Calcium Release from Aortic Sarcoplasmic Reticulum

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Calcium Release from Aortic Sarcoplasmic Reticulum. Journal of Molecular and Cellular Cardiology (1989) 21 (Suppl I) 125-130. The ability of inositol 1,4,5-trisphosphate (IP3) and other inositol phosphates to induce calcium release from canine aortic sarcoplasmic reticulum vesicles was examined. Using the calcium indicator chlorotetracycline or antipyrylazo III, aortic vesicles were shown to accumulate calcium in the presence of ATP, and then release ~25% of the intravesicular calcium upon addition of 7 μM IP3. Inositol 2-phosphate, inositol 1,4-bisphosphate, and inositol 1,3,4,5-tetrakisphosphate did not induce calcium release from these vesicles, and GTP[γ-S] did not affect the IP3-induced calcium release. Aortic IP3-induced calcium release was not affected by ruthenium red, but was inhibited by Mg2+ and Ca2+, and thus differs from the Mg2+-insensitive IPs-induced calcium release in platelets and the ruthenium red-sensitive IP3-induced calcium pathway in skeletal muscle sarcoplasmic reticulum. Stopped-flow analyses showed that aortic IP3-induced calcium release was much slower than the caffeine-induced calcium release from skeletal muscle sarcoplasmic reticulum. Moreover, the aortic IP3-induced calcium release was biphasic, suggestive of heterogeneity of the putative calcium channels.

KEY WORDS: Sarcoplasmic reticulum; Inositol 1,4,5-trisphosphate; Calcium; Aorta; Smooth muscle; Calcium release.

Introduction

Several investigators have shown that inositol 1,4,5-trisphosphate (IP3) is an important stimulus for calcium release from the sarcoplasmic reticulum of smooth muscle, and that this calcium release probably involves activation of a channel rather than inhibition of the calcium pump [1, 3, 19]. Smith et al. [14] have also shown that IP3-induced calcium release from permeabilized aortic cells is nucleotide dependent, analogous to the Ca2+-sensitive and caffeine-sensitive calcium channels in skeletal muscle sarcoplasmic reticulum [13]. The latter observation contrasts with a report on platelet reticular membranes [2], in which IP3-induced calcium release was shown to be nucleotide-independent, and thus raises the possibility that IP3-sensitive calcium pathways may differ between tissues, analogous to the heterogeneity of the voltage-dependent calcium channel [8]. The present study was undertaken to extend the comparison of the IP3-sensitive calcium release system in vascular smooth muscle with the IP3-sensitive calcium pathway in platelet reticular membranes and the well characterized caffeine-sensitive calcium channel in skeletal muscle sarcoplasmic reticulum. With the aid of a stopped-flow apparatus, the rate constant(s) of aortic IP3-induced calcium release were also determined.

Methods

Aortic sarcoplasmic reticulum vesicles were isolated from canine aortic smooth muscle by differential centrifugation, as described previously [17]. Skeletal sarcoplasmic reticulum vesicles (presumably enriched in terminal cisternae) were isolated from rabbit hindlimb muscle, as described by Kim et al. [6].

Calcium uptake and release by aortic sarcoplasmic reticulum vesicles was monitored fluorimetrically, using the calcium indicator chlorotetracycline (380 nm excitation, 520 nm emission). Reaction media contained 120 mM KCl, 1.5 mM Na2ATP, 0.5 mM
MgCl₂, 10 mM creatine phosphate, 8 u/ml creatine phosphokinase, 10 μM chlorotetracycline, 20 mM HEPES (pH 6.8, 25°C). Calcium uptake was initiated by addition of aortic sarcoplasmic reticulum vesicles (0.05 mg/ml), then at a specified time calcium release was initiated by addition of IP₃ (<0.004 vol.). Total intravesicular calcium content (in relative fluorescence units) was determined by addition of the ionophore alamethicin.

Calcium uptake and release by aortic sarcoplasmic reticulum vesicles was also monitored by dual wavelength spectrophotometry, using the calcium indicator antipyrylazo III (720 nm, 790 nm). Reaction media were similar to those described above, except that 400 μM antipyrylazo III was used in place of chlorotetracycline. Calcium uptake was initiated by addition of aortic vesicles (0.35 mg/ml) to media containing the specified free Mg²⁺ concentration (0.06–5.6 mM), then 7 min later calcium release was initiated by addition of IP₃ (0.004 vol.). Total intravesicular calcium content was determined by addition of the calcium ionophore A23187 (2 μM). Reactions were calibrated by four serial additions of CaCl₂ (0.004 vol.).

Fast kinetic analyses of calcium release were accomplished using an Aminco stopped flow apparatus attached to the dual wavelength spectrophotometer. Reaction media were as described above, with 400 μM antipyrylazo III and 1.5 mM 2,3-diphosphoglycerate. Preliminary experiments were conducted to determine the amount of time needed for calcium accumulation to reach a plateau, then the calcium-loaded aortic vesicles were placed in syringe 1. Syringe 2 contained the same media, except for the omission of aortic vesicles, and the inclusion of 20 μM IP₃. Calcium release was initiated by rapid mixing (1:1) of the solutions in the two syringes (~8 ms dead time), with the output signal from the spectrophotometer displayed on a Tektronix storage oscilloscope and an Omniscribe chart recorder. The photograph of the oscilloscope trace was used for rate constant determinations. Stopped-flow analyses of caffeine-induced calcium release from skeletal muscle sarcoplasmic reticulum vesicles were done similarly, except that syringe 2 contained 20 mM caffeine instead of 20 μM IP₃. Rate constants of calcium release were calculated from graphical analysis of ln ((Ct - C)/C₀ - Ct) vs. time, where C₀ = calcium content at the time of agonist addition, Cₜ = calcium content at any time t, and Cᵢ = calcium content at the time of maximal calcium release.

Concentrations of free Mg²⁺ and free Ca²⁺ were calculated using published association constants [5,20].

Results

Using the fluorescent calcium indicator chlorotetracycline, aortic sarcoplasmic reticulum vesicles were shown to accumulate calcium in the presence of ATP, and then release ~20% of the accumulated calcium upon addition of 1 μM IP₃ [Figure 1(a)]. A second addition of IP₃ did not cause calcium release, suggesting that 7 μM IP₃ was sufficient to cause maximal calcium release. A gradual calcium reaccumulation followed the IP₃-induced calcium release, then the ionophore alamethicin was added to determine total intravesicular calcium.

Inositol 1,3,4,5-tetrakisphosphate (7–27 μM), inositol 4,5-bisphosphate (7–27 μM), and inositol 1,3,4,5-tetrakisphosphate (7 μM) could not induce calcium release from these aortic vesicles [Figure 1(b), and data not shown]. Subsequent addition of 7 μM IP₃ caused calcium release which was indistinguishable from the IP₃-induced calcium release obtained in the absence of these other inositol phosphates [cf. Figure 1(a), (b)]. To assess the possibility that activation of G-proteins may be needed to obtain a greater IP₃-induced calcium release, 100 μM GTP[γ - S] was included in the reaction media [Figure 1(c)]. Contrary to a previous report [17], however, GTP[γ - S] had no effect on the IP₃-induced calcium release [cf. Figure 1(a), (c)]. The hypothesis that micromolar calcium concentrations may inhibit IP₃-induced calcium release [16] was also tested. In this case, 100 μM CaCl₂ was added to the reaction media just before addition of IP₃, increasing the calculated free calcium concentration from 2 to 30 μM. As shown in Figure 1(d), this 15-fold increase in free calcium concentration inhibited the rate of IP₃-induced calcium release.

The IP₃-induced calcium release from aortic vesicles was also examined spectropho-
Aortic IP$_3$-induced Calcium Release

FIGURE 1. Time course of calcium uptake and calcium release by aortic reticular vesicles, measured by chlorotetracycline fluorescence in the absence [(a), (b), (d)] or presence (c) of 100 μM GTP[y-35S] IP$_1$, IP$_2$, IP$_3$, and IP$_4$ represent addition of inositol 2 monophosphate, inositol 1,4-bisphosphate, inositol 1,4,5-trisphosphate, and inositol 1,3,4,5-tetrakisphosphate, respectively (7 μM final concentration). AL and CA represent addition of alamethicin and 100 μM CaCl$_2$, respectively. The ordinate represents fluorescence intensity (arbitrary units), with an upward deflection indicative of calcium accumulation by the aortic vesicles.

Tomometrically, using the calcium indicator antipyrylazo III. Consistent with the fluorimetric assays (described above), 7 μM IP$_3$ caused a rapid, partial release of calcium from aortic vesicles [cf. Figures 1(a), 2(a)]. The calcium ionophore A23187 was then added to determine total intravesicular calcium. Maximal IP$_3$-induced calcium release amounted to

FIGURE 2. Effects of Mg$^{2+}$ on IP$_3$-induced calcium release from aortic reticular vesicles, measured by antipyrylazo III absorbance. Media contained 0.5 mM (a) or 7.5 mM (b) MgCl$_2$. Calcium content at the time of IP$_3$ addition (c) and the percentage of calcium released by IP$_3$ (d) were measured in the presence (x) or absence (O) of 1.5 mM 2,3-diphosphoglycerate. IP$_3$ and A2 represent addition of 7 μM IP$_3$ and 2 μM A23187, respectively.
~25% of the intravesicular Ca\(^{2+}\) [20], although the preparation used in Figure 2 shows a slightly greater release (~40%). The aortic IP\(_3\)-induced calcium release was insensitive to azide and ruthenium red, but was inhibited by Mg\(^{2+}\) [20]. As shown in Figure 2, aortic IP\(_3\)-induced calcium release was reduced ~60\% when assayed in the presence of 5.6 mM Mg\(^{2+}\) [Figure 2(b)] relative to that obtained in the presence of 0.06 mM Mg\(^{2+}\) [Figure 2(a)]. This Mg\(^{2+}\)-dependent inhibition of aortic IP\(_3\)-induced calcium release could not be ascribed to differences in intravesicular Ca\(^{2+}\) since aortic Ca\(^{2+}\) content at the time of IP\(_3\) addition was constant over this Mg\(^{2+}\) range [Figure 2(c)]. Mg\(^{2+}\) activation of IP\(_3\) hydrolysis was also unable to explain the observed Mg\(^{2+}\) inhibition of aortic IP\(_3\)-induced calcium release since inclusion of 1.5 mM 2,3-diphosphoglycerate (which inhibited IP\(_3\) hydrolysis 80\% [20]) had no effect on the Mg\(^{2+}\) inhibition of calcium release [Figure 9(d)]. Under the same reaction conditions (with 0.06 to 7.9 mM Mg\(^{2+}\)), IP\(_3\)-induced calcium release from platelet reticular membranes was Mg\(^{2+}\) independent (data not shown), consistent with a previous report on platelet reticular membranes [9].

The rate of IP\(_3\)-induced calcium release was too fast to measure using techniques shown in Figures 1 and 2; consequently, stopped-flow analysis (capable of millisecond resolution) was employed (Figure 3). The aortic IP\(_3\)-induced calcium release exhibited a half-time of ~3 s, though graphic analysis showed that the calcium release was biphasic, with rate constants of 1.66 and 0.12 s\(^{-1}\) for 33\% and 67\% of the IP\(_3\)-sensitive calcium pool, respectively [Figure 3(a)]. This contrasts with caffeine-induced calcium release from skeletal muscle sarcoplasmic reticulum vesicles, which exhibited a monophasic calcium release with a much faster rate constant (12.3 s\(^{-1}\); 56 ms half-time). Aortic vesicles did not exhibit caffeine-induced calcium release, and skeletal muscle vesicles did not exhibit IP\(_3\)-induced calcium release.

**Discussion**

The present study provides evidence from two independent techniques that sarcoplasmic reticulum vesicles isolated from canine aortic smooth muscle contain an IP\(_3\)-sensitive calcium release pathway. Chlorotetracycline fluorescence purportedly monitors intravesicular calcium concentration [a, and thereby provides a means of continuously monitoring aortic IP\(_3\)-induced calcium release in the presence or absence of a Ca\(^{2+}\)-EGTA buffer. This differs markedly from the extravesicular Ca\(^{2+}\) indicator antipyrylazo III [12], which is sensitive to micromolar changes in extravesicular free Ca\(^{2+}\) concentration. Both Ca\(^{2+}\) indicators showed
that IP₃ caused rapid release of ~25% of the aortic intravesicular calcium, whereas three other inositol phosphates had no such effect. Moreover, GTP[γ - S] had no effect on the aortic IP₃-induced calcium release, consistent with a report which ruled out involvement of G-proteins in IP₃-induced calcium release from platelet reticular vesicles [10]. A recent study on permeabilized mesenteric arterial fibers, however, suggested that GTP (or a nonhydrolyzable GTP analog) was required for IP₃-induced calcium release [11]. The reason for this discrepancy is not clear, but may involve tissue differences since several permeabilized tissues exhibit substantial IP₃-induced calcium release in the absence of GTP analogs [15, 16, 19, 21]. Alternatively, the GTP requirement for aortic IP₃-induced calcium release may have been lost during membrane preparation, though further analyses are necessary to resolve this issue.

Previous analyses showed that the calcium accumulation in this membrane preparation was due mainly to sarcoplasmic reticulum vesicles, with little or no contribution by possible mitochondrial and sarcolemmal contaminants [20]. The calcium ionophore A23187 released the accumulated calcium, whereas IP₃ released ~25% of this intravesicular (A23187 sensitive) calcium pool. This partial release of intravesicular calcium by IP₃ may reflect the existence of two populations of sarcoplasmic reticulum (i.e., IP₃-sensitive, and IP₃-insensitive) in this aortic membrane preparation, perhaps due to specialization of a portion of the sarcoplasmic reticulum [18].

Aortic IP₃-induced calcium release was partially inhibited by both micromolar concentrations of Ca²⁺ and millimolar concentrations of Mg²⁺. This contrasts with the platelet IP₃-induced calcium release, which is Mg²⁺-independent [9]. It also differs from the IP₃-induced calcium release in permeabilized arterial fibers, which was completely inhibited by 10 μM Ca²⁺ [16]. Ruthenium red, on the other hand, had no effect on aortic IP₃-induced calcium release, but has been reported to inhibit IP₃-induced calcium release from platelet and skeletal muscle reticular preparations [2, 18]. The IP₃-sensitive calcium pathway in aortic sarcoplasmic reticulum, therefore, differs from that in either platelet or skeletal muscle reticular membranes, at least in terms of Mg²⁺ and ruthenium red sensitivity.

Efforts have been directed at elucidating the mechanism by which Mg²⁺ inhibits aortic IP₃-induced calcium release. In contrast to a previous report [17], Mg²⁺ stimulation of IP₃ hydrolysis could not explain the Mg²⁺-dependent inhibition of IP₃-induced calcium release. Instead, Mg²⁺ appeared to inhibit the calcium release noncompetitively [20], perhaps by lodging within the pore [7]. Alternatively, Mg²⁺ could inhibit IP₃ binding noncompetitively. Further analysis, using electrophysiological and radiochemical techniques, is necessary to distinguish between these possibilities.

Stopped-flow analysis indicated that aortic IP₃-induced calcium release occurred with a half-time of ~3 s, and showed that the calcium release was biphasic. This contrasts with the caffeine-induced calcium release from skeletal muscle sarcoplasmic reticulum, which exhibited a monophasic calcium release with a half-time of ~56 ms. The marked differences in the half-times of these calcium release processes may be attributable to a lower density of calcium channels in the aortic preparation, although differences in conductance, mean open time, and frequency of opening of the calcium channels cannot be discounted. The reason for the biphasic IP₃-induced calcium release is also unclear, but may reflect the existence of two populations of IP₃-sensitive calcium channels in this aortic preparation. Alternatively, the rise in extravesicular Ca²⁺ concentration during the IP₃-induced calcium release may serve as a negative feedback mechanism, reducing calcium eflux. Additional experiments (using electrophysiological and/or chlorotetacycline stopped-flow analyses in Ca-EGTA buffered media) are necessary to resolve these issues.

In summary, two independent techniques (chlorotetacycline fluorescence and dual wavelength spectrophotometry) showed that canine aortic sarcoplasmic reticulum vesicles released ~25% of the intravesicular calcium upon addition of IP₃. The aortic IP₃-induced calcium release appears to be distinct from that in platelet and skeletal muscle reticular vesicles, at least in terms of Mg²⁺ and ruthenium red sensitivity. The aortic IP₃-induced
calcium release is also much slower than caffeine-induced calcium release from skeletal muscle reticular vesicles, and appears to be biphasic. The cause of the biphasicity is not clear, but may reflect heterogeneity of the putative IP₃-sensitive calcium channel.

Acknowledgements

The authors wish to thank Drs A. M. Katz and D. O. Levitsky for helpful discussions during the course of this study. Supported by a grant-in-aid from the American Heart Association (Connecticut Affiliate), NIH grant HL-33036, and the US-USSR Joint Program in Cardiovascular Diseases.

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