Biphasic Ca$^{2+}$ Dependence of Inositol 1,4,5-Trisphosphate-induced Ca Release in Smooth Muscle Cells of the Guinea Pig Taenia Caeci

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ABSTRACT Ca$^{2+}$ dependence of the inositol 1,4,5-trisphosphate (IP$\text{$_3$}$)-induced Ca release was studied in saponin-skinned smooth muscle fiber bundles of the guinea pig taenia caeci at 20–22°C. Ca release from the skinned fiber bundles was monitored by microfluorometry of fura-2. Fiber bundles were first treated with 30 μM ryanodine for 120 s in the presence of 45 mM caffeine to lock open the Ca-induced Ca release channels which are present in ~40% of the Ca store of the smooth muscle cells of the taenia. The Ca store with the Ca-induced Ca release mechanism was functionally removed by this treatment, but the rest of the store, which was devoid of the ryanodine-sensitive Ca release mechanism, remained intact. The Ca$^{2+}$ dependence of the IP$\text{$_3$}$-induced Ca release mechanism was, therefore, studied independently of the Ca-induced Ca release. The rate of IP$\text{$_3$}$-induced Ca release was enhanced by Ca$^{2+}$ between 0 and 300 nM, but further increase in the Ca$^{2+}$ concentration also exerted an inhibitory effect. Thus, the rate of IP$\text{$_3$}$-induced Ca release was about the same in the absence of Ca$^{2+}$ and at 3 μM Ca$^{2+}$, and was about six times faster at 300 nM Ca$^{2+}$. Hydrolysis of IP$\text{$_3$}$ within the skinned fiber bundles was not responsible for these effects, because essentially the same effects were observed with or without Mg$^{2+}$, an absolute requirement of the IP$\text{$_3$}$ phosphatase activity. Ca$^{2+}$, therefore, is likely to affect the gating mechanism and/or affinity for the ligand of the IP$\text{$_3$}$-induced Ca release mechanism. The biphasic effect of Ca$^{2+}$ on the IP$\text{$_3$}$-induced Ca release is expected to form a positive feedback loop in the IP$\text{$_3$}$-induced Ca mobilization below 300 nM Ca$^{2+}$, and a negative feedback loop above 300 nM Ca$^{2+}$.

INTRODUCTION
It has been postulated that inositol 1,4,5-trisphosphate (IP$\text{$_3$}$) formed in response to external stimuli releases Ca from the internal store and serves as the second messenger in signal transduction in many types of cells (Berridge and Irvine, 1984). Ability of IP$\text{$_3$}$ to release Ca from the permeabilized smooth muscle cells has been demon-
strated (Suematsu et al., 1984; Somlyo et al., 1985). Production of IP₃ after agonist stimulation has been measured in smooth muscle as in many other cells (for review see Berriidge and Irvine, 1984; Abdel-Latif, 1986). Walker et al. (1987) showed that IP₃ rapidly formed in a skinned smooth muscle fiber bundle by photolysis of a photo-labile inactive precursor of IP₃ (caged IP₃) caused a rapid development of tension, the time course of which was comparable to the tension rise in intact muscle after agonist stimulus. These experimental results suggest that IP₃ is the intracellular second messenger for agonist-stimulated Ca release from the store in smooth muscle cells.

Among the factors that have been reported to influence IP₃-induced Ca release mechanism, Ca²⁺ is of potential importance because Ca²⁺ dependence of the Ca release mechanism is expected to constitute a feedback mechanism in the intracellular Ca mobilization and to regulate the effectiveness of IP₃. However, the results so far remain in apparent contradiction as to the effect of Ca²⁺ on the IP₃-induced Ca release. It was shown that the amount of Ca released by IP₃ decreased at Ca²⁺ concentrations above 1 μM in macrophage and coronary artery smooth muscle cells while no effect was found below 1 μM Ca²⁺ (Hirata et al., 1984; Suematsu et al., 1984). On the other hand, Iino (1987) reported that the rate of IP₃-induced Ca release was enhanced by Ca²⁺ at concentrations of around 100 nM, but in that work Ca²⁺ dependence was studied only below 1 μM in order to avoid the interference of the Ca-induced Ca release mechanism that is activated by Ca²⁺ above 1 μM (Iino, 1989). In the present study a new protocol was used to study the effect of a wide range of Ca²⁺ concentration in the absence of the Ca-induced Ca release. In this protocol, IP₃-induced Ca release is biphasically dependent on Ca²⁺ in the concentration range where the contractile system is controlled; the Ca release mechanism is enhanced by Ca²⁺ below 300 nM, and above this concentration Ca²⁺ exerts an inhibitory effect as well. Some of these results have been presented in preliminary form (Iino and Endo, 1989).

**METHODS**

Details of the method and apparatus used to study properties of the Ca store in skinned smooth muscle fibers have been described elsewhere (Iino, 1989). In brief, thin fiber bundles (150–250 μm in width and 5 mm in length) were obtained from guinea pig taenia caeci and the surface membranes were rendered permeable by treatment with saponin (50 μg/ml) in a relaxing solution for 30–35 min. Fiber bundles were placed in a capillary cuvette (400 μm internal diameter) through which solutions can be rapidly flushed on the stage of an epifluorescence microscope. The Ca stores of skinned fiber bundles were loaded with Ca, then various Ca-releasing test stimuli were applied in the absence of ATP. After the removal of the test stimulus, Ca remaining in the store was thoroughly released with high concentration of IP₃, and the amount of Ca was assayed by a microfluorometry of fura-2 (Gryniewicz et al., 1985) and then was compared with that of a control run conducted without the test procedure to estimate the magnitude of Ca release due to the test stimulus.

**Solutions and Experimental Protocol**

Compositions of the experimental solutions were calculated by solving multiequilibrium equations based on the stability constants compiled by Martell and Smith (1974–1982). The solutions used in this study are described in Table I. To prepare solutions of various Ca²⁺...
TABLE I
Composition of the Solutions

<table>
<thead>
<tr>
<th>Condition</th>
<th>EGTA or EDTA*</th>
<th>CaEGTA or CaEDTA*</th>
<th>MgMs</th>
<th>KMg</th>
<th>ATP</th>
<th>AMP</th>
<th>Mg^2+</th>
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</thead>
<tbody>
<tr>
<td>G1</td>
<td>1</td>
<td>0</td>
<td>5.54</td>
<td>106.8</td>
<td>4.76</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>pCa 6-loading</td>
<td>0.298</td>
<td>0.702</td>
<td>5.51</td>
<td>106.6</td>
<td>4.76</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>G0R</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
<td>137.6</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>G0RMg0</td>
<td>0</td>
<td>0</td>
<td>142.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Assay</td>
<td>0</td>
<td>0</td>
<td>84.1</td>
<td>0</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>0.171</td>
<td>0.829</td>
<td>84.1</td>
<td>0</td>
<td>22.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G1R</td>
<td>1</td>
<td>0</td>
<td>1.54</td>
<td>134.5</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>G10R</td>
<td>10</td>
<td>0</td>
<td>1.90</td>
<td>107.1</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>CaG10R</td>
<td>0</td>
<td>10</td>
<td>1.50</td>
<td>107.5</td>
<td>0</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
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<td>0</td>
<td>139.0</td>
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<td>0</td>
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<tr>
<td>G10RMg0</td>
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<td>111.8</td>
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<td>0</td>
</tr>
<tr>
<td>CaG10RMg0</td>
<td>0</td>
<td>10</td>
<td>112.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D1R</td>
<td>1*</td>
<td>0</td>
<td>136.5</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<tr>
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<td>0</td>
</tr>
<tr>
<td>CaD10R</td>
<td>0</td>
<td>10*</td>
<td>112.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Concentrations are in millimolar. All solutions contained 20 mM PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid)) and 20 mM NaNs. Assay contained 10 μM IP₃ unless otherwise described. Ryanodine contained 45 mM caffeine and 30 μM ryanodine. pH was adjusted to 7.0 at 20°C with KOH. Mg^2+ concentration was estimated by the numerical solution of multiequilibrium between metals and ligands in the solution. When ATP was present, MgATP₂⁻ concentration was calculated to be 4.0 mM. Total ionic strength was 200 mM in all the solutions. EGTA, ethyleneglycol-bis[α-aminoethyl ether] N,N,N',N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid, Ms, methanesulfonic acid.

concentrations, two solutions containing 10 mM EGTA (or EDTA) without Ca and 10 mM CaEGTA (or CaEDTA) were mixed (see Table II). To obtain CaEGTA or CaEDTA stock solution, CaCO₃ and equimolar EGTA or EDTA were mixed and neutralized with KOH. IP₃ and 2,3-bisphosphoglycerate were simply added to the solutions, and the pH of such solutions was readjusted to pH 7.0 when necessary.

Fiber bundles were preincubated in a relaxing solution (G1, for the code of solutions see Tables I and II) for 120 s, before Ca store was loaded with Ca for 180 s in a solution containing 1 μM Ca^2+ (pCa 6-loading). Then both Ca^2+ and ATP were washed out for 120 s with G1R (end of Ca loading). After the Ca loading, 30–40 μM fura-2 was introduced in G1R for 60 s, and in the continued presence of fura-2 a pre-assay solution without both Mg and

TABLE II
Total Ca Concentrations in the Ca-Containing Solutions

<table>
<thead>
<tr>
<th>Condition</th>
<th>pCa (x)</th>
<th>Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;8</td>
<td>7.5</td>
</tr>
<tr>
<td>pCaR</td>
<td>0.00</td>
<td>0.69</td>
</tr>
<tr>
<td>pCaRMs</td>
<td>0.00</td>
<td>0.71</td>
</tr>
<tr>
<td>pCaDR</td>
<td>0.00</td>
<td>3.56</td>
</tr>
</tbody>
</table>

Ca-containing solutions of various pCa were prepared by mixing two solutions indicated in the rightmost column in such a ratio that the total Ca concentration was equal to the values (in millimolar) shown in this table.
EGTA (G0RMg0) was applied for 60 s, which was followed by the application of an assay solution for 70 s that contained 10 μM IP₃ and 25 mM AMP but no EGTA ("Assay"). AMP was added to the assay solution because adenine nucleotides enhance IP₃-induced Ca release mechanism (manuscript in preparation). The Ca assay was carried out in the absence of ATP so that unidirectional Ca release could be observed without the movement of the fiber bundles. Because fura-2 is a high affinity chelator of Ca, almost all the Ca released from the skinned fiber bundle would bind to the dye in the assay solution with 1:1 stoichiometry, resulting in a proportional change in the fluorescence intensity (see Fig. 1 of Iino, 1989). To distinguish genuine response due to Ca release of the fiber bundle from disturbances such as small differences in the level of Ca contamination, any direct effect of ingredients of the assay solution on fura-2 fluorescence, and slow liberation of Ca passively trapped in the fiber bundle, Ca assay was repeated in the same protocol except for the omission of the Ca-loading procedure, and the difference in the paired fluorescence intensity change was obtained as Ca signal (see Fig. 2 of Iino, 1989). The amount of Ca released can be evaluated from the concentration of fura-2 in the assay solution and the proportion of the fluorescence intensity change due to Ca release to the maximum fura-2 fluorescence intensity change induced by a saturating concentration of Ca under the same condition. In some occasions the composition of the assay solution was changed, e.g., IP₃ was replaced with 50 mM caffeine (see Fig. 3) or AMP was omitted (see Fig. 1 A).

In order to study Ca²⁺ dependence of the IP₃-induced Ca release mechanism, a test solution that contained IP₃ and the desired concentration of Ca²⁺ was applied in the absence of ATP for various lengths of time after the Ca loading, and then the amount of remaining Ca in the store was assayed as described above. The difference in the amount of Ca in the store with and without the application of the test solution corresponded to the amount of Ca released during the test period. The details of applying test solutions were as follows. After the Ca loading, the condition of the solution was changed for 60 s to that of the test solution except that IP₃ was absent and the EGTA concentration was 1 mM without added Ca (e.g., G1R, GIRMg0, or D1R). The Ca²⁺ concentration was then changed to the desired value, using 10 mM EGTA or EDTA as a Ca²⁺ buffer (Table II), and 15 s later IP₃ was added. After a 0–360-s application of 0–30 μM IP₃, both Ca²⁺ and IP₃ were removed by washing the preparation for 60 s with a solution that contained 10 mM EGTA, 1.5 mM Mg²⁺, and no ATP (G10R).

The whole sequence of the protocol could be repeated several times in one fiber bundle. Control runs without the test procedure were inserted every four to five runs as internal control to allow for rundown. The first ryanodine treatment was carried out using solution "Ryanodine" (see Table I) for 120 s at the concentration of 30 μM at pCa 5.7 in the presence of 45 mM caffeine and 22.5 mM AMP, potentiators of the Ca-induced Ca release mechanism (Iino, 1989). The effect of ryanodine lasts 1 h or so at 20°C. However, since the whole experiment on one fiber bundle took 3–5 h, a brief 30-s ryanodine treatment was inserted between runs to ensure that the effect of the drug was complete. In some experiments lack of responsiveness to caffeine of skinned fiber bundles was confirmed at the end of the experiments. Temperature was controlled between 20 and 22°C.

In most of the experiments in this paper, the fluorescence intensity of fura-2 was measured with double wavelength excitation at 340 and 360 nm alternating either at 200 or 400 Hz using a light source system (CAX-100; Nihon Bunko Kogyo, Tokyo, Japan) attached to the microscope. There was little Ca²⁺-dependent change in the fluorescence intensity at 360 nm excitation, and the results agreed well between single (340 nm) wavelength excitation and the ratio (340/360 nm) measurement.

**Mg²⁺ Concentration Measurements**

In order to measure Mg²⁺ concentration in the solutions containing 2,3-bisphosphoglycerate, differential absorbance spectra of a metallochromic indicator antipyrylazo III (Scarpa et al.,...
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Iino 1978) were recorded using a double-beam spectrophotometer (type 340; Nissei Sangyo, Tokyo, Japan). The reference cuvette contained a solution with the dye but without added Mg. The difference between the differential absorbance at 520 nm and that at 600 nm was used as a Mg$^{2+}$-dependent absorbance change, and varied with Mg$^{2+}$ concentration in a hyperbolic manner as expected from one-to-one binding between the dye and the cation.

**Chemicals**

Inositol 1,4,5-trisphosphate and 2,3-bisphosphoglycerate were purchased from Sigma Chemical Co. (St. Louis, MO). Saponin and antipyrylazo III were obtained from ICN Pharmaceuticals Inc. (Cleveland, OH), ryanodine was from Agrysystems International (Wind Gap, PA), and fura-2 was from Molecular Probes, Inc. (Eugene, OR).

**RESULTS**

*Effect of Mg$^{2+}$ on the IP$_3$-induced Ca Release*

Fig. 1A shows the time courses of the fluorescence intensity change of fura-2 due to Ca release induced by the application of assay solutions containing 10 µM IP$_3$ and either 0, 0.5, or 1.5 mM Mg$^{2+}$ (prepared from G0RMg0, 2:1 mixture of G0RMg0 and G0R, and G0R of Table I, respectively). Ca was loaded at pCa 6 for 180 s, and after the removal of both Ca and MgATP, IP$_3$ was applied at the time indicated by the arrow. As a control experiment, response to an "assay solution" without IP$_3$ is also shown. IP$_3$ induced a rapid release of Ca in the absence of Mg$^{2+}$, but with increasing concentration of Mg$^{2+}$, the rate of Ca release declined, while the plateau values seem nearly constant.

Since skinned smooth muscle fibers contain a high activity of intrinsic IP$_3$ phosphatase (Walker et al., 1987) with Mg$^{2+}$-dependent hydrolysis rate (Downes et al., 1982), the inhibitory effect of Mg$^{2+}$ on the rate of the IP$_3$-induced Ca release could be due either to a direct inhibition of the Ca release mechanism or to enhanced hydrolysis of IP$_3$ by the IP$_3$ phosphatase. In order to explore this problem, the effect of 2,3-bisphosphoglycerate, an inhibitor of IP$_3$ phosphatase (Downes et al., 1982), was examined.

When 2,3-bisphosphoglycerate was present during 10 µM IP$_3$ application in the presence of 1.5 mM Mg$_{total}$, IP$_3$-induced Ca release was markedly increased (Fig. 1A). Although 2,3-bisphosphoglycerate may bind Mg$^{2+}$, the Mg$^{2+}$ concentration of the solution with 2 mM 2,3-bisphosphoglycerate was >1 mM (1.2–1.4 mM), based on the assay of absorbance of antipyrylazo III. Therefore, most of the enhancing effect of 2,3-bisphosphoglycerate is not due to a simple Mg$^{2+}$-binding effect.

The effects of Mg$^{2+}$ and 2,3-bisphosphoglycerate were further examined using a different protocol. In these experiments, the Ca store was loaded with Ca at pCa 6 for 180 s, then 10 µM IP$_3$ was applied for 60 s in the absence of ATP and the presence of 0, 0.5, and 1.5 mM Mg$^{2+}$ at pCa 7.0 (buffered with 10 mM EGTA; pCa7RMg0, 2:1 mixture of pCa7RMg0 and pCa7R, and pCa7R of Table II, respectively). And finally, the amount of Ca remaining in the Ca store was assayed as described in Methods. As shown in Fig. 1B, IP$_3$-induced Ca release depended on Mg$^{2+}$, and the amount of Ca that remained unreleased after a 15-s application of IP$_3$ either at 0.5 or 1.5 mM Mg$^{2+}$ was significantly greater than that after IP$_3$ application in the absence of Mg$^{2+}$ (P < 0.001, paired t test was used unless otherwise
The inhibitory effect of Mg$^{2+}$ was reversed by 2 mM 2,3-bisphosphoglycerate ($P < 0.001$), and the amount of Ca release was not significantly different from that in the absence of Mg$^{2+}$ ($P > 0.5$).

The possibility of 2,3-bisphosphoglycerate having a direct effect on the IP$_3$-induced Ca release was examined under the condition where Mg$^{2+}$ concentration was lowered virtually to null with 10 mM EDTA so that IP$_3$ phosphatase was not active with or without 2,3-bisphosphoglycerate. In the presence of a high concentration of EDTA, Ca released from the store will bind to EDTA rather than to 30–40 μM fura-2. Therefore, experiments similar to those shown in Fig. 1 A were not feasible, and the type of experiment shown in Fig. 1 B was carried out. Fig. 2 shows the relative amount of Ca remaining in the store either after a 45-s application of 10 μM IP$_3$ in the presence of 1.5 mM M$_{\text{final}}$ at pCa 7 (buffered with 10 mM EGTA, pCa7R of Table II) or after a 15-s application of 0.3 μM IP$_3$ without Mg$^{2+}$ at pCa 7 (buff-

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**FIGURE 1.** (A) Time course of the fluorescence intensity change of fura-2 due to IP$_3$-induced Ca release from a skinned fiber bundle of taenia in the absence of ATP. Ratio of the fluorescence intensity at 340 nm excitation to that at 360 nm is shown. IP$_3$ was applied at the arrow and the concentration was 10 μM except for the bottom trace (without IP$_3$). Mg$^{2+}$ concentration was varied as indicated by the label next to each trace. In one of the traces, the solutions contained both 2 mM 2,3-bisphosphoglycerate (BPG) and 1.5 mM Mg$^{2+}$. All the assay solutions contained 31 μM fura-2 but no AMP. Vertical calibration bar corresponds to one-tenth of the maximum fluorescence intensity ratio change or 3.1 μM Ca (= 31 μM/10). Fiber bundle widths: 150 × 200 μm.

(B) Relative amount of remaining Ca in the store after a 15-s application of 10 μM IP$_3$ at pCa 7 in the presence of 0, 0.5, or 1.5 mM Mg after constant Ca loading. (Rightmost column) IP$_3$ was applied in the presence of 1.5 mM M$_{\text{final}}$ and 2 mM 2,3-bisphosphoglycerate. (Leftmost column) A control run. ATP was absent during IP$_3$ application. Mean and SEM of the results from six fiber bundles. Each sample was tested in all the conditions.
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mechanism is also present in the smooth muscle Ca store and is activated by Ca\(^{2+}\) above 1 \(\mu\)M (Iino, 1989). It would have been difficult to separate the effect of Ca\(^{2+}\) on the IP\(_3\)-induced Ca release from that on the Ca-induced Ca release at the higher Ca\(^{2+}\) concentrations.

It has been proposed that the Ca store of smooth muscle consists of two compartments: one (Sa) is both caffeine- and IP\(_3\)-releasable, and the other (S\(_b\)) is releasable with IP\(_3\) but not with caffeine (Iino, 1987, 1989; Iino et al., 1988). Because caffeine releases Ca through enhancement of the Ca-induced Ca release mechanism (Iino, 1989), this Ca release mechanism is thought to be present only in Sa. Results shown in Fig. 3 support this view. Panels a to e of Fig. 3A were obtained from the same fiber bundle after constant Ca loading at pCa 6 for 180 s. In each panel two Ca

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**Figure 3.** (A) Ca release in the absence of ATP from a skinned fiber bundle before (a–c) and after (d and e) ryanodine treatment. In each panel Ca loading was carried out at pCa 6 for 180 s. The left trace was obtained in the first Ca assay after the Ca loading, and Ca assay was repeated as in the right trace. Between the two traces assay solution was washed away but no Ca loading was carried out. In a, 50-mM caffeine assays were repeated twice. In b and d, a caffeine assay was followed by a 10-\(\mu\)M IP\(_3\) assay. In c and e, a caffeine assay followed an IP\(_3\) assay. 30 \(\mu\)M ryanodine was treated for 120 s at pCa 5.7 in the presence of 45 mM caffeine and 22.5 mM AMP. Ratio of the fluorescence intensity changes of 32.5 \(\mu\)M fura-2 at 340 and 360 nm excitation is shown, and the vertical calibration corresponds to one-fifth of the maximum change or 6.5 \(\mu\)M Ca. Fiber bundle widths: 150 × 200 \(\mu\)m. (B) Size of Ca store before and after ryanodine treatment plotted against the size of IP\(_3\)-releasable Ca store before ryanodine treatment. IP\(_3\)-releasable Ca store after ryanodine treatment (crosses) was examined in 58 fiber bundles, and in 10 of these samples caffeine-releasable Ca store before (solid squares) and after (open squares) ryanodine treatment was also studied. Ca loading, ryanodine treatment, and Ca assays were carried out as in A. "Size" of Ca store was expressed by the increase in the Ca\(^{2+}\) concentration in the capillary cuvette due to Ca release in response to the application of assay solutions. For explanation of the straight lines, see text.
assays either with 10 μM IP₃ (Assay) or with 50 mM caffeine (Assay without IP₃ but with caffeine) were carried out in succession with 220-s intervals, but without intervening Ca loading. In a, two caffeine assays were carried out. There was no Ca response for the second caffeine assay, and this indicates that the caffeine-releasable store was depleted by the first assay. However, as shown in b, IP₃ assay could release a considerable amount of Ca when caffeine-releasable stores had been depleted by the preceding caffeine assay. If IP₃ assay was carried out first, it released about two times more Ca than the caffeine assay did, and the following caffeine application failed to release Ca (c). These results clearly indicate that the caffeine-releasable store overlaps with the IP₃-sensitive Ca store, but makes up only a part of it.

Ryanodine, a plant alkaloid, has been shown to bind to the Ca-induced Ca release channels preferentially in their open state and to lock open these channels in skeletal muscle sarcoplasmic reticulum (Fleischer et al., 1985; Rousseau et al., 1987). This drug has been shown to have the same effect which is virtually irreversible at 20°C on the Ca-induced Ca release mechanism of smooth muscle, and if ryanodine is treated under a condition that the Ca-induced Ca release is activated, Sα loses its capacity to hold Ca because of the “permanent holes” in this compartment of the store, but Sβ remains intact (Iino et al., 1988). Fig. 3 A, a and e were obtained after the skinned fiber bundle was treated with 30 μM ryanodine together with 45 mM caffeine and 22.5 mM AMP, potentiators of the Ca-induced Ca release mechanism (Iino, 1989), at pCa 5.7 for 120 s. It is clear that caffeine could no longer release Ca after the ryanodine treatment, whereas IP₃ could. However, IP₃-releasable Ca was decreased by ~40% after the ryanodine treatment (cf. e and e). The difference in the size of IP₃-releasable Ca between e and d can be explained by leakage of Ca from Sβ at a rate of ~0.17 min⁻¹, which is within the range of leakage rate encountered in the absence of caffeine (cf. Fig. 4).

The effect of ryanodine on the capacity of IP₃-releasable Ca store was studied in 58 samples (Fig. 3 B). In 10 fiber bundles the effect of ryanodine on the caffeine-releasable store was also examined. The value on the abscissa of Fig. 3 B indicates the increase in the Ca concentration within the capillary cuvette due to Ca release from the skinned fiber bundle upon application of the IP₃ assay solution before ryanodine treatment. Ca loading was carried out at pCa 6 for 180 s. The value plotted on the ordinate indicates the same parameter upon application of either IP₃ after the ryanodine treatment (crosses), caffeine before the ryanodine treatment (solid squares), or caffeine after the ryanodine treatment (open squares). The size of the IP₃-releasable store after ryanodine treatment, and of the caffeine-releasable store before and after the ryanodine treatment, when normalized to each fiber bundle by the size of IP₃-releasable store before the ryanodine treatment, are 0.625 ± 0.055, 0.382 ± 0.069, and 0.047 ± 0.020 (mean ± SD, n = 10), respectively. Straight lines having the slope of these mean values are drawn on Fig. 3 B. These results show that the decrease in the capacity of the IP₃-releasable Ca store due to the ryanodine treatment is mostly attributable to the loss in the caffeine-releasable store (Sα). The effect of ryanodine on the capacity of Sα was statistically significant (P < 0.001) and the size of Sα decreased from 38% to ~7.5% of the total (remaining) Ca store due to the ryanodine treatment. The response to caffeine after the ryanodine treatment may include passive Ca leakage from Sβ (note the creep in Fig. 3 A, d), and the size of Sα could have been overestimated. From these results, it can
be safely concluded that the Ca store of ryanodine-treated skinned fiber bundles consists mostly of $S\beta$, which does not have the Ca-induced Ca release mechanism.

The amount of Ca in the store immediately after Ca loading at pCa 6 for 180 s, which can be estimated considering the size of fiber bundle and the rate of Ca leakage as described in my previous paper (Iino, 1989), was $171.2 \pm 32.5 \mu$mol/liter cell water before ryanodine treatment, and was reduced to $108.3 \pm 20.1 \mu$mol/liter cell water after the treatment (mean $\pm$ SD of 58 fiber bundles, $P < 0.001$). Maximum capacity of the Ca store would be 20–30% greater than these values as estimated from the time course of Ca loading (cf. Fig. 3 of Iino, 1989).

**Effect of Ca$^{2+}$ on the Time Course of IP$_3$-induced Ca Release**

Fig. 4 shows the time course of IP$_3$-induced Ca release measured at three different Ca$^{2+}$ concentrations in the absence of both ATP and Mg$^{2+}$ (pCaRMg0 of Table II) in ryanodine-treated skinned fiber bundles of taenia. After Ca loading at pCa 6 for 180 s, 1 $\mu$M IP$_3$ was applied in the virtual absence of Ca$^{2+}$ (pCa > 8, solid triangles), at pCa 6.5 (solid circles), or at pCa 5.5 (solid squares) for the time indicated in the abscissa (test procedure). The Ca$^{2+}$ concentration in the test procedure was strongly buffered with 10 mM EGTA and was changed to the respective value 15 s before the application of IP$_3$. Ca remaining in the store (mainly $S\beta$) after these treatments was released in the following application of 10 $\mu$M IP$_3$ in the presence of 30–40 $\mu$M fura-2, which replaced EGTA as the Ca$^{2+}$ buffer (assay procedure). The amount of Ca released in the assay procedure was estimated from the change in the fluorescence intensity of the dye. The complete time course of Ca release at one or two conditions was obtained from each fiber bundle, and some data were paired between different time-course curves.

The rate of Ca release induced by 1 $\mu$M IP$_3$ was rather slow at pCa > 8, with a half-time of 60 s or more. When IP$_3$ was applied at pCa 6.5, Ca release was significantly enhanced compared with that at pCa > 8 ($P < 0.001$ at 15 and 45 s,
Ca Dependence of IP₃-induced Ca Release

0.05 < P < 0.1 at 120 s, unpaired t test) and the half-time was ~10 s. These results are consistent with the time course of 10 μM IP₃-induced Ca release observed in ryanodine untreated skinned fibers (Iino, 1987). If the Ca²⁺ concentration during IP₃ application was further increased to pCa 5.5, the rate of Ca release declined again and the time course was roughly the same as that obtained in the absence of Ca²⁺ (P > 0.3 at 15, 45, and 120 s, unpaired t test) but was significantly slower than that at pCa 6.5 (P < 0.01 at 15 and 45 s, P < 0.02 at 120 s, four paired samples). As control experiments, the same test procedure was carried out without IP₃ and the results are shown by the open symbols. The IP₃-independent Ca leakage rate was much less dependent on the Ca²⁺ concentration.

Effect of Ca²⁺ on the Amount of Ca Released by a Brief Treatment of IP₃

In order to obtain the profile of the Ca²⁺ dependence of the IP₃-induced Ca release, Ca release induced by 1 μM IP₃ in the absence of both ATP and Mg was examined in the wide range of Ca²⁺ concentrations using pCa₆Mg₀ of Table II (Fig. 5). Complete Ca²⁺ dependence either with or without IP₃ was obtained from each fiber bundle, and the results from eight fiber bundles are compiled in Fig. 5. The duration of IP₃ application was fixed to 15 s so that the amount of Ca released during this period should roughly represent the initial rate of IP₃-induced Ca release (solid circles). The amount of Ca leakage without IP₃ treatment within 15 s was independent of Ca²⁺ concentration as shown by the open symbols. The difference between the open and solid circles represents Ca release induced by IP₃ and has a clear biphasic dependence on the Ca²⁺ concentration during the IP₃ application. The data points with 15-s applications of IP₃ at pCa 7.0 and 6.5 were significantly different from that at pCa > 8 (P < 0.001), and the data points at pCa 5.5 and 5.0 were greater than that at pCa 6.5 (P < 0.01). Thus, the IP₃-induced Ca

![Figure 5. pCa dependence of the IP₃-induced Ca release in ryanodine-treated skinned fiber bundles of taenia. Solid circles show the relative amount of Ca remaining in the Ca store after a 15-s application of 1 μM IP₃ at the Ca²⁺ concentration indicated in the abscissa (n = 5). Both ATP and Mg²⁺ were absent during the IP₃ application. Open circles represent the control experiments with the same protocol but without IP₃ obtained from a different set of fiber bundles (n = 3). Within each set every fiber bundle was used to obtain complete Ca²⁺ dependence so that the data were paired within the same symbols. Vertical lines indicate SEM.](image)
release rate is enhanced with the increase of Ca²⁺ up to 300 nM, while the rate declines with Ca²⁺ concentrations that exceed 300 nM.

To see whether the same range of Ca²⁺ is effective on the IP₃-induced Ca release in the presence of Mg²⁺, experiments were also carried out at 1.5 mM Mg²⁺ and in the absence of ATP (pCaₓR of Table II). Because Mg²⁺ decreased the effectiveness of IP₃, the concentration of IP₃ was increased to 10 μM and the duration of the application was 45 s. Fig. 6 shows the results of such experiments obtained from eight fiber bundles in the same manner as in Fig. 5. The results are in general agreement with those obtained in the absence of Mg²⁺, and data points with IP₃ treatment at pCa 7.0 and 6.5 were significantly smaller than that in the absence of Ca²⁺ (P < 0.01), and IP₃ released less Ca at pCa 5.5 (P < 0.02) and at pCa 5.0 (P < 0.01) than at pCa 6.5. These results clearly indicate that essentially the same Ca²⁺ dependence is present in the absence or presence of 1.5 mM Mg²⁺.

**Figure 6.** pCa dependence of IP₃-induced Ca release in the presence of 1.5 mM Mg²⁺. Solid circles show the relative amount of Ca remaining in the Ca store of ryanodine-treated skinned fiber bundles after a 45-s application of 10 μM IP₃ in the absence of ATP at the Ca²⁺ concentration indicated in the abscissa (n = 4). Open circles represent the control experiments with the same protocol but without IP₃ obtained from a different set of fiber bundles (n = 4). Data points were paired within the same symbols. Vertical lines indicate SEM.

**Dose-Response Relation of IP₃-induced Ca Release at Three Different Ca²⁺ Concentrations**

Dependence of the IP₃-induced Ca release on the ligand concentration was determined in the absence of Ca²⁺, at pCa's 6.5 and 5.5 (pCaₓRMg₀). IP₃ at concentrations of 0–30 μM was applied for 15 s in the absence of both ATP and Mg²⁺, and the amount of Ca remaining in S3 is plotted in Fig. 7. Each fiber bundle was used to obtain a full dose-response relation at one Ca²⁺ concentration, and the data from the fiber bundles used to construct either Fig. 4 or Fig. 5 were not included in Fig. 7. There was no significant difference between the dose-response curve at pCa > 8 and that at pCa 5.5 (P > 0.05 at all the IP₃ concentrations, unpaired t test), and very high concentrations of IP₃ were required to induce Ca release at these extreme Ca²⁺ concentrations. However, IP₃ was considerably more effective at pCa 6.5, and the Ca release was significantly enhanced at or above 1 μM IP₃ compared with those at either pCa > 8 or pCa 5.5 (P < 0.01, unpaired t test).
Rate of IP$_3$-induced Ca Release vs. the Size of Fiber Bundles

Lack of an effective buffering system for IP$_3$ may affect the effectiveness of IP$_3$ because of limited control of diffusion of IP$_3$ within skinned fiber bundles. Care was taken to use preparations of similar size, because comparison between the fiber bundles was necessary, but the cross-sectional area, roughly estimated assuming ellipsoidal cross section, of the fiber bundles used in this study varied from 0.019 to 0.031 mm$^2$. To see whether such variation in the size of fiber bundles should affect the main results, the amount of Ca remaining in the store after 15 s application of 1 $\mu$M IP$_3$ at pCa > 8 (open squares), pCa 6.5 (solid circles), and pCa 5.5 (crosses) in the absence of Mg was plotted against the size of the capacity of the store in each fiber bundle (Fig. 8). All the corresponding results used in Figs. 4, 5, and 7 were pooled and plotted in Fig. 8. The values along the abscissa indicate the change in the Ca$^{2+}$ concentration within the capillary cuvette in response to the application of the assay solution with 10 $\mu$M IP$_3$ after Ca loading (at pCa 6 for 180 s) after the ryanodine treatment; this parameter should be proportional to the fiber size. Correlation coefficients ($r$) between the fiber "size" and IP$_3$-induced Ca release and their probability of being equal to zero were $-0.054$ ($P > 0.5$), $-0.151$ ($P > 0.5$), and $-0.093$ ($P > 0.5$) at pCa > 8, pCa 6.5, and pCa 5.5, respectively. Therefore, there is no clear dependence of the rate of IP$_3$-induced Ca release at least within the range of the fiber bundle size used in this study. Similar results were obtained if the rate of IP$_3$-induced Ca release was plotted against the estimation of the cross-sectional area. Hence, the biphasic dependence of the rate of IP$_3$-induced Ca release on Ca$^{2+}$ does not seem to be affected by the diffusion problem.

The range along the ordinate of the data points shown in Fig. 8 for a 15-s appli-
cation of 1 μM IP₃ at pCa 6.5 obtained from 13 fiber bundles did not overlap with those obtained either at pCa > 8 (n = 13) or at pCa 5.5 (n = 15). The differences are statistically significant (P < 0.001, unpaired t test), and the results from pooled data also support the main results of the present study, i.e., biphasic dependence of IP₃-induced Ca release on Ca²⁺ concentration.

DISCUSSION

The major finding of the present study is the biphasic dependence of the rate of IP₃-induced Ca release on the Ca²⁺ concentration in the range of intracellular Ca²⁺ concentration during the contraction-relaxation cycle of the smooth muscle (Iino, 1981; Yagi et al., 1988). It has been shown that the Ca store of taenia has the Ca-induced Ca release mechanism in addition to the IP₃-induced Ca release, but with different distributions: the Ca-induced Ca release is present in only ~40% of the store while the IP₃-induced Ca release is present in all the store (Fig. 3; Iino, 1987, 1989; Iino et al., 1988). Both the Ca release mechanisms have now been shown to be Ca²⁺ dependent. Similar results have been obtained in portal vein of the guinea pig (unpublished observation).

Compartments of the Ca Store and the Effect of Ryanodine

The notion that the Ca store of smooth muscle consists of two compartments in terms of the distribution of Ca release mechanisms is based on the following observations. (a) Caffeine, a potentiator of the Ca-induced Ca release mechanism (Iino, 1989), is able to release only ~40% of IP₃-releasable Ca (Fig. 3 and Iino, 1987). (b) IP₃ can release a considerable amount of Ca after application of caffeine, although the amount of released Ca is about half of that without caffeine pretreatment, whereas caffeine-releasable Ca is completely depleted after IP₃ application (Fig. 3 A, b and c). (c) Ca-induced Ca release from the total Ca store takes place in two distinct
phases, with an early rapid Ca release whose magnitude and time course are almost identical to the Ca-induced Ca release from the caffeine-releasable store and a much slower Ca leakage (Iino, 1989). (d) After Ca loading in the presence of 50 mM caffeine, caffeine fails to release Ca but IP$_3$ releases ~55% of the amount released after control loading without caffeine (Iino, 1989). a, b and d indicate that only a fraction of the Ca store is sensitive to caffeine and possesses the Ca-induced Ca release mechanism. b suggests that the IP$_3$-induced Ca release mechanism is present not only in the caffeine-insensitive pool but also in the caffeine-sensitive pool. From these results it has been postulated that the smooth muscle Ca store consists of two components, one (Sa, ~40% of the total Ca store in taenia caeci) with both the Ca-induced Ca release and the IP$_3$-induced Ca release mechanisms, and the rest (Sb) with only the IP$_3$-induced Ca release mechanism. The relative amount of Sa and Sb differs in different organs (Iino et al., 1988). An alternative notion may be that Sa has only the Ca-induced Ca release mechanism and the IP$_3$ releases Ca from Sa through modulation of the Ca-induced Ca release channel. However, this seems unlikely because the time course of IP$_3$-induced Ca release from the caffeine-releasable store (Sa) is almost identical to that from the total Ca store even in the absence of Ca$^{2+}$, which is required to activate the Ca-induced Ca release mechanism (Iino, 1987).

Ryanodine locks open Ca-induced Ca release channels of skeletal muscle sarcoplasmic reticulum (Fleischer et al., 1985; Rousseau et al., 1987). The effect of ryanodine on the smooth muscle Ca store (Fig. 3 and Iino et al., 1988) can be best explained by the same effect of the drug on the Ca-induced Ca release channels in the Sa, i.e., production of “permanent holes” in Sa. Ryanodine blocks the Ca release channels at much higher doses (Meissner, 1986). Although such blockade of the Ca-induced Ca release channels by ryanodine may explain the lack of caffeine-induced Ca release after the ryanodine treatment, it fails to explain the reduction of IP$_3$-releasable Ca. Ryanodine had scarcely any effect on the sizes of both caffeine and IP$_3$-releasable Ca store, even when the drug was applied under such conditions that the IP$_3$-induced Ca release mechanism was highly activated (Iino et al., 1988). Thus, the effect of ryanodine is specific to the Ca-induced Ca release channels.

In summary, ryanodine selectively locks open the Ca-induced Ca release channel in Sa and removes the function of this compartment. Sb remains intact after ryanodine treatment and the effect of Ca$^{2+}$ on the IP$_3$-induced Ca release mechanism can be studied independently of the Ca-induced Ca release mechanism.

Mechanism of the Ca Dependence of the IP$_3$-induced Ca Release

Possible mechanisms for the Ca$^{2+}$ dependence of the IP$_3$-induced Ca release may be: (a) direct effect of Ca$^{2+}$ on the IP$_3$-gated Ca release mechanism, (b) Ca$^{2+}$-dependent change in the affinity of the IP$_3$ receptor for the ligand, or (c) Ca$^{2+}$-dependent metabolism of IP$_3$ within the skinned fiber. In the following, the mechanism and potential physiological significance of the Ca$^{2+}$ dependence of the IP$_3$-induced Ca release are discussed.

Metabolism of IP$_3$ within the Skinned Fiber

IP$_3$ phosphatase may be activated by C-kinase (Connoly et al., 1986), and IP$_3$-kinase, which converts IP$_3$ to inositol 1,3,4,5-tetrakisphosphate, is Ca$^{2+}$ dependent (Biden
and Wollheim, 1986). But neither kinase was functioning in the present condition, because ATP had been withdrawn during IP₃ application. The effect of Ca²⁺ on the IP₃ phosphatase has been controversial. The activity of the enzyme from platelet has been reported to be inhibited by Ca²⁺ with a Kᵢ of 70 μM (Connoly et al., 1985). Similar results have been obtained in the enzyme purified from brain (Hansen et al., 1987). On the other hand, IP₃ phosphatase activity from macrophage and coronary artery smooth muscle was activated by Ca²⁺ between 0.1 and 1 μM (Sasaguri et al., 1985; Kukita et al., 1986). Further study is required for the Ca²⁺ dependence of the IP₃ phosphatase. However, as shown by Figs. 5 and 6, almost the same Ca²⁺ dependence was obtained with or without Mg²⁺ which is an absolute requirement of the IP₃ phosphatase activity (Downes et al., 1982). Therefore, Ca²⁺ dependence of the enzyme is unlikely to be responsible for the mechanism of the Ca²⁺ dependence of the IP₃-induced Ca release mechanism in the present study.

**Enhancement of IP₃-induced Ca Release by Ca Below 300 nM**

The rate of the IP₃-induced Ca release is markedly enhanced by Ca²⁺ near 100 nM but below 300 nM. This effect was first described in a previous report (Iino, 1987). Here the same effect is observed in ryanodine-treated skinned fiber bundles in which So or the part of the Ca store that possesses the Ca-induced Ca release mechanism was functionally removed. Therefore, the results of the present study strengthen the argument that the Ca²⁺ dependence of the IP₃-induced Ca release is not the consequence of the simultaneous activation of the Ca-induced Ca release mechanism.

To my knowledge, a Ca²⁺-dependent increase in the affinity of the IP₃ receptor has not been reported. In fact, binding of IP₃ and membrane fraction obtained from rat cerebellum has been shown to be inhibited by submicromolar concentrations of Ca²⁺ (Worley et al., 1987). Therefore, the potentiating effect of Ca²⁺ on the IP₃-induced Ca release is likely to be exerted through a direct influence of Ca²⁺ on the IP₃-gated Ca release mechanism. If that is a Ca channel, IP₃ is expected to produce either increased open probability or greater channel conductance.

It has been reported that IP₃ modulates ryanodine-sensitive Ca channels from frog skeletal muscle incorporated into planar bilayer, and that the potentiation by IP₃ is Ca²⁺ dependent (Suarez-Isla et al., 1989). Although there seems to be some resemblance between the present results and those of Suarez-Isla et al., these two studies refer to different Ca release mechanisms. The Ca channels of Suarez-Isla and colleagues are opened by Ca²⁺ and are claimed to be sensitive to ryanodine. IP₃ is only a modulator for the ryanodine-sensitive channels, and is ineffective in the absence of Ca²⁺. However, the IP₃-induced Ca release mechanism in this study is activated by IP₃ in the absence of Ca²⁺, and is insensitive to ryanodine. Ca²⁺ is a modulator of the IP₃-induced Ca release mechanism, but Ca²⁺ alone cannot activate the Ca release mechanism.

**Inhibitory Effect on IP₃-induced Ca Release of Ca²⁺ Above 300 nM**

The present study demonstrates that Ca²⁺ has an inhibitory effect on the rate of IP₃-induced Ca release above 300 nM. This is in general agreement with the finding of Hirata and his coworkers (Hirata et al., 1984; Suematsu et al., 1984), who
reported that the amount of Ca released by IP₃ becomes smaller with the increase of Ca²⁺ concentration above ~1 μM in macrophage and coronary artery smooth muscle cells. However, they did not find potentiation of the IP₃-induced Ca release by submicromolar concentrations of Ca²⁺. It is not clear whether their preparation had a Ca-induced Ca release mechanism, although it is an important point for the determination of the size of the IP₃-sensitive store. If Ca²⁺ itself released Ca from the store, the amount of IP₃-induced Ca release (difference between the amount of Ca release with and without IP₃) may well appear smaller at high Ca²⁺ concentrations. In this study, complication of the Ca-induced Ca release was removed by pretreating the fiber bundle with ryanodine so that only the store without the Ca-induced Ca release mechanism could be examined.

A similar inhibitory effect of Ca²⁺ on Ca channels has been noted in skeletal muscle Ca-induced Ca release mechanism (Endo, 1985), but the range of Ca²⁺ concentration for such depression is almost two orders of magnitude greater. I doubt that the same mechanism is responsible for the inhibitory effect of Ca²⁺ on the IP₃-induced Ca release channels. "Time-dependent Ca-induced release of Ca" described in cardiac muscle (Fabiato, 1985) shows Ca²⁺- and time-dependent inactivation. However, evidence for the presence of such inactivation in the IP₃-induced Ca release mechanism has not been found, and there was no significant difference between the time course of Ca release at pCa > 8 and at pCa 5.5 (Fig. 4). It has been shown that submicromolar concentrations of Ca²⁺ inhibit binding between IP₃ and membrane fraction prepared from rat brain (Worley et al., 1987), but the IP₃ binding to purified receptor is unaffected by Ca²⁺ (Supattapone et al., 1988). Calmodin, a membrane protein, has been shown to restore Ca²⁺ sensitivity of the IP₃ binding when it is added with the purified receptor (Danoff et al., 1988). We still do not know whether the IP₃ receptor found in the brain is similar to that of peripheral tissues, and there has been no report that the affinity of the peripheral IP₃ receptor is Ca²⁺ dependent. However, it is a distinct possibility that IP₃ binding is also inhibited by Ca²⁺ in smooth muscle IP₃ receptor, because calmodin activity is low but present in peripheral organs (Danoff et al., 1988).

Possible Physiological Significance of the Ca²⁺ Dependence of IP₃-induced Ca Release

Acceleration of the rate of IP₃-induced Ca release by Ca²⁺ below 300 nM is expected to form a positive feedback loop, and the Ca release mechanism may behave as an apparent "Ca-induced Ca release mechanism." In other words, a small increase in Ca²⁺ concentration may result in a Ca release from the store through the IP₃-induced Ca release mechanism even if IP₃ concentration remains unchanged. Since the effective range of Ca²⁺ concentrations is more than an order of magnitude lower than that of the Ca-induced Ca release mechanism, which requires Ca²⁺ above 1 μM (Iino, 1989), and is close to the intracellular Ca²⁺ concentration of relaxed muscle, this mechanism may have physiological significance. It is an interesting possibility that Ca release due to a small change in the IP₃ concentration is amplified by this mechanism, or that the influx of the extracellular Ca²⁺ may influence Ca release from the store through this Ca²⁺ dependence.

The present study makes it clear that the Ca²⁺ concentration is a very important
factor in the determination of dose-response relation of the IP₃-induced Ca release. Meyer et al. (1988) has shown that the rate of IP₃-induced Ca release in the permeabilized rat basophilic leukemia cell has a very steep dependence on the IP₃ concentration. These authors have postulated that binding of at least three IP₃ molecules is necessary for channel opening. Because Ca²⁺ was only weakly buffered with 1.5 μM fura-2 in their experiments, it is possible that the released Ca potentiated the rate of Ca release in the positive feedback manner mentioned above. And at least a part of the apparently cooperative dependence of IP₃-induced Ca release on IP₃ concentration might have been due to the Ca²⁺ dependence of IP₃-induced Ca release, provided that the properties of the IP₃-induced Ca release mechanism are the same between the smooth muscle and the mast cell line. Although I could not obtain a steep dose-response relation of the IP₃-induced Ca release while Ca²⁺ concentration was strongly buffered with 10 mM EGTA (Fig. 7), IP₃ concentration was not buffered in the present study and further study is necessary to obtain a more precise dose-effect curve. There is some uncertainty as to the concentration of IP₃ in the center of the fiber bundles, and the real dose-response relation may very well be shifted toward lower IP₃ concentrations. Notwithstanding this uncertainty, the Ca²⁺ dependence of the IP₃-induced Ca release still holds, because the effect of Ca²⁺ was clearly observed when IP₃ was applied in the same fiber bundle under the same conditions except for changes in the Ca²⁺ concentration.

The inhibitory effect of Ca²⁺ on the IP₃-induced Ca release above 300 nM forms a negative feedback loop, and cuts down Ca release when Ca²⁺ concentration exceeds the limiting value. This can be a useful safety valve and can limit excess Ca release. Williams et al. (1987) have reported that there is a ceiling to Ca²⁺ concentration rise in agonist-induced contractions of single smooth muscle cells. The Ca²⁺-dependent negative feedback mechanism may be an important candidate for the underlying mechanism of the ceiling effect.

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