Cornwell et al., 1991), there is a species and tissue variability in the amount of phospholamban expressed (Raeymaekers and Jones, 1986). There is also variability in the sensitivity of SERCA isoforms to this regulatory protein; although SERCA2b is regulated by both phospholamban and calmodulin kinase, SERCA3 is not regulated by either of these proteins. In addition, SERCA3 is more resistant to reactive oxygen species than SERCA2b (Grover and Samson, 1997).

Unphosphorylated phospholamban 1) lowers the apparent turnover rate (global V\(_{\text{max}}\) of SERCA2 through interactions with its cytoplasmic domain (likely by decreasing the Ca\(^{2+}\) transport portion of the cycle E\(_1\)P-[Ca\(_2\)] → ... → E\(_2\)P (Hughes et al., 1994, 1996) and 2) lowers its apparent affinity for Ca\(^{2+}\), without affecting the true chemical affinity, through interactions with its transmembrane domain (James et al., 1989; Sasaki et al., 1992a; Cantilina et al., 1993). Its phosphorylation causes its dissociation from SERCA (Tada, 1992) and increases SERCA apparent affinity for Ca\(^{2+}\) by reducing the activation energy for a slow transition triggered by Ca\(^{2+}\) binding in the Ca\(^{2+}\) pumping cycle (Fig. 3), making the pump more “reactive” to cytoplasmic Ca\(^{2+}\) (Cantilina et al., 1993).

When phospholamban is phosphorylated, e.g., by cAMP or Ca\(^{2+}\)/calmodulin kinase, SERCA activity is increased resulting in an enhanced uptake of Ca\(^{2+}\) by the SR, although it should be noted that the extent of cAMP-stimulated phospholamban activity is considerably lower than in cardiac tissue (Watras, 1988). Phospholamban is also an excellent substrate for PKG (Raeymaekers et al., 1988). cGMP is more effective in reducing cytoplasmic Ca\(^{2+}\) and is thus a potent mediator of
smooth muscle relaxation (Felbel et al., 1988). Indeed, it is likely involved in NO-induced SERCA activation; NO activation of guanylate cyclase would increase cGMP concentration, which would activate SR membrane-located PKG, which would then phosphorylate phospholamban (Felbel et al., 1988; Raeymaekers et al., 1988; Twort and van Breemen, 1989; Cornwell et al., 1991; Karczewski et al., 1992; Andriantsitohaina et al., 1995).

Several groups have also described a novel means of activation of SERCA by direct phosphorylation by a Ca\(^{2+}\)-calmodulin kinase (CaM kinase)—for example in heart (Xu et al., 1993), HEK cells (Toyofuku et al., 1994), skeletal muscle (Hawkins et al., 1994), and coronary arteries (Grover et al., 1996).

2. Ca\(^{2+}\) Release Channels. As mentioned earlier, release of Ca\(^{2+}\) from the SR can occur via a basal leak that is removed from the cytoplasm via a concerted action of mitochondrial uptake, SERCA activity, and plasma membrane extrusion through the Na\(^{+}\)-Ca\(^{2+}\) exchanger and PMCA. The most physiologically relevant SR Ca\(^{2+}\) release, however, occurs through activation of InsP\(_3\)R and RyR. Interestingly, in many smooth muscle preparations, the SR Ca\(^{2+}\) pools released through these channels overlap (Missiaen et al., 1992b). Furthermore, there could be functional interactions between SERCA and the SR Ca\(^{2+}\) release channels (Gomez-Vquez et al., 2003).

There are three genes that encode InsP\(_3\)R and also three genes that encode RyR, each generating a specific isoform. The sequences encoded by InsP\(_3\)R and RyR genes house a basic similarity in structure, sharing fragmental amino acid residue homology concentrated in the ligand-binding and Ca\(^{2+}\) channel domains, implying fundamental roles of these domains in the activity of the SR Ca\(^{2+}\) channels (Yoshida and Imai, 1997) and suggesting a common ancestral history (Sorrentino and Rizzuto, 2001). Each of these release channels is indeed configured in as a tetrameric formation, with the RyR being homotetrameric, the InsP\(_3\)R being heterotetrameric, and the molecular weights of purified InsP\(_3\)R (500 kDa) and RyR (300 kDa) indicating large protein structures (Furuichi et al., 1989, 1994). However, despite the above molecular similarities, the three-dimensional structure at 24-Å resolution of these two classes of channels is quite different (Jiang et al., 2002) (see the two following sections).

Local accessory proteins tailor the functional properties of these channels within particular cells and subcellular domains (Mackrill et al., 1996). Some of these proteins modulate activity of all SR Ca\(^{2+}\) release channels, whereas others have class- or even isoform-specific effects. Some proteins exert both direct and indirect regulation, sometimes with opposing effects, whereas others are themselves modulated by \([\text{Ca}\(^{2+}\)\] \(_{\text{cyt}}\) changes, thus being part of feedback loops.

a. Ca\(^{2+}\)-Gated Channel/Ryanodine Receptor. The RyR channel is activated when surrounding \([\text{Ca}\(^{2+}\)\] \(_{\text{cyt}}\) increases sufficiently to trigger CICR (Fig. 4) (see below). The RyR is a homotetrameric protein approximately 2 MDa in molecular weight. In mammalian tissues (including smooth muscle), it forms a family of three isoforms, RyR1, RyR2, and RyR3, each encoded by a distinct gene, either \(\text{ryr1}\) (initially cloned and sequenced from skeletal muscle), \(\text{ryr2}\) (initially cloned and sequenced from cardiac muscle), and \(\text{ryr3}\) (initially cloned and sequenced from the brain) (Sutko et al., 1997). Two alternatively splice variants of RyR1 and one variant of RyR2 have also been identified. RyR knockout mice have also been developed; in mice lacking RyR3, caffeine and NE maintain their contractile effects (Yamazawa et al., 1996), whereas Ca\(^{2+}\) activity is significantly increased (Lohn et al., 2001). RyR2 knockout is lethal due to cardiac malformation (Takehisa et al., 1998). An interesting approach was used by Drega et al. (2001) to minimize the function of RyR: Using organ culture techniques (4 days, 10–100 \(\mu\)M ryanodine), RyR protein was recovered but RyR were nonfunctional. An interesting finding of this study is that although intracellular stores recover following chronic ryanodine treatment, RyR activity is essential for Ca\(^{2+}\)-spark activity but not for Ca\(^{2+}\) waves/oscillations (Dreja et al., 2001).

Expression patterns of RyR subtypes show variable distribution, with RyR1 and RyR2 being present in skeletal muscle and cardiac muscle, respectively; RyR2 is the predominant isoform in neural tissue. RyR3 is expressed mainly in embryonic tissue, and levels decrease during development (Rossi and Sorrentino, 2002). In smooth muscle cells, RyR2 and RyR3 are the primary isoforms (Sanders, 2001). Interestingly, although all three RyR isoforms are present in vascular smooth muscle of neonatal mice, where the SR content appears normal, these RyR do not become fully functional until further maturity of the animals (Gollasch et al., 1998). There is also a regional variability in the expression combination of the three RyR isoforms in smooth muscle from different organs. RyR2 is required for generation of Ca\(^{2+}\) sparks, with either a minimal (Mironneau et al., 2001) or inhibitory (Lohn et al., 2001) contribution for RyR3.

The channel is an assembly of four RyR subunits (protomers) of the same isoform (thus an homotetramer) forming a central Ca\(^{2+}\)-conducting pore, which has a diameter of 2 to 3 nm. RyR1 and RyR3 have been shown to differ significantly in vitro in terms of gating and activation. Topologically, various studies suggest that the RyR spans the membrane 4, 10, or 12 times (Michikawa et al., 1996), with highly conserved ion-channel-forming membrane-spanning regions that appear to be localized to the carboxyl terminal (20% of the protein), while the remaining amino-terminal region of the protein forms a large cytoplasmic foot domain that assumes a quatrefoil shape (Sutko et al., 1997; Welch et al., 1997; Wagenknecht and Samso, 2002). The RyR is anchored to the SR by interaction with the Ca\(^{2+}\) binding storage protein calsequestrin (see Section I.E.).
The distribution pattern of RyR follows that of the SR such that in where there is a patch distribution of SR in the cytoplasm, as in the guinea pig aorta, there is a sparse labeling with ryanodine markers, whereas tissues with a more prominent peripheral SR have a rich marking of RyR in the periphery—in other words, RyR distribution parallels that of the SR (Lesh et al., 1998). The physiological roles of RyR in smooth muscle cells are still being elucidated (Guerrero-Hernandez et al., 2002). Although its endogenous gating ligand is Ca\(^{2+}\)/H\(^{100}\), leading to CICR, as mentioned above, it appears that its basal Ca\(^{2+}\)/H\(^{100}\) sensitivity is in the micromolar range, a [Ca\(^{2+}\)]\(_{\text{cyt}}\) not globally reached in the bulk of the cytoplasm (Sanders, 2001). Hence, RyR appears to be activated in proximity to the plasma membrane (e.g., on the SR face of the plasma membrane-SR junctional space) by extracellular Ca\(^{2+}\) influx through Ca\(_{\text{in}}\), to produce CICR of Ca\(^{2+}\) sparks, which in turn modulates plasma membrane excitability (e.g., hyperpolarization) through activation of small conductance K\(_{\text{Ca}}\) channels (SK), large conductance K\(_{\text{Ca}}\) (BK), and depolarization through Cl\(_{\text{Ca}}\) (Jaggar et al., 2000; Sanders, 2001). In cerebral artery smooth muscle cells, for instance, Ca\(^{2+}\) sparks-activated BK openings promote relaxation (Nelson et al., 1995). There may be a role, however, for regional vascular differences with regard to the role of Ca\(^{2+}\) sparks. For example, spontaneous transient outward currents (STOC), and hence Ca\(^{2+}\) sparks, are very active in fetal pulmonary arteries, and this activity diminishes with maturation (Pratusevich and Balke, 1996; Jaggar et al., 2000). This contrasts completely with the nearly 100-fold increase in Ca\(^{2+}\) spark activity and STOC frequency during maturation of systemic arterial cells (Gollasch et al., 1998). Thus, in pulmonary artery cells, endothelin, the potent endogenous vasoconstrictor, activates Ca\(^{2+}\) sparks by causing the cross-signaling of RyR and InsP\(_3\)R—in this case, increased smooth muscle Ca\(^{2+}\) activity is associated with constriction (Ge et al., 2003; Zhang et al., 2003). Another example of the physiological relevance of Ca\(^{2+}\) sparks is the finding that stretching of urinary bladder smooth muscle cells generates Ca\(^{2+}\) sparks from RyR sites on the SR (Ji et al., 2002).

The finer details of the organization of microdomains that underlie Ca\(^{2+}\) sparks are being unraveled with
insightful experimental analysis. It is now apparent that KCa channels that underpin STOC are exposed to a mean Ca\(^{2+}\) concentration on the order of 10 \(\mu\)M during a Ca\(^{2+}\) spark (ZhuGe et al., 2002). The membrane area over which a concentration of 10 \(\mu\)M or more (range 12–21 \(\mu\)M) is achieved has an estimated radius of 15 to 30 nm, corresponding to an area that is a fraction of one square micron (0.07–0.28 \(\mu\)m\(^2\)). It is also apparent that KCa channels are not uniformly distributed over the membrane but exist as clusters at sites of frequent discharge of Ca\(^{2+}\) sparks, where the KCa channels and RyR mediating Ca\(^{2+}\) sparks is in the order of 25 nM (ZhuGe et al., 2000, 2002). Indirect support for an intimate relationship between K\(_{\text{Ca}}\) channels and RyR comes from findings in smooth muscle-excised patches of portal vein (Xiong et al., 1992) and vas deferens (Ohi et al., 2001b), where there is evidence for clustering of K\(_{\text{Ca}}\) channels and an apparent attachment of SR membrane.

An emerging role for RyR is the development of hypertension, where Ca\(^{2+}\) spark regulation of BK channels is altered. Although the pore-forming \(\alpha\) subunit is conserved and ubiquitously expressed, there are four distinct \(\beta\) regulatory subunits (Nelson and Quayle, 1995). The \(\beta_1\) subunit is selectively expressed in smooth muscle. In a recent study of angiotensin I-induced hypertension, expression of the \(\beta_1\) subunit (and not the \(\alpha\) subunit) was shown to be reduced, thus uncoupling K\(_{\text{Ca}}\) channels from Ca\(^{2+}\) sparks from the RyR (Amberg et al., 2003). The reduced efficacy of vascular K\(_{\text{Ca}}\) channels is associated with greater depolarization and increased vascular tone (Amberg et al., 2003).

In contrast, CICR from RyR at SR locations further away from the plasma membrane could propagate Ca\(^{2+}\) waves that induce contractile activity (Collier et al., 2000). For instance, CICR could be triggered by IICR (Fig. 5) (see below), such as in the IICR-triggered and CICR-propagated Ca\(^{2+}\) waves induced by NE in portal vein (Boittin et al., 1999) and inferior vena cava (Lee et al., 2002b) smooth muscle cells. CICR could also be triggered by the spatially restricted [Ca\(^{2+}\)\(_{\text{cyt}}\)]\(\text{cyt}\) bursts induced by the newly identified Ca\(^{2+}\) releaser nicotinic acid adenine dinucleotide phosphate, a pyridine nucleotide derived from \(\beta\)-NADP\(^{+}\), such as in the nicotinic acid adenine dinucleotide phosphate-triggered and CICR-propagated global Ca\(^{2+}\) wave and contractile activity in pulmonary artery smooth muscle cells (Boittin et al., 2002).

Smooth muscle CICR is a function of the net flux of Ca\(^{2+}\) ions into the cytoplasm rather than the single channel amplitude of Ca\(_{\text{L}}\). Unlike CICR in cardiac muscle, RyR channel opening in smooth muscle is not tightly linked to the gating of Ca\(_{\text{L}}\), and unlike CICR in striated muscle, Ca\(^{2+}\) release is completely eliminated by cytoplasmic Ca\(^{2+}\) buffering. Thus, Ca\(_{\text{L}}\) is loosely coupled to RyR through an increase in global [Ca\(^{2+}\)\(_{\text{cyt}}\)]\(\text{cyt}\) due to an increase in the effective distance between Ca\(_{\text{L}}\) and RyR compared with striated muscle.

The RyR sensitivity (hence CICR sensitivity) to Ca\(^{2+}\) could be increased by the endogenous cyclic ADP ribose (the only endogenous RyR agonist identified so far) (see Section II.B.1.), although its sensitivity could also be altered by many other endogenous factors (Ca\(^{2+}\), Mg\(^{2+}\), H\(^+\), adenine nucleotide, calmodulin, and protein kinases A and C (PKC) (Rossi and Sorrentino, 2002). As CICR itself, this alteration of RyR sensitivity could promote either relaxation or contractile activity depending on the circumstances. For instance, it could relax through Ca\(^{2+}\) sparks generation, such as in \(\beta\)-adrenoreceptor-activated (Boittin et al., 2003) or resting (Cheung, 2003) arterial smooth muscle. In contrast, this phenomenon could induce (i.e., without requiring extracellular Ca\(^{2+}\) influx) or amplify (i.e., by augmenting an initial [Ca\(^{2+}\)\(_{\text{cyt}}\)]\(\text{cyt}\) rise mediated by Ca\(^{2+}\) influx) contractile activity through global [Ca\(^{2+}\)\(_{\text{cyt}}\)]\(\text{cyt}\) increases. Examples of this are the Ca\(^{2+}\)-influx-independent M\(_{\text{1}}\) muscarinic receptor-induced coronary artery smooth muscle contractile activity (Ge et al., 2003) and the Ca\(^{2+}\)-influx-triggered cholecystokinin-A receptor-induced longitudinal intestinal smooth muscle contractile activity (Kuemmerle and Makhlof, 1995).

b. Inositol 1,4,5-Trisphosphate-Gated Channel/Inositol 1,4,5-Trisphosphate Receptor. The InP\(_{\text{3}}\)R family is formed in mammalian tissues of at least three isoforms, type 1 (InP\(_{\text{3}}\)R1), type 2 (InP\(_{\text{3}}\)R2), and type 3 (InP\(_{\text{3}}\)R3), each encoded by a distinct gene and sharing 60 to 70% amino acid residue homology (Wilcox et al., 1993; Nakanode et al., 1994; Michikawa et al., 1996; Yoshida and Imai, 1997; Taylor, 1998; Patel et al., 1999; Yule, 2001; Jiang et al., 2002). These isoforms, each ~300 kDa, share the same structural and functional organization (Yoshida and Imai, 1997; Wilcox et al., 1998): a bulbous ligand-binding domain in the N-terminal portion (~24% of the whole receptor molecule), a large coupling (a.k.a. regulatory, modulatory) domain in the middle portion (~60% of the molecule) that transduces the signal of ligand binding and contains sites for Ca\(^{2+}\) binding, ATP binding, and phosphorylation by protein kinases, and a short Ca\(^{2+}\) channel domain in the C-terminal portion. Only InP\(_{\text{3}}\)R1 is known to undergo alternative splicing with two segments (Yoshida and Imai, 1997): the 15-residue-long S1 segment located in the ligand-binding domain predominantly expressed in peripheral tissues and the 40-residue long S2 segment located in the coupling domain and predominantly expressed in the nervous system. As is the case for the majority of cell types examined so far (Wilcox et al., 1993, 1998), smooth muscle cells express multiple InP\(_{\text{3}}\)R isoforms, although some could express none, such as the longitudinal intestinal smooth muscle cells (Kuemmerle et al., 1994).

The proportion of amino acid residue identity among isoforms is 68% in the ligand-binding domain, 53% in the coupling domain, and 59% in the Ca\(^{2+}\) channel domain (Yoshida and Imai, 1997). Poor conservation in the coupling domain suggests the possible operation of differential
regulation in that region among the three isoforms, as illustrated by their sensitivity to protein kinase A-mediated phosphorylation (InsP$_3$R1 $\gg$ InsP$_3$R3 $>$ InsP$_3$R2) (Wojcikiewicz and Luo, 1998b; Murthy and Zhou, 2003). Isoforms were also shown to differ in their sensitivities to breakdown by cellular proteases (InsP$_3$R2 relatively resistant versus InsP$_3$R1 and InsP$_3$R3) (Wojcikiewicz, 1995) and in their spatial distribution (Sugiyama et al., 2000; Tasker et al., 2000) (see Section II.C.1.).

The three-dimensional structure of InsP$_3$R1 has recently been determined by electron cryomicroscopy and single-particle reconstitution using immunopurified and functional bovine cerebellar InsP$_3$R1 (Serysheva et al., 2003). As expected, the channel forms a 4-fold symmetric structure divided into a peripheral large pinwheel (ca. 80% total tetramer volume), created by four centrally interconnected radial curved spokes and a central smaller square (remaining tetramer volume). The InsP$_3$R-binding core domain (likely corresponding to the “coupling domain” mentioned above) was localized within each spoke of the pinwheel region through three-dimensional reconstruction. Putative assignment of the InsP$_3$R1 protomer amino acid sequence to domains within the three-dimensional map was also done, concurrent with the topological model deduced from biochemical, electrophysiological, and molecular biology experiments (Joseph et al., 1997; Galvan et al., 1999; Ramos-Franco et al., 1999; Galvan and Mignery, 2002).
The pinwheel region is likely exposed to the cytoplasm, whereas the square region likely includes the transmembrane domain and faces the ER lumen, putting about 89% of the tetramer in the cytoplasm, with the remaining portion constituting the transmembrane channel domain. A model was also proposed where binding of InsP3 on the pinwheel region far from the transmembrane channel domain induces long range conformational changes in the cytoplasmic region of InsP3R1 to trigger channel opening.

The InsP3R isoform protomers, each traversing the SR membrane six times, much like voltage-gated and ligand-activated ion channels of the plasma membrane (Jan and Jan, 1992), associate via their C termini into homotetrameric, or in contrast with the RyR isoform protomers, heterotetrameric channels; at present, it is unclear to what extent differences in isoform compositional influence ligand binding or Ca2+ permeation. The density of InsP3R is over 100 times greater in smooth muscle than in brain, or contractile activity with ClCa) as with Ca2+ sparks. This water-soluble InsP3 then diffuses into the cytoplasm away from the plasma membrane to bind to InsP3R in the SR membrane, where it induces the opening of the Ca2+ channel that they are forming leading to Ca2+ release (IICR). The diffusion coefficient for InsP3 is 283 μm/2s without metabolism by specific phosphatases and kinases; the time scale of InsP3 action is limited by its degradation to ~1 s, making the effective domain of second messenger action an area of ~24 μm (Michikawa et al., 1996). The InsP3R has a single high-affinity binding site (K_D value of 80 nM), and it is estimated that half-maximal release of Ca2+ from the SR requires 40 nM of InsP3. The InsP3 sensitivities of the isoforms vary to a limited extent and are ranking InsP3R1 > InsP3R2 > InsP3R3 (K_D values of 1.5, 2.5, and 22.4 nM, respectively), consistent with the sensitivities for IICR of cell lines expressing predominantly homotetrameric forms of either of these isoforms (Wojcikiewicz and Luo, 1998a). This sensitivity is controlled by the SR luminal Ca2+ content such that a reduced content also reduces IICR sensitivity (Missiaen et al., 1992c). Likewise, moderate increases in [Ca2+]_cyt sensitize IICR, whereas higher [Ca2+]_cyt has an inhibitory effect (Iino, 1990; Iino and Endo, 1992). Pharmacological modulation of InsP3R sensitivity occurs with thimerosal, which increases the affinity of the receptor for InsP3 (Bootman et al., 1992b; Michelangeli et al., 1995). Of notable interest is that IICR is quantal in nature (Bootman et al., 1992a; Ferris et al., 1992), whereby maximal InsP3 concentrations release 40% of SR Ca2+ content in freshly isolated canine smooth muscle cells (Hashimoto et al., 1985) and 84% of SR Ca2+ store in cultured human smooth muscle cells (Twort and van Bremmen, 1989).

This IICR can sum with agonist-activated Ca2+ entry mechanisms and contribute to global [Ca2+]_cyt transients and ensuing contractile activity. IICR can also result in very spatially localized increases in [Ca2+]_cyt (Ca2+ puffs) analogous to Ca2+ sparks (Bayguinov et al., 2000; Boittin et al., 2000; Burdyga and Wray, 2002). The response to these Ca2+ puffs could be either initiation of Ca2+ waves, which can then be propagated by RyR-induced CICR leading to contractile activity (see above), or alteration of plasma membrane Ca2+-activated conductances (SK and BK channels) promoting relaxation (or contractile activity with ClCa) as with Ca2+ sparks. As for CICR, the type of response to Ca2+ puffs depends on the spatial location of InsP3R, which may vary between smooth muscle cell types (see Section II.C.1.c.).
An interesting development is the proposal by McCarron et al. (2002) that IP₃ evokes contractile activity of smooth muscle by suppressing STOC, which would promote Ca²⁺ entry via membrane depolarization and activation of voltage-gated Ca²⁺ channels.

II. Physiological and Pharmacological Agents

Physiological and pharmacological agents targeting SERCA, RyR, or InsP₃R have been characterized mostly using isolated ER/SR membrane-enriched cell membrane fractions (lipidic spheres called microsomes) or the molecular target of interest partially purified from these microsomes and reconstituted into lipid membrane bilayers. Although the microsome approach allows the measurement of Ca²⁺ fluxes either using ⁴⁵Ca²⁺ loaded into the microsomes or high-affinity Ca²⁺ indicators outside the microsomes, the lipid bilayer reconstitution approach allows the measurement of specific membrane currents. However, both approaches may suffer from the loss of important cofactors or accessory proteins associated with the harshness of the preparatory methods and the unavoidable loss of cytoplasm and non-ER/SR subcellular structures. This could generate results conflicting with data obtained with the same agents in isolated smooth muscle cells or tissues. It could also lead to the oversight of important nonselective effects requiring the presence of other molecular targets in the cytoplasm or on non-ER/SR subcellular structures. It should also be considered that there might be variations in the nature and quantitative aspects of the response to an agent related to the nature of the cell type/tissue considered as well as the animal species. Finally, more than one cell type in an isolated tissue may respond the same way to a given agent, but the effect on one type of cell may inhibit or accentuate the effect of the agent on a neighboring cell of another type (e.g., endothelial cells versus vascular smooth muscle cells). Thus, each agent must be carefully evaluated with all these caveats in mind. One must also remain aware that selectivity versus all major relevant molecular targets involved in the Ca²⁺ handling process has not always been thoroughly assessed for every agent, and caution must be exerted accordingly in experimental design using these agents.

It should be noted that 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), chlorpromazine, and tetracaine, although affecting some of the molecular targets of interest here, are not discussed in this review because of their high degree of nonselectivity (Prozialeck et al., 1987; Ishihara and Karaki, 1991). Similarly, AlF₄⁻ and vanadate, although SERCA inhibitors, are not considered here because of their nonselectivity for P-type ATPases in general (e.g., PMCA, Na⁺/K⁺-ATPase) (Missiaen et al., 1988), with AlF₄⁻ also activating heterotrimeric G-proteins (Bigay et al., 1987) and vanadate-inhibiting protein-phosphotyrosine phosphatases (Gordon, 1991). Likewise, dantrolene is not discussed because of its important inhibition of Ca²⁺ influx, reducing its usefulness as a RyR inhibitor (Sanz et al., 1990; Satoh et al., 1994; Nasu et al., 1996). Although they have been reported as RyR agonists, 4-chloro-methyl-phenol and 4-chloro-3-ethylphenol are not considered because of their too-low potency (Larini et al., 1995; Yusufi et al., 2002). The InsP₃R inhibitors flunarizine (difluorinated derivative of cinnarizine) and decavanadate are not reviewed either, the former because of its dominant calmodulin inhibitory effect and the latter because of its rapid decomposition in solution into vanadate species (Fohr et al., 1989; Strupish et al., 1991). Finally, the use of neutralizing rabbit polyclonal anti-RyR (Boittin et al., 1999, 2000) and anti-RyR3 (Mironneau et al., 2002) antibodies has been reported but is not reviewed here since all published studies so far originate from only one laboratory.

A. Ca²⁺ Pump (Sarco/Endoplasmic Reticulum Ca²⁺ ATPase)

1. Thapsigargin.

a. Source and Chemical Structure. Thapsigargin is a sesquiterpene lactone isolated from the root and fruit of the Mediterranean umbelliferous (Apiaceae) plants Thapsia gargarica (Linnaeus) and Thapsia gymnhesca (Rosselo and Pujadas) (Fig. 6) (Rasmussen et al., 1981; Christensen et al., 1997). It is part of a group of naturally occurring hexaoyxgenated 6,12-guaianolides found in several plant species of the genus Thapsia and often designated “thapsigargins” as a group (Christensen et al., 1997; Treiman et al., 1998). It is highly lipophilic and thus cell-permeant (Treiman et al., 1998).

b. Mechanism of Action. Following the seminal observation by Thastrup et al. (1990) that thapsigargin selectively inhibits SERCA in a variety of cells, it was subsequently shown that it does so by locking SERCA in its Ca²⁺-free E₂ conformations (see Section I.G.1.) by forming a dead-end complex with them (Fig. 7) (Inesi and Sagara, 1992; Sagara et al., 1992). Indeed, although this reaction is in principle reversible, its negligible dissociation constant [K_D = 2.2 pM or less (Davidson and Varhol, 1995)] makes it irreversible in practice, hence the name “dead-end complex”. Accordingly, high concentrations of free Ca²⁺ attenuate SERCA inhibition by thapsigargin (Kijima et al., 1991). Interestingly, in this respect, the fluorescent analog 8-O-(4-aminocinnamoyl)-8-O-debutanoylthapsigargin is almost as potent as thapsigargin in inhibiting ⁴⁵Ca²⁺ uptake by rabbit SERCA1-expressing skeletal muscle microsomes (IC₅₀ = 168 versus 123 nM, respectively), and its fluorescence is sensitive to the E₁-E₂ conformational equilibrium, thus making it a conformational probe (Procida et al., 1998). This mechanism of action is also consistent with the localization of the thapsigargin-specific binding site on the SERCA within the S3 stalk segment, as shown in chimerical studies (Ma et al., 1999). The CPA-specific
binding site was also shown to significantly overlap it, making CPA a competitor of thapsigargin at the molecular level. It is proposed that the perturbation induced by binding of these inhibitors interferes with the long-range functional linkage between ATP utilization in the SERCA cytoplasmic region and Ca\(^{2+}\) binding in the membrane-bound region (uncoupling) (Ma et al., 1999).

SERCA1a, SERCA2a, SERCA2b, and SERCA3 expressed in COS cells are equally sensitive to thapsigargin (but see Section II.A.1.c.) (Lytton et al., 1991). Also, studies of chimeras made from SERCA1 and Na\(^+/\)K\(^-\)ATPase have revealed that thapsigargin binding involves the third transmembrane segment (S3-M3) (Norregaard et al., 1993, 1994; Andersen and Vilsen, 1995; Zhong and Inesi, 1998), whereas studies of interaction between SERCA1 and a fluorescent thapsigargin analog have shown that thapsigargin is at less than 19 Å from tryptophan residue 272 (Hua et al., 1995). Consistent with these findings, the S3-M3 region is highly conserved among SERCA isoforms (Norregaard et al., 1994).

Thapsigargin is 4 to 10,000 times more potent than the two other selective SERCA inhibitors, CPA and 2,5-di-(tert-butyl)-1,4-benzohydroquinone (tBuBHQ) (see Sections II.A.2. and II.A.3.) (Mason et al., 1991; Foskett and Wong, 1992; Luo et al., 1993; Inesi and Sagara, 1994), potentially explaining the relatively more widespread use of thapsigargin. However, its potency is quite variable across experimental systems (IC\(_{50}\) values ranging from subnanomolar to micromolar) (Treiman et al., 1998). Since the interaction between SERCA1 and thapsigargin is stoichiometric (Lytton et al., 1991; Sagara et al., 1992), it would follow that, if this relationship is also applied to the other SERCA isoforms, the apparent inhibitory potency of thapsigargin would be influenced by the number of SERCA molecules per cell or per microgram of microsomal preparation, as shown in various preparations (Papp et al., 1991; Caspersen and Treiman, 1995; Hussain et al., 1995). Likewise, as for other highly lipophilic drugs, thapsigargin interaction with SERCA should be highly dependent on the ratio (lipid + SERCA protein)/thapsigargin (Heirwegh et al., 1988). In keeping with this, indeed, inhibition of purified SERCA by thapsigargin occurs more effectively when the membrane concentration is reduced (Sagara and Inesi, 1991).

Although its metabolism has not been studied thoroughly, thapsigargin is known to be quickly degraded by carboxyesterases at C(2) and C(8) in hepatocytes (Nielsen et al., 1994) (see Fig. 6 for numeration).

In terms of structure-activity relationships (SAR), very small changes in structure have very profound effects on the analogs’ potency in inhibiting purified SERCA ATPase activity, suggesting that that the thapsigargin binding site is very restrictive (Christensen et al., 1992, 1997; Andersen et al., 1994; Nielsen et al., 1995). In particular, epimerization of C(8) decreases potency by more than 3000 times, and epimerization at C(3) reduces it by 40 times. Likewise, the acyl residue at O(10) is critical, as hydrolysis of this ester decreases potency by 40 times. The carboxylic acid residue at O(3) (angelic acid in thapsigargin) appears to have some importance, since its replacement with the larger octanoic acid reduces potency by 11 times. In contrast, the hydroxyl groups at C(7) and C(11), as well as the lactone carbonyl at C(12) and the octanoic acid residue at O(2), have marginal roles in potency as their modifications are well tolerated.

c. Selectivity. Although it has been proposed that thapsigargin inhibitory potency may vary to a small extent among the currently identified SERCA isoforms (see Section I.G.) (Papp et al., 1993; Cavallini et al., 1995; Engelender et al., 1995; Waldron et al., 1995), it is not sufficient to allow for a truly discriminating concentration-dependent pattern (Treiman et al., 1998).

![Figure 6](image-url)
Interestingly, there is circumstantial evidence suggesting the existence of thapsigargin-insensitive Ca\textsuperscript{2+}-ATPase pumps in nonmitochondrial Ca\textsuperscript{2+} stores of various cultured cell lines (Bian et al., 1991; Tanaka and Tashjian, 1993). Furthermore, functional evidence indicates that long-term exposure of cell cultures to thapsigargin promotes the selection of cells with dominance of this thapsigargin-insensitive type of pump (Hussain et al., 1995; Waldron et al., 1995). In isolated vascular cells from the rat aorta, bradykinin releases Ca\textsuperscript{2+} from a thapsigargin-insensitive store (Neusser et al., 1999).

Recent evidence suggests that the thapsigargin-insensitive pump may be the mammalian homolog of Pmr1, a P-type Ca\textsuperscript{2+}-ATPase pump of the Golgi apparatus of C. elegans (Sorin et al., 1997). Transfection of Pmr1 in COS cells (which possess a functional ER) showed that 1) it localizes itself to the Golgi apparatus; 2) it transports Ca\textsuperscript{2+} and Mg\textsuperscript{2+} with high affinity; 3) its mediation of Ca\textsuperscript{2+} uptake is insensitive to thapsigargin, relatively insensitive to tBuBHQ (IC\textsubscript{50} > 1 mM versus 1 \mu M for ER-mediated Ca\textsuperscript{2+} uptake), and about 400 times less sensitive to CPA than the ER (IC\textsubscript{50} value ~300 \mu M; steeper concentration-response curve than for ER-mediated Ca\textsuperscript{2+} uptake); 4) its Ca\textsuperscript{2+} store is less leaky and less sensitive to InsP\textsubscript{3} and ATP than the ER; and 5) it allows generation of Ca\textsuperscript{2+} signals such as caffeine-sensitive regenerative oscillations and ilimaquinone-sensitive baseline spiking (Missiaen et al., 2001b; Wuytack et al., 2002). In A7r5 and 16HBE140- cell lines, which possess an endogenous nonmitochondrial thapsigargin-sensitive Ca\textsuperscript{2+} store (~10% of total cell Ca\textsuperscript{2+} uptake) and express a mammalian homolog of Pmr1, Ca\textsuperscript{2+} accumulation in this store displays the same sensitivity pattern to the SERCA inhibitors as Pmr1-transfected COS cells (as well as a similar IC\textsubscript{50} value), and although the store could be released by InsP\textsubscript{3} or an InsP\textsubscript{3}-generating receptor agonist such as arginine vasopressin) in A7r5 cells (EC\textsubscript{50} ~5 \mu M versus ~1 \mu M for ER-mediated Ca\textsuperscript{2+} release), it is InsP\textsubscript{3}-insensitive in 16HBE140- cells (Wuytack et al., 2002).

Despite the fact that thapsigargin does not affect the various plasma membrane ATPases (PMCA, Na\textsuperscript{+}/K\textsuperscript{+}-ATPase) or mitochondrial membrane pumps (Thastrup et al., 1989, 1990; Lytton et al., 1991), it can inhibit capacitative Ca\textsuperscript{2+} entry (see Section I.G.1.) (Mason et al., 1991; Geiszt et al., 1995). Unlike CPA, but similarly to tBuBHQ (see Section II.A.3.c.), thapsigargin can inhibit Ca\textsubscript{d} when used in the micromolar range (Rossier et al., 1993; Nelson et al., 1994; Buryi et al., 1995). However, in many preparations, maximal SERCA inhibition is obtained with lower concentrations of the compound, thus providing a selectivity window. In contrast, concentrations of CPA and tBuBHQ that inhibit SERCA also partially inhibit capacitative Ca\textsuperscript{2+} entry (see sections on these agents below and Section I.C.) (Mason et al., 1991).

Missiaen et al. (1992a) have shown that thapsigargin (2 \mu M), in contrast with CPA and tBuBHQ (50 \mu M) (see Sections II.A.2.c. and II.A.3.c.), does not alter the SR Ca\textsuperscript{2+} permeability in A7r5 cells (Missiaen et al., 1992a). Interestingly, Darby et al. (1996) observed that thapsigargin (3 \mu M) slightly decreases the slow phase of \textsuperscript{45}Ca\textsuperscript{2+} efflux from smooth muscle membrane vesicles in an oxalate-dependent manner, as does CPA (30 \mu M). The authors proposed that this could be due to the specific obstruction of a putative transmembrane channel within the SERCA (de Meis and Inesi, 1992).

**d. Use in Smooth Muscle Preparations.** Thapsigargin has been successfully used to uncover the influence of SERCA activity in a variety of phasic and tonic vascular and nonvascular smooth muscle preparations. Thus, thapsigargin can be used effectively to deplete the SR stores of Ca\textsuperscript{2+}. The effective concentration range is between 0.1 to 10 \mu M, and the inhibition of SERCA isoforms occurs indiscriminately. Due to the highly lipophilic nature of the compound, the inhibition is long-lasting and difficult to overcome in intact preparations (Table 1). There is intriguing evidence of a thapsigargin-insensitive Ca\textsuperscript{2+} storage site, and it is therefore advisable to confirm findings with structurally unrelated SERCA inhibitors. Thapsigargin can inhibit capacitative Ca\textsuperscript{2+} entry, but at the concentrations used to inhibit SERCA, it does not affect other plasmaemmal and mitochondrial transporters. Thapsigargin can release relaxing factors from intact tissues with endothelium or epithelium by its interaction with SERCA in these cells. Rise in endothelial or epithelial Ca\textsuperscript{2+} has a faster time course (<1 min) than in smooth muscle (15–20 min). In some tissues, thapsigargin can release both endothelium-derived relaxing and contracting factors. Thapsigargin may have multiple sites of action in the endothelium of intact arteries. For example, in rat aorta, thapsigargin causes an endothelium-dependent NO-mediated vaso-
<table>
<thead>
<tr>
<th>Tissue</th>
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<th>Reference</th>
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<td>Cattle</td>
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<td>Berger et al., 2001</td>
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<tr>
<td>Airways</td>
<td>Human</td>
<td>Isolated smooth muscle cells; fura-2 cellular loading, fluorometry; $[Ca^{2+}]_{cyt}$</td>
<td>0.3 μM</td>
<td>Ethier and Madison, 2002</td>
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<tr>
<td>Airways</td>
<td>Mouse</td>
<td>Lung slices; Oregon Green cellular loading, confocal fluorescence microscopy; $[Ca^{2+}]_{cyt}$</td>
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<td>Berger and Sanderson, 2002</td>
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<td>Rat</td>
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<td>Karkanis et al., 2001</td>
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<tr>
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<td>Adachi et al., 2001</td>
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<tr>
<td>Aorta</td>
<td>Rabbit</td>
<td>Microsomes; $[Ca^{2+}]_{cyt}$, cellular loading</td>
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<td>Luo et al., 2000</td>
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<td>Aorta</td>
<td>Rabbit</td>
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<td>2 μM</td>
<td>Missiaen et al., 2001</td>
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<tr>
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<td>Aorta</td>
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<td>Isolated smooth muscle cells; fura-2 cellular loading, fluorometry; $[Ca^{2+}]_{cyt}$</td>
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<td>Vallot et al., 2001</td>
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<td>Isolated smooth muscle cells; fura-2 cellular loading, fluorometry; $[Ca^{2+}]_{cyt}$</td>
<td>1 μM</td>
<td>Neuss et al., 1999</td>
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<tr>
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<td>Rat</td>
<td>Isolated smooth muscle cells; fura-2 cellular loading, fluorometry; $[Ca^{2+}]_{cyt}$</td>
<td>2 μM</td>
<td>Neylon et al., 1992</td>
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<td>Carotid artery</td>
<td>Rabbit</td>
<td>Isolated smooth muscle cells and endothelium-denuded strips; fura-2 cellular loading, fluorometry, isotropic dynamometry; $[Ca^{2+}]_{cyt}$, contractile activity</td>
<td>1-2 μM</td>
<td>Szado et al., 2001</td>
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<td>Rat</td>
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<td>100 nM</td>
<td>Nomura and Asano, 2000</td>
</tr>
<tr>
<td>Cerebellar resistance arteries</td>
<td>Rat</td>
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<td>Cheranov and Jaggar, 2004</td>
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<td>Cerebral arterioles</td>
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<td>Isolated smooth muscle cells; fura-PE3 cellular loading, fluorometry; $[Ca^{2+}]_{cyt}$</td>
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<td>Guibert et al., 2002</td>
</tr>
<tr>
<td>Cerebral arterioles</td>
<td>Rat</td>
<td>Isolated smooth muscle cells; indo-1 cellular loading, confocal fluorescence microscopy; $[Ca^{2+}]_{cyt}$</td>
<td>1 μM</td>
<td>Saino et al., 2002</td>
</tr>
<tr>
<td>Cerebral artery</td>
<td>Rat</td>
<td>Isolated smooth muscle cells; whole-cell patch-clamping; $I_{C(Ca)}$</td>
<td>(no effect)</td>
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<tr>
<td>Cerebral artery</td>
<td>Rat</td>
<td>Isolated, cannulated and pressurized segments; fluo-3 cellular loading, confocal fluorescence microscopy, diameter monitoring by video microscopy; $[Ca^{2+}]_{cyt}$</td>
<td>100 nM</td>
<td>Jaggar, 2001</td>
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<td>Colon</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle cells; whole-cell patch-clamping, fluo-3 or fura-2 cellular loading, fluorometry; $I_{K(Ca)}$, $[Ca^{2+}]_{cyt}$</td>
<td>500 nM</td>
<td>Bradly et al., 2002</td>
</tr>
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<td>Colon</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle cells; whole-cell patch-clamping, fluo-3 cellular loading, epifluorescence microscopy; membrane currents, $[Ca^{2+}]_{cyt}$</td>
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<td>Flynn et al., 2001</td>
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<td>Colon</td>
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<td>Bradly et al., 2003</td>
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<td>Coronary artery</td>
<td>Pig</td>
<td>F3 microsomal membranes fraction, isolated cultured smooth muscle cells and endothelium-denuded rings; fluo-3 loading (cells), $[Ca^{2+}]<em>{cyt}$ loading (microsomal membranes), epifluorescence microscopy (cells), isotropic dynamometry (rings); $[Ca^{2+}]</em>{cyt}$ (cells), $[Ca^{2+}]<em>{cyt}$, $[Ca^{2+}]</em>{mem}$, contractile activity (rings)</td>
<td>1 μM</td>
<td>Walia et al., 2003</td>
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<td>Cremaster muscle arterioles</td>
<td>Hamster</td>
<td>Isolated, cannulated and pressurized segments isolated from 1st- and 2nd-order branches; fura-2 cellular loading, fluorometry, diameter monitoring by video microscopy; $[Ca^{2+}]_{cyt}$, conducted vasomotor responses</td>
<td>100 nM</td>
<td>Yashiro and Duling, 2003</td>
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</tbody>
</table>
dilation (1–100 nM), whereas at low concentrations (3 nM), it diminishes endothelium-dependent vasodilation to A23187 and Ach (Huang et al., 2000).

2. Cyclopiazonic Acid.

a. Source and Chemical Structure. CPA is a mycotoxin produced by some strains of the molds Penicillium cyclopium and Aspergillus flavus (Holzapfel, 1968). It is an indole tetracarboxylic acid metabolite derived from the amino acid tryptophan (Fig. 6) (Holzapfel, 1968). CPA is a natural contaminant of some cereal products and mold-fermented cheese or meat and is considered a toxic hazard to humans and animals. The usual clinical signs of its toxicity are related to skeletal muscle dysfunction, consistent with its accumulation in this tissue (Norred et al., 1985).

<table>
<thead>
<tr>
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<th>Experimental Details (Preparation; Measurement Method; Measured Response)</th>
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<th>Reference</th>
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<tr>
<td>Cremaster muscle arterioles</td>
<td>Rat</td>
<td>Isolated, cannulated and pressurized endothelium-denuded segments; fura-2 cellular loading, fluoro microscopy, diameter monitoring by video microscopy; [Ca^{2+}]_I, contractile activity</td>
<td>1 μM</td>
<td>Potocnik and Hill, 2001</td>
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<td>Esophagus (lower sphincter, circular layer)</td>
<td>Cat</td>
<td>Isolated saponin-permeabilized smooth muscle cells; cell length monitoring by phase-contrast microscopy; contractile activity</td>
<td>3 μM</td>
<td>Sohn et al., 1993</td>
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<td>Femoral artery</td>
<td>Rat</td>
<td>Isolated endothelium-denuded strips; isometric dynamometry; contractile activity</td>
<td>100 nM</td>
<td>Nomura and Asano, 2000</td>
</tr>
<tr>
<td>Pulmonary artery Rat</td>
<td>Isolated endothelium-denuded strips; isometric dynamometry; contractile activity</td>
<td>100 nM</td>
<td>Asano and Nomura, 2001</td>
<td></td>
</tr>
<tr>
<td>Gall bladder Guinea pig</td>
<td>Isolated smooth muscle cells; whole-cell patch-clamping, fluo-4 cellular loading, confocal fluorescence microscopy, isometric dynamometry; [Ca^{2+}]_I, imaging, contractile activity</td>
<td>1 μM</td>
<td>Pozo et al., 2002</td>
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<td>Lung</td>
<td>Rat</td>
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<td>Mesenteric artery Dog</td>
<td>Microsomes; 45Ca^{2+} loading; Ca^{2+} fluxes</td>
<td>3 μM</td>
<td>Darby et al., 1996</td>
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<td>Mesenteric resistance arteries Rat</td>
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<td>1 μM</td>
<td>Lagaud et al., 1999</td>
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<td>Pulmonary artery Cattle</td>
<td>Microsomes; extramicrosomal fluo-3 and fluorometry or 45Ca^{2+} loading; Ca^{2+} fluxes</td>
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<td>Stout et al., 2002</td>
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<tr>
<td>Pulmonary artery Dog</td>
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<td>Doi et al., 2000</td>
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<td>Pulmonary artery Rat</td>
<td>Isolated intact or β-escin-permeabilized strips; isometric dynamometry; contractile activity</td>
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<td>Gonzalez De La Fuente et al., 2005</td>
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<tr>
<td>Pulmonary artery Rat</td>
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<td>100 nM</td>
<td>Nomura and Asano, 2000</td>
<td></td>
</tr>
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<td>Renal artery Pig</td>
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<td>Ihara et al., 1999</td>
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<td>Malysz et al., 2001</td>
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<td>Stomach (antrum) Cattle</td>
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<td>Stout et al., 2002</td>
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<td>Dreja et al., 2001</td>
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<td>Ito et al., 2002</td>
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<td>Ito et al., 2000</td>
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<td>Isolated smooth muscle cells; whole-cell patch-clamping, fura-2 (for [Ca^{2+}]_I) or mag-fura-2 (for [Ca^{2+}]_I) cellular loading, fluorometry; STOCs, [Ca^{2+}]_syn, [Ca^{2+}]_les, contractile activity</td>
<td>100 nM-10 μM</td>
<td>Gomez-Viquez et al., 2003</td>
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<td>Vas deferens Rat</td>
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<td>3 μM</td>
<td>Darby et al., 1996</td>
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</table>
b. Mechanism of Action. CPA was originally shown to be a potent inhibitor of both SERCA activity and ATP-dependent Ca\(^{2+}\) sequestration in SR vesicles isolated from mammalian fast skeletal muscle (Goeger et al., 1988). This finding is consistent with its inhibition of oxalate-stimulated \(^{45}\)Ca\(^{2+}\) uptake in mammalian visceral and vascular smooth muscle microsomal vesicles (Darby et al., 1996)—oxalate-stimulated uptake is an exclusive property of the SR-derived components of smooth muscle membrane fractions (Grover, 1985; Kwan, 1985). Maximal inhibition is achieved with 10 to 30 \(\mu\)M CPA (Darby et al., 1996), which corresponds with the concentrations required to prevent SR Ca\(^{2+}\) release and the accompanying contractile activity in different intact and permeabilized smooth muscle preparations. Furthermore, CPA inhibition of vesicular \(^{45}\)Ca\(^{2+}\) uptake has a rapid onset and is rapidly and completely reversible both in intact and permeabilized smooth muscle preparations (Darby et al., 1996).

CPA mechanism of action was recently elucidated using mammalian fast skeletal muscle SR vesicles (Plenge-Tellechea et al., 1997). It decreases the SERCA affinity (\(K_p\)) for ATP under nonturnover conditions by approximately one order of magnitude. The net effect results in inhibition of enzymatic hydrolytic activity and hence, its Ca\(^{2+}\) pumping ability. Although the number of CPA binding sites on the ATPase is equivalent to that of the high-affinity ATP binding sites, they are distinct. Earlier studies (Seidler et al., 1989; Karon et al., 1994) proposed that CPA could compete for ATP-binding sites, but later findings reported that the decrease in the \(K_p\) for ATP is independent of CPA (Plenge-Tellechea et al., 1997). Furthermore, CPA does not modify the 2′(or 3′)-O-(trinitrophenyl)adenosine-5′-triphosphate (TNP-ATP) fluorescence signal, further supporting the absence of direct competition (Plenge-Tellechea et al., 1997). TNP-ATP fluorescence increases upon its binding to the SERCA in the absence of Ca\(^{2+}\) and competition by ATP for the binding site occupied by TNP-ATP decreases the fluorescence signal because of TNP-ATP displacement.

Early confusion was caused by the observations that CPA blockade of the SERCA turnover could be overcome if the ATP concentration is sufficiently increased. However, recent evidence suggests that CPA does not bind at the catalytic site, a conclusion supported by the observation that autophosphorylation of the Ca\(^{2+}\)-bound enzyme by ATP is not inhibited by CPA (Plenge-Tellechea et al., 1997). Nonetheless, CPA effect on the enzyme is likely not restricted to the ATP-binding process since it causes additional inhibition of the ATPase at concentrations above the stoichiometric levels (Plenge-Tellechea et al., 1997). With an ATP concentration of 1 mM, SERCA is completely inhibited at a CPA/SERCA molar ratio of approximately 10 (Soler et al., 1998). Furthermore, enzyme activity is observed to recover at a CPA/SERCA molar ratio of 1 when the Ca\(^{2+}\) concentration is raised, which is consistent with the competitive character of CPA and Ca\(^{2+}\) (Soler et al., 1998). These results suggest that ATP and Ca\(^{2+}\) can protect against CPA inhibition. Whether this mechanism of action documented in the SERCA1a isoform also applies to the other SERCA isofoms has not been shown.

In terms of the Ca\(^{2+}\) pumping cycle, CPA appears to stabilize the E\(_2\) SERCA conformation (Seidler et al., 1989; Karon et al., 1994; Plenge-Tellechea et al., 1997; Soler et al., 1998). The onset of inhibition during SERCA enzymatic turnover is slow, implying that numerous cycles are required (Plenge-Tellechea et al., 1997). Under turnover conditions, the \(K_D\) for CPA is 7 nM (Soler et al., 1998). This suggests the existence of a slow isomerization step between binding of CPA to the SERCA E\(_2\) conformations and the formation of E\(_2\) conformations with lower ATP-binding affinity. This is consistent with the localization of the CPA-specific binding site on the SERCA within the S3 stalk segment, as shown in chimeric studies (Ma et al., 1999). The thapsigargin-specific binding site was also shown to significantly overlap it, thus making thapsigargin a competitor of CPA at the molecular level. It is proposed that perturbation induced by binding of these inhibitors interferes with the long-range functional linkage between ATP utilization in the SERCA cytoplasmic region and Ca\(^{2+}\) binding in the membrane-bound region (uncoupling) (Ma et al., 1999).

A model for the inhibition of the SERCA by CPA in the substoichiometric/stoichiometric range has been developed (Fig. 8) (Plenge-Tellechea et al., 1997). In the presence of a stoichiometric CPA concentration, all enzyme molecules would eventually bind CPA, such that hydrolytic activity could only be maintained by a high ATP concentration. However, the enzyme turnover displays a lower ATP affinity (\(K'_{\text{ATP}}\)) and a lower maximal velocity (\(V'_{\text{max}}\)). Consequently, at substoichiometric CPA and high ATP concentrations, the observed kinetic parameters correspond to the contribution of two different reaction cycles sustained by the CPA-free form of the enzyme (\(K_{\text{ATP}}\) and \(V_{\text{max}}\)) and its CPA-bound form (\(K'_{\text{ATP}}\) and \(V'_{\text{max}}\)).

As discussed earlier (see Section I.G.1.), the SERCA-inhibiting accessory protein phospholamban, which needs to be phosphorylation to be inactivated, is an excellent substrate for PKG (Raeymaekers et al., 1988), and as such, is likely involved in NO-induced SERCA activation. NO activation of guanylate cyclase would increase cGMP concentration, which would activate SR membrane-located cGMP-dependent PKG, which would then phosphorylate phospholamban (Raeymaekers et al., 1988; Twort and van Bremen, 1989; Cornwell et al., 1991; Karaczewski et al., 1992; Andriansitosohaina et al., 1995). Indeed, the role of SERCA in this scheme is supported by the fact that NO donors suppress 1) CPA-induced extracellular Ca\(^{2+}\)-dependent contractile activity of the feline gastric fundus smooth muscle (Petkov and Boev, 1996) and 2) CPA-induced Cl\(_{\text{Ca}}\) activation in the mouse anococcygeus (Westerduin et al., 1992). In
addition, CPA inhibits the relaxation of phenylephrine-induced contractile activity triggered by NO donors in the endothelium-denuded rabbit aorta (Luo et al., 1993). Finally, CPA inhibits the relaxation of carbachol (Cch)-induced contractile activity triggered by an NO donor and by 8-bromo-cGMP in the canine tracheal smooth muscle (McGrogan et al., 1995).

It was proposed that phospholamban would act as an indirect competitor of CPA (Westerduin et al., 1992; Petkov and Boev, 1996), as shown in cardiac muscle for both CPA and thapsigargin (Mahaney et al., 1999). This is consistent with data in bladder from mice in which the phospholamban gene was ablated (phospholamban-knockout mice) showing that CPA amplifies the increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) and the associated contractile activity induced by Cch, as it does to a more limited extent in the bladder from wild-type mice, and that this effect is virtually abolished in bladder from mice where phospholamban had been overexpressed in a smooth muscle-specific manner (Nobe et al., 2001).

c. Selectivity.

There is no direct evidence indicating that SERCA1a and SERCA2a/b isofoms have different sensitivities to CPA; likewise, the three mammalian-homolog avian SERCA isoforms (SERCA1, SERCA2a, and SERCA2b), which are encoded by three distinct cDNAs, have identical sensitivities to CPA (Campbell et al., 1991). However, it should be kept in mind that CPA-insensitive/MgATP-independent SR Ca\(^{2+}\) uptake may exist in parallel with a CPA-sensitive/MgATP-dependent mechanism in some vascular (Stout, 1991; Low et al., 1992) and airway (Bourreau et al., 1993) smooth muscles. A direct but regulated communication between the extracellular space and the SR lumen has been postulated but seems unlikely based on existing structural and biophysical data.

CPA has no effect on cation ATPases other than the SERCA. It does not interact with brain and kidney Na\(^+/\)K\(^+\)-ATPase pumps, the gastric H\(^+\)/K\(^+\)-ATPase pump, the mitochondrial F\(_{1}\) ATPase, or the erythrocyte and skeletal muscle PMCA (Seidler et al., 1989). However, CPA can inhibit Ca\(^{2+}\) uptake by the thapsigargin-insensitive Ca\(^{2+}\) store (IC\(_{50}\) value ~300 \(\mu\)M in A7r5 cells; steeper concentration-response curve than for ER-mediated Ca\(^{2+}\) uptake with threshold at ~100 \(\mu\)M, likely mediated by a Pmr1-like P-type Ca\(^{2+}\)-ATPase pump located in the Golgi apparatus (see Section II.A.1.c.) (Wuytack et al., 2003).

In smooth muscle, CPA has no effect on Ca\(_{L}\)- (in contrast with the inhibitory effect of thapsigargin and tBuBHQ) (see Sections II.A.1.c. and II.A.3.c.) and delayed rectifier K\(^+\) channel-mediated currents in visceral smooth muscle (Suzuki et al., 1992). However, a supramaximal concentration of CPA for inhibition of the SERCA (300 \(\mu\)M) appears to inhibit Ca\(^{2+}\) influx through Ca\(_{L}\) in the myometrium (Imai et al., 1984). Whether this is due to a direct action on the channel is not known. CPA has no direct effect on the single-channel conductance or the open probability (\(P_0\)) of BK from visceral smooth muscle (Suzuki et al., 1992). In addition, CPA has no effect on plasmalemmal ATP-dependent Ca\(^{2+}\) extrusion; indeed, it does not alter saponin-sensitive microsomal Ca\(^{2+}\) uptake, believed to reflect the plasmalemmal-derived component of smooth muscle membrane fractions (Grover, 1985). Finally, CPA has no effect on the sensitivity of the contractile apparatus to Ca\(^{2+}\) in \(\beta\)-escin permeabilized visceral (Uyama et al., 1992) and vascular (Gonzalez De La Fuente et al., 1995) smooth muscle preparations. In the latter permeabilized preparations, CPA inhibits NE- and/or caffeine-induced contractile activity in absence of Ca\(^{2+}\) only if it is...
<table>
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**TABLE 2 Continued**
present during the loading of the intracellular Ca\textsuperscript{2+}
stores. This indicates that the inhibition of contractile activity occurs via the effects of CPA on SR loading.

Missiaen et al. (1992a) proposed that CPA, at 50 μM, like tBuBHQ (see Section II.A.3.c.), nonselectively decreases Ca\textsuperscript{2+} permeability of isolated smooth muscle cells. This is based on the fact that CPA inhibits passive Ca\textsuperscript{2+} efflux from permeabilized A7r5 cells. The non-
specificity of this effect was suggested by the fact that it persists even after prior exposure to 2 μM of the SERCA inhibitor thapsigargin, which has no effect on passive Ca\textsuperscript{2+} efflux at this concentration. However, these findings have to be reconciled with the absence of effect of CPA on d-[\textsuperscript{14}C(U)]sorbitol efflux in skeletal muscle SR vesicles (Goeger and Riley, 1989). Indeed, this latter finding indicates that CPA does not affect SR permeability nonselectively. Interestingly, Darby et al. (1996) observed that both CPA (30 μM) and thapsigargin (3 μM) slightly decrease the slow phase of \textsuperscript{45}Ca\textsuperscript{2+} efflux from smooth muscle membrane vesicles in an oxalate-dependent manner. The authors proposed that this could be due to the specific obstruction of a putative transmembrane channel within the SERCA (de Meis and Inesi, 1992).

Finally, CPA (10 μM) inhibits ecto-ATPase activity in the guinea pig urinary bladder and vas deferens to some extent (<25%) (Ziganshin et al., 1994). It also substantially potentiates neuronal ATP release induced by transmural electrical field stimulation of the vas deferens (15-fold). However, these effects did not significantly influence the ATP-induced P\textsubscript{2α}-purinoceptor-mediated contractile activity of these smooth muscle preparations (Ziganshin et al., 1994).

d. Use in Smooth Muscle Preparations. CPA is a watersoluble inhibitor of all SERCA isoforms that is effective in concentrations of 10 to 30 μM (Table 2). The inhibition of Ca\textsuperscript{2+} uptake by the SR is rapid in onset and fully reversible. CPA inhibits the ATP hydrolytic activity of SERCA, thus reducing its pumping ability. CPA does not affect the other pumps, such as the Na\textsuperscript{+}/K\textsuperscript{+}-ATPases and PMCA, the Ca\textsuperscript{2+} and K\textsuperscript{+} channels, or contractile protein activity. In intact mesenteric arteries, CPA causes an initial endothelium-dependent dilation and a subsequent endothelium-dependent maintained rhythmic vasomotion that may be the result of released hyperpolarizing factors that activate K\textsuperscript{+} channels on the smooth muscle cells (Huang and Cheung, 1997).

3. 2,5-Di-(tert-butyl)-1,4-benzoquinone.

a. Source and Chemical Structure. 2,5-Di-(tert-butyl)-1,4-benzoquinone (tBuBHQ) is a synthetic phenolic compound, originally studied for its antioxidant properties (Wilson and Poley, 1960; Ershoff, 1963, 1969), characterized by a repeated bifunctionality consisting of a hydrophilic group associated with a lipophilic area (the latter making it cell-permeant) (Fig. 6). In aqueous solution at physiological pH, as a hydroquinone, it very easily undergoes superoxide dismutase-inhibitable spontaneous oxidation (auto-oxidation) to form semiquinone radicals and donate an electron to molecular oxygen, thus generating the membrane-impermeant superoxide anion (Marklund and Marklund, 1974; Fusi et al., 1999).

A caged form of tBuBHQ, O-[o-nitromandeloyloxycarbonyl]-tBuBHQ, has been designed (Rossi and Kao, 1997), although it is not available commercially. Its carboxylate group can be used to attach an acetoxyethyl ester moiety to make the caged compound cell-permeant (Rossi and Kao, 1997). Irradiation of O-[o-nitromandeloyloxycarbonyl]-tBuBHQ with UV light (<400 nm wavelength) results in the formation of tBuBHQ-bicarbonate, which would rapidly decompose under physiological conditions to tBuBHQ and carbon dioxide (Rossi and Kao, 1997).

b. Mechanism of Action. tBuBHQ was originally shown to impair ER/SR-mediated Ca\textsuperscript{2+} sequestration in hepatocytes (expressing SERCA2b and 3) by relatively potent inhibition of SERCA activity (Moore et al., 1987). In rabbit skeletal muscle microsomes, containing SERCA1a, and canine cardiac muscle microsomes, containing SERCA2a, tBuBHQ inhibits ATP hydrolysis by up to 80 and 90%, respectively, at less than 10 μM with an IC\textsubscript{50} value ~1.5 μM for both preparations (Nakamura et al., 1992b). More specifically, at 5 to 10 μM, tBuBHQ inhibits by 35 to 40% the decomposition rate of the phosphorylated SERCA intermediate (EP), probably due to a decreased rate of conversion of high Ca\textsuperscript{2+} affinity (E\textsubscript{P}) to the low Ca\textsuperscript{2+} affinity (E\textsubscript{P}) form. It also inhibits, by almost 90%, the formation of EP by decreasing the rate of Ca\textsuperscript{2+} binding to the Ca\textsuperscript{2+}-free, nonphosphorylated SERCA (mostly E\textsubscript{1}) (Nakamura et al., 1992b) (see Fig. 3 and Section I.G.1.). This suggests that tBuBHQ has at least two sites of action in the SERCA Ca\textsuperscript{2+} pumping cycle (Fig. 9).

Another comparative study looking at the inhibitory activity of tBuBHQ in microsomes from platelets [human; SERCA2b and 3 (Bobe et al., 1994; Kovacs et al., 1997)], smooth muscle (pig and rat aorta; mostly SERCA2b), cardiac muscle, and skeletal muscle showed that tBuBHQ inhibits EP formation in the 0.1- to 1-μM range with maximal inhibition at 5 μM, whereas hydroquinone (lacking the two tertiary butyl groups of tBuBHQ; see Section II.A.2.a.) is inactive, supporting the selectivity of effect of tBuBHQ (Papp et al., 1992). However, there is a clear insensitivity of the smooth muscle microsomes to this effect (SERCA2b; ~5% at 10 μM), while the amplitude of this effect in the other preparations is ranking as platelet (SERCA3; ~70%) > skeletal muscle (SERCA1a; ~60%) > cardiac muscle (SERCA2a; ~45%).

c. Selectivity. As is the case for thapsigargin and CPA, tBuBHQ can inhibit all three families of SERCA isoforms, although SERCA2b may be resistant (Moore et al., 1987; Nakamura et al., 1992b; Papp et al., 1992). tBuBHQ is also without effect on mitochondrial Ca\textsuperscript{2+} fluxes and PMCA activity (Moore et al., 1987) and on the Ca\textsuperscript{2+} sensitivity of contractile proteins (Westerblad and Allen, 1994). How-
ever, at high concentrations (50 μM), within the minimal range required for maximal SERCA inhibition, it can partially inhibit capacitative Ca$^{2+}$ entry presumably through a direct effect on the plasma membrane permeability for divalent cations (Mason et al., 1991; Foskett and Wong, 1992). Furthermore, also at high concentrations (IC$_{50}$ value ~67 μM), in contrast with CPA but similar to thapsigargin, tBuBHQ inhibits the Ca$_L$ currents in freshly isolated rat tail artery smooth muscle cells (Fusi et al., 2001). It does so in a partially reversible and voltage-dependent manner; its auto-oxidation into superoxide anion impairs channel function from the extracellular side of the plasma membrane (possibly by stabilizing it in its inactivated state) (Fusi et al., 2001). This effect is consistent with the Ca$_L$ blockade observed in GH3 pituitary cells (Nelson et al., 2000). tBuBHQ (50 μΜ), like CPA (see Section II.A.3.c.), nonselectively decreases SR Ca$^{2+}$ permeability as it inhibits passive Ca$^{2+}$ efflux from permeabilized A7r5 cells (Missiaen et al., 1992a). Evidence for a nonspecific effect was suggested by the observation that the inhibition persists after cells are exposed to thapsigargin (2 μM) at a concentration that does not affect passive Ca$^{2+}$ efflux.

**d. Use in Smooth Muscle Preparations.** tBuBHQ is a SERCA inhibitor, the use of which is limited by its rapid conversion to superoxide radicals in solution. This compound can inhibit capacitative Ca$^{2+}$ entry while not affecting mitochondrial Ca$^{2+}$ fluxes, PMCA activity, or contractile protein sensitivity. By formation of free radicals, tBuBHQ can readily inhibit various Ca$^{2+}$ channels. SERCA inhibition in various smooth muscle preparations is fast and readily reversible and requires 50 to 100 μM (Table 3).

**B. Ca$^{2+}$-Gated Ca$^{2+}$ Release Channel/Ryanodine Receptor**

1. **Cyclic ADP-Ribose and Analogs.** Cyclic ADP-ribose (cADPR) is a pyridine nucleotide metabolite derived from nicotinamide adenine dinucleotide (NAD$^+$, β-NAD$^+$) (Lee et al., 1989). Cyclization is at the N1-position of the adenine ring linked to the anomeric carbon C1’ of the terminal ribose with the linkage in β-configuration (Lee et al., 1994) (Fig. 10, Fig. 11). The NAD nicotinamide group is released during the cyclization reaction.

Several cADPR analogs with either agonistic or antagonistic activity have been synthesized by enzymatic and chemoenzymatic methods owing to the broad substrate specificity of ADP-ribosylecylase from *Aplysia californica*, the mollusk where cADPR was first discovered (Lee et al., 1989). However, new enzyme-independent methods (Fukuoka et al., 2001; Shuto et al., 2001) should allow for more analogs with structural diversity.

To date, five significantly potent agonist analogs have been designed (in decreasing order of potency): 3-deaza-cADPR, cADP-carbocyclic-ribose (cADPcR), 2’A-deoxy-
cADPR, cyclic aristeromycin diphosphate ribose (cAris-DPR), and 3’-deoxy-cADPR (Fig. 11). 3-Deaza-cADPR differs from cADPR by the substitution of carbon for nitrogen at the purine ring 3-position (Wong et al., 1999). In cADPcR, the oxygen atom in the cADPR N-1-ribose ring is substituted by a methylene group (Fukuoka et al., 1999; Shuto et al., 2001). 2’-A-Deoxy-cADPR has a deletion of the cADPR 2’-position hydroxyl group of the ribose moiety linked to the adenine, i.e., the adenosine ribose (Ashamu et al., 1997). cArisDPR was the first carbocyclic cADPR analog synthesized, wherein the adenosine ribosyl moiety is replaced with a carbocyclic 5-membered ring (Bailey et al., 1996). Finally, 3’-OMe-cADPR has a methoxyl moiety substituting for cADPR 3’-position hydroxyl group of the adenosine ribose (Ashamu et al., 1997).

A caged form of cADPR has also been designed (Aarhus et al., 1995a; Walseth et al., 1997) and contains one photolabile (2-nitrophenyl)ethyl group on either of the two phosphates of cADPR (Fig. 12). The two resulting isomers have similar photolysis efficiency [quantum yield of 0.11 (Walseth et al., 1997)] and are both biologically inactive until photolysis with UV light (<400-nm wavelength), and therefore, do not need to be separated for biological applications (Aarhus et al., 1995a).

b. Mechanism of Action.

i. Synthesis and Degradation. The cell-impermeant cADPR is generated from NAD$^+$ by an ADP-ribosyl cyclase (ADPRC) and is degraded to ADP-ribose by a
cADPR hydrolase (cADPRH) (Guse, 2002) (Fig. 10). Presently, there are no details on the physiological mechanisms that lead to activation of ADPRC. In mammals, ADPRC and cADPRH activities are expressed as ectoenzymes by the type II transmembrane glycoprotein CD38 (Lee, 2000, 2001; Higashida et al., 2001). For instance, ADPRC and cADPRH activities were both demonstrated in plasma membrane-enriched tracheal smooth muscle crude membranes but not in the SR-enriched fractions (White et al., 2000). It is possible, however, that in smooth muscle cells, CD38 or closely related enzymes are expressed in nuclear membranes or at other intracellular sites (Adebanjo et al., 1999; Khoo et al., 2000). Both activities of ADPRC and cADPRH also comigrate at a molecular weight of ~40 kDa on SDS-PAGE, which was confirmed by sucrose gradient density fractionation and gel filtration chromatography. Kinetic analysis has yielded $K_m$ values of 30.4 and 695.3 $\mu$M and $V_{max}$ values of 330.4 and 102.8 nmol/mg/h for ADPRC and cADPRH activities, respectively (White et al., 2000). This is consistent with the observation that both ADPRC and cADPRH activities are associated with a single-membrane protein fraction in rat myometrial smooth muscle (Dogan et al., 2002). Interestingly, in membranes from rat aortic smooth muscle cells, an ADPRC is detected on Western blot with an anti-CD38 antibody, but...
it was shown to be pharmacologically distinct from the classical CD38 (de Toledo et al., 2000).

The bifunctional activity of CD38 is regulated in smooth muscle cells, thus influencing cADPR levels. For instance, in porcine tracheal smooth muscle cell membranes, NO donors produce a concentration-dependent decrease in ADPRC but not cADPRH activity through a cGMP-independent pathway involving S-nitrosylation of thiols, resulting in reduced cADPR synthesis and in part mediating NO-induced inhibition of intracellular Ca\(^{2+}\) mobilization (White et al., 2002). NO inhibits the Ca\(^{2+}\) mobilization induced by endogenous cADPR in bovine coronary arterial smooth muscle cells (Yu et al., 2000). In rat myometrial smooth muscle, estradiol-17β increases CD38 mRNA and protein expression and significantly enhances ADPRC, but not cADPRH, activity. This differential regulation could result in increased cADPR synthesis and influence myometrial Ca\(^{2+}\) regulation and contractility (Dogan et al., 2002). cADPRH activity was also shown to be differentially regulated in rabbit pulmonary arterial smooth muscle where, in homogenates, cADPR levels are increased by reduction of the β-NAD\(^+\)/β-NADH ratio, at least in part due to the inhibition of cADPRH by β-NADH (Wilson et al., 2001). This mechanism, reflecting the cellular redox state, may mediate the SR Ca\(^{2+}\) release-induced hypoxic pulmonary vasoconstriction and associated hypertension (Wilson et al., 2001).

An important contrast with other second messenger molecules, such as adenylate cyclase or PLC, that act on plasma membrane or intracellular substrates is that CD38 is an ecto-NADase, as alluded above; thus, it acts on extracellular substrates (Lee, 2000, 2001). Such an extracellular active site makes its role still a matter of debate given the intracellular function of cADPR. However, an evolving model proposes that CD38 ADPRC activity is sensitive to extracellular stimuli through receptor-mediated phosphorylation and/or G-protein- or ATP-mediated modulation (Guse, 2002). In this model, NAD\(^+\) needs to be exported from the cytoplasm, probably by connexin 43 hemichannels (Bruzzone et al., 2001).

The cell-impermeant cADPR subsequently formed must then be transported back into the cell, probably by dimeric or oligomeric CD38 itself or by an unknown transport system (Zocchi et al., 1999).

Consistent with such a model is that tracheal mucosa strips release NAD\(^+\) and increase [Ca\(^{2+}\)]\(_{cyt}\) in a cADPR-dependent manner, suggesting the existence of a paracrine mechanism whereby mucosa-released extracellular NAD\(^+\) plays a hormone-like function and where cADPR behaves as second messenger (Franco et al., 2001). Additional support for the model is that extracellularly applied cADPR (100 μM) potentiates Ach-induced [Ca\(^{2+}\)]\(_{cyt}\) increases and contractile activity in tracheal smooth muscle cells (Franco et al., 2001). Furthermore, this latter effect is mimicked by extracellular application of the more potent and metabolically stable cell-impermeant analog 3-deaza-cADPR (Wong et al., 1999) (see below). This suggests that, by formation of NAD\(^+\), cADPR could play an autocrine/paracrine role in addition to its second messenger role.

ii. Interaction with Ca\(^{2+}\)-Gated Ca\(^{2+}\) Release Channel/Ryanodine Receptor. cADPR is the only known endogenous RyR agonist apart from Ca\(^{2+}\). Its ability to activate smooth muscle RyR was directly shown using RyR from bovine coronary arterial smooth muscle cells SR reconstituted into planar lipid bilayers; cADPR (0.01–1 μM) increases RyR \(P_o\) up to 8 times in a ryanodine-sensitive manner and sensitizes RyR activation by Ca\(^{2+}\) (Li et al., 2001). cADPR (10 μM) reduces \([^{3}H]\)ryanodine binding to microsomes from freshly cultured rat aortic smooth muscle cells that express RyR1–3 (Yusufi et al., 2002). However, a direct interaction of cADPR with the RyR has not been demonstrated in any type of cellular preparation thus far.

In cultured bovine coronary arterial smooth muscle cells, cADPR activation of RyR requires the tacrolimus (FK-506)-binding protein 12.6 (FKBP12.6) (Tang et al., 2002), a RyR accessory protein. This protein was shown to bind in a stoichiometry of four RyR protomers: four FKBP12.6 molecules using either RyR1 from skeletal muscle, RyR2 from cardiac muscle, or RyR3 cloned from lung epithelial cells (Timerman et al., 1996; Bultynck et al., 2001; Jeyakumar et al., 2001). FK-506, an immunosuppressant drug, increases the \(P_o\) of reconstituted RyR channels from the SR of these cells by interacting with FKBP12.6 (Tang et al., 2002). FK-506 dissociates FKBP12.6 from RyR, and this is believed to activate or delay the inactivation/closing of the Ca\(^{2+}\) channel; it is assumed that cADPR acts similarly (Higashida et al., 2001). The lower sensitivity of RyR to cADPR in microsomal and permeabilized smooth muscle preparations (micromolar) compared with that observed in sea urchin egg homogenates (nanomolar—the standard assay for cADPR) may be due to the fact that small accessory proteins, such as FKBP12.6 or calmodulin, which would be lost in membrane or permeabilized preparations, are necessary for expression of cADPR full potency [either directly (Lee et al., 1994; Tanaka and Tashjian, Jr., 1995) or indirectly (Takasawa et al., 1995)].

It was recently demonstrated in preparations from striated muscles (rabbit skeletal and canine cardiac) that 1) RyR is insensitive to cADPR (up to 10 μM) when reconstituted into planar lipid bilayers (channel \(P_o\)) or in purified or crude microsomes (energized Ca\(^{2+}\) uptake or passive Ca\(^{2+}\) leak), 2) \(^{32}P\)cADPR does not bind significantly (<1/10,000 equivalent of binding) to purified or crude microsomes, and 3) cADPR does not affect FKBP12 or 12.6 association to crude microsomes (Copello et al., 2001). Care was taken to use cADPR from several sources to optimize the experimental conditions for cADPR activity (Iino et al., 1997; Thomas et al., 2001), verify retention of FKBP in crude microsomes, and demonstrate the classical effects of known RyR modulators.
(Ca\(^{2+}\), caffeine, ruthenium red). Thus, this study calls attention to the need to appreciate the molecular details of cADPR action in other mammalian tissues, including smooth muscles, and to take caution in extrapolating results.

Caged cADPR has been successfully used in non-smooth muscle mammalian cell types (Varadi and Rutter, 2002), including cardiac muscle cells (Guo et al., 1996; Cui et al., 1999). However, only one report has been published so far on its use in a smooth muscle preparation. In this study, caged cADPR was used to examine the effect of cADPR on Ca\(^{2+}\) signaling in voltage-clamped isolated guinea pig colon smooth muscle cells that express RyR but lack FKBP12.6 (Bradley et al., 2003). Photoreleased cADPR (50–500 \(\mu\)M) does not alter global or subsarcolemmal [Ca\(^{2+}\)]\(_{cyt}\) through CICR, nor does it enhance caffeine-induced CICR, despite the fact that it is releasing Ca\(^{2+}\) in sea urchin eggs. Furthermore, this lack of cADPR activity on Ca\(^{2+}\) release is unchanged by introduction of recombinant FKBP12.6 into the smooth muscle cells. However, photoreleased cADPR increases the rate of Ca\(^{2+}\) removal from the cytoplasm, an effect that is slowed by the cADPR antagonist 8-bromo-cADPR (see Section II.B.1.b.3.). These results suggest that cADPR modulates [Ca\(^{2+}\)]\(_{cyt}\) by promoting Ca\(^{2+}\) extrusion but not via RyR.

Structure-Activity Relationships. In terms of SAR, the design of five noticeably potent, but cell-impermeant, cADPR full agonists has been possible. The most potent of these is 3-deaza-cADPR (Fig. 11), which can induce Ca\(^{2+}\) release at a threshold concentration of 0.3 nM and with an EC\(_{50}\) value of 1 nM (in sea urchin egg homogenates), making it ~70 times more potent that cADPR (Wong et al., 1999). This activity is potentiated by caffeine (1 mM) and concentration-dependently (60–300 nM) inhibited by the cADPR antagonist 8-NH\(_2\)-cADPR (Wong et al., 1999). An increased lag time for Ca\(^{2+}\) release by 3-deaza-cADPR compared with cADPR suggests that binding of cADPR may involve formation of a hydrogen bond or electrostatic interaction between N3 of adenine and its receptor (Foskett and Wong, 1992; Wong et al., 1999).

The second most potent cADPR agonist is cADPcR (Fig. 11). In intact sea urchin eggs, injection of cADPcR (30–500 nM) increases [Ca\(^{2+}\)]\(_{cyt}\) by more than the levels produced with equivalent concentrations of injected cADPR, making cADPcR a superagonist (Shuto et al., 2001). This is very likely related to its almost complete enzymatic stability compared with cADPR when assayed in rat brain extract in the presence of recombinant CD38 (Shuto et al., 2001).

The third most potent cADPR agonist is 2’A-deoxy-cADPR (Fig. 11), which acts as a full agonist (in a sea urchin egg homogenate Ca\(^{2+}\) releasing assay) with an EC\(_{50}\) value of 58 nM compared with 32 nM for cADPR (Ashamu et al., 1997). Furthermore, 2’A-deoxy-cADPR (200 nM) desensitizes Ca\(^{2+}\) release induced by cADPR (100 nM), suggesting a similar mechanism of action for the two ligands (Ashamu et al., 1997). Enzymatically, its stability is similar to cADPR (Ashamu et al., 1997). The SAR suggests the importance of potential hydrogen bonding with cADPR binding sites by proton donation from the 2’-position hydroxyl moiety as a means of gaining potency.

Interestingly, substitution of the hydroxyl moiety in position 2’ by a phosphate group results in an analog inactive in sea urchin egg homogenates (Aarhus et al., 1995b; Ashamu et al., 1997) but which is at least as effective as cADPR in releasing Ca\(^{2+}\) from rat brain microsomes (Vu et al., 1996) and permeabilized T-lymphocytes (Guse et al., 1997). This may reflect subtle differences between cADPR-sensitive Ca\(^{2+}\)-releasing mechanisms for the RyR of sea urchin eggs and mammalian cells (Ashamu et al., 1997; Lee, 1997), and it is unclear whether inactivity in the sea urchin eggs is the result of charge addition and/or increase in steric volume (Ashamu et al., 1997). In this regard, it is worth mentioning the discovery of a new Ca\(^{2+}\) current activated by cADPR in sea urchin egg microsomes reconstituted into planar lipid bilayers; this current has different unitary conductance than RyR, despite the fact that it is similarly modulated by caffeine, ruthenium red, and calmodulin (Perez et al., 1998).

The fourth agonist of significant potency is cArisDPR (Fig. 11). In the sea urchin egg homogenate Ca\(^{2+}\) releasing assay, cArisDPR is a full agonist with an EC\(_{50}\) value of 80 nM compared with 30 nM for cADPR, thus having about one-third the potency of cADPR (Bailey et al., 1996). However, it is degraded significantly more slowly in the homogenates, with a \(t_{1/2}\) of 170 min versus 15 min for cADPR (Bailey et al., 1996).

The last agonist of noticeable potency is 3’A-deoxy-cADPR (Fig. 11), with an EC\(_{50}\) value of ~5 \(\mu\)M, which is a potency of ~100-fold lower than that of 2’A-deoxy-cADPR (EC\(_{50}\) value of 58 nM) and ~150-fold lower than that of cADPR (EC\(_{50}\) value of 32 nM) (Ashamu et al., 1997). Consistent with these findings, like 2’A-deoxy-cADPR, 3’A-deoxy-cADPR also desensitizes Ca\(^{2+}\) release induced by cADPR (100 nM) but at a concentration 100 times larger (20 \(\mu\)M) (Ashamu et al., 1997). This difference in potency does not appear to be due to a difference in enzymatic stability, suggesting that the 3’-position hydroxyl group of the adenosine ribose is more critical to cADPR that the 2’-position one for agonist activity (Ashamu et al., 1997).
The design of significantly potent antagonists has been quite successful using position-8 substitutions, with potency roughly decreasing with increasing size of the substituting groups, thus highlighting the crucial importance of the cADPR hydrogen atom on adenine ring 8-position in its agonistic effect. For instance, it has led to 8-N3-cADPR (Fig. 11), the most potent antagonist known, which competitively and fully antagonizes at a nearly maximally effective concentration of cADPR (135 nM) with an IC50 value ~10 nM in the sea urchin egg homogenate Ca2+-releasing assay (Walseth and Lee, 1993). Furthermore, this effect is immediate, as 8-NH2-cADPR is effective even when added after cADPR-induced Ca2+ release had been initiated (Walseth and Lee, 1993). In contrast, this analog is ineffective in blocking either ryanodine- or caffeine-induced Ca2+ release, demonstrating the selective nature of its antagonist activity and suggesting that the cADPR binding site is distinct from those of ryanodine and caffeine (Walseth and Lee, 1993).

The second most potent antagonist designed so far is 8-N3-cADPR with an IC50 value ~0.45 μM against cADPR (135 nM) in the sea urchin egg microsomal Ca2+-releasing assay (Walseth and Lee, 1993). 8-N3-cADPR and cADPR reciprocally displace each other in binding to the microsomes. However, its most remarkable property is that it is a photoaffinity probe for cADPR binding sites; photolysis of microsomes preincubated with [32P]8-N3-cADPR resulted in specific labeling of proteins of 140 and 100 kDa, which could be specifically prevented by 8-N3-cADPR or by nanomolar concentrations of cADPR (Walseth and Lee, 1993). Interestingly, caffeine preferentially inhibits the labeling of the 100-kDa protein as compared with the 140-kDa protein. In keeping with the cADPR requirement for the protein FKBP12.6 in cultured coronary arterial smooth muscle cells (discussed above), these results also suggest that cADPR may not directly interact with the RyR to alter its activity but could do so by acting through accessory proteins (Walseth and Lee, 1993).

The third most potent antagonist is 7-deaza-8-Br-cADPR (Fig. 11), which has an IC50 value ~0.7 μM against cADPR (100 nM) (Sethi et al., 1997). However, its most remarkable properties, in contrast to the other 8-substituted antagonists, are its resistance to hydrolysis (heat- and enzymatically-mediated), owing to its substitution of the 7-position nitrogen atom by a carbon atom (Bailey et al., 1997), and its cell permeability, owing to the lipophilic character of its 8-bromo and 7-CH moieties (Sethi et al., 1997).

The fourth most potent antagonist is 8-Br-cADPR (Fig. 11), with an IC50 value ~1 μM against cADPR (100 nM) (Sethi et al., 1997). Like 7-deaza-8-Br-cADPR, this analog is also cell-permeant due to the lipophilicity of its 8-bromo moiety; however, it lacks the enzymatic stability of 7-deaza-8-Br-cADPR (Sethi et al., 1997).

3′-OMe-cADPR (Fig. 11) is the last antagonist of significant potency with an IC50 value of ~5 μM against cADPR (100 nM) (Ashamu et al., 1997). In contrast with the OH moiety in the 3′-position of the adenosine ribose in cADPR, the methoxyl group is larger and cannot donate a proton for potential hydrogen bonding with cADPR binding site, although it can still act as an acceptor (Ashamu et al., 1997). Thus, it appears that proton donation may indeed be critical for agonist activity, whereas an oxygen atom is sufficient for recognition of the cADPR binding site (Ashamu et al., 1997).

c. Selectivity. It is unknown whether cADPR can affect the activity of all three known mammalian RyR isoforms. For instance, cADPR (1 μM) augments Ca2+-induced (100 μM) Ca2+ release and reduces its inhibition by ryanodine (50 μM) in brain and cardiac microsomes (which express RyR2). In addition, cADPR (1 μM) increases [3H]ryanodine binding to cardiac microsomes in the presence of Ca2+ (100 μM) (Meszaros et al., 1993), which concurs with the observation that cADPR (10 μM) augments the P0 of sheep cardiac SR Ca2+ channels (presumably RyR2) reconstituted into planar lipid bilayers (Sitsapesan et al., 1994). Interestingly, the latter effect is inhibited by physiologic concentrations of ATP (100 μM-1 mM), suggesting that a direct interaction of cADPR with RyR2 is unlikely to occur in the intact cardiac muscle cell (Sitsapesan et al., 1994). However, both studies are at odds with the lack of effect of cADPR (0.1–5 μM) on the activity of SR Ca2+ channel reconstituted into planar lipid bilayers or on [3H]ryanodine binding to microsomes from procone cardiac muscle (Fruen et al., 1994).

In sheep skeletal muscle SR Ca2+ channel (RyR1) reconstituted into planar lipid bilayers, cADPR (1–10 μM) increases P0 with a [Ca2+]i of 10 μM (Sitsapesan and Williams, 1995). However, this effect is proportional to luminal [Ca2+] (1–50 mM), and channel conductance is still susceptible to partial inhibition by ryanodine (1 μM), suggesting that the cADPR effect is on the RyR and not on another type of SR channel (Sitsapesan and Williams, 1995). This may explain why other groups have been unable to show an effect of cADPR on CICR in skeletal muscle microsomes (Meszaros et al., 1993; Morrise et al., 1993) since their luminal [Ca2+] may have been too low (e.g., 1–4 μM) (Sitsapesan and Williams, 1995).

Although the lack of effect of cADPR (0.1–5 μM) on the activity of SR Ca2+ channel reconstituted into planar lipid bilayers or on [3H]ryanodine binding to microsomes from procone cardiac muscle (Fruen et al., 1994) still remains to be reconciled, more recent studies support a stimulatory role of cADPR on RyR1. Indeed, cADPR enhances a ruthenium red (1 μM)- and ryanodine (100 μM)-sensitive CICR in rabbit skeletal muscle (Yamaguichi and Kasai, 1997); however, cADPR increases [Ca2+]i in a ruthenium red-resistant manner when microinjected in intact porcine skeletal muscle fibers.
without changes in plasma membrane potential (Lopez et al., 2000). Also, in RyR1-expressing microsomes from RyR3 knockout mouse diaphragm muscle (which normally only expresses RyR1 and RyR3), cADPR (0.1–2 μM) amplifies caffeine-induced (10 mM) Ca^{2+} release (Fulceri et al., 2001), although at 1 μM, it does not affect the P_0 of the SR Ca^{2+} channel when reconstituted into planar lipid bilayers (Sonnleitner et al., 1998). In contrast, cADPR (1 μM) increases the P_0 of reconstituted SR Ca^{2+} channels from wild-type mouse diaphragm muscle (10-fold leftward shift of the Ca^{2+}/P_0 curve) (Sonnleitner et al., 1998), suggesting that it also affects RyR3 activity.

cADPR appears selective for CICR versus IICR in smooth muscle. For instance, in microsomes from freshly cultured rat aortic smooth muscle cells that express RyR1–3, cADPR (1–10 μM) induces Ca^{2+} release that is fully inhibited by 8-Br-cADPR (40 μM) and ruthenium red (10 μM), whereas InsP_3 stimulates (1–8 μM) Ca^{2+} release that is fully inhibited by heparin (1 mg/ml) (Yusufi et al., 2002). Although both agents induce maximal Ca^{2+} release of similar magnitude, the cADPR effect is insensitive to heparin (Yusufi et al., 2002). Likewise, in saponin-permeabilized freshly cultured seminiferous tubular smooth muscle cells from rat testis that express InsP_3R1–3 but only RyR2 among RyR isoforms, cADPR-induced (10 μM) Ca^{2+} release from a SR Ca^{2+} store was shown to be independent from that released by InsP_3 (10 μM) but fully inhibited by 8-Br-cADPR (50 μM) or ryanodine (5 μM) (Barone et al., 2002). In contrast, cADPR (10 μM)-induced Ca^{2+} release was shown to be resistant to heparin (10 μg/ml), which is nevertheless able to fully inhibit the InsP_3 effect (Barone et al., 2002). It was observed in inside-out plasma membrane patches that cADPR (1–10 μM) concentration-dependently reduces K_{Ca} P_0 by up to 75% (Li et al., 1997).

d. Use in Smooth Muscle Preparations. The role of cADPR in the regulation of smooth muscle function is unclear; there are no studies indicating that the inhibition of the synthesis or activity of this endogenous ligand for the RyR causes alteration in either mechanical or electrical activity under basal conditions. In an exhaustive study of smooth muscle from three animal species (human, guinea pig, and rabbit), Izuka et al. (1998) were unable to detect any Ca^{2+} release even with supramaximal concentrations of cADPR, either under resting or stimulated conditions. The synthesis of membrane-permeant analogs has assisted in the use of cADPR agonists and antagonists. Some success in smooth muscle has been reported with the use of 10 μM cADPR or 100 μM 8Br-cADPR (Table 4). However, the constriction with exogenously administered cADPR requires high concentrations and is delayed in onset, with a plateau response occurring some 25 min after administration.

The evidence that cADPR has a functional role in smooth muscle is relatively weak. For example, Nixon et al. (1994) were unable to elicit responses with cADPR in permeabilized rat aorta and vas deferens. Important persuasive evidence that cADPR is ineffective as an RyR modulator in smooth muscle comes from the detailed study by McCarron’s group (Bradley et al., 2003), discussed above (see Section II.B.1.b.), where caged cADPR failed to cause Ca^{2+} release, with no detectable changes in either subsarcolemmal or global [Ca^{2+}]_cyt. As discussed by Bradley et al. (2003), cADPR is also ineffective in other nonvascular (bronchial, intestinal, and vas deferens) and vascular (aorta, coronary, and cerebral arteries) smooth muscle preparations. Rather than mediating constriction, cADPR is proposed to increase the rate of Ca^{2+} removal from the cytoplasm, likely through an action on the PMCA (Bradley et al., 2003).

2. Caffeine and 9-Methyl-7-bromoeudistomin D.

a. Source and Chemical Structure. Caffeine is an alkaloid occurring in plants that are geographically widely distributed (e.g., Thea sinensis L). It is a methylated xanthine derivative (1,3,7-trimethylxanthine; Fig. 13). Xanthine is a dioxypurine structurally related to uric acid. 9-Methyl-7-bromoeudistomin D (MBED; Fig. 13) is a synthesized compound (Nakamura et al., 1986; Kobayashi et al., 1989a; Seino et al., 1991; Takahashi et al., 1995) derived from a natural product, eudistomin D (Fig. 13), from the Caribbean tunicate Eudistoma olivaceum (Kobayashi et al., 1984). However, in terms of physicochemical properties, MBED is a larger molecule than caffeine and possesses a much higher affinity for phospholipid membranes, which may explain its distinct effect compared with caffeine (see Section II.B.2.b.).

b. Mechanism of Action. The primary site of action for caffeine is believed to be the RyR (Zucchi and Ronca-Testoni, 1997). Essentially, caffeine increases the RyR Ca^{2+} sensitivity by increasing its P_0 without changing its conductance, as shown in single-channel experiments done with RyR purified from cardiac (Rousseau et al., 1987; Rousseau and Meissner, 1989; Sitsapesan and Williams, 1990) and skeletal (Rousseau et al., 1988) muscles and reconstituted into planar lipid bilayers. At low concentrations (0.5 to 2 mM), RyR activation requires submicromolar Ca^{2+} and P_0 is increased by a reduction of the channel closed-state lifetime, whereas at higher concentrations (>5 to 10 mM), RyR activation requires picomolar Ca^{2+} and P_0 is increased by a prolongation of the open-state lifetime. The specific binding site of caffeine on the RyR has not yet been established. However, it is unlikely to be that of adenine nucleotides, despite the structural resemblance of caffeine to these molecules (Zucchi and Ronca-Testoni, 1997). In terms of its pharmacological action on the RyR, caffeine is a class II activator as are Ca^{2+} and adenine nucleotides (Zucchi and Ronca-Testoni, 1997); they all increase RyR P_0, without altering its conductance, and they increase its affinity for [3H]ryridine. It has been proposed that their binding sites are distinct but interacting synergistically (Pessah et al., 1987).
as assessed by fura-2-reported \([\text{Ca}^{2+}]_{\text{cyt}}\) (Herrmann-Frank et al., 1991). By comparison, 5 mM caffeine increases the sensitivity of CICR by \(\approx 30\) times, as assessed by fura-2-reported \([\text{Ca}^{2+}]_{\text{cyt}}\), in saponin-permeabilized guinea pig taenia caeci (Iino, 1989). Caffeine also increases the rate of SR \(\text{Ca}^{2+}\) release in a concentration-dependent manner (1 to 25 mM). Furthermore, caffeine by itself (i.e., without \(\text{Ca}^{2+}\)) has no \(\text{Ca}^{2+}\)-releasing effect.

Interestingly, low temperature amplifies CICR induced by caffeine as monitored by the \([\text{Ca}^{2+}]_{\text{cyt}}\) transient reported by fura-2 in endothelium-free rat aorta strips.

### Table 4: Effective concentrations of cADPR and analogs in smooth muscle

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Animal Species</th>
<th>Experimental Details (Preparation; Measurement Method; Measured Response)</th>
<th>Effective Concentrations of cADPR and Analogs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Airways</td>
<td>Human</td>
<td>Isolated (\alpha)-toxin-permeabilized strips; isometric dynamometry; contractile activity</td>
<td>No effect at up to 300 (\mu)M (cADPR)</td>
<td>Iizuka et al., 1998</td>
</tr>
<tr>
<td>Aorta</td>
<td>Guinea pig</td>
<td>Isolated (\alpha)-toxin-permeabilized strips; isometric dynamometry; contractile activity</td>
<td>100 (\mu)M (no effect) (cADPR)</td>
<td>Nixon et al., 1994</td>
</tr>
<tr>
<td>Aorta</td>
<td>Rat</td>
<td>Microsomes; (\text{45Ca}^{2+}) loading; (\text{Ca}^{2+}) fluxes</td>
<td>10 and 40 (\mu)M (8-Br-cADPR)</td>
<td>Yusufi et al., 2002</td>
</tr>
<tr>
<td>Colon</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle cells; whole-cell patch-clamping; fluo-3 cellular loading; fluorescence or wide-field fluorescence digital imaging; (\text{Ca}^{2+}) Imaging</td>
<td>50-500 (\mu)M (caged cADPR)</td>
<td>Bradley et al., 2003</td>
</tr>
<tr>
<td>Coronary artery</td>
<td>Cattle</td>
<td>Microsomal membranes, stripped or not of FKB-12.6, incorporated into planar lipid bilayers; bilayer clamp amplification; single (\text{Ca}^{2+}) channel currents</td>
<td>10 (\mu)M (cADPR)</td>
<td>Tang et al., 2002</td>
</tr>
<tr>
<td>Coronary artery</td>
<td>Cattle</td>
<td>Microsomal membranes, stripped or not of FKB-12.6, incorporated into planar lipid bilayers; bilayer clamp amplification; single (\text{Ca}^{2+}) channel currents</td>
<td>0.01-1 (\mu)M (cADPR)</td>
<td>Li et al., 2001</td>
</tr>
<tr>
<td>Coronary artery</td>
<td>Cattle</td>
<td>Isolated (\alpha)-toxin-permeabilized smooth muscle cells; fura-2 cellular loading, fluorescence; ([\text{Ca}^{2+}]_{\text{cyt}}) Imaging</td>
<td>5 (\mu)M (cADPR)</td>
<td>Yu et al., 2000</td>
</tr>
<tr>
<td>Coronary artery</td>
<td>Cattle</td>
<td>Isolated smooth muscle cells; fura-2 cellular loading, fluorescence; ([\text{Ca}^{2+}]_{\text{cyt}}) Imaging</td>
<td>100 (\mu)M (8-Br-cADPR)</td>
<td>Geiger et al., 2000</td>
</tr>
<tr>
<td>Coronary artery</td>
<td>Pig</td>
<td>Isolated (\beta)-escin-permeabilized smooth muscle cells; fura-2 cellular loading, ([\text{Ca}^{2+}]_{\text{cyt}}) Imaging</td>
<td>2 (\mu)M (cADPR)</td>
<td>Kannan et al., 1996</td>
</tr>
<tr>
<td>Ileum</td>
<td>Guinea pig</td>
<td>Isolated (\alpha)-toxin-permeabilized strips; isometric dynamometry; contractile activity</td>
<td>No effect at up to 300 (\mu)M (cADPR)</td>
<td>Iizuka et al., 1998</td>
</tr>
<tr>
<td>Mesenteric resistance arteries</td>
<td>Rabbit</td>
<td>Isolated 2nd- and 3rd-order branches endothelium-intact or -denuded rings and saponin-permeabilized smooth muscle cells; whole-cell patch-clamping (cells), isometric dynamometry (rings); membrane potential (cells), contractile activity (rings)</td>
<td>30 (\mu)M (cADPR)</td>
<td>Wilson et al., 2001</td>
</tr>
<tr>
<td>Pulmonary artery</td>
<td>Cattle</td>
<td>Microsomes; extramicrosomal fluo-3 and fluoro or (\text{45Ca}^{2+}) loading; (\text{Ca}^{2+}) fluxes</td>
<td>10 nM-1 (\mu)M (cADPR)</td>
<td>Stout et al., 2002</td>
</tr>
<tr>
<td>Pulmonary resistance arteries</td>
<td>Rabbit</td>
<td>Isolated 2nd- and 3rd-order branches endothelium-intact or -denuded rings and saponin-permeabilized smooth muscle cells; whole-cell patch-clamping (cells), isometric dynamometry (rings); membrane potential (cells), contractile activity (rings)</td>
<td>30 and 300 (\mu)M (8-Br-cADPR)</td>
<td>Wilson et al., 2001</td>
</tr>
<tr>
<td>Pulmonary resistance arteries</td>
<td>Rat</td>
<td>Isolated endothelium-denuded rings; isometric dynamometry; contractile activity</td>
<td>300 (\mu)M (8-Br-cADPR)</td>
<td>Dipp and Evans, 2001</td>
</tr>
<tr>
<td>Renal resistance arteries</td>
<td>Rat</td>
<td>Isolated, cannulated and pressurized segments and (\beta)-escin-permeabilized cells; fura-2 cellular loading, fluorescence; diameter monitoring by video microscopy; ([\text{Ca}^{2+}]_{\text{cyt}}), contractile activity</td>
<td>10 (\mu)M (cADPR)</td>
<td>Li et al., 2000</td>
</tr>
<tr>
<td>Stomach (antrum)</td>
<td>Pig</td>
<td>Microsomes; extramicrosomal fluo-3 and fluoro or (\text{45Ca}^{2+}) loading; (\text{Ca}^{2+}) fluxes</td>
<td>10 nM-1 (\mu)M (cADPR)</td>
<td>Stout et al., 2002</td>
</tr>
<tr>
<td>Testicular peritubules</td>
<td>Rat</td>
<td>Isolated saponin-permeabilized smooth muscle cells; fura-2 cellular loading, fluorescence; ([\text{Ca}^{2+}]_{\text{cyt}}) Imaging</td>
<td>10 and 50 (\mu)M (cADPR)</td>
<td>Barone et al., 2002</td>
</tr>
<tr>
<td>Trachea</td>
<td>Cattle</td>
<td>Isolated smooth muscle cells; fura-2 cellular loading, fluorescence; ([\text{Ca}^{2+}]_{\text{cyt}}) Imaging</td>
<td>100 (\mu)M (cADPR)</td>
<td>Franco et al., 2001</td>
</tr>
<tr>
<td>Trachea</td>
<td>Pig</td>
<td>Isolated (\beta)-escin-permeabilized smooth muscle cells; furo-3 cellular loading, confocal fluorescence microscopy; ([\text{Ca}^{2+}]_{\text{cyt}}) Imaging</td>
<td>1-10 (\mu)M (cADPR)</td>
<td>Prakash et al., 1998</td>
</tr>
<tr>
<td>Trachea</td>
<td>Rabbit</td>
<td>Isolated (\alpha)-toxin-permeabilized strips; isometric dynamometry; contractile activity</td>
<td>No effect at up to 300 (\mu)M (cADPR)</td>
<td>Iizuka et al., 1998</td>
</tr>
<tr>
<td>Vas deferens</td>
<td>Guinea pig</td>
<td>Isolated (\alpha)-toxin-permeabilized strips; isometric dynamometry; contractile activity</td>
<td>100 (\mu)M (no effect) (cADPR)</td>
<td>Nixon et al., 1994</td>
</tr>
</tbody>
</table>
In contrast, RyR2 is present only in pregnant human myometrium and in TGF-β-exposed cultured human myometrial cells (Awad et al., 1997). TGF-β exposure creates a caffeine-induced $^{45}$Ca$^{2+}$ efflux from these cultured cells that are normally unresponsive to caffeine, constitutively expressing only RyR3. This is consistent with the fact that the RyR3 isoform expressed in mink lung epithelial cells is caffeine insensitive (Giannini et al., 1992; Morgan and Gillespie, 1995). Curiously, caffeine (10 mM) amplifies the ryanodine-induced $^{45}$Ca$^{2+}$ efflux in permeabilized human myometrial cells, which may indicate the action of caffeine on the RyR1 isoform to promote the actions of ryanodine on $P_o$ (Lynn et al., 1995).

In β-escin-permeabilized guinea pig mesenteric artery and longitudinal ileum smooth muscles, caffeine causes a contractile activity and Ca$^{2+}$ transient that is accompanied by STOC through BK channels (Imaizumi et al., 1993, 1996). Interestingly, MBED, a compound structurally related to caffeine (see Section II.B.2.a) and originally shown to be a potent releaser of Ca$^{2+}$ from skeletal muscle microsomes (Seino et al., 1991) and to bind to RyR like caffeine (Fang et al., 1993; Adachi et al., 1994), fails to activate Ca$^{2+}$ transients or contractile activity, even at high concentrations (up to 300 μM), despite the fact that it activates BK channels (at 30 μM) like caffeine (5–10 mM) (Imaizumi et al., 1993, 1996). The basis for these contrasting profiles was recently elucidated using confocal Ca$^{2+}$ imaging and whole-cell voltage-clamp methods in guinea pig urinary bladder isolated smooth muscle cells (Ohi et al., 2001a). It was shown that MBED increases $\left[Ca^{2+}\right]_{cyt}$, mainly in the subplasmalemmal space synchronously with BK channels activation. This suggests that MBED selectively induces superficial SR Ca$^{2+}$ release that activates BK channels but does not stimulate the contractile apparatus. Since MBED does not directly activate BK channels (Imaizumi et al., 1996), it is likely that this selective action is due to its high lipophilicity compared with caffeine that may concentrate it in the junctional SR areas (see Section I.B.) (Ohi et al., 2001a)—this is consistent with the longer exposure of isolated smooth muscle cells (2 min versus few seconds) required to decrease the amount of Ca$^{2+}$ releasable by a subsequent exposure to caffeine (Ohi et al., 2001a).

c. Selectivity. Caffeine has several nonselective effects in smooth muscle. First, the SR Ca$^{2+}$ release induced by InsP$_3$ could be inhibited by caffeine, as shown in permeabilized A7r5 cells (Missiaen et al., 1994). However, in these cells, caffeine does not affect the binding of InsP$_3$ to its purified receptor, isocaffeine (differing from caffeine by the position of one methyl group) is ineffective in inhibiting InsP$_3$-induced SR Ca$^{2+}$ release, and ATP prevents the inhibitory effect of caffeine. This suggests that caffeine has to bind to a specific site on the InsP$_3$R different from that of InsP$_3$ but very likely that of ATP, given the structural resemblance of the two
molecules. Caffeine binding at this latter site probably induces a conformational change in the InsP_{3}R modifying its gating properties and inhibiting its opening upon InsP_{3} binding, hence inhibiting IICR. Consistent with these findings, caffeine decreases the P_{o} of purified InsP_{3}R reconstituted into planar lipid bilayers without reducing specific [{}^{3}H]InsP_{3} binding to its receptor (Bezprozvannya et al., 1994).

Second, caffeine can inhibit plasmalemmal Ca_{L} in cultured myometrial cells from pregnant rats (Martin et al., 1989). In these cells, caffeine inhibits the depolarization-induced Ca^{2+} current with an IC_{50} value of 35 mM. This effect shows no use-dependence, and caffeine does not alter the steady-state inactivation of the Ca^{2+} current. Furthermore, caffeine inhibits the specific binding of [{}^{3}H]Irsadipine, a specific Ca_{L} blocker, to myometrial membranes with a similar IC_{50} value without altering the dissociation constant. Caffeine also causes a rapid and reversible concentration-dependent (1 to 30 mM) blockade of the Ba^{2+} current (occurring through Ca_{L}) in freshly isolated smooth muscle cells from the rabbit ear artery (Hughes et al., 1990). This effect has no voltage- or use-dependence, and caffeine does not alter the steady-state inactivation of the Ba^{2+} current. Also, inhibition of the Ba^{2+} current is unaffected by ryanodine and intracellular Ca^{2+} buffering with EGTA or 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′′-tetraacetic acid. The effect on the Ba^{2+} current is not due to caffeine-induced cAMP phosphodiesterase (PDE) inhibition (Poch and Umfahrer, 1976), as nonmethylxanthine cAMP PDE inhibitors do not inhibit the Ba^{2+} current. These findings are consistent with data obtained in freshly isolated smooth muscle cells from the guinea pig ileum (Zholos et al., 1991a, b), where caffeine (10 mM) substantially inhibits depolarization pulse-induced Ca^{2+} currents. This latter effect occurs in a biphasic manner: a transient component, associated with Ca^{2+}-dependent inactivation of Ca^{2+}_{L} resulting from Sr Ca^{2+} release, and a tonic component. This latter tonic component is not due to activation of additional ionic currents of opposite direction or to inhibition of PDE activity, and it is unaffected by Ca^{2+} cyt buffering by EGTA, RyR blockers procaine and ruthenium red, or by ryanodine. In summary, results obtained in three different types of smooth muscles suggest that caffeine can block Ca_{L} by specifically occupying a binding site on the channel independently of its SR Ca^{2+} releasing ability or of its potential cAMP PDE inhibition.

Third, caffeine can activate a plasmalemmal Ca^{2+}-permeant nonselective cation channel as shown in the freshly isolated toad gastric smooth muscle cells (Guerrero et al., 1994). Current through such a channel (~20% of this current was estimated to be carried by Ca^{2+}) is elicited by caffeine (20 mM) in 85% of the cells studied; ryanodine only partially prevents the increase in [Ca^{2+}]_{cyt} accompanying exposure to caffeine. Almost complete blockade of the current and the associated [Ca^{2+}]_{cyt} increase occurs with Gd^{3+}, a nonselective cation channel and Ca_{L} blocker, or by decreasing the Ca^{2+} influx electrochemical driving force. Intracellular Ca^{2+} buffering with 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′′-tetraacetic acid does not prevent the activation of the current by caffeine, although this maneuver eliminates the associated [Ca^{2+}]_{cyt} increase. Finally, the cAMP PDE-resistant cAMP analog 8-bromo-cAMP is not able to induce the current, nor is it able to inhibit the current activation by caffeine. These results suggest that caffeine can activate a plasmalemmal nonselective cation channel independent of its SR Ca^{2+}-releasing ability or of its potential cAMP PDE inhibition. It has been proposed (Guerrero et al., 1994) that this proceeds either through a direct interaction between caffeine and the channel or through a linkage between RyR on the peripheral SR and the channel. Caffeine then acts on the latter indirectly through its direct interaction with the RyR. Whether this channel or its analog exists in mammalian smooth muscle is unknown. Related to this observation, caffeine can increase a voltage-sensitive Ca^{2+} influx in freshly isolated guinea pig jejunal smooth muscle cells (Pacaud and Bolton, 1991b). However, the identity of the plasmalemmal structure involved in this effect of caffeine is unknown.

Fourth, caffeine directly inhibits the contractile apparatus in Triton X-100-permeabilized chicken gizzard smooth muscle (Ozaki et al., 1990). In this tissue, caffeine (1 to 40 mM) inhibits the contractile activity induced by 3 μM of free Ca^{2+}. These concentrations of caffeine also inhibit the phosphorylation of the regulatory 20-kDa myosin light chain (LC_{20}) in native actomyosin preparations. LC_{20} steady-state phosphorylation is the result of the activity ratio of myosin light chain kinase and myosin light chain phosphatase (MLCP), and Ca^{2+}-induced LC_{20} phosphorylation is triggered by activation of MLCK through binding of Ca^{2+}-activated calmodulin (Walsh, 1994). However, calmodulin activity is not affected by 20 mM of caffeine. Likewise, time-dependent dephosphorylation of LC_{20} upon removal of Ca^{2+}, an indicator of MLCP activity, is not affected by caffeine. These results suggest that caffeine inhibits MLCK directly. Caffeine also inhibits the Ca^{2+}-independent contractile activity in thiophosphorylated tissue, where contractile activity is due to the irreversible thio-phosphorylation of LC_{20} (MLCP cannot hydrolyze thio-phosphate bonds). Thus, caffeine also inhibits the basic actin-myosin interactions leading to force production.

In contrast, data obtained in saponin-permeabilized guinea pig mesenteric artery shows that caffeine (25 mM) does not alter either the minimum free [Ca^{2+}] required to induce contractile activity, the maximal Ca^{2+}-induced contractile activity, or the slope of the free [Ca^{2+}]-force relationship (Itoh et al., 1981). Likewise, caffeine (5 mM) does not alter the free [Ca^{2+}]-force relationship in saponin-permeabilized porcine coronary artery (Itoh et al., 1982a). However, caffeine (10 mM)
slightly suppresses contractile activity of saponin-permeabilized rabbit mesenteric artery (Itoh et al., 1983), and high concentrations of caffeine (50 mM) inhibit the maximal Ca$^{2+}$-induced contractile activity in saponin-permeabilized longitudinal myometrial smooth muscle from pregnant rats (Savineau et al., 1988). It is possible that the inhibitory effects of caffeine reported in chicken gizzard smooth muscle are avian-specific and/or variable among mammalian smooth muscles.

It is unlikely that inhibition of contractile activity with caffeine is due to inhibition of CiMP PDE, although cAMP concentration was increased by caffeine (≤5 mM) during relaxation of intact gizzard smooth muscle (Ozaki et al., 1990). cAMP-induced relaxation of phenylephrine- or histamine-induced contractile activity in the porcine carotid artery is not associated with an alteration of the Ca$^{2+}$ dependence of LC$_{20}$ phosphorylation or the LC$_{30}$ phosphorylation dependence of force production (McDaniel et al., 1991).

It should be mentioned that caffeine could affect other molecular targets such as 5′-nucleotidase (inhibition), adenosine receptors (nonselective antagonism), and GABA/benzodiazepine receptors (antagonism) (Sawynok and Yaksh, 1993), although the significance of these effects in smooth muscle preparations is not likely to be significant.

Caffeine also has secondary effects in smooth muscle either directly associated with Ca$^{2+}$ released from the SR or associated with the subsequent Ca$^{2+}$ depletion of the SR. First, caffeine increases [Ca$^{2+}$]$_{cyt}$ in the subplasmalemmal space. For instance, the [Ca$^{2+}$]$_{cyt}$ increase induced by caffeine (20 mM) through SR Ca$^{2+}$ release in the freshly isolated toad gastric smooth muscle cell rises ~15 times faster as reported by the membrane-associated low-affinity fluorescent Ca$^{2+}$ probe FFP-18 (>65% being located near or at the plasmalemma) than as reported by fura-2 (uniformly distributed throughout the cytoplasm) (Etter et al., 1996). Also, [Ca$^{2+}$]$_{cyt}$ reported by FFP-18 reaches levels about 3 times higher than that reported by fura-2. In freshly isolated bovine, porcine, and guinea pig coronary artery smooth muscle cells (Stehno-Bittel and Sturek, 1992; Ganitkevich, 1996), the $K_{Ca}$-mediated current was used to report subplasmalemmal [Ca$^{2+}$]$_{cyt}$. It was monitored simultaneously with fura-2 "global" [Ca$^{2+}$]$_{cyt}$ signals. It was shown that caffeine-induced (5 or 10 mM) SR Ca$^{2+}$ release raises $K_{Ca}$-mediated current faster than the fura-2 signal. The current also peaks and decays faster. This could be interpreted as a faster caffeine-induced Ca$^{2+}$ release from the peripheral SR, because caffeine reaches peripheral SR before it can stimulate central SR (Ganitkevich, 1996). The transient increase in $K_{Ca}$-mediated current could explain the transient plasmalemmal hyperpolarization associated with exposure to caffeine (10 mM) (Itoh et al., 1992). It also reflects the potential ability of caffeine to activate plasmalemmal Ca$^{2+}$-dependent processes through Ca$^{2+}$ release from the peripheral SR.

Currents mediated by Cl$_{Ca}$ (for review, see Carl et al., 1996) and Ca$^{2+}$-activated nonspecific cation channels (Lorand et al., 1991; Janssen and Sims, 1992; Sims, 1992; Wang and Kotlikoff, 1997) are transiently induced by caffeine in vascular and visceral smooth muscle cells. Caffeine also transiently inhibits Ca$^{2+}$,L-mediated currents in freshly isolated smooth muscle cells from the guinea pig ileum (Zholos et al., 1991), an effect that is inhibited by the RyR blockers procaine and ruthenium red, suggesting that this transient inhibition of Ca$^{2+}$,L-mediated current is due to Ca$^{2+}$,L-dependent inactivation of Ca$^{2+}$,L caused by the caffeine-induced SR Ca$^{2+}$ release.

Second, a long exposure time to caffeine (more than several minutes) prevents access of Ca$^{2+}$ influx to the myofilaments in the porcine carotid artery (Rembold et al., 1995). In this tissue, caffeine (20 mM) first induces synchronized transient increases in aequorin-reported [Ca$^{2+}$]$_{cyt}$, fura-2-reported [Ca$^{2+}$]$_{cyt}$, LC$_{20}$ phosphorylation, and force peaking within 1.5 min. Both the aequorin and the fura-2 signals decline to steady-state levels within 10 min of exposure to higher than resting values, whereas LC$_{30}$ phosphorylation and force go back to resting values. The transient in aequorin signal is larger than that in fura-2 signal. After 10 min of exposure to caffeine, the tissue was depolarized in the presence of caffeine for 10 more minutes. In the presence of caffeine (20 mM), high [K$^+$/H$^+$]-induced (109 mM) depolarization lead to Ca$^{2+}$ influx through Ca$^{2+}$,L associated with a maintained increase in the aequorin signal but in only a transient increase in the fura-2 signal. This is not accompanied by LC$_{20}$ phosphorylation of force production. However, withdrawal of caffeine leads to an increase in fura-2 signal within 5 min to a steady-state level comparable to that reached by the transient peak initially induced, whereas the aequorin signal is not significantly altered. Also, within 10 min, LC$_{20}$ phosphorylation and force increase to steady-state levels. Based on these observations, the authors proposed that a ratio of the aequorin and fura-2 light signals could reflect the relative spatial heterogeneity of [Ca$^{2+}$]$_{cyt}$ distribution within the smooth muscle cells; the aequorin signal would report local changes in [Ca$^{2+}$]$_{cyt}$, whereas fura-2 would report average changes in [Ca$^{2+}$]i, assuming that both Ca$^{2+}$ probes are distributed in the same tissue volume and that the cellular responses across the tissue are synchronized and identical (Rembold et al., 1995). The following scheme is suggested by the results: 1) the initial transient increase in [Ca$^{2+}$]$_{cyt}$ induced by caffeine promotes LC$_{20}$ phosphorylation and subsequent force production; 2) the sustained increase in [Ca$^{2+}$]$_{cyt}$ persisting after the fading of both LC$_{20}$ phosphorylation and force could be due to persistent leakage of Ca$^{2+}$ taken up by the SR caused by caffeine-promoted opening of RyR (and also potentially to stimulated Ca$^{2+}$ influx indirectly induced by caffeine, see above); 3) the increase in the aequorin/fura-2 ratio accompanying these events
### TABLE 5

**Effective concentrations of caffeine and 9-methyl-7-bromoeadostin D (MBED) in smooth muscle**

<table>
<thead>
<tr>
<th>Tissue</th>
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suggested that caffeine induces a nonuniform increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in the smooth muscle cells; 4) the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by high $[\text{K}^+]_0$ in the presence of caffeine occurs away from the myofilaments since it does not increase LC$_{20}$ phosphorylation; 5) the sustained increase in the aequorin/fura-2 ratio upon exposure to high $[\text{K}^+]_0$ suggests further spatial heterogeneity in $[\text{Ca}^{2+}]_{\text{cyt}}$ distribution; and 6) upon removal of caffeine, the ratio value collapses because of an even redistribution of $[\text{Ca}^{2+}]_{\text{cyt}}$, which is associated with an increase of

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[Ca\(^{2+}\)]\(_{\text{c}}\) in the vicinity of the myofilaments as revealed by the increase in LC\(_{20}\) phosphorylation. Thus, these results suggest that caffeine can prevent access of Ca\(^{2+}\) to the myofilaments by localizing the increase in [Ca\(^{2+}\)]\(_{\text{c}}\) at remote sites. This could explain the often observed dissociation between Ca\(^{2+}\) influx or [Ca\(^{2+}\)]\(_{\text{c}}\) increase from LC\(_{20}\) phosphorylation and force production in the presence of caffeine (for review, see Karaki et al., 1997).

Third, caffeine could affect smooth muscle by depleting SR Ca\(^{2+}\). For instance, spontaneous Ca\(^{2+}\)-activated currents mediated by K\(_{\text{Ca}}\) and Cl\(_{\text{Ca}}\), termed STOCs and STICs (for spontaneous transient inward currents), respectively, are inhibited following caffeine-induced SR Ca\(^{2+}\) depletion in vascular and visceral smooth muscle cells (for review, see Carl et al., 1996). Inhibition of such spontaneous currents by caffeine leads to plasmalemmal depolarization and increased smooth muscle excitability (Itoh et al., 1981, 1982b; Savineau, 1988; Savineau and Mironneau, 1990). Caffeine-induced SR Ca\(^{2+}\) depletion can also lead to capacitative Ca\(^{2+}\) entry, a Ca\(^{2+}\) store depletion-activated Ca\(^{2+}\) influx (Janssen and Sims, 1993; Ohta et al., 1995).

Little information is available on MBED selectivity for RyR. However, it has been shown that its parent compound, bromo-eudistomin D (up to 30 \(\mu\)M), has no effect on the activity of skeletal muscle SERCA or Na\(^+/K\)\(^{-}\)-ATPase (Nakamura et al., 1986). On the other hand, it was shown that, in bovine aortic smooth muscle homogenates, \(^{3}H\)MBED has non-negligible soluble binding sites, suggesting that it may interact with non-RyR caffeine molecular targets such as phosphodiesterases (Adachi et al., 1994).

d. Use in Smooth Muscle Preparations. Caffeine has long been used to deplete SR Ca\(^{2+}\) stores in smooth muscle; this is frequently done in a Ca\(^{2+}\)-free medium containing Ca\(^{2+}\)-chelators such as EGTA. The transient contractile response obtained under such conditions is a qualitative estimate of the average size of the Ca\(^{2+}\) stores in the SR, assuming a linear relationship between Ca\(^{2+}\) released from the SR and the size of the contractile activity. Effective concentrations for caffeine range from 5 to 25 mM (Table 5). The use of caffeine in smooth muscle comes with several concerns: 1) it inhibits PDE and therefore raises cAMP, 2) it inhibits voltage-gated Ca\(^{2+}\) entry and activates a nonspecific inward current, 3) it has the potential to augment capacitative Ca\(^{2+}\) entry by depletion of the SR, and 4) it can also redistribute Ca\(^{2+}\) within cells such that it predominately remains at superficial sites.

3. Ryanodine.

a. Source and Chemical Structure. Ryanodine is a complex polycyclic, polyhydroxylic diterpene (\(+\)-ryanodol esterified at C3 with pyrrole-2-carboxylic acid (Fig. 14). Although this molecule is electrically neutral, it has a hydrophilic face with five hydroxyl groups at C2, C4, C6, C12, and C10 and a lipophilic surface defined by the isopropyl group and the hydrogens attached to C14, C20, C7, C8, and C21 (Sutko et al., 1997). Ryanodine is an alkaloid found in members of the genus R vanilla, shrubs or slender trees growing in several tropical locations in Central and South America, including Trinidad and the Amazon basin (Sutko et al., 1997). In acidic medium, ryanodine dehydrates easily to give anhydroyranodine with loss of activity (Fig. 14).

b. Mechanism of Action. The pharmacology of ryanodine receptors has been elegantly reviewed by Guerrero-Hernandez et al. (2002). Ryanodine has complex effects on the conductance and gating of single RyR channels that are concentration-dependent (Sutko et al., 1997). At submicromolar concentrations, it has one of two effects. In the first instance, ryanodine increases channel activity to a full conductance state (Pessah and Zimanyi, 1991). Alternately, and more typically, ryanodine makes the channel partially conducting (Roussel et al., 1987), and although multiple subconductance states have been observed (Ding and Kasai, 1996), the most common state is one near 50% full conductance (Sutko et al., 1997). Finally, at micromolar or greater concentrations, ryanodine induces the channel to adopt a closed state (Meissner, 1994). Micro- to millimolar concentrations of ryanodine induce a permanently closed state in RyR partially purified from canine and porcine aortic microsomal protein fractions and reconstituted into planar lipid bilayers (Herrmann-Frank et al., 1991), whereas a subconductance state is observed with lower concentrations of ryanodine (0.1–10 \(\mu\)M) in similar preparation from the bovine coronary arteries (Li et al., 2001). Higher concentrations of ryanodine (20 and 50 \(\mu\)M) close the channel. It is still unclear whether ryanodine modifies conductance by stabilizing the RyR channel in a specific conformation via allosteric effects or whether it physically interferes with the flux of ions through the pore of the channel (Sutko et al., 1997), although recent structure-activity data from experiments on the RyR2 channel favor an allosteric mechanism (Welch et al., 1997) as discussed below.

There is consensus that \(^{3}H\)ryanodine binds to high- \((K_{D} \sim 1–10 \text{ nM})\) and low-affinity sites \((K_{D} \sim 1–10 \mu\text{M})\) on the RyR channel, likely localized to the C-terminal 76-kDa fragment of the receptor (Sutko et al., 1997). There is also agreement that high-affinity binding results in channel activation or subconductivity, whereas low-affinity binding leads to channel inhibition (Sutko et al., 1997). The density of \(^{3}H\)ryanodine binding is \sim 100 fmol/mg protein, although in the rat portal vein it is reported to reach nearly 6 pmol/mg protein (Boittin et al., 1999). In general, the RyR density in smooth muscle is about 10 times lower than that in striated muscle (Guerrero-Hernandez et al., 2002), this presumably being a reflection of a lower SR volume in smooth muscle. The binding of \(^{3}H\)ryanodine to smooth muscle microsomes is increased by agents that also modulate RyR activity, such as Ca\(^{2+}\), caffeine, ATP, and pH, whereas it is decreased by...
ruthenium red and Mg²⁺ (for review, see Guerrero-Hernandez et al., 2002).

Ryanodine-induced activation also exhibits use-dependence; high-affinity binding occurs in the RyR channel open state. Furthermore, there is suggestive evidence that the tetrameric form of the channel is required for high-affinity binding (Lai et al., 1989), consistent with a stoichiometry of 1 mol of [³H]ryanodine/1 mol of RyR tetramer (Pessah and Zimanyi, 1991). In contrast, stoichiometries of either 3:1 (Lai et al., 1989) or 1:1 (Wang et al., 1993) have been reported for the low-affinity binding site.

These variable stoichiometries allow for at least two models of ryanodine binding to its receptor: the distinct site model and the interconvertible site model (Sutko et al., 1997). The distinct site model states that the two binding sites are physically distinct on the RyR tetramer in a 1:1 stoichiometry, with binding of ryanodine to the high-affinity site stabilizing the channel in a subconductance state, and subsequent binding of ryanodine to the low-affinity site leading to channel closure (Wang et al., 1993). Although both sites are distinct, it is proposed that ryanodine binding to the low-affinity site reduces the dissociation rate of ryanodine from the high-affinity site (Wang et al., 1993), although the nature of this interaction, be it steric or allosteric, has not been determined (Sutko et al., 1997). In contrast, the interconvertible site model envisions four initially identical (i.e., high-affinity) interacting binding sites per RyR tetramer that can be either high- or low-affinity sites; binding of ryanodine to one site exerts a negatively cooperative effect on the remaining sites, lowering their affinity. It has been shown that this decrease in affinity is equivalent among the three remaining sites, with channel closure requiring binding of ryanodine to all three (Carroll et al., 1991). Alternatively, the decrease was proposed to be sequential, with binding of ryanodine to each site successively lowering the affinity of the remaining unbound sites, thus leading to four classes of sites with different $K_D$ values, with binding to each site successively lowering channel conductance to reach closure once the four sites are occupied by ryanodine (Pessah and Zimanyi, 1991). It remains to be resolved how switching between conductance states occurs, as it may be an important regulator of the average quantity of $Ca^{2+}$ released per unit of time by a RyR channel (Sutko et al., 1997).

In terms of SAR, naturally occurring ryanoids (i.e., ryanodine congeners) have been found to differ in their biological activity (Sutko et al., 1997), with the most interesting case being that of ester A, which has a methoxyl moiety instead of a hydroxyl in the 10-position (Fig. 14). Ryanodine ester A only activates the RyR channel at concentrations up to 3 mM (Sutko et al., 1990) and binds to the high-affinity site with a $K_D$ of 110 nM (Welch et al., 1994), although its binding to the low-affinity site is unknown. Quantitative SAR analysis of various ryanoids has revealed that both physical bulk (van der Waals contacts in the pyrrole region) and electrostatic interactions (localized in the hydroxyl regions) correlate with high-affinity binding to the RyR channel (Welch et al., 1994). These correlations have led to a binding model for ryanodine with the pyrrole locus buried within the high-affinity binding site and 9- and 10-positions extending outside beyond the pyrrole carbonyl group (Welch et al., 1994) and possibly involving hydrogen bonding (Sutko et al., 1997). One end of ryanodine (3-position) appears to primarily determine binding affinity, whereas the opposite end (9- and 10-positions) seems to primarily control channel behavior (e.g., conductivity) (Sutko et al., 1997).

c. Selectivity. Although there are no RyR-isoform-specific ryanodine analogs, affinity of ryanoids for RyR2 is generally 2- to 3-fold higher than for RyR1 (ryanodine being close to 3-fold selective), with the most discriminating ryanoid reaching a 10-fold selectivity (Sutko et al., 1997).

d. Use in Smooth Muscle Preparations. Ryanodine is frequently used to deplete the SR by causing the $Ca^{2+}$-release channels to remain in a semiconducting state. This leak of $Ca^{2+}$ from the SR has several consequences, such as preventing the SR from storing any $Ca^{2+}$ it may...
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Accumulate (loss of buffering activity), loss of $K_{Ca}$ regulation by $Ca^{2+}$ sparks, and loss of regulation of $Cl_{Ca}$ activity. Ryanodine effectively depletes caffeine-sensitive $Ca^{2+}$ stores in both isolated smooth muscle cells and intact smooth muscle. Ryanodine actions are complicated by having distinct actions on the RyR that are concentration-dependent, such that at low-intermediate concentrations (submicromolar/micromolar range) the channel is locked in a semiconducting state, whereas at higher concentrations the channel remains in a closed state (Table 6). A comprehensive review of ryanodine receptor properties and functions has recently been published (Guerrero-Hernandez et al., 2002).

4. Procaine.

a. Source and Chemical Structure. Procaine is a synthetic amphiphilic ($p_K = 8.9$) local anesthetic tertiary amine, which exists predominantly in its cationic (versus neutral) form at physiological pH (Burdyga and Magura, 1986) (Fig. 15). It was originally derived from cocaine (Fig. 15), which was used medicinally for its local anesthetic and vasoconstrictive effects in an attempt to isolate the anesthesiophoric part of the latter molecule to eliminate its undesirable hallucinogenic and euphoric effects (Virji et al., 1969). It was initially found that the cocaine complex ring system was not essential to its local anesthetic activity (Virji et al., 1969). By comparing results obtained from alkyl para-aminobenzoates with the degradation residues of cocaine, the anesthesiophoric group was identified as the aromatic acid esterified with a tertiary amino alcohol. This led to the synthesis of procaine.

b. Mechanism of Action. Procaine was one of the first agents to be identified as an inhibitor of caffeine and CICR from the skeletal muscle SR (Feinstein, 1963). Its mechanism of action has been characterized on partially purified RyR from dog cardiac muscle microsomes reconstituted into planar lipid bilayers (Zahradnikova and Palace, 1993). In contrast with other RyR inhibitors, it does not reduce single-channel conductance, nor does it significantly shorten the channel $P_o$, but rather it in-

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<tr>
<td>Taenia coli</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle cells; whole-cell amphotericin B-perforated patch-clamping; STOCs</td>
<td>$10 \mu M$</td>
<td>Kong et al., 2000</td>
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<tr>
<td>Tail artery</td>
<td>Rat</td>
<td>Isolated freshly or cultured endothelium-denuded rings; fura-2 cellular loading, fluorometry, isometric dynamometry; $[Ca^{2+}]}_i$, contractile activity</td>
<td>$10 \mu M$</td>
<td>Drea et al., 2001</td>
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<tr>
<td>Testicular peritubules</td>
<td>Rat</td>
<td>Isolated saponin-permeabilized smooth muscle cells; fura-2 cellular loading, fluorometry; $[Ca^{2+}]}_i$</td>
<td>$5 \mu M$</td>
<td>Barone et al., 2002</td>
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<td>Trachea</td>
<td>Cat</td>
<td>Isolated smooth muscle cells; whole-cell nystatin perforated patch-clamping; $I_{Ca}$</td>
<td>$4 \mu M$</td>
<td>Wanshish et al., 1998</td>
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<tr>
<td>Trachea</td>
<td>Rat</td>
<td>Isolated smooth muscle cells and strips; fura-2 cellular loading, fluorometry, isometric dynamometry; $[Ca^{2+}]}_i$, contractile activity</td>
<td>$30 \mu M$</td>
<td>Tao et al., 2000</td>
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<tr>
<td>Trachea</td>
<td>Dog</td>
<td>Isolated cultured smooth muscle cells; fura-2 cellular loading, fluorometry; $[Ca^{2+}]}_i$</td>
<td>$50 \mu M$</td>
<td>Mitchell et al., 2000</td>
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<tr>
<td>Trachea</td>
<td>Rabbit</td>
<td>Isolated a-toxin-permeabilized strips; isometric dynamometry, contractile activity</td>
<td>$30 \mu M$</td>
<td>Iizuka et al., 1988</td>
</tr>
<tr>
<td>Ureter</td>
<td>Guinea pig</td>
<td>Isolated a-toxin- or b-toxin-permeabilized strips; isometric dynamometry, contractile activity</td>
<td>$10 \mu M$</td>
<td>Burdyya et al., 1998</td>
</tr>
<tr>
<td>Ureter</td>
<td>Rat</td>
<td>Isolated smooth muscle cells; fluo-4 cellular loading, confocal fluorescence microscopy; $[Ca^{2+}]}_i$ imaging</td>
<td>$50 \mu M$</td>
<td>Burdyya et al., 2003</td>
</tr>
<tr>
<td>Ureter</td>
<td>Rat</td>
<td>Isolated smooth muscle cells; fluo-3 cellular loading, confocal fluorescence microscopy; $[Ca^{2+}]}_i$ imaging</td>
<td>$50 \mu M$</td>
<td>Boitinin et al., 2000</td>
</tr>
<tr>
<td>Ureter</td>
<td>Rat</td>
<td>Isolated o-toxin- or p-toxin-permeabilized strips; isometric dynamometry, contractile activity</td>
<td>$10 \mu M$</td>
<td>Burdyya et al., 1998</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle cells; whole-cell amphotericin B-perforated patch-clamping; $I_{Ca},[Ca^{2+}]}<em>i, I</em>{Ca}$</td>
<td>$10 \mu M$</td>
<td>Herrera and Nelson, 2002</td>
</tr>
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<td>Urinary bladder</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle cells; fura-2 cellular loading, fluorometry; $[Ca^{2+}]}_i$, contractile activity</td>
<td>$10 \mu M$</td>
<td>Rueda et al., 2002b</td>
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<tr>
<td>Urinary bladder</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle cells; whole-cell and outside-out patch-clamping; $I_{Ca}$</td>
<td>$10 \mu M$</td>
<td>Inaimuzumi et al., 1996</td>
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<tr>
<td>Urinary bladder</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle cells; whole-cell amphotericin B-perforated patch-clamping, fluo-3 cellular loading, confocal fluorescence microscopy; $I_{Ca}$; $[Ca^{2+}]}_i$ imaging</td>
<td>$10 \mu M$</td>
<td>Herrera et al., 2001</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>Mouse</td>
<td>Isolated smooth muscle cells; fura-2 cellular loading, confocal fluorescence microscopy; $I_{Ca}$; $[Ca^{2+}]}_i$ imaging</td>
<td>$10 \mu M$</td>
<td>Ji et al., 2002</td>
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<tr>
<td>Uterus</td>
<td>Human</td>
<td>Isolated myometrial strips; indo-1 cellular loading, fluorometry, isometric dynamometry; $[Ca^{2+}]}_i$, contractile activity</td>
<td>$50 \mu M$</td>
<td>Kupittayanant et al., 2002</td>
</tr>
<tr>
<td>Vas deferens</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle cells; fluo-4 cellular loading, confocal fluorescence microscopy; local $[Ca^{2+}]}_i$ imaging</td>
<td>$100 \mu M$</td>
<td>White and McGown, 2003</td>
</tr>
<tr>
<td>Vena cava (inferior)</td>
<td>Rabbit</td>
<td>Isolated smooth muscle cells; fluo-3 cellular loading, confocal fluorescence microscopy; local $[Ca^{2+}]}_i$ imaging</td>
<td>$100 \mu M$</td>
<td>Ruchmann et al., 2000</td>
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</tbody>
</table>
creases the longest closed time. This suggests that procaine interacts selectively with a closed state of the channel rather than with an open state, consistent with computer simulation of RyR gating (Zahradnikova and Palade, 1993). This mechanism of action is also consistent with the long-known inhibitory effect of procaine on caffeine-induced contractile activity in vascular and visceral smooth muscles. For instance, in the porcine coronary artery, procaine (5 mM) inhibits caffeine (20 mM)-induced contractile activity of isolated strips in the absence of extracellular Ca$^{2+}$ (Itoh et al., 1982a) and inhibits (1–10 mM) the associated reduction in cellular Ca$^{2+}$ content in saponin-permeabilized smooth muscle cells (Ueno et al., 1987). Likewise, in guinea pig taenia caeci, procaine (1 mM) inhibits caffeine-induced (1–25 mM) contractile activity in the absence of extracellular Ca$^{2+}$ (Yagi et al., 1985), consistent with the inhibition of CICR rate by procaine (millimolar concentrations) in fura-2-loaded saponin-permeabilized smooth muscle fiber bundles (Ino, 1989).

c. Selectivity. Procaine has no effect on the pCa-force relationship in permeabilized arterial smooth muscle cells (Itoh et al., 1981, 1982b), and at up to 10 mM, it does not affect basal permeability of intracellular Ca$^{2+}$ stores as shown in dispersed and saponin-permeabilized porcine coronary artery smooth muscle cells (Ueno et al., 1987). Likewise, procaine (at up to 1 mM) does not affect PMCA activity in membranes from pig coronary artery and human myometrium, in contrast with its concentration-dependent (at 0.1–1 mM) inhibition of human erythrocyte PMCA (Popescu et al., 1987).

However, in reserpinized rat vas deferens, procaine (10 mM) prevents NE-induced increase in Ca$^{2+}$ efflux (NE = 100 μM), increases tissue Ca$^{2+}$ content (NE = 10 μM), and completely inhibits NE-induced (10 μM) contractile activity in the absence of extracellular Ca$^{2+}$ (Khoyi et al., 1993). Similarly, in the absence of extracellular Ca$^{2+}$, procaine (1 mM) inhibits Cch-induced (1–1000 μM) guinea pig taenia caeci contractile activity (Yagi et al., 1985). The same concentration of procaine also inhibits Ach-induced (10 μM) contractile activity of the porcine coronary artery in absence of extracellular Ca$^{2+}$. Furthermore, in dispersed and saponin-permeabilized smooth muscle cells from this blood vessel, the reduction in cellular Ca$^{2+}$ content induced by Ach (3 μM) is prevented by 1 mM of procaine (Ueno et al., 1987). This is consistent with the complete inhibition by procaine (5 mM) of the intracellular Ca$^{2+}$ transients elicited by Ach (10 μM) in intact cells (Ueno et al., 1987).

Although all of the above effects of procaine on agonist-induced responses might be related to the fact that, as shown in permeabilized coronary artery cells, it concentration-dependently (1–10 mM) inhibits InsP$_3$-induced (3 μM) release of cellular Ca$^{2+}$ content (although with only partial effectiveness (Ueno et al., 1987)), they are more likely due to inhibition of agonist-induced InsP$_3$ production. Indeed, in intact strips of porcine coronary artery, procaine (1–10 mM) concentration-dependently inhibits and eventually completely suppresses Ach-induced (10 μM) production of phosphatidyl inositol 4,5-bisphosphate, a surrogate marker of InsP$_3$ (Ueno et al., 1987). Likewise, in reserpinized rat vas deferens, procaine (10 mM) prevents NE-induced (10 μM) tissue increase in total inositol phosphates and in InsP$_3$ in particular (Khoyi et al., 1993). Whether this effect is mediated by a direct inhibition of phospholipase C and/or of an upstream event (e.g., by affecting plasma membrane fluidity, see below) is still undetermined.

Procaine (1–20 mM) also inhibits Ca$^{2+}$ influx (through Ca$_{L}$) associated with K$^+$-induced depolarization both in spontaneously active [e.g., guinea pig taenia caeci (Spedding and Berg, 1985; Ahn and Karaki, 1988), urinary bladder (Kurihara and Sakai, 1976a), rat duodenum (Ozturk et al., 1990)] and quiescent smooth muscle [e.g., rabbit aorta (Ahn and Karaki, 1988), and sheep carotid artery (Jacobs and Krettinge, 1974)]. Similarly, procaine (10 mM) increases Ca$^{2+}$ influx induced by NE (10 μM) in reserpinized rat vas deferens (Khoyi et al., 1993) and by Cch in guinea pig taenia caeci (Ahn and Karaki, 1988), occurring in both cases through Ca$_{L}$. This Ca$^{2+}$-channel inhibition may be a consequence of procaine membrane stabilizing effect on depolarized plasma membrane that it shares with other local anesthetics, an effect that can be overcome by extracellular Ca$^{2+}$ (Ahn and Karaki, 1988) but not by activators of Ca$_{L}$ (Ahn and Karaki, 1988). Salicylic acid, which incorporates itself in the plasma membrane to increase negative surface charge and therefore favors the incorporation of cationic drugs (McLaughlin, 1973), potentiates the membrane effects of procaine (Spedding and Berg, 1985).

Procaine (>1 mM) depolarizes tissues mainly by inhibiting K$^+$ conductance, thus enhancing spike frequency and amplitude in spontaneously active smooth muscles [e.g., guinea pig urinary bladder (Kurihara, 1975; Kurihara and Sakai, 1976a,b; Fujii et al., 1990), ureter (Burdyga and Magura, 1986), and portal vein (Hara et al., 1980)] and enabling outward current pulses to evoke action potentials in electrically quiescent smooth muscles [e.g., rabbit (Casteels et al., 1977; Ito et al., 1977) and guinea pig (Hara et al., 1980) pulmonary artery, dog trachea (Imaizumi and Watanabe, 1982), and sheep carotid artery (Jacobs and Krettinge, 1974)].

In contrast, procaine (up to 0.1 mM) was shown to be unable to displace radiolabeled batrachotoxin, a Na$^+$ channel selective blocker, or to inhibit specific guanidine uptake, a surrogate marker of Na$^+$ fluxes, in rat brain crude synaptosomal preparations (Pauwels et al., 1986), suggesting that procaine does not affect Na$^+$ conductance.

d. Use in Smooth Muscle Preparations. Procaine interacts with RyR by prolonging their closed state, and thus it is able to deplete caffeine-sensitive Ca$^{2+}$ stores. Procaine also inhibits IICR and has little effect Ca$^{2+}$ extrusion by the PMCA. Although it does not affect basal
Ca2+ permeability, it inhibits voltage-gated Ca2+ channel activity. In intact rat aorta, procaine causes a vaso-dilation that may reflect a multitude of effects, including release of NO, direct inhibition of extracellular Ca2+ entry, and reduction in the release of intracellular Ca2+ (Huang et al., 1999). The concentrations of procaine used in smooth muscle are shown in Table 7.

5. Ruthenium Red.

a. Source and Chemical Structure. Ruthenium red is a water-soluble and intensely colored inorganic synthetic dye that is obtained from the reaction of RuCl3 and NH3 in solution (Joly, 1892), is defined as ammoniated ruthenium oxychloride, and is a hexavalent cation in an aqueous environment (Carrondo et al., 1980) (Fig. 16). It is a complex compound of relatively high molecular weight that was originally used as a histochemical stain for its ability to bind to acidic glycosaminoglycans and other negatively charged polyanions (Charuk et al., 1990). Ruthenium red has an electron-dense character (Dierichs, 1979). This dye is relatively cell-impermeant.

b. Mechanism of Action. Ruthenium red (30 μM) fully inhibits RyR channels partially purified from canine and porcine aortic microsomal protein fractions and reconstituted into planar lipid bilayers (see Guerrero-Hernandez et al., 2002). Binding of [3H]ryanodine to microsomes obtained from cultured rat aortic smooth muscle cells is inhibited by ruthenium red (10 μM) (Yusufi et al., 2002). This is consistent with its inhibition, at 20 μM, of caffeine (3–10 mM)-induced contractile activity of saponin-permeabilized rabbit mesenteric artery smooth muscle (Kamcura et al., 1989) and with its inhibition (at 10 μM) of cADPR (10 μM)- and caffeine (20 mM)-induced Ca2+ release from rat aortic microsomes (Yusufi et al., 2002).

c. Selectivity. Relatively low concentrations (1–20 μM) of ruthenium red inhibit RyR1, RyR2, and RyR3 isoforms equally well (Sonleitner et al., 1998; Marx et al., 2001). Neither basal Mg2+-ATPase, nor the Na+/K+-ATPase, are affected by ruthenium red in erythrocyte membranes (Watson et al., 1971). However, in various tissues and cell types, ruthenium red has several non-selective effects: 1) it inhibits voltage-gated Ca2+ channels (most likely by its binding in a 1:1 stoichiometry at a site in the extracellular entrance of the pore (Cibulsky and Sather, 1999)—e.g., millimolar concentrations block depolarization-induced Ca2+ uptake (Greenberg et al., 1973), inhibition of Ica in isolated smooth muscle cells from guinea pig urinary bladder by externally applied ruthenium red (IC50 = 5.6 μM) (Sasaki et al., 1992; Hamilton and Lundy, 1995; Hirano et al., 1998); 2) it inhibits PMCA—e.g., K1/2 = 25 μM in purified PMCA from pig gastric antral smooth muscle—it reduces Vmax for Ca2+ without affecting affinity for Ca2+ by inhibiting the stimulant effect of negatively charged (i.e. acidic) phospholipids on the cytoplasmic face of the pump (Mis-siaen et al., 1990); 3) it inhibits BK—e.g., at 10 μM in guinea pig urinary bladder dissociated smooth muscle cells, likely by interacting with BK cytoplasmic Ca2+-binding site (Hirano et al., 1998); 4) it inhibits SERCA—e.g., at 10 to 100 μM, ruthenium red inhibits oxalate-dependent Ca2+ uptake in pig gastric antral smooth muscle microsomes (Kamcura et al., 1989), possibly through adsorption to negatively charged phospholipids in the SR membrane (Voelker and Smejtek, 1996a,b) resulting in altered phosphorylation kinetics (Meszaros and Ikemoto, 1985); 5) it inhibits IICR—e.g., ruthenium red (20 μM) inhibits InsP3 (20 μM)-induced contractile activity in saponin-permeabilized rabbit mesenteric artery smooth muscle (Kamcura et al., 1989), but it is inactive at 10 μM on the InsP3 (8 μM)-induced Ca2+ release from microsomes from freshly cultured rat aortic smooth muscle cells (Yusufi et al., 2002); 6) it inhibits the mitochondrial Ca2+- uniporter responsible for energy-dependent Ca2+ uptake (noncompetitive inhibition in rat liver mitochondria, K1/2 = 30 nM) (Moore, 1971; Ash and Bygrave, 1977; Matlib et al., 1998); and 7) it inhibits various Ca2+-binding proteins, including actin-activated myosin Mg2+-ATPase (it specifically interacts with F-actin binding site competitively (K1/2 = 4.4 μM with purified enzyme from chicken gizzard smooth muscle), thus preventing ATPase activation) (Nakamura et al., 1992), calmodulin (K1/2 = 18 μM) (Sasaki et al., 1992), and calsequestrin (K1/2 = 72 μM versus Ca2+ with purified enzyme from rabbit skeletal muscle) (Charuk et al., 1990).

Ruthenium red also increases the Ca2+ sensitivity of contractility in β-escin-permeabilized porcine smooth muscle from urinary bladder (EC50 = 60 μM at pCa 6.0), ileal longitudinal layer, and mesenteric artery without affecting Emax (at pCa 4.5). These effects are associated with increased LC20 phosphorylation (Yamada et al.,

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**Fig. 15. Molecular structure of procaine (1) and cocaine (2).**
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Animal Species</th>
<th>Experimental Details (Preparation; Measurement Method; Measured Response)</th>
<th>Effective Concentrations of Procaine</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anococcygeus</td>
<td>Rat</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>10 μM-1 mM</td>
<td>Babulova et al., 1981</td>
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<tr>
<td>Aorta</td>
<td>Rabbit</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>10 mM</td>
<td>Ahn and Karaki, 1988</td>
</tr>
<tr>
<td>Aorta</td>
<td>Rat</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>10 mM</td>
<td>Huang et al., 1999</td>
</tr>
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<td>Carotid artery</td>
<td>Sheep</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>up to 50 mM</td>
<td>Jacobs and Kretinge, 1974</td>
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<td>Coronary artery</td>
<td>Dog</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>10 mM</td>
<td>Imai et al., 1984</td>
</tr>
<tr>
<td>Coronary artery</td>
<td>Pig</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>10 mM</td>
<td>Ueno et al., 1987</td>
</tr>
<tr>
<td>Femoral artery</td>
<td>Rabbit</td>
<td>Isolated strips; isotonic dynamometry; contractile activity</td>
<td>10 mM</td>
<td>Yagi et al., 1985</td>
</tr>
<tr>
<td>Mesenteric artery</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle cells; microelectrode, alumina adsorption; membrane potential, excitatory junction potentials</td>
<td>5 mM</td>
<td>Fujii et al., 1985</td>
</tr>
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<td>Nasal mucosa</td>
<td>Dog</td>
<td>Isolated septal mucosa; dynamometry; contractile activity</td>
<td>100 μM</td>
<td>Wang and Jackson, 1988</td>
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<td>Portal vein</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>5 mM</td>
<td>Somlyo et al., 1992</td>
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<tr>
<td>Portal vein</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>1 mM</td>
<td>Nanjo, 1984</td>
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<tr>
<td>Portal vein</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>1 mM</td>
<td>Hara et al., 1980</td>
</tr>
<tr>
<td>Portal vein</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>10 mM</td>
<td>Yagi et al., 1985</td>
</tr>
<tr>
<td>Pulmonary artery</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>1 mM</td>
<td>Hara et al., 1980</td>
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<td>Stomach (antrum, circular layer)</td>
<td>Guinea pig</td>
<td>Isolated circular muscle strips; isotonic dynamometry; contractile activity</td>
<td>10 mM</td>
<td>Itoh et al., 1982b</td>
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<td>Taenia caeci</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>10 mM</td>
<td>Ahn and Karaki, 1988</td>
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<td>Taenia caeci</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>10 mM</td>
<td>Ueno et al., 1987</td>
</tr>
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<td>Trachea</td>
<td>Cat</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>10 mM</td>
<td>Nakahara et al., 2000</td>
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<tr>
<td>Trachea</td>
<td>Cattle</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>10 mM</td>
<td>Ito and Itoh, 1984</td>
</tr>
<tr>
<td>Trachea</td>
<td>Dog</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>1-10 mM</td>
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<td>Trachea</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>10 μM-1 mM</td>
<td>Babulova et al., 1981</td>
</tr>
<tr>
<td>Trachea</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>7 μM-6 mM</td>
<td>Okumura and Denborough, 1980</td>
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<tr>
<td>Trachea</td>
<td>Rabbit</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>20 mM</td>
<td>Iizuka et al., 1998</td>
</tr>
<tr>
<td>Ureter</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>10 mM</td>
<td>Burdyga and Magura, 1986</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>10 mM</td>
<td>Fujii et al., 1990</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>1, 7, and 15 mM</td>
<td>Kurihara and Sakai, 1976b</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>Guinea pig</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>15 mM</td>
<td>Kurihara, 1975</td>
</tr>
<tr>
<td>Vas deferens</td>
<td>Rat</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>10 mM</td>
<td>Khoyi et al., 1993</td>
</tr>
<tr>
<td>Vas deferens</td>
<td>Rat</td>
<td>Isolated smooth muscle fibers; isotonic dynamometry; contractile activity</td>
<td>10 mM</td>
<td>Huang, 1995</td>
</tr>
</tbody>
</table>
Consistent with the selective noncompetitive inhibition of capsaicin-induced (10–10,000 nM) contractile activity of isolated rat vas deferens (with 3 μM) and urinary bladder (with 10–30 μM) (Maggi et al., 1993).

d. Use in Smooth Muscle Preparations. Ruthenium red is a water-soluble inhibitor of RyR that is relatively impermeant in intact cells. It inhibits all isoforms of RyR and effectively competes for binding with [3H]ryanodine in isolated membranes. Although ruthenium red depletes caffeine-sensitive Ca2+ stores, its use is made unattractive due to its myriad of nonspecific effects that can occur at concentrations normally used to inhibit RyR. Included in the list of nonspecific effects are ruthenium red-induced inhibition of voltage-gated Ca2+ channel activity, PMCA, KCa, ICaR, mitochondrial Ca2+ uniporter, and calmodulin activity. There are also important direct effects on MLCK and MLCP. Table 8 summarizes the use of ruthenium red in various smooth muscle preparations.

C. Inositol 1,4,5-Triphosphate-Gated Ca2+ Release Channel/Inositol 1,4,5-Triphosphate Receptor

1. Inositol 1,4,5-Triphosphate.
   a. Source and Chemical Structure. d-myo-Inositol 1,4,5-triphosphate [Ins(1,4,5)P3, InsP3, IP3] is a phosphorylated species of the myo-stereoisomeric form of inositol 1,2,3,4,5,6-hexahydroxycyclohexane, a monosaccharide that is a member of the vitamin B complex (Fig. 17). This myo isomer is the most abundant of nine naturally occurring stereoisomers found in plant and animal tissues.

   A caged form of InsP3 has also been designed (Walker et al., 1987); it contains one photolabile (2-nitrophenyl) ethyl group esterified with either 4- or 5-position phosphates of InsP3 that confers cell permeability and are hydrolyzed once the compound is inside the cells, and a photolabile group on the 6–position hydroxyl, 4,5-dimethoxy-2-nitrobenzyl.

   A new form of caged InsP3 analog, 2,3-methoxymethylene InsP3, has been designed with the advantage of being cell-permeant, although not available commercially (Li et al., 1998). It bears propionyloxymethyl groups on 4– and 5–positions phosphates of InsP3 that confer cell permeability and are hydrolyzed once the compound is inside the cells, and a photolabile group on the 6–position hydroxyl, 4,5-dimethoxy-2-nitrobenzyl. The caged compound was shown to accumulate in the cytoplasm in 1321N1 astrocytoma cells at concentrations of hundreds of micromolar without InsP3R activation (Li et al., 1998). UV illumination uncages the InsP3 analog that is nearly as potent as InsP3. Because of the large accumulation of compound, repetitive flashes

![Molecular structure of ruthenium red](image-url)
could be used to generate oscillations in \([\text{Ca}^{2+}]_{\text{cyt}}\) at approximately physiological rates (Li et al., 1998).

\textit{b. Mechanism of Action.} The cell-impermeant \textit{InsP}_3 is an important second messenger produced simultaneously with diacylglycerol from the phosphoinositide-specific PLC (also known as phosphoinositidase C or phosphoinositidase)-mediated hydrolysis of plasma membrane phosphatidylinositol 4,5-bisphosphate (phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol 4,5-bisphosphate) (Berridge and Irvine, 1984). PLC, also found in the nucleus (Cocco et al., 2001), is a multidomain phosphodiesterase forming a family of more than 11 isoforms grouped into four subtypes (\(\beta, \gamma, \delta, \) and \(\epsilon\)) (Rebecchi and Pentyala, 2000; Rhee, 2001; Fukami, 2002). In the plasma membrane, depending on its subtype, PLC can be activated either through interactions with the heterotrimeric G proteins \(G_\alpha\) or \(G_{11}\) following stimulation of numerous GPCR or through direct interactions with receptor and nonreceptor tyrosine kinases (Rhee, 2001). Plasma membrane-generated \textit{InsP}_3 then

<table>
<thead>
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<td>up to 20 (no effect)</td>
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<td>Microsomes; (\text{Ca}^{2+}) loading; (\text{Ca}^{2+}) fluxes</td>
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<td>Colon (circular layer)</td>
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<td>Isolated non-inflamed and inflamed saponin-permeabilized smooth muscle cells; fura-2 cellular loading or (\text{Ca}^{2+}) cellular loading, cell length monitoring by phase-contrast microscope; (\text{Ca}^{2+}) fluxes, ([\text{Ca}^{2+}]_{\text{cyt}}), contractile activity</td>
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<td>Microsomal membranes, stripped or not of FKBP-12.6, incorporated into planar lipid bilayers; bilayer clamp amplification; single (\text{Ca}^{2+}) channel currents</td>
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<td>Guinea pig</td>
<td>Isolated smooth muscle cells; whole-cell patch-clamping; (I_{\text{sAC}}),(I_{\text{LAC}}), STOCs</td>
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<td>Urinary bladder</td>
<td>Rat</td>
<td>Isolated strips; isometric dynamometry; contractile activity</td>
<td>3-30</td>
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<td>Uterus</td>
<td>Rat</td>
<td>Isolated digitonin-permeabilized smooth muscle cells; (\text{Ca}^{2+}) mitochondrial loading; mitochondrial (\text{Ca}^{2+}) fluxes</td>
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<td>Isolated myometrial strips; isometric dynamometry; contractile activity</td>
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<td>Vas deferens</td>
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<td>Isolated strips; isometric dynamometry; contractile activity</td>
<td>3-30</td>
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diffuses in the cytoplasm to induce SR Ca\(^{2+}\) release by binding to InsP\(_3\)R (see Section II.C.).

In mammalian cells, the InsP\(_3\) signal is rapidly terminated by metabolism through two known routes (Connolly et al., 1987; Shears, 1989): dephosphorylation to d-myo-inositol 1,4-diphosphate [Ins(1,4)P\(_2\)] by an inositol polyphosphate 5-phosphatase located in both cytoplasmic and membrane cellular fractions (Downes et al., 1982; Verjans et al., 1994) or phosphorylation by a predominantly cytoplasmic ATP-dependent InsP\(_3\) 3-kinase to d-myo-inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P\(_4\), Fig. 17], which is further metabolized by the above 5-phosphatase and a 3-phosphatase (Irvine et al., 1986; Hoer et al., 1988). In its substrate interactions, the InsP\(_3\)3-kinase exhibits high stereo- and positional selectivity and constitutes a family of at least three isoforms (Communi et al., 1995), whereas the inositol polyphosphate 5-phosphatase seems relatively nonspecific (Nahorski and Potter, 1989) and forms a family of at least 10 mammalian members (Mitchell et al., 2002; Whisstock et al., 2002). However, little is known about the expression and regulation of these enzymes in smooth muscle. Interestingly, although Ins(1,4)P\(_2\) is inactive in terms of Ca\(^{2+}\) movements, Ins(1,3,4,5)P\(_4\) is a weak agonist at the InsP\(_3\)R (Wilcox et al., 1994a) and has a controversial role in the regulation of extracellular Ca\(^{2+}\) influx (Hermosura et al., 2000).

In terms of SAR (Fig. 18), the interaction of InsP\(_3\) with its receptor is highly stereospecific, with the d-isomer being over 1000 times more potent than the l-isomer (Nahorski and Potter, 1989). The most critical structural feature of InsP\(_3\) is its vicinal d-4,5-bisphosphate motif, with the 5-phosphate being the dominant partner in receptor interaction (Wilcox et al., 1997). The 1-phosphate also contributes to the receptor interaction specificity (Nahorski and Potter, 1989), potency (Willocks et al., 1989; Jenkinson et al., 1992), and efficacy (Wilcox et al., 1995, 1997). The hydroxyl group in 6-position appears to make a major contribution to the binding interactions with the receptor (Safrany et al., 1991), whereas...
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<tr>
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<th>Experimental Details (Preparation; Measurement Method; Measured Response)</th>
<th>Effective Concentrations of InsP3 (µM)</th>
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<td>Microsomal membranes incorporated into planar lipid bilayers, microsomes; bilayer clamp amplification (for membranes), extra-microsomal anti-pyridylazo-III (for microsomes); single Ca2+ channel currents (for membranes), Ca2+ fluxes (for microsomes)</td>
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<td>Pig</td>
<td>Microsomes; extramicrosomal fluo-3, fluorometry; Ca2+ fluxes</td>
<td>10 and 30</td>
<td>Tovey et al., 2000</td>
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<td>Aorta</td>
<td>Rabbit</td>
<td>Isolated saponin-permeabilized smooth muscle cells; magnesium-fura-2 cellular loading; [Ca2+]cyt, [Ca2+]mito, bilayer clamp amplification; single Ca2+ channel currents</td>
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<td>Gurney et al., 2000</td>
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<td>Purified InsP3R incorporated into planar bilayers; wide-field fluorescence digital imaging; [Ca2+]cyt, imaging</td>
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<td>10 and 30</td>
<td>Missiaen et al., 2001a</td>
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<td>Guinea pig</td>
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<td>25 (caged InsP3)</td>
<td>McCarron et al., 2004</td>
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<td>Isolated smooth muscle cells; whole-cell patch-clamping, fluo-3 cellular loading, fluorometry or wide-field fluorescence digital imaging; [Ca2+]cyt, imaging</td>
<td>20 (caged InsP3)</td>
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<td>Isolated non-inflamed and inflamed saponin-permeabilized smooth muscle cells; fura-2 cellular loading or 45Ca2+ cellular loading, cell length monitoring by phase-contrast microscope; Ca2+ fluxes, [Ca2+]cyt, contractile activity</td>
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<td>10 and 30</td>
<td>Tovey et al., 2000</td>
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<td>25</td>
<td>Somlyo et al., 1992</td>
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the axial 2-hydroxyl and the equatorial 3-hydroxyl groups do not seem as important for either binding or Ca$^{2+}$ release (Hirata et al., 1989). In fact, the InsP$_3$R has a remarkable tolerance for electronic and steric changes in either axial or equatorial substituents of the InsP$_3$ 2-position, which suggests that the 2-hydroxyl is not closely associated with the binding pocket (Hirata et al., 1993). Interestingly, InsP$_3$R is intracellularly localized in structures other than the ER/SR. For instance, it has been found in the nuclear envelope in many cell types, (Newton et al., 1994; Mackrill et al., 1996).

Unfortunately, despite this relatively detailed SAR information, the rational design of potent agonist and antagonist InsP$_3$ analogs has not been successful, making the design of InsP$_3$R isoform-specific analogs even less tenable. Progress in the development of pharmacological tools has relied mostly on the discovery of natural products, such as the adenophostins (agonists) and xestospongin-insensitive/CPA-sensitive Ca$^{2+}$ store (~10% of
total cell Ca\(^{2+}\) uptake), likely corresponding to the Golgi apparatus (see Section II.1.c.) where InsP\(_3\)R has also been localized (Surroca and Wolff, 2000), InsP\(_3\) (or an InsP\(_3\)-generating receptor agonist such as arginine vasopressin) can release Ca\(^{2+}\) in A7r5 cells (EC\(_{50}\) values \(~5 \mu \text{M} \) versus \(~1 \mu \text{M} \) for ER-mediated Ca\(^{2+}\) release) but not in 16HEB14o- cells (Missiaen et al., 2002). It is unclear what role this type of IICR has and whether it is of significance in differentiated smooth muscle cells.

InsP\(_3\) appears very selective for IICR versus CICR in smooth muscle. For instance, in microsomes from freshly cultured rat aortic smooth muscle cells that express RyR1–3, InsP\(_3\) induces concentration-dependent (1–8 \mu M) Ca\(^{2+}\) release that is fully inhibited by heparin (1 mg/ml), whereas cADPR induces concentration-dependent (1–10 \mu M) Ca\(^{2+}\) release that is fully inhibited by 8-Br-cADPR (40 \mu M) and ruthenium red (10 \mu M). Although both agents induced maximal Ca\(^{2+}\) release of similar order of magnitude, InsP\(_3\) effect is insensitive to ruthenium red (Yusufi et al., 2002). Likewise, InsP\(_3\) (10 \mu M) is ineffective at changing the P, of RyR partially purified from bovine coronary arteries microsomal protein fractions and reconstituted into planar lipid bilayers, which is fully sensitive to caffeine (0.5–5 mM), ry-anodine (0.1–50 \mu M), ruthenium red (40 and 80 \mu M), and cADPR (0.01–1 \mu M) (Li et al., 2001).

In saponin-permeabilized freshly cultured seminiferous peritubular smooth muscle cells from rat testis expressing RyR1–3, InsP\(_3\) induces concentration-dependent (1–8 \mu M) Ca\(^{2+}\) release that is fully inhibited by heparin (1 mg/ml), whereas cADPR induces concentration-dependent (1–10 \mu M) Ca\(^{2+}\) release that is fully inhibited by 8-Br-cADPR (40 \mu M) and ruthenium red (10 \mu M). Although both agents induced maximal Ca\(^{2+}\) release of similar order of magnitude, InsP\(_3\) effect is insensitive to ruthenium red (Yusufi et al., 2002). Likewise, InsP\(_3\) (10 \mu M) is ineffective at changing the P, of RyR partially purified from bovine coronary arteries microsomal protein fractions and reconstituted into planar lipid bilayers, which is fully sensitive to caffeine (0.5–5 mM), ry-anodine (0.1–50 \mu M), ruthenium red (40 and 80 \mu M), and cADPR (0.01–1 \mu M) (Li et al., 2001).

d. Use in Smooth Muscle Preparations. InsP\(_3\) does not cross cell membranes, complicating its routine use in either isolated smooth muscle cells or intact tissue. As shown in Table 9, the preferred methods of use of this endogenous SR Ca\(^{2+}\) releaser are via either direct injection through intracellular application (e.g., with a patch pipette) or photolytic release of caged InsP\(_3\). When caged InsP\(_3\) is released, there is a delay of \(~5 \) to 10 ms before Ca\(^{2+}\) release occurs (Somlyo et al., 1992). This release raises [Ca\(^{2+}\)]\(_{\text{cyt}}\) from 100 nM (basal) to 175 nM in guinea pig portal vein (Somlyo et al., 1992). Nonetheless, there is variable affinity of InsP\(_3\) for its receptors: 2 nM for InsP\(_3\)R1 and InsP\(_3\)R2 and 22 nM for InsP\(_3\)R3. The d-isomer of InsP\(_3\) is \(~1000\) times more potent than the l-isomer. Smooth muscle (e.g., intestinal) has at least 10 times more InsP\(_3\)R than RyR (Bolton et al., 1999). The EC\(_{50}\) value for InsP\(_3\) is reportedly 1 \mu M, with maximal Ca\(^{2+}\) release requiring 4 \mu M (Somlyo et al., 1992).

The general applicability of this differential density profile in smooth muscle (vascular and nonvascular) that differ in location (different vascular beds or various hollow organs), size (resistance versus conduit arteries), orientation (circular versus longitudinal), and function (phasic versus tonic) has not been examined so far.

2. Adenophostins.

a. Source and Chemical Structure. The gluconucleosides adenophostins A (3’-O-(α-d-glucopyranosyl)-adenosine-2’,3’,4’-trisphosphate) and B (the 6’-O-acetyl derivative) are metabolic products of the fungus Penicillium brevicompactum (Takahashi et al., 1993) (Fig. 17). Apart from a vicinal bisphosphate group and a third phosphate, adenophostins bear little resemblance to InsP\(_3\), two of the major differences being the adenine component and the hydroxymethyl substituent (acyetylated in adenophostin B).

b. Mechanism of Action. The cell-impermeant adenophostins are the most potent InsP\(_3\)R agonists known. They are full InsP\(_3\)R agonists in rat cerebellar microsomes (which express InsP\(_3\)R1 almost exclusively) (Wojcikiewicz, 1995) with potencies about 100 times higher than InsP\(_3\) potency (EC\(_{50}\) values: adenophostin A = 1.4 nM, adenophostin B = 1.5 nM, and InsP\(_3\) = 170 nM). In binding data, adenophostin A has an affinity about seven times higher than InsP\(_3\) in rat cerebellar microsomes (K\(_D\) values: 0.91 nM versus 6.75 nM) (Marchant et al., 1997a) and about five times higher in hepatic membranes (Beecroft et al., 1999). Consistent with this, the displacement of [\(^{3}H\)]InsP\(_3\) by adenophostin has an IC\(_{50}\) value about 20 to 50 times lower than InsP\(_3\) in rat cerebellar microsomes (Takahashi et al., 1994; Murphy et al., 1997) and about 10 times lower than in porcine cerebellar microsomes (Shuto et al., 1998). At the purified InsP\(_3\)R1, adenophostin B is about 10 times more potent than InsP\(_3\) (EC\(_{50}\) values: 11 nM versus 100 nM) and exhibits a positive cooperativity in binding that is not observed with InsP\(_3\) (Hirota et al., 1995). Adenophostin A is also about a 10-fold more potent Ca\(^{2+}\) releaser than InsP\(_3\) in permeabilized hepatocytes (Marchant et al., 1997b; Beecroft et al., 1999) that have predominance of InsP\(_3\)R2 versus InsP\(_3\)R1 (\(>80\%) \) versus \(<20\%) \) (Wojcikiewicz, 1995; De Smedt et al., 1997).

Adenophostins are resistant (Takahashi et al., 1993) to the metabolic enzymes inositol polyphosphate 5-phosphatase and InsP\(_3\) 3-kinase (Nahorski and Potter, 1989) that are involved in the generation of the full but weaker SR InsP\(_3\)R agonist InsP\(_4\) (Wilcox et al., 1993) (see Section II.C.1.b.). As such, adenophostins are able to produce a sustained Ca\(^{2+}\) release in rat cerebral microsomes (Takahashi et al., 1994) and rabbit permeabilized platelets (Murphy et al., 1997) with potencies 10 to 100 times higher than InsP\(_3\).

Adenophostins possess important structural features for agonism at the InsP\(_3\)R. The 4- and 3-positions of the glucose 3,4-bisphosphate moiety and the 2-position hydroxyl group were shown by molecular modeling to be similar to 4-, 5-, and 6-positions, respectively, of InsP\(_3\) (Takahashi et al., 1994; Wilcox et al., 1995), and both structural features are as essential for Ca\(^{2+}\)-releasing activity in rabbit permeabilized platelets (Murphy et al., 1997). The third phosphate of adenophostins (2’-phos-
phate) is essential for high potency (Shuto et al., 1998); its removal causes a 1000-fold decrease in binding affinity in rat cerebellar microsomes (Takahashi et al., 1994) and a 2000-fold lower affinity to purified rat cerebellar InsP₃R (Takahashi et al., 1993). The adenosine component at the 1’-position of the glucopyranose ring is also necessary for the high potency of adenophostins (Wilcox et al., 1995; Murphy et al., 1997; Shuto et al., 1998; de Kort et al., 2000).

c. Selectivity. Adenophostins, at a concentration of 10 μg/ml (−15 μM), do not bind to Ca₄ or to a series of receptors of functional significance for smooth muscle function (α₁ and β adrenergic, angiotensin II, cholecystokinin A and B, dopamine D2, leukotriene B4 and D4, Ach muscarinic, neurokinin, NMDA, serotonin 5-HT1, 5-HT2 and 5-HT3, and thromboxane receptors) (Takahashi et al., 1993).

d. Use in Smooth Muscle Preparations. The adenophostins (A and B) are cell-impermeant and represent the most potent agonists for InsP₃R. They are 10 to 100 times more potent than InsP₃ in binding and Ca²⁺ release studies. Unlike InsP₃, the adenophostins are metabolically stable and produce lasting effects. As can be seen from Table 10, the use of these compounds in smooth muscle has not been favorably received thus far.

3. Xestospongins.

a. Source and Chemical Structure. The macrocyclic bis-1-oxaquinolizidines xestospongins (xestospongins A, C, and D, araguspongine B, and demethylxestospongion B) are alkaloids from the Australian marine sponge Xestospongia sp. (Nakagawa et al., 1984; Vassas et al., 1996; Gafni et al., 1997) (Fig. 19).

b. Mechanism of Action. The xestospongins are potent blockers of IICR in rabbit cerebellar microsomes with IC₅₀ values ranging from ~300 nM to ~6 μM, with the most potent form, xestospongion C, being cell-permeant (Gafni et al., 1997). The more potent xestospongins (xestospongion C, araguspongine B, and xestospongion D) produce a multiphasic inhibition of IICR in these microsomes, which may be explained by the existence of multiple InsP₃R isoforms in preparations from whole cerebella. This would imply that these receptor isoforms have different affinities for these xestospongins (Gafni et al., 1997). There is indeed evidence that the cerebellum contains more than one of the three known InsP₃R isoforms (Nakanishi et al., 1991; Ross et al., 1992). The blockade appears to be noncompetitive for InsP₃ as xestospongion C (7.5 μM) completely blocks Ca²⁺ release from rabbit cerebellar microsomes but cannot decrease [³H]InsP₃-specific binding to the same microsomes (at 10 μM) (Gafni et al., 1997). Although not yet elucidated, the specific mechanism could be either blockade of the Ca²⁺ channel pore or an allosteric interaction uncoupling InsP₃ binding from Ca²⁺ release (Gafni et al., 1997). Consistent with the above findings, xestospongion C concentration-dependently (6–10 μM) inhibits ATP-induced transient increase in [Ca²⁺]c in bovine aortic endothelial cells (Bishara et al., 2002).

In terms of SAR, the combination of a cis-fused and trans-fused oxaquinolizidines, such as in xestospongion C, is about 10 times more potent than a trans/trans system, such as in xestospongion A (Gafni et al., 1997). Because of purity issues, it is still not clear whether a cis/trans system would be superior in potency (about 2 times) to a cis/cis system, such as in araguspongine B (Gafni et al., 1997). The addition of a hydroxyl group to C9 of xestospongion C, such as in xestospongion D, reduces the potency about two times (Gafni et al., 1997). Substitution of the cis-fused oxaquinolizidine of xestospongion D for a trans-fused one, such as in demethylxestospongion B, further reduces the potency about 7 times (Gafni et al., 1997). Interestingly, xestospongions A, C, and D were recognized as vasodilators at the time of their discovery (Nakagawa et al., 1984), likely as a consequence of their InsP₃R blocking activity.

c. Selectivity. Xestospongins display a high selectivity for the InsP₃R over RyR1 (Gafni et al., 1997). In rabbit skeletal muscle, xestospongion C (10 μM) decreases [³H]ryanodine binding to SR preparations by 22% and caffeine-induced Ca²⁺ release by 46%. The IC₅₀ value for blocking caffeine-induced Ca²⁺ release in skeletal muscle is more than 30 times greater than that for blocking Ca²⁺ release in rabbit cerebellar microsomes. Likewise, in smooth muscle, xestospongion inhibits InsP₃R and not RyR (Ozaki et al., 2002). In permeabilized smooth muscle, xestospongion C inhibits both K⁺-and Cch-induced contractile activity (3–10 μM), whereas in isolated smooth muscle cells from this tissue, the inhibition of voltage-gated currents has an IC₅₀ value of 0.63 μM (Ozaki et al., 2002). Thus, at least in the ileum, xestospongion is selective for InsP₃R when applied to permeabilized tissue but not when applied to intact cells or tissue (Bishara et al., 2002; Ozaki et al., 2002).

Much like 2-APB (see Section II.C.4.c.), but with greater potency, xestospongion C (3–10 μM) inhibits agonist (ATP)- and receptor-independent (thapsigargin, ionomycin)-induced capacitative Ca²⁺ entry in bovine aortic endothelial cells (Bishara et al., 2002). Interestingly, in human platelets, xestospongion C, but not 2-APB, disrupts the thapsigargin-induced structural coupling (Rosado and Sage, 2000) between InsP₃R2 and

<table>
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<th>Tissue</th>
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<th>Experimental Details (Preparation; Measurement Method; Measured Response)</th>
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<td>Tovey et al., 2000</td>
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the putative capacitative Ca$^{2+}$ protein Trp1 (see Section I.C.) (Rosado et al., 2002).

Also like 2-APB (see Section II.C.4.c.), xestospongin C was shown to inhibit SERCA in saponin-permeabilized A7r5 cells with a potency (IC$_{50}$ value = 67 nM) similar to its potency to inhibit InsP$_3$R in this smooth muscle preparation (IC$_{50}$ value = 55 nM), resulting in depletion of SR Ca$^{2+}$ stores (De Smet et al., 1999). Such a depletion of ER/SR Ca$^{2+}$ stores in the presence of xestospongin C has also been observed in nonpermeabilized A7r5 cells (Broad et al., 1999) and PC12 cells (Gafni et al., 1997).

d. Use in Smooth Muscle Preparations. The xestospongin s are a composite of several structures including xestospongin A, C, and D. They are cell-permeant, potent inhibitors of InsP$_3$R with little effect on RyR, with the most potent being xestospongin C. The various isoforms of InsP$_3$R differ in their sensitivities to xestospongin C. Xestospongin s are vasodilators in intact tissues, but this may be due to a multitude of actions, including inhibition of voltage-gated Ca$^{2+}$ channels, since the selectivity for inhibition of InsP$_3$R appears to be unique for permeabilized cells and microsomal preparations (Table 11).

4. 2-Aminoethoxy-Diphenylborate.

a. Source and Chemical Structure. 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 (Strang et al., 1989) (Fig. 20). This heterocyclic ring species would be more hydrophobic than the primary amine open-chain species that could be protonated at physiological pH; it should thus permeate cells more rapidly (Dobrydneva and Blackmore, 2001). The hydrogen bonding ability is likely the basis of 2-APB water solubility (Dobrydneva and Blackmore, 2001).

b. Mechanism of Action. The cell-permeant 2-APB was originally characterized as an inhibitor of IICR in rat cerebellar microsomes (Maruyama et al., 1997). Furthermore, high concentrations of 2-APB (up to 1 mM) do not affect [${}^{3}$H]InsP$_3$ binding to its receptor, which is consistent with the inability of 2-APB (200 nM) to affect [${}^{3}$H]InsP$_3$ binding to microsomes from the A7r5 cell line (Missiaen et al., 2001a), suggesting that 2-APB inhibits InsP$_3$R channel opening allosterically (Maruyama et al., 1997). In human platelets and neutrophils, 2-APB (3–100 nM) inhibits thrombin- and 9,11-epithio-11,12-methano-thromboxane A$_2$-[synthetic thromboxane A$_2$ (STA$_2$)]-induced [Ca$^{2+}$]$_{cyt}$ increase but does not affect (at 100 nM) STA$_2$-induced InsP$_3$ production in human platelets (Maruyama et al., 1997; Diver et al., 2001; Dobrydneva and Blackmore, 2001). Likewise, 2-APB (100 nM) does not affect ATP-induced InsP$_3$ production in bovine aortic endothelial cells (Bishara et al., 2002).

The inhibitory effect of 2-APB on IICR in A7r5 cells is independent of the concentration of InsP$_3$, ATP, or [Ca$^{2+}$]$_{cyt}$ (Missiaen et al., 2001a). In rabbit thoracic aorta, 2-APB (10 nM–1 mM) blocks angiotensin II-, NE-, and STA$_2$-induced contractile activity in a noncompetitive manner (Maruyama et al., 1997).

c. Selectivity. 2-APB (up to 100 nM) has no effect on caffeine-induced Ca$^{2+}$ release from ryanodine-sensitive Ca$^{2+}$ stores of rat cardiac and skeletal SR vesicles (Maruyama et al., 1997). It also has no effect, at concentrations up to 1 mM, on K$^+$-induced rabbit thoracic aorta contractile activity (Maruyama et al., 1997).

However, more recently, 2-APB was shown to affect the function of several molecular targets involved in Ca$^{2+}$ handling besides the InsP$_3$R. First, it inhibits (1–100 nM) thapsigargin-activated store-operated (i.e., capacitative) Ca$^{2+}$ entry in human platelets (Diver et al.,...
2001; Dobrydneva and Blackmore, 2001), consistent with its concentration-dependent (3–300 μM) inhibition of agonists (ATP, bradykinin)- and receptor-independent (thapsigargin, ionomycin)-induced capacitative Ca\(^{2+}\) entry in bovine aortic and rat cardiac endothelial cells (Bishara et al., 2002). 2-APB (1–100 μM) also readily blocks Sr\(^{2+}\), Ba\(^{2+}\), and Mn\(^{2+}\) entry in unstimulated human platelets following SR Ca\(^{2+}\) store depletion by thapsigargin, suggesting a direct action of 2-APB on store-operated Ca\(^{2+}\) channels (SOCC) (Diver et al., 2001; Dobrydneva and Blackmore, 2001), although it cannot be discounted that it also interacts with a non-InsP\(_3\)R protein that regulates SOCC. However, complete inhibition by 2-APB of single-channel recordings of a putative SOCC in excised plasma membrane patches of rat basophilic leukemia cells (RBL-2H3 m1), an immortalized mucosal mast cell line, supports a direct action on the channel (Braun et al., 2001). However, the effects on capacitative Ca\(^{2+}\) entry may not be simple though. Indeed, in Jurkat human T cells, DT40 chicken B-lymphocytes, and RBL cells, 2-APB has a dichotomous action; at low concentrations (1–5 μM), it enhances the size of the \(I_{\text{CRAC}}\) and speeds up its fast Ca\(^{2+}\)-dependent inactivation, whereas at higher concentrations (≥10 μM), it inhibits \(I_{\text{CRAC}}\) and blocks its Ca\(^{2+}\)-dependent inactivation (Prakriya and Lewis, 2001). This latter inhibition is likely mediated through an extracellular site, as 2-APB is more potent when applied extracellularly and as this inhibition is unaffected by increased protonation of 2-APB (which reduces its cell permeability) (Prakriya and Lewis, 2001). The mechanism underlying these concentration-dependent effects is unknown, but different binding sites (i.e., high versus low affinity) could be involved by analogy with RyR (see Section II.B.1.b.) (Sutko et al., 1997), or different 2-APB species may exist at different concentrations (i.e., monomer at low concentrations versus dimer at high concentrations; see Section II.C.4.a.; Fig. 20) (Prakriya and Lewis, 2001). In terms of SAR, the inhibition of capacitative Ca\(^{2+}\) entry is dependent on diphenyl groups attached to a tetrahedral atom of a five-membered ring (e.g., 2-APB heterocyclic ring; see Section II.C.4.a.) (Dobrydneva and Blackmore, 2001). Interestingly, in human platelets, thapsigargin induces structural coupling (Rosado and Sage, 2000) between InsP\(_3\)R2 and the putative SOCC Trp1 (Rosado et al., 2002) that is unchanged by 2-APB despite abolition of capacitative Ca\(^{2+}\) entry (Diver et al., 2001). This is contrasting with the effects of xestospongins C (see above). In this respect, it is relevant to note that 2-APB blocks capacitative Ca\(^{2+}\) entry and \(I_{\text{CRAC}}\) in both wild-type DT40 chicken B-lymphocytes and a variant where all three types of InsP\(_3\)R are knocked out (Broad et al., 2001; Prakriya and Lewis, 2001), suggesting that an interaction of 2-APB with an InsP\(_3\)R is not required for its inhibition of capacitative Ca\(^{2+}\) entry.

### Table 11

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Animal Species</th>
<th>Experimental Details (Preparation; Measurement Method; Measured Response)</th>
<th>Effective Concentrations of Xestospongins</th>
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<td>Cattle</td>
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<td>Etherie and Madison, 2002</td>
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<td>Berger et al., 2001</td>
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<td>Aorta</td>
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<td>10 (Xestospongin C)</td>
<td>Mitchell et al., 2000</td>
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PHARMACOLOGICAL MODULATION OF SMOOTH MUSCLE SR 493
Second, 2-APB (5–50 \mu M) can inhibit Ca\(^{2+}\) efflux from mitochondria [believed to be mostly mediated by a Na\(^{+}\)-Ca\(^{2+}\) exchanger on the mitochondrial inner membrane (Gunter et al., 2000)] based on \([\text{Ca}^{2+}]_{\text{cyt}}\) measurements made in the absence of extracellular Ca\(^{2+}\) in Jurkat human T cells (Prakriya and Lewis, 2001). This effect needs to be confirmed more directly, as well as the finding that 2-APB may electrically uncouple smooth muscle cells in an intact blood vessel (Lamont and Wier, 2004).

Third, 2-APB can inhibit Ca\(^{2+}\) pumps (e.g., SERCA) and the nonspecific Ca\(^{2+}\) leak from nonmitochondrial Ca\(^{2+}\) stores in saponin-permeabilized A7r5 cells (Missiaen et al., 2001a). In this smooth muscle preparation, the IC\(_{50}\) values for these effects are 91 \mu M and >100 \mu M, respectively, compared with an IC\(_{50}\) value of 36 \mu M for inhibition of IICR.

Finally, it was shown recently that 2-APB completely and reversibly blocks gap junctional intercellular communication in monolayers of normal rat kidney cells (NRK/49F) with an IC\(_{50}\) value of 5.7 \mu M (maximal effect at 50 \mu M), the same concentration range required for inhibition of PGF\(_{2\alpha}\)-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) increases (Harks et al., 2003). It displays a similar potency in human embryonic kidney epithelial cells (HEK293/tsA201) with an IC\(_{50}\) value of 10.3 \mu M (Harks et al., 2003).

d. Use in Smooth Muscle Preparations. There is increasing usage of 2-APB as a selective inhibitor of InsP\(_3\)R, as summarized in Table 12. 2-APB inhibits the InsP\(_3\)R channel opening without affecting InsP\(_3\) synthesis or binding. Although 2-APB does not interact with RyR or voltage-gated Ca\(^{2+}\) entry, it reduces capacitative Ca\(^{2+}\) entry, possibly by directly inhibiting store-operated Ca\(^{2+}\) channels. 2-APB also reduces Ca\(^{2+}\) efflux from mitochondria by inhibition of the Na\(^{+}\)-Ca\(^{2+}\) exchanger and may potentially block gap junctional intercellular communication.

5. mAb18A10 and Other Neutralizing Anti-Inositol 1,4,5-Triphosphate Receptor Antibodies.

a. Source and Chemical Structure. A monoclonal antibody, mAb18A10, has been raised in rats against the partially purified 2749-residue-long mouse cerebellar InsP\(_3\)R produced by fusing spleen cells with mouse Sp2 myeloma cells (Maeda et al., 1988). Its epitope is located within residues 2736 and 2747 at the very end of the mouse InsP\(_3\)R1 C-terminal tail (Swiss-Prot Access Number P11881) (Furuichi et al., 1989; Nakade et al., 1991). A polyclonal antiserum, named 3\(^\beta\)_1, has also been raised in rabbits against a 95-residue synthetic polypeptide corresponding to residue sequence 2604–2698 of InsP\(_3\)R, but its epitope has not been defined (Sullivan et al., 1995). Another neutralizing monoclonal antibody, named IPR.1, has been produced in mice using an 11-residue synthetic polypeptide corresponding to residue sequence 2546–2556 of the mouse InsP\(_3\)R1 (Bourguignon et al., 1993a), and a neutralizing rabbit polyclonal antibody has also been raised against the same polypeptide (Boittin et al., 2000).

b. Mechanism of Action. The cell-impermeant mAb18A10 has two effects in mouse cerebellar microsomes (Nakade et al., 1991): it suppresses IICR and increases the ability of the InsP\(_3\)R to bind InsP\(_3\). Suppression of Ca\(^{2+}\) release is only observed at submaximally effective InsP\(_3\) concentrations (manifested by a rightward shift of InsP\(_3\) threshold concentration to ~60 nM from a basal value of ~20 nM and a 5-fold increase in the EC\(_{50}\) values, which are 500 nM versus 110 nM) as the antibody does not affect maximal response. Binding of InsP\(_3\) to the InsP\(_3\)R is maximally increased by ~25%
with a $K_D$ value decreased to $\sim 25$ nM from a basal value of $\sim 45$ nM (Nakade et al., 1991).

Since the effective concentration range of the antibody (2–50 $\mu$g/ml) is the same for both suppressing IICR and decreasing the ability of the InsP$_3$R to bind InsP$_3$, it is likely that these effects are related (Nakade et al., 1991). Because the InsP$_3$R C-terminal tail is located close to the central membrane pore of the tetrameric Ca$^{2+}$ release channel, it may be that inhibition of channel activity occurs either as an allosterically-mediated change in channel conformation suppressing activity, or as steric hindrance of the pore by the InsP$_3$R-bound antibody (Nakade et al., 1991). The increased InsP$_3$ binding to the InsP$_3$R may be due to an allosterically induced conformational change (Nakade et al., 1991). However, an explanation as to why mAb18A10 can only inhibit Ca$^{2+}$ release at submaximally effective concentrations of InsP$_3$, which would reconcile the Ca$^{2+}$ release inhibition with InsP$_3$ binding promotion, may be that channel opening is delayed after InsP$_3$ binds to the receptor (Nakade et al., 1991). At low concentrations, InsP$_3$ may be degraded by inositol polyphosphate 5-phosphatase faster than the time required for channel opening under the influence of the antibody. This effect would be overcome if the initial InsP$_3$ concentration was sufficiently high and remained above threshold level long enough. Detailed kinetic studies are required to test this hypothesis. However, one might predict that, if this hypothesis was correct, mAb18A10 would be ineffective against Ca$^{2+}$ release by nonhydrolysable InsP$_3$ agonists, such as the adenophostins, or by InsP$_3$ in presence of a 5-phosphatase inhibitor (Qazi and Trimmer, 1999).

Interestingly, the polyclonal antiserum 3' $\beta_1$ is able to inhibit ICR from a Xenopus oocyte membrane fraction, but in contrast with mAb18A10, does not alter receptor affinity ($K_D$) for InsP$_3$ (Sullivan et al., 1995). This suggests that 3' $\beta_1$ and mAb18A10 inhibit InsP$_3$R channel activity by different mechanisms.

Although mAb18A10 has been extensively used in ascidian, hamster, and mouse oocytes (Miyazaki et al., 1992; Fujiwara et al., 1993; Miyazaki and Shirakawa, 1993; Xu et al., 1994; Shirakawa and Miyazaki, 1995; Mehlmann et al., 1996; Pesty et al., 1998; Yoshida et al., 1998), to our knowledge, neither mAb18A10 nor the polyclonal antibody 3' $\beta_1$ has been used in smooth muscle preparations.

c. Selectivity. Although selectivity against other relevant molecular targets, such as RyR, has not been experimentally demonstrated, the 12-residue-long sequence containing the epitope to which mAb18A10 binds
is shared only by the mouse, rat, and human InsP$_3$R1. This sequence is not present in InsP$_3$R1 and InsP$_3$R2.

Interestingly, although the polypeptide used to raise the neutralizing monoclonal antibody IPR.1 corresponds to a residue sequence also found in the mouse, rat, and human InsP$_3$R1 (Bourguignon et al., 1993a,b), homologs of this sequence also occur in the InsP$_3$R2 and InsP$_3$R3 from these species. IPR.1 thus recognizes all three known InsP$_3$R isoforms. In enzymatically dissociated smooth muscle cells from porcine trachea, IPR.1 inhibits the $C_{1_{Ca}}$ oscillations induced by Ach, as does heparin (5 mg/ml in pipette solution), another InsP$_3$R channel blocker (Table 13; see Section II.C.6.b.) (Liu and Farley, 1996). In contrast, neither IPR.1 nor heparin affect the caffeine-induced transient $C_{1_{Ca}}$ that is presumably caused by RyR-induced SR Ca$^{2+}$ release (Liu and Farley, 1996) (Table 13; see Section II.B.2.b.), suggesting that IPR.1 is selective for InsP$_3$R versus RyR. In agreement with this finding, a rabbit polyclonal antibody raised against the same polypeptide used to raise IPR.1 inhibits Ach- and caged InsP$_3$-induced Ca$^{2+}$ waves in enzymatically dissociated rat ureteric smooth muscle cells, an effect shared by heparin (1 mg/ml in pipette solution) (Boittin et al., 2000).

d. Use in Smooth Muscle Preparations. mAb18A10, a targeted monoclonal antibody against InsP$_3$R and the polyclonal antiserum 3’β1, have not been used extensively in smooth muscle; only results with IPR.1 and the associated polyclonal antibody have been published (see Table 13). mAb18A10 has two important effects: it reduces IICR (possibly by delaying InsP$_3$R channel opening) and also increases InsP$_3$ binding to its receptor by ~25%.

6. Heparin.

a. Source and Chemical Structure. Heparin is a component of various tissues (especially liver and lung) and mast cells in man and several mammalian species; its principle and active constituent is a highly sulfated (thus highly anionic), polydisperse polysaccharide (glucosaminoglycan) of molecular weight 4 to 20 kDa consisting of alternating d-glucosamine and d-glucuronic acid residues, both sulfated, in a 1,4-linkage (Ueno and Shimada, 2001). Sulfation has been characterized as mainly 2-N,6-O-disulfated d-glucosamine, and 2-O-sulfated d-iduronic acid and d-glucuronic acid (Lindahl and Hook, 1978). A subset of heparin molecules in the lower end of its molecular weight range (4 to 6 kDa), termed low molecular weight heparin, is usually used in the experiments cited below, and it is presently unknown whether this is critical to its mechanism of action and selectivity as an InsP$_3$R inhibitor. Although recent advances in carbohydrate analysis and biochemistry have shed light on heparin fine structure, its precise chemical structure remains undetermined.

b. Mechanism of Action. Heparin, which is cell-impermeant, potently and competitively inhibits InsP$_3$ binding to the InsP$_3$R, as initially shown in rat cerebellar membranes (Worley et al., 1987) [IC$_{50}$ value ~2–5 μg/ml, similar values in human myometrium (Varney et al., 1990), rat vas deferens (Mourey et al., 1990), and bovine aortic smooth muscle (Chadwick et al., 1990)]. It has a similar potency in isolated membranes and for inhibition of InsP$_3$-induced contractile activity in β-escin-permeabilized rabbit main pulmonary artery (IC$_{50}$ = 5 μg/ml) (Kobayashi et al., 1988). Heparin (20 μg/ml) prolongs the delay, slows the rate, and reduces the amplitude of SR Ca$^{2+}$ release induced by photolysis of caged InsP$_3$ in β-escin-permeabilized guinea pig portal vein, as the kinetics of caged InsP$_3$ are similar to that of low concentrations of InsP$_3$ (Somlyo et al., 1992).

In terms of SAR, the effects of heparin appear to be specific as equivalent concentrations of chondroitin sulfate, a highly sulfated glycosaminoglycan of very similar chemical composition, and are ineffective in rat cerebellar membranes (Worley et al., 1987). Likewise, neither de-N-sulfated heparin, obtained by specific de-N-sulfation of native heparin, nor heparin sulfate (a structurally similar molecule to heparin but without 2-N,6-O-disulfated d-glucosamine and no 2-O-sulfated d-iduronic acid, are effective in blocking IICR in saponin-permeabilized aortic smooth muscle cells (Yamamoto et al., 1990). Consistent with these findings, de-N-sulfated heparin was also shown to be unable to inhibit IICR in the DDT$_2$MF-2 smooth muscle cell line (Ghosh et al., 1988). These results suggest that the sulfate groups at C-2-N and C-6 of d-glucosamine and at C-2 of L-iduronic acid are important for the inhibitory effect (Yamamoto et al., 1990, 1991).

An activity profile similar to heparin was described for low molecular weight dextran sulfate (average 5 kDa), the salt of sulfuric acid esters of the glucose polymer dextran having an average of two sulfate groups per glucose unit. This compound inhibits InsP$_3$-induced decrease in cellular Ca$^{2+}$ content in saponin-permeabilized primary cultured rat aortic smooth muscle cells with an IC$_{50}$ value of ~173 μg/ml ([versus IC$_{50}$ ~7 μg/ml for heparin in this preparation (Yamamoto et al., 1990)]
without affecting the caffeine-induced decrease in cellular Ca$^{2+}$ content (at up to 10 mg/ml) (Yamamoto et al., 1991). This suggests selectivity for IICR versus CICR, as it is the case for heparin (see Selectivity section). Consistent with this functional effect, dextran sulfate also concentration-dependently inhibits InsP$_3$ binding to these permeabilized cells, although here too it has lower potency than heparin (IC$_{50}$ ~ 120 µg/ml versus 10 µg/ml for heparin) (Yamamoto et al., 1991). Unlike heparin, dextran sulfate has no sulfate group at C-6 of the d-glucose, because the d-glucose units are α-(1→6)-linked to each other. In contrast, the d-glucosamine of heparin is α-(1→4)-linked to L-iduronic acid. Thus, there may be differences between these two compounds regarding the relative orientation of the sulfate groups on two adjacent units. In any case, the inhibitory activity of both compounds suggests that at least two sulfate groups, one presumably at C-2 of a d-glucose unit and the other on the adjacent unit, are essential for interaction with the InsP$_3$R (Yamamoto et al., 1991).

In bovine adrenal cortex membranes, heparin does not to change the affinity of binding sites for InsP$_3$ but decreases the number of sites available for InsP$_3$ binding (Guillemette et al., 1989). Heparin inhibits InsP$_3$-dependent gating of InsP$_3$R Ca$^{2+}$-channel conductance in rat aortic smooth muscle microsomes incorporated into planar lipid bilayers (Ehrlich and Watras, 1988). Interestingly, the affinity of InsP$_3$R for heparin does not always correlate with the affinity of InsP$_3$R for InsP$_3$ and is not species-specific (Varney et al., 1990). However, heparin is a much larger molecule than InsP$_3$, which may give rise to access difficulties creating artifactual differences between tissues and preparations (Varney et al., 1990).

c. Selectivity. At a concentration maximally effective in inhibiting InsP$_3$ action (300 µg/ml (Kobayashi et al., 1988), heparin is ineffective at preventing caffeine-induced (20 mM) contractile activity in saponin-permeabilized rabbit main pulmonary artery (Kobayashi et al., 1988) or caffeine-induced (20 mM) Ca$^{2+}$ release and contractile activity in permeabilized rabbit main pulmonary artery and ileum longitudinal smooth muscle layer (Kobayashi et al., 1988, 1989b).

Furthermore, heparin (25 mg/ml) is ineffective at changing the P$_o$ of RyR partially purified from bovine coronary arteries microsomal protein fractions and reconstituted into planar lipid bilayers, which is fully sensitive to caffeine (0.5–5 mM), ryanodine (0.1–50 µM), ruthenium red (40 and 80 µM), and cADPR (0.01–1 µM) (Li et al., 2001). These results suggest that heparin inhibition of SR Ca$^{2+}$ release is selective for InsP$_3$R versus RyR. However, heparin induces Ca$^{2+}$ release from skeletal muscle microsomes (Ritov et al., 1985). In rabbit skeletal muscle microsomes incorporated into planar lipid bilayers, heparin (0.1–1 µg/ml on cis chamber, equivalent to the cytoplasmic side) increases the P$_o$ of RyR in a ryanodine (5 µM)- and ruthenium red (2 µM)-sensitive manner (characteristic of RyR1) and does so in a Ca$^{2+}$-dependent manner (requires free [Ca$^{2+}$] $\geq$ 80 nM in cis chamber, inactive if <20 nM) (Bezprozhvanny et al., 1993). This effect is mimicked, but with reduced potency, by other polyanions such as pentosan polysulfate and polyvinyl sulfate, suggesting that these agents act by increasing the local concentration of Ca$^{2+}$ near regulatory sites on the RyR-channel complex (Bezprozhvanny et al., 1993). That this potentiating effect is relevant for smooth muscles, where RyR2 and/or RyR3 isoforms can be expressed instead of or in addition to RyR1, is evidenced by the fact that heparin (300 µg/ml), which inhibits histamine (100 µM)- and InsP$_3$ (40 µM)-induced contractile activity, tends to potentiate subsequent caffeine (20 mM)-induced contractile activity in β-escin-permeabilized guinea pig ileum longitudinal smooth muscle layer (Fukami et al., 1993), although this effect could also be explained by a functional overlap of InsP$_3$- and caffeine-sensitive SR Ca$^{2+}$ stores.

Heparin (300 µg/ml) does not affect K$^+$ (143 mM)-induced contractile activity in reversibly permeabilized rabbit ileum longitudinal smooth muscle layer (Kobayashi et al., 1988), suggesting that it does not affect Ca$^{2+}$ sensitivity of contractile proteins. Consistent with this finding, heparin, at a concentration that maximally inhibits InsP$_3$ action (300 µg/ml), is ineffective in blocking the potentiation of Ca$^{2+}$-induced contractile activity by Cch or GTPγS in β-escin-permeabilized rabbit ileum longitudinal smooth muscle, suggesting that it does not affect G-protein-dependent sensitization of the contractile apparatus to Ca$^{2+}$ (Kobayashi et al., 1989b). Likewise, heparin (300 µg/ml) does not affect calmodulin-induced potentiation of Ca$^{2+}$-induced contractile activity in β-escin-permeabilized rabbit tracheal smooth muscle (Iizuka et al., 1998).

However, although heparin has no effect on $I_{\text{CRAC}}$ in mast cells (Hoht and Penner, 1992), its highest molecular weight fraction (14–17 kDa) inhibits capacitative Ca$^{2+}$ entry in human neutrophils (100 µg/ml on cytoplasmic side) (Davies-Cox et al., 2001). Neither microinjected neutralizing monoclonal antibody IPR.1 (250 kDa) nor albu- min-conjugated heparin (≥200 kDa) inhibits capacitative Ca$^{2+}$ entry, whereas all three agents reduce intracellular Ca$^{2+}$ release induced by the peptide formylmethionyl-leucylphenylalanine (f-Met-Leu-Phe) (Davies-Cox et al., 2001). This suggests that the capacitative Ca$^{2+}$ entry mechanism in these cells involves a heparin-sensitive step that is not accessible to higher molecular weight InsP$_3$R inhibitors. Consistent with these findings, heparin inhibits an InsP$_3$-activated Ca$^{2+}$ current in plasma membranes by inhibiting the $P_o$ of 1) an InsP$_3$R-like Ca$^{2+}$ channel in excised plasma-membrane patches from cultured bovine aortic epithelial cells (1 µg/ml) (Vaca and Kunze, 1995), 2) an InsP$_3$R purified from rat liver plasma membrane (IC$_{50}$ value ~20 µg/ml; effective concentration range = 10–40 µg/ml) (Mayreitner et al., 1995), and 3) $I_{\text{CRAC}}$-like channels, termed miniature Ca$^{2+}$ channels, in excised plasma-membrane patches from mouse macrophage (Kiselyov et
al., 1999; Semenova et al., 1999) and from A431 cells (human carcinoma cell line) (Kiselyov et al., 1997) (100–500 µg/ml on cytoplasmic side).

At high concentrations (≥1 mg/ml), heparin significantly chelates free Ca2+ (K_D = 0.64 mM = 3.2 mg/ml), which likely explains its inhibition of K+ (143 mM) and Ch (10 µM)-induced contractile activity (IC50 values ~20 µg/ml and >50 µg/ml, respectively) of intact rabbit ileum longitudinal smooth muscle (Kobayashi et al., 1989b).

Heparin also inhibits bovine adrenal cortex InsP3 3-kinase in a noncompetitive manner with an IC50 value of ~0.4 µg/ml (versus an IC50 value of ~10–20 µg/ml for blocking InsP3R in this tissue). In contrast, it is without effect on inositol polyphosphate 5-phosphatase (at up to 2 mg/ml) (Guillemette et al., 1989). Given that these enzymes are responsible for InsP3 inactivation in the cytoplasm (see Section II.C.1.b.), these results raise the possibility that, at low concentrations, heparin might potentiate the effect of InsP3 by inhibiting its inactivation, although the relevance of this mechanism in smooth muscle preparations is unknown.

d. Use in Smooth Muscle Preparations. Heparin is considered prototypical inhibitor of InsP3R (Table 14). Although it is cell-impermeant, it is frequently used as a competitive inhibitor of InsP3 binding to microsomes and IICR in permeabilized smooth muscle. Heparin does not inhibit voltage-gated Ca2+ entry or G-protein-mediated intracellular Ca2+ sensitization, although it has other selectivity problems. The IC50 value for heparin in reducing IICR in permeabilized vascular smooth muscle is 5 µg/ml (Kobayashi et al., 1989b), a fairly low potency. At high concentrations however, heparin chelates Ca2+. In intact tissue, low concentrations of heparin (up to 2 mg/ml) do not affect contractile responses to K+ depolarization or agonist (Ch) stimulation, whereas higher concentrations (>20 µg/ml) are inhibitory (Kobayashi et al., 1989b).

III. Conclusions and Perspectives

We provide an overview of the pharmacology of research tools useful in understanding SR function in smooth muscle. Although we attempted to be comprehensive, there undoubtedly are oversights and omissions, which we hope will not detract from the usefulness of this overview. This review is aimed as a starting point for those who are new to the field and also as a summative document for more established investigators. Advances in our understanding of the physiology and pharmacology of the SR in smooth muscle has benefitted greatly from the availability of an array of agents that interact with various elements affecting SR function. Although such compounds have revealed the complex role of this organelle in regulating cell function, there is no doubt much more to be unearthed. Thus, there is a continuing need to develop improved ligands, coupled with a greater understanding of the strengths and limitations of currently available compounds. This, together with the application with more direct techniques, will greatly assist in the design of insightful experiments that will yield more detailed information.

Until relatively recently, contraction of smooth muscle was thought to depend almost exclusively on the entry of Ca2+ from extracellular sources, mainly through the activation of voltage-gated Ca2+ channels. However, the combined use of high-resolution imaging techniques and a variety of pharmacological modulators of SR function has revealed a complex system that has a far greater reliance on SR Ca2+ for the initiation, maintenance, and decay of smooth muscle tone. As recently summarized by Wier and Morgan (2003), smooth muscle SR serves a multitude of functions with regard to smooth muscle excitation-contraction coupling, including: 1) regulation of contraction (activation of contractile proteins) and relaxation (activation of KCa channels, SERCA-mediated Ca2+ uptake), 2) interception of a portion of Ca2+ that enters a cell (superficial barrier function), 3) generation of Ca2+ waves and oscillations, and 4) interaction with Ca2+-store-operated channels (possibly through the interaction of InsP3R and Trp channels). Smooth muscle contains isoforms of RyR as well as isoforms of InsP3R, and there is a clear interaction between release events occurring at these receptor sites (Iino, 2002). The Ca2+ stores in smooth muscle have variously been described as those containing only a single Ca2+-release channel (RyR or IP3; or both. Thus, RyR and InsP3R can work cooperatively as Ca2+ is a ligand at both sites (Iino, 2002).

Elementary Ca2+ release events, termed Ca2+ sparks, frequently occur in discreet but preferred areas of the cell. These preferred areas are adjacent to the superficially located SR and lie within ~15 nm of the plasma membrane (Somlyo and Franzini-Armstrong, 1985; Gordienko et al., 2001). Both RyR1 and RyR2 are required for Ca2+ spark activity, with the role of RyR3 thought to be minimal (Boittin et al., 2000) or inhibitory (Lohn et al., 2001). It has been proposed that microsparks, which are smaller in duration, point spread, and amplitude, may form the basis of Ca2+ waves (see Bradley et al., 2003). The average Ca2+ concentration recorded, e.g., with intracellular recorder dyes, represents the summation of the asynchronous Ca2+ waves of the individual smooth muscle cells. Therefore, an important consequence of SR Ca2+ release/depletion during the generation of Ca2+ waves is the stimulation of Ca2+ influx.

In addition to the now familiar concept of Ca2+ sparks, there are other modalities of spontaneously oc-
curing Ca^{2+} release events. Two such types are Ca^{2+} ripples and Ca^{2+} flashes, as observed in individual smooth muscle cells within intact segments of rat tail artery (Asada et al., 1999). Ca^{2+} ripples, which are modest in amplitude and frequency, are thought to be InsP_{3R}-generated. On the other hand, Ca^{2+} flashes are described as large Ca^{2+} discharges occurring in small areas (<20 μm) of the cell and that spread in a passive manner. Although ripples occur in about half the cell population studied, Ca^{2+} flashes are considerably more infrequent (Asada et al., 1999). These different patterns of Ca^{2+} delivery within the cell (sparks, waves, oscillations, etc.) encodes signaling information that determines the characteristics (e.g., amplitude, frequency,
and duration) of smooth muscle cell function at level as diverse as excitability/contractility, secretion, proliferation, migration, and cell cycling.

It is clear that Ca$^{2+}$ released from the SR can activate either contraction or relaxation. As reviewed by Wier and Morgan (2003), Ca$^{2+}$ released via InsP$_3$R channels initiates contraction, whereas Ca$^{2+}$ release through RyR channels mediates smooth muscle relaxation. The spread of the Ca$^{2+}$ wave generated by IICR is sufficiently slow (−20 μm sec$^{-1}$) to allow for activation of the contractile machinery. Smooth muscle activation generates asynchronous Ca$^{2+}$ waves, the frequency of which is increased with agonist concentration (Ruehlmann et al., 2000). When first observed by Hirose and Iino (1994), it was suggested that asynchronous Ca$^{2+}$ waves were likely due to regenerative IICR. Support for this comes from more recent observations in arteries lacking functional RyR, where plasma membrane receptor activation was still able to produce apparently normal Ca$^{2+}$ waves (Dreja et al., 2001). There is also evidence for a central role for RyR in the establishment of agonist-generated Ca$^{2+}$ waves (Peng et al., 2001). In contrast to the asynchronous Ca$^{2+}$ waves generated by agonists through discrete openings of InsP$_3$R, arteries can also undergo vasomotion or rhythmic contractions that are due to spatially uniform changes in Ca$^{2+}$, most likely generated by oscillatory changes in membrane potential (Mauban et al., 2001).

Regardless of the events leading to the generation of the Ca$^{2+}$ signal, to be effective as a messenger, mechanisms must exist that allow for this ion to cause discrete and targeted cellular activation in a manner that has spatial and temporal characteristics consistent with physiological events. The assembly of various combinations of SR, ion transporters, exchangers, pumps, and channels makes feasible the creation of gradients of Ca$^{2+}$ in various domains within the cell. A restricted space (15–30 nm) is created by the close opposition of a portion of the SR that extends toward the cell membrane (superficial SR) (see Section 1.D.). This superficially located SR acts as a Ca$^{2+}$ buffer by accumulating a component of Ca$^{2+}$ that enters the cell; the SERCA pump thus diverts part of the Ca$^{2+}$ that enters the cell away from the deeper elements of the cell for storage and subsequent extrusion (Poburko et al., 2004). Ca$^{2+}$ removal mechanisms, primarily comprised of the Na$^{+}$-Ca$^{2+}$ exchanger, and the PMCA maintain the buffering capacity of the SR.

There are currently no promising leads that implicate a therapeutic potential for reagents that disturb SR function in smooth muscle, be it vascular or non-vascular. One intriguing possibility may be in the manipulation of the basal leak of the relatively high basal leak of Ca$^{2+}$ in unstimulated smooth muscle—this would require characterization of the Ca$^{2+}$ leak pathway and selective inhibitors thereof. Presently, this would appear to be a somewhat distant possibility, since the Ca$^{2+}$ leak may not necessarily require specific structural elements in cell membrane. A more likely therapeutic application lies in the manipulation of the “spontaneous Ca$^{2+}$ release” from the SR—Ca$^{2+}$ sparks—in vascular (e.g., small arteries) and nonvascular (e.g., bladder) smooth muscles. It is highly likely that the properties of Ca$^{2+}$ sparks are altered in hyperactive smooth muscle. One approach to this would be to target the response elements associated with Ca$^{2+}$ sparks, since the sites of release (RyR) may be a more problematic site of manipulation as these receptors required for a host of normal functions including those related to organ development.

The development of more selective reagents will enable us to ask more refined and better-focused questions related to cell function. Included among these is better definition of the precise role of the PMCA in health and disease states. In addition, it may be possible to gain a greater understanding of possible cross-talk by the various elements regulating SR function, for instance, between the RyR and InsP$_3$R. Uncovering the distribution patterns of these Ca$^{2+}$ release sites and appreciating their interaction will generate fruitful insights in spontaneous contractile activity, such as pacemaker currents in the gut, spontaneous fluctuations of resistance artery tone, and the spread and function of intracellular Ca$^{2+}$ waves. Likewise, availability of newer reagents with known specificities will help to establish the possibility of dynamic interactions of the peripheral SR with elements of the plasma membrane such as caveolae (and possibly gap junctions). Such compounds will be a valuable tool in ongoing research on the structure-function aspects regulating the activity of phasic versus tonic smooth muscle.

The urgent need to develop cell-permeant reagents cannot be understated since the goal is to integrate these findings in intact, functional systems. This is particularly evident in the study of the functional roles InsP$_3$ and, more so, cADPR. Those who have advanced technologies generate meaningful data with agents that regulate these endogenous Ca$^{2+}$ releasing agents. One example is the need to understand the role of cADPR in smooth muscle—it produces no electrical or mechanical events when applied under acute conditions in smooth muscle, even though smooth muscle has the biochemical machinery for the synthesis and degradation of cADPR. With appropriate cell-permeant pharmacological tools, one can explore the possibility that cADPR has a more permissive or accessory role in excitation-contraction coupling, or even that it may have some trophic effects.

An emerging area is in the understanding of capacitative Ca$^{2+}$ entry and the pharmacology of Ca$^{2+}$ entry pathways that are activated by a depleted SR, e.g., trp channels. This is complicated immensely by several factors, one of which remains an overarching enigma—what is the sensing mechanism that couples SR content...
with extracellular Ca\textsuperscript{2+} entry? How does the luminal content of the SR signal the plasma membrane? Resolution of these details will undoubtedly spur the development of pharmacological strategies aimed at augmenting or attenuating the actions of such processes. This then leads to another layer of complexity in multiple isoforms of trp channels and combination patterns, which again may vary regionally. These issues will increase in importance if there is evidence linking capacitive Ca\textsuperscript{2+} entry and trp channel function/density to disease states.

It is apparent that these pharmacological tools, which in many cases were in routine use for striated muscle research, have found homes in smooth muscle research. There are some notable exceptions in the literature; for example, the dearth of reports on the use of adenophostins in smooth muscle as muscle is evident from this review of the available literature. Important strides are being made through innovative approaches with existing reagents, e.g., down-regulation of RyR using organ culture techniques.

As the possibilities made available by advances in techniques in molecular biology are better integrated with functional correlates, important insights in the (many) physiological roles of SR will emerge. Some efforts in this direction have produced some unexpected findings, such as the embryonic importance of RyR. Other opportunities may lie in manipulation of storage findings, such as the embryonic importance of RyR. With functional correlates, important insights in the techniques in molecular biology are better integrated

made through innovative approaches with existing reagents in smooth muscle as is evident from this review of the pharmacological strategies aimed at augmenting these details will undoubtedly spur the development of these findings. It is important to recognize the complex nature of this intracellular organelle. Findings that the SR that is associated with the nuclear membrane may also regulate transcriptional events are just one promissory note for radical changes in our appreciation of the role of the SR in various aspects of smooth muscle function.

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