Modulation of Evoked Contractions in Rat Arteries by Ryanodine, Thapsigargin, and Cyclopiazonic Acid

Hiroki Shima and Mordecai P. Blaustein

The contribution of sarcoplasmic reticulum (SR) Ca\(^{2+}\) release to evoked tension in rat arterial rings was studied by comparing the effects of ryanodine (an SR Ca\(^{2+}\) channel opener) and thapsigargin and cyclopiazonic acid (CPA) (two Ca\(^{2+}\)-ATPase inhibitors). Isometric tension was evoked by serotonin (5-HT), 30–50 mM external K\(^+\), and 10 mM caffeine in rings of aorta and a small (second-order) branch of the superior mesenteric artery (SMA). Resting tension was unaffected by 10 \(\mu\)M ryanodine or 1–5 \(\mu\)M thapsigargin, but 20 \(\mu\)M CPA raised resting tension in aortic rings and evoked spontaneous contractions in some SMA rings. Ryanodine (10 \(\mu\)M) or 1–5 \(\mu\)M thapsigargin partially depleted the SR Ca\(^{2+}\) stores (indicated by reduced caffeine-evoked contractions) and attenuated 5-HT– and high K\(^+\)–evoked contractions in aortic rings but augmented 5-HT– and high K\(^+\)–evoked contractions in SMA. Caffeine completely emptied the SR Ca\(^{2+}\) stores in the presence of ryanodine but not thapsigargin in both the aorta and SMA; thus, thapsigargin may selectively affect one component of a heterogeneous SR. When the aortic SR Ca\(^{2+}\) stores were empty (i.e., caffeine contractions were abolished), the 5-HT– and high K\(^+\)–evoked contractions in the aorta were also augmented. CPA rapidly emptied the SR Ca\(^{2+}\) stores in both the aorta and SMA. CPA augmented the 5-HT–evoked contractions in the SMA and in five of nine aortic rings but attenuated evoked contractions in the remaining aortic rings. The attenuation or abolition of the caffeine contractions implies that ryanodine, thapsigargin, and CPA all deplete the SR Ca\(^{2+}\) stores. The attenuated responses to 5-HT and high K\(^+\) observed when the aortic SR Ca\(^{2+}\) stores were only partially depleted are consistent with the idea that evoked SR Ca\(^{2+}\) release is a large component of the Ca\(^{2+}\) transient in the aorta. The augmentation of 5-HT– and high K\(^+\)–evoked responses after partial (SMA) or complete (aorta) depletion of the SR Ca\(^{2+}\) stores suggests that evoked release of SR Ca\(^{2+}\) normally regulates Ca\(^{2+}\) entry by negative feedback and/or that the SR normally buffers the evoked rise in cytosolic Ca\(^{2+}\). (Circulation Research 1992;70:968–977)

KEY WORDS • caffeine • cyclopiazonic acid • ryanodine • thapsigargin • sarcoplasmic reticulum • serotonin • vascular smooth muscle

Contraction of vascular smooth muscle (VSM) is normally activated by a rise in the cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) that is evoked by vasoconstrictors.\(^1\)\(^2\) The vasoconstrictors interact with plasma membrane receptors and thereby promote the influx of Ca\(^{2+}\) via agonist receptor–operated and/or voltage-gated Ca\(^{2+}\) channels in the plasma membrane.\(^3\)\(^4\) The vasoconstrictors also activate the formation of inositol-1,4,5-trisphosphate (IP\(_3\)), which, in turn, evokes the release of Ca\(^{2+}\) from intracellular stores located mainly in the sarcoplasmic reticulum (SR).\(^5\)\(^6\)\(^7\)

The relative contributions of vasoconstrictor-evoked Ca\(^{2+}\) entry and SR Ca\(^{2+}\) release to the changes in [Ca\(^{2+}\)]\(_i\) and tension depend on several factors that are likely to vary in VSM cells in different parts of the arterial tree. These factors include the relative size of the SR and the amount of Ca\(^{2+}\) (i.e., fractional saturation) in the SR store. For example, the VSM cells in at least some small muscular arteries have a significantly less extensive SR than do VSM cells in large conduit arteries.\(^8\)\(^9\)\(^10\) Thus, the SR may have only a relatively limited role as a source of Ca\(^{2+}\) for excitation–contraction coupling in small muscular arteries in which the movement of Ca\(^{2+}\) across the sarcolemma may dominate. Furthermore, the amount of Ca\(^{2+}\) in the store can be modulated by such factors as the Na\(^+\) gradient across the sarcolemma.\(^11\)\(^12\)

Removal of Ca\(^{2+}\) from the cytosol, by extrusion across the sarcolemma and by sequestration in the SR, is also important for the control of [Ca\(^{2+}\)]\(_i\) (and tension). Indeed, sequestration of Ca\(^{2+}\) by the SR may buffer [Ca\(^{2+}\)]\(_i\)\(^11\)\(^13\)\(^14\) and thereby modulate tension in those VSM cells in which Ca\(^{2+}\) entry across the sarcolemma is a major source of Ca\(^{2+}\) for excitation–contraction coupling.

Functional assessment of the role of SR in contraction is difficult. It has often involved measurements of
contraction in Ca\textsuperscript{2+}-free media, which eliminates Ca\textsuperscript{2+} entry\textsuperscript{9} but may also deplete Ca\textsuperscript{2+} stores and, in the presence of caffeine, unloads the SR Ca\textsuperscript{2+} store.\textsuperscript{15} Unfortunately, caffeine also inhibits cyclic adenosine 3',5'-monophosphate (cAMP) phosphodiesterase,\textsuperscript{16} thereby eliciting a rise in cAMP levels; this promotes smooth muscle relaxation by phosphorylating myosin light chain kinase, thus reducing its sensitivity to Ca\textsuperscript{2+} \textsuperscript{17,18}.

Ryanodine, a plant alkaloid, depletes the SR Ca\textsuperscript{2+} store by opening SR Ca\textsuperscript{2+} release channels.\textsuperscript{19} Ryanodine does not affect plasma membrane Ca\textsuperscript{2+} channels,\textsuperscript{20} nor does it affect the contractile apparatus or the sarcoplasmic Ca\textsuperscript{2+} transport mechanisms.\textsuperscript{21} Therefore, this alkaloid appears to be useful for assessing the relative role of the SR in VSM contraction.

Recently, two other agents that affect Ca\textsuperscript{2+} storage in the endoplasmic reticulum (of which the SR is a specialized form) have been described: thapsigargin and cyclopiazonic acid (CPA). There are, however, no published reports on the effects of these agents on VSM contraction.

Thapsigargin is a naturally occurring sesquiterpene lactone that causes the net loss of Ca\textsuperscript{2+} from ER by inhibiting the ATP-driven uptake of Ca\textsuperscript{2+}.\textsuperscript{22} Thapsigargin does not inhibit ATP-driven Ca\textsuperscript{2+} transport in plasma membrane vesicles,\textsuperscript{23} nor does it stimulate IP\textsubscript{3} formation,\textsuperscript{24} but it selectively blocks IP\textsubscript{3}-releasable Ca\textsuperscript{2+} sequestration in some cells.\textsuperscript{25-28} Moreover, although thapsigargin is a tumor promoter, it does not activate protein kinase C and thereby differs from phorbol esters.\textsuperscript{29}

CPA is an indole tetramic acid metabolite of Aspergillus and Penicillium. CPA was found to be a selective inhibitor of the SR Ca\textsuperscript{2+}-ATPase in skeletal muscle.\textsuperscript{30} Thus, the mechanism of modulation of Ca\textsuperscript{2+} stores by ryanodine, which promotes Ca\textsuperscript{2+} release, appears to be different from that of thapsigargin and CPA, which both inhibit the ATP-driven sequestration system.

We tested the effects of ryanodine, thapsigargin, and CPA on small rings of rat thoracic aorta (a conduit artery) and a small (second-order) branch of the superior mesenteric artery (SMA, a small muscular artery). Our aims were to compare the actions of these three agents and to determine the relative contribution of the SR to contractions induced by serotonin (5-HT), K\textsuperscript{+}, and caffeine in the two types of arteries.

**Materials and Methods**

**Experimental Methods**

Rings of rat thoracic aorta and SMA were used for these experiments. Details of the experimental procedures and statistical methods have been described;\textsuperscript{31} unless otherwise noted, statistical data refer to Student’s paired t test.

**Reagents and Solutions**

The composition of the standard physiological salt solution (PSS) and other solutions used for these studies has been described.\textsuperscript{31} CPA was purchased from Sigma Chemical Co., St. Louis, Mo.; thapsigargin was obtained from LC Services Corp., Woburn, Mass.; ryanodine was obtained from Calbiochem Corp., La Jolla, Calif. Sources of other reagents and drugs are given in the preceding article.\textsuperscript{31}

All drug solutions were made on the day of use. Ryanodine and thapsigargin were made as 1 mM stock solutions in 10% dimethyl sulfoxide; CPA was made as a 10 mM stock solution in 100% dimethyl sulfoxide; caffeine was directly dissolved in PSS. The preparation of other solutions is described elsewhere.\textsuperscript{31} The final concentration of dimethyl sulfoxide in PSS was always less than 0.1%.

**Results**

**Control Responses of Rat Aorta and Small Mesenteric Artery to Serotonin, K\textsuperscript{+}, and Caffeine**

Figure 1 illustrates the effects of 5-HT on tension in rings of rat aorta and SMA. During superfusion with control PSS, short exposures to 5-HT induced brief contractions in both types of rings, followed by relaxation back to the original "resting" tension as the 5-HT was washed out.

When the external media contained elevated (30 or 50 mM) K\textsuperscript{+} concentrations ([K\textsuperscript{+}]\textsubscript{o}), tension in SMA rose rapidly and approached a plateau (Figure 2B). Tension rose substantially more slowly in the aorta and did not reach a steady level during the 5-minute exposure to high [K\textsuperscript{+}]\textsubscript{o} (Figure 2A), but peak amplitude was constant with repetitive exposure to the same high [K\textsuperscript{+}]\textsubscript{o} solution. When the normal (4.7 mM) [K\textsuperscript{+}]\textsubscript{o} was restored, tension in both types of rings rapidly declined to control levels. The aorta and SMA were equally sensitive to elevated [K\textsuperscript{+}]\textsubscript{o}, but the SMA was substantially more sensitive to 5-HT than was the aorta (Figure 1; see also Reference 31).

Superfusion of aortic rings with PSS containing 10 mM caffeine for 5 minutes evoked brief contractions that were invariably followed by a reversible caffeine-induced relaxation to below the original resting tension (Figures 1A and 1B). Caffeine also induced a transient contraction in SMA, but tension then returned to the original baseline and no further relaxation was observed (Figure 1C).

**Effects of Ryanodine on Responses to Serotonin, K\textsuperscript{+}, and Caffeine**

Ryanodine (10 \mu M) had no effect on resting tension in either aorta or SMA (Figure 1). It markedly suppressed the contractile responses to caffeine in both types of arteries but did not inhibit the caffeine-induced relaxation in the aorta (Figure 1).

The effects of ryanodine on caffeine-evoked contractions from several such experiments are summarized in Figure 3. Ryanodine almost completely blocked the initial contractile response to caffeine in the SMA. In the aorta, however, ryanodine reduced the first response to caffeine by only 27±7% (n=6), but it completely blocked the second response to caffeine. This partial block of the contraction during the first exposure to caffeine in the aorta was not influenced by prior exposure to 5-HT; for example, it did not matter whether the ring had been activated by 5-HT only twice (Figure 1A) or seven times (Figure 1B), so that the caffeine-sensitive SR Ca\textsuperscript{2+} store did not seem to be unloaded by 5-HT in the presence of ryanodine. Moreover, this store was also not reloaded by 5-HT in the
presence of ryanodine; when the contractile response to caffeine was completely blocked, subsequent activation by 5-HT did not restore the caffeine response (Figure 1B). After washout of ryanodine, the caffeine contractions slowly recovered in both the aorta\(^4\) and SMA (Figure 1C).

Figures 1A and 1B show that 5-HT–induced contractions in aortic rings were significantly diminished by ryanodine before the application of caffeine (62±6% of control, \(n=7\), \(p<0.01\)). However, when the caffeine-sensitive \(\text{Ca}^{2+}\) stores were depleted by the application of caffeine in the presence of ryanodine, the 5-HT–evoked responses were significantly augmented (144±11% of control, \(n=6\), \(p<0.05\)). In contrast, in the SMA, 5-HT–evoked contractions were invariably augmented by ryanodine, even before the application of caffeine (153±7% of control, \(n=8\), \(p<0.01\); see Figure 1C), although in this case, as already noted, the caffeine-sensitive stores were markedly depleted by ryanodine alone. Data from several such experiments on rings of aorta and SMA are summarized in Figure 4 (left three bars).

Under control conditions a large fraction (perhaps half) of the aortic response to agonists is due to \(\text{Ca}^{2+}\) release from internal stores.\(^5\) In contrast, as illustrated in Figure 1B, the 5-HT–evoked responses in the presence of ryanodine (after caffeine treatment) were dependent on external \(\text{Ca}^{2+}\). In the SMA, too, the augmented 5-HT–evoked responses in the presence of ryanodine were totally dependent on external \(\text{Ca}^{2+}\) (Figure 1D).

Aortic contractions elicited by 30–50 mM [K\(^+\)], before the introduction of caffeine were also reduced by ryanodine (Figures 2A and 5), whereas those in the SMA were enhanced by ryanodine (Figures 2B and 5). Indeed, ryanodine appeared to shift the K\(^+\) dose–response curve toward higher [K\(^+\)], in the aorta and toward lower [K\(^+\)], in the SMA. After the introduction of caffeine and the
disappearance of the caffeine-evoked contractions, however, high [K⁺]₀-evoked contractions in the aorta were also augmented relative to control responses (Figure 2C). In other words, K⁺- and 5-HT-evoked contractions in the aorta were affected comparably by ryanodine and subsequent caffeine treatment.

**Figure 2.** Effects of ryanodine on high K⁺-evoked contractions in rings of rat aorta (panels A and C) and small mesenteric artery (panel B). In panels A and B, the thin and thick black bars below the tension recordings indicate, respectively, the periods of superfusion with physiological salt solution (PSS) containing 30 and 50 mM K⁺. In panel C, the thin black bars just below the tension recordings indicate the periods of superfusion with 30 mM K⁺ PSS, and the short open bars indicate the periods of superfusion with PSS containing 10 mM caffeine. The long open bars show the periods of superfusion with PSS containing 10 μM ryanodine. The results shown in panel C are representative; ryanodine decreased the 30 mM K⁺-evoked tension in four aortic rings to 68±7% of control before caffeine (p<0.05 vs. control by paired t test) and increased the evoked tension to 199±20% of control after caffeine (p<0.05 vs. control and p<0.01 vs. before caffeine, by paired t test). Temperature, 37°C; resting tension, 500 mg (panels A and C) and 300 mg (panel B); tissue wet weight, 2.0 mg (panel A), 0.06 mg (panel B), and 2.3 mg (panel C).

**Figure 3.** Effects of ryanodine, thapsigargin, and cyclopiazonic acid on the relative amplitudes of the caffeine-evoked contractions in rat aorta (solid symbols) and small mesenteric artery (SMA, open symbols). The data are normalized to the amplitudes of the caffeine-evoked contractions before introduction of the Ca²⁺-ATPase inhibitors (=1.0); results obtained during the first and second (and in some cases the third) exposure to caffeine after the introduction of the Ca²⁺-ATPase inhibitors are indicated. The data are mean±SEM (where the SEM values extend beyond the symbols) for the number of rings indicated in parentheses (n).
Effects of Thapsigargin on Resting Tension and on Serotonin-, Caffeine-, and K⁺-Evoked Tension

Some effects of thapsigargin were very similar to those observed with ryanodine. First, thapsigargin had no effect on resting tension (Figure 6). Second, thapsigargin reduced the contractile responses to caffeine in both the aorta and SMA but did not affect the caffeine-induced late relaxation response in the aorta. The attenuation of the caffeine contraction by thapsigargin appeared to be use dependent in the SMA but not in the aorta. In SMA, the first response to caffeine was reduced by only 4±14% relative to control (n=7); inhibition increased progressively during second and third exposures to caffeine (Figure 3, open triangles). In contrast, thapsigargin reduced the initial response of the aorta to caffeine by 57±8% relative to control (n=6), but little further reduction was observed with subsequent exposures to caffeine (Figure 3, solid triangles). The effects of thapsigargin and the other SR inhibitors were additive, however: in the presence of thapsigargin, ryanodine (Figure 6A) and CPA (not shown) both blocked the residual (thapsigargin-insensitive) caffeine-induced contractions in the aorta.

Thapsigargin (1 μM) diminished the 5-HT–evoked responses in aortic rings to 29±5% of control (n=6, p<0.01). In contrast to the effects observed with ryanodine, however, the 5-HT–evoked responses in the aorta were attenuated by thapsigargin even after exposure to caffeine (Figures 6A and 6C and hatched bars in Figure 4) and even when the thapsigargin concentration was increased to 5 μM (not shown). However, when either ryanodine (Figure 6A) or CPA (not shown) was added in the presence of thapsigargin and the residual caffeine-induced contractions were blocked, the 5-HT–evoked contractions in the aorta were augmented. On the other hand, SMA rings exhibited augmented 5-HT–

---

**Figure 4.** Effects of ryanodine, thapsigargin, and cyclopiazonic acid on the relative amplitudes of the serotonin (5-HT)–evoked contractions in rat aorta and small mesenteric artery (SMA). In all instances, the contractions are normalized to the mean amplitudes of the two 5-HT contractions observed before the introduction of the Ca²⁺-ATPase inhibitors (=1.0); these two “control” contractions never differed from one another by more than 5%. For the aorta, the bars indicate the relative amplitudes of the 5-HT–evoked contractions before (open bars) and after (hatched bars) exposure to caffeine. Note that five aortic rings exhibited increased 5-HT–evoked contractions in cyclopiazonic acid–containing physiological salt solution both before and after exposure to caffeine; four other rings exhibited reduced 5-HT–evoked contractions in cyclopiazonic acid–containing physiological salt solution both before and after exposure to caffeine. The numbers of rings tested are indicated in parentheses (n); thin bars indicate ±SEM. Significance of the differences from control contractions was determined by Student’s paired t test: *p<0.05; **p<0.01.

---

**Figure 5.** Effects of ryanodine and thapsigargin on the 30 mM and 50 mM K⁺–evoked contractions in rat aorta and small mesenteric artery (SMA). The contractions are normalized to the mean amplitudes of the 30 or 50 mM K⁺–evoked contractions, respectively, observed before the introduction of the Ca²⁺-ATPase inhibitors (=1.0). The numbers of rings tested are indicated in parentheses (n); thin bars indicate ±SEM. Significance of the differences from control contractions was determined by Student’s paired t test: *p<0.05; **p<0.01.
evoked responses (156±6% of control \( n=7, p<0.01 \)) in the presence of 1 \( \mu \text{M} \) thapsigargin alone, even under conditions in which large caffeine-evoked contractions could still be observed (Figures 4 and 6B). These augmented contractions in the SMA, like those observed in the presence of ryanodine, were totally dependent on external Ca\(^{2+}\) (Figure 6D).

Thapsigargin, like ryanodine, shifted the K\(^+\) dose-response curve slightly toward higher [K\(^+\)], in the aorta and toward lower [K\(^+\)], in the SMA; i.e., responses at [K\(^+\)], levels of 30–50 mM were reduced in the aorta and augmented in the SMA. Representative original recordings are presented in Figure 7, and the data from three such experiments are summarized in Figure 5 (hatched bars).

**Effects of Cyclopiazonic Acid on Resting Tension and on Serotonin-, Caffeine-, and K\(^+\)-Evoked Tension**

In contrast to ryanodine and thapsigargin, CPA increased unstimulated tension in the aorta, at least transiently (Figures 8A and 8B). On the average, 20 \( \mu \text{M} \) CPA raised aortic resting tension by about 16.3±4.8 mg/mg wet wt \( (n=9) \). The effect of CPA on resting tension in SMA was variable: 5–20 \( \mu \text{M} \) CPA induced large, unstable increases in resting tension in three of seven rings (Figure 8C) but had no measurable effect in the other four rings (not shown).

CPA, like ryanodine, virtually abolished the contractile responses to caffeine in both the aorta and SMA (Figure 3, squares) and had no effect on caffeine-induced relaxation in the aorta (Figures 8A and 8B). Unlike the other agents, however, the effect of CPA on the aorta was relatively rapid, and even the first response to caffeine was markedly reduced. CPA could also be washed out more readily than ryanodine or thapsigargin; after washout, large caffeine-induced contractions reappeared in both the aorta and SMA (Figure 8).

The effect of CPA on 5-HT-evoked contractions in SMA (Figures 4 and 8C) was similar to the effects observed with ryanodine and thapsigargin; CPA markedly and reversibly increased the responses to 219±33% of control \( (n=4) \).

In four of nine aortic rings exposed to CPA for 30 minutes, the tension evoked by 5-HT was reduced to 74±7% of control \( (p<0.05) \); the 5-HT-evoked tension in these rings was much further reduced, to 20±3% of control \( (p<0.01) \), after exposure to caffeine (Figures 4 and 8B). In the other five aortic rings, however, after 30 minutes of superfusion with CPA, the tension evoked by the initial application of 5-HT was increased to 218±23% of control \( (p<0.01) \). Tension was still substantially augmented, albeit to a smaller extent, in all of these rings after exposure to caffeine (171±21% of control; \( p<0.05 \)) (Figures 4 and 8A).

CPA also augmented the K\(^+\)-evoked contractions in both SMA (Figure 9) and the single aortic ring in which it was tested (not shown).

**Discussion**

**Ryanodine, Thapsigargin, and Cyclopiazonic Acid All Deplete Ca\(^{2+}\) Stores in Vascular Smooth Muscle**

In non-smooth muscle cells, thapsigargin and CPA reduce the endoplasmic reticulum (or SR) Ca\(^{2+}\) stores by
inhibiting the ATP-driven Ca\(^{2+}\) sequestration mechanisms (i.e., the Ca\(^{2+}\)-ATPases).\(^{22,26}\) In contrast, ryanodine depletes the stores by opening the Ca\(^{2+}\) release channels and making the endoplasmic reticulum (or SR) leaky to Ca\(^{2+}\).\(^{19,32,33}\) In some cells, the IP\(_3\)-releasable Ca\(^{2+}\) stores are thapsigargin sensitive but ryanodine insensitive.\(^{25-28}\)

Figure 7. Effects of thapsigargin on 30 mM and 50 mM K\(^{+}\)-evoked contractions in rings of rat aorta (panel A) and small mesenteric artery (panel B). The thin and thick black bars below the tension recordings indicate, respectively, the periods of superfusion with physiological salt solution (PSS) containing 30 and 50 mM K\(^{+}\). The long open bars show the periods of superfusion with PSS containing 1 \(\mu\)M thapsigargin. Temperature, 37\(^{\circ}\)C; resting tension, 500 mg (panel A) and 300 mg (panel B); tissue wet weight, 2.0 mg (panel A) and 0.05 mg (panel B).

Figure 8. Effects of cyclopiazonic acid on caffeine- and serotonin (5-HT)-evoked contractions in rings of rat aorta (panels A and B) and small mesenteric artery (SMA, panel C). Bolus injections of 5-HT into the superfusion inflow line are indicated by arrowheads; periods of superfusion with physiological salt solution (PSS) containing 10 mM caffeine are indicated by the black bars just below the tension recordings. The large open bars under the tension recordings indicate the periods during which the standard superfusion fluid (PSS) contained 20 \(\mu\)M (panels A and B) or 5 \(\mu\)M (panel C) cyclopiazonic acid. Recordings A and B illustrate, respectively, data from aortic rings in which cyclopiazonic acid reversibly augmented and attenuated the 5-HT-evoked contractions. Recording C illustrates data from an SMA ring in which cyclopiazonic acid spontaneously increased "resting" (unstimulated) tension. Temperature, 37\(^{\circ}\)C; resting tension, 500 mg (panels A and B) and 300 mg (panel C); tissue wet weight, 2.5 mg (panel A), 2.3 mg (panel B), and 0.06 mg (panel C).
this fits with the evidence that there are (at least) two classes of endoplasmic reticulum Ca$^{2+}$-ATPases, only one of which is inhibited by thapsigargin.\textsuperscript{34}

The SR in VSM is apparently neither structurally nor functionally homogeneous; e.g., it may be divided into peripheral or "junctional" SR and central or "perinuclear" SR.\textsuperscript{10} Moreover, SR Ca$^{2+}$ may be released by a Ca$^{2+}$-induced mechanism or by an IP$_{3}$-activated mechanism, and these two mechanisms may reside in different portions of the smooth muscle SR.\textsuperscript{23} Our observations (and see Reference 28) raise the possibility that these VSM Ca$^{2+}$ stores may also be pharmacologically heterogeneous. All three agents inhibited the caffeine-evoked increase in tension in both aortic and SMA rings (Figure 3), presumably by depleting the VSM caffeine-sensitive SR Ca$^{2+}$ stores. However, whereas CPA and ryanodine blocked almost all of the caffeine-evoked tension in both types of rings, thapsigargin reduced these responses by a maximum of only about 70%. The residual, thapsigargin-insensitive component may represent a different (perhaps IP$_{3}$-insensitive) SR Ca$^{2+}$ store.

None of the three agents affected the caffeine-induced relaxation response in the aorta. Thus, they apparently have no effect on the caffeine-sensitive cyclic nucleotide phosphodiesterase.

Effects of Ryanodine, Thapsigargin, and Cyclopiazonic Acid on Resting Tension

Ryanodine and thapsigargin apparently released stored Ca$^{2+}$ without raising resting tension or reducing the sensitivity of the contractile apparatus to Ca$^{2+}$. In the presence of these agents, 5-HT- and K$^{+}$-evoked contractions were enhanced when caffeine-evoked contractions were reduced or abolished. The implication is that ryanodine and thapsigargin release Ca$^{2+}$ relatively slowly\textsuperscript{28} from the SR into the cytosol; Ca$^{2+}$ extrusion can then prevent [Ca$^{2+}$], from rising and elevating resting tension unless extrusion is slowed, e.g., by removing external Na$^{+}$ to block Na-Ca exchange (in the absence of external Ca$^{2+}$, to prevent Ca$^{2+}$ influx).\textsuperscript{8}

In contrast, CPA presumably raises resting tension in both aorta and SMA because it unloads the SR Ca$^{2+}$ store more rapidly than either ryanodine or thapsigargin but not as rapidly as caffeine. These transient CPA-induced changes in resting tension were more long lasting than those induced by caffeine, which terminated as a result of the rise in cyclic nucleotide levels.\textsuperscript{17,18}

Effects of Ryanodine, Thapsigargin, and Cyclopiazonic Acid on Serotonin- and K$^{+}$-Evoked Contractions

Mesenteric Artery. In SMA, all three agents augmented the contractions evoked by submaximal concentrations of 5-HT and by modestly elevated [K$^{+}$], even when the caffeine-sensitive Ca$^{2+}$ stores were not completely empty. In other words, the vasoconstrictor dose-response curves were shifted toward lower concentrations by all three agents in the SMA; the maximal vasoconstrictor-evoked responses were apparently unaltered. These data suggest that SR may modulate contractile responses in SMA in part by sequestering (buffering) some of the Ca$^{2+}$ that enters the cells during excitation. In vivo, however, these VSM cells may normally be tonically contracted, so there may be a more dynamic exchange of Ca$^{2+}$ between the SR and the cytosol as the level of cell activation is altered. In our in vitro experiments, the rings were only transiently activated during long periods of rest. Under these conditions, it appears that relatively little Ca$^{2+}$ for the 5-HT- and K$^{+}$-evoked contractions in SMA came from the caffeine-sensitive Ca$^{2+}$ stores (as in bovine tail artery).\textsuperscript{9} It is unlikely that these contractions were due to the release of Ca$^{2+}$ from a caffeine-insensitive portion of the SR, because such contractions are markedly reduced by removal of external Ca$^{2+}$ and by Ca$^{2+}$ channel blockers.\textsuperscript{8}

Aorta. When the caffeine-sensitive Ca$^{2+}$ store was only partially depleted by ryanodine or thapsigargin in the aorta, both 5-HT- and K$^{+}$-evoked (submaximal) contractions were significantly attenuated. After complete loss of the caffeine response (in ryanodine), however, the contractions of aortic rings evoked by submaximal concentrations of 5-HT and [K$^{+}$], were significantly augmented, relative to control responses; i.e., as in SMA, the dose–response curves were shifted toward lower vasoconstrictor concentrations. These augmented contractions were totally dependent on extracellular Ca$^{2+}$.

The attenuated responses can be explained if 1) much of the Ca$^{2+}$ for activation of the evoked contractions in the aorta is normally derived from the caffeine-sensitive store\textsuperscript{8} and/or 2) Ca$^{2+}$ can still be buffered by the SR when the caffeine-sensitive stores are not totally depleted.

The augmented responses in the aorta, like those in the SMA, may be attributed to the loss of the Ca$^{2+}$ buffering activity of the SR because the SR either is very leaky to Ca$^{2+}$ (in the case of ryanodine) or is unable to sequester Ca$^{2+}$ because of inhibition of the Ca$^{2+}$-dependent ATPase (in the case of thapsigargin and CPA). The implication is that, even in the aorta, Ca$^{2+}$ buffering by the SR helps to modulate the amplitude of vasoconstrictor-evoked contractions. Alternatively, since there are (at least) two types of SR,\textsuperscript{25,26,28}
complete (but not partial) emptying of Ca\(^{2+}\) from the caffeine-sensitive SR might lead to markedly increased loading of Ca\(^{2+}\) into a caffeine-insensitive (as well as CPA- and thapsigargin-insensitive) store. Ca\(^{2+}\) from the caffeine-insensitive store might then be released when the VSM is stimulated, by either 5-HT, which should increase IP\(_3\) levels, or high [K\(^+\)], which should not, but which might initiate Ca\(^{2+}\)-induced Ca\(^{2+}\) release. However, the fact that 5-HT responses in the presence of ryanodine or thapsigargin are virtually completely dependent on external Ca\(^{2+}\), as are some K\(^{+}\)-evoked responses,\(^8\) appears to rule out this alternative hypothesis.

An intriguing possibility that may tie together all the aforementioned observations is that there is feedback between the junctional SR and the sarcoclemmal Ca\(^{2+}\) entry mechanisms.\(^9\) Release of Ca\(^{2+}\) from this SR may, for example, inactivate sarcoclemmal Ca\(^{2+}\) entry\(^9\) and, thereby, attenuate tension development. When the junctional SR is empty and no longer able to sequester Ca\(^{2+}\), however, the regulation of [Ca\(^{2+}\)]\(_i\) and tension may then be dominated by the sarcoclemmal Ca\(^{2+}\) entry and the SR may be unable either to supply Ca\(^{2+}\) or to help buffer the entering Ca\(^{2+}\) and modulate tension development. The junctional SR may contain all the thapsigargin- and IP\(_3\)-sensitive Ca\(^{2+}\) stores because IP\(_3\) synthesis and degradation occur at or near the plasmalemma; the thapsigargin-insensitive SR Ca\(^{2+}\) stores may then be located more centrally.

The variable aortic responses to 5-HT observed in the presence of CPA, when the caffeine-sensitive stores were depleted, are difficult to explain. The action of CPA on the SR Ca\(^{2+}\)-ATPases was apparently less selective than thapsigargin,\(^34\) and perhaps CPA also has other effects, such as alteration of feedback between the junctional SR and the sarcoclemna, or direct inhibition of contractile activation. This may have been the dominant effect in some aortic rings, but not in others.

**Conclusions**

Our data indicate that ryanodine, thapsigargin, and CPA all deplete VSM SR Ca\(^{2+}\) stores, albeit by different mechanisms. Furthermore, the results with thapsigargin provide evidence for the functional heterogeneity of VSM SR. Especially important, however, is the evidence that the SR in VSM may play a critical role as a Ca\(^{2+}\)-buffering organelle. In addition to serving as a source of Ca\(^{2+}\) for contractile activation, the SR may help to modulate tension by limiting the rises in [Ca\(^{2+}\)]\(_i\) induced by rapid Ca\(^{2+}\) entry, perhaps by feedback inactivation of sarcoclemmal Ca\(^{2+}\) channels as well as by rapid (re)sequestration of Ca\(^{2+}\) after evoked release. This dynamic role of the SR in sequestering as well as releasing Ca\(^{2+}\) in VSM now needs to be assessed during tonic activity to gain a better understanding of its physiological significance.

The spatial, functional, and pharmacological heterogeneity of VSM SR, the possible feedback control of Ca\(^{2+}\) entry, and the differences between SR in different blood vessels are all evidence of the complexity of VSM SR and the control of Ca\(^{2+}\) transients. Moreover, multiple factors regulate cell Ca\(^{2+}\) and contraction in VSM: 1) the fractional saturation of SR Ca\(^{2+}\) stores, 2) the fraction of Ca\(^{2+}\) released from the SR stores, 3) the amount of Ca\(^{2+}\) that enters via sarcoclemmal Ca\(^{2+}\) channels, and 4) the sensitivity of the contractile apparatus to the available Ca\(^{2+}\). Several of the underlying mechanisms may be modulated by protein kinase C, and there may be qualitative and quantitative differences in this modulation in different blood vessels. For example, activation of protein kinase C by phorbol esters may diminish SR Ca\(^{2+}\) stores,\(^37\) increase plasma membrane Ca\(^{2+}\) conductance, and enhance the sensitivity of the contractile apparatus to Ca\(^{2+}\) (see Reference 31). In the preceding report,\(^31\) we described differences in the effects of phorbol esters on the agonist-evoked versus K\(^{+}\)-evoked responses of the aorta and SMA. The fact that thapsigargin attenuated both K\(^{+}\)-evoked and 5-HT-evoked contractions in the aorta while phorbols augmented these responses is consistent with the idea that the dominant effect of the phorbols on the aorta is to enhance Ca\(^{2+}\) sensitivity and/or Ca\(^{2+}\) entry. In SMA, phorbol esters and thapsigargin both increased K\(^{+}\)-evoked contractions, but only thapsigargin increased 5-HT-evoked contractions. We have attributed the latter effect to a reduction in Ca\(^{2+}\) buffering; it cannot be explained by depletion of SR Ca\(^{2+}\) stores even though thapsigargin did reduce caffeine-evoked contractions. Thus, it appears that the inhibitory effect of phorbols on 5-HT-evoked contractions in SMA\(^31\) may be due primarily to a large reduction in the availability of Ca\(^{2+}\) and perhaps also in part to an increase in Ca\(^{2+}\) buffering.

**Acknowledgments**

We thank Dr. Sergio Grinstein for very worthwhile suggestions regarding the use of thapsigargin and CPA. We also thank Dr. William F. Goldman for many fruitful discussions throughout the course of this study and for his very insightful critique of the manuscript and Dr. Dora M. Berman for very helpful discussions about the pharmacology of calcium stores.

**References**

Modulation of evoked contractions in rat arteries by ryanodine, thapsigargin, and cyclopiazonic acid.

H Shima and M P Blaustein

_Circ Res._ 1992;70:968-977
doi: 10.1161/01.RES.70.5.968

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/70/5/968

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/