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Different roles of ryanodine receptors and inositol (1,4,5)-trisphosphate receptors in adrenergically stimulated contractions of small arteries

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Lamont, Christine, and W. Gil Wier. Different roles of ryanodine receptors and inositol (1,4,5)-trisphosphate receptors in adrenergically stimulated contractions of small arteries. *Am J Physiol Heart Circ Physiol* 287: H617–H625, 2004. First published April 8, 2004; 10.1152/ajpheart.00708.2003.—The functions of ryanodine receptors (RyRs) and inositol (1,4,5)-trisphosphate receptors [Ins(1,4,5)P_3Rs] in adrenergically activated contractions of pressurized rat mesenteric small arteries were investigated. Caffeine (20 mM) but not phenylephrine (PE; 10 μM) facilitated the depletion of smooth muscle sarcoplasmic reticulum (SR) Ca^{2+} stores by ryanodine (40 μM). In ryanodine-treated SR-depleted arteries, *1*) Ca^{2+} sparks were absent, *2*) low concentrations of PE failed to elicit either vasoconstriction or normal asynchronous propagating Ca^{2+} waves, and *3*) high [PE] induced abnormally slow oscillatory contractions (vasomotion) and synchronous Ca^{2+} oscillations. In ryanodine-treated SR-depleted arteries denuded of endothelium, high [PE] induced steady contraction and steady elevation of intracellular [Ca^{2+}]. In contrast, 2-aminoethyl diphenylborate (2-APB), a putative blocker of Ins(1,4,5)P_3Rs, produced opposite effects to ryanodine: *1*) Ca^{2+} sparks were present; *2*) Ca^{2+} waves were absent; *3*) caffeine-releasable Ca^{2+} stores were intact; and 4) PE, even at high concentrations on endothelial-denuded arteries, failed to elicit contraction, asynchronous Ca^{2+} waves, or synchronous Ca^{2+} oscillations or maintained elevated [Ca^{2+}]. We conclude that *1*) Ins(1,4,5)P_3Rs are essential for adrenergically induced asynchronous Ca^{2+} waves and the associated steady vasoconstriction, *2*) RyRs are not appreciably opened during adrenergic activation (because PE did not facilitate the development of the effects of ryanodine), and *3*) Ins(1,4,5)P_3Rs are not essential for Ca^{2+} sparks. This provides an explanation of the fact that adrenergic stimulation decreases the frequency of Ca^{2+} sparks (previously reported) while simultaneously increasing the frequency of asynchronous propagating Ca^{2+} waves; different SR Ca^{2+}-release channels are involved.

ryanodine; 2-aminoethyl diphenylborate; vasomotion; smooth muscle; confocal microscopy

IN SMALL ARTERIES, α_1-adrenoceptor agonists [phenylephrine (PE)] elicit vasoconstriction by elevating intracellular calcium ion concentration ([Ca^{2+}]$_i$) and by stimulating biochemical mechanisms that "sensitize" the contractile apparatus to Ca^{2+} (44). We have shown previously (30) that in pressurized arteries with intact endothelium under "near-physiological" conditions, three types of intracellular Ca^{2+} "signals" are involved: *1*) propagating Ca^{2+} waves that are asynchronous among the individual smooth muscle cells of the vascular wall, *2*) Ca^{2+} sparks, and *3*) Ca^{2+} oscillations that are uniform within individual cells and synchronous in all cells. Ca^{2+} signaling is similarly heterogeneous in veins (see Ref. 26 for a recent review of Ca^{2+} signaling in vascular smooth muscle). In general, low concentrations of agonist elicit steady vasoconstriction and asynchronous Ca^{2+} waves; high concentrations elicit oscillatory vasomotion and synchronous Ca^{2+} oscillations. Both types of Ca^{2+} signals should produce contraction through activation of Ca^{2+}/calmodulin-dependent myosin light chain kinase. The frequency of Ca^{2+} sparks decreases during adrenergic stimulation (30). This would be expected to aid contraction, because Ca^{2+} sparks tend to hyperpolarize the membrane potential, thus reducing voltage-dependent Ca^{2+} entry.

The role of the sarcoplasmic reticulum (SR) and its Ca^{2+}-release channels {ryanodine receptors (RyRs) and inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] receptors [Ins(1,4,5)P_3Rs]} in these Ca^{2+} signals is not completely known. Whereas there is little doubt that Ca^{2+} sparks in smooth muscle involve the release of Ca^{2+} through RyRs, as they do in striated muscle, the role of RyRs and InsP_3Rs in asynchronous Ca^{2+} waves and synchronous Ca^{2+} oscillations is still uncertain. Furthermore, the ability of adrenergic stimulation to increase the frequency of Ca^{2+} waves and their speed of propagation while simultaneously decreasing the frequency of Ca^{2+} sparks (through a PKC-mediated inhibition of RyR) (4) is paradoxical if Ca^{2+} waves also involve RyRs, as they do in cardiac muscle. Therefore, we sought to test the hypothesis that RyRs are involved predominantly in Ca^{2+} sparks and not in Ca^{2+} waves.

We used three pharmacological tools: ryanodine, caffeine, and the putative blocker of Ins(1,4,5)P_3R 2-aminoethyl diphenylborate (2-APB). While ryanodine is completely specific for RyRs, it leads to depletion of sarcoplasmic reticulum (SR) Ca^{2+} stores in smooth muscle (16, 23), and it may thereby indirectly abolish Ins(1,4,5)P_3R-mediated Ca^{2+} release. Abolition of propagating Ca^{2+} waves by ryanodine would therefore not be proof that RyRs are directly involved. Ryanodine has a very useful property, however, in that it binds irreversibly to open RyRs (18) and not to closed RyRs. Agents or circumstances that increase the open probability (P_o) of RyRs, such as caffeine (18, 20), should lead to a rapid action of ryanodine (i.e., promote its effects), whereas agents or circumstances that do not increase P_o should not. Therefore, we compared the efficacies of an adrenergic agonist and caffeine in opening RyRs, as judged by their ability to promote the effects of ryanodine. To directly study the involvement of Ins(1,4,5)P_3Rs, we used a relatively new pharmacological agent, 2-APB, an inhibitor of Ins(1,4,5)P_3Rs (2, 27, 28, 45) and store-operated channels (5, 38). This substance is potentially useful, compared with ryanodine, because it is not expected to lead to depletion of SR Ca^{2+} stores. We used confocal imaging

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of cellular Ca^{2+} signaling in individual smooth muscle cells because neither global $[Ca^{2+}]_i$ nor contraction are representative of Ca^{2+} in individual smooth muscle cells.

METHODS

Animals

All experiments were carried out according to the guidelines of the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine. Male Sprague-Dawley rats, weighing 196.7 ± 5.0g (mean ± SE, $n = 52$), were anesthetized with intramuscular ketamine (50–100 mg/kg) and killed by cervical dislocation. The mesenteric arcade was dissected from the abdominal cavity, rinsed free of blood, and placed in a temperature-controlled dissection chamber containing a dissection solution (5°C) of the following composition (in mmol/l): 3.0 MOPS, 145.0 NaCl, 5.0 KCl, 2.5 $CaCl_2$, 1.0 $MgSO_4$, 1.0 KH_2PO_4, 0.02 EDTA, 2.0 pyruvate, and 5.0 glucose with 1.0% albumin (pH 7.4).

Loading of Resistance Arteries with Calcium Indicators

Isolated arteries were dissected by methods similar to those described previously (8). Dissected segments of the third- or fourth-order arteries, 1–2 mm in length, were transferred to a recording chamber, where their ends were mounted on glass pipettes (tip diameter 60–100 μm) and secured by 10-0 sutures. One pipette was attached to a servo-controlled pressure-regulating device (Living Systems; Burlington, VT), whereas the other was attached to a closed stopcock to study the pressure-dependent effects in the absence of intraluminal flow. The vessel was then loaded with a calcium indicator in dissection solution containing fluo-4 AM at 15 μM, 1.5% (vol/vol) DMSO, and 0.03% (vol/vol) cremophor EL. Loading was allowed to proceed for 3 h at room temperature with the intraluminal pressure set to 40 mmHg. The arteries were equilibrated over an hour to initial experimental conditions (room temperature, 70 mmHg); those with significant leaks or branches were discarded. During this time, the arteries were continuously superfused with gassed Krebs solution containing (in mmol/l) 112.0 NaCl, 25.7 $NaHCO_3$, 4.9 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 1.2 $KHPO_4$, 11.5 glucose, and 10.0 HEPES (pH 7.4) (gas composition of 5% O_2-5% CO_2-90% N_2). Chamber P_{O_2} = 90–100 mmHg, measured with an oxygen electrode (Microelectrodes; Londonderry, NH). There was no statistically significant difference between the magnitude of constrictions of loaded and unloaded arteries, suggesting that there was not substantial calcium buffering by the calcium indicator. For 10 arteries loaded with fluo-4, the response to 1 μM PE was a constriction to 61.0 ± 5.7% of the resting diameter; for 20 unloaded arteries, the response was a constriction to 60.3 ± 6.2% (mean ± SD) of the resting diameter.

Drugs and Solutions

PE, ryanodine, prazosin, acetylcholine (ACh), 2-APB, tetraethylammonium chloride (TEA), scopolamine, capsaicin, nifedipine, and guanethidine were prepared as concentrated stock solutions and diluted in the superfusate reservoir. PE, prazosin, ACh, 2-APB, TEA, scopolamine, capsaicin, guanethidine, nifedipine, and cremophor EL were obtained from Sigma Chemical (St. Louis, MO); fluo-4 AM was purchased from Molecular Probes (Eugene, OR); and ryanodine was purchased from Calbiochem (La Jolla, CA). In the experiments where a "zero" calcium solution was used, it had the same composition as the standard Krebs with the omission of $CaCl_2$ and the addition of 1 mM Na_2EGTA. In the experiments where high-potassium solutions were used, NaCl was replaced by KCl on a mole-for-mole basis.

Removal of the Endothelium

In some experiments, the endothelium was functionally removed using an air bubble. An air bubble was introduced into the lumen of the artery. The bubble was removed after 30 min. We deemed the protocol successful if >90% of the ACh (10 μM) induced dilation of the artery, preconstricted with PE (10 μM), was abolished and the constriction induced by PE (10μM) was greater after the deendothelialization than before. Arteries with attenuated PE-induced constrictions were discarded, as this probably reflects damage to the smooth muscle layer of the artery wall. In some experiments, the protocol had to be repeated to functionally remove the endothelium.

Measurement of Fluorescence and Arterial Diameter

We used a custom-built confocal laser scanning microscope described previously in detail (35, 43). The confocal images were collected using a ×60 water objective (numerical aperture 1.2). This objective provided excellent spatial resolution but a small field of view on the custom-built confocal microscope. To obtain a larger field of view during larger contractions, we used a "dry" lower-powered objective lens (×20, 0.4 numerical aperture) (see Fig. 2). Two types of optical sections, "radial" and "tangential," were used as described previously (30). Radial sections through the center of the artery are relatively uninfluenced by arterial wall motion and show individual smooth muscle cells in cross section. With the use of this optical plane, individual smooth muscle cells can be "tracked" during vasomotion. Tangential sections through the base of the arterial wall are strongly affected by motion but more easily reveal Ca^{2+} sparks and are required to investigate propagating Ca^{2+} waves. To improve temporal resolution, smooth muscle cells were imaged in the tangential line-scan mode in which the same line, 50 μm in length, was scanned once every 3 ms for ~0.8 s, thus creating a single line-scan image. Tangential line-scan mode was also used in a manner where between successive line scans the position of the line was moved randomly within a 50 × 25-μm planar area of the muscle, a technique referred to as "randomized confocal line scanning" (30). Measurements of arterial wall position were made either by using the edges of the fluorescence image or in arteries not loaded with fluo-4, from transmitted light images recorded at 2/s, using a ×20 objective. All image analysis was done with custom computer procedures written in IDL (Research Systems; Boulder, CO). Diameter measurements were made on-line using a custom-built LabView program (National Instruments; Austin, TX). SigmaPlot 2000 (SPSS; St. Louis, MO) was used to graph the data.

Statistical Analysis

Differences between groups were evaluated with the use of a Student's *t*-test or Mann-Whitney Rank sum test.

RESULTS

Ryanodine Treatment

In the presence of ryanodine (40 μM), a brief exposure to caffeine (20 mM, 1 min) caused transient contraction of a pressurized artery, whereas a second exposure to caffeine elicited a much-reduced contraction, and a third failed to elicit any contraction at all (Fig. 1*A*). We interpret the final lack of contraction in response to caffeine to indicate that release of Ca^{2+} through RyRs on the SR was no longer possible due to depletion of SR Ca^{2+} content and/or the locking of RyRs in an open state. We refer to this protocol (Fig. 1*A*), designed to produce a full effect of ryanodine, as "ryanodine treatment." This protocol was used consistently to develop the full effect of ryanodine quickly; all preparations treated in this way are referred to as "ryanodine treated." The concentration of ryanodine used (40 μM) is equal to or higher than that reported to

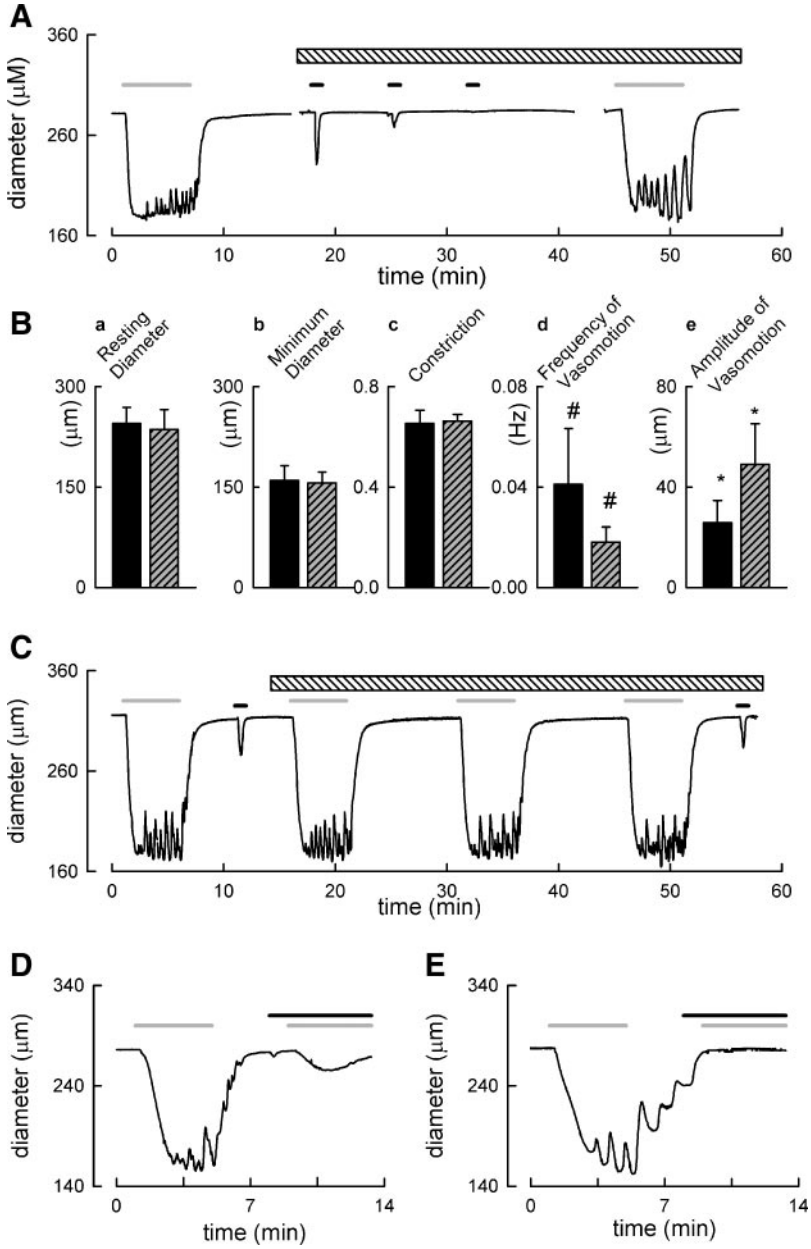

Fig. 1. Effects of ryanodine on adrenergic contraction of pressurized rat mesenteric arteries at room temperature (23°C). *A*: ryanodine treatment. The vessel was first exposed to 10 μM phenylephrine (PE) for 6 min; this produced a strong constriction with associated vasomotion. Then followed three 1-min exposures to caffeine (20 mM; indicated by the solid bars) in the continuous presence of ryanodine (40μM; indicated by the hatched bar above the trace). A final exposure to 10 μM PE produced a constriction of similar amplitude to the initial exposure, but with vasomotion greatly increased in amplitude and decreased in frequency. Ryanodine treatment was judged complete when caffeine failed to elicit any contraction. Shaded bars indicate presence of PE. *B*: summarized effects of ryanodine on pressurized arteries at 22–25°C. Data are from 9 arteries; values are means ± SD. *Paired data that are statistically different with a paired *t*-test ($P < 0.05$); #paired data that are significantly different with a rank sum test ($P < 0.05$). Each parameter was determined before and after ryanodine treatment. *a*, Diameter at an internal pressure of 70 mmHg (resting diameter); *b*, minimum diameter achieved during the exposure to PE (1 μM); *c*, constriction (minimum diameter divided by resting diameter); *d*, frequency of vasomotion; *e*, amplitude of vasomotion. *C*: example of the protocol used to measure the ability of PE to render ryanodine effective. A control response was obtained by applying 10 μM PE for 5 min (shaded bars), followed by an application of 20 mM caffeine (solid bars). No contraction was observed when 40 μM ryanodine was applied (indicated by the hatched bar above the trace). In the continuing presence of ryanodine, PE (10 μM) was applied three more times for 3 min each time. Finally, a second 1-min exposure to 20 mM caffeine was made to compare the response before and after ryanodine application. For 4 arteries, the constriction to 20 mM caffeine decreased from 34.6 ± 4.0% of the maximal PE response to 27.0 ± 3.7% (mean ± SD) of the maximal PE response (difference not statistically significant, $P \leq 0.05$) during this protocol. *D*: effects of removal of extracellular Ca^{2+}. The response to PE (10 μM; shaded bars) was changed from a maintained constriction with vasomotion superimposed to a transient constriction when the external Ca^{2+} was removed (solid bar indicates the absence of external Ca^{2+}). *E*: response to PE in the absence of external Ca^{2+} was abolished after ryanodine treatment (shaded bars indicate presence of 10 μM PE; solid bars indicate absence of external Ca^{2+}).

lock RyRs in smooth muscle in an open state (16, 18, 23). The open state of the RyR would be produced, in this protocol, by the combined actions of caffeine and cytoplasmic Ca^{2+}. The effectiveness of the protocol in abolishing Ca^{2+} release through RyRs was confirmed by the abolition of Ca^{2+} sparks in fluo-4-loaded preparations observed using confocal microscopy, which is described later (see Fig. 3, *B* and *C*).

After ryanodine treatment, the maximum constriction in response to PE was unchanged, but the frequency of the (adrenergic) vasomotion was greatly reduced and its amplitude was increased (Fig. 1, *A* and *B*). The responses of nine arteries to 1 μM PE before and after ryanodine treatment are compared in Fig. 1*B*. The response to 1 μM PE was a constriction to 65.3 ± 5.7% of the resting diameter and oscillations of 10.5 ± 3.6% of resting diameter with a frequency of 0.041 ± 0.022 Hz (mean ± SD; Fig. 1*B*). The response in the same arteries after ryanodine treatment was a constriction to 66.3 ± 2.7% of the resting diameter and oscillations of 20.8 ± 6.8% of resting diameter with a frequency of 0.018 ± 0.006 Hz (mean ± SD; Fig. 1*B*).

At a lower concentration of PE (300 nM), which under control conditions elicits small, maintained constrictions (16.4 ± 2.5% of the constriction to 1 μM PE, mean ± SE, $n = 11$) with no vasomotion, ryanodine-treated preparations exhibited two behaviors. In 5 of the 11 preparations examined, no contraction at all was observed to 300 nM PE, while the 6 other preparations went directly into a strong contraction with associated vasomotion. The viability of the arteries that did not respond to 300 nM PE after ryanodine treatment was confirmed; all arteries produced a strong constriction and vasomotion at higher concentrations of PE (1 μM).

RyR Activation During α_1-Adrenergic Activation

We made use of the properties of ryanodine to investigate the extent to which RyRs open during α_1-adrenergic activation by agonists such as PE. Because ryanodine binds irreversibly to open RyRs (18) and not to closed RyRs, agents that increase the P_o of RyR should lead to a rapid action of ryanodine (i.e., promotion of the effects shown above), whereas agents that do not increase P_o should not. The binding of ryanodine can therefore be used as a probe for the functional state of the channel (14). Transient exposure to caffeine was used to gauge the extent of the effects of ryanodine, by its (caffeine's) action to release Ca^{2+} from the SR. In four preparations, exposed to 40 μM ryanodine for an hour, during which time they were also exposed to 10 μM PE three times for 5 min, the caffeine contraction declined to only ~75% of control [declining from 34.6 ± 4.0% of the maximal PE response to 27.0 ± 3.7% of the maximal PE response (difference not statistically significant, $P \leq 0.05$, means ± SD)]. An example of this protocol is shown in Fig. 1*C*, in which it can be seen that the response to caffeine after three exposures to PE in the presence of ryanodine is only slightly smaller than that before any PE. In contrast, a single 1-min exposure to 20 mM caffeine, in the presence of ryanodine, reduced the subsequent caffeine-induced constriction to ~20% of control [declining from 37.6 ± 7.48% of the maximal PE response to 8.24 ± 3.6% of the maximal PE response (difference statistically significant, $P \leq 0.05$, $n = 5$, means ± SD)]. The inability of PE to promote the effects of ryanodine suggests that α_1-adrenoceptor activation produces only a small increase in the P_o of RyRs and that activation of RyRs is therefore not involved in the normal responses to PE.

Effect of Removal of External Ca^{2+}

Our working assumption is that release of Ca^{2+} from the SR is not possible after ryanodine treatment. We assume that the SR calcium content is depleted by the action of ryanodine as has been suggested previously (16, 23). If this assumption was wrong, Ca^{2+} could still be released in response to PE in the absence of external Ca^{2+} through other SR Ca^{2+}-release channels or from intracellular Ca^{2+} stores not affected by ryanodine. Therefore, we examined the responses of control and ryanodine-treated arteries to 10 μM PE in the absence of external Ca^{2+}. When external Ca^{2+} was removed under control conditions before ryanodine treatment, the response to PE was changed from a maintained contraction with vasomotion superimposed to a transient contraction (Fig. 1*D*). The transient constriction in the absence of external Ca^{2+} was 38.3 ± 13.94% ($n = 5$) of the constriction in the presence of external Ca^{2+}. In the same five preparations after ryanodine treatment, this response was always abolished (Fig. 1*E*). Thus α_1-adrenoceptor-induced contractions in ryanodine-treated arteries are entirely dependent on extracellular Ca^{2+}.

Ca^{2+} Imaging

We next sought to determine the changes in intracellular Ca^{2+} signals that might underlie contractions in arteries in which the effects of ryanodine were fully developed. To permit simultaneous recording of artery wall position and fluo-4 fluorescence, we used an optical section through the center of the artery. In such central sections, arterial wall motion is entirely horizontal, allowing the cells to be imaged in cross section, during vasomotion (30).

Ca^{2+} signals in response to [PE] ≥1 μM. Under control conditions, concentrations of PE ≥1 μM produced a brief period of asynchronous calcium waves, followed by spatially uniform synchronous Ca^{2+} oscillations (Fig. 2*A*) and vasomotion. During the oscillatory vasomotion, the peaks of the intracellular Ca^{2+} transients in different cells all coincided with each other and with the point of maximum diameter (relaxation), as reported previously (30).

After ryanodine treatment, the large amplitude and slow vasomotion elicited by PE were accompanied by much slower, spatially uniform oscillations in Ca^{2+} (Fig. 2*B*) throughout the PE exposure. Again, during oscillatory vasomotion, the peaks of the intracellular Ca^{2+} transients in different cells were coincident. However, they did correspond to the point of maximum diameter (relaxation) but were close to the point of minimum diameter (constriction). The apparent shift in the relationship between calcium and constriction produced by ryanodine treatment reflects only the greatly slowed calcium oscillations. Ryanodine treatment produced no significant change in the delay between calcium rise and constriction. The

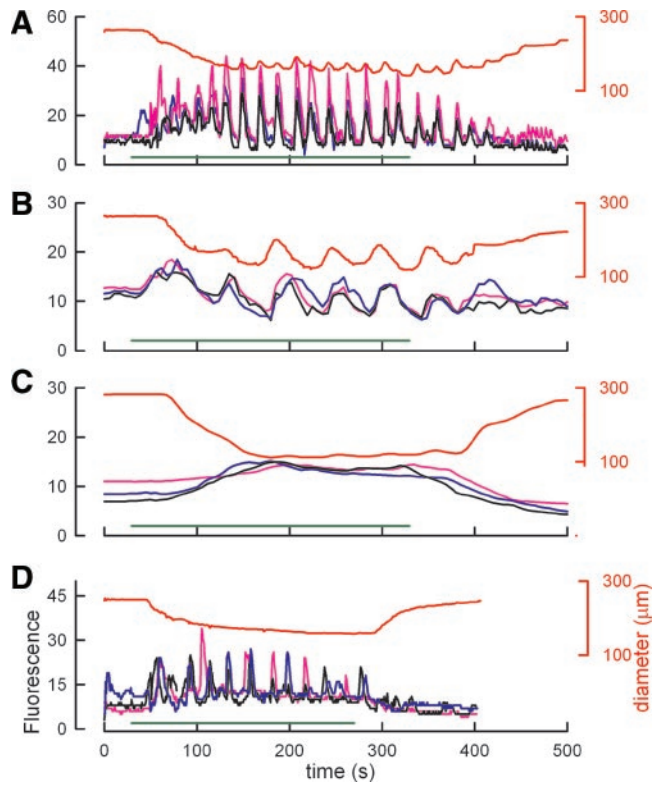

Fig. 2. Effects of ryanodine on adrenergically induced Ca^{2+} transients in single smooth muscle cells within the wall of a pressurized artery. In confocal images, individual smooth muscle cells were identified within the wall of pressurized artery from a "radial section" through the center of the artery, as described previously (29). In *A–D*, the red, blue, and black traces represent the fluo-4 fluorescence from 3 cells identified and followed during vasoconstriction induced by PE (10 μM). The *top* traces (dark red) represent the simultaneous recording of artery wall position. *A*: in control conditions; *B*: after treatment with ryanodine; *C*: after ryanodine treatment (same artery) in the absence of the endothelium; *D*: in a different artery, the response to PE in the absence of the endothelium (and no ryanodine). The results are typical of 6, 4, 3, and 3 arteries, respectively.

average delay between the peak calcium level and the peak level of constriction was 9.57 ± 1.74 s (14 observations from 4 arteries) in control and 10.12 ± 1.80 s (8 observations from 3 arteries) after ryanodine treatment.

The presence of oscillatory vasomotion in ryanodine-treated arteries could be regarded as puzzling, if such oscillatory vasomotion is thought to be dependent on the SR. Adrenergic vasomotion may be dependent on the presence of an intact endothelium, however. In fact, removal of the endothelium in our ryanodine-treated arteries did abolish the vasomotion and the synchronous Ca^{2+} oscillations, as has been shown previously (11) under control conditions. Ca^{2+} signals from the same ryanodine-treated artery before and after removal of the endothelium are shown in Fig. 2, *B* and *C*. The maximum constriction always increased after removal of the endothelium. Finally, Fig. 2*D* illustrates the response to PE in a deendothelialized vessel when ryanodine had not been used. In this case, a strong contraction was elicited, but the calcium signals in individual cells were asynchronous and there was no vasomotion.

In summary, relatively high levels of α_1-adrenoceptor activation, produced by [PE] $\geq$ 1 μM, in arteries devoid of endothelium and functional RyRs, elicits only steady, uniform increases in cytoplasmic [Ca^{2+}] of smooth muscle cells, and this increase depends entirely on extracellular Ca^{2+}.

Ca^{2+} signals in ryanodine and low [PE]. Lower levels of α_1-adrenoceptor activation, in response to [PE] $<$ 1 μM, normally produced asynchronous propagating Ca^{2+} waves and steady vasoconstriction ([PE] = 300 nM; Fig. 3*D*) (30, 31, 46). As mentioned above, after ryanodine treatment, 5 of 11 arteries failed to contract at all in response to [PE] of 300 nM. The remaining six arteries of this group responded with oscillatory vasomotion. We next sought to examine the Ca^{2+} signals underlying this behavior. In 14 ryanodine-treated arteries loaded with fluo-4 and exposed to 300 nM PE, 9 arteries did not constrict and 5 arteries went into a strong constriction with associated vasomotion. Those arteries that constricted developed synchronous oscillations in calcium identical to that seen at higher [PE]. Of the nine arteries that did not constrict, seven arteries exhibited a novel type of Ca^{2+} transient (Fig. 3*G*). We refer to this type of Ca^{2+} transient as a "Ca^{2+} flash," as it is similar to a novel Ca^{2+} transient recorded once before in vascular tissue (1). The remaining two arteries showed no change in calcium with the application of PE. By decreasing the [PE] applied to the arteries that went directly in to full constrictions and vasomotion and increasing the [PE] applied to those arteries that showed no calcium change upon application of PE, Ca^{2+} flashes could be induced in all ryanodine-treated arteries somewhere in the range of 50–400 nM PE. In some cases (Fig. 3*F*), the frequency of Ca^{2+} flashes increased as "background" levels of Ca^{2+} rose, signifying the development of uniform synchronous Ca^{2+} oscillations that produced oscillatory vasomotion. Ca^{2+} flashes were not associated with contraction except in such cases. We tested the hypothesis that the flashes were Ca^{2+} transients that reflected entry of Ca^{2+} through voltage-dependent L-type Ca^{2+} channels during action potentials. Action potentials could be produced in the presence of ryanodine, by the loss of Ca^{2+} sparks and their hyperpolarizing influence. In three arteries, Ca^{2+} flashes were elicited in ryanodine-treated preparations exposed to [PE] between 200 and 400 nM. For these three arteries, the frequency of flashes was 0.250 ± 0.031 flashes/s (9.2-min recording). The subsequent addition of 300 nM nifedipine abolished the flashes (6-min recording; Fig. 3*H*). To test this hypothesis further, we inhibited Ca^{2+}-activated K^+ channels by adding 7.5 mM TEA and applying brief (1 ms) electrical stimuli using platinum electrodes running the length of the muscle chamber to stimulate action potentials directly. To eliminate the possibility of electrically evoked neurotransmission, the arteries were treated with 30 μM guanethidine for 1 h before the experiment was started (39). Under these conditions, flashes with similar characteristics to those produced by ryanodine treatment were observed (not shown), strengthening the possibility that Ca^{2+} flashes are the calcium signals underlying depolarization-induced muscle action potentials. The flashes ceased after TEA was removed.

Spontaneous Ca^{2+} Sparks and Ca^{2+} Waves

After ryanodine treatment, the spontaneous Ca^{2+} sparks and occasional propagating Ca^{2+} waves normally seen in resting conditions (30) were abolished. The frequency of Ca^{2+} sparks was measured using randomized line scanning (30). Ca^{2+} spark frequency fell from $1.80 \times 10^{-2} \pm 0.1 \times 10^{-2}$ $\mu m^{-1} \cdot s^{-1}$ (770 scans, 6 vessels) to $1.32 \times 10^{-4} \pm 1.01 \times 10^{-4}$ $\mu m^{-1} \cdot s^{-1}$ (644 scans, 6 vessels, means ± SE) after ryanodine treatment (Fig. 3*C*). Examples of line-scan images before and after ryanodine treatment are illustrated in Fig. 3, *A* and *B*. The frequency of spontaneous asynchronous propagating Ca^{2+} waves declined from 1.47 waves·$cell^{-1}$·min^{-1} (50 min of sampling from 4 arteries) to 0 waves·$cell^{-1}$·min^{-1} (no waves were observed in 54 min of sampling).

Effect of the Ins(1,4,5)P_3 Inhibitor 2-APB

Contraction studies. To investigate the role of Ins(1,4,5)P_3Rs in Ca^{2+} signals associated with α_1-adrenergic activation, we used the Ins(1,4,5)P_3R blocker 2-APB (30 μM). Because 2-APB might be expected to affect intact arteries via the endothelium, these experiments were carried out on deendothelialized preparations. 2-APB had little effect on the contractions elicited by caffeine (Fig. 4*A*) or on the contraction produced by exposure to elevated external K^+ (Fig. 4*B*). Thus the effects of 2-APB do not involve directly RyRs or L-type Ca^{2+} channels, nor does 2-APB cause depletion of SR Ca^{2+} stores (which are releasable by caffeine). The effect of 2-APB on resting diameter was variable. In eight preparations, the average constriction produced by 30 μM 2-APB was 3.4 ± 1.2% . 2-APB almost completely inhibited PE-induced contractions, as shown in Fig. 4*C* (10 μM PE; 5.20 ± 1.00% of the constriction remained, $n = 7$).

Effect of 2-APB on cellular calcium signals. Application of 2-APB did not cause any change in the frequency or other characteristics of Ca^{2+} sparks. Ca^{2+} spark frequency in control conditions was 0.0109 ± 0.0009 $\mu m^{-1} \cdot s^{-1}$ ($n = 539$ scans, 5 preparations, mean ± SE) and 0.0110 ± 0.0008 $\mu m^{-1} \cdot s^{-1}$ ($n = 489$ scans, 5 preparations, mean ± SE) in the presence of 30 μM 2-APB (Fig. 5*D*). No propagating asynchronous Ca^{2+} waves or synchronous Ca^{2+} oscillations were ever observed. Instead, only Ca^{2+} flashes were observed in eight arteries (Fig. 5*A*). As with ryanodine, the flashes were uniform within each cell (see Fig. 5*C*). In contrast to the Ca^{2+} flashes elicited by PE in the presence of ryanodine, however, the Ca^{2+} flashes elicited by PE in the presence of

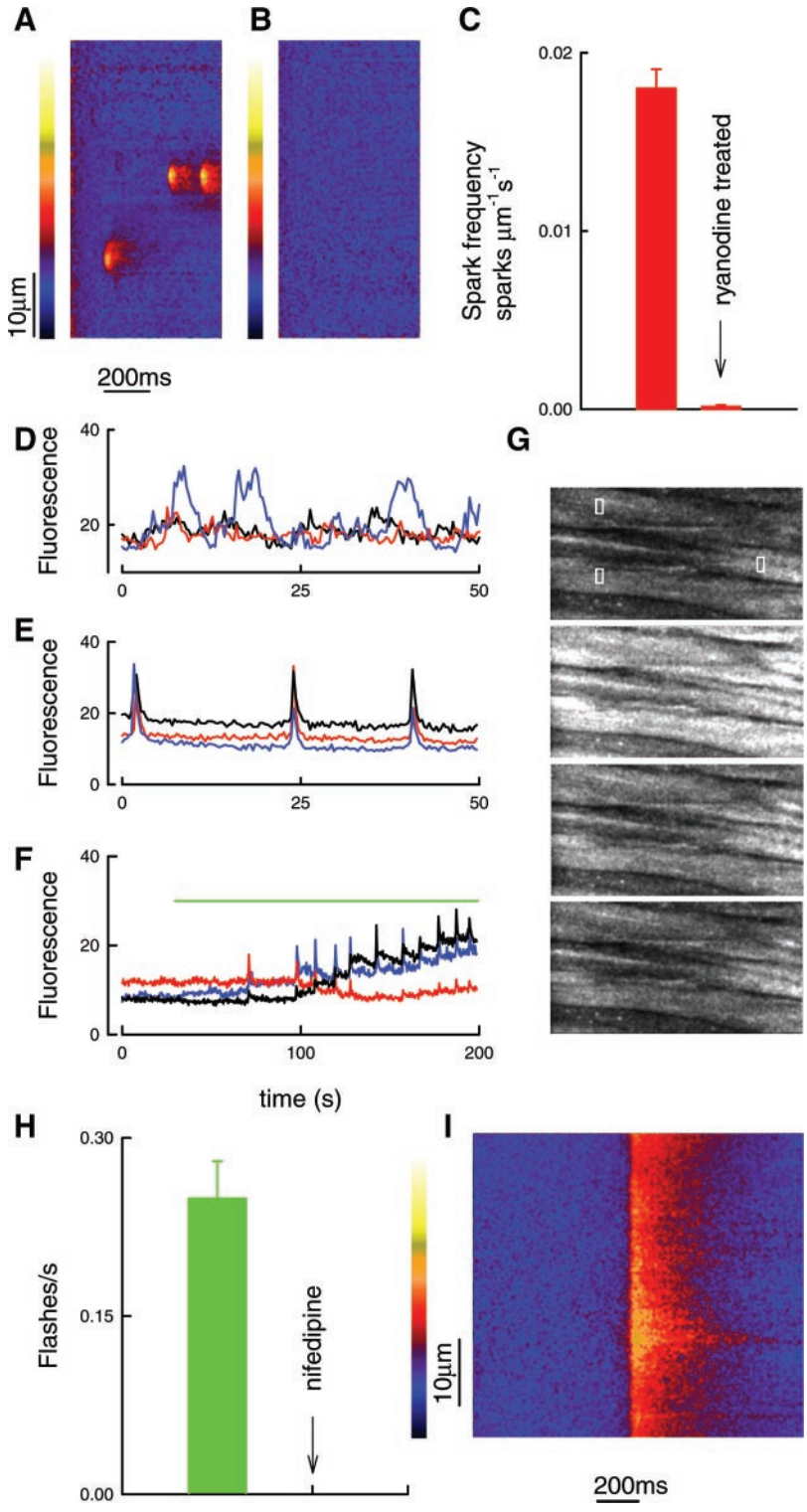

Fig. 3. Effect of ryanodine treatment on Ca^{2+} signals. *A*: example of a random line-scan image taken in control conditions showing Ca^{2+} sparks. *B*: example of a random line-scan image taken from the same artery after ryanodine treatment. *C*: Ca^{2+} spark frequency in 6 arteries before and after ryanodine treatment. *D*: control data. The 3 traces in *D* first show the typical asynchronous Ca^{2+} transients (waves) elicited by exposure to 300 nM PE, measured as average fluorescence within an area of interest (AOI; size of AOI: 1 × 3.3 μm) from 3 cells from an image similar to that in *G*. This artery constricted to ~15% of the maximal constriction evoked by 1 μM PE. *E*: same artery after ryanodine treatment and exposed again to 300 nM PE. The artery did not constrict upon application of the agonist; however, Ca^{2+} flashes were present and were synchronous in all cells. The white boxes shown on the *top* frame of *G* indicate the AOIs selected. *F*: different ryanodine-treated artery. In this example, the frequency of the Ca^{2+} flashes increased as the "background" levels of Ca^{2+} rose, signifying the development of uniform synchronous Ca^{2+} oscillations that eventually produced vasomotion. The green bar indicates the application of 300 nM PE. *G*: four images (0.332 s/frame, 50 × 25 μm) of the fluo-4 fluorescence, illustrating Ca^{2+} flashes. *H*: frequency of flashes in 3 arteries before and after the application of 300 nM nifedipine. *I*: confocal line-scan image of the fluo-4 fluorescence pseudoratio (F/F_0) illustrating a Ca^{2+} flash at higher temporal resolution.

2-APB were not always synchronous between cells (Fig. 5*B*, *bottom*). In some preparations, the flashes were initially synchronous, but after 5 min of exposure to 2-APB, the flashes became asynchronous between cells in all preparations. No change in the characteristics of these flashes could be detected with the addition of 10 μM PE (n = 4). Flash frequency with 30 μM 2-APB was 0.080 ± 0.010 flashes·cell^{-1}·s^{-1} (40 cells, total 54-min sample, 4 preparations, mean ± SE) and 0.076 ± 0.013 flashes·cell^{-1}·s^{-1} (35 cells, total 48-min sample, 4 preparations, mean ± SE) in the presence of 30 μM 2-APB and 10 μM PE (Fig. 5*E*). Chemical abolition of perivascular nerve function had no effect on the flashes. The sympathetic nerves were destroyed using guanethidine (30 μM, 1 h), sensory nerves were inhibited with capsaicin (1 μM, 1 h), and any cholinergic effects were antagonized with scopolamine (1 μM).

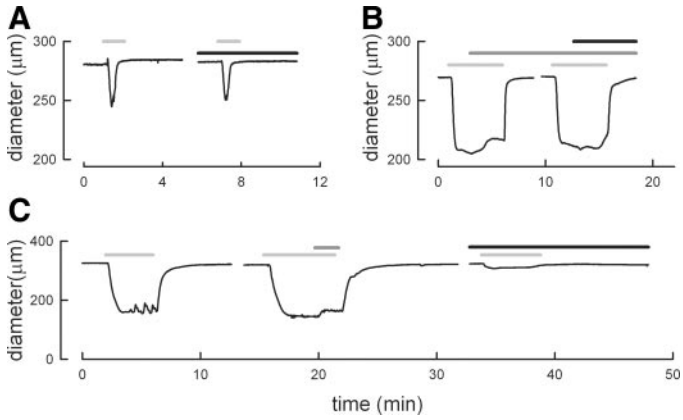

Fig. 4. Effects of the putative blocker of inositol (1,4,5)trisphosphate [Ins(1,4,5)P_3] receptors 2-aminoethyl diphenylborate (2-APB). *A*: effect of 2-APB (30 μM) on the contractions elicited by caffeine. Shaded bars indicate the duration of the caffeine applications (20 mM). The solid bars indicated the presence of 2-APB (30 μm). 2-APB had little effect on the contraction elicited by caffeine. In 3 preparations, the caffeine contraction in 30 μM 2-APB was 83.0 ± 7.01% of that in control. *B*: effect of 2-APB on contractions elicited by depolarization. Depolarization was produced by elevating the extracellular [K^+] to 33 mM (light shaded bars). To eliminate any contribution of adrenergic neurotransmitters released from intrinsic nerves, 10 μM prazosin, an α_1-antagonist, was applied (dark shaded bar). Prasozin slightly reduced the potassium-induced contraction in control conditions. The potassium contraction was unaffected by the application of 2-APB (solid bar). The potassium contraction in the presence of 30 μM 2-APB was 93.5 ± 3.70% of that in control (n = 4, mean ± SE) *C*: effect of 2-APB on a PE-induced constriction. The first constriction was elicited by the application of 10 μM PE (light shaded bar). During the first break in the trace, the vessel was deendothelialized. The second PE constriction was slightly greater, and the application of 10 μM ACh (dark shaded bar) produced only a small reduction in the PE-induced constriction, indicating deendothelialization. The vasoconstriction elicited by 10 μM PE was almost abolished in the presence of 2-APB; 5.2 ± 1.0% (mean ± SD) of the control PE response remained in the presence of 30 μM 2-APB (n = 7). 2-APB had been present for 5 min before PE was applied. The solid bar indicates the presence of 30 μM 2-APB.

DISCUSSION

Adrenergic activation, either via neurally released norepinephrine (25) or bath-applied agonists, elicits maintained vasoconstriction that is associated with asynchronous propagating Ca^{2+} waves in smooth muscle cells and, at higher levels of activation, in arteries with intact endothelium, synchronous Ca^{2+} oscillations and vasomotion (30, 44). In general, both RyRs and Ins(1,4,5)P_3Rs have been thought to be involved in adrenergically induced propagating Ca^{2+} waves (3, 17, 19). In fact, blockade of RyRs did deplete SR Ca^{2+} stores and abolish both Ca^{2+} sparks and adrenergically induced asynchronous Ca^{2+} waves. Nevertheless, our observation that exposure to an adrenergic agonist (PE) rendered ryanodine effective only very slowly compared with caffeine is a strong indication that RyRs are not, in fact, open (i.e., in a state to which ryanodine can bind) during the agonist-induced Ca^{2+} waves. Blockade of Ins(1,4,5)P_3Rs with 2-APB also abolished adrenergically induced propagating Ca^{2+} waves but did not deplete caffeine-releasable Ca^{2+} stores, nor did it affect Ca^{2+} sparks. Taken together, these results point to a primary involvement of Ins(1,4,5)P_3Rs, and not RyRs, in adrenergically induced propagating Ca^{2+} waves. Therefore, we suggest here, as have others previously (16, 23), that ryanodine abolishes propagating Ca^{2+} waves by depleting a Ca^{2+} store from which Ca^{2+} is also released by Ins(1,4,5)P_3 during Ca^{2+} waves. Interestingly, it has been observed that the Ca^{2+} stores in rat tail arteries recover during long-term (4 days) treatment with ryanodine (7). In that condition, it was reported that Ca^{2+} sparks and responses to caffeine were completely absent (i.e., RyRs were not functional), but the agonist-induced Ca^{2+} waves were indistinguishable from those under control conditions (7).

We propose that the Ca^{2+} flashes seen in ryanodine-treated arteries at low levels of adrenergic activation result entirely from voltage-dependent Ca^{2+} entry during action potentials, because they were blocked by nifedipine. Action potentials might occur more frequently in ryanodine-treated arteries compared with controls due to the loss of the spontaneous outward currents that are normally activated by Ca^{2+} sparks (32). It is known that action potentials do occur spontaneously in mesenteric arteries in vivo (40) and can be elicited in vitro (as can Ca^{-2+} flashes in our experiments) by blocking Ca^{2+}-activated K^+ channels with TEA (13).

High levels of adrenergic activation are associated with spontaneous, spatially uniform Ca^{2+} oscillations that generate vasomotion (30). Vasomotion has been shown to be dependent on an intact endothelium (11) and is at least partly mediated by EDHF (34). It is known to be abolished by removal of extracellular calcium (12, 34) and by blockers of voltage-dependent calcium channels (12, 33). Nevertheless, the mechanism(s) underlying such Ca^{2+} oscillations is not clear, but oscillations in membrane potential are known to occur (9, 10, 15). Therefore, most models involve oscillatory entry of Ca^{2+} through voltage-dependent Ca^{2+} channels. Ryanodine decreased the frequency and increased the amplitude of the Ca^{2+} oscillations markedly (Fig. 2). We speculate that this effect is somehow due to the loss of the "buffer-barrier" function of the SR (34, 42) and to loss of the moderating influence of Ca^{2+} sparks on membrane potential changes. In this respect, our results are different to those of a recent study (36) in which ryanodine abolished adrenergic vasomotion.

2-APB

2-APB is a small-molecular-weight membrane-permeable modulator of the Ins(1,4,5)P_3R. This molecule has been shown to inhibit agonist-induced [Ins(1,4,5)P_3 mediated] calcium release and capacitative calcium entry in a number of cell types including myometrial, skeletal, and large artery smooth muscle (2, 28, 29, 37, 45). Importantly, 2-APB abolished asynchronous propagating Ca^{2+} waves without depleting caffeine-releasable Ca^{2+} stores (Fig. 4*A*). This would be consistent with an action of 2-APB to block Ins(1,4,5)P_3R. This result strengthens our conclusion that waves are dependent on Ins(1,4,5)P_3Rs and not RyRs. Nevertheless, 2-APB had unusual, and previously unreported, effects on Ca^{2+} signals stimulated by PE, particularly asynchronous, Ca^{2+} flash-like Ca^{2+} transients. In contrast to the Ca^{2+} flashes occurring in some ryanodine-treated preparations in the presence of low levels of adrenergic stimulation (Fig. 3), these flashes are distinctly asynchronous between cells (Fig. 5*B*, *bottom*). We speculate that this drug may, therefore, have electrically uncoupled the smooth muscle cells from each other. The inward currents normally elicited by exposure to α_1-adrenergic agonists might have triggered action potentials in the individual cells. We cannot determine whether or not such an action is related to the inhibition of the Ins(1,4,5)P_3R or to some other

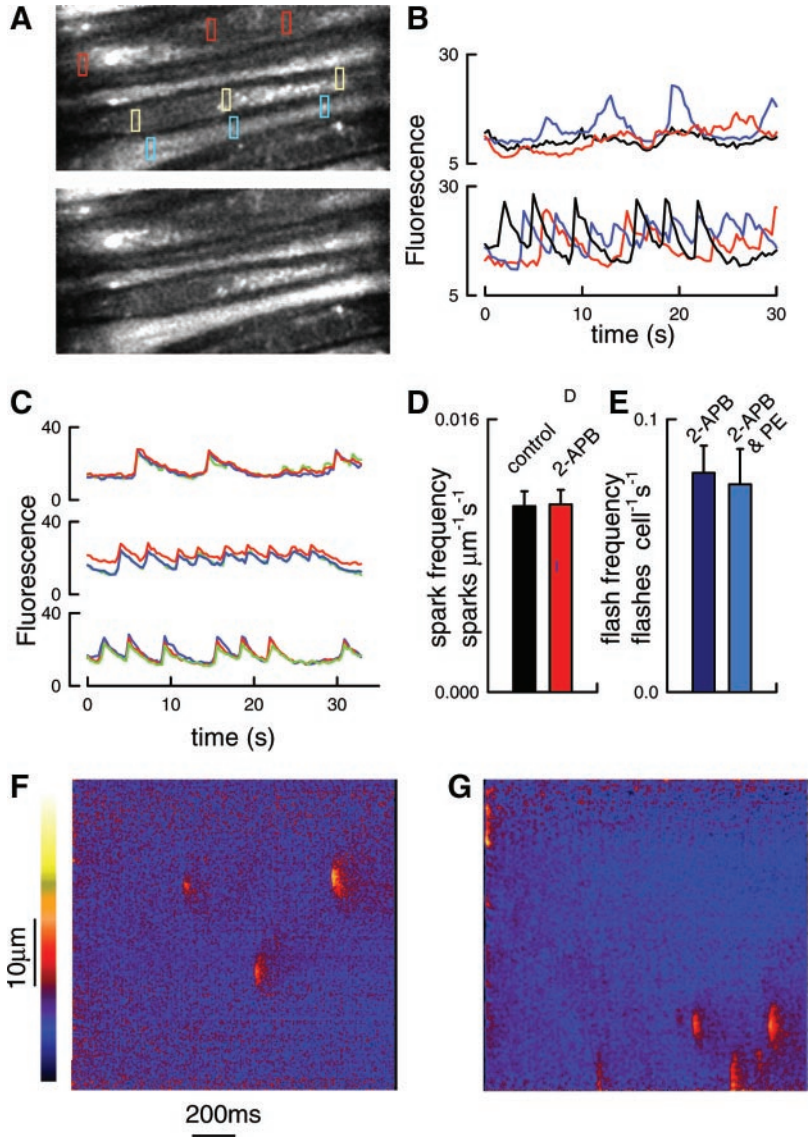

Fig. 5. Ca^{2+} signals observed in the presence of 2-APB and PE (asynchronous Ca^{2+} flashes). *A*: two sequential images (50 × 25 μm, 0.332 s/frame) from a vessel loaded with fluo-4 and exposed to 250 nM PE and 30 μM 2-APB. The colored boxes indicate the AOIs used to create the plots of mean fluorescence against time plotted in *C* and *B*, *bottom*. *B*, *top*: fluorescence from 3 AOIs from images like those shown in *A* in the presence of 250 nM PE but before exposure to 2-APB. The signals are asynchronous propagating Ca^{2+} waves normally seen under these conditions. *Bottom*, fluorescence from the same vessel in the same conditions in the presence of 30 μM 2-APB. In this condition, asynchronous Ca^{2+} flashes can be seen. *C*: fluorescence signals from 3 AOIs in 3 cells in the area shown in *A*. This illustrates that fluorescence signals between cells are asynchronous but that the fluorescence within individual cells is uniform, i.e., that it is not a wave. *D*: spark frequency before and after the application of 30 μM 2-APB. Application of 2-APB did not cause any change in the frequency or other characteristics of Ca^{2+} sparks. Ca^{2+} spark frequency in control conditions was 0.0109 ± 0.0009 $\mu m^{-1} \cdot s^{-1}$ (n = 539 scans, 5 preparations, mean ± SE) and 0.0110 ± 0.0008 $\mu m^{-1} \cdot s^{-1}$ (n = 489 scans, 5 preparations, mean ± SE) in the presence of 30 μM 2-APB. *E*: flash frequency with 30 μM 2-APB before and after the application of 10 μM PE. Flash frequency in the absence of 10 μM PE (dark blue bar) was 0.080 ± 0.010 flashes·$cell^{-1} \cdot s^{-1}$ (40 cells, total 54-min sample, 4 preparations, mean ± SE) and 0.076 ± 0.013 flashes·$cell^{-1} \cdot s^{-1}$ (35 cells, total 48-min sample, 4 preparations, mean ± SE) in the presence of 10 μM PE. There was no statistically significant difference between the two groups. *F*: example of a random line-scan image taken in control conditions showing Ca^{2+} sparks. *G*: example of a random line-scan image taken from the same artery in the presence of 30 μM 2-APB.

unknown effect of this substance. A complete investigation of these phenomena would be beyond the scope of the present work, but the occurrence of these phenomena, which we report here for the first time, mandates caution in interpretation of any experimental results obtained with this compound.

Temperature

In pressurized cerebral arteries studied at mammalian temperature (24), application of ryanodine caused an immediate contraction, different to its effects in the present study.

We speculate that this difference may arise from at least two causes. First, arteries with myogenic tone at the higher temperature are more depolarized, leading to a higher frequency of Ca^{2+} sparks (21) due to the activation of Ca^{2+} sparks by Ca^{2+} entering via voltage-dependent Ca^{2+} channels (22). This would lead to more rapid binding of ryanodine to its receptors at the high temperature. Second, the "Ca^{2+} sensitivity of contraction" is higher in arteries with myogenic tone (41), and thus small changes in Ca^{2+} at the higher temperature may be more efficacious in eliciting contraction.

In summary, the results are consistent with a scheme in which RyRs are involved mainly in Ca^{2+} sparks; Ca^{2+} sparks are important in controlling membrane potential (and thus membrane excitability). Ins(1,4,5)P_3Rs, on the other hand, are involved mainly in agonist-induced asynchronous propagating Ca^{2+} waves. In arteries with intact endothelium, agonist-induced vasomotion occurs and is dependent mainly on Ca^{2+} entry through nifedipine-sensitive Ca^{2+} channels during oscillations in membrane potential but is influenced by the SR (12, 34). Because Ca^{2+} sparks (RyRs) are not involved in asynchronous propagating Ca^{2+} waves, adrenergic stimulation can decrease Ca^{2+} spark frequency while increasing the frequency of Ca^{2+} waves, as previously reported (30).

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