

# Effects of Adenine Nucleotides on Inositol 1,4,5-Trisphosphate-induced Calcium Release in Vascular Smooth Muscle Cells

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**ABSTRACT** Effects of adenine nucleotides on the inositol 1,4,5-trisphosphate ( $IP_3$ )-induced Ca release (IICR) mechanism were studied in smooth muscle cells of the guinea pig portal vein. A microfluorometry method of fura-2 was used to measure Ca release from saponin-skinned thin muscle strips (width  $\sim 200$   $\mu\text{m}$ , thickness 50–70  $\mu\text{m}$ , length 2–3 mm). About 80% of ionomycin-releasable Ca store was sensitive to  $IP_3$ , of which  $\sim 20\%$  was also sensitive to caffeine. The rate of Ca release by 0.1  $\mu\text{M}$   $IP_3$  depended biphasically on ATP concentration in the absence of  $Mg^{2+}$ ; it was dose-dependently enhanced by ATP up to  $\sim 0.5$  mM, and above this concentration the enhancement became smaller. However, the decline of enhancement of the IICR at the higher ATP concentrations was absent at  $IP_3$  concentrations  $> 1$   $\mu\text{M}$ . This suggests competitive antagonism between  $IP_3$  and ATP. Clear effects of ATP were observed not only at pCa 7 or 8, where the Ca-induced Ca release was not activated, but after a ryanodine treatment to excise the functional compartment that possessed the Ca-induced Ca release mechanism. ATP had no effect on the rate of Ca leakage in the absence of  $IP_3$  even at pCa 5.5 after the ryanodine treatment. Therefore, ATP has direct biphasic effects on the  $IP_3$ -induced Ca release mechanism. The Ca release induced by 0.1  $\mu\text{M}$   $IP_3$  at pCa 7 was potentiated not only by ATP, but by 0.5 mM ADP, AMP, or  $\beta,\gamma$ -methyleneadenosine 5'-triphosphate. 0.5 mM GTP had only a little effect on the  $IP_3$ -induced Ca release. These results extend the functional similarities between Ca- and  $IP_3$ -induced Ca release mechanisms in that adenine nucleotides enhance Ca release. Millimolar concentration of ATP, which is present physiologically, will shift the dose-response relation of  $IP_3$  toward the higher  $IP_3$  concentration and enhance the maximal effect of  $IP_3$ . Thus, ATP is expected to assist the Ca release by higher concentrations of  $IP_3$  while having less effect on the Ca release by low levels of  $IP_3$ . These effects of ATP may be important in the switching of Ca release from the intracellular Ca store by  $IP_3$ .

## INTRODUCTION

Inositol 1,4,5-trisphosphate ( $IP_3$ ) is formed in receptor-coupled signal transduction and is capable of releasing Ca from intracellular stores. This receptor- $IP_3$  pathway

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has been proposed to be an important mechanism for Ca mobilization in agonist-induced responses in many types of cells, including smooth muscle cells (Berridge and Irvine, 1984). Many studies have then been initiated to look into the physiological modulating factors of the IP<sub>3</sub>-induced Ca release (IICR) mechanism, such as Ca<sup>2+</sup>, pH, and nucleotides. It has been shown that Ca<sup>2+</sup> itself modulates the IICR in a biphasic manner; i.e., Ca<sup>2+</sup> stimulates the IICR below 300 nM and depresses the IICR above 300 nM (Iino, 1990).

Adenine nucleotides are the important intracellular constituents, and have been reported to modulate the IICR, although some of the results remain controversial. Smith, Smith, and Higgins (1985) noted that ATP or its analogues are required for IP<sub>3</sub>-induced Ca efflux from permeabilized cultured smooth muscle cells. On the contrary, it has been demonstrated that IP<sub>3</sub> induces Ca release from the smooth muscle Ca store of skinned smooth muscle fiber bundles in the absence of any nucleotide (Iino, 1987, 1990). In accordance with these results, both aortic smooth muscle sarcoplasmic reticulum vesicles incorporated into planar bilayer and purified cerebellar IP<sub>3</sub> receptor incorporated into lipid vesicles showed Ca channel activities in response to IP<sub>3</sub> in the complete absence of nucleotides (Ehrlich and Watras, 1988; Ferris, Haganir, Supattapone, and Snyder, 1989). On the other hand, adenine nucleotides have been reported to enhance the IICR. Hirata, Kukita, Sasaguri, Suematsu, Hashimoto, and Koga (1985) have shown that ATP enhances the IICR in permeabilized macrophages. In general accordance with their results, I have noted that AMP enhances the IICR in smooth muscle cells (Iino, 1990). This study was conducted, therefore, to further look into the effects of adenine nucleotides on the IICR in saponin-permeabilized smooth muscle cells.

A preliminary account of the study was presented at the Japanese Pharmacological Society Meeting (Iino and Endo, 1989).

#### METHODS

Apparatus and procedures of the experiments have been described in previous papers (Iino, 1989, 1990). Saponin-skinned thin smooth muscle fiber bundles were fixed in a capillary cuvette (internal diameter 400 μm) through which solutions can be perfused. The amount of Ca released from the specimen was measured by a microfluorometry of ~35 μM fura-2 at 20–22°C. The capillary cuvette was illuminated at 340 and 360 nm (or 345 and 365 nm) alternating at 200 Hz, and the fluorescence intensity near 500 nm was measured. Fluorescence intensity with 340 nm (or 345 nm) excitation or the ratios of the fluorescence intensity with 340 nm (or 345 nm) to that with 360 nm (or 365 nm) were used as the Ca signals. The results did not depend on the type of these fluorescence signals.

#### *Fiber Bundles*

Fiber bundles of portal vein of guinea pig were used in this study. Segments of portal vein (length ~10 mm) adjacent to the liver were removed and cut open by a longitudinal incision in a dissecting trough filled with the following external solution (in mM): 150 NaCl, 4 KCl, 2 CaMs<sub>2</sub> (calcium methanesulfonate), 1 MgMs<sub>2</sub>, 5 HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid), pH 7.4, adjusted with NaOH. The opened wall of the vein was pinned down onto the Silgard bottom of the trough and the endothelial side was rubbed. A thin longitudinal strip (width ~200 μm) was then cut and the adventitial layer was removed using fine forceps and scissors. Both ends of a ~2.5-mm-long segment with uniform width and thickness (50–70 μm) were then tied with fine silk filament to a stainless steel wire (diameter

100  $\mu\text{m}$ , length  $\sim 15$  mm). Fiber bundles were treated with saponin (50  $\mu\text{g}/\text{ml}$ ) in a relaxing solution (solution 1, Table I) for 35 min and then inserted into the capillary cuvette.

*Protocol to Measure Nucleotide Dependence of IP<sub>3</sub>-induced Ca Release*

The experimental protocols are schematically presented in Fig. 1. Protocol 1 was used to study the size of the Ca stores and protocol 2 to analyze the effect of nucleotides on the IICR.

An example of the experiments following protocol 2 is shown in Fig. 2. The records show the fluorescence intensity with 340 nm excitation. At the small vertical bars or arrows the solutions were flushed into the capillary cuvette. Following the flush marked with the bars, there was a slow continuous flow of the same solution to keep the environment constant. After the flush indicated by the arrows, flow of the solution was completely halted to observe the change in the

TABLE I  
*Composition of the Solutions*

Protocol	Solution No.	Name	EGTA or EDTA*	CaEGTA or CaEDTA*	MgMs <sub>2</sub>	KMs	ATP-Na <sub>2</sub>	AMP	Mg <sup>2+</sup>	K <sup>+</sup>
Loading	1	G1	1	0	5.54	108.6	4.76	0	1.5	149
	2	pCa6-loading	0.298	0.702	5.51	108.6	4.76	0	1.5	149
Wash	3	G1R	1	0	1.54	134.5	0	0	1.5	166
Test	4	D1R	1.0*	0	0	136.5	0	0	0	168
	5	pCa8RD	8.51*	1.49*	0	90.7	0	0	0	147
	6	pCa7RD	3.64*	6.36*	0	102.9	0	0	0	155
	6'	pCa5.5RD	0.18*	9.83*	0	111.6	0	0	0	151
	7	pCa7R	8.11	1.89	1.82	107.2	0	0	1.5	156
	8	G10R	10	0	1.90	107.1	0	0	1.5	157
Assay	8'	D10R	10*	0	0	86.9	0	0	0	144
	9	GORMg0	0	0	0	142.1	0	0	0	171
	10	Assay	0	0	0	84.1	0	25	0	155

Concentrations are in millimolar. All solutions contained 20 mM PIPES (piperazine-*N,N'*-bis(2-ethanesulfonic acid)) and 20 mM NaN<sub>3</sub>. Solution 10 contained 10  $\mu\text{M}$  IP<sub>3</sub>, unless otherwise described. pH was adjusted to 7.0 at 20°C with KOH. Mg<sup>2+</sup> and K<sup>+</sup> concentrations were estimated by the numerical solution of multi-equilibrium between metals and ligands in the solution. When ATP was present, MgATP<sup>2-</sup> concentration was calculated to be 4.0 mM. Total ionic strength was 200 mM in all the solutions. EGTA, ethyleneglycol-bis[ $\beta$ -aminoethyl ether] *N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Ms, methanesulfonic acid.

Ca concentration within the cuvette. For the numbering of the solutions, see Table I. 33  $\mu\text{M}$  fura-2 was added to the solutions underlined by the hatched horizontal bars in Fig. 2. The microcuvette was illuminated only when the solutions contained fura-2 to avoid unnecessary irradiation of the skinned fiber bundles with the UV light. Data acquisitions and solution changes were controlled by a microcomputer (Iino, 1989) so that they were precisely synchronized.

The experimental runs proceeded in the following manner. For the solution numbers, refer to Table I. Step 1 (loading): After a 2–3-min preincubation in a relaxing solution (solution 1), Ca store was loaded with Ca at pCa 6 for 3 min in the presence of 4 mM MgATP (solution 2). Step 2 (wash): Both Ca and ATP were removed by a flush of solution 3 (a solution with 1 mM EGTA, 1.5 mM Mg, and no ATP) and perfusion of the same solution for 1 min. This procedure was repeated twice. Step 3 (test): This step was optional, and was included only in protocol 2.

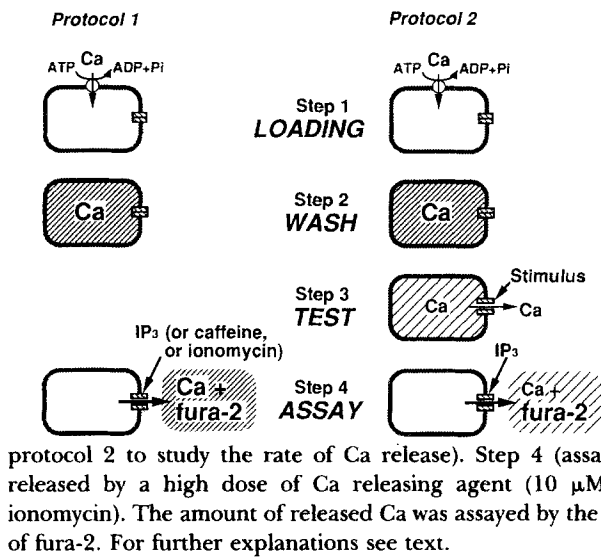


FIGURE 1. Scheme of the experimental protocol. Rounded boxes represent intracellular Ca store. Step 1 (loading): In situ Ca pump ATPase was used to load the store in the presence of 1  $\mu\text{M}$   $\text{Ca}^{2+}$  and 4 mM MgATP for 3 min. Step 2 (wash): Both  $\text{Ca}^{2+}$  and ATP were washed. Step 3 (test): Ca releasing test stimulus was applied in the presence of a high concentration of Ca buffer (EDTA or EGTA). This step is optional (i.e., included only in

protocol 2 to study the rate of Ca release). Step 4 (assay): Ca in the store was thoroughly released by a high dose of Ca releasing agent (10  $\mu\text{M}$   $\text{IP}_3$ , 50 mM caffeine, or 10  $\mu\text{M}$  ionomycin). The amount of released Ca was assayed by the change in the fluorescence intensity of fura-2. For further explanations see text.

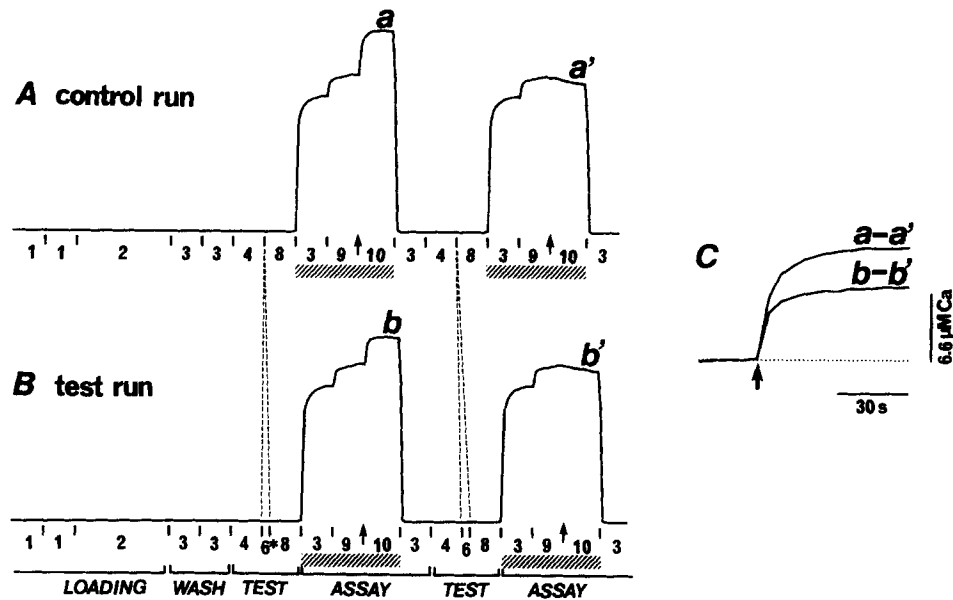


FIGURE 2. A typical record of experiments following protocol 2. Fluorescence intensity with 340 nm excitation is shown. Small vertical bars and arrows below the traces indicate the timing of solution changes. The numbers below the traces refer to the solutions listed in Table I. 33  $\mu\text{M}$  fura-2 was added to the solutions only during assay as indicated by the hatched bar, during which the capillary cuvette was illuminated. (A) Control run without test  $\text{IP}_3$  application. (B) A test run with a 15-s application of 0.1  $\mu\text{M}$   $\text{IP}_3$  at pCa 7 as indicated (6\*). (C) Amount of Ca release during assay in the control run (a-a') and in the test run (b-b'). Vertical calibration indicates the amount of Ca increase in the cuvette due to Ca release from the skinned fiber bundle. For further explanations see text.

Mg<sup>2+</sup> was removed by the perfusion for 1 min of solution 4, which contained 1 mM EDTA with no added Mg. Then a test solution that contained IP<sub>3</sub> on top of either solution 6 (or solution 5) was applied for 5–45 s (Fig. 2 B). This IP<sub>3</sub> application was omitted in the control runs (Fig. 2 A). Solution 4 and the test solution contained the same concentration of a nucleotide. IP<sub>3</sub> was then removed by the application of solution 8 that contained 10 mM EGTA instead of EDTA, 1.5 mM Mg<sup>2+</sup>, and no Ca. Step 4 (assay): Fura-2 was then introduced in solution 3 for 1 min. Then both EGTA and Mg<sup>2+</sup> were removed by solution 9 in the continued presence of fura-2 (1 min). Because fura-2 was the only Ca buffer in this solution, the fluorescence intensity increased here due to contaminating ~3 μM total Ca. Finally, 10 μM IP<sub>3</sub> (or in some experiments 50 mM caffeine or 10 μM ionomycin) was applied to discharge Ca in the store, and the resulting change in the fluorescence intensity of fura-2 was measured (for 70–180 s). The assay solution was then replaced by solution 3.

To obtain the baseline Ca level of the assay solution, steps 3 and 4 were repeated without the Ca loading. The resulting Ca signal (*a'* and *b'* of Fig. 2) was subtracted from the initial assay (*a* and *b* of Fig. 2) to obtain the amount of Ca released from the store (Fig. 2 C). At the end of the experiment the fluorescence intensities of the assay solutions with 100 μM Ca and 1 mM EGTA were separately obtained. The difference between these measurements corresponded to the Ca concentration change equal to the fura-2 concentration, and was used to calibrate the increase in the total Ca concentration within the cuvette due to Ca release from the skinned fiber. Linearity of the measurement is excellent and has been discussed elsewhere (Iino, 1989).

Experiments following these protocols can be repeated many times in one fiber bundle unless ionomycin is used, which causes irreversible loss of the Ca uptake capacity. In protocol 2, if one compares the amount of Ca assayed at step 4 with and without the application of the test solution in step 3 as shown in Fig. 2 C, the amount of Ca released during the test procedure can be measured. The advantage of this protocol is that the condition for the IICR can be strictly controlled in step 3, especially with a high concentration of a Ca buffer.

### *Solutions*

The main experimental solutions are listed in Table I. Compositions of the solutions were calculated as described previously (Iino, 1989, 1990). The total ionic strength of the solutions was adjusted to 200 mM. Nucleotides were simply added to the appropriate solutions when their concentration was less than or equal to 0.5 mM. Above this concentration, the amount of potassium methanesulfonate (KMs) was reduced to keep the total ionic strength constant. ATP and ADP were obtained from Boehringer Mannheim GmbH, Mannheim, Germany. AMP, adenine, β,γ-methyleneadenosine 5'-triphosphate (AMPOPCP), and IP<sub>3</sub> were purchased from Sigma Chemical Co. (St. Louis, MO), ionomycin from Calbiochem Corp. (La Jolla, CA), saponin from ICN Pharmaceuticals Inc. (Cleveland, OH), ryanodine from Agrysystems International (Wind Gap, PA), and fura-2 from Molecular Probes, Inc. (Eugene, OR). All other chemicals were of the highest reagent grade.

### *Statistics*

Statistical significance was tested using the *t* test. When paired observations were to be compared, the paired *t* test was used.

## RESULTS

### *Compartments of the Ca Store*

Our previous studies demonstrated the presence of two functional compartments in the smooth muscle Ca store (Iino, 1987, 1989, 1990; Iino, Kobayashi, and Endo,

1988). One of the functional compartments, designated as  $S\alpha$ , has both Ca-induced Ca release (CICR) and IICR mechanisms. Since caffeine induces Ca release by increasing the Ca sensitivity of the CICR mechanism,  $S\alpha$  is sensitive to both caffeine and  $IP_3$  (Iino, 1989). The rest of the store,  $S\beta$ , has only the IICR, and therefore is insensitive to caffeine. The traces without asterisks in Fig. 3A show the superimposed traces of Ca release by either 10  $\mu M$   $IP_3$  or 50 mM caffeine in skinned fiber bundles

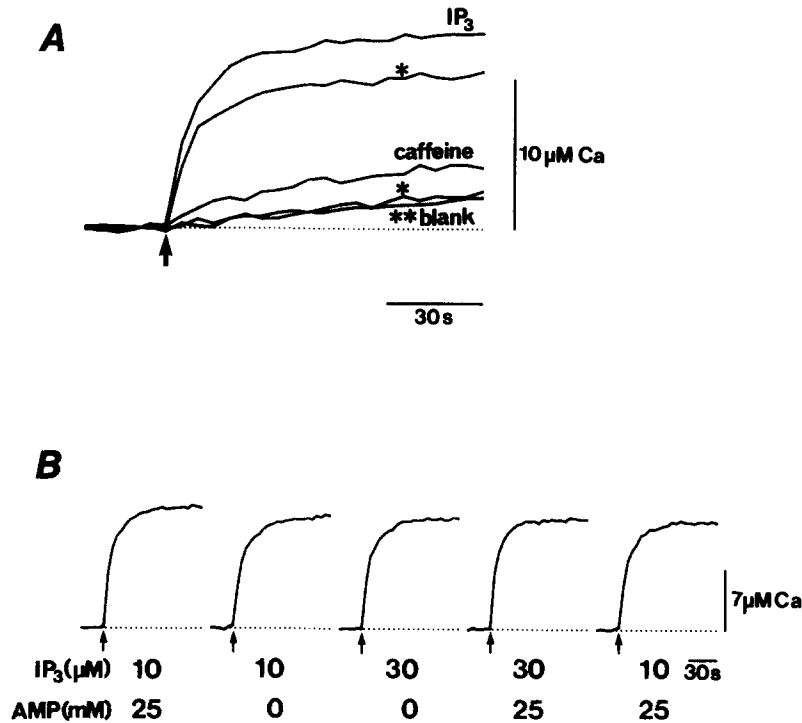


FIGURE 3. (A) Effect of ryanodine treatment on the Ca release by 50 mM caffeine and 10  $\mu M$   $IP_3$  in the presence of 25 mM AMP. Traces marked with \* were obtained after a 2-min treatment of 30  $\mu M$  ryanodine in the presence of 45 mM caffeine and 22.5 mM AMP at pCa 5.7. The trace marked with \*\* shows the results obtained by the application of the assay solution that contained neither caffeine nor  $IP_3$  (blank) after the ryanodine treatment. Representative record of eight separate experiments. (B) Effect of the concentrations of  $IP_3$  and AMP on the Ca release during assay. Either 10 or 30  $\mu M$   $IP_3$  was applied at the arrows in either solution 9 (without AMP) or solution 10 (25 mM AMP). The same results were obtained in the duplicate experiment.

of portal vein after the Ca loading at pCa 6 for 3 min (protocol 1, see Methods). Caffeine releases  $\sim 20\%$  or less ( $18.5 \pm 11.2\%$ , mean  $\pm$  SD,  $n = 12$ ) of the  $IP_3$ -sensitive store and the remaining  $> 80\%$  of the store is sensitive only to  $IP_3$ .

A plant alkaloid, ryanodine has been shown to lock the CICR channels of the striated muscle in an open state (Fleischer, Ogunbunmi, Dixon, and Fleer, 1985; Rousseau, Smith, and Meissner, 1987). Ryanodine seems to have the same effect on the CICR channels of smooth muscle, and removes the Ca uptake capacity of  $S\alpha$  in

guinea pig taenia caeci by making this functional compartment permanently hyperpermeable to Ca (Iino et al., 1988; Iino, 1990). The effect of ryanodine on the smooth muscle cells of guinea pig portal vein was examined. In Fig. 3 A, caffeine- and IP<sub>3</sub>-releasable Ca were compared before and after a 30- $\mu$ M ryanodine treatment for 2 min at pCa 5.7 in the presence of 45 mM caffeine and 22.5 mM AMP. After the ryanodine treatment (traces marked with \*), caffeine failed to induce rapid Ca release. The remaining creeping signal was not due to enhancement of the CICR, because the same rate of Ca release was obtained by the application of a blank assay solution without caffeine or IP<sub>3</sub> (trace marked with \*\*) and would represent simple Ca leakage from S $\beta$ . A similar rate of Ca leakage was observed before the ryanodine treatment or with a blank assay solution without AMP (not shown). After the ryanodine treatment, the amount of Ca released by IP<sub>3</sub> declined by almost the same extent as the decrease in the caffeine-releasable Ca. These results show that the ryanodine treatment specifically removes the function of S $\alpha$  in the portal vein smooth muscle cells.

Since Ca in S $\alpha$  may be released through the two Ca release mechanisms, the interpretation of the results may become complicated when one tries to study the IICR mechanism at Ca<sup>2+</sup> concentrations > 1  $\mu$ M, where the CICR mechanism becomes activated (Iino, 1989). To circumvent this problem, fiber bundles obtained from guinea pig taenia caeci were treated in a previous study with ryanodine to excise the function of S $\alpha$  at the beginning of the experiments so that the IICR mechanism could be studied independently of the CICR (Iino, 1990). In this study portal vein smooth muscle cells were used to study the effect of nucleotides on the IICR. Because the proportion of S $\alpha$  is smaller than that of taenia (~20% vs. ~40% in taenia caeci), and because the effect on the IICR was studied at either pCa 8 or 7, which was too low for the CICR to become active (Iino, 1989), no ryanodine pretreatment was carried out in most of the experiments. The presence of the CICR did not affect the conclusions as will be shown later.

Fig. 3 B compares the amount of Ca released within 2 min by 10 or 30  $\mu$ M IP<sub>3</sub> in the presence and the absence of 25 mM AMP; i.e., solutions 10 and 9 (Table I), respectively. If a small amount of run-down is allowed for, 10  $\mu$ M IP<sub>3</sub> with 25 mM AMP releases about the same amount of Ca that is released by 30  $\mu$ M IP<sub>3</sub> with or without 25 mM AMP. 10  $\mu$ M IP<sub>3</sub> without AMP may release a few percent less Ca within this period than the other stimuli. In the following, therefore, 10  $\mu$ M IP<sub>3</sub> with 25 mM AMP was used as a standard stimulus to deplete the IP<sub>3</sub>-sensitive Ca store. The reason why AMP enhances 10  $\mu$ M IP<sub>3</sub>-induced Ca release will be presented later.

Since IP<sub>3</sub> is a polyanion, its binding to the receptor is expected to be highly dependent on the ionic strength. To keep the total ionic strength constant, there were inevitable changes in the osmolarity and K<sup>+</sup> concentration among the experimental solutions. The solution with the greatest osmolarity was solution 10, and its osmolarity was ~15% greater than that of solution 9 with the smallest value. There was a ~10% difference in the K<sup>+</sup> concentration between the two solutions. However, as shown in Fig. 3 B, the amount of IP<sub>3</sub>-releasable Ca was the same in either solution when the maximum dose of IP<sub>3</sub> was used. Therefore, the variation in the osmolarity and K<sup>+</sup> concentration within the range of the current study had no effect on the size of Ca release.

To estimate the proportion of the IP<sub>3</sub>-sensitive compartment in the total Ca store,

the amount of Ca released by  $\text{IP}_3$  was compared with that released by a high concentration of a Ca ionophore using protocol 1. As shown in Fig. 4A, the size of  $\text{IP}_3$ -releasable Ca was  $\sim 80\%$  of the  $10 \mu\text{M}$  ionomycin-releasable Ca in skinned fiber bundles of portal vein smooth muscle. After the ionomycin treatment,  $\text{IP}_3$  failed to release Ca even after the same Ca loading step. This indicated that ionomycin irreversibly destroyed the Ca holding capacity of the Ca stores. In Fig. 4, B and C, the difference between the sizes of  $\text{IP}_3$ - and ionomycin-releasable stores was studied in a different way using modified protocol 1. After Ca loading at  $1 \mu\text{M}$  Ca for 3 min, Ca assay (step 4) with  $10 \mu\text{M}$   $\text{IP}_3$  was immediately followed by a repetition of the Ca assay with either  $10 \mu\text{M}$   $\text{IP}_3$  (B) or  $10 \mu\text{M}$  ionomycin (C). The second application of  $\text{IP}_3$  did not release Ca (because Ca loading was not carried out between the assays), but under the same condition ionomycin released Ca which was left unreleased after the

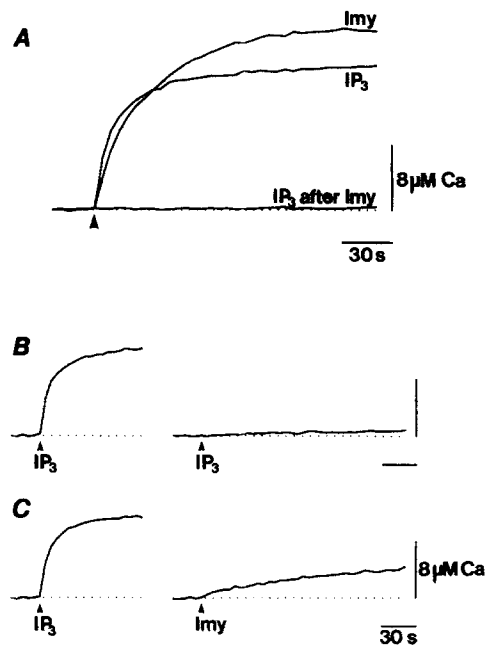


FIGURE 4. Comparison between the sizes of  $\text{IP}_3$ -sensitive and ionomycin-releasable Ca store in portal vein measured using protocol 1. (A) Ca release during assay (step 4) with either  $10 \mu\text{M}$   $\text{IP}_3$  (+25 mM AMP) or  $10 \mu\text{M}$  ionomycin (without AMP) was superimposed. The bottom trace was obtained with an  $\text{IP}_3$  assay after the ionomycin treatment. (B, C) Immediately after the completion of step 4 with  $10 \mu\text{M}$   $\text{IP}_3$  (left traces), another step 4 with either  $10 \mu\text{M}$   $\text{IP}_3$  (right trace of B) or with  $10 \mu\text{M}$  ionomycin (right trace of C) was repeated. B and C were obtained from the same fiber bundle, which was different from that used in A. Representative records of duplicate experiments.

application of  $\text{IP}_3$ . The amount of this Ca release was equal to  $\sim 20\%$  of the  $\text{IP}_3$ -releasable Ca (Fig. 4C). This again indicates that not all of the total Ca store, but a large fraction of it, was sensitive to  $\text{IP}_3$ . A 50-mM caffeine assay carried out immediately after the  $\text{IP}_3$  assay failed to release Ca (not shown), as was the case in taenia caeci (Iino, 1987, 1990).

#### *Effect of ATP on the Time Course of the IICR*

Fig. 5 shows the time course of  $1 \mu\text{M}$   $\text{IP}_3$ -induced Ca release at pCa 7 obtained using protocol 2. In each skinned fiber, three test runs with  $1 \mu\text{M}$   $\text{IP}_3$  application in step 3 were bracketed by two control runs without the test  $\text{IP}_3$  applications as shown in Fig. 5A. Compared with the control runs, the amounts of Ca assayed were smaller in the



test runs. If the duration of IP<sub>3</sub> application during step 3 was increased, less Ca was assayed. The data points were normalized to the average amount of Ca assayed in the control runs. The compiled results are shown in Fig. 5 B. These results indicate that Ca was released roughly exponentially during step 3 upon application of IP<sub>3</sub> (open circles). The time course of Ca release was faster when the test IP<sub>3</sub> was applied in the presence of 0.5 mM ATP without Mg (solid circles). If the test solution did not contain IP<sub>3</sub>, the Ca in the store declined only very slowly and was independent of the presence or absence of ATP (solid and open triangles, respectively). This slow Ca leakage seems to correspond to the creeping signal marked with \*\* in Fig. 3 A. These results demonstrate that ATP enhances the IICR, although ATP neither induces Ca release by itself nor enhances the CICR at pCa 7.

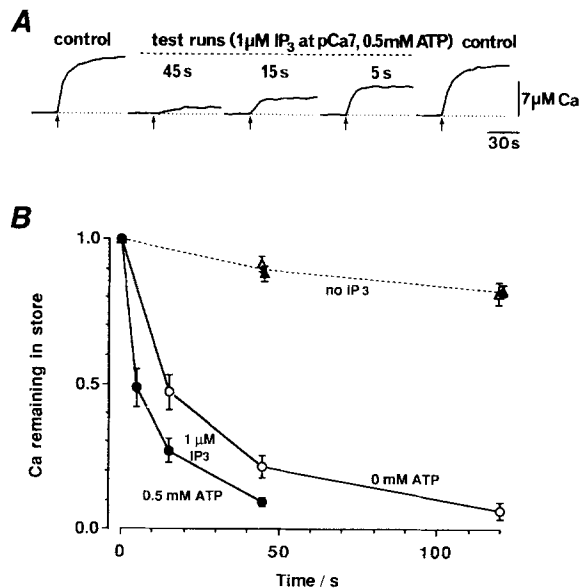


FIGURE 5. Effects of ATP on the time course of IP<sub>3</sub>-induced Ca release. (A) An example of the experiments (protocol 2) to study the time course of the IP<sub>3</sub>-induced Ca release. Ca release records during step 4 of 5 consecutive runs are shown. The first and the fifth runs were the controls without stimulus application in step 3. From the second to the fourth runs, 1 μM IP<sub>3</sub> was applied for 45, 15, or 5 s at pCa 7 (solution 6) in the presence of 0.5 mM ATP in step 3. The order of the test runs was shuffled among different experiments. (B) The amount of Ca assayed in step 4 after the application of a test

solution for the duration shown in the abscissa is shown. The data points were normalized to the average of the control runs in each fiber. The test solution contained 1 μM IP<sub>3</sub> without ATP (open circles,  $n = 3$ ), 1 μM IP<sub>3</sub> with 0.5 mM ATP (solid circles,  $n = 4$ ), no IP<sub>3</sub> without ATP (open triangles,  $n = 5$ ), or no IP<sub>3</sub> with 0.5 mM ATP (solid triangles,  $n = 5$ ). All the test solutions were at pCa 7 (solution 6). Mean  $\pm$  SEM.

In this and the following figures, the test IP<sub>3</sub> application in protocol 2 was carried out in the absence of Mg<sup>2+</sup>. This was to inhibit the intrinsic IP<sub>3</sub> phosphatase which absolutely requires Mg<sup>2+</sup> for its activity, so that the secondary effect of the nucleotides through its possible effect on the enzyme can be excluded.

#### *Dose-Response Relation of the ATP Effect on the IICR*

The range of ATP concentration effective on the rate of IICR was then studied. Fig. 6 shows the amount of Ca released from the store by a 15-s application of 0.1 μM IP<sub>3</sub> at pCa 7 in the presence of ATP concentration shown on the abscissa. The data were

normalized within each skinned fiber by dividing the amount of Ca release by that without ATP. A total of 19 fibers were used to construct the curve, and on average a 15-s application of  $\text{IP}_3$  without ATP released  $37 \pm 7\%$  (mean  $\pm$  SD) of Ca in the store. The effect of ATP (circles) was found to be biphasic. Up to  $\sim 0.2\text{--}0.5$  mM, ATP enhanced the Ca release induced by  $0.1 \mu\text{M}$   $\text{IP}_3$  ( $P < 0.001$ , 0 vs. 0.2 or 0.5 mM ATP). At concentrations  $> 0.5$  mM, ATP enhancement of IICR declined ( $P < 0.001$ , 0.5 vs. 5 mM ATP).

AMP mimicked the enhancing effect of ATP (Fig. 6, squares). The results are expressed in the same way as for ATP, and a 15-s application of  $0.1 \mu\text{M}$   $\text{IP}_3$  without AMP released  $38 \pm 4\%$  (mean  $\pm$  SD,  $n = 6$ ) of Ca in the store in the fibers used for this analysis. Compared with ATP, nearly 100 times higher concentrations of AMP were required to obtain a similar potentiating effect. Up to 25 mM, the highest concentration tested, no obvious inhibitory effect was detected for AMP. This effect of AMP seems to be responsible for the potentiation of  $10 \mu\text{M}$   $\text{IP}_3$ -induced Ca release by the nucleotide seen in Fig. 3 B.

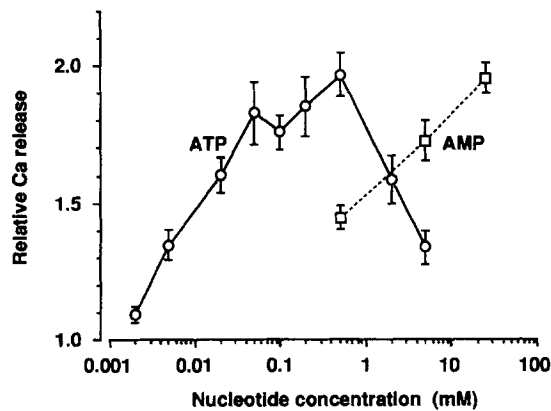


FIGURE 6. Effects of ATP and AMP on the rate of IICR.  $0.1 \mu\text{M}$   $\text{IP}_3$  was applied for 15 s at  $p\text{Ca } 7$  with either ATP (circles) or AMP (squares) at the concentration indicated on the abscissa in step 3 of protocol 2. The amount of Ca release was normalized within each fiber by that released with  $0.1 \mu\text{M}$   $\text{IP}_3$  in the absence of any nucleotide. Each data point represents the mean of five to six experiments and SEM.

#### *The Effect of ATP on the IICR with a Higher Concentration of EDTA or after a Ryanodine Treatment*

Adenine nucleotides have been shown to enhance the CICR, another Ca release mechanism present in the smooth muscle (Iino, 1989). Removal of  $\text{Mg}^{2+}$  during the test  $\text{IP}_3$  application is also expected to enhance the CICR. Because the experiments here were carried out in the presence of a high concentration of Ca buffer and at very low concentrations of  $\text{Ca}^{2+}$  where the CICR was not active, the effect of adenine nucleotides on the IICR is unlikely to be affected by their effect on the CICR. Furthermore, the fraction of  $\text{S}\alpha$  that has the CICR was only  $\sim 20\%$  or less of the  $\text{IP}_3$ -sensitive store (Fig. 3 A). To strengthen these arguments, the effect of ATP was observed either in the presence of a higher concentration of EDTA (20 mM) to obtain a better control of  $\text{Ca}^{2+}$  during the test  $\text{IP}_3$  application, or after a ryanodine treatment to reduce the fraction of  $\text{S}\alpha$  that possessed the CICR mechanism to zero (see Fig. 3 A; Iino, 1990).

The amount of Ca released by a 15-s application of 0.1  $\mu\text{M}$  IP<sub>3</sub> in the presence of 0, 0.5, and 5 mM ATP at pCa 7 was compared in Fig. 7 under three conditions; i.e., 10 mM EDTA used as the Ca buffer (open columns), 20 mM EDTA used as the Ca buffer (hatched columns), and 10 mM EDTA used after the same ryanodine treatment used in Fig. 3 A (shaded columns). Under any of these conditions 0.1  $\mu\text{M}$  IP<sub>3</sub> released a greater amount of Ca with 0.5 mM ATP than without ATP ( $P < 0.001$ ). In addition, the amount of Ca release declined when the ATP concentration was increased from 0.5 to 5 mM ( $P < 0.01$ ).

To examine the completeness of the ryanodine treatment, CICR at pCa 5.5 (solution 6') was studied before and after the ryanodine treatment (Fig. 8). Experiments were carried out following protocol 2. The test solutions contained 3  $\mu\text{M}$  Ca<sup>2+</sup>, no IP<sub>3</sub>, and either 0, 0.05, 0.5, or 5 mM ATP, and were washed with solution 8'. As shown by the open circle in Fig. 8, 3  $\mu\text{M}$  Ca released  $\sim 7\%$  of Ca in the store within 15 s in the absence of ATP and Mg. The CICR was enhanced with ATP at 5 mM

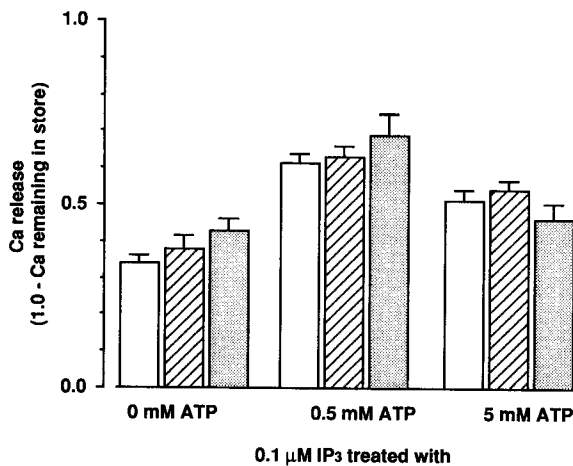


FIGURE 7. Amount of Ca release by 0.1  $\mu\text{M}$  IP<sub>3</sub> in the presence of 0, 0.5, or 5 mM ATP (expressed as a fraction of the total IP<sub>3</sub>-releasable Ca in the store). Experiments were carried out under three different conditions: 10 mM EDTA to buffer Ca<sup>2+</sup> during the test (open columns,  $n = 6$ ), 20 mM EDTA to buffer Ca<sup>2+</sup> (hatched columns,  $n = 7$ ), or pretreatment of the fibers with ryanodine to remove the function of S $\alpha$  (shaded columns,  $n = 5$ ). Mean  $\pm$  SEM.

( $P < 0.01$ ) in accordance with previous results (Iino, 1989). The amount of Ca-induced Ca release is small even with 5 mM ATP, because the average size of the functional compartment with the CICR mechanism (S $\alpha$ ) is only  $\sim 18\%$  of the IP<sub>3</sub>-sensitive store. However, the same Ca treatment failed to induce Ca release after the ryanodine treatment (solid circles) even at 5 mM ATP. None of the data points after the ryanodine treatment were statistically different from zero ( $P > 0.1$ ). Similar results were obtained when a nonhydrolyzable ATP analogue (AMPOPCP) was used in the place of ATP (not shown). Together with the absence of caffeine-induced Ca release after the ryanodine treatment (Fig. 3 A), these results provide clear evidence that the ryanodine treatment completely abolished the function of the compartment with the CICR.

The above results strongly indicate that the biphasic effect of ATP on the IICR is independent of the effect of ATP on the CICR, and that ATP has a direct influence on the IICR.

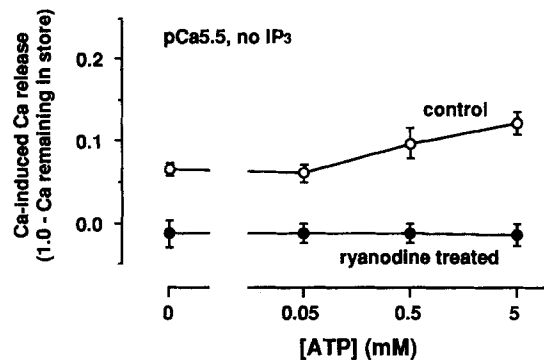


FIGURE 8. Amount of Ca release by a 15-s application of 3  $\mu\text{M}$  Ca without  $\text{IP}_3$  (solution 6') in the presence of 0, 0.05, 0.5, or 5 mM ATP (expressed as a fraction of the total  $\text{IP}_3$ -releasable Ca in the store). Experiments were carried out without ryanodine pretreatment (*open symbols*,  $n = 6$ ) or after the same treatment with ryanodine as in Fig. 3 A to remove the function of  $\text{S}\alpha$  (*solid symbols*,  $n = 6$ ). Mean  $\pm$  SEM.

#### The Effects of ATP on the Dose-Response Relation of the IICR

To find out if the inhibitory component of the ATP effect could be overcome by the increase in the  $\text{IP}_3$  concentration, the amount of Ca release within the 15-s application of 0.1–10  $\mu\text{M}$   $\text{IP}_3$  was determined at 0, 0.5 and 5 mM ATP (Fig. 9). The ATP concentrations were such that the enhancement and inhibition of the IICR were clearly seen at 0.1  $\mu\text{M}$   $\text{IP}_3$ . The  $\text{Ca}^{2+}$  concentration during the test  $\text{IP}_3$  application in step 3 of protocol 2 was pCa 8, so that the rate of IICR was about three times smaller than that at pCa 7 (Iino, 1990). This was necessary for reliable measurement of Ca release with the higher doses of  $\text{IP}_3$ .

At all the  $\text{IP}_3$  concentrations tested, 0.5 mM ATP enhanced the IICR ( $P < 0.001$ ). At 0.1  $\mu\text{M}$   $\text{IP}_3$ , the increase in the ATP concentration from 0.5 to 5 mM reduced the amount of Ca release ( $P < 0.02$ ), in accordance with the results at pCa 7 (Fig. 6). However, the difference between the effects of 0.5 and 5 mM ATP became insignificant at 0.3 and 1  $\mu\text{M}$   $\text{IP}_3$  ( $P > 0.3$  and  $P > 0.05$ , respectively). Indeed, the

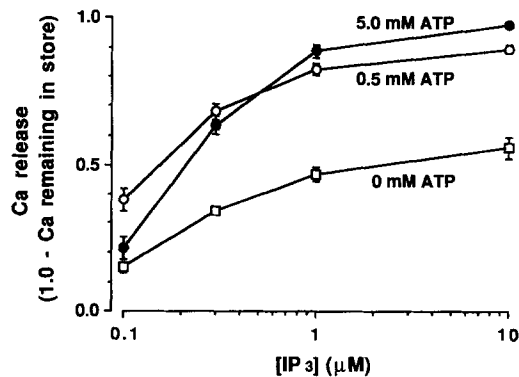


FIGURE 9. Effects of ATP on the relation between the dose of  $\text{IP}_3$  and the rate of Ca release.  $\text{IP}_3$  was applied in step 3 of protocol 2 for 15 s at pCa 8 (solution 5) with either 0 (*squares*,  $n = 6$ ), 0.5 (*open circles*,  $n = 6$ ), or 5 mM ATP (*solid circles*,  $n = 6$ ). Amount of Ca release by the application of  $\text{IP}_3$  was normalized by the size of total  $\text{IP}_3$ -releasable store. The full dose-response relation at one ATP concentration was obtained in each fiber bundle. Mean  $\pm$  SEM. Error bars are not shown when they are less than the symbol size.

effect was clearly reversed at 10  $\mu\text{M}$  IP<sub>3</sub>, where the amount of Ca release was greater with 5 mM ATP than with 0.5 mM ATP ( $P < 0.001$ ). Because the reduction of the potentiating effect at the higher ATP concentrations was overcome by the increase in the IP<sub>3</sub> dose, the negative component of the ATP effect appears to be that of a competitive inhibitor.

#### *Effects of Other Nucleotides on the IICR*

Effects of other nucleotides (0.5 mM) on the IICR were studied. The results were normalized in each fiber bundle and compiled in Fig. 10. A 15-s application of 0.1  $\mu\text{M}$  IP<sub>3</sub> at pCa 7 in the absence of Mg<sup>2+</sup> released, on average, 40% of Ca from the store in the fibers studied ( $40 \pm 11\%$ , mean  $\pm$  SD,  $n = 13$ ). ATP, ADP, AMP, and AMPOPCP had a clear enhancing effect ( $P < 0.001$ ). ATP alone had no Ca releasing action (see Fig. 5). Adenine and GTP had only a very small effect. Therefore, the enhancing effect on IICR seems to be rather specific to adenine nucleotides.

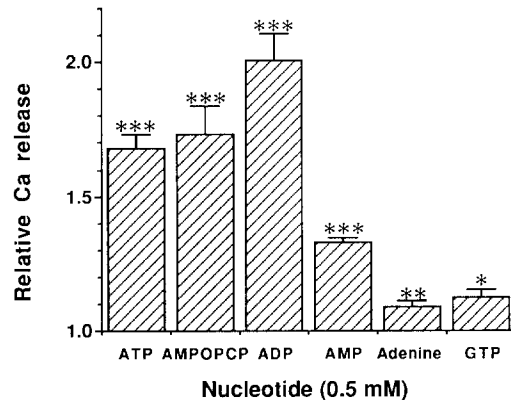


FIGURE 10. The effect of nucleotides on the IICR. The amount of Ca released by 0.1  $\mu\text{M}$  IP<sub>3</sub> with the nucleotides (0.5 mM) at pCa 7 was normalized in each fiber bundle by that without nucleotide before compilation of the results. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $0.01 < P < 0.02$ . Number of observations was six or seven for each nucleotide. Mean  $\pm$  SEM.

#### *Effect of ATP on the IICR in the Presence of Mg<sup>2+</sup>*

So far the effects of ATP on the IICR have been studied in the absence of Mg<sup>2+</sup>. Effects of ATP on the IICR were also studied in the presence of 1.5 mM Mg<sup>2+</sup> at pCa 7 in six fiber bundles. 1  $\mu\text{M}$  IP<sub>3</sub> released  $46 \pm 3\%$  (mean  $\pm$  SEM) of Ca in the store within 45 s. The same dose of IP<sub>3</sub> released  $69 \pm 5\%$  and  $81 \pm 3\%$  of Ca in the store in the presence of 0.5 and 5 mM ATP, respectively.

#### DISCUSSION

This study has demonstrated that adenine nucleotides potentiate the Ca release induced by IP<sub>3</sub> in vascular smooth muscle cells. Evidence has been presented that the results are not indirect consequences of the effects of the nucleotides on the CICR. (a) Clear effects of adenine nucleotides on the IICR are observed at pCa 7 or 8, one to two orders of magnitude lower than the Ca<sup>2+</sup> concentration required to activate the CICR (Iino, 1989), while ATP alone does not induce Ca release under these conditions. (b) Increasing EDTA concentration during test IP<sub>3</sub> application to 20 mM

from 10 mM did not affect the enhancement of IICR by ATP (Fig. 7). This argues against the possibility that Ca buffering during IP<sub>3</sub>-induced Ca release was insufficient. (c) The same results were obtained (Fig. 7) even when the experiments were carried out after the ryanodine treatment which was shown to completely abolish the CICR (Figs. 3A and 8).

Since clear effects of the nucleotides were seen in the absence of Mg<sup>2+</sup>, the potentiation seems to be independent of the effect of adenine nucleotides on the metabolism of IP<sub>3</sub> by IP<sub>3</sub>-phosphatase, which requires Mg<sup>2+</sup> for its activity (Downes, Mussat, and Michell, 1982). Nor is it likely to be due either to IP<sub>3</sub>-kinase activity or protein kinase activities, because adenine nucleotides other than ATP, including nonhydrolyzable AMPOPCP, were also effective. Therefore, there seem to be adenine nucleotide binding sites either on the IP<sub>3</sub> receptor/channels or on the associated proteins. While this manuscript was in preparation, nucleotide dependence of Ca flux into the lipid vesicles reconstituted with purified IP<sub>3</sub> receptors prepared from rat cerebellum was reported (Ferris, Haganir, and Snyder, 1990). Their results are in general agreement with the present results in terms of the enhancement of the IICR, and suggest that the nucleotide binding site is located on the IP<sub>3</sub> receptor itself. The enhancing effect of ATP was also observed in the presence of 1.5 mM Mg<sup>2+</sup>, although a quantitative comparison was difficult because of the presence of intrinsic Mg-dependent IP<sub>3</sub>-phosphatase (Iino, 1990). Since ATP has been reported to inhibit IP<sub>3</sub>-phosphatase (Hansen, Johanson, Williamson, and Williamson, 1987), the nucleotide may partly enhance the IICR by increasing the effective IP<sub>3</sub> concentration in the presence of Mg<sup>2+</sup>.

We must assume at least one ATP binding site associated with the enhancement of the Ca release mechanism. At higher ATP concentrations (> 0.5 mM) the enhancing effect on the rate of Ca release induced by 0.1 μM IP<sub>3</sub> became smaller. This suggests that there is a secondary ATP binding site with a lower affinity that exerts a negative effect on the IICR. One of the possibilities is that ATP binding to the secondary site may just neutralize the potentiating effect of the high affinity site. However, the decrease in the enhancing effect of ATP became less at higher IP<sub>3</sub> concentrations and was absent at 10 μM IP<sub>3</sub> (Fig. 9). Therefore, a simple neutralizing site cannot explain the negative effect. The disappearance of the negative effect of ATP at higher IP<sub>3</sub> concentrations suggests that ATP may compete with IP<sub>3</sub> on the IP<sub>3</sub> binding site of the receptor. It has been reported that ATP near millimolar concentrations inhibits binding of IP<sub>3</sub> to the receptors (Guillemette, Balla, Baukal, and Catt, 1987). On the contrary, Ferris et al. (1990) found that the decrease in the enhancing effect of ATP on the IP<sub>3</sub>-induced Ca flux in reconstituted vesicles remained even at 100 μM IP<sub>3</sub>, and concluded that the effect was not due to competitive antagonism. The discrepancy could be due to differences between smooth muscle and cerebellum. It could also be due to differences between a physiological system and a reconstituted system. It is conceivable that inhibition of IP<sub>3</sub> binding by ATP requires separate associate protein(s) that are lost during the purification procedure.

I cannot provide a definite reason why Smith et al. (1985) found the adenine nucleotide "requirement" for the IICR. One of the possibilities may be that the potency of IP<sub>3</sub> was rather low in their experimental system and Ca release was detected only when the IICR was potentiated by the nucleotides. Since they measured

the IICR in the presence of 5 mM total Mg, there could have been at least three mechanisms for the potentiation of the IICR by ATP; i.e., (a) direct potentiating effect on the IICR, (b) direct inhibition of intrinsic IP<sub>3</sub>-phosphatase (Hansen et al., 1987), and (c) indirect inhibition of IP<sub>3</sub>-phosphatase by the decrease in the free Mg<sup>2+</sup> concentration (Downes et al., 1982) due to chelation of the metal ions by ATP.

*Possible Physiological Significance of the Effects of ATP*

<sup>31</sup>P nuclear magnetic resonance spectra of smooth muscle bundles were measured to study nucleotide concentration within the cells (Kushmerick, Dillon, Meyer, Brown, Krisanda, and Sweeney, 1986). Intracellular concentration of ATP within the smooth muscle cells was 1–2 mM, and remained nearly constant even during maximal agonist stimulation under an aerobic condition. Therefore, it is unlikely, at least under physiological conditions, that the intracellular ATP concentration should change greatly and regulate the IICR. However, the dual effects of ATP on the IICR may influence the switching of the Ca release by IP<sub>3</sub>. Thus, a physiological concentration of ATP may enhance the effect of massively released IP<sub>3</sub> during agonist–receptor coupling, while it has a less enhancing effect on the low levels of IP<sub>3</sub> produced by a basal turn over of the phosphatidylinositol cycle.

*Comparison of CICR and IICR Mechanisms*

It is interesting that another type of Ca release channel in the Ca store is also dependent on adenine nucleotides at millimolar concentrations. The CICR mechanism is enhanced by adenine nucleotides but not by guanine, cytosine, or thymidine nucleotides in skeletal muscle (Endo, 1985). The CICR in smooth muscle was also potentiated by AMPOPCP with a  $K_m$  of ~0.3 mM (Iino, 1989). The effect of adenine nucleotides on the CICR channels seems to be exerted by simple ligand binding, because nonhydrolyzable analogues of ATP are also effective.

Having characterized both Ca- and IP<sub>3</sub>-induced Ca release mechanisms in a series of experiments on skinned smooth muscle fiber bundles (Iino, 1989, 1990), we find interesting correspondence between the two Ca release mechanisms in the intracellular Ca store (Table II). Ca and IP<sub>3</sub> are the activators of the CICR and the IICR, respectively. Although Ca is not essential for the IICR because IP<sub>3</sub> can induce Ca release in the presence of 10 mM EGTA without added Ca, Ca can modulate the IICR at submicromolar concentrations (Iino, 1990). On the other hand, IP<sub>3</sub> has been shown to modulate at least skeletal muscle CICR in the presence of Ca<sup>2+</sup>, although IP<sub>3</sub> alone cannot activate the channel (Suárez-Isla, Iribarra, Oberhauser, Larralde, Bull, Hidalgo, and Jaimovich, 1988). So there is a remarkable resemblance between the activators and modulators. The present study extended the similarity in the properties, and both Ca release mechanisms have been shown to be enhanced by adenine nucleotides but little by guanine nucleotides. For both mechanisms G proteins do not seem to be involved, because both Ca release mechanisms are switched on and off in the absence of GTP. Of course, these Ca release mechanisms are quite distinct as noted by the differential effect of ryanodine or caffeine (Iino et al., 1988; Iino, 1990).

Recently, proteins responsible for the CICR mechanism (ryanodine receptor) in striated muscles and the IICR mechanism (IP<sub>3</sub> receptor) in cerebellum have been

purified and their primary structures have been determined (Imagawa, Smith, Coronado, and Campbell, 1987; Inui, Saito, and Fleischer, 1987; Lai, Erickson, Rousseau, Liu, and Meissner, 1988; Supattapone, Worley, Baraban, and Snyder, 1988; Furuichi, Yoshikawa, Miyawaki, Wada, Maeda, and Mikoshiba, 1989; Takeshima, Nishimura, Matsumoto, Ishida, Kangawa, Minamino, Matsuo, Ueda, Hanaoka, Hirose, and Numa, 1989). both proteins have high molecular weight ( $\sim 500$  and  $\sim 300$  kD, respectively) and a large putative cytoplasmic domain as well as putative transmembrane segments located toward the COOH terminus end. Segmental similarities in the amino acid sequence have been noted. Furthermore, both proteins seem to form tetramers exhibiting fourfold symmetry on electron microscopic examination (Wagenknecht, Grassucci, Frank, Saito, Inui, and Fleischer, 1989; Chadwick, Saito, and Fleischer, 1990).

We now know that both CICR channels and IICR channels function as Ca channels in the intracellular Ca store, and share not only structural resemblance but also closely related properties in terms of activators and modulators. The two Ca release

TABLE II  
*Comparison between CICR and IICR in Smooth Muscle*

	Ca-induced Ca release	IP <sub>3</sub> -induced Ca release
Ca <sup>2+</sup>	Activator ( $> 1 \mu\text{M}$ ) (a)	Positive modulator ( $< 300$ nM) (b, c) Negative modulator ( $> 300$ nM) (c, d)
IP <sub>3</sub>	(Positive modulator) (e)	Activator
Adenine nucleotides	Potentiator (a)	Potentiator (f, g) Inhibitor for low IP <sub>3</sub> (g)
Guanine nucleotides	Not required (a)	Not required (a, b, c, g, h)
Ryanodine	Open-lock (c, i)	No effect (c, i)

(a) Iino, 1989; (b) Iino, 1987; (c) Iino, 1990; (d) Suematsu, Hirata, Hashimoto, Kuriyama, 1984; (e) Suárez-Isla et al., 1988 (parentheses indicate that this has so far been reported only in skeletal muscle); (f) Hirata et al., 1985; (g) this work; (h) Ehrlich and Watras, 1988; Ferris et al., 1989; (i) Iino et al., 1988.

mechanisms seem to distribute differently among intracellular Ca stores (Iino, 1987, 1989, 1990; Iino et al., 1988). Assignment of physiological roles to these mechanisms awaits further clarification.

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