Inositol 1,4,5-trisphosphate receptors modulate Ca\(^{2+}\) sparks and Ca\(^{2+}\) store content in vas deferens myocytes

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White, Carl, and J. Graham McGeown. Inositol 1,4,5-trisphosphate receptors modulate Ca\(^{2+}\) sparks and Ca\(^{2+}\) store content in vas deferens myocytes. Am J Physiol Cell Physiol 285: C195–C204, 2003.—Spontaneous Ca\(^{2+}\) sparks were observed in fluo 4-loaded myocytes from guinea pig vas deferens with line-scan confocal imaging. They were abolished by ryanodine (100 μM), but the inositol 1,4,5-trisphosphate (IP\(_3\)) receptor (IP\(_3\)R) blockers 2-aminoethoxydiphenyl borate (2-APB; 100 μM) and intracellular heparin (5 mg/ml) increased spark frequency, rise time, duration, and spread. Very prolonged Ca\(^{2+}\) release events were also observed in ~20% of cells treated with IP\(_3\)R blockers but not under control conditions. 2-APB and heparin abolished norepinephrine (10 μM; 0 Ca\(^{2+}\))-evoked Ca\(^{2+}\) transients but increased caffeine (10 mM; 0 Ca\(^{2+}\)) transients in fura 2-loaded myocytes. Transients evoked by ionomycin (25 μM; 0 Ca\(^{2+}\)) were also enhanced by 2-APB. Ca\(^{2+}\) sparks and transients evoked by norepinephrine and caffeine were abolished by thimerosal (100 μM), which sensitizes the IP\(_3\)R to IP\(_3\). In cells voltage clamped at −40 mV, spontaneous transient outward currents (STOCs) were increased in frequency, amplitude, and duration in the presence of 2-APB. These data are consistent with a model in which the Ca\(^{2+}\) store content in smooth muscle is limited by tonic release of Ca\(^{2+}\) via an IP\(_3\)-dependent pathway. Blockade of IP\(_3\)Rs elevates sarcoplasmic reticulum store content, promoting Ca\(^{2+}\) sparks and STOC activity.

IT IS WELL ESTABLISHED THAT release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) can be evoked by activating receptors sensitive to inositol 1,4,5-trisphosphate (IP\(_3\)Rs) or ryanodine (RyRs) (2). The resulting Ca\(^{2+}\) signal may be generalized or restricted to a specific region of the cell. Transient, local release events known as “Ca\(^{2+}\) sparks” are generated by the spontaneous opening of a cluster of RyR channels (3, 15). These sparks represent fundamental signaling events whose spatial and temporal summation generates global increases in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) during excitation-contraction coupling in cardiac and skeletal muscle (6, 29). In smooth muscle sparks may also modulate membrane conductances (for review see Ref. 14), either opening Ca\(^{2+}\)-activated Cl\(^{−}\) channels to cause depolarization and contraction (40) or promoting relaxation by activating large-conductance Ca\(^{2+}\)-activated K\(^+\) channels and so hyperpolarizing the membrane (24, 41). Understanding the control of Ca\(^{2+}\) spark activity is crucial, therefore, to our understanding of smooth muscle function.

The [Ca\(^{2+}\)]\(_i\) within the cytosol and the SR lumen may both play an important role in spark modulation. An elevation in [Ca\(^{2+}\)]\(_i\), increases the open probability of the RyR channel, increasing the frequency of Ca\(^{2+}\) sparks (7), whereas changes in the SR load can affect both spark frequency and amplitude (41). The filling state of the SR must reflect the balance between the rate of loss through leak pathways and the rate of Ca\(^{2+}\) uptake via Ca\(^{2+}\)-ATPase. Recent studies in cardiac myocytes have suggested that Ca\(^{2+}\) leak is more important in setting the SR load than the thermodynamic limits of the Ca\(^{2+}\) pump (9, 16–18). It has also been postulated that the resting SR load in smooth muscle is also limited by Ca\(^{2+}\) leak (33). There is little experimental evidence concerning the nature of this leak pathway, however, and this issue is addressed directly in this study. We investigated the effect of IP\(_3\)R blockade on SR store content and Ca\(^{2+}\) sparks in smooth muscle from the vas deferens. Our results indicate that basal activation of IP\(_3\)Rs provides a Ca\(^{2+}\) leak pathway limiting Ca\(^{2+}\) stores in this tissue. Blockade of this leak pathway increased store content and Ca\(^{2+}\) spark activity. Evidence is presented to suggest that raising SR [Ca\(^{2+}\)] upregulates both open probability and mean duration of opening in RyR clusters responsible for sparks. This leads, in turn, to an increase in spontaneous transient outward currents (STOCs), thus providing a negative-feedback mechanism that might limit further Ca\(^{2+}\) influx via voltage-sensitive ion channels (24).

MATERIALS AND METHODS

Cell isolation and [Ca\(^{2+}\)]\(_i\) recording. Adult male Duncan-Hartley guinea pigs were anesthetized with CO\(_2\) and killed by cervical dislocation, as approved under United Kingdom Home Office regulations. The epididymal portions of the vasa deferentia were dissected out, and myocytes were isolated enzymatically by incubating the tissues at 32°C for 10–15 min in a Ca\(^{2+}\)-free Hanks’ solution containing collagenase (Sigma type IA; 10 mg/5 ml), protease (Sigma type XXIV; 1...
mg/5 ml), bovine serum albumin (8 mg/5 ml) and trypsin inhibitor (8 mg/5 ml). Single relaxed smooth muscle cells were released during a subsequent wash in enzyme-free solution (10–45 min). These cells were stored at 4°C and used within 4 h. Myocytes were loaded with Ca2+ indicator by incubation with the acetoxyethyl ester (AM) of either fluo 4 (10 μM) or fura 2 (5 μM) for 20 min at 37°C. They were then plated out in a glass-bottomed organ bath and viewed with an inverted microscope (Nikon Eclipse TE300; ×60 oil immersion objective, NA 1.4) and superfused with experimental solutions at 37°C.

Changes in cytoplasmic [Ca2+] were recorded with microfluorimetry or confocal microscopy. Global [Ca2+] was measured by exciting fura 2 alternately at 340 and 380 nm and recording the ratio (R) of the emitted fluorescence (filtered at 510 nm) with a microfluorimeter (Photon Technology International). [Ca2+]c was calculated from R by applying the equations of Grynkiewicz et al. (11) with experimentally determined values of maximum R (Rmax), minimum R (Rmin), β (ratio of maximum-to-minimum fluorescence excited at 380 nm) and a Kf of 287 nM (35). Local changes in [Ca2+]c were imaged in Ca2+-free solution with a confocal scanning laser microscope (Bio-Rad, MR-A1) used in line-scan mode at a rate of 500 scans/s. Confocally imaged cells were treated with wortmannin (5 μM) to prevent movement (5) and excited at 488 nm. Emitted light was filtered with a 530- to 560-nm band-pass filter before detection, and data acquisition was controlled with Timecourse software (Bio-Rad). Images were analyzed in Lasersharp (Bio-Rad) and further processed with Scion Image graphic software (shareware; Scion). Confocal fluorescence data (F) were normalized with the average resting fluorescence (F0) for control periods in line scan regions that showed no spontaneous [Ca2+]c rises.

For experiments that required intracellular application of heparin or recording of spontaneous ionic currents, cells were voltage clamped with the whole cell patch-clamp technique as previously described in detail elsewhere (35). Changes in STOC activity were recorded at a holding potential of –40 mV with consecutive 1-s recording sweeps and a data sampling rate of 2 kHz. The output was then analyzed to determine the average frequency of STOCs per second and the average amplitude and duration of spontaneous outward current events under control and treatment conditions. Data acquisition and analysis were controlled with shareware written for the Windows operating system by Dr. J. Dempster of Strathclyde University, Glasgow, UK (Whole Cell Analysis Program v3.1.8).

**Solutions and drugs.** The bath solution had the following composition (mM): 125 NaCl, 5.36 KCl, 10 glucose, 2.9 succrose, 4.17 NaHCO3, 0.44 KH2PO4, 0.33 Na2HPO4, 0.5 MgCl2, 1.8 CaCl2, 0.4 MgSO4, and 10 HEPES, pH adjusted to 7.4 with NaOH. In Ca2+-free solutions MgCl2 was substituted for CaCl2 and EGTA (5 mM) was included. The whole cell patch pipette solution had the following composition (mM): 53 KCl, 80 K-gluconate, 1.0 MgCl2, 1.0 Na2ATP, 0.1 NaGTP, 2.5 phosphocreatine, 0.5 EGTA, and 10 HEPES, pH adjusted to 7.2 with KOH.

Ryanodine was dissolved in 50% ethanol to give a 10-2 M stock solution that was further diluted in extracellular perfusate to achieve the final concentration. Ionomycin was dissolved in DMSO to give a stock solution of 10-2 M. Ethanol and DMSO alone had no effects on the cell's excitability (0.5% vol/vol). 2-Aminoethoxydiphenyl borate (2-APB) was dissolved in ethanol to give a stock solution of 10-3 M. All other drugs were dissolved directly in extracellular solution at the required concentrations. Solutions were delivered with a rapid solution changer (Intracel) that allowed complete exchange of the superfusate around a cell in <1 s. Chemicals were obtained from Sigma-Aldrich (Poole, UK) with the exceptions of fura 2-AM and fluo 4-AM (Molecular Probes, Eugene, OR) and 2-APB (Acros Organics).

**Analysis and statistics.** Data are summarized as means ± SE. Ca2+ spark amplitude was taken as the maximum increase in normalized fluorescence (ΔF/F0). Spark duration was measured along a line through the peak fluorescence as the time in milliseconds between half the maximum spark amplitude during the rising and falling phases, i.e., the full time at half-maximal fluorescence (FTHM). Spatial spread was similarly defined as the distance in micrometers between the half-maximal fluorescence rise on either side of the peak fluorescence, i.e., the full width at half-maximum (FWHM). These values were averaged for all sparks seen at a given site in a single cell, and the averages were then used to generate the mean value for all the cells exposed to a given protocol. Differences between means were accepted as statistically significant at the 95% significance level, as assessed with the paired Student’s t-test or Wilcoxon signed-rank test, as stated in RESULTS. Average spark images for individual cells (e.g., Fig. 1) were generated by sampling data from a rectangle of fixed size and shape. This rectangle was fitted to the same number of sparks recorded at a single site before and during drug application. These image samples were then used to produce composite stacks for that spark site under control and test conditions (Scion Image software). In some experiments, sparks were observed under whole cell voltage clamp in different groups of cells with either heparin-containing or heparin-free pipette solutions. On these occasions, sparks were analyzed as above and the statistical significance of any differences seen was assessed with an unpaired t-test or Mann-Whitney U-test, as stated in RESULTS. The STOC data are also summarized as means ± SE, and the statistical significance of differences between means was assessed with a paired or unpaired t-test as indicated.

**RESULTS.**

**Effects of IP3R blockade on spontaneous Ca2+ sparks.**

Line-scene images of myocytes from the vas deferens loaded with fluo 4 and superfused with physiological external [Ca2+] often demonstrated spontaneous and localized [Ca2+]c rises with spatiotemporal characteristics similar to those of Ca2+ sparks described in other smooth muscle cells (14). These were completely blocked in four cells during bath perfusion with ryanodine (100 μM) (data not shown). In contrast, however, 2-APB (100 μM), which is believed to be a cell-permeant inhibitor of IP3Rs (19), increased the mean frequency of Ca2+ sparks from 0.89 ± 0.18 to 1.36 ± 0.13 s–1 at 11 spark sites in 9 different cells (P < 0.02, Student’s paired t-test). From the example shown in Fig. 1A, it can also be seen that the spatiotemporal characteristics of these spontaneous release events were also modified by 2-APB, individual sparks showing increased spread and duration. This is clearly seen when an averaged image for six control sparks is compared with the image for six sparks observed at the same site in the same cell during superfusion with 2-APB (Fig. 1B). Summary data for all 11 spark sites studied (Fig. 1C) show that spark duration (FTHM) was increased from 42.4 ± 5.6 to 66.9 ± 7.0 ms (P < 0.01, paired t-test). Although less obvious on the time scale used in Fig. 1C, the increase in overall duration
was also associated with considerable prolongation of the rising phase of the spark, the average rise time from basal to peak fluorescence increasing from 19.8 ± 2.0 ms under control conditions to 37.4 ± 3.4 ms in the presence of 2-APB (P < 0.005, paired t-test; n = 11 spark sites). Spark width (FWHM) was also increased from 2.30 ± 0.28 to 3.22 ± 0.35 μm (P < 0.05, paired t-test). There was, however, no statistically significant change in spark amplitude (ΔF/F₀; not significant (NS), Wilcoxon signed-rank test).

Additional experiments were carried out with the competitive IP₃R blocker heparin (10). Because heparin is membrane impermeant, myocytes were loaded by diffusion from the patch pipette solution (5 mg/ml) under whole cell voltage-clamp conditions with the membrane potential set at −60 mV. Sparks were recorded 4–5 min after the membrane patch was ruptured subjacent to the electrode, to allow diffusion of heparin into the cytoplasm. Sample line-scan images for a typical cell dialyzed with heparin-free pipette solution (Fig. 2A) and for a cell dialyzed with heparin-containing solution (Fig. 2B) are shown in Fig. 2. The summary data (Fig. 2C) compare 101 sparks recorded in 6 cells dialyzed with heparin-free pipette solution and 77 sparks recorded in 6 heparin-dialyzed cells. Spark frequency was increased from 1.75 ± 0.25 to 2.65 ± 0.28 s⁻¹ (P < 0.05, unpaired t-test), spark duration (FWHM) was increased from 25.9 ± 1.3 to 39.1 ± 1.8 ms (P < 0.01, unpaired t-test), and spark width (FWHM) was increased from 2.03 ± 0.06 to 2.38 ± 0.10 μm (P < 0.01, unpaired t-test) in the presence of heparin. As for 2-APB, most of the increase in overall spark duration could be accounted for by a prolongation of the spark rise time. This rose from a control value of 15.2 ± 0.6 ms to 27.5 ± 1.4 ms after heparin dialysis (P < 0.0001, unpaired t-test). Again, there was no statistically significant change in spark amplitude (ΔF/F₀; NS, Mann-Whitney U-test).

The increase in spark duration produced by 2-APB and heparin, particularly given that the average rise time for these sparks was almost doubled, suggests that the mean open time of RyRs was increased after IP₃R blockade. This interpretation is further supported by the observation that, on occasion, these agents gave rise to very prolonged Ca²⁺ release events (Fig. 3). These events had the same amplitude as the Ca²⁺ sparks but were maintained for up to several seconds rather than <100 ms in the case of sparks. This phenomenon, which presumably resulted from persistent opening of the relevant RyRs in their full conductance state, was observed in 3 of 14 cells superfused with 2-APB and in 2 of 8 cells dialyzed with heparin. It was never seen in a total of 41 cells observed under control conditions.

Effect of IP₃R blockade on Ca²⁺ store content. One possible mechanism that might explain why IP₃R blockade promoted spark activity in these cells would be an increase in the Ca²⁺ content of the SR,

Fig. 1. Effects of 2-aminoethoxydiphenyl borate (2-APB) on Ca²⁺ sparks in myocytes from the vas deferens. A: consecutive confocal line-scan images from a cell loaded with fluo 4 under control conditions (i) and during superfusion with 2-APB (100 μM; ii). The pseudo-colored scale bar represents fluorescence normalized to resting fluorescence (F/F₀), and the position of the scan line is indicated on the cell outline. B: top 2 panels show averaged line-scan images from a single spark site in A for 6 sparks before and 6 sparks during superfusion with 2-APB. The graph, bottom, shows the time course of these averaged sparks on the same time scale. C: effect of 2-APB on the mean ± SE values for spark amplitude (ΔF/F₀), frequency, spread (full width at half-maximal fluorescence; FWHM), and duration (full time at half-maximal fluorescence; FTHM) for all sparks seen in 9 cells. *Statistically significant differences between means (see text for P values).
Fig. 2. Effects of heparin on Ca\textsuperscript{2+} sparks in myocytes voltage clamped at \(-60\) mV. A: consecutive confocal line-scan images from a cell loaded with fluo 4 recorded after 4- to 5-min dialysis with normal pipette solution. B: consecutive confocal line-scan images from a cell after 4- to 5-min dialysis with pipette solution containing 5 mg/ml heparin. The pseudocolored scale bar represents normalized fluorescence. C: mean \pm SE values for spark amplitude (\(\Delta F/F_0\)), frequency, spread (FWHM), and duration (FTHM) for all sparks observed with heparin-free (\(n = 6\) cells) and heparin-containing (\(n = 6\) cells) pipettes. *Statistically significant differences between means (see text for \(P\) values).

the open probability of RyRs is a function of luminal as well as cytoplasmic [Ca\textsuperscript{2+}] (12, 20, 31). This was tested in cells loaded with fura 2 that were superfused with a zero-Ca\textsuperscript{2+} solution containing 10 mM caffeine to open the ryanodine channels. The resulting increase in [Ca\textsuperscript{2+}], which was used as a measure of Ca\textsuperscript{2+} store content, was considerably enhanced during superfusion with 2-APB (100 \(\mu\)M; Fig. 4A). In contrast, the norepinephrine (10 \(\mu\)M) response, which is believed to be mediated via IP\textsubscript{3} production (2), was completely blocked, a fall in [Ca\textsuperscript{2+}], being seen because norepinephrine was also applied in zero Ca\textsuperscript{2+} (Fig. 4A). In a series of eight cells, the average caffeine transient was increased in amplitude from 63 \(\pm\) 16 nM under control conditions to 223 \(\pm\) 46 nM in the presence of 2-APB (\(P < 0.005\), paired \(t\)-test; Fig. 4B). In the same experiments, however, the mean transient evoked by norepinephrine was 60 \(\pm\) 9 nM under control conditions but no response to norepinephrine was seen in any cell in the presence of 2-APB. After washout, both responses returned to control values (Fig. 4B). As exemplified in Fig. 4A, 2-APB also produced a small but consistent fall in basal [Ca\textsuperscript{2+}], from a mean control value of 99 \(\pm\) 13 nM to 82 \(\pm\) 13 nM (\(P < 0.01\), paired \(t\)-test). This suggests that the increased spark activity seen in the presence of 2-APB cannot be explained on the basis of activation of intracellular Ca\textsuperscript{2+} channels by increased cytoplasmic [Ca\textsuperscript{2+}].

These findings with 2-APB were also confirmed in additional experiments using intracellular dialysis of heparin to block IP\textsubscript{3}Rs. Control responses to norepinephrine or caffeine were recorded at \(-60\) mV immediately after rupture of the cell membrane, i.e., before there was time for appreciable heparin diffusion into the cytoplasm (Fig. 5). The agonists were then reapplied three times at 100-s intervals. After 5 min, the norepinephrine response was reduced to 17 \(\pm\) 4% of control (\(P < 0.001\), paired \(t\)-test; \(n = 6\) whereas, in a separate series of cells, the caffeine response was increased to 246 \(\pm\) 35% of control (\(P < 0.01; n = 6\)).

In an attempt to rule out the possibility that 2-APB promoted caffeine responses through a direct action on RyRs, changes in the Ca\textsuperscript{2+} store content were also assessed by using the Ca\textsuperscript{2+} ionophore ionomycin (25 \(\mu\)M) applied in zero-external Ca\textsuperscript{2+} solution (Fig. 6) to dump the stores (36). The mean amplitude of the resulting Ca\textsuperscript{2+} transients was increased from 67 \(\pm\) 7 nM in control conditions to 164 \(\pm\) 35 nM in the presence of 2-APB (\(P < 0.05\), paired \(t\)-test; \(n = 6\)). This supports the conclusion that the increased caffeine responses following IP\textsubscript{3}R blockade resulted from increased Ca\textsuperscript{2+} storage (Figs. 4 and 5).

Effects of thimerosal. The results presented so far have demonstrated that blockade of IP\textsubscript{3}Rs elevates Ca\textsuperscript{2+} store content in guinea pig vas deferens myocytes. This implies that background activation of IP\textsubscript{3}Rs by endogenous IP\textsubscript{3} normally limits store content by activating a leak pathway. If so, then thimerosal, which increases the affinity and sensitivity of the IP\textsubscript{3}R for IP\textsubscript{3} (21), would be expected to enhance SR Ca\textsuperscript{2+} leak and reduce store content. In fura 2-loaded myocytes, thimerosal (100 \(\mu\)M) completely abolished the responses to both norepinephrine and caffeine (Fig. 7A), suggesting that it had indeed depleted the SR of Ca\textsuperscript{2+}. Thimerosal also increased [Ca\textsuperscript{2+}], from a resting value of 67 \(\pm\) 8 nM to 97 \(\pm\) 13 nM (\(P < 0.01\), \(n = 6\)). In fluo 4-loaded cells imaged with line-scan techniques, Ca\textsuperscript{2+} sparks were also blocked during superfusion with thimerosal (Fig. 7B; typical of 4 cells tested). Overall, these results provide tentative evidence for IP\textsubscript{3} production even in the absence of agonist stimulation and confirm that modulation of store content by an IP\textsubscript{3}-dependent pathway can alter Ca\textsuperscript{2+} spark activity.
Effects of raising extracellular [Ca\(^{2+}\)]. Heparin and 2-APB produced increases in spark activity under conditions in which the Ca\(^{2+}\) store content was increased but the cytoplasmic [Ca\(^{2+}\)] was either little changed or reduced (see Figs. 4 and 5). It was of interest, therefore, to contrast this with the effects of raising [Ca\(^{2+}\)], directly by elevating the extracellular [Ca\(^{2+}\)]. Raising the [Ca\(^{2+}\)] in the superfusate from 1.8 to 5 mM increased the resting [Ca\(^{2+}\)] in fura 2-loaded cells from a mean value of 63 ± 18 nM to 80 ± 13 nM in a series of seven cells (P < 0.005, paired t-test). Although there were associated increases in the mean amplitudes of the [Ca\(^{2+}\)] transients evoked by caffeine (10 mM) and norepinephrine (10 \(\mu\)M), these changes were not statistically significant (paired t-test; n = 7, data not shown). Thus the Ca\(^{2+}\) stores were not demonstrably overloaded under these conditions. When the effects of elevating extracellular [Ca\(^{2+}\)] on Ca\(^{2+}\) spark activity were investigated with confocal microscopy in line-scan mode, spark frequency was increased from 1.25 ± 0.14 s\(^{-1}\) with physiological bathing solution to 2.10 ± 0.34 s\(^{-1}\) when the extracellular [Ca\(^{2+}\)] was raised to 5 mM (P < 0.05, paired t-test; n = 5 spark sites in 5 cells, data not shown). In contrast with the results obtained with 2-APB and heparin, however, the spark spread (FWHM) and duration (FTHM) were unaltered (NS, paired t-test; data not shown). Somewhat surprisingly, spark amplitude (ΔF/F\(_{0}\)) was actually decreased from an average of 1.01 ± 0.09 under control conditions to 0.82 ± 0.07 when extracellular [Ca\(^{2+}\)] was raised to 5 mM (P < 0.01, Wilcoxon signed-rank test; n = 5).

Effects of 2-APB on spontaneous outward currents. It is widely accepted that near-membrane Ca\(^{2+}\) release events can activate [Ca\(^{2+}\)]-sensitive conductances (40). In smooth muscle, Ca\(^{2+}\) sparks can lead to the opening of Ca\(^{2+}\)-activated K\(^{+}\) channels, resulting in STOCs. It was of interest, therefore, to investigate the effects of 2-APB on STOC activity in isolated myocytes from the vas deferens. Very few STOCs were seen under control conditions at membrane potentials negative to −40 mV, so cells were voltage clamped at this potential throughout these experiments. The current record from one experiment (Fig. 8) demonstrates clearly that STOC frequency, amplitude, and duration were all increased during superfusion with 2-APB (100 \(\mu\)M). These effects were readily reversed on washout (Fig. 8C). Data from eight similar experiments were summarized by analyzing 20 consecutive 1-s sweeps recorded under control conditions and another 20 consecutive sweeps recorded after at least 100-s exposure to 2-APB. Only outward current events with an amplitude >20 pA were included in this analysis because they were of largest amplitude and most reliable for evaluation. The mean amplitude of Ca\(^{2+}\) sparks increased by 10.2 ± 3.3 on April 19, 2017 http://ajpcell.physiology.org/ Downloaded from

![Figure 3](image_url)  
**Fig. 3.** Long-duration Ca\(^{2+}\) release events recorded during inositol 1,4,5-trisphosphate receptor (IP\(_3\)) blockade. A: confocal line-scan image from a fluo 4-loaded cell perfused with 2-APB (100 \(\mu\)M) (top). Average ΔF/F\(_{0}\) for the marked region of interest (ROI) is plotted at bottom; the dashed lines indicate the level of resting Ca\(^{2+}\) and the amplitude of the long-duration events. A Ca\(^{2+}\) wave has been initiated toward the end of the recording. B: confocal line-scan images recorded from a cell loaded with fluo 4 after 4- to 5-min dialysis with a heparin (5 mg/ml)-containing pipette solution. Average ΔF/F\(_{0}\) for the marked ROI is plotted at bottom.

![Figure 4](image_url)  
**Fig. 4.** Effects of 2-APB on transients evoked by caffeine and norepinephrine. A: intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) recorded from a single myocyte loaded with fura 2 and exposed to norepinephrine (10 \(\mu\)M; NE) and caffeine (10 mM; Caff), both applied in 0 Ca\(^{2+}\) solution. Superfusion with 2-APB (100 \(\mu\)M) blocked the response to norepinephrine but enhanced the caffeine transient. B: summary of mean amplitudes of norepinephrine and caffeine responses in 8 myocytes under control conditions, during application of 2-APB, and after washout. *P < 0.01.
this was twice the peak-to-peak noise in the current records. STOC frequency was increased from 3.92 ± 0.16 s⁻¹ under control conditions to 4.82 ± 0.20 s⁻¹ in the presence of 2-APB (P < 0.001, unpaired t-test; n = 160 sweeps each for control and 2-APB). The average STOC amplitude was also increased from 47.0 ± 1.9 pA under control conditions (n = 630 STOCs) to 56.4 ± 2.1 pA in the presence of 2-APB (n = 782 STOCs; P < 0.001, unpaired t-test), and the duration of outward current events rose from an average of 59.9 ± 1.6 ms in control conditions (n = 536 events) to a mean value of 70.0 ± 1.7 ms (n = 640 events) in the presence of 2-APB (P < 0.0001, unpaired t-test). When the outward current was integrated relative to the holding current for each recording sweep, the total charge movement was increased from 6.59 ± 0.16 pC per sweep under control conditions to 11.62 ± 3.91 pC per sweep in the presence of 2-APB (P < 0.0001, unpaired t-test; n = 160 sweeps for both treatments), a net increase of >75%.

**DISCUSSION**

**IP₃R blockade and Ca²⁺ store content.** The present study was stimulated by the initial observation that blockade of IP₃Rs promoted Ca²⁺ spark activity (Figs. 1–3). We hypothesized that an increase in store loading might explain the changes in spark activity seen, and this was supported by the increased responses to caffeine in the presence of 2-APB and heparin (Figs. 4 and 5). Store release with ionomycin was similarly potentiated (Fig. 6), suggesting that 2-APB was not simply promoting release via a direct action on RyRs. These effects cannot easily be explained on the basis of 2-APB’s other reported effects, which include inhibition of Ca²⁺ uptake by the SR and stimulation of nonspecific leak from the SR (22) as well as blockade of store-operated Ca²⁺ influx by an IP₃R-independent mechanism (27). Such actions would be expected to deplete stores and so are unlikely to explain the enhanced responses to caffeine and ionomycin seen in the present study (Fig. 6). In addition, heparin had effects on sparks and caffeine transients similar to those of 2-APB (Figs. 2 and 5), further strengthening the conclusion that Ca²⁺ store content was increased after IP₃R blockade. It should be noted, however, that these experiments do not rule out the possibility that spontaneous Ca²⁺ release via RyRs may also affect Ca²⁺ store content, as recently described in vascular smooth muscle (8). Experiments were also attempted with a third putative IP₃R blocker, xestospongin C (1). Unlike 2-APB (Fig. 4) and heparin (Fig. 5), however, xestospongin C (2 μM) did not block Ca²⁺ transients evoked by norepinephrine in isolated myocytes from the vas (data not shown). This suggested that results obtained with xestospongin C could add little to the current study because it appeared to be ineffectual as an IP₃R-blocker under our experimental conditions.
It was suggested previously that release of Ca\textsuperscript{2+}/H\textsubscript{11001}
from the SR via IP\textsubscript{3}Rs limits the store content (33), but the current study provides direct evidence for such a model. This implies that there is sufficient IP\textsubscript{3} even in the unstimulated cell to maintain IP\textsubscript{3}R activation (23).

We tested for the presence of IP\textsubscript{3} with thimerosal, a reagent that enhances the affinity and sensitivity of the IP\textsubscript{3}R to IP\textsubscript{3} (21). Thimerosal abolished Ca\textsuperscript{2+}/H\textsubscript{11001} transients evoked by caffeine and norepinephrine in fura\textsubscript{2}-loaded cells and caused an elevation in basal [Ca\textsuperscript{2+}/H\textsubscript{11001}]\textsubscript{i} (Fig. 7). Ca\textsuperscript{2+} sparks were also suppressed, again consistent with store depletion. If thimerosal exerted these effects by increasing receptor sensitivity to IP\textsubscript{3} it would be consistent with the notion that there was endogenous IP\textsubscript{3} production in these cells. This evidence is indirect at best, and there must be many potential target molecules for an organomercurial oxidizing agent like thimerosal. Because IP\textsubscript{3} production is dependent on phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (2), preliminary experiments were also carried out with various putative phospholipase C blockers. These included 2-nitro-4-carboxyphenyl N,N-diphenylcarbamate (NCDC), U-73122, neomycin, and 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphorylcholine (ET-18-OCH\textsubscript{3}). NCDC increased caffeine transients but failed to block norepinephrine responses, U-73122 blocked responses to both norepinephrine and caffeine, and both neomycin and ET-18-OCH\textsubscript{3} produced gradual and irreversible increases in [Ca\textsuperscript{2+}]\textsubscript{i}. We regard these results as inconclusive, and so the role of basal phospholipase C activity and IP\textsubscript{3} production remains to be determined.

**IP\textsubscript{3}R blockade and Ca\textsuperscript{2+} spark characteristics.** The Ca\textsuperscript{2+} sparks recorded under control conditions in the present study are similar to those previously reported for guinea pig vas deferens (25) and other smooth muscles (14). Blockade of the IP\textsubscript{3} channel with either 2-APB or heparin produced a significant increase in spark frequency (Figs. 1 and 2). This confirms previous reports that RyR channel activity can be modulated in smooth muscle after acute changes in SR [Ca\textsuperscript{2+}] (8, 41) and suggests that the mean open probability for the RyRs was increased under conditions in which intraluminal [Ca\textsuperscript{2+}] was increased but cytoplasmic [Ca\textsuperscript{2+}] was not (Figs. 4 and 5). Single-channel recordings from cardiac RyRs with planar lipid bilayer techniques have also revealed that the open probability does increase as Ca\textsuperscript{2+} is elevated on the luminal side (31, 37). This is believed to be mediated via an intraluminal domain of the channel that senses increases in SR [Ca\textsuperscript{2+}] (12).

As well as increasing spark frequency, 2-APB and heparin also altered the spatiotemporal characteristics of individual sparks. The spark rise time was prolonged by 89% and 81%, respectively, in the presence of 2-APB and heparin. This was further reflected in in-

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**Fig. 6.** Effects of 2-APB on store content as assessed by ionomycin-evoked release. *A*: the [Ca\textsuperscript{2+}] transient evoked from a fura\textsubscript{2}-loaded myocyte by ionomycin (25 mM in 0 Ca\textsuperscript{2+}; Iono) was increased in the presence of 2-APB (100 mM). *B*: summary of mean ± SE amplitude of the ionomycin-dependent transient in 6 cells under control conditions and in the presence of 2-APB. *P* < 0.05.

**Fig. 7.** Effects of thimerosal on evoked responses and Ca\textsuperscript{2+} sparks. *A*: typical record from a single myocyte loaded with fura\textsubscript{2} showing the effects of superfusion with thimerosal (100 mM) on both basal [Ca\textsuperscript{2+}], and the responses to norepinephrine (10 mM) and caffeine (10 mM). *B*: consecutive confocal line scans from a myocyte loaded with fluo 4 both before and during superfusion with thimerosal (100 mM). Background [Ca\textsuperscript{2+}] levels were raised, but all sparks were eliminated.
increased spark duration (FTHM; Figs. 1 and 2). In the case of heparin, nearly all of the increase in mean FTHM, which was 13.2 ms longer, could be accounted for by the associated increase in spark rise time, which was 12.3 ms. Again, in the presence of 2-APB, the prolongation of the rising phase (17.6 ms) was not statistically significantly different from the corresponding increase in FTHM (24.5 ms; NS, paired t-test; \( n = 11 \) cells). These observations suggest that the channel clusters responsible for each spark remained open longer under conditions in which the SR load was increased. This hypothesis was further supported by the observation of very prolonged \( \text{Ca}^{2+} \) release events that were seen only under conditions in which stores were overloaded (Fig. 3). Long-duration \( \text{Ca}^{2+} \) release events, coined “calcium glows,” were observed previously in skeletal muscle treated with ryanodine (13). Those events had an amplitude approximately one-half that of sparks in the same cell, suggesting that, at the concentration used, ryanodine had locked RyRs open in a subconductance state (28). The prolonged release events recorded in the present study, however, had the same amplitude as \( \text{Ca}^{2+} \) sparks (Fig. 3). To the best of our knowledge, this is the first report of prolonged, full-conductance RyR openings in smooth muscle, or indeed any other cells.

Prolongation of RyR opening might also explain the increase in spark spread (FWHM) seen with 2-APB and heparin (Figs. 1 and 2) because this would be expected to increase the total \( \text{Ca}^{2+} \) release during each spark. If this \( \text{Ca}^{2+} \) is mainly cleared from the cytoplasm by diffusion in smooth muscle cells (38), then the increased \( \text{Ca}^{2+} \) load should also increase the spark width. Thus, although we cannot rule out the possibility that increased spark duration and spread may reflect recruitment of additional RyRs after an increase in their \( \text{Ca}^{2+} \) sensitivity (7), it seems that an increase in open probability and mean time to closure could adequately explain most of the changes seen. It is also worth noting that no increases in spark rise time, spread, or duration were observed when spark frequency was increased by elevating extracellular \( [\text{Ca}^{2+}] \). Under these conditions there were no statistically significant increases in the caffeine- or norepinephrine-evoked transients, either. This leads us to speculate that although a rise in cytoplasmic \( [\text{Ca}^{2+}] \) increases RyR open probability and thus spark frequency, it has little effect on the mean open time in a channel cluster and so does not affect duration. Changes in SR \( [\text{Ca}^{2+}] \), on the other hand, appear to affect both parameters.

It may at first seem surprising that the amplitude of \( \text{Ca}^{2+} \) sparks was not increased under conditions of increased store loading (Figs. 1 and 2). Similar results were also reported, however, for studies on vascular myocytes in which the caffeine-releasable store was increased acutely by using tetracaine to block RyRs. During the subsequent tetracaine washout spark frequency was increased relative to control but spark amplitude was unaffected (8). The effects of persistent increases in SR load on \( \text{Ca}^{2+} \) sparks were previously examined in arterial smooth muscle from transgenic mice in which expression of phospholamban (30), an SR protein that inhibits \( \text{Ca}^{2+} \) uptake by the SR \( \text{Ca}^{2+} \)-ATPase, was reduced (34). These experiments also showed that there was increased spark frequency but no change in spark amplitude in these phospholamban-deficient knockout mice, even though the SR \( \text{Ca}^{2+} \) load was increased relative to controls. Acute reduction of the SR \( [\text{Ca}^{2+}] \) in amphibian gastric myocytes by as little as 10%, on the other hand, has been shown to decrease spark amplitude by up to 40% (41). It may be, therefore, that the resting SR \( [\text{Ca}^{2+}] \) in smooth muscle is delicately poised close to a value that maximizes spark amplitude. Local reduction in store \( \text{Ca}^{2+} \) levels near release sites secondary to increased spark frequency may explain why spark amplitude was actually reduced in cells exposed to 5 mM external \( [\text{Ca}^{2+}] \) given that, in contrast with IP3R blockade, the total releasable store was not increased under these conditions.

Fig. 8. Effects of 2-APB on spontaneous transient outward current (STOC) activity. Membrane current records for a myocyte voltage clamped at −40 mV. In each case the current is displayed on both slow (top traces) and faster (bottom traces) time bases. A: STOCs recorded under control conditions. B: record from the same cell during superfusion with 2-APB (100 μM). This record was taken after 100 s of drug application. C: record from the same cell after washout of 2-APB.

AJP-Cell Physiol • VOL 285 • JULY 2003 • www.ajpcell.org
2-APB and STOC activity. It is well established that Ca$^{2+}$ sparks can activate Ca$^{2+}$-sensitive conductances in the adjacent plasma membrane of smooth muscle cells. In the case of Ca$^{2+}$-activated K$^+$ channels, the high [Ca$^{2+}$] generated by localized Ca$^{2+}$ release into the microdomain adjacent to the membrane increases K$^+$ channel open probability, resulting in a STOC (38, 39). It follows, therefore, that interventions that increase spark activity are also likely to promote STOCs. The electrophysiological findings were consistent with this expectation, in that 2-APB increased STOC frequency, amplitude, and duration in voltage-clamped myocytes (Fig. 8). These changes probably reflect temporal and spatial summation of individual outward current events and correlate well with the increases in spark frequency, spread, and duration produced by 2-APB in this cell type (Fig. 1). Overall, 2-APB increased the mean outward current by 75% in the cells studied. In a cell that is not voltage-clamped this would be expected to favor polarization in response to store overload. Polarization would, in turn, be expected to reduce net Ca$^{2+}$ influx through voltage-activated channels. Indeed, this may have contributed to the fall in mean resting [Ca$^{2+}$] levels produced by 2-APB (Fig. 4). The current results also demonstrate that increased store content can increase spark and STOC activity even when the global cytoplasmic [Ca$^{2+}$] is reduced. This is consistent with the negative feedback model proposed to regulate store content in arterial smooth muscle. Polarization would, in voltage-clamped this would be expected to favor polarization.


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