ATP Regulation of Type 1 Inositol 1,4,5-Trisphosphate Receptor Channel Gating by Allosteric Tuning of Ca\textsuperscript{2+} Activation*

(Received for publication, April 1, 1999, and in revised form, May 17, 1999)

Don-On Daniel Mak\dagger, Sean McBride\dagger, and J. Kevin Foskett\ddagger§

From the \daggerDepartment of Physiology and \ddaggerInstitute for Human Gene Therapy, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6100

Inositol 1,4,5-trisphosphate (InsP\textsubscript{3}) mobilizes intracellular Ca\textsuperscript{2+} by binding to its receptor (InsP\textsubscript{3}R), an endoplasmic reticulum-localized Ca\textsuperscript{2+} release channel. Patch clamp electrophysiology of Xenopus oocyte nuclei was used to study the effects of cytoplasmic ATP concentration on the cytoplasmic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) dependence of single type 1 InsP\textsubscript{3}R channels in native endoplasmic reticulum membrane. Cytoplasmic ATP free-acid ([ATP])\textsubscript{i}, but not the MgATP complex, activated gating of the InsP\textsubscript{3}R-ligated InsP\textsubscript{3}R, by stabilizing open channel state(s) and destabilizing the closed state(s). Activation was associated with a reduction of the half-maximal activating [Ca\textsuperscript{2+}]\textsubscript{i} concentrations, to 500 ± 50 nM in 0 [ATP]\textsubscript{i}, to 29 ± 4 nM in 9.5 mM [ATP]\textsubscript{i}, with apparent ATP affinity = 0.27 ± 0.04 mM, similar to in vivo concentrations. In contrast, ATP was without effect on maximum open probability or the Hill coefficient for Ca\textsuperscript{2+} activation. Thus, ATP enhances gating of the InsP\textsubscript{3}R by allosteric regulation of the Ca\textsuperscript{2+} sensitivity of the Ca\textsuperscript{2+} activation sites of the channel. By regulating the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release properties of the InsP\textsubscript{3}R, ATP may play an important role in shaping cytoplasmic Ca\textsuperscript{2+} signals, possibly linking cell metabolic state to important Ca\textsuperscript{2+}-dependent processes.

Modulation of free cytoplasmic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) is a ubiquitous cellular signaling system. In many cell types, binding of ligands to plasma membrane receptors activates the hydrolysis of phosphatidylinositol 4,5-bisphosphate by membrane-bound phospholipase C, generating inositol 1,4,5-trisphosphate (InsP\textsubscript{3}). InsP\textsubscript{3} causes the release of Ca\textsuperscript{2+} from the endoplasmic reticulum (ER) by binding to its receptor (InsP\textsubscript{3}R), which itself is a Ca\textsuperscript{2+} channel (1–3). Complex control of Ca\textsuperscript{2+} release through the InsP\textsubscript{3}R by various intracellular factors, including cooperative activation by InsP\textsubscript{3} (4–8) and biphasic feedback from the permeant Ca\textsuperscript{2+} ion (6, 8–11) generates intricate [Ca\textsuperscript{2+}]\textsubscript{i} signals that can be manifested temporally as repetitive spikes or oscillations, with frequencies often tuned to the level of stimulation, and spatially as propagating waves or highly localized events (2, 12, 14) and display properties of “adaptation” and “quanta release,” which are poorly understood (15). Several types of InsP\textsubscript{3}R as products of different genes with alternatively spliced isoforms have been identified and sequenced (16, 17). The InsP\textsubscript{3}Rs have about 2700 amino acid residues in InsP\textsubscript{3} binding, regulatory (modulatory) and transmembrane channel domains (16–18). The sequences of the regulatory domains of all InsP\textsubscript{3}R isoforms include putative ATP-binding site(s) (17). ATP was shown to bind to the InsP\textsubscript{3}R (19) and regulate InsP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release (20–24), although the detailed mechanisms of this regulation remain unclear.

Several studies have demonstrated that mitochondria and the ER are in close physical and functional proximity in many cell types, including neurons (24–27). [Ca\textsuperscript{2+}]\textsubscript{i}, signals generated by InsP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release from the ER appear to be rapidly and efficiently transmitted to mitochondria (28–30), acutely affecting mitochondrial functions (31–33), including ATP synthesis (34). It is unknown, however, whether the communication between these two organelles is reciprocal. ATP release from mitochondria, globally into the cytoplasm and locally into the vicinity of the InsP\textsubscript{3}R channels that are in close apposition, may provide a signaling pathway for communication from the mitochondria back to the ER. Thus, regulation of the InsP\textsubscript{3}R by ATP could have considerable significance for intracellular signaling, particularly if the channel is sensitive to ATP levels in normal physiological as well as pathological conditions, including ischemia.

Most previous studies of ATP regulation of the InsP\textsubscript{3}R have been limited to indirect measurements, i.e. Ca\textsuperscript{2+} fluxes or concentrations, to infer InsP\textsubscript{3}R channel activity, because the intracellular location of the Ca\textsuperscript{2+} release channel has limited its accessibility to electrophysiological approaches. Furthermore, only a limited range of [Ca\textsuperscript{2+}]\textsubscript{i}, was examined in previous studies, despite the fact that the InsP\textsubscript{3}R is intricately regulated by [Ca\textsuperscript{2+}]\textsubscript{i} (6, 8–11) and that the primary known regulator of the channel, InsP\textsubscript{3}, mediates its effects by modulating the [Ca\textsuperscript{2+}]\textsubscript{i} dependence of channel gating (8). Therefore, in the present study, we have systematically investigated the effects of cytoplasmic ATP concentration on the [Ca\textsuperscript{2+}]\textsubscript{i} response of single InsP\textsubscript{3}R channels. We applied the patch clamp technique to isolated Xenopus oocyte nuclei (35–37) to study the single channel activities of the type 1 InsP\textsubscript{3}R (InsP\textsubscript{3}R-1), the major brain isoform (38, 39), in its native ER membrane environment under rigorously defined conditions on both the cytoplasmic and luminal sides of the channel. Our results demonstrate that cytoplasmic ATP free-acid, but not cytoplasmic MgATP complex, activates the gating of the InsP\textsubscript{3}R primarily by allosteric regulation of the [Ca\textsuperscript{2+}]\textsubscript{i} sensitivity of the Ca\textsuperscript{2+} activation sites of the channel. By regulating the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release properties of the InsP\textsubscript{3}R, ATP may play an important role in shaping the extent and duration of [Ca\textsuperscript{2+}]\textsubscript{i} signals, possibly linking cell metabolic state to important Ca\textsuperscript{2+}-dependent processes including synaptic plasticity.

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¶ To whom correspondence and reprint requests should be addressed: Dept. of Physiology, University of Pennsylvania, B39 ANAT-CHEM, Philadelphia, PA 19104-6085. Tel.: 215-888-1354; Fax: 215-573-6808; E-mail: foskett@mail.med.upenn.edu.

† The abbreviations used are: InsP\textsubscript{3}, inositol 1,4,5-trisphosphate; InsP\textsubscript{3}R, InsP\textsubscript{3} receptor; ER, endoplasmic reticulum; BAPTA, 1,2-bis(O-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; LTD, long term depression; mGluR, metabotropic glutamate receptor(s).
EXPERIMENTAL PROCEDURES

Patch Clamping the Oocyte Nucleus—Patch clamp experiments were performed as described in Refs. 8, 35, 36, and 40. Briefly, stage V or VI oocytes were opened mechanically just prior to use. The nucleus was separated from the cytoplasm and transferred to a dish on the stage of a microscope for patch-clamping. The oocyte expresses only a single InsP3R isoform (type 1) and lacks other (e.g., ryanodine receptor) Ca2+ release channels (41). Experiments were done in "on-nucleus" configuration, with the solution in the perinuclear lumen between the outer and inner nuclear membranes in apparent equilibrium with the bath solution (35) and with the cytoplasmic aspect of the InsP3R channel facing into the patch pipette. Following standard conventions, the applied potential is that of the pipette electrode minus the reference bath electrode (positive current flows from pipette outward). Experiments were performed at room temperature with the pipette electrode at +20 mV relative to the reference bath electrode.

Data Acquisition and Analysis—Single channel currents were amplified with an Axopatch-1D amplifier (Axon Instruments, Foster City, CA) with antialiasing filtering at 1 kHz, transferred to a Power Macintosh 8100 via an ITC-16 interface (Instrutech Corp., Great Lake, NY), digitized at 5 kHz, and written directly onto the hard disc by Pulse+PulseFit software (HEKA Elektronik, Lambrecht, Germany). Data were analyzed to identify channel opening and closing events and evaluate channel open probabilities using MacTac 3 (Bruxton, Seattle, WA). Each data point shown is the mean of results from at least four separate patch clamp experiments performed under the same conditions. Error bars indicate the S.E. Theoretical curves were fitted to the experimental data using Igor Pro 3 (WaveMetrics, Lake Oswego, OR).

Solutions for Patch Clamp Experiments—All patch clamp experiments were performed with solutions containing 140 mM KCl and 10 mM HEPES with pH adjusted to 7.1 using KOH. Since the luminal (Ca2+) or [ATP] have no systematic effects on the open probability response of the InsP3R (8), a bath solution containing no ATP and 250 mM NaCl was used in all experiments. Pipette solutions contained various concentrations of nucleotides (sodium salts of ATP, ADP, AMP, GTP, and UDP and adenosine, from Sigma) as specified. Because of chelation of Mg2+ by ATP, the actual free [Mg2+] and free [ATP] in the solutions containing Mg2+ and ATP were calculated by the MaxChelator software (C. Patton, Stanford University, Stanford, CA). By using K as the current carrier and appropriate quantities of the high affinity Ca2+ chelator, BAPTA (100–500 μM; Molecular Probes, Inc., Eugene, OR), the low affinity Ca2+ chelator, 5,5'-dibromo-BAPTA (100–350 μM; Molecular Probes), or just ATP (0 or 0.5 mM) to buffer [Ca2+] in the experimental solutions, [Ca2+] was tightly controlled in our experiments. Total Ca2+ content (60–370 μM) in the solutions was determined by induction-coupled plasma mass spectrometry (Mayo Medical Laboratory, Rochester, MN). Free [Ca2+] was calculated using the MaxChelator software. Pipette solutions contained 10 μM InsP3 (Molecular Probes).

RESULTS

Activation of Channel Gating by ATP—To examine the effects of ATP on the permeation and gating properties of the InsP3R, we included 10 μM InsP3, 250 mM free Ca2+, and 0.5 mM free ATP in the pipette solution. Under these conditions, the endogenous Xenopus type 1 InsP3R channel exhibited channel conductance properties and kinetics similar to those observed previously under similar conditions (8, 40). The channels gated with a moderately high open probability (Popen) of ~0.5 (Fig. 1A). In similar experiments employing pipette solutions that lacked ATP, the InsP3R channel Popen was significantly lower (~0.2) in either the absence or presence of 5 mM Mg2+ (Fig. 1, B and C). To determine whether the ATP activation of the channel Popen was mediated by MgATP, which could suggest a role for ATP hydrolysis or phosphorylation, similar experiments were undertaken in total Mg2+ and total MgATP in the pipette. Under these conditions, [MgATP] is approximately 0.5 mM, and the free Mg2+ concentration ([Mg2+]free) and the cytoplasmic free ATP concentration ([ATP]i) were calculated to be 2.5 and 0.012 mM, respectively. Nevertheless, Popen remained low (Fig. 1D). The low Popen in the presence of MgATP (Fig. 1) was solely caused by ATP complexation by Mg2++, since it was fully reversed by adding more ATP to the pipette solution to restore [ATP]i (Fig. 1E). Thus, MgATP has no effect, stimulatory or inhibitory, on InsP3R activity. We previously demonstrated that the Popen of the Xenopus type 1 InsP3R is independent of [Mg2+]up to 9.5 mM (40). Taken together, these results suggest that ATP free acid (ATP3− or ATP4−) was the relevant ionic species and that ATP hydrolysis was not involved in the stimulation of InsP3R channel gating.

Effects of ATP on the [Ca2+]i Dependence of InsP3R Gating—InsP3R activates the InsP3R by modulating the sensitivity of the channel to [Ca2+]i. To determine the mechanism of ATP activation of the InsP3R channel gating, we investigated in detail the effects of ATP on the channel kinetics of the InsP3R over a wide range of [Ca2+]i. A systematic series of patch clamp experiments were performed using pipette solutions containing various [Ca2+]i, with 0.5 mM ATP alone, 3 mM Mg2+ alone, 0.5 mM ATP and 3 mM Mg2+ (calculated [ATP]i = 0.012 mM; calculated [Mg2+]i = 2.5 mM) or no ATP or Mg2+. To avoid possible effects of Ca2+ on InsP3, binding, a functionally saturating InsP3 concentration of 10 μM was used (8). The [Ca2+]i sensitivity of the InsP3R in the absence of cytoplasmic free ATP was biphasic (Fig. 2) and could be well fitted with a biphasic Hill equation similar to the following one previously derived for the InsP3R in the presence of 0.5 mM cytoplasmic free ATP (8).

\[
P_{act} = P_{max}[1 + (K_{Ca}(Ca^{2+})^n)^{-1}](1 + (Ca^{2+}/K_{Ca})^{Hill})^{-1}. \tag{1}
\]

Similar results were obtained independent of the presence or absence of either Mg2+ or MgATP in the pipette solutions. This result indicates that the InsP3R can achieve a maximum open probability Pmax of 0.79 in the absence of cytoplasmic free ATP, a level of activity very similar to the Pmax of 0.81 found in the presence of 0.5 mM cytoplasmic free ATP (8). Thus, ATP does not activate the channel by increasing Pmax. The Hill coefficient for Ca2+ activation Hhill was 2.4 ± 0.6 in the absence of free ATP, similar to Hhill, and was calculated to be 1.9 ± 0.3 in the presence of 0.5 mM free ATP. This result suggests that Ca2+ probably activates the InsP3R via the same cooperative process in either the presence or absence of cytoplasmic free ATP. Thus, ATP does not activate the channel by modulating Hhill. The observed activation of the InsP3R by cytoplasmic free ATP (Fig. 1) was associated with a reduction of the half-maximal activating [Ca2+]i (KCa) from 500 ± 50 nM in the absence of free ATP to 190 ± 20 nM in the presence of 0.5 mM free ATP. Thus, ATP activates the
channel by sensitizing it to $\text{Ca}^{2+}$. ATP therefore enhances $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$ release (CICR) by the InsP$_3$R.

Because InsP$_3$ activates channel gating by modifying the $[\text{Ca}^{2+}]$, inhibition phase of the channel $[\text{Ca}^{2+}]$, dependence (8), we were also interested in examining the effects of ATP on this aspect of the response. However, investigations of InsP$_3$R channel activity in the absence of free ATP at $[\text{Ca}^{2+}]$, which inhibit channel gating (>20 mM; Ref. 8) were not possible because of the unavailability of a $\text{Ca}^{2+}$ chelator with the appropriate $\text{Ca}^{2+}$ affinity. In our previous experiments that examined the effects of high $[\text{Ca}^{2+}]$, on InsP$_3$R channel gating (8), ATP was used as the $\text{Ca}^{2+}$ chelator for buffering $[\text{Ca}^{2+}]$ at high $[\text{Ca}^{2+}]$. The data we were able to obtain in the absence of ATP in the present study indicated that $P_o$ began to decrease as $[\text{Ca}^{2+}]$ was increased beyond 10 mM, but the inhibitory half-maximal $[\text{Ca}^{2+}]$, $K_{inh}$, or Hill coefficient, $H_{inh}$, could not be determined accurately (Fig. 2).

**Effects of ATP on InsP$_3$R Channel Kinetics—**Analysis of the mean open and closed durations of the InsP$_3$R revealed that the mean open duration ($\tau_o$) in the absence of free ATP lay within a narrow range between 5 and 15 ms over a wide range of $[\text{Ca}^{2+}]$, (1–10 μM). At both very low (<400 mM) or very high (>10 μM) $[\text{Ca}^{2+}]$, $\tau_o$ was shorter (~3 ms) (Fig. 3). In contrast, the mean closed duration ($\tau_c$) in the absence of free ATP decreased about 2 orders of magnitude, from 200 to 3 ms, as $[\text{Ca}^{2+}]$, was increased from 200 to 1 μM. $\tau_c$ remained low between 1 and 10 μM $[\text{Ca}^{2+}]$, (Fig. 4). These same basic kinetics were observed in all experiments conducted in the absence of cytoplasmic free ATP, regardless of the presence or absence of free Mg$^{2+}$ or MgATP complex. Similar $[\text{Ca}^{2+}]$, dependences of $\tau_o$ and $\tau_c$ (Figs. 3 and 4) were also observed in the presence of 0.5 mM free ATP (8).

An examination of the differences in the $[\text{Ca}^{2+}]$, dependences of $\tau_o$ and $\tau_c$, or either the presence and absence of cytoplasmic free ATP reveals that the mechanism whereby free ATP enhances channel activity is by stabilization of the open channel state(s) and destabilization of the closed channel state(s) in the low $[\text{Ca}^{2+}]$, regime (30–500 μM). Our more limited data indicate that free ATP may also stabilize the open channel state(s) at very high $[\text{Ca}^{2+}]$, (>15 μM).

**ATP Concentration Dependence of $\text{Ca}^{2+}$ Activation of InsP$_3$R Gating—**We undertook a systematic study of the activation of the InsP$_3$R by $[\text{Ca}^{2+}]$, over a wide range of free [ATP]$. In the presence of 10 μM InsP$_3$, the activating Hill equation (Equation 2) agreed well the experimental data for [ATP], 4.8 and 9.5 mM, with no significant effects of [ATP] on $H_{act}$ or $P_{max}$ (Fig. 5).
Importantly, the gating of the channel is regulated by both optimal conditions, gating of the channel is robust, with maximal activity being dependent on the presence of a high concentration of MgATP in the cytoplasm. The regulation of the channel is biphasic, with half-maximal activity occurring at 10 μM MgATP, and in the presence of 0.5 mM MgATP, the channel is activated. The effects of nucleotides on InsP3-induced Ca2+ fluxes were also studied, and the results indicate that ATP stimulates gating of the InsP3R by modulating the Ca2+ sensitivity of the Ca2+ activation sites.

**DISCUSSION**

We previously described the detailed permeation and gating properties of the Xenopus type 1 InsP3R channel by patch clamp studies of isolated oocyte nuclei (8, 35, 36, 40). Under optimal conditions, gating of the channel is robust, with maximum open probability of ~80% over a wide range of [Ca2+]i (8). Importantly, the gating of the channel is regulated by both InsP3 as well as by [Ca2+]i. The regulation of the InsP3-ligated channel activity by [Ca2+]i is biphasic, with half-maximal activation at 210 nM and half-maximal inhibition at 45 μM in 10 μM InsP3. InsP3 binds noncooperatively to a high affinity site (KD ~ 50 nM) on each monomer of the channel tetramer. Binding of InsP3 to the channel has the sole effect of decreasing the Ca2+ affinity of the Ca2+ inhibition site on each monomer, in a process which has high cooperativity (Hill coefficient of 4) (8). When cytoplasmic InsP3 concentration ([InsP3]i) is low, under conditions of no or weak stimulation, the channel is inhibited by relatively low [Ca2+]i, whereas it becomes much less sensitive to Ca2+ inhibition at higher [InsP3]i, enabling it to become activated. Thus, InsP3 activates the channel by tuning the inhibition efficacy of the Ca2+ ligand. Of particular significance, [Ca2+]i, activation of the channel was not modified by InsP3. However, it is unknown whether other modulators of InsP3R activity similarly impinge on the Ca2+ inhibition properties of the channel or whether the Ca2+ activation properties of the channel are exploited as an alternate method of channel regulation. The results from the present study suggest that ATP stimulates gating of the InsP3R by modulating the Ca2+ sensitivity of the Ca2+ activation sites.

**Regulation of InsP3-mediated Ca2+ Release by ATP**—We performed a systematic investigation of the effects of nucleotides on gating of the Xenopus type 1 InsP3R Ca2+ release channel. Our study focused on the effects of ATP on single channel activity, and included additional examination of effects of other nucleotides for comparison. Stimulation of InsP3R by nucleotides in the presence of InsP3 has been previously reported (20–23, 42, 43). The majority of published studies measured the effects of nucleotides on InsP3-induced Ca2+ fluxes either from intracellular stores in permeabilized cells or into lipid vesicles in vitro. ATP stimulation of InsP3-induced Ca2+ fluxes was reported in all these studies, but the considerable qualitative as well as quantitative variability among them makes comparison with our results difficult. Maximum stimulation by ATP of Ca2+ release by the InsP3R has been reported to range from 1.5- to 2-fold (20–22) the activity with InsP3 alone. Considerable discrepancies exist regarding the ATP concentration required for maximal stimulation, ranging from 10 μM (20) to 1 mM (21, 22). In terms of the nucleotide specificity, ADP was reported to be as potent as ATP (21, 22) or to be only 40% as effective (20) or ineffective (24); AMP has been reported to stimulate by ~70% (21, 22) or 10% (20) of the ATP stimulation level; GTP was ineffective (21) or stimulated to 30% of the ATP stimulation level (22). Regarding the specific ATP species, MgATP complex was as effective as free ATP (20) or only 50% as effective (21).

Some reports indicate that the effects of ATP on InsP3R activity are biphasic, being stimulatory at low [ATP], and inhibitory at high [ATP] (20–23). The concentrations at which increased [ATP], starts to reduce InsP3R activity have varied from 0.1 (20) to 1 mM (21, 22) in Ca2+ flux experiments. In
bilayer experiments, ATP concentrations of >5 mM inhibited channel activity with a half-maximal concentration of 11 mM (23). In contrast, recent Ca$$^{2+}$$ flux studies found no inhibitory effects of ATP at 5 (24) or 10 mM (42) on InsP$_3$-induced Ca$$^{2+}$$ release from the ER. The results from our nuclear patch clamp experiments are in agreement with these latter studies. We detected no inhibitory effects of cytoplasmic ATP up to 10 mM. It has been suggested that the inhibitory effects of high [ATP]$_i$ are caused by competitive inhibition of InsP$_3$ binding to the InsP$_3$R (23). Therefore, the different observations may be caused by different [InsP$_3$]$_i$ used in these studies, ~5 ~mM in studies reporting ATP inhibition (20–23) and >5 ~mM in studies reporting no ATP inhibition (Refs. 24 and 42 and this study).

Stimulation of Single Channel Gating of the InsP$_3$R by ATP—Many of the discrepancies in the results of Ca$$^{2+}$$ flux studies may have been caused by species differences, different concentrations of InsP$_3$, or other important parameters, including Ca$$^{2+}$$ and other divalent cations (i.e. Mg$$^{2+}$$) whose concentrations in the vicinity of the InsP$_3$R might not have been adequately controlled. Furthermore, the measurements of Ca$$^{2+}$$ fluxes involved populations of unknown numbers and multiple types of InsP$_3$R, which may also have contributed to the discrepant results (42). Importantly, the effects of nucleotides on the activity of the InsP$_3$R on the single molecule level can only be inferred from these studies. There has been only one previous detailed investigation of the effects of ATP on single channel activity of the InsP$_3$R (23). In the presence of 0.2 ~mM [Ca$$^{2+}$$], and 2 ~mM InsP$_3$, ATP enhanced the Po of canine cerebellum type 1 InsP$_3$R reconstituted from microsomes into artificial planar lipid bilayers with a binding coefficient of 40 ~mM and Hill coefficient of 1. The MgATP complex was as effective as free ATP, whereas GTP was only 20% as effective and AMP was ineffective.

The effects of nucleotides on the Xenopus type 1 channel observed by nuclear patch clamp in the present study have some similarities but also differ in several important respects. In agreement with the results from the bilayer study, the Hill coefficient for ATP activation was also 1 in our study, although in agreement with the results from the bilayer study, the Hill coefficient for ATP activation was also 1 in our study, although it is interesting to consider the similarities involved in the regulation of channel gating by ATP and InsP$_3$. As mentioned above, InsP$_3$ is a channel activator because it decreases the affinity of the Ca$$^{2+}$$ inhibition site of the channel. It is important to note, however, that it is not the absolute magnitude of the Ca$$^{2+}$$ affinity of that site that is critical for InsP$_3$R activity, but rather its relationship to that of the Ca$$^{2+}$$ activation site.
The higher Ca$^{2+}$ affinity of the inhibition site keeps the channel inactive in the absence of InsP$_3$. However, the Ca$^{2+}$ affinity of the inhibition site becomes less than that of the activation site when the channel binds InsP$_3$. It follows, therefore, that an alternate mechanism to activate the channel would be to increase the Ca$^{2+}$ affinity of the activation site. Because this is a major effect of ATP, we speculate that suitable conditions could be defined in which channel gating could be activated by an increase in [ATP], without any change in [InsP$_3$]. Because the relative affinities for Ca$^{2+}$ of the InsP$_3$R activation and inhibition sites is the critical factor in determining the level of channel activity, it follows that allosteric regulation of antagonistic Ca$^{2+}$-binding sites by ATP and InsP$_3$, by together tuning the Ca$^{2+}$ dependence of channel gating, render the Ca$^{2+}$ dependence of Ca$^{2+}$ release by the InsP$_3$R a dynamic property, dependent upon stimulus intensity and cell metabolic state.

**Physiological Implications**—The results of our study suggest that complex features of InsP$_3$-induced [Ca$^{2+}$], signals will be dependent upon an elaborated regulation of Ca$^{2+}$ release through the InsP$_3$R by [Ca$^{2+}$], [InsP$_3$], and [ATP]. Importantly, modulation of channel activity by both ATP and InsP$_3$ is achieved by regulating the [Ca$^{2+}$]$_i$ dependence of channel gating. The interplay between [ATP], and [Ca$^{2+}$]$_i$, in the control of InsP$_3$R channel activities observed in the present study probably has important physiological significance, particularly if the InsP$_3$R actually experiences various [ATP]. Our results indicate that the apparent affinity of the ATP-binding site on the InsP$_3$R is physiologically relevant, since it approximates the free ATP concentration in the cytoplasm. Estimates of total Mg$^{2+}$ (44, 45) and total ATP (46–48) concentrations in cells are each in the range of 5–10 mM. Assuming equal concentrations of each, the free ATP concentration is calculated to vary roughly from 420 $\mu$M (5 mM total Mg$^{2+}$) to 540 $\mu$M (8 mM total Mg$^{2+}$). For comparison, the apparent affinity of the ATP-binding site on the InsP$_3$R was determined in the present study to be 270 $\mu$M. Interestingly, not only is the apparent affinity of the ATP-binding site on the InsP$_3$R coincident with the normal cytoplasmic free ATP concentration, but changes in free ATP concentration are very sensitive to changes in total ATP concentration in the cytoplasm. For example, at 5 mM total Mg$^{2+}$, an increase of total ATP concentration from 5 to 5.5 mM (10% change) will result in a 1.5-fold increase in the concentration of free ATP (from 420 $\mu$M to 600 $\mu$M). Thus, relatively small changes in total cytoplasmic ATP can have pronounced effects on free ATP concentration and, therefore, on the CICR properties of the InsP$_3$R. Thus, our results suggest that the channel is poised in vivo to respond to changes in the free ATP concentration, for example those that may occur during ischemia. Therefore, the nucleotide sensitivity may enable Ca$^{2+}$ release properties of the InsP$_3$R to be tuned to the metabolic state of the cell.

It has become evident from several recent studies that mitochondria and the ER form a tightly coupled, complex signaling unit. Imaging studies have revealed that mitochondria are in close physical proximity to the ER (25), especially to sites of Ca$^{2+}$ release (26). In cerebellar Purkinje cells, ER cisternae containing high densities of InsP$_3$R-1 are often wrapped around or closely apposed to mitochondria (49, 50). A physiological implication of this structural arrangement is that it enables the rise in [Ca$^{2+}$] in the ER, to locally high [Ca$^{2+}$] in the microdomain of the release channels and rapid uptake of released Ca$^{2+}$ by the mitochondria (28–30, 33, 51). The resulting changes in mitochondrial matrix [Ca$^{2+}$] affect the mitochondrial membrane potential (32) and the activities of the mitochondrial dehydrogenases that are crucial in ATP synthesis and intracellular ATP levels (27, 31, 33, 34, 52). The cytosolic ATP concentration in turn affects processes that contribute to [Ca$^{2+}$]$_i$ regulation, including InsP$_3$-induced Ca$^{2+}$ release (Refs. 20–24 and 43 and this study), passive leak from Ca$^{2+}$ stores (53), plasma membrane store-operated Ca$^{2+}$ entry (24, 54, 55), and Ca$^{2+}$ extrusion and uptake into the ER by Ca$^{2+}$-ATPases (24, 56, 57). Thus, in addition to buffering [Ca$^{2+}$], directly by active Ca$^{2+}$ sequestration and export (58–62), mitochondria indirectly participate in intracellular Ca$^{2+}$ signaling, using cytosolic ATP as a global cytoplasmic messenger. We speculate that the close physical proximity of mitochondria and ER may enable local changes in ATP concentration, due to release from mitochondria into the microdomains of close ER-mitochondria apposition, to rapidly effect local InsP$_3$R-mediated Ca$^{2+}$ release. Of significance, the ATP released by mitochondria is free ATP, the InsP$_3$R ligand, not MgATP (63). Thus, communication between these two organelles may be two-way, with local Ca$^{2+}$ release as the currency of communication from ER to mitochondria and local ATP release providing the cross-talk from mitochondria to ER.

The phosphoinositide signaling system is highly expressed throughout the brain (64, 65). Recent observations suggest that Ca$^{2+}$ release from type 1 InsP$_3$ receptors is involved in nerve growth (66) and synaptic plasticity, including long term potentiation (67–69) and depression (LTD) (67, 67, 70–72). Disruption of the mouse InsP$_3$R-1 gene eliminates LTD in the cerebellum (70), and the competitive InsP$_3$R inhibitor heparin blocks LTD in the neocortex (73). Metabotropic glutamate receptors (mGluR), which couple to the InsP$_3$ signaling pathway, have been implicated in synaptic plasticity (65, 69, 73), and mice with targeted disruption of mGluR1 show impaired LTD (74, 75). InsP$_3$-mediated LTD in Purkinje cell dendrites was recently shown to be spatially restricted to sites where both mGluR and InsP$_3$R are located (71). Ca$^{2+}$ influx through N-methyl-d-aspartate receptors and voltage-gated channels is considered to be of major importance in synaptic plasticity (65). Synaptic plasticity in several different brain regions requires both Ca$^{2+}$ entry and mGluR/InsP$_3$R (72, 76, 77), and it been suggested that Ca$^{2+}$ influx might serve to trigger Ca$^{2+}$ release by CICR (65, 69, 78), with InsP$_3$Rs therefore playing a critical role in amplifying the Ca$^{2+}$ influx signal (65). Importantly, [Ca$^{2+}$]$_i$, signaling in nonexcitable cells is also associated with both Ca$^{2+}$ release from stores and Ca$^{2+}$ influx, and Ca$^{2+}$ influx has been demonstrated to play a similar role in amplifying and modifying InsP$_3$-mediated [Ca$^{2+}$]$_i$ signals (79). By demonstrating that the Ca$^{2+}$ sensitivity of CICR by the InsP$_3$R-1 can be regulated, our data raise the possibility that synaptic plasticity and other cellular processes involving InsP$_3$Rs may be modulated by physiological stimuli that impinge on the Ca$^{2+}$ sensitivity of the release channel. The results of the present study suggest that cytosolic ATP, and therefore the metabolic status of the cell, may be relevant in this respect. Of note, phospholipase C activity in brain tissue is enhanced in response to ischemia (80), and exposure of hippocampal slices to anoxia (81) or 2-deoxyglucose (13), manipulations expected to alter cytoplasmic ATP concentrations, induce long term potentiation. Directed studies of the role of cell metabolic state and cytosolic ATP concentrations in controlling InsP$_3$-mediated Ca$^{2+}$ release will be required to determine the relevance of ATP regulation of InsP$_3$R gating for synaptic plasticity as well as other cellular processes.

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