Regulation of the Type III InsP$_3$ Receptor by InsP$_3$ and ATP

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ABSTRACT   Many hormones and neurotransmitters raise intracellular calcium (Ca^{2+}) by generating InsP$_3$ and activating the inositol 1,4,5-trisphosphate receptor (InsP$_3$R). Multiple isoforms with distinct InsP$_3$ binding properties (Cardy et al., 1997) have been identified (Morgan et al., 1996). The type III InsP$_3$R lacks Ca^{2+}-dependent inhibition, a property that makes it ideal for signal initiation (Hagar et al., 1998). Regulation of the type III InsP$_3$R by InsP$_3$ and ATP was explored in detail using planar lipid bilayers. In comparison to the type I InsP$_3$R, the type III InsP$_3$R required a higher concentration of InsP$_3$ to reach maximal channel activity (EC$_{50}$ of 3.2 μM versus 0.5 μM for the types III and I InsP$_3$R, respectively). However, the type III InsP$_3$R did reach a 2.5-fold higher level of activity. Although activation by InsP$_3$ was isoform-specific, regulation by ATP was similar for both isoforms. In the presence of 2 μM InsP$_3$, low ATP concentrations (<6 mM) increased the open probability and mean open time. High ATP concentrations (>6 mM) decreased channel activity. These results illustrate the complex nature of type III InsP$_3$R regulation. Enhanced channel activity in the presence of high InsP$_3$ may be important during periods of prolonged stimulation, whereas allosteric modulation by ATP may help to modulate intracellular Ca^{2+} signaling.

INTRODUCTION

The inositol 1,4,5-trisphosphate receptor (InsP$_3$R) is an important component of intracellular Ca^{2+} signaling in most cells (Berridge, 1993; Clapham, 1995). Activation of the InsP$_3$R opens a Ca^{2+}-permeable channel that leads to an increase in cytoplasmic Ca^{2+} by releasing Ca^{2+} from the endoplasmic reticulum. InsP$_3$-mediated Ca^{2+} release regulates many cellular processes, such as the expression of transcription factors (Negulescu et al., 1994), the formation of the fertilization envelope during egg activation (Nuccitelli et al., 1993), stimulus-contraction coupling in smooth muscle (Walker et al., 1987), and the development of long-term depression (Finch and Augustine, 1998; Inoue et al., 1998; Khodakhah and Armstrong, 1997).

The InsP$_3$R exists as a tetramer in which each subunit is 260 kD. Three isoforms have been cloned (Blondel et al., 1993; Furuichi et al., 1989; Maranto, 1994; Mignery et al., 1989; Morgan et al., 1996; Sudhof et al., 1991). Both the expressed isoform and the extent of expression depend upon the cell type. Cerebellar Purkinje cells, for example, express almost exclusively the type I InsP$_3$R. Pancreatic acinar cells express types II and III InsP$_3$R, whereas several epithelia express all three isoforms (Bush et al., 1994; Nathanson et al., 1994; Wojcikiewicz, 1995). Additional receptor diversity is achieved through the formation of heterotetramers due to the association of different isoforms (Joseph et al., 1995; Monkawa et al., 1995).

Ca^{2+} release by the type I InsP$_3$R is regulated by a variety of cofactors and cellular processes, including cytosolic and intraluminal free Ca^{2+}, phosphorylation of the InsP$_3$R, and intracellular pH (Ferris et al., 1991a,b; Finch et al., 1991; Iino, 1990; Missiaen et al., 1992; Supattapone et al., 1988). Regulation by Ca^{2+} is bell-shaped with maximal channel activity occurring at 300 nM free Ca^{2+} (Bezprozvanny et al., 1991). ATP has been shown to enhance the gating of the type I InsP$_3$R by allosteric regulation (Bezprozvanny and Ehrlich, 1993; Mak et al., 1999). In *Xenopus* oocyte nuclei, ATP was shown to alter the Ca^{2+} sensitivity of Ca^{2+}-activation sites on the InsP$_3$R (Mak et al., 1999). This type of regulation allows ATP to shape the extent and duration of cytoplasmic Ca^{2+} signals depending upon stimulus intensity and a cell's metabolic state.

The InsP$_3$R contains a large regulatory domain between the InsP$_3$ binding site (at the NH2-terminal end) and the pore-forming region (the COOH-terminal end, which contains six membrane-spanning regions) where these modulators are able to exert their effects. Additional cytoplasmic factors and associated proteins (MacKrill, 1999; Thrower et al., 1998) contribute important regulatory functions that permit the wide range of responses that are seen for InsP$_3$-induced Ca^{2+} release. Pancreatic cells, for example, display markedly different Ca^{2+} responses depending on the agonist (such as acetylcholine and cholecystokinin) that is used to stimulate the cells (Lawrie et al., 1993; Osipchuk et al., 1990; Petersen et al., 1991; Thorn et al., 1993; Yule et al., 1991). These agonists can produce different Ca^{2+} oscillation patterns by affecting the phosphorylation state of the InsP$_3$R (LeBeau et al., 1999).

Recently, the single-channel properties of the types II and III InsP$_3$R have been determined in planar lipid bilayers. The types II and III InsP$_3$R do not show the same bell-shaped Ca^{2+}-dependence curve as the type I InsP$_3$R (Hagar et al., 1998; Ramos-Franco et al., 1998). Despite this difference in Ca^{2+} regulation, ion permeation and channel gating properties for the type III InsP$_3$R are similar to the type I InsP$_3$R (Mak et al., 2000). The types II and III

InsP$_3$R, however, are ideal for signal initiation because they lack Ca^{2+}-dependent negative feedback—a characteristic that supports the generation of Ca^{2+} oscillations. Differences in the Ca^{2+}-dependence of the three isoforms may underlie the spatial and temporal patterns of cytosolic Ca^{2+} signals that are important for many cellular responses. Further, the subcellular distribution of the different isoforms may help to coordinate Ca^{2+} signals within the cell.

The types II and III InsP$_3$R have an overall sequence homology with the type I InsP$_3$R of 69 and 64%, respectively (Blondel et al., 1993; Sudhof et al., 1991). Despite their similarity, the three isoforms possess different affinities for InsP$_3$: the relative order of affinity is type II $>$ type I $>$ type III (Maranto, 1994). A specific binding site for ATP on each subunit of the InsP$_3$R has been identified through biochemical studies (Maeda et al., 1991). For the type I InsP$_3$R, low concentrations of ATP ($<$4 mM) increase channel open probability (Bezprozvanny and Ehrlich, 1993). Concentrations of ATP above 4 mM cause a decrease in channel activity.

In the current study, the effects of both InsP$_3$ and ATP on the type III InsP$_3$R were investigated. Channel activity and mean open time were measured over a range of InsP$_3$ concentrations. The type III InsP$_3$R required a 10-fold higher InsP$_3$ concentration (with respect to the type I InsP$_3$R) to be maximally activated. As found for the type I InsP$_3$R, low concentrations of ATP ($<$6 mM) increased the open probability of the type III InsP$_3$R, and high concentrations of ATP ($>$6 mM) inhibited channel activity. Elevated InsP$_3$ levels restored channel activity inhibited by ATP, suggesting that ATP can displace InsP$_3$ from the InsP$_3$ binding site. Thus, the effects of ATP on the types I and III InsP$_3$R are similar, whereas the activation by InsP$_3$ is isoform-specific.

METHODS

Isolation of microsomes

Endoplasmic reticulum microsomes were isolated from RIN-m5F cells using the gradient centrifugation protocol for cerebellum as described previously (Bezprozvanny and Ehrlich, 1994). All solutions used for the isolation and storage of microsomes included a protease cocktail (5 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin A, 10 μg/ml trypsin inhibitor, and 1 mg/ml Pefabloc Plus) to minimize proteolysis.

Single-channel recordings

Microsomal vesicles were fused into planar lipid bilayers composed of the synthetic lipids phosphatidylethanolamine and phosphatidylserine (3:1, wt:wt; Avanti Polar Lipids, Alabaster, AL) dissolved in decane (20 mg/ml) so that the cis and trans chambers corresponded to the cytosol and lumen of the endoplasmic reticulum, respectively. Cytoplasmic bilayer solutions contained 110 mM TRIS and 250 mM HEPES (pH 7.35), and lumenal solutions contained 53 mM Ba(OH)$_2$ and 250 mM HEPES (pH 7.35). The trans chamber was held at virtual ground, and the transmembrane voltage was maintained at 0 mV. Single-channel currents were recorded under voltage clamp conditions using a bilayer clamp amplifier (BC-525B,

Warner Instruments Corp., Hamden, CT) and stored on VHS tape (Instrutech Corp., Great Neck, NY). Data were filtered at 1 kHz and digitized at 4 kHz for computer analysis using pClamp 6.0.3 (Axon Instruments, Foster City, CA). Additions of InsP$_3$ (Calbiochem, La Jolla, CA), ATP (disodium salt, Sigma, St. Louis, MO), and GTP (disodium salt, Sigma) were made to the cis chamber to obtain the desired concentrations of each compound. The cytosolic Ca^{2+} concentration was fixed at 160 nM (pCa = 6.8) using calibrated amounts of CaCl$_2$ and EGTA in the cis chamber (Fabiato, 1988). At least 3 min of continuous recording was analyzed to determine mean open times, mean closed times, open probability, and current amplitude. The number of active channels in each bilayer was estimated using a statistical model that is dependent on the maximum number of channels that are observed simultaneously during the course of an experiment (Horn, 1991).

RESULTS

Open probability of the type III InsP$_3$R as a function of InsP$_3$ concentration

After incorporation of RIN-m5F endoplasmic reticulum microsomes with planar lipid bilayers, the type III InsP$_3$R was activated with InsP$_3$ concentrations ranging from 0.1 to 200 μM (Fig. 1, A and B). In these experiments, 0.5 mM ATP and 2 μM ruthenium red were present. The ATP was added to act as a cofactor in the activation of the channel, and ruthenium red was added to inhibit any ryanodine receptors that may have co-incorporated into the bilayer. A 10-fold increase in InsP$_3$ concentration (2 to 20 μM) caused a threefold increase in channel open probability (2.2 to 7.6%). To fit the data with the Hill equation, a value of 1.9 was used for the Hill coefficient, suggesting that at least 2 molecules of InsP$_3$ are needed to bind to the receptor complex before the channel will open. Over this range of InsP$_3$ concentrations, the mean open time was unchanged (Fig. 1 C) for the type III InsP$_3$R. The open probability was maximal once the InsP$_3$ concentration was raised to 20 μM (Fig. 1, A and B, circles and solid line). In contrast, the open probability for the type I InsP$_3$R was maximal with 2 μM InsP$_3$ (Fig. 1 B, dashed line; data from Moraru et al., 1999). As shown in Fig. 1 B, the EC$_{50}$ for the types I and III InsP$_3$R were 0.5 and 3.2 μM, respectively. Finally, the type III InsP$_3$R remained very active at 10 nM free cytoplasmic Ca^{2+} in the presence of 20 μM InsP$_3$ (see Fig. 4 D; open probability was 6.8 $\pm$ 0.8%, n = 3). This high level of activity at high InsP$_3$ and low Ca^{2+} was predicted by a recent model that considers the effect of Ca^{2+} on InsP$_3$ binding for the type III InsP$_3$R (LeBeau et al., 1999).

Activation of the type III InsP$_3$R by low concentrations of ATP

In the presence of 2 μM InsP$_3$ and 2 μM ruthenium red, the channel open probability was observed with increasing amounts of ATP (Fig. 2, A and C). Variation in the single-channel open probability in separate experiments was minimized by normalizing the single-channel open probabilities
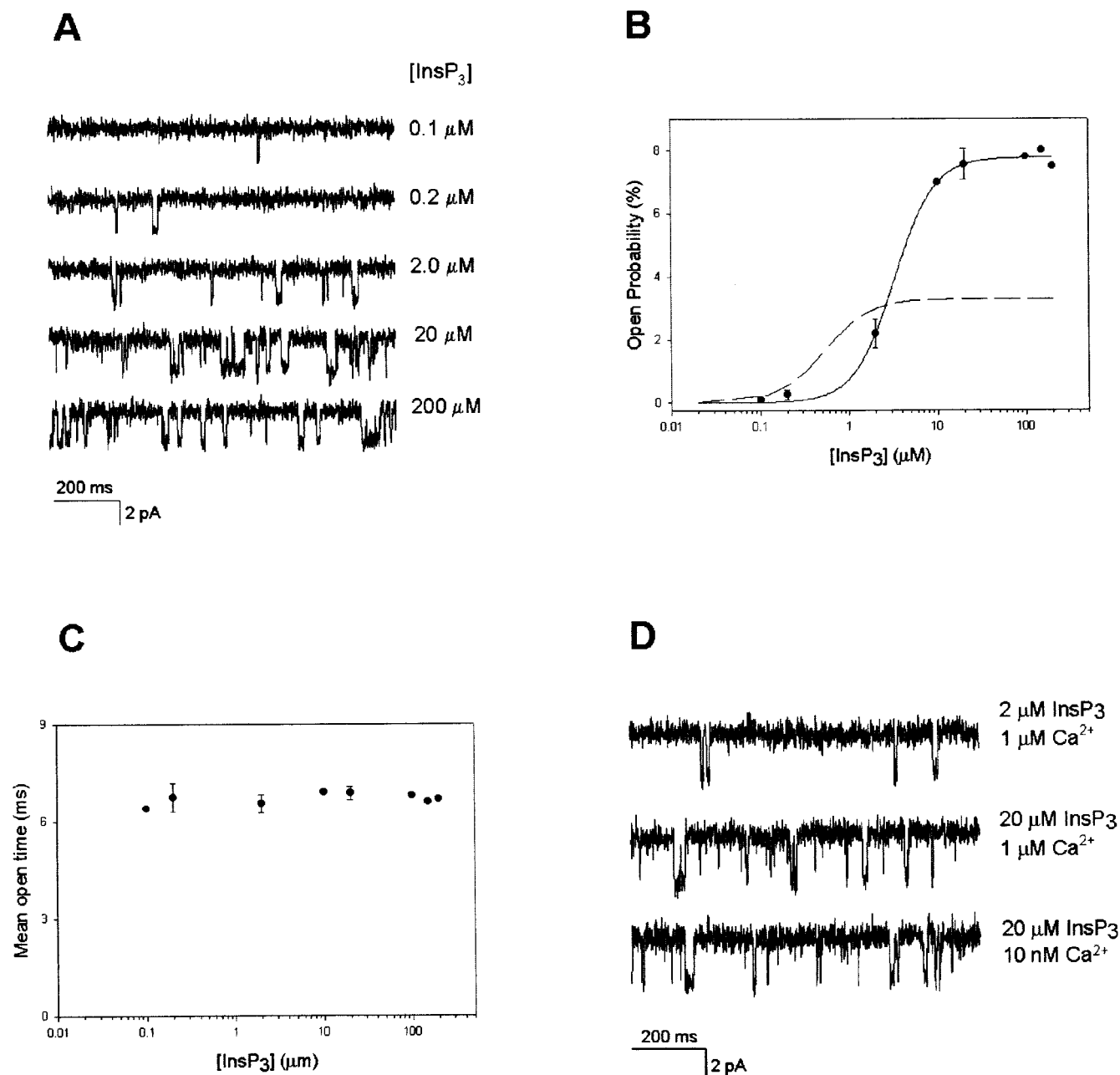
A

B

C

D

FIGURE 1 InsP$_3$ dependence of the types I and III InsP$_3$R. (*A*) Channel activity for the type III InsP$_3$R was observed at several InsP$_3$ concentrations (indicated at the right of each trace) with 0.5 mM ATP and 150 nM free Ca^{2+}. Channel openings are shown as downward deflections from the baseline. (*B*) Data for the type III InsP$_3$R were plotted as open probability (*circles*, mean ± SEM, $n = 4$) and fit using the Hill equation. A value of 1.9 for the Hill coefficient resulted, indicating that two molecules of InsP$_3$ must bind for the channel to open. Data for the type I InsP$_3$R (*dashed line*) were taken from Moraru et al. (1999). The type III InsP$_3$R was activated at higher InsP$_3$ concentrations (the EC$_{50}$ was 3.2 μM versus 0.5 μM for the type I InsP$_3$R). (*C*) Mean open time for the type III InsP$_3$R (mean ± SEM, $n = 4$) is not affected by the InsP$_3$ concentration. (*D*) In the presence of 20 μM InsP$_3$, the type III InsP$_3$R remains active at 10 nM free cytoplasmic Ca^{2+}.

in each experiment to the value obtained at 6 mM. The data were then fit using a modified Hill equation:

$$P(x) = P(o) + [P_{max} * x^n]/[k^n + x^n], \qquad (1)$$

where $P(x)$ is the single-channel open probability, $P(o)$ is the channel open probability in the absence of added ATP, k is

the apparent dissociation constant for the InsP$_3$R–ATP complex, and x corresponds to the ATP concentration. The best fit was obtained with $k = 2.8$ mM and $n = 2.9$ (the line in Fig. 2 *C* uses these parameters). When two concentrations of GTP were used, the open probability was unchanged (Fig. 2, *B* and *C*, *squares*; $n = 3$). This result shows that
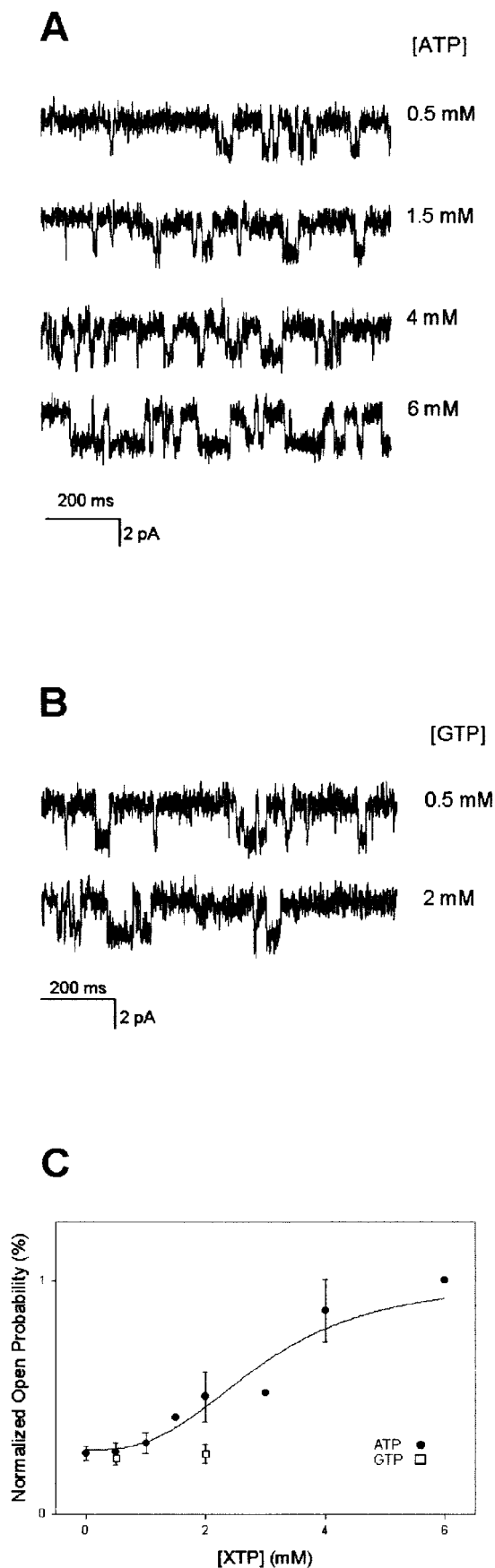
activation of the type III InsP$_3$R by nucleotide triphosphates is specific for the adenosine derivative. Similar specificity has been observed for the activation of type I InsP$_3$R by ATP (Bezprozvanny and Ehrlich, 1993).

As the ATP concentration was raised from 0.5 to 6 mM, the mean open time increased (Fig. 3 *A*), whereas mean closed time decreased (Fig. 3 *B*). Over this range of ATP concentrations, the current amplitude was unchanged (Fig. 3 *C*). A similar result was obtained for the type I InsP$_3$R in *Xenopus* oocyte nuclei; ATP activated gating by stabilizing the open state and destabilizing the closed state (Mak et al., 1999). As a result, it is likely that ATP activates the type III InsP$_3$R by increasing both the duration of channel openings (mean open time) and the frequency of the openings (by decreasing the mean closed time).

Inactivation of the type III InsP$_3$R by high concentrations of ATP

High ATP concentrations ($>$6 mM) inhibited the type III InsP$_3$R (Fig. 4, *A* and *C*). The single-channel open probability in each experiment was normalized to the channel open probability at 6 mM ATP. The steep inhibitory effect of high ATP concentrations suggests cooperative binding of ATP to a low affinity site as found previously for the type I InsP$_3$R ($k = 10.6$ mM, $n = 5$; Bezprozvanny and Ehrlich 1993). Although channel activity was completely inhibited by these high levels of ATP, activity was restored by raising the InsP$_3$ concentration (Fig. 4 *B*). This finding supports previous studies that have found a competitive interaction between InsP$_3$ and ATP (Guillemette et al., 1987; Iino, 1991; Maeda et al., 1991; Spat et al., 1987).

DISCUSSION

In this paper, two properties of the type III InsP$_3$R were examined: the effects of different InsP$_3$ concentrations and regulation by ATP. The channel open probability increased threefold as the InsP$_3$ concentration was increased from 2 to 20 μM, and open probability was maximal at 20 μM InsP$_3$ (Fig. 1, *A* and *B*). The type III InsP$_3$R reached a maximal level of channel activity that was $\sim$2.5-fold higher than the maximal level for the type I InsP$_3$R (Fig. 1 *B*, *dashed line*;

FIGURE 2   Activation of the type III InsP$_3$R by low concentrations of ATP. (*A*) Channel activity for the type III InsP$_3$R was monitored at several ATP concentrations (indicated at the right of each trace) in the presence of 2 μM InsP$_3$. (*B*) Channel activity at two GTP concentrations was similar. (*C*) Open probability was plotted as a function of nucleotide triphosphate concentration. Open probability at each ATP concentration was normalized to the open probability in the presence of 6 mM ATP. Activation of the type III InsP$_3$R is specific for ATP (*circles*, mean $\pm$ SEM, $n = 3$); GTP (*squares*, mean $\pm$ SEM, $n = 3$) did not change the open probability. The best fit for the ATP data was obtained using Eq. 1 with $k = 2.8$ mM and $n = 2.9$.
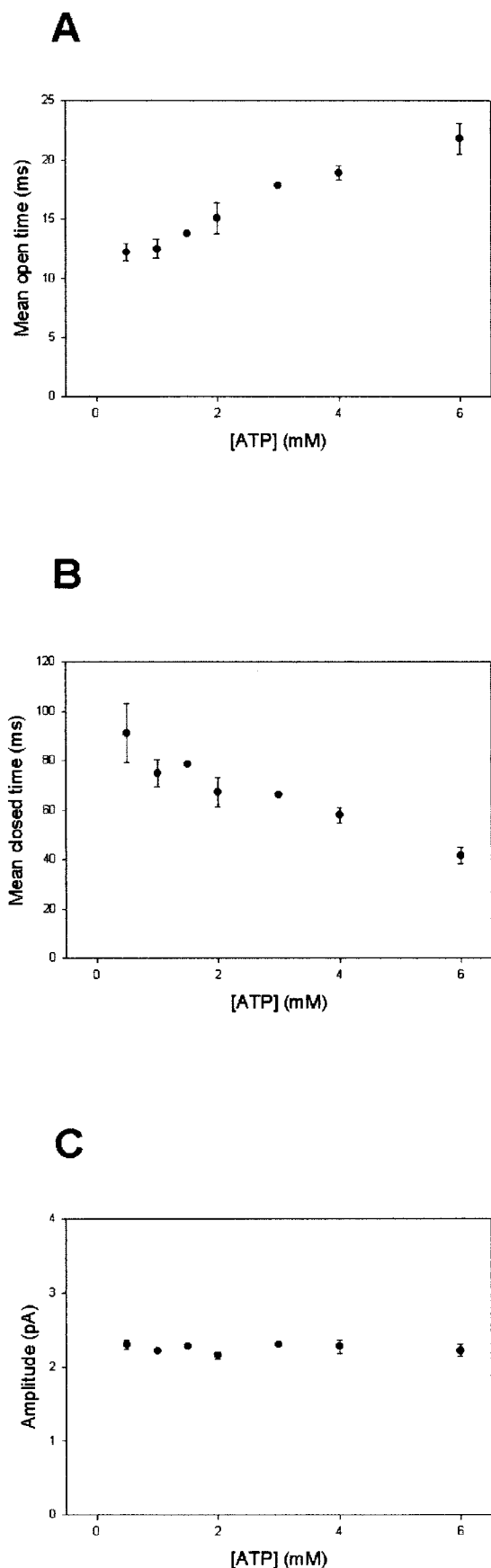
data from Moraru et al., 1999). Although both isoforms required the binding of at least two molecules of InsP$_3$ to open the channel, the type I InsP$_3$R activated at lower InsP$_3$ concentrations (the EC$_{50}$ was 0.5 μM for the type I InsP$_3$R and 3.2 μM for the type III InsP$_3$R). For these two reasons, activation by InsP$_3$ is an isoform-specific characteristic.

The EC$_{50}$ for the type III InsP$_3$R (3.2 μM) is much higher than the InsP$_3$ binding affinity of the type III InsP$_3$R (K_d = 151 nM) (Maranto, 1994). However, this value for the EC$_{50}$ is consistent with the InsP$_3$ concentration that is necessary for half-maximal release in both pancreatic islet cells (2.5 μM) (Wolf et al., 1985) and insulinoma microsomes (3.0 μM) (Prentki et al., 1984). In addition, InsP$_3$ concentrations needed to induce half-maximal Ca^{2+} release are often in the μM range (Berridge, 1987) even though InsP$_3$ binding affinities in a variety of tissues are in the low nM range (Taylor and Richardson, 1991). This discrepancy is likely due to the very different conditions under which InsP$_3$ binding and Ca^{2+} release measurements are generally performed. Frequently, InsP$_3$ binding is done on ice, in the absence of ATP, and at pH 8.5, whereas Ca^{2+} mobilization is done at 37°C, in the presence of ATP and at physiological pH, 7.4. When conditions for binding and release were matched, similar values for the K$_d$ and EC$_{50}$ were obtained (Mauger et al., 1989; Nunn and Taylor, 1990).

Calcium release from rat insulinoma microsomes (containing predominately the type III InsP$_3$R) showed a similar threefold increase in open probability when the InsP$_3$ concentration was raised from 2 to 20 μM (Prentki et al., 1984). Interestingly, a second addition of InsP$_3$ did not elicit a subsequent release of Ca^{2+}. In light of the fact that the type III InsP$_3$R can deplete intracellular stores in RIN-m5F cells (Hagar et al., 1998), it is likely that activation of the type III InsP$_3$R allowed depletion of the microsomal Ca^{2+} stores during the first stimulation so that a second response did not occur.

Despite the change in open probability as the InsP$_3$ concentration was increased from 0.1 to 200 μM, the mean open time did not change (Fig. 1 *C*). The increase in channel activity is unlikely to involve other cellular processes that have been implicated in the regulation of InsP$_3$R (Cameron et al., 1995). Phosphorylation of the channel by protein kinase C, for example, could not occur because the experimental protocol uses Na-ATP and does not include a kinase. A low affinity InsP$_3$ binding site (K_d = 10 μM) has recently been identified for the type I InsP$_3$R (Kaftan et al., 1997) and explains persistent channel activity at high Ca^{2+} and InsP$_3$ levels. An alternate explanation is that an elevated

FIGURE 3 Effect of ATP on the single-channel properties of the type III InsP$_3$R. (*A*) Mean open time increased and (*B*) mean closed time decreased as the ATP concentration was raised in the presence of 2 μM InsP$_3$. However, the amplitude of channel openings (*C*) was unaffected. Individual points with error bars are the mean $\pm$ SEM for n = 3.
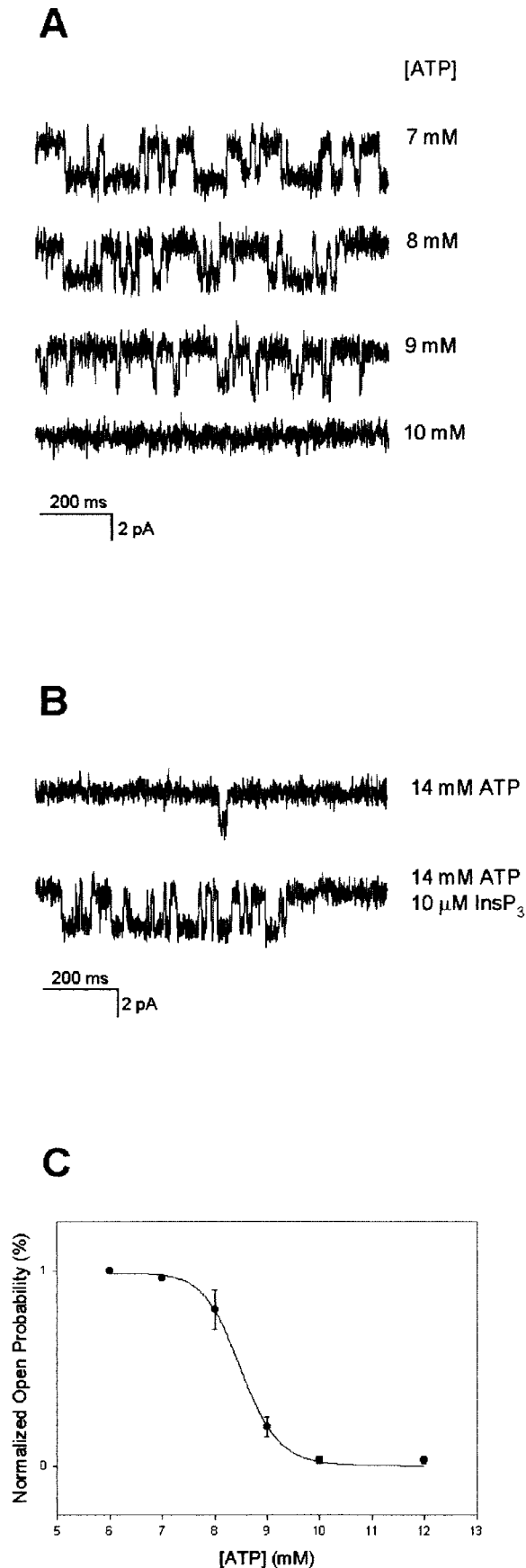
InsP$_3$ concentration relieves Ca^{2+} inhibition by decreasing the affinity of Ca^{2+} for a Ca^{2+} inhibitory site on the InsP$_3$R (Mak et al., 1998). It is presently unknown whether the type III InsP$_3$R also possesses the low affinity InsP$_3$ binding site, or if elevated InsP$_3$ relieves Ca^{2+} inhibition. Calmodulin, a Ca^{2+}-dependent regulatory protein with four Ca^{2+}-binding sites (Means and Dedman, 1980), has recently been shown to mediate Ca^{2+}-dependent inactivation of the type I InsP$_3$R (Michikawa et al., 1999). Although RIN-m5F cells contain high levels of calmodulin, which are not affected by glucose stimulation (Nelson et al., 1983), the type III InsP$_3$R is unique among the InsP$_3$R isoforms because it does not bind calmodulin (Yamada et al., 1995).

InsP$_3$ binding to the type III InsP$_3$R is enhanced by an increase in cytoplasmic Ca^{2+} (Cardy et al., 1997; Yoneshima et al., 1997). Modeling that considers the effect of Ca^{2+} on InsP$_3$ binding predicts a leftward shift of the steady-state open probability curve for the type III InsP$_3$R as the InsP$_3$ concentration is increased (LeBeau et al., 1999). Indeed, a high level of channel activity exists in the presence of 10 nM Ca^{2+} and 20 μM InsP$_3$ (Fig. 1 D). Under these conditions, the type I InsP$_3$R is not active (Kaftan et al., 1997). Likewise, open probability for the type III InsP$_3$R is very low at this Ca^{2+} concentration when the InsP$_3$ concentration is 2 μM (Hagar et al., 1998). Consequently, high InsP$_3$ concentrations can strongly activate the type III InsP3R even when cytoplasmic Ca^{2+} levels are low. High concentrations of InsP$_3$ have been found in a variety of cell types under both basal (0.1–3 μM InsP$_3$) and agonist-stimulated (1–20 μM) conditions (Putney, 1990).

Two putative ATP binding sites are located in the region between the InsP$_3$ binding site and the transmembrane region in the primary sequence of the InsP$_3$R (Furuichi et al., 1989; Mignery et al., 1990; Mignery and Sudhof, 1990). Both high- and low-affinity sites are present and mediate the effects of ATP. For the type I InsP$_3$R, the high affinity site provides allosteric regulation of channel activity (Iino, 1991; Maeda et al., 1991; Smith et al., 1985); ATP concentrations (<4 mM) increase the intrinsic efficacy of InsP$_3$ (Bezprozvanny and Ehrlich, 1993). Low concentrations of ATP are also able to increase channel open probability for the type III InsP$_3$R (Fig. 2, A and C). The increase in channel activity reflects an increase in the mean open time

FIGURE 4   Inhibition of the type III InsP$_3$R by high concentrations of ATP. (*A*) Channel activity was observed at several ATP concentrations (>6 mM) in the presence of 2 μM InsP$_3$. Channel openings were very infrequent when the ATP concentration was above 10 mM. (*B*) Raising the InsP$_3$ concentration to 10 μM restored channel activity. (*C*) Open probability was plotted as a function of ATP concentration for the type III InsP$_3$R (mean $\pm$ SEM, $n = 3$). The open probability at each ATP concentration was normalized to the open probability in the presence of 6 mM ATP. High ATP concentrations had a strong inhibitory effect on channel activity due to the cooperative binding of ATP to a low affinity site ($k = 8.5$ mM) on the type III InsP$_3$R.

and a decrease in mean closed time with no change in current amplitude (Fig. 3, *A–C*) as found for the type I InsP$_3$R of dog cerebellum (Bezprozvanny and Ehrlich, 1993) and *Xenopus* oocyte nuclei (Mak et al., 1999). In addition, this effect is specific for ATP; GTP is unable to increase channel activity (Fig. 2, *B* and *C*, *squares*).

High ATP concentrations inhibit InsP$_3$-induced Ca^{2+} release from permeabilized smooth muscles if low InsP$_3$ concentrations are used (Iino, 1991). Other studies also suggest a competitive interaction between InsP$_3$ and ATP (Guillemette et al., 1987; Maeda et al., 1991; Spat et al., 1987) and imply that the low affinity site is the InsP$_3$ binding site. High ATP concentrations inhibit channel activity for both the type I (Bezprozvanny and Ehrlich, 1993) and type III (Fig. 4, *A* and *C*) InsP$_3$R. In both cases, raising the InsP$_3$ concentration restores activity in the presence of an inhibitory amount of ATP (Fig. 4 *B*).

In summary, InsP$_3$ and ATP both regulate the activity of the type III InsP$_3$R in important ways. High InsP$_3$ concentrations (10–100 μM) maintain channel activity and may allow a cell to raise cytoplasmic Ca^{2+} levels during periods of prolonged stimulation. Regulation of the types I and III InsP$_3$R by ATP is similar: both isoforms are activated by low concentrations of ATP ($<$6 mM) and inhibited by high concentrations of ATP ($>$6 mM). From a mechanistic point of view, it is likely that ATP allosterically regulates each isoform at low concentrations and competes for the InsP$_3$ binding site at high concentrations. Allosteric regulation of the type III InsP$_3$R by ATP may be important for the modulation of intracellular Ca^{2+} signaling (Ferris et al., 1990) and the maintenance of cell viability during conditions of energy starvation (Katz, 1992).

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