Temperature and Nucleotide Dependence of Calcium Release by myo-Inositol 1,4,5-Trisphosphate in Cultured Vascular Smooth Muscle Cells*

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myo-Inositol 1,4,5-trisphosphate (IP3) rapidly increased 
$^{45}$Ca$^{2+}$ efflux from a nonmitochondrial organelle in cultured vascular smooth muscle cells that were permeabilized with saponin. A nucleotide, preferably ATP, was essential for IP3-evoked $^{45}$Ca$^{2+}$ release. Two nonhydrolyzable ATP analogues satisfied the nucleotide requirement for IP3-evoked $^{45}$Ca$^{2+}$ release. IP3 strongly stimulated $^{45}$Ca$^{2+}$ efflux at low temperatures (1 to 15°C). Decreasing the temperature from 37 to 4°C inhibited the rate of IP3-stimulated efflux by only about 33%. The failure of such low temperatures to strongly inhibit IP3-induced $^{45}$Ca$^{2+}$ efflux suggests that IP3 activated a Ca$^{2+}$ channel, rather than a carrier, by a ligand-binding, rather than a metabolic, reaction.

Agonist-receptor activation of phosphoinositol metabolism has been known for many years (1, 2), but the link between the inositol lipids and calcium, as originally envisioned and later revised by Michell (3, 4), was only recently clarified (5). myo-Inositol 1,4,5-trisphosphate (IP3), which appears to be a mixture of two isomers (6), releases calcium from an intracellular organelle (7) that is probably the endoplasmic reticulum (8, 9). The mechanism by which IP3 releases intracellular calcium is unknown. Two laboratories (10, 11) have reported that IP3 stimulates protein phosphorylation, which raised the possibility that an IP3-dependent protein kinase plays a role in the release of sequestered calcium. If a protein kinase is involved in the action of IP3, then ATP would be required for calcium release. Here we report that a nucleotide is in fact required for IP3-induced $^{45}$Ca$^{2+}$ efflux from permeabilized smooth muscle cells cultured from rat aorta. The present observations suggest, however, that IP3 activates a calcium channel by a ligand-binding mechanism that is independent of the metabolism of ATP or IP3.

EXPERIMENTAL PROCEDURES

We cultured smooth muscle cells from rat aortas (12) and permeabilized them with saponin by a previous method (13) that we adapted for use with attached cell cultures. Nongrowing cultures (approximately 0.44 mg of total cell protein/35-mm diameter dish) were rinsed 4 times with a cytosol-like buffer (CB) containing (mM): 20 NaCl, 100 KCl, 5 MgSO$_4$, 1 Na$_2$HPO$_4$, 1 KEGTA, 20 Hepes adjusted to pH 7.2 with Tris. The cultures were incubated for 10 min at 37°C with CB containing 70 μg/ml saponin, 0.1 mM Ca$^{2+}$Cl$_2$, 0.2% defatted bovine serum albumin, rinsed 3 times with CB, and incubated with CB containing 0.1 mM Ca$^{2+}$Cl$_2$, 0.2% bovine serum albumin, 4.5 mM ATP, and $^{45}$Ca$^{2+}$ (6 μCi, 17.4 μCi/μg Ca$^{2+}$, Amersham). The cultures accumulated $^{45}$Ca$^{2+}$ via an ATP-dependent reaction which was inhibited less than 10% by the combination of 20 μM antimycin A and 1.25 μg/ml oligomycin, which were routinely present during the $^{45}$Ca$^{2+}$ loading for the efflux experiments. A steady state level of approximately 1.5 nmol of Ca$^{2+}$/mg of protein was reached after 30 min. Efflux of $^{45}$Ca$^{2+}$ from the nonmitochondrial store was assayed by rinsing the cultures 8 times with CB (20 μl s total) at the end of the loading with $^{45}$Ca$^{2+}$. The cultures were incubated, usually for 10-s intervals, in 0.5 ml of efflux medium which was entirely removed for measuring $^{45}$Ca$^{2+}$ and immediately replaced with 0.8 ml of fresh efflux medium as described previously (14). Total $^{45}$Ca$^{2+}$ in the saponin-treated cells at the start of the efflux (usually about 100,000 cpm/culture or 1.7 nmol of Ca$^{2+}$/mg of total cell protein) was calculated from the total $^{45}$Ca$^{2+}$ lost during the efflux and the amount left in the cells at the end of the experiment (14). The efflux rate coefficient was calculated as described by Borle (15): $^{45}$Ca$^{2+}$ released in a given time interval/mean cellular $^{45}$Ca$^{2+}$ present during the time interval × the time interval. Efflux data are also presented as semilog plots of the percentage of $^{45}$Ca$^{2+}$ remaining intravesicular after each sampling interval. First-order rate constants were calculated with an exponential curve fitting program from the linear portion of the curve after the addition of IP3 and for basal efflux in the absence of IP3. The difference between the two rate constants equals the IP3-stimulated rate. The assumption that IP3 does not change the basal efflux rate appears to be valid since efflux rapidly returned to the basal rate in the continuous presence of IP3 as indicated below.

To measure the rate of IP3 hydrolysis we permeabilized cultures by the same protocol used to study efflux, but we omitted $^{45}$Ca$^{2+}$. At the end of the incubation with ATP and 0.1 mM $^{45}$Ca$^{2+}$, the cultures were rinsed as usual for assaying efflux and incubated at 37°C with 3 mM ATP and 5 μM [γ-32P]IP3 (62,500 cpm; 110 Ci/mmol; New England Nuclear) in CB containing 0.2% bovine serum albumin and 0.1 mM Ca$^{2+}$Cl$_2$. One ml of ice-cold 10% trichloroacetic acid was added to the dish to stop the incubation. After 10 min the extract was removed, and the extraction was repeated with 2 ml of 5% trichloroacetic acid. Culture dishes without cells were subjected to the same protocol. The combined extracts were chromatographed on Dowex 1-formate columns after the removal of trichloroacetic acid as previously described (16).

RESULTS AND DISCUSSION

IP3 rapidly increased the release of $^{45}$Ca$^{2+}$ from a nonmitochondrial pool in saponin-treated vascular smooth muscle cells (Fig. 1A). Fig. 1B shows the efflux rate coefficients (ERC) calculated from the data in Fig. 1A. The plots of cpm released/culture and ERC are very similar in appearance since the loading of the nonmitochondrial organelle with $^{45}$Ca$^{2+}$ differed by less than 10% for different cultures. Twenty μM IP3 maximally increased $^{45}$Ca$^{2+}$ efflux, whereas 20 μM myo-inositol, myo-inositol 2-monophosphate, mositol bisphosphate purified from erythrocytes (17), phytic acid, 2,3-diphosphoglycerate, or fructose 1,6-diphosphate, all had no effect on $^{45}$Ca$^{2+}$ efflux. IP3 had no effect on the rate of ATP-dependent $^{45}$Ca$^{2+}$ uptake by the nonmitochondrial organelle.

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1 The abbreviations used are: IP3, myo-inositol 1,4,5-trisphosphate; CB, cytosol-like buffer; ERC, efflux rate coefficient; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; AppNHp, adenylyl imidodiphosphate; AppCH2p, adenylyl (8,9-methylenedioxy) diposphonate; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.

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indicating that IP₃ selectively increased calcium efflux without affecting influx.

Omitting ATP from the efflux buffer prevented IP₃ from stimulating ⁴⁵Ca²⁺ efflux (Fig. 1). A nucleotide requirement for calcium release by IP₃ has not previously been reported, although ATP was present in the previous studies of calcium release by IP₃ from a variety of different cell types (7-9, 18-28) including vascular smooth muscle (29-31). ATP by itself failed to induce ⁴⁵Ca²⁺ release and had no significant effect on the basal ⁴⁵Ca²⁺ efflux (Fig. 1).

Three mM ATP supported almost maximal ⁴⁵Ca²⁺ release by IP₃. The calcium releasing mechanism appears to selectively require ATP since 3 mM AMP or CTP was inactive, and 3 mM ADP and GTP were less effective than 3 mM ATP in restoring IP₃-evoked ⁴⁵Ca²⁺ efflux (Fig. 1C). Two non-hydrolyzable ATP analogues, a β,γ-imido derivative (AppNHp) and a β,γ-methylene derivative (AppCH₂p), were tested at 1.5 mM and found to be almost as effective as 1.5 mM ATP in restoring IP₃-evoked ⁴⁵Ca²⁺ efflux (Fig. 1D). The fact that AppNHp and AppCH₂p supported calcium release by IP₃ suggests that the transfer of the γ phosphate from ATP to a protein is not involved in calcium release by IP₃.

To obtain additional evidence regarding the apparent independence of calcium release from the metabolism of either IP₃ or ATP, we assayed calcium efflux at low temperatures. Between 1 and 37 °C IP₃ evoked approximately the same peak increase in the efflux rate coefficient (Fig. 2A). A semilog plot of the same data (Fig. 2B) shows that IP₃ increased the first-order rate constant of ⁴⁵Ca²⁺ efflux to approximately the same value at each temperature as indicated by the nearly parallel slopes of the curves after the addition of IP₃. In contrast to IP₃-stimulated efflux, basal efflux was considerably faster at 37 °C than at the lower temperatures (Fig. 2B). Therefore, approximately 80% of the initial intravesicular ⁴⁵Ca²⁺ remained at the time of IP₃ addition at the lower temperatures compared to 70% at 37 °C (Fig. 2B).

In order to have the same amount of intravesicular ⁴⁵Ca²⁺ at the time of addition of IP₃, we added IP₃ 40 s after initiating efflux at 37 °C and 160 s after initiating efflux at 4 °C (Fig. 3). Additionally we used 20 μM IP₃ to obtain the maximal stimulation of efflux since at 37 °C 40 μM IP₃ gave the same increase in ⁴⁵Ca²⁺ efflux as 20 μM. Therefore, at 20 μM IP₃ it is unlikely that the stimulation of efflux is limited by the rate of diffusion of IP₃ from the external medium to its receptor in the cytoplasm of the permeabilized cells. Five- instead of 10-s sampling intervals were used to assay efflux since it was faster under these conditions (Fig. 3). The rate of IP₃-evoked efflux (stimulated rate minus the basal rate) was approximately 33% slower at 4 °C than at 37 °C (Fig. 3). The fact that there was less intravesicular ⁴⁵Ca²⁺ at 37 °C at the time
The addition of IP3 began at the times indicated by the arrows and every 5 s thereafter until the end of the experiment. Efflux data from cultures that did not receive IP3 is indicated by open circles (4°C) and open squares (37°C). The data are presented in panel A as efflux rate coefficients and in panel B as the percentage of the initial intravesicular Ca2+. At the start of the efflux assay there was approximately 0.59 nmol of Ca2+/culture or 1.5 nmol of Ca2+/mg of protein. Values are mean of duplicates for 1 of 3 experiments which gave similar results.

The temperature dependence of IP3-evoked 45Ca2+ efflux was explored at 4 and 37°C (Fig. 2). Efflux was carried out at 37°C (A) or 4°C (B) under the conditions indicated in the Fig. 2 legend except that the sampling time was 5 s and the concentration of IP3 was 20 μM. The addition of IP3 began at the times indicated by the arrows and every 5 s thereafter until the end of the experiment (solid circles). Some cultures did not receive IP3 (open circles). At the start of the efflux there was approximately 0.84 nmol of intravesicular Ca2+/culture or 1.9 nmol/mg of protein. Values are mean of duplicates for 1 of 3 experiments which gave similar results. The first-order rate constants of IP3-stimulated efflux were 0.016 ± 0.001 and 0.0243 ± 0.001 (mean ± S.E., n = 6) at 4 and 37°C, respectively.

The increase in 45Ca2+ efflux rate that was evoked by IP3, even in the absence of ATP, occurred 10-20 s later at 1°C than it did at 37°C (Fig. 2). Decreasing the temperature progressively decreases the activity of carrier-type antibiotics as the viscosity of the lipid bilayer increases (32, 33). Ion carriers apparently have to diffuse freely in the membrane and become virtually inactive when the phospholipids are in the solid state. In contrast, ion channels, for example, gramicidin (32) or bacteriorhodopsin (33), are almost fully active even at temperatures below the transition state of the membrane lipids. The temperature dependence of the IP3-activated Ca2+ transporter, which was highly active at 1 and 4°C (Figs. 2 and 3), resembles that of a channel rather than a carrier.

Conceivably, the rapid return to the basal rate of 45Ca2+ efflux may be caused by the depletion of 45Ca2+ from the IP3-sensitive pool, although at 4°C about half of the initial 45Ca2+ remained intravesicular at the time when efflux returned to the basal rate. Accordingly it may be that only 50% of the total 45Ca2+ in the nonmitochondrial store is releasable by IP3. Alternatively, the transient nature of 45Ca2+ release may be due to a desensitization of the IP3 receptor as noted previously (9).

The temperature dependence of IP3-evoked 45Ca2+ efflux suggests that calcium release is mediated by a channel rather than a carrier. Decreasing the temperature progressively decreases the activity of carrier-type antibiotics as the viscosity of the lipid bilayer increases (32, 33). Ion carriers apparently have to diffuse freely in the membrane and become virtually inactive when the phospholipids are in the solid state. In contrast, ion channels, for example, gramicidin (32) or bacteriorhodopsin (33), are almost fully active even at temperatures below the transition state of the membrane lipids. The temperature dependence of the IP3-activated Ca2+ transporter, which was highly active at 1 and 4°C (Figs. 2 and 3), resembles that of a channel rather than a carrier.

It is noteworthy that ATP was required for 45Ca2+ release in the presence (Fig. 1, A and B) as well as in the absence of calcium (Fig. 1C). The reported calcium contamination of the ATP used for the present experiments was 3 ppm (Sigma), so the addition of 3 mM ATP to efflux buffer containing 0.1 mM total calcium (Fig. 1, A and B) would not significantly change its calcium content. These data show that extragranellar calcium was not essential for 45Ca2+ release by IP3, although
low external calcium (20 to 60 nM) increased IP₃-induced ⁴⁴Ca²⁺ efflux somewhat (Fig. 1).²

The present results show that IP₃ releases ⁴⁴Ca²⁺ from a nonmitochondrial organelle at low temperatures (1 to 4 °C) by a nucleotide-dependent mechanism. The finding that IP₃ is highly active at such low temperatures suggests that IP₃ activates an intracellular calcium channel, not a carrier, by a ligand-binding reaction that does not depend on metabolism. Perhaps the binding of the nucleotide transforms the channel from a mode that is unopenable to one that can be opened by IP₃. Cardiac Ca²⁺ channels seem to have three modes of gating behavior: mode 0 with no openings; mode 1 with brief openings; and mode 2 with long-lasting openings, which is promoted by a dihydropyridine Ca²⁺ agonist (34). Single channel analysis with the patch clamp technique is needed to define the modes which occurred in the presence of a continuous supply of IP₃, dihydropyridine Ca²⁺ antagonists; mode 1 with brief openings; free calcium elicited by phenylephrine (21), and angiotensin (36) in vascular smooth muscle.

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REFERENCES


² J. B. Smith, L. Smith, and B. L. Higgins, unpublished data.