Calcium flux mediated by purified inositol 1,4,5-trisphosphate receptor in reconstituted lipid vesicles is allosterically regulated by adenine nucleotides

(ryanodine/ATP/calcium oscillations/ Ca^{2+} -ATPase/calcium pump)

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ABSTRACT When incorporated into lipid vesicles, the purified inositol 1,4,5-trisphosphate (IP_3) receptor protein mediates $^{45}\text{Ca}^{2+}$ flux. We observe a potent, selective allosteric regulation by ATP of IP_3 actions on Ca^{2+} flux. The action of ATP is selective for adenine nucleotides with ADP and AMP less potent and GTP inactive. At 1–10 μM , ATP increases maximal IP_3 -induced flux by 50% with no change in IP_3 potency. The enhancing effect of ATP diminishes between 0.1 and 1 mM. Concentration–response curves are steep for both the increasing and the decreasing effects of ATP on IP_3 actions, suggesting a physiological regulatory role of ATP in IP_3 -induced Ca^{2+} release. Diminishing local ATP concentrations coincident with filling of Ca^{2+} stores by the Ca^{2+} -ATPase may enhance IP_3 release of Ca^{2+} , an effect that would decline as ATP returns to physiological levels. ATP regulation of Ca^{2+} release may also play a role in oscillations of intracellular Ca^{2+} concentration.

Inositol 1,4,5-trisphosphate (IP_3), a major second messenger for actions of hormones, neurotransmitters, and growth factors, releases Ca^{2+} from nonmitochondrial intracellular stores (1, 2). An IP_3 receptor protein has been purified from brain membranes (3) and localized by immunohistochemical techniques to specific sites in the endoplasmic reticulum (4). IP_3 -stimulated Ca^{2+} release is regulated selectively via stoichiometric phosphorylation by cAMP-dependent protein kinase (5). When reconstituted into lipid vesicles, the purified IP_3 receptor mediates IP_3 -induced Ca^{2+} flux, indicating that the purified protein contains both an IP_3 recognition site and its associated Ca^{2+} channel (6). The proposed structure of the IP_3 receptor protein (7, 8), molecularly cloned from a mouse brain cDNA library (7), resembles the ryanodine receptor of skeletal muscle sarcoplasmic reticulum, which also mediates Ca^{2+} flux (9).

Physiologically, IP_3 can release the majority of Ca^{2+} stored within the IP_3 -sensitive pools of the endoplasmic reticulum (1, 2). A rapid signaling system between the ATP-driven Ca^{2+} pump and the IP_3 release mechanism may regulate the rapid refilling of IP_3 -depleted Ca^{2+} pools. In the present study we demonstrate a potent and selective allosteric regulation of IP_3 -induced Ca^{2+} flux by ATP. This regulation is conferred through an ATP binding site on the IP_3 receptor protein, which we have detected by radioligand binding with [^{32}P]ATP and adenosine 5'-[^{35}S]thio]diphosphate ([^{35}S]ADP[βS]).

MATERIALS AND METHODS

Materials. [^3H] IP_3 , [α - ^{32}P]ATP, [γ - ^{32}P]ATP, [^{35}S]ADP[βS], and $^{45}\text{Ca}^{2+}$ were obtained from DuPont/NEN. IP_3 was

obtained from Calbiochem. Concanavalin A-Sepharose was purchased from Pharmacia. All other chemicals were from Sigma.

Purification and Reconstitution of IP_3 Receptor. IP_3 receptor was purified (3, 6) and reconstituted into lipid vesicles (6) as described. Briefly, IP_3 receptor was purified using two affinity chromatography steps, heparin-agarose and concanavalin A-Sepharose. Purified receptor protein was used for either radioligand binding or reconstitution. For reconstitution, the purified receptor protein was mixed with sonicated solubilized lipids, and the mixture was dialyzed to effect detergent removal and vesicle formation.

$^{45}\text{Ca}^{2+}$ Flux. Reconstituted proteoliposomes were assayed for IP_3 -stimulated $^{45}\text{Ca}^{2+}$ flux as described (6). Briefly, vesicles were incubated (for 8 sec in routine experiments) in the presence of tracer amounts of $^{45}\text{Ca}^{2+}$ in the presence or absence of IP_3 and ATP or other nucleotides. The flux reaction was stopped by the addition of excess buffer containing unlabeled divalent cations and heparin (100 $\mu\text{g}/\text{ml}$). Finally, intravesicular $^{45}\text{Ca}^{2+}$ content was determined by immediately passing the vesicle/buffer mixture over a cation-exchange column (Dowex 50W, Sigma). The vesicles were collected and their intravesicular $^{45}\text{Ca}^{2+}$ content was measured by scintillation spectrometry.

Radioligand Binding. All ligand binding was assayed by precipitation of solubilized proteins with polyethylene glycol using gamma globulin as carrier protein. For [^3H] IP_3 binding, purified receptor (0.5 μg) was mixed with 3 nM [^3H] IP_3 in 50 mM Tris (pH 8.4, 25°C) plus 1 mM EDTA and incubated at 25°C for 15 min in a final volume of 250 μl . The incubation was stopped by the addition of 50 μl of 0.5% gamma globulin and 600 μl of ice-cold 30% polyethylene glycol (average molecular weight, 8000). This mixture was then centrifuged for 5 min at 4°C. The supernatant was aspirated, and the radioactivity in the protein pellet was measured by liquid scintillation spectrometry. Nonspecific binding was measured in the presence of 1 μM IP_3 . [^{35}S]ADP[βS] binding was assayed as described above except that more receptor was used (15 μg), the concentration of radioligand was 0.4 nM, the pH of the Tris buffer was 7.4 (25°C), and nonspecific binding was defined in the presence of 50 μM ADP[βS] or ATP. [^{32}P]ATP (α or γ labeled) binding was measured exactly as for [^{35}S]ADP[βS] except that the concentration of radioligand was 10 nM.

RESULTS

Adenine Nucleotide Enhancement of IP_3 -Stimulated $^{45}\text{Ca}^{2+}$ Flux in Reconstituted Lipid Vesicles. As we have previously

Abbreviations: IP_3 , inositol 1,4,5-trisphosphate; ADP[βS], adenosine 5'-[β -thio]diphosphate (other nucleotide analogues are abbreviated similarly).

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shown (6), purified IP₃ receptor protein reconstituted into lipid vesicles catalyzes IP₃-dependent ⁴⁵Ca²⁺ flux. In the presence of micromolar concentrations of IP₃, Ca²⁺ flux into the reconstituted vesicles is enhanced severalfold (Fig. 1). At concentrations up to 1 mM, ATP by itself has no effect on ⁴⁵Ca²⁺ flux. By contrast, at low micromolar concentrations, ATP considerably augments the effect of IP₃. The maximal enhancement of Ca²⁺ flux by IP₃ is about 4-fold, whereas in the presence of ATP, IP₃ stimulates flux 6-fold. The time course of ⁴⁵Ca²⁺ flux is similar in the presence or absence of ATP, with at least half-maximal effects within 2 sec and a full plateau by 4–8 sec (Fig. 1).

The effect of ATP is strikingly concentration-dependent (Fig. 2). At a threshold concentration of 1 μM, ATP increases IP₃-induced Ca²⁺ flux 10–15%, while maximal enhancement of flux occurs at 10 μM ATP. At higher concentrations, the effect of ATP diminishes with no augmentation of IP₃ actions at 1 mM ATP. The concentration–response curves for both increased and decreased effects of ATP are precipitous. Almost all the enhancement of flux is apparent between 1 and 10 μM ATP. The great majority of the decline in the effect of ATP occurs between 0.1 and 1 mM ATP.

The influence of ATP on IP₃-elicited Ca²⁺ flux is similar with 5 μM and 100 μM IP₃. Thus, the diminished effect of ATP on IP₃-induced flux with increasing concentrations of ATP does not reflect a simple competition with IP₃.

As observed previously (6), IP₃-elicited Ca²⁺ flux is detectable at 3 nM and near maximal at 0.5 μM, with half-maximal effects at about 50 nM (Fig. 3). A similar concentration–response relationship occurs for IP₃ in the presence of 50 μM ATP, which augments the IP₃ effect to the same extent at all IP₃ concentrations.

The influence of ATP on IP₃-induced Ca²⁺ flux is the same in the presence of various cations (Table 1). The nonmetabolized analogue ATP[γS] has the same potency as ATP. Other nonmetabolized analogues of ATP, adenosine 5'-[β,γ-imido]triphosphate (ATP[β,γ-NH]) and adenosine 5'-[β,γ-methylene]triphosphate (ATP[β,γ-CH₂]), are somewhat weaker. ADP provides only about 40% of the response of ATP, whereas ADP[βS] is more active than ADP. AMP is virtually inactive, and AMP[αS] produces a slightly greater response than AMP. The effect of ATP is specific for adenine nucleotides, as GTP and GTP[γS] are ineffective (Table 1).

FIG. 1. Time course of IP₃-stimulated ⁴⁵Ca²⁺ flux in the presence or absence of ATP. Reconstituted proteoliposomes were incubated for the times indicated with 5 μM IP₃ by itself or plus 50 μM ATP. ATP (50 μM) alone did not influence ⁴⁵Ca²⁺ flux. Measurements in duplicate or triplicate varied less than 10%. This experiment has been repeated with similar results.

FIG. 2. Concentration–response relationship for ATP enhancement of IP₃-stimulated ⁴⁵Ca²⁺ flux. Vesicles were incubated for 8 sec with 5 or 100 μM IP₃ and various concentrations of Na₂ATP. ATP alone did not stimulate ⁴⁵Ca²⁺ flux at concentrations as high as 1 mM. Measurements in duplicate or triplicate varied less than 10%. This experiment has been performed four times with the same results.

The nucleotide structure is required, as adenosine is inactive (Table 1).

[³²P]ATP and [³⁵S]ADP[βS] Binding to Purified IP₃ Receptor. We have detected the ATP recognition site with [³²P]ATP or [³⁵S]ADP[βS]. Radioligand binding was assayed by incubating purified IP₃ receptor protein with the radioligands, precipitating protein with polyethylene glycol/gamma globulin, and determining the radioactivity in the pellet. The binding of [³⁵S]ADP[βS] to purified IP₃ receptor is saturable and reversible. Typical incubations were for 15 min at room temperature, since we determined that equilibrium is obtained rapidly (5–15 min) at 25°C. Binding is similar at 25°C and 4°C, though equilibrium is attained more slowly at 4°C (data not shown).

[³⁵S]ADP[βS] binding is inhibited by micromolar concentrations of ATP (Fig. 4). The pseudo-Hill coefficient is not significantly different from unity, indicating that [³⁵S]ADP[βS] binds to a single population of sites. The calculated dissociation constant (*K_d*) is 5.0 μM, and the *B_{max}* value is 1.4 nmol/mg of protein, identical to the *B_{max}* of [³H]IP₃ for the IP₃ receptor protein measured in parallel experiments. Thus, there seem to be equivalent numbers of binding sites for IP₃ and ATP in the purified protein preparation. However, it is

FIG. 3. Concentration–response relationship for IP₃-stimulated ⁴⁵Ca²⁺ flux in the presence or absence of 50 μM ATP. Reconstituted vesicles were incubated for 8 sec with various concentrations of IP₃ in the presence or absence of 50 μM ATP. ⁴⁵Ca²⁺ flux was determined in duplicate or triplicate with replicates varying less than 10%. This experiment has been performed four times with the same results.

Table 1. Nucleotide specificity for enhancement of IP₃-mediated ⁴⁵Ca²⁺ flux

Nucleotide	% maximum ATP stimulation	
	10 μ M	1 μ M
Na ₂ ATP	100	38
MgATP	99	25
TrisATP	100	25
K ₂ ATP	101	33
ATP[γ S]	102	35
ATP[β , γ -NH]	78	33
ATP[β , γ -CH ₂]	65	10
ADP	40	12
ADP[β S]	92	20
AMP	12	0
AMP[α S]	38	0
Adenosine	0	—
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Nucleotide	100 μ M	10 μ M
Na ₂ GTP	0	0
GTP[γ S]	0	0
cGMP	0	0

Vesicles were incubated in the presence or absence of IP₃ and ATP or other nucleotides as indicated. Measurements of ⁴⁵Ca²⁺ flux were made in duplicate with replicates varying less than 10%. This experiment has been repeated with the same results. None of the nucleotides had any effect in the absence of IP₃, even at the highest concentrations indicated.

conceivable that degradation of IP₃ and/or ATP recognition sites takes place during purification so that their apparent number in the pure receptor preparation may not necessarily reflect the *in situ* condition. ATP[γ S] displays about the same potency as ATP for the [³⁵S]ADP[β S] binding sites, whereas ADP and AMP are progressively less potent (Table 2). The relative affinities of ATP, ADP, and AMP for the [³⁵S]ADP[β S] binding sites closely resemble their relative potencies in enhancing IP₃-stimulated Ca²⁺ flux.

[³²P]ATP also binds saturably to the IP₃ receptor protein. The association rate and temperature dependence of

 Table 2. Specificity of [³²P]ATP and [³⁵S]ADP[β S] binding to purified IP₃ receptor

Competitor Conc.	μ M	% inhibition	
		[³² P]ATP	[³⁵ S]ADP[β S]
ATP[γ S]	50	100	100
	20	98	100
	2	56	51
ADP	0.2	0	2
	200	100	100
	20	78	75
AMP	2	24	20
	200	86	75
	20	58	50
IP ₃	2	4	0
	10	0	0

Radioligand binding was measured in duplicate with replicates varying less than 5%. This experiment has been repeated with similar results.

[³²P]ATP binding are similar to those of [³⁵S]ADP[β S] binding (data not shown). The *K_d* for [³²P]ATP binding is 2 μ M, similar to the affinity of ATP for sites labeled by [³⁵S]ADP[β S] (Fig. 4). The *B_{max}* for [³²P]ATP binding is 1.4 nmol/mg of protein, the same as the *B_{max}* for both [³⁵S]ADP[β S] and [³H]IP₃. The relative affinities of ATP, ATP[γ S], ADP, and AMP at the sites labeled by [³²P]ATP are essentially the same as at [³⁵S]ADP[β S] binding sites (Table 2). IP₃ (5 μ M) has no effect on the binding of either [³²P]ATP or [³⁵P]ADP[β S] (Table 2).

To ensure that the effects of ATP on IP₃-induced Ca²⁺ flux and the binding of [³²P]ATP were not associated with ATP hydrolysis, we incubated 10 μ M [³²P]ATP for 30 min at 25°C with either purified IP₃ receptor protein or the receptor reconstituted into lipid vesicles under the binding or flux assay conditions, respectively. Intact ATP was precipitated with charcoal under conditions in which any free phosphate does not precipitate. Hydrolysis of ATP was not detected in either the purified protein preparation itself or the protein incorporated into vesicles in either the presence or the absence of 5 μ M IP₃.

DISCUSSION

The major finding of this study is that IP₃-induced Ca²⁺ flux is regulated selectively and potently by ATP via an allosteric site on the IP₃ receptor protein. This effect cannot involve ATP metabolism for several reasons. The influence of ATP is maximal in incubations conducted for 2 sec at 4°C. Also, MgATP, the form metabolized by ATPases, has the same effect as Na₂ATP. Moreover, nonmetabolized derivatives of ATP as well as other adenine nucleotides produce similar effects as ATP itself. Finally, we cannot detect ATP hydrolysis under the assay conditions in these preparations. The selectivity of the ATP enhancement of IP₃ for adenine rather than guanine nucleotides differs markedly from actions of nucleotides on ligand binding to neurotransmitter and hormone receptors linked to GTP-binding proteins. While the ATP effects observed here employed a purified and reconstituted receptor protein, ATP is likely to play a similar regulatory role *in vivo*. Thus, in a permeabilized smooth muscle cell preparation, the ability of IP₃ to release Ca²⁺ was not detectable when ATP was removed from the incubation medium, and Ca²⁺ release could be restored with nonmetabolized analogues of ATP (10). Moreover, Ehrlich and Watras (11) observed a doubling in the open probability of an IP₃-regulated Ca²⁺ channel from aortic smooth muscle microsomes in the presence of 100 μ M ATP or a nonmetabolized ATP analogue.

What might be the physiological role for ATP regulation of IP₃-induced Ca²⁺ release? IP₃ releases Ca²⁺ from stores that

FIG. 4. Concentration-response relationship for ATP inhibition of [³²P]ATP and [³⁵S]ADP[β S] binding to purified IP₃ receptor. Radioligand binding was determined by polyethylene glycol precipitation. Nonspecific binding was measured in the presence of 50 μ M ATP or ATP[γ S]. Total binding for 10 nM [³²P]ATP was typically 1000 cpm, while nonspecific binding was 600 cpm; for 0.4 nM ADP[β S] total binding was typically 3000 cpm and nonspecific binding about 2500 cpm. Results shown were obtained with [γ -³²P]-ATP; similar results were obtained with [α -³²P]ATP. Measurements were made in duplicate with replicates varying less than 5%. This experiment has been performed three times with similar results.

FIG. 5. Regulation of IP_3 receptors by ATP and Ca^{2+} may contribute to intracellular Ca^{2+} oscillations. IP_3 binding to its receptor triggers Ca^{2+} release, lowering endoplasmic reticulum stores of Ca^{2+} and thereby stimulating hydrolysis of ATP by the Ca^{2+} pump. Local depletion of ATP by the Ca^{2+} pump reduces ATP concentration to micromolar levels that facilitate IP_3 release of Ca^{2+} . Ca^{2+} released by IP_3 binds to the membrane protein calmodulin (17), which inhibits IP_3 interactions with its receptor, thereby lowering the concentration of free Ca^{2+} .

are normally filled by the Ca^{2+} -ATPase pump. Autoradiographic studies show that IP_3 releases Ca^{2+} from approximately two-thirds of the sites in the brain wherein Ca^{2+} is accumulated via this pump mechanism (12). Sites of IP_3 -induced Ca^{2+} release correspond closely to the localization of IP_3 receptor binding sites as well as to one type of Ca^{2+} pump localized by *in situ* hybridization (12). Physiological levels of IP_3 can release the majority of Ca^{2+} stored within the IP_3 -sensitive pools in peripheral tissues as well as brain (1, 2). For dynamic cellular signaling, these pools must be rapidly refilled by the ATP-requiring Ca^{2+} pump. ATP modulation of IP_3 -induced Ca^{2+} release could provide a signaling mechanism between the Ca^{2+} -ATPase and the IP_3 receptor. ATP maximally enhances IP_3 -induced Ca^{2+} flux at concentrations of 10–100 μM ATP, which are lower than resting physiological levels. Physiological concentrations of ATP, about 1 mM, no longer enhance IP_3 -induced Ca^{2+} flux. Rapid filling of Ca^{2+} stores by the Ca^{2+} -ATPase may deplete local ATP levels to submillimolar values, facilitating IP_3 -induced Ca^{2+} release. As the local ATP levels return to physiological values, IP_3 release of Ca^{2+} would diminish.

Receptor-mediated intracellular Ca^{2+} release often elicits marked oscillations in the cytoplasmic level of free Ca^{2+} (13, 14), which may be elicited by fixed concentrations of IP_3 or its nonmetabolized thiol derivative (15). Variations in Ca^{2+} release by IP_3 in response to fluctuating intracellular ATP levels may contribute to these oscillations. Another important factor in such oscillations may be the potent inhibition of IP_3 binding to its receptors by submicromolar concentrations of Ca^{2+} (16, 17). These two mechanisms might act in concert (Fig. 5). Following agonist-induced Ca^{2+} release by IP_3 , the Ca^{2+} -ATPase would be activated to refill the Ca^{2+} stores.

Diminished ATP levels associated with ATPase pump activity would then further augment Ca^{2+} release by IP_3 . The precipitous, positively cooperative concentration-response relationship between ATP concentration and IP_3 -induced Ca^{2+} release may contribute to the sharp increase in intracellular Ca^{2+} associated with Ca^{2+} oscillations. Released Ca^{2+} , acting via the membrane protein calmodulin (17), would inhibit IP_3 receptor binding and Ca^{2+} release, initiating a decline in intracellular free Ca^{2+} . As endoplasmic reticulum stores of Ca^{2+} increase, Ca^{2+} pump activity would diminish, raising local ATP concentration and further reducing IP_3 release of Ca^{2+} . The concentration-response relationship for this aspect of ATP regulation of IP_3 actions is also precipitous, helping to explain the abrupt declines in free Ca^{2+} concentration that are associated with its oscillations.

The ryanodine receptor, which mediates Ca^{2+} release by sarcoplasmic reticulum and structurally resembles the IP_3 receptor protein (7, 9), is also regulated by ATP (18–21). Although ATP by itself does not stimulate Ca^{2+} flux from reconstituted IP_3 receptors, ATP alone does open the Ca^{2+} channel of ryanodine receptors. The action of ATP on ryanodine receptors occurs at micromolar Ca^{2+} levels, consistent with the ryanodine receptor mediating excitation-contraction coupling of skeletal muscle by a Ca^{2+} -induced Ca^{2+} release (22). Thus, both ryanodine and IP_3 receptors are regulated by Ca^{2+} , but whereas Ca^{2+} inhibits IP_3 receptor interactions, it facilitates Ca^{2+} release at the ryanodine receptor. Both IP_3 and ryanodine receptors are very large proteins of similar native molecular mass (1000–1500 kDa) composed of four identical subunits (3, 23). Both bind to heparin and concanavalin A with high affinity. The three-dimensional structures of both receptors comprise at least four C-terminal membrane-spanning regions with a very large cytoplasmic N-terminal region and significant homology in the membrane-spanning region that may contribute to the channel structure (7, 9). The present work provides further evidence that IP_3 and ryanodine receptors are members of a new class of intracellular ligand-gated ion channels.

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