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

MASAMITSU IINO

From the Department of Pharmacology, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

ABSTRACT Ca^{2+} dependence of the inositol 1,4,5-trisphosphate (IP_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_3-induced Ca release mechanism was, therefore, studied independently of the Ca-induced Ca release. The rate of IP_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_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_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_3 phosphatase activity. Ca^{2+}, therefore, is likely to affect the gating mechanism and/or affinity for the ligand of the IP_3-induced Ca release mechanism. The biphasic effect of Ca^{2+} on the IP_3-induced Ca release is expected to form a positive feedback loop in the IP_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_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_3 to release Ca from the permeabilized smooth muscle cells has been demon-

Address reprint requests to Dr. Masamitsu Iino, Department of Pharmacology, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan.

strated (Suematsu et al., 1984; Somlyo et al., 1985). Production of IP_3 after agonist stimulation has been measured in smooth muscle as in many other cells (for review see Berridge and Irvine, 1984; Abdel-Latif, 1986). Walker et al. (1987) showed that IP_3 rapidly formed in a skinned smooth muscle fiber bundle by photolysis of a photo-labile inactive precursor of IP_3 (caged IP_3) 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 IP_3 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 IP_3-induced Ca release mechanism, Ca^{2+} is of potential importance because Ca^{2+} dependence of the Ca release mechanism is expected to constitute a feedback mechanism in the intracellular Ca mobilization and to regulate the effectiveness of IP_3. However, the results so far remain in apparent contradiction as to the effect of Ca^{2+} on the IP_3-induced Ca release. It was shown that the amount of Ca released by IP_3 decreased at Ca^{2+} concentrations above 1 μM in macrophage and coronary artery smooth muscle cells while no effect was found below 1 μM Ca^{2+} (Hirata et al., 1984; Suematsu et al., 1984). On the other hand, Iino (1987) reported that the rate of IP_3-induced Ca release was enhanced by Ca^{2+} at concentrations of around 100 nM, but in that work Ca^{2+} dependence was studied only below 1 μM in order to avoid the interference of the Ca-induced Ca release mechanism that is activated by Ca^{2+} above 1 μM (Iino, 1989). In the present study a new protocol was used to study the effect of a wide range of Ca^{2+} concentration in the absence of the Ca-induced Ca release. In this protocol, IP_3-induced Ca release is biphasically dependent on Ca^{2+} in the concentration range where the contractile system is controlled; the Ca release mechanism is enhanced by Ca^{2+} below 300 nM, and above this concentration Ca^{2+} 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 IP_3, and the amount of Ca was assayed by a microfluorometry of fura-2 (Grynkiewicz 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 Ca^{2+}

TABLE I

Composition of the Solutions

Name	EGTA or EDTA*	CaEGTA or CaEDTA*	$MgMs_2$	KMs	ATP	AMP	Mg^{2+}
G1	1	0	5.54	108.6	4.76	0	1.5
pCa 6-loading	0.298	0.702	5.51	108.6	4.76	0	1.5
G0R	0	0	1.5	137.6	0	0	1.5
G0RMg0	0	0	0	142.1	0	0	0
Assay	0	0	0	84.1	0	25	0
Ryanodine	0.171	0.829	0	84.1	0	22.5	0
G1R	1	0	1.54	134.5	0	0	1.5
G10R	10	0	1.90	107.1	0	0	1.5
CaG10R	0	10	1.50	107.5	0	0	1.5
G1RMg0	1	0	0	139.0	0	0	0
G10RMg0	10	0	0	111.8	0	0	0
CaG10RMg0	0	10	0	112.0	0	0	0
D1R	1*	0	0	136.5	0	0	0
D10R	10*	0	0	86.9	0	0	0
CaD10R	0	10*	0	112.0	0	0	0

Concentrations are in millimolar. All solutions contained 20 mM PIPES (piperazine-*N*-*N'*-bis[2-ethanesulfonic acid]) and 20 mM NaN_3. Assay contained 10 μM IP_3 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, $MgATP^{2-}$ concentration was calculated to be 4.0 mM. Total ionic strength was 200 mM in all the solutions. EGTA, ethyleneglycol-bis[β-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, $CaCO_3$ and equimolar EGTA or EDTA were mixed and neutralized with KOH. IP_3 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

Condition	pCa (*x*)								Mix
	>8	7.5	7.0	6.5	6.0	5.5	5.0	4.5	
pCaxR	0.00	0.69	1.89	4.24	7.00	8.81	9.60	9.91	G10R and CaG10R
pCaxRMg0	0.00	0.71	1.96	4.34	7.09	8.85	9.61	9.90	G10RMg0 and GaG10RMg0
pCaxDR	0.00	3.56	6.36	8.47	9.46	—	—	—	D10R and CaD10R

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 IP_3 and 25 mM AMP but no EGTA ("Assay"). AMP was added to the assay solution because adenine nucleotides enhance IP_3-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., IP_3 was replaced with 50 mM caffeine (see Fig. 3) or AMP was omitted (see Fig. 1 *A*).

In order to study Ca^{2+} dependence of the IP_3-induced Ca release mechanism, a test solution that contained IP_3 and the desired concentration of Ca^{2+} 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 IP_3 was absent and the EGTA concentration was 1 mM without added Ca (e.g., G1R, G1RMg0, or D1R). The Ca^{2+} concentration was then changed to the desired value, using 10 mM EGTA or EDTA as a Ca^{2+} buffer (Table II), and 15 s later IP_3 was added. After a 0–360-s application of 0–30 μM IP_3, both Ca^{2+} and IP_3 were removed by washing the preparation for 60 s with a solution that contained 10 mM EGTA, 1.5 mM Mg^{2+}, 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 Ca^{2+}-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.

Mg^{2+} Concentration Measurements

In order to measure Mg^{2+} concentration in the solutions containing 2,3-bisphosphoglycerate, differential absorbance spectra of a metallochromic indicator antipyrylazo III (Scarpa et al.,

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. 1 *A* 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. 1 *A*). 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. 1 *B*, 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 stated). 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 concentra-

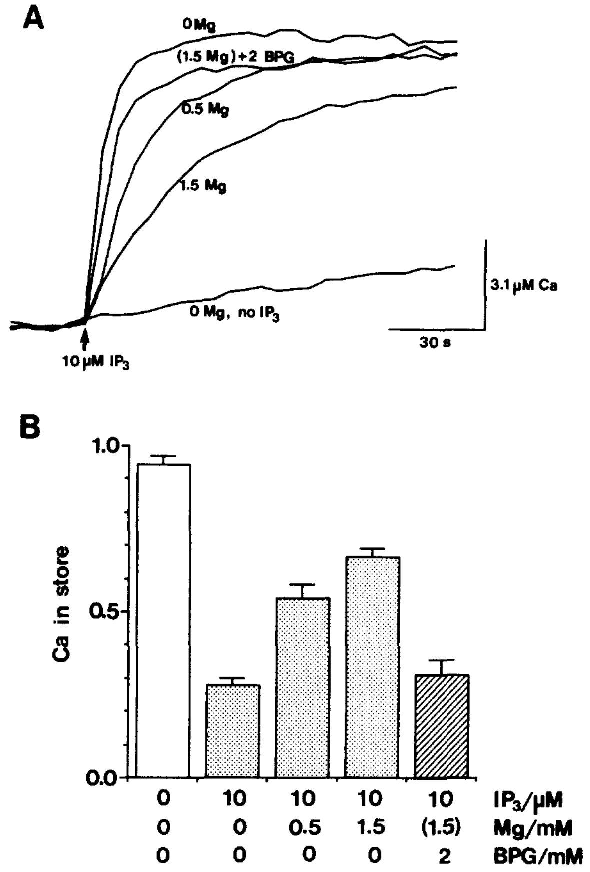

FIGURE 1. (*A*) Time course of the fluorescence intensity change of fura-2 due to IP_3-induced Ca release from a skinned fiber bundle of taenia in the absence of ATP. Ratio of the fluorescence intensity at 340 nm excitation to that at 360 nm is shown. IP_3 was applied at the arrow and the concentration 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_{total}. 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 Mg_{total} 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.

tion 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 Mg_{total} 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 (buffered with 10 mM EDTA, pCa7DR of Table II). IP_3 was applied in the absence of ATP. 2,3-Bisphosphoglycerate clearly enhanced Ca release in the presence of Mg^{2+} ($P < 0.001$) but had no such effect in the absence of Mg^{2+} and, therefore, the effect of 2,3-bisphosphoglycerate in the presence of Mg^{2+} was likely to be due to an inhibition of IP_3 phosphatase, but not to a direct effect on the IP_3-induced Ca release. In the absence of Mg^{2+}, 2,3-bisphosphoglycerate showed a tendency to inhibit the IP_3-induced Ca release ($0.02 < P < 0.05$). This could be a consequence of an inhibition of IP_3 binding to the receptor, because such an effect of 2,3-bisphosphoglycerate has been noted in rat brain IP_3 receptor (Worley et al., 1987).

The above results make it likely that at least the main part of the inhibitory effect of Mg^{2+} on the IP_3-induced Ca release is exerted through activation of IP_3 phosphatase, because the effect of Mg^{2+} is almost completely reversed with 2,3-bisphosphoglycerate. However, the present study does not rule out the possibility that there is a direct effect of Mg^{2+} on the IP_3-induced Ca release mechanism.

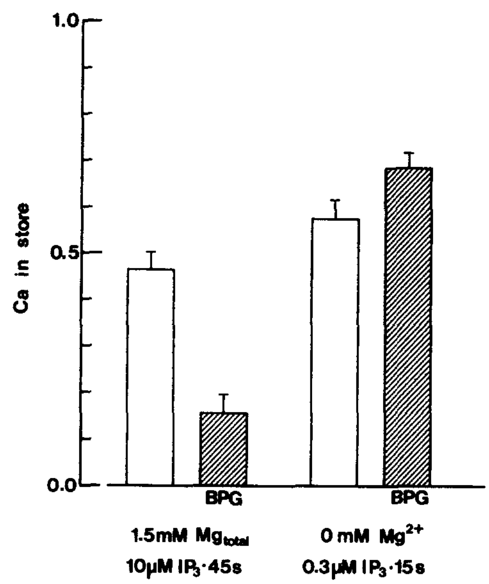

FIGURE 2. Relative amount of remaining Ca in the store after a brief application of IP_3 in the absence of ATP at pCa 7 with (*hatched columns*) or without (*open columns*) 2 mM 2,3-bisphosphoglycerate (BPG) after constant Ca loading. (*Left-hand side two columns*) 10 μM IP_3 application for 45 s in the presence of 1.5 mM Mg_{total}. (*Right-hand side two columns*) 0.3 μM IP_3 application for 15 s in the absence of Mg^{2+} (buffered with 10 mM EDTA). Average of data from four fiber bundles that were tested in all the conditions; the vertical lines indicate SEM.

In the following, Mg^{2+} was excluded from the test solutions containing IP_3 so that the IP_3-induced Ca release could be studied in the greatly reduced IP_3 phosphatase activity. But some experiments were carried out with Mg^{2+} in the test solutions to see if the same phenomenon is observable in the presence of physiological concentrations of Mg^{2+}.

Functional Removal of Sα by Ryanodine Treatment

In a previous report (Iino, 1987) it was shown that the rate of the IP_3-induced Ca release was potentiated by Ca^{2+} at concentrations of ~100 nM in the absence of Mg^{2+}. The highest Ca^{2+} concentration examined in the previous study was 300 nM. Higher concentrations of Ca^{2+} were not tested, because Ca-induced Ca release mechanism is also present in the smooth muscle Ca store and is activated by Ca^{2+} above 1 μM (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 ($S\alpha$) is both caffeine- and IP_3-releasable, and the other ($S\beta$) is releasable

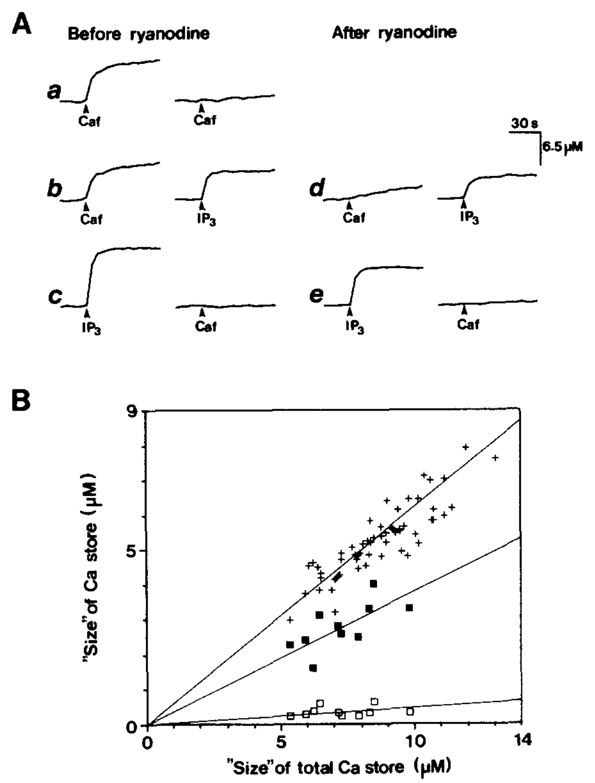

FIGURE 3. (*A*) Ca release in the absence of ATP from a skinned fiber bundle before (*a–c*) and after (*d* and *e*) ryanodine treatment. In each panel Ca loading was carried out at pCa 6 for 180 s. The left trace was obtained in the first Ca assay after the Ca loading, and Ca assay was repeated as in 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-μM IP_3 assay. In *c* and *e*, a caffeine assay followed an IP_3 assay. 30 μM 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 μM 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 μM Ca. Fiber bundle widths: 150 × 200 μm. (*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.

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 $S\alpha$. Results shown in Fig. 3 support this view. Panels *a* to *e* of Fig. 3 *A* were obtained from the same fiber bundle after constant Ca loading at pCa 6 for 180 s. In each panel two Ca assays either with 10 μM IP_3 (Assay) or with 50 mM caffeine (Assay without IP_3 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*, IP_3 assay could release a considerable amount of Ca when caffeine-releasable stores had been depleted by the preceding caffeine assay. If IP_3 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 IP_3-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, $S\alpha$ loses its capacity to hold Ca because of the "permanent holes" in this compartment of the store, but $S\beta$ remains intact (Iino et al., 1988). Fig. 3 *A*, *d* 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, while IP_3 could. However, IP_3-releasable Ca was decreased by ~40% after the ryanodine treatment (cf. *c* and *e*). The difference in the size of IP_3-releasable Ca between *e* and *d* can be explained by leakage of Ca from $S\beta$ 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 IP_3-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 IP_3 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 IP_3 after the ryanodine treatment (*crosses*), caffeine before the ryanodine treatment (*solid squares*), or caffeine after the ryanodine treatment (*open squares*). The size of the IP_3-releasable store after ryanodine treatment, and of the caffeine-releasable store before and after the ryanodine treatment, when normalized in each fiber bundle by the size of IP_3-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 IP_3-releasable Ca store due to the ryanodine treatment is mostly attributable to the loss in the caffeine-releasable store ($S\alpha$). The effect of ryanodine on the capacity of $S\alpha$ was statistically significant ($P < 0.001$) and the size of $S\alpha$ 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 $S\beta$ (note the creep in Fig. 3 *A*, *d*), and the size of $S\alpha$ 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 ± 32.5 μmol/liter cell water before ryanodine treatment, and was reduced to 103.3 ± 20.1 μmol/liter cell water after the treatment (mean ± 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+} (pCa*x*RMg0 of Table II) in ryanodine-treated skinned fiber bundles of taenia. After Ca loading at pCa 6 for

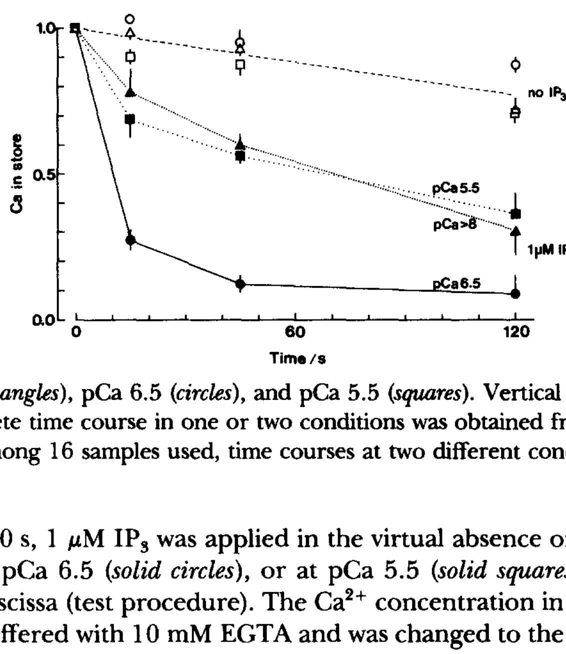

FIGURE 4. Time course of IP_3-induced Ca release in ryanodine-treated skinned fiber bundles of taenia. Relative amount of Ca remaining in the store after the application of a test solution containing either 1 μM IP_3 (*solid symbols*) or no IP_3 (*open symbols*) in the absence of both ATP and Mg^{2+} for the time indicated in the abscissa is shown. Ca^{2+} concentration of the test solution was pCa > 8 (*triangles*), pCa 6.5 (*circles*), and pCa 5.5 (*squares*). Vertical lines indicate SEM (n = 4–5). Complete time course in one or two conditions was obtained from each sample. In 9 fiber bundles among 16 samples used, time courses at two different conditions were studied.

180 s, 1 μM 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 μM IP_3 in the presence of 30–40 μM 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 μM 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,

$0.05 < P < 0.1$ at 120 s, unpaired t test) and the half-time was ~10 s. These results are consistent with the time course of 10 μM IP_3-induced Ca release observed in ryanodine untreated skinned fibers (Iino, 1987). If the Ca^{2+} concentration during IP_3 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 Ca^{2+} ($P > 0.3$ 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 IP_3 and the results are shown by the open symbols. The IP_3-independent Ca leakage rate was much less dependent on the Ca^{2+} concentration.

Effect of Ca^{2+} on the Amount of Ca Released by a Brief Treatment of IP_3

In order to obtain the profile of the Ca^{2+} dependence of the IP_3-induced Ca release, Ca release induced by 1 μM IP_3 in the absence of both ATP and Mg was

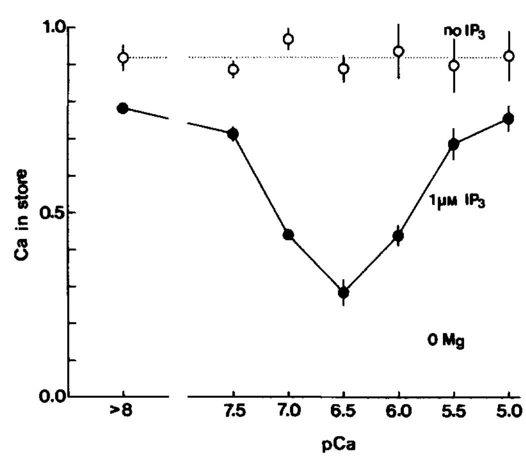

FIGURE 5. pCa dependence of the IP_3-induced Ca release in ryanodine-treated skinned fiber bundles of taenia. Solid circles show the relative amount of Ca remaining in the Ca store after a 15-s application of 1 μM IP_3 at the Ca^{2+} concentration indicated in the abscissa ($n = 5$). Both ATP and Mg^{2+} were absent during the IP_3 application. Open circles represent the control experiments with the same protocol but without IP_3 obtained from a different set of fiber bundles ($n = 3$). Within each set every fiber bundle was used to obtain complete Ca^{2+} dependence so that the data were paired within the same symbols. Vertical lines indicate SEM.

examined in the wide range of Ca^{2+} concentrations using pCaxRMg0 of Table II (Fig. 5). Complete Ca^{2+} dependence either with or without IP_3 was obtained from each fiber bundle, and the results from eight fiber bundles are compiled in Fig. 5. The duration of IP_3 application was fixed to 15 s so that the amount of Ca released during this period should roughly represent the initial rate of IP_3-induced Ca release (*solid circles*). The amount of Ca leakage without IP_3 treatment within 15 s was independent of Ca^{2+} concentration as shown by the open symbols. The difference between the open and solid circles represents Ca release induced by IP_3 and has a clear biphasic dependence on the Ca^{2+} concentration during the IP_3 application. The data points with 15-s applications of IP_3 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 IP_3-induced Ca release rate is enhanced with the increase of Ca^{2+} up to 300 nM, while the rate declines with Ca^{2+} concentrations that exceed 300 nM.

To see whether the same range of Ca^{2+} is effective on the IP_3-induced Ca release in the presence of Mg^{2+}, experiments were also carried out at 1.5 mM Mg^{2+} and in the absence of ATP (pCa*x*R of Table II). Because Mg^{2+} decreased the effectiveness of IP_3, the concentration of IP_3 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 Mg^{2+}, and data points with IP_3 treatment at pCa 7.0 and 6.5 were significantly smaller than that in the absence of Ca^{2+} ($P < 0.01$), and IP_3 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 Ca^{2+} dependence is present in the absence or presence of 1.5 mM Mg^{2+}.

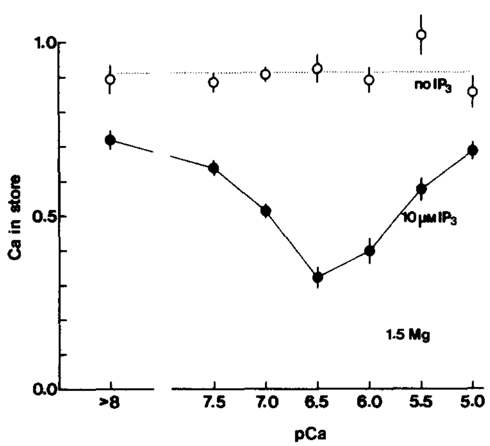

FIGURE 6. pCa dependence of IP_3-induced Ca release in the presence of 1.5 mM Mg^{2+}. Solid circles show the relative amount of Ca remaining in the Ca store of ryanodine-treated skinned fiber bundles after a 45-s application of 10 μM IP_3 in the absence of ATP at the Ca^{2+} concentration indicated in the abscissa ($n = 4$). Open circles represent the control experiments with the same protocol but without IP_3 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 IP_3-induced Ca Release at Three Different Ca^{2+} Concentrations

Dependence of the IP_3-induced Ca release on the ligand concentration was determined in the absence of Ca^{2+}, at pCa's 6.5 and 5.5 (pCa*x*RMg0). IP_3 at concentrations of 0–30 μM was applied for 15 s in the absence of both ATP and Mg^{2+}, and the amount of Ca remaining in $S\beta$ is plotted in Fig. 7. Each fiber bundle was used to obtain a full dose-response relation at one Ca^{2+} 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 IP_3 concentrations, unpaired *t* test), and very high concentrations of IP_3 were required to induce Ca release at these extreme Ca^{2+} concentrations. However, IP_3 was considerably more effective at pCa 6.5, and the Ca release was significantly enhanced at or above 1 μM IP_3 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 μM 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+}

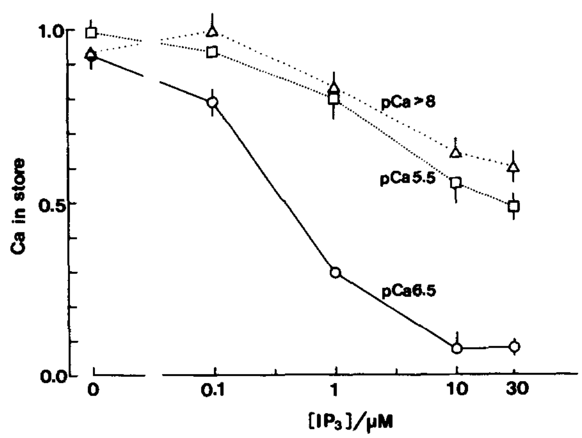

FIGURE 7. Dose-response relation of the IP_3-induced Ca release in ryanodine-treated skinned fibere bundles of taenia. Data points represent the relative amount of Ca remaining in the store after a 15-s application of IP_3 in the absence of both ATP and Mg^{2+} at three different Ca^{2+} concentrations, pCa > 8 (*triangles*, $n = 4$), pCa 6.5 (*circles*, $n = 4$), and pCa 5.5 (*squares*, $n = 5$). Three sets of fiber bundles were used to obtain results in the different Ca^{2+} concentrations, and within each set a complete dose-response relation was studied in every fiber bundle. Vertical lines indicate SEM.

concentration within the capillary cuvette in response to the application of the assay solution with 10 μM 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 application of 1 μM IP_3 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 IP_3-induced Ca release on Ca^{2+} concentration.

DISCUSSION

The major finding of the present study is the biphasic dependence of the rate of IP_3-induced Ca release on the Ca^{2+} concentration in the range of intracellular Ca^{2+} 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 IP_3-induced Ca release, but with different distributions: the Ca-induced Ca release is present in only ~40% of the store while the IP_3-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 Ca^{2+} dependent. Similar results have been obtained in portal vein of the guinea pig (unpublished observation).

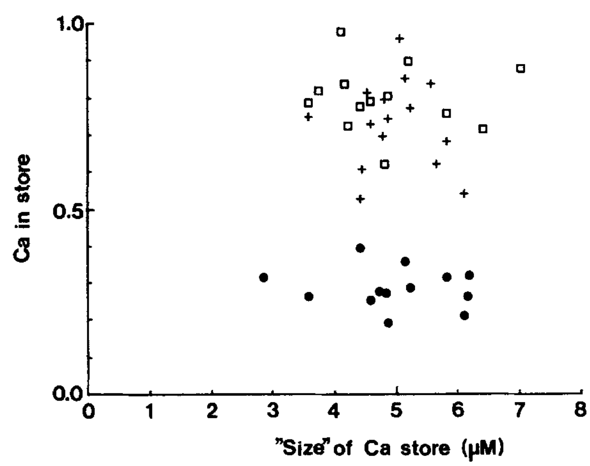

FIGURE 8. Relative amount of Ca remaining in the store after a 15-s application of 1 μM IP_3 at pCa > 8 (*open squares*, $n = 13$), pCa 6.5 (*solid circles*, $n = 13$), and pCa 5.5 (*crosses*, $n = 15$) in the absence of both ATP and Mg following constant Ca loading plotted against the "size" of IP_3-releasable Ca store after the ryanodine treatment. Increase in the Ca concentration of the assay solution in the capillary cuvette due to Ca release upon application of 10 μM IP_3 was plotted along the abscissa.

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 IP_3-releasable Ca (Fig. 3 and Iino, 1987). (*b*) IP_3 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 IP_3 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 ($S\alpha$, ~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 ($S\beta$) with only the IP_3-induced Ca release mechanism. The relative amount of $S\alpha$ and $S\beta$ differs in different organs (Iino et al., 1988). An alternative notion may be that $S\alpha$ has only the Ca-induced Ca release mechanism and the IP_3 releases Ca from $S\alpha$ 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 ($S\alpha$) 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 $S\alpha$, i.e., production of "permanent holes" in $S\alpha$. 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 $S\alpha$ and removes the function of this compartment. $S\beta$ 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 IP_3 application. The effect of Ca^{2+} on the IP_3 phosphatase has been controversial. The activity of the enzyme from platelet has been reported to be inhibited by Ca^{2+} with a K_i 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, IP_3 phosphatase activity from macrophage and coronary artery smooth muscle was activated by Ca^{2+} between 0.1 and 1 μM (Sasaguri et al., 1985; Kukita et al., 1986). Further study is required for the Ca^{2+} dependence of the IP_3 phosphatase. However, as shown by Figs. 5 and 6, almost the same Ca^{2+} dependence was obtained with or without Mg^{2+} which is an absolute requirement of the IP_3 phosphatase activity (Downes et al., 1982). Therefore, Ca^{2+} dependence of the enzyme is unlikely to be responsible for the mechanism of the Ca^{2+} dependence of the IP_3-induced Ca release mechanism in the present study.

Enhancement of IP_3-induced Ca Release by Ca Below 300 nM

The rate of the IP_3-induced Ca release is markedly enhanced by Ca^{2+} 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 $S\alpha$ 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 Ca^{2+} dependence of the IP_3-induced Ca release is not the consequence of the simultaneous activation of the Ca-induced Ca release mechanism.

To my knowledge, a Ca^{2+}-dependent increase in the affinity of the IP_3 receptor has not been reported. In fact, binding of IP_3 and membrane fraction obtained from rat cerebellum has been shown to be inhibited by submicromolar concentrations of Ca^{2+} (Worley et al., 1987). Therefore, the potentiating effect of Ca^{2+} on the IP_3-induced Ca release is likely to be exerted through a direct influence of Ca^{2+} on the IP_3-gated Ca release mechanism. If that is a Ca channel, IP_3 is expected to produce either increased open probability or greater channel conductance.

It has been reported that IP_3 modulates ryanodine-sensitive Ca channels from frog skeletal muscle incorporated into planar bilayer, and that the potentiation by IP_3 is Ca^{2+} 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 Ca^{2+} and are claimed to be sensitive to ryanodine. IP_3 is only a modulator for the ryanodine-sensitive channels, and is ineffective in the absence of Ca^{2+}. However, the IP_3-induced Ca release mechanism in this study is activated by IP_3 in the absence of Ca^{2+}, and is insensitive to ryanodine. Ca^{2+} is a modulator of the IP_3-induced Ca release mechanism, but Ca^{2+} alone cannot activate the Ca release mechanism.

Inhibitory Effect on IP_3-induced Ca Release of Ca^{2+} Above 300 nM

The present study demonstrates that Ca^{2+} has an inhibitory effect on the rate of IP_3-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 IP_3 becomes smaller with the increase of Ca^{2+} concentration above ~ 1 μM in macrophage and coronary artery smooth muscle cells. However, they did not find potentiation of the IP_3-induced Ca release by submicromolar concentrations of Ca^{2+}. 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 IP_3-sensitive store. If Ca^{2+} itself released Ca from the store, the amount of IP_3-induced Ca release (difference between the amount of Ca release with and without IP_3) may well appear smaller at high Ca^{2+} 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 Ca^{2+} on Ca channels has been noted in skeletal muscle Ca-induced Ca release mechanism (Endo, 1985), but the range of Ca^{2+} concentration for such depression is almost two orders of magnitude greater. I doubt that the same mechanism is responsible for the inhibitory effect of Ca^{2+} on the IP_3-induced Ca release channels. "Time-dependent Ca-induced release of Ca" described in cardiac muscle (Fabiato, 1985) shows Ca^{2+}- and time-dependent inactivation. However, evidence for the presence of such inactivation in the IP_3-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 Ca^{2+} inhibit binding between IP_3 and membrane fraction prepared from rat brain (Worley et al., 1987), but the IP_3 binding to purified receptor is unaffected by Ca^{2+} (Supattapone et al., 1988). Calmedin, a membrane protein, has been shown to restore Ca^{2+} sensitivity of the IP_3 binding when it is added with the purified receptor (Danoff et al., 1988). We still do not know whether the IP_3 receptor found in the brain is similar to that of peripheral tissues, and there has been no report that the affinity of the peripheral IP_3 receptor is Ca^{2+} dependent. However, it is a distinct possibility that IP_3 binding is also inhibited by Ca^{2+} in smooth muscle IP_3 receptor, because calmedin activity is low but present in peripheral organs (Danoff et al., 1988).

Possible Physiological Significance of the Ca^{2+} Dependence of IP_3-induced Ca Release

Acceleration of the rate of IP_3-induced Ca release by Ca^{2+} 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 Ca^{2+} concentration may result in a Ca release from the store through the IP_3-induced Ca release mechanism even if IP_3 concentration remains unchanged. Since the effective range of Ca^{2+} concentrations is more than an order of magnitude lower than that of the Ca-induced Ca release mechanism, which requires Ca^{2+} above 1 μM (Iino, 1989), and is close to the intracellular Ca^{2+} 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 IP_3 concentration is amplified by this mechanism, or that the influx of the extracellular Ca^{2+} may influence Ca release from the store through this Ca^{2+} dependence.

The present study makes it clear that the Ca^{2+} concentration is a very important factor in the determination of dose-response relation of the IP_3-induced Ca release. Meyer et al. (1988) has shown that the rate of IP_3-induced Ca release in the permeabilized rat basophilic leukemia cell has a very steep dependence on the IP_3 concentration. These authors have postulated that binding of at least three IP_3 molecules is necessary for channel opening. Because Ca^{2+} 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 IP_3-induced Ca release on IP_3 concentration might have been due to the Ca^{2+} dependence of IP_3-induced Ca release, provided that the properties of the IP_3-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 IP_3-induced Ca release while Ca^{2+} concentration was strongly buffered with 10 mM EGTA (Fig. 7), IP_3 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 IP_3 in the center of the fiber bundles, and the real dose-response relation may very well be shifted toward lower IP_3 concentrations. Notwithstanding this uncertainty, the Ca^{2+} dependence of the IP_3-induced Ca release still holds, because the effect of Ca^{2+} was clearly observed when IP_3 was applied in the same fiber bundle under the same conditions except for changes in the Ca^{2+} concentration.

The inhibitory effect of Ca^{2+} on the IP_3-induced Ca release above 300 nM forms a negative feedback loop, and cuts down Ca release when Ca^{2+} 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 Ca^{2+} concentration rise in agonist-induced contractions of single smooth muscle cells. The Ca^{2+}-dependent negative feedback mechanism may be an important candidate for the underlying mechanism of the ceiling effect.

The author thanks Prof. M. Endo for discussion and encouragement.

Supported by grants from the Ministry of Education, Science and Culture, Japan and by the Shimabara Science Promotion Foundation.

Original version received 16 May 1989 and accepted version received 23 October 1989.

REFERENCES

Abdel-Latif, A. A. 1986. Calcium-mobilizing receptors, polyphosphoinositides, and the generation of second messengers. *Pharmacological Reviews.* 38:227–272.

Berridge, M. J., and R. F. Irvine. 1984. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature.* 312:315–321.

Biden, T. J., and C. B. Wollheim. 1986. Ca^{2+} regulates the inositol tris/tetrakisphosphate pathway in intact and broken preparation of insulin-secreting RINm5F cell. *Journal of Biological Chemistry.* 261:11931–11934.

Connoly, T. M., T. E. Bross, and P. W. Majerus. 1985. Isolation of a phosphomonoesterase from human platelets that specifically hydrolyzes the 5-phosphate of inositol 1,4,5-trisphosphate. *Journal of Biological Chemistry.* 260:7868–7874.

Connoly, T. M., W. J. Lawing, Jr., and P. W. Majerus. 1986. Protein kinase C phosphorylates human platelet inositol trisphosphate 5'-phosphomonoesterase increasing the phosphatase activity. *Cell.* 46:951–958.

Danoff, S. K., S. Supattapone, and S. H. Snyder. 1988. Characterization of a membrane protein from brain mediating the inhibition of inositol 1,4,5-trisphosphate receptor binding by calcium. *Biochemical Journal.* 254:701–705.

Downes, C. P., M. C. Mussat, and R. H. Michell. 1982. The inositol trisphosphate phosphomonoesterase of the human erythrocyte membrane. *Biochemical Journal.* 203:169–177.

Endo, M. 1985. Calcium release from sarcoplasmic reticulum. *Current Topics in Membranes and Transport.* 25:181–230.

Fabiato, M. 1985. Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *Journal of General Physiology.* 85:247–289.

Fleischer, S., E. M. Ogunbunmi, M. C. Dixon, and E. A. M. Fleer. 1985. Localization of Ca^{2+} release channels with ryanodine in junctional terminal cisternae of sarcoplasmic reticulum of fast skeletal muscle. *Proceedings of the National Academy of Sciences.* 82:7256–7259.

Grynkiewicz, P., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry.* 260:3440–3450.

Hansen, C. A., R. A. Johanson, M. T. Williamson, and J. R. Williamson. 1987. Purification and characterization of two types of soluble inositol phosphate 5-phosphomonoesterase from rat brain. *Journal of Biological Chemistry.* 262:17319–17326.

Hirata, M., E. Suematsu, T. Hashimoto, T. Hamachi, and T. Koga. 1984. Release of Ca^{2+} from a non-mitochondrial store site in peritoneal macrophages treated with saponin by inositol 1,4,5-trisphosphate. *Biochemical Journal.* 223:229–236.

Iino, M. 1981. Tension responses of chemically skinned fibre bundles of the guinea-pig taenia caeci under varied ionic environments. *Journal of Physiology.* 142:47–52.

Iino, M. 1987. Calcium dependent inositol trisphosphate-induced calcium release in the guinea-pig taenia caeci. *Biochemical and Biophysical Research Communications.* 142:47–52.

Iino, M. 1989. Calcium-induced calcium release mechanism in guinea pig taenia caeci. *Journal of General Physiology.* 94:363–383.

Iino, M., and M. Endo. 1989. Biphasic effect of calcium on the inositol trisphosphate-induced calcium release in guinea-pig smooth muscle. *Japanese Journal of Pharmacology.* 49(Suppl.):96P. (Abstr.)

Iino, M., T. Kobayashi, and M. Endo. 1988. Use of ryanodine for functional removal of the calcium store in smooth muscle cells of the guinea-pig. *Biochemical and Biophysical Research Communications.* 152:417–422.

Kukita, M., M. Hirata, and T. Koga. 1986. Requirement of Ca^{2+} for the production and degradation of inositol 1,4,5-trisphosphate in macrophages. *Biochimica et Biophysica Acta.* 885:121–128.

Martell, A. E., and R. E. Smith. 1974–1982. Critical stability constants. Vols. 1–5. Plenum Publishing Corp., New York.

Meissner, G. 1986. Ryanodine activation and inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. *Journal of Biological Chemistry.* 261:6300–6306.

Meyer, T., D. Holowka, and L. Streyer. 1988. Highly cooperative opening of calcium channels by inositol 1,4,5-trisphosphate. *Science.* 240:653–656.

Rousseau, E., J. S. Smith, and G. Meissner. 1987. Ryanodine modifies conductance and gating behavior of single Ca^{2+} release channel. *American Journal of Physiology.* 253:C364–C368.

Sasaguri, T., M. Hirata, and H. Kuriyama. 1985. Dependence on Ca^{2+} of the activities of phosphatidylinositol 4,5-bisphosphate phosphodiesterase and inositol 1,4,5-trisphosphate phosphatase in smooth muscle of porcine coronary artery. *Biochemical Journal.* 231:497–503.

Scarpa, A., F. J. Brinley, and G. Dubyak. 1978. Antipyrylazo III, a "middle range" Ca^{2+} metallochromic indicator. *Biochemistry.* 17:1378–1386.

Somlyo, A. V., M. Bond, A. P. Somlyo, and A. Scarpa. 1985. Inositol trisphosphate-induced calcium release and contraction in vascular smooth muscle. *Proceedings of the National Academy of Sciences.* 82:5231–5235.

Suarez-Isla, B. A., R. Bull, and J. J. Marengo. 1989. Activation of ryanodine-sensitive calcium channels from frog sarcoplasmic reticulum (SR) by inositol (1,4,5)-trisphosphate (IP_3) is strongly calcium dependent. *Biophysical Journal.* 55:487*a.* (Abstr.)

Suematsu, E., M. Hirata, T. Hashimoto, and H. Kuriyama. 1984. Inositol 1,4,5-trisphosphate releases Ca^{2+} from intracellular store sites in skinned single cells of porcine coronary artery. *Biochemical and Biophysical Research Communications.* 120:481–485.

Supattapone, S., P. F. Worley, J. M. Baraban, and S. H. Snyder. 1988. Solubilization, purification, and characterization of an inositol trisphosphate receptor. *Journal of Biological Chemistry.* 263:1530–1534.

Walker, J. W., A. V. Somlyo, Y. E. Goldman, A. P. Somlyo, and D. R. Trentham. 1987. Kinetics of smooth muscle and skeletal muscle activation by laser pulse photolysis of caged inositol-1,4,5-trisphosphate. *Nature.* 327:249–252.

Williams, D. A., P. L. Becker, and F. S. Fay. 1987. Regional changes in calcium underlying contraction of single smooth muscle cells. *Science.* 235:1644–1648.

Worley, P. F., J. M. Baraban, S. Supattapone, V. Wilson, and S. H. Snyder. 1987. Characterization of inositol trisphosphate receptor binding in brain. Regulation by pH and calcium. *Journal of Biological Chemistry.* 262:12132-12136.

Yagi, S., P. L. Becker, and F. S. Fay. 1988. Relationship between force and Ca^{2+} concentration in smooth muscle as revealed by measurements on single cells. *Proceedings of the National Academy of Sciences.* 85:4109–4113.