# Inositol 1,4,5-Trisphosphate ($InsP_3$) and Calcium Interact to Increase the Dynamic Range of $InsP_3$ Receptor-dependent Calcium Signaling

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ABSTRACT The inositol 1,4,5-trisphosphate ($InsP_3$)-gated Ca channel in cerebellum is tightly regulated by Ca (Bezprozvanny, I., J. Watras, and B.E. Ehrlich. 1991. *Nature (Lond.).* 351:751–754; Finch, E.A., T.J. Turner, and S.M. Goldin. 1991. *Science (Wash. DC).* 252:443–446; Hannaert-Merah, Z., J.F. Coquil, L. Combettes, M. Claret, J.P. Mauger, and P. Champeil. 1994. *J. Biol. Chem.* 269:29642–29649; Iino, M. 1990. *J. Gen. Physiol.* 95:1103–1122; Marshall, I., and C. Taylor. 1994. *Biochem. J.* 301:591–598). In previous single channel studies, the Ca dependence of channel activity, monitored at 2 μM $InsP_3$, was described by a bell-shaped curve (Bezprozvanny, I., J. Watras, and B.E. Ehrlich. 1991. *Nature (Lond.).* 351:751–754). We report here that, when we used lower $InsP_3$ concentrations, the peak of the Ca-dependence curve shifted to lower Ca concentrations. Unexpectedly, when we used high $InsP_3$ concentrations, channel activity persisted at Ca concentrations as high as 30 μM. To explore this unexpected response of the channel, we measured $InsP_3$ binding over a broad range of $InsP_3$ concentrations. We found the well-characterized high affinity $InsP_3$ binding sites (with $K_d < 1$ and 50 nM) (Maeda, N., M. Niinobe, and K. Mikoshiba. 1990. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:61–67; Mignery, G., T.C. Sudhof, K. Takei, and P. De Camilli. 1989. *Nature (Lond.).* 342:192–195; Ross, C.A., J. Meldolesi, T.A. Milner, T. Satoh, S. Supattapone, and S.H. Snyder. 1989. *Nature (Lond.).* 339:468–470) and a low affinity $InsP_3$ binding site ($K_d$ = 10 μM). Using these $InsP_3$ binding sites, we developed a new model that accounts for the shift in the Ca-dependence curve at low $InsP_3$ levels and the maintained channel activity at high Ca and $InsP_3$ levels. The observed Ca dependence of the $InsP_3$-gated Ca channel allows the cell to abbreviate the rise of intracellular Ca in the presence of low levels of $InsP_3$, but also provides a means of maintaining high intracellular Ca during periods of prolonged stimulation.

KEY WORDS: cerebellum • ligand binding • intracellular calcium channel • channel regulation

## INTRODUCTION

The inositol 1,4,5-trisphosphate ($InsP_3$)[1] receptor is an intracellular calcium (Ca) release channel found in virtually all cell types (Berridge, 1993; Bezprozvanny and Ehrlich, 1995; Clapham, 1995; Divecha and Irvine, 1995). Activation of the $InsP_3$-gated channel causes an increase in cytoplasmic Ca by releasing Ca from the endoplasmic reticulum. $InsP_3$-mediated Ca release is important for many cellular processes, including the expression of transcription factors (Negulescu et al., 1994), the formation of the fertilization envelope during egg activation (Nuccitelli et al., 1993), nuclear membrane reformation in mitosis (Sullivan et al., 1995), stimulus-contraction coupling in smooth muscle (Walker et al., 1987), and the development of long term depression (Kasono and Hirano, 1995).

The $InsP_3$-gated channel exists as a complex comprised of four subunits of 260 kD each. To date, three isoforms of the subunits have been cloned (Furuichi et al., 1989; Mignery et al., 1989; Sudhof et al., 1991; Blondel et al., 1993; Maranto, 1994; Morgan et al., 1996). The isoform type and extent of expression is cell-type specific. The cerebellar Purkinje cell expresses almost exclusively type 1 receptor at levels at least 10× greater than other cell types. Hepatocytes express both type 1 and type 2 receptors, pancreatic acinar cells express type 2 and type 3 receptors, and several epithelia express all three receptor types (Bush et al., 1994; Nathanson et al., 1994; Wojcikiewicz, 1995). Further diversity may exist in tissues where different isoforms associate to form heterotetramers (Joseph et al., 1995; Monkawa et al., 1995).

Each subunit of the tetrameric channel complex contains an $InsP_3$ binding site near the $NH_2$ terminus (Mignery and Sudhof, 1990). $InsP_3$ binds to $InsP_3$-gated channels from cerebellum with high affinity ($K_d$ ranging from 5 to 50 nM). An additional low affinity site for $InsP_3$ was described using the $InsP_3$ analog $InsP_3S_3$, but the location of the site was unclear because crude microsomes were used (Challiss et al., 1991). Ca inhibits $InsP_3$ binding to the $InsP_3$-gated channel (Worley et al., 1987; Danoff et al., 1988). The Ca-dependent inhibi-

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[1]*Abbreviation used in this paper:* $InsP_3$, inositol 1,4,5-trisphosphate.

tion of $InsP_3$ binding can be reversibly removed by purifying the channel by heparin affinity chromatography, suggesting that Ca sensitivity is conferred by an accessory protein (Danoff et al., 1988).

$InsP_3$ is the only known physiological activator of the $InsP_3$-gated channel. Activation of the channel with $InsP_3$ is reversible: channels stop opening if $InsP_3$ is washed out and readdition of $InsP_3$ reactivates the channels (Ehrlich and Watras, 1988). In the presence of $InsP_3$, Ca acts as an allosteric regulator of the $InsP_3$-gated Ca channel (Iino, 1990; Bezprozvanny et al., 1991; Finch et al., 1991; Hannaert-Merah et al., 1994; Marshall and Taylor, 1994). Previous studies using permeabilized smooth muscle (Iino, 1990), Ca release from cerebellar microsomes (Finch et al., 1991), and single channel recordings (Bezprozvanny et al., 1991) showed that the Ca dependence of channel activity is described by a bell-shaped curve. With single channel recordings, maximal channel activity was observed in the presence of 0.25 μM free Ca and there was a steep decline in channel activity on either side of the maximum (Bezprozvanny et al., 1991). Complete inhibition of channel activity occurred when cytoplasmic Ca reached 5 μM. The Ca-dependent activation provides an amplification of the initial signal and the Ca-dependent inhibition of the $InsP_3$-gated channel allows for fast negative feedback of the cytoplasmic Ca concentration.

A number of models have been proposed to describe the regulation of the $InsP_3$-gated channel. Most models assume three regulatory sites on the channel: one site for $InsP_3$, one site for activating Ca, and one site for inhibitory Ca. Under steady state conditions, these models make different predictions of the shape of the Ca-dependence curve as $InsP_3$ concentrations are varied. As the $InsP_3$ concentration is increased, some models predict that the peak of the Ca-dependence curve will (*a*) shift to lower Ca concentrations (Othmer and Tang, 1993), (*b*) shift to higher Ca concentrations (De Young and Keizer, 1992), or (*c*) remain unchanged (Atri et al., 1993; Bezprozvanny and Ehrlich, 1994).

In this paper, we measured the Ca dependence of $InsP_3$-gated channel activity as a function of $InsP_3$ concentration. We also compared $InsP_3$ binding and channel activity using the same experimental conditions. Using our measured values, we constructed a model that accounts for the interaction of Ca and $InsP_3$ in regulating the $InsP_3$ receptor. The model is distinct from other models currently applied to the $InsP_3$-gated Ca channel and is consistent with the observed leftward shift of the curve at low $InsP_3$ concentrations. A novel feature of the model is the inclusion of a low affinity $InsP_3$ binding site, which broadens the range of regulation of the channel by $InsP_3$ and explains the observed maintained activity of the channel at high concentrations of Ca and $InsP_3$.

## METHODS

### *Single Channel Recordings*

Canine cerebellar endoplasmic reticulum vesicles were prepared as previously described (Ehrlich and Watras, 1988) and fused with planar lipid bilayers composed of phosphatidylethanolamine and phosphatidylserine (3:1, wt:wt; Avanti Polar Lipids, Alabaster, AL) dissolved in decane (20 mg/ml). Cytoplasmic bilayer solutions contained 500 μM ATP, 500 μM EGTA, 110 mM Tris, and 250 mM HEPES, pH 7.35, and luminal solutions contained 53 mM $Ba(OH)_2$, 250 mM HEPES, pH 7.35. Calibrated $CaCl_2$ was added to the cytoplasmic solution to obtain the desired free Ca concentration (Fabiato, 1988). Because estimation of free Ca was critical, calculations were routinely checked spectrofluorometrically with BTC (Molecular Probes, Inc., Eugene, OR). The number of channels in each experiment was estimated from the maximum number of channels observed simultaneously in the bilayer (Horn, 1991). The $InsP_3$ dependence of the open probability, measured at a fixed Ca concentration, was used to correct for variations in the maximum open probability among individual channels. Transmembrane voltage was maintained at 0 mV and the single channel current amplified (Warner Instruments, Hamden, CT) and stored on VHS tape (Instrutech Corp., Great Neck, NY). Data were filtered at 1 kHz and digitized at 5 kHz for computer analysis using pClamp 6.0 (Axon Instruments, Foster City, CA).

### *$InsP_3$ Binding Measurements*

[$^3$H]$InsP_3$ binding was measured as previously described (Benevolensky et al., 1994) except that binding was measured using solutions similar to the cytoplasmic solution in single channel experiments (500 μM ATP, 500 μM EGTA, 110 mM Tris, 250 mM HEPES, pH 7.35, with the specified free Ca concentration). Radioligand concentrations were varied from 0.4 nM to 30 μM. To achieve this range, stock radioligand (480 nM; New England Nuclear, Boston, MA) was diluted with unlabeled $InsP_3$ (Calbiochem, La Jolla, CA). To assure sufficient reliability in the measurement, the protein concentration was increased as the specific activity decreased. Nonspecific binding was measured in the presence of 2.5 mM unlabeled $InsP_3$ or 1–10 mg/ml heparin. These conditions generated the same value for nonspecific binding. To be included in the analysis, specific binding had to exceed 50% of the nonspecific binding. As Scatchard analysis of $InsP_3$ binding showed the presence of three binding sites, $InsP_3$ binding was modeled as shown:

$$b = \frac{b_H \cdot S}{K_H + S} + \frac{b_M \cdot S}{K_M + S} + \frac{b_L \cdot S}{K_L + S} \quad (1)$$

where $K_H$, $K_M$, and $K_L$ are the apparent dissociation constants for the 1-nM, 50-nM, and 10-μM sites and $b_H$, $b_M$, and $b_L$ refer to the maximum binding at the respective sites.

In most published reports, $InsP_3$ binding experiments were done using conditions that maximize binding, pH 8.0, at 4°C. In contrast, measurements of $InsP_3$-gated channel function have generally been done using conditions closer to physiological conditions, pH 7.3, at 22–37°C. In the experiments described here, measurements were done using similar conditions when possible to compare in vitro channel function with biochemical properties. In addition, $InsP_3$ saturation binding curves were generated at 0 and 22°C to determine the temperature coefficient of binding ($Q_{10}$). The $Q_{10}$ was determined to be 2.3 for the 50-nM site and was estimated to be 1.0 for the 10-μM site. Although the $Q_{10}$ value for the 10-μM site is consistent with our data, it must be called an estimate due to low signal-to-noise ratio in binding mea-

surements at 22°C in combination with the low specific activity obtained when very high $InsP_3$ concentrations are used.

### *Modeling of Channel Function Using Single Channel Data and Binding*

The "2-$IP_3$/2-Ca" model used for the analysis of the open probability data assumes that the $InsP_3$-gated Ca channel complex contains four monomers, and that each monomer of the tetrameric channel complex has two $InsP_3$ binding sites (with apparent dissociation constants $K_{50nM}$ and $K_{10\mu M}$), one Ca binding site for activation of the channel ($Ca_{AC}$), and one Ca binding site for inhibition of the channel ($Ca_{IN}$). The affinity of the 50-nM site depends upon the occupancy of the $Ca_{IN}$ site, as determined from binding experiments in the presence and absence of Ca (see Fig. 3 *C* and Benevolensky et al., 1994). To fit the model to the data, it was not necessary to include cooperativity of binding among the sites or sequential binding steps.

The presence of four ligands associated with each monomer means there are 16 possible states ($2^4$) of each monomer of the tetrameric Ca channel complex. To fit the open probability data over a wide range of $InsP_3$ and Ca concentrations, we had to assume that more than 1 of the 16 states was able to conduct Ca. The simplest model that fit the open probability data and the $InsP_3$ binding data required that 3 of the 16 possible states be capable of conducting Ca. The three possible conducting states are: $S_1$ = $InsP_3$ bound to the $K_{50nM}$ site, and Ca bound to the $Ca_{AC}$ site; $S_2$ = $InsP_3$ bound to the $K_{50nM}$ and $K_{10\mu M}$ sites, and Ca bound to the $Ca_{AC}$ site; and $S_3$ = $InsP_3$ bound to the $K_{50nM}$ and $K_{10\mu M}$ sites, and Ca bound to the $Ca_{AC}$ and $Ca_{IN}$ sites.

The relative abundance (*RA*) of the three possible conducting states is determined as follows:

$$RA = \frac{a_1 + a_2 + a_3}{\sum_{x=1}^{16} a_i} \quad (2)$$

where $a_i$ is the calculated fractional abundance of the $i^{th}$ state of the channel based on equilibrium binding constants of the transitions among the 16 states, assuming mass action kinetics. Then, the single channel open probability ($P_o$) is calculated as follows:

$$P_o = RA^4 P_4 + 4RA^3 \cdot (1 - RA) P_3 + 6RA^2 \cdot (1 - RA)^2 P_2 + 4RA \cdot (1 - RA)^3 P_1 \quad (3)$$

where $P_i$ is the probability over time that the channel complex will open assuming that i of the monomers in the channel complex are in one of the conducting states. An iterative curve fitting routine (Sigmaplot; Jandel Scientific, San Rafael, CA) was used to calculate the equilibrium constants of the various transitions that best fit both the single channel data and the $InsP_3$ binding data.

## RESULTS

### *$InsP_3$ Shifts the Bell-shaped Calcium-dependence Curve*

The Ca dependence of the $InsP_3$-gated Ca channel was previously monitored at the single channel level using 2 μM $InsP_3$, a concentration 10× the $K_d$ previously determined for $InsP_3$-gated release and channel activity (Watras et al., 1991). Measurements of $InsP_3$-dependent Ca release from vesicles showed that inhibition by Ca varied with $InsP_3$ concentration (Joseph et al., 1989; Combettes et al., 1994; Bootman et al., 1995; Hannaert-Merah et al., 1995). Specifically, as the $InsP_3$ concentration was increased, Ca-dependent inhibition of $InsP_3$-induced Ca release occurred at higher Ca concentrations (Joseph et al., 1989; Combettes et al., 1994; Bootman et al., 1995; Hannaert-Merah et al., 1995). We now report a similar response at the single channel level (Fig.

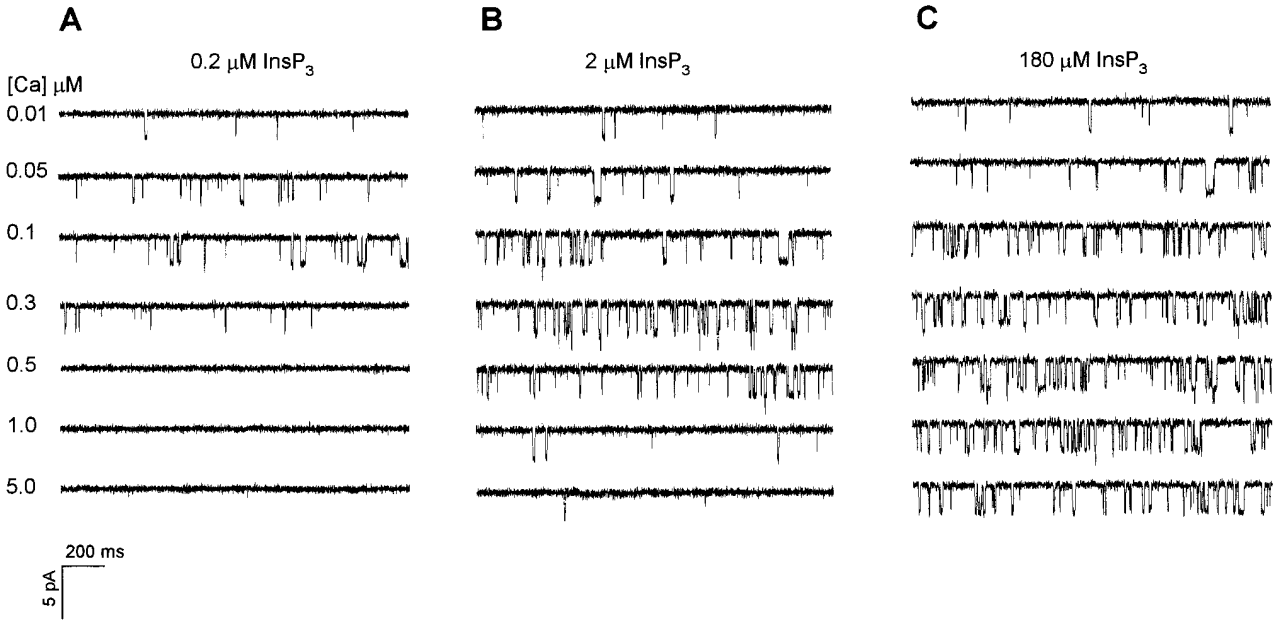


FIGURE 1. Ca dependence of cerebellar $InsP_3$-gated Ca channels at several concentrations of $InsP_3$. Channel activity was observed in the presence of (*A*) 0.2, (*B*) 2, and (*C*) 180 μM $InsP_3$. Free Ca concentrations (μM) are given to the left of each trace. Channel openings are shown as downward deflections. Each panel is taken from one experiment. Activity from multiple channels is seen in many experiments, as in the third and fourth traces of *B* and *C*. The single channel conductance is the same at all concentrations of $InsP_3$ tested.

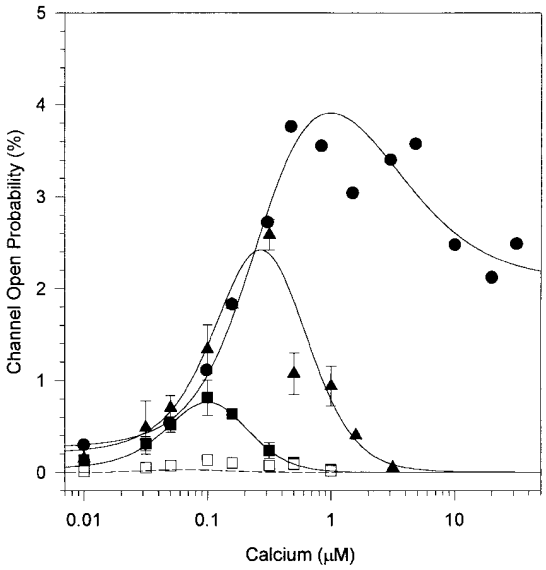


FIGURE 2. Open probability of $InsP_3$-gated channels as a function of Ca concentration. Ca-dependence curves were generated at 0.02 (□), 0.2 (■), 2 (▲), and 180 (●) μM $InsP_3$. Data points represent the mean ± SEM of five experiments for 0.2 and 2 μM $InsP_3$ and one experiment for 0.02 μM $InsP_3$. Data points pooled from three experiments are displayed for 180 μM $InsP_3$ without error bars because the same series of Ca concentrations was not obtained in all experiments. The ordinant shows the open probability for a single channel. Curves through the data were generated by the model described in the text. A dotted line is used to predict channel activity at 0.02 μM $InsP_3$ and solid lines show the fit to the single channel open probability measured at 0.2, 2, and 180 μM $InsP_3$.

1). In the presence of either 0.2 or 2 μM $InsP_3$, cytoplasmic Ca activates single channel currents over a similar concentration range (compare Fig. 1, *A* and *B, top 3 traces*). As the $InsP_3$ concentration is elevated, activity is maintained at higher cytoplasmic Ca concentrations (Fig. 1, *A* and *B, bottom 4 traces*). A comparison of the open probability of the $InsP_3$-gated channel as a function of cytoplasmic Ca concentration shows that the peak channel activity shifts from 0.1 to 0.25 μM Ca when the $InsP_3$ concentration is increased from 0.2 to 2 μM (Fig. 2, ■ and ▲). However, the activating phase of each curve is quite similar. The predominant effect of raising the $InsP_3$ concentration is an increase in channel activity above 0.1 μM Ca. For example, the channel is essentially closed at 1 μM free Ca in the presence of 0.2 μM $InsP_3$, but there is substantial channel activity in the presence of 2 μM $InsP_3$.

If the $InsP_3$ concentration is increased further, an unexpected response of the channel is observed. At 180 μM $InsP_3$, channel activity remains robust at all Ca concentrations tested (Fig. 1 *C*). Note that the activity measured at 30 μM Ca is essentially the same as that observed at 5 μM Ca (Fig. 2, ●). The relatively large open probability of the channel in the presence of 5–30 μM Ca and high $InsP_3$ concentrations was not predicted in published models (De Young and Keizer, 1992; Bezprozvanny, 1994; Tang et al., 1996) of the regulation of the $InsP_3$-gated channel by Ca and $InsP_3$. The persistent activity of the channel at high levels of $InsP_3$ provides a means for the cell to maintain intracellular Ca beyond 1 μM during periods of continued stimulation of the phosphoinositide cascade.

### *Analysis of [$^3$H]$InsP_3$ Binding Reveals a Low Affinity Site*

To investigate the persistent elevation of channel activity in the presence of high concentrations of Ca and $InsP_3$, a series of $InsP_3$ binding experiments were undertaken. When $InsP_3$ concentrations spanning a broad range are used (0.4 nM to 30 μM), the binding data shows three distinct slopes indicating at least three binding sites for $InsP_3$ (Fig. 3 *A*). Three sites are evident in all four cerebellar preparations tested. The $InsP_3$-gated Ca channel purified by either immunoprecipitation with a type 1-specific $InsP_3$ receptor antibody or by heparin-sepharose column chromatography (Fig. 3 *B*) also had three distinct binding sites, which indicates that these sites are integral parts of the channel complex. The high affinity (<1 nM) binding site is least abundant, representing <1% of the total sites in all preparations tested. This high affinity site has been observed previously in cerebellum and vascular smooth muscle (Hingorani and Agnew, 1992; Benevolensky et al., 1994). Two binding sites of approximately equal abundance account for the remaining 99% of the $InsP_3$ binding in this tissue; the $K_d$'s of the sites when measured at 0°C are 54 nM and 10.2 μM. In this text, these $InsP_3$ binding sites are called the 1-nM, 50-nM, and 10-μM sites. The 50-nM site is the $InsP_3$ binding site that has been shown previously to be concentrated in cerebellar Purkinje cells (Mignery et al., 1989; Ross et al., 1989; Maeda et al., 1990). We report here the existence of a low affinity site that saturates above 10 μM $InsP_3$ (Fig. 3, *A* and *B*). The $K_d$ of this site is more than 200× higher than those previously shown for the purified $InsP_3$ receptor. The 10-μM site identified in this report may be the site responsible for low affinity $InsP_3$ binding described indirectly in crude cerebellar microsomes (Challiss et al., 1991).

To investigate the inositol phosphate specificity of the two predominant $InsP_3$ binding sites, competition binding was done at two concentrations of $InsP_3$, 10 nM [$^3$H]$InsP_3$ to examine the 50-nM site and 6 μM [$^3$H]$InsP_3$ to examine the 10-μM site (Fig. 4). For each concentration of $InsP_3$, three competitors were examined: 1,3,4,5-$InsP_4$, 1,4,5-$InsP_3$, and 2,4,5-$InsP_3$. At the 50-nM site, 1,4,5-$InsP_3$ is at least 30× more effective than 1,3,4,5-$InsP_4$ at displacing 10 nM [$^3$H]1,4,5-$InsP_3$ from the receptor (Fig. 4 *A*). The ability of 2,4,5-$InsP_3$ and 1,3,4,5-$InsP_4$ to displace 30 nM [$^3$H]1,4,5-$InsP_3$ from the 50-nM site was indistinguishable. Thus, 1,4,5-$InsP_3$

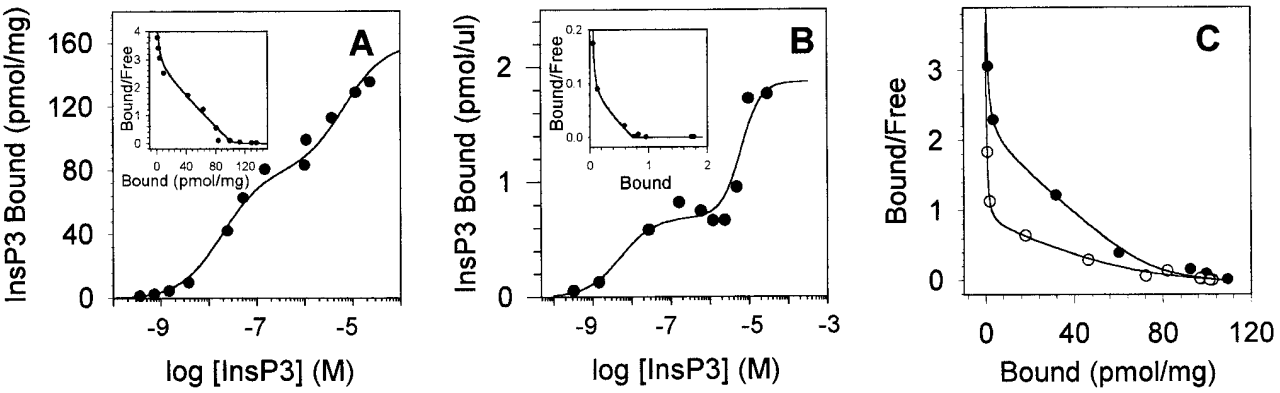


FIGURE 3. $InsP_3$ binding reveals three binding sites. (*A*) Saturation-binding isotherm of $InsP_3$ binding to cerebellar reticular vesicles. $InsP_3$ binding was conducted in the absence of Ca with $InsP_3$ concentrations varying between 0.4 nM and 30 μM. Inset shows the Scatchard analysis. Data were fit assuming the presence of three binding sites for $InsP_3$. Four different vesicle preparations were used; one experiment is displayed. In the absence of Ca, the $K_d$'s for the vesicle preparations are 0.47 ± 0.19 nM, 54 ± 5 nM, and 10.2 ± 3.2 μM; $\bar{x}$ ± SEM, $n = 4$. (*B*) Saturation-binding isotherm of $InsP_3$ binding to the type 1 $InsP_3$ receptor purified by heparin-agarose chromatography. Inset shows the Scatchard analysis. Similar results were obtained with a receptor purified from cerebellar microsomes by immunoprecipitation using a type 1-specific antibody (C-19). The purity of the receptor, as assessed from $InsP_3$ binding, was 94% ($InsP_3$ binding was 3,612 pmol/mg for the 50-nM site, compared with a theoretical maximum binding of 3,846 pmol/mg for a molecular weight of 260 kD). In this panel, the abscissa is expressed as picomoles per microliter of purified $InsP_3$ receptor. $K_d$'s for this preparation are 0.5 nM, 29 nM, and 6.8 μM. For comparison, one preparation of $InsP_3$ receptor purified by immunoprecipitation generated $K_d$'s of 0.1 nM, 35 nM, and 12 μM. (*C*) Scatchard analysis of $InsP_3$ binding in the absence of Ca (●) and 10 μM free Ca (○). Increasing the free Ca results in a decrease in the apparent affinity of $InsP_3$ binding from 35 nM in the absence of Ca to 91 nM in the presence of 10 μM Ca without altering binding to the 1-nM or 10-μM sites. The Ca-induced decrease in $InsP_3$ affinity of the 50-nM site was completely reversed upon addition of EGTA.

has the highest affinity at the 50-nM site and both 2,4,5-$InsP_3$ and 1,3,4,5-$InsP_4$ have 30-fold lower affinity for this site. In contrast, 1,3,4,5-$InsP_4$ appears more effective than 1,4,5-$InsP_3$ in its ability to displace 6 μM [$^3$H]1,4,5-$InsP_3$ from the 10-μM site and 6 μM 2,4,5-$InsP_3$ was unable to displace [$^3$H]1,4,5-$InsP_3$ from the 10-μM site (Fig. 4 *B*). Therefore, the 10-μM site can be distinguished from the 50-nM site by its relative specificity for inositol phosphates.

The possibility that other isoforms of the $InsP_3$ receptor were responsible for the 10-μM site was ruled out by comparing the amount of the three $InsP_3$ receptor isoforms in both purified preparations using Western analysis. We obtained strong staining for type 1 and, despite using 10× more protein, virtually no type 2 or 3 $InsP_3$ receptors, confirming published reports that cerebellum contains >90% type 1 $InsP_3$ receptor (Sudhof et al., 1991; Wojcikiewicz, 1995; Morgan et al., 1996). These values need to be compared with the observation that the 50-nM and 10-μM sites are present in approximately equal abundance.

Micromolar concentrations of Ca alter $InsP_3$ binding in a variety of tissues (Pietri et al., 1990; Marshall and Taylor, 1994; Watras et al., 1994). In cerebellum and vascular smooth muscle, Ca decreases $InsP_3$ binding (Worley et al., 1987; Danoff et al., 1988; Benevolensky et al., 1994). Both of these tissues contain predominantly the type 1 $InsP_3$ receptor (Marks et al., 1990; Furuichi et al., 1993; Newton et al., 1994). We find that $InsP_3$ binding to cerebellar membranes under conditions identical to those used for the single channel measurements also shows Ca-dependent inhibition (Fig. 3 *C*). Only binding to the 50-nM site appears to be Ca sensitive. Elevation of Ca to 10 μM decreases, but does not completely inhibit, $InsP_3$ binding (Fig. 3 *C*, ○). Similarly, in vascular smooth muscle, Ca concentrations as high as 150 μM failed to completely inhibit $InsP_3$ binding (Benevolensky et al., 1994). In contrast, $InsP_3$ binding to both the 1-nM and 10-μM sites appears insensitive to Ca (Fig. 3 *C*).

### *Model of $InsP_3$-gated Channel Function Needs the Novel $InsP_3$ Binding Site*

We created a model of $InsP_3$-gated channel function that accounts for the Ca dependence of $InsP_3$ binding (Fig. 3) and the Ca dependence of channel activity (Fig. 2) over a broad range of $InsP_3$ concentrations. This model is named the 2-$InsP_3$/2-Ca model because it incorporates two $InsP_3$ binding sites (50 nM and 10 μM) and two calcium regulatory sites (activating and inhibitory) on each monomer of the tetrameric channel complex. The 1-nM site for $InsP_3$ is not included in the model due to its low abundance. A model incorporating one $InsP_3$ site and two Ca sites previously proposed (De Young and Keizer, 1992) is able to predict channel activity when $InsP_3$ levels are <2 μM, but the one $InsP_3$ binding site model requires parameters that

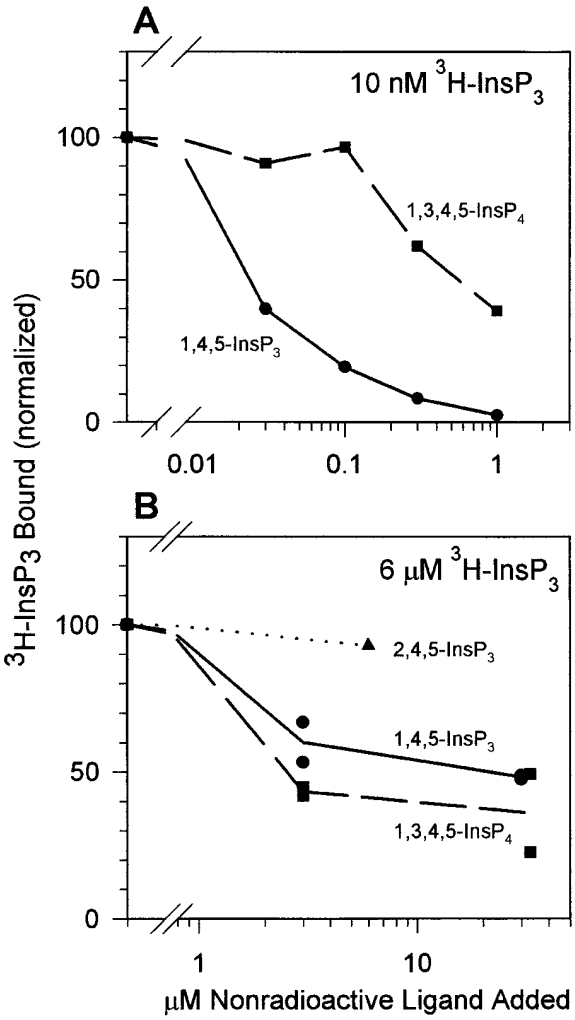


FIGURE 4. Specificity of inositol phosphate binding to the purified $InsP_3$ receptor. (*A*) Displacement of 10 nM [$^3$H]1,4,5-$InsP_3$ by unlabeled 1,4,5-$InsP_3$ (●) and 1,3,4,5-$InsP_4$ (■). This experiment shows that 1,4,5-$InsP_3$ is 30× more effective than 1,3,4,5-$InsP_4$ at the 50-nM site. One of two similar experiments is shown. (*B*) Displacement of 6 μM [$^3$H]1,4,5-$InsP_3$ by unlabeled 2,4,5-$InsP_3$ (▲), 1,4,5-$InsP_3$ (●), and 1,3,4,5-$InsP_4$ (■). The two points at each concentration represent two experiments on two different preparations. The points at 30 μM 1,3,4,5-$InsP_4$ were displaced slightly to the right to show that one of the data points falls in the same location as the data points for 30 μM 1,4,5-$InsP_3$. This experiment shows that 2,4,5-$InsP_3$ is ineffective and 1,3,4,5-$InsP_4$ is more effective than 1,4,5-$InsP_3$ at displacing 6 μM [$^3$H]1,4,5-$InsP_3$ from the 10-μM site.

are inconsistent with $InsP_3$ binding data. In addition, this model with one $InsP_3$ binding site cannot predict the persistent activity observed at high concentrations of $InsP_3$ and Ca. In our 2-$InsP_3$/2-Ca model, $InsP_3$ binding to the 10-μM site allows channel activity to be sustained at cytoplasmic Ca concentrations above 5 μM.

Values for the affinity of $InsP_3$ in the absence of Ca were determined from binding experiments (Fig. 3); other parameters were predicted from fits of the model

TABLE I

*"2-$InsP_3$/2-Ca" Model Parameters*

| | |
|---|---|
| Affinity for $InsP_3$ binding to the 50-nM site* (−Ca) | 0.3 μM |
| Affinity for $InsP_3$ binding to the 50-nM site* (+Ca) | 1.5 μM |
| Affinity for $InsP_3$ binding to the 10-μM site* | 10 μM |
| Affinity for Ca binding to its activating site | 0.03 μM |
| Affinity for Ca binding to its inhibitory site | 1.0 μM |

*The name of the site corresponds to the apparent affinity obtained at 0°C. The values shown in the table and used for fits to the data of Fig. 2 were, when possible, measured directly at 22°C or calculated from the fits to the data of Fig. 2. The experiments shown in Fig. 2 were performed at 22°C.

to both the single channel and binding data (Table I). All curves through the experimental points (Figs. 2 and 3) were generated by the 2-$InsP_3$/2-Ca model. Using the parameters generated by the fit of the model to the data, the open probability of the channel can be predicted over a wide range of both $InsP_3$ and Ca. Predictions of channel activity at concentrations of $InsP_3$ and Ca up to 1 mM are shown in Fig. 5.

An outcome of the 2-$IP_3$/2-Ca model is that at least two of the four monomers of the tetrameric Ca channel complex must be in one of the three possible conducting states for the channel complex to conduct Ca. That is, if only one of the monomers in the tetrameric complex is in one of the conducting states ($P_1$ in Eq. 3), the predicted value for the probability that the channel will open was $10^{-11}$, suggesting that the singly occupied channel rarely opens. In contrast, if two or three monomers are occupied, the predicted values for the probability that the channel will open were 0.06 and 0.04, respectively. The requirement for at least two $InsP_3$ molecules to bind to the receptor is supported by experimental findings. An extension of our earlier experiments describing the $InsP_3$ concentration dependence of the open probability of the channel (Watras et al., 1991) to lower concentration of $InsP_3$ (10 nM, data not shown) generates a curve with a Hill coefficient of 1.8. This result and additional reports (Somlyo et al., 1992; Marchant et al., 1997) support the suggestion that multiple molecules of $InsP_3$ bind to the channel before it opens.

As a further test of the model, single channel behavior was measured at 20 nM $InsP_3$ (Fig. 2, □). At this very low $InsP_3$ concentration, channel activity was difficult to measure because openings were infrequent, but the shape of the Ca dependence and the peak in channel activity were similar to the predicted values. Although other models could be generated, the ability of the 2-$InsP_3$/2-Ca model to fit both the single channel and binding data and to predict $InsP_3$-gated channel function under a variety of conditions lends support for this model of channel function.

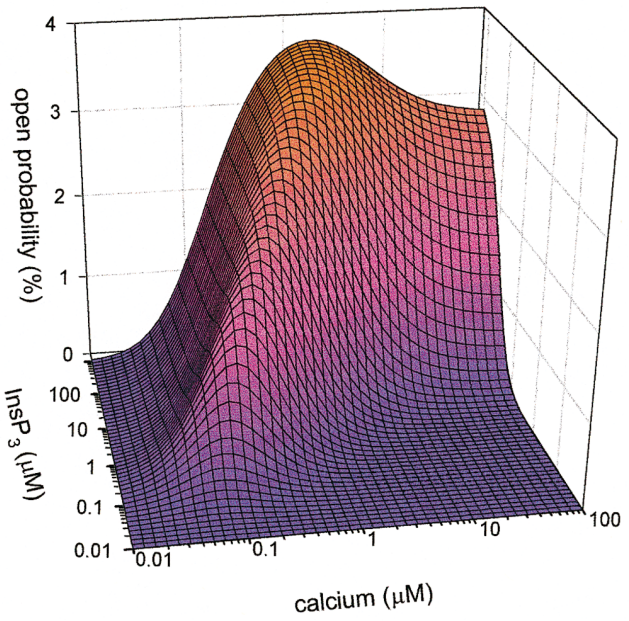


FIGURE 5. Prediction of the Ca and $InsP_3$ dependence of the open probability using the data in Figs. 2 and 3. The 2-$IP_3$/2-Ca model used for the analysis of the open probability data assumes that the $InsP_3$-gated Ca channel complex contains four monomers, and that each monomer of the tetrameric channel complex has two $InsP_3$ binding sites, one Ca binding site for activation of the channel, and one Ca binding site for inhibition of the channel. Details of the model are included in METHODS.

## DISCUSSION

In this paper, we tested the effect of changing the $InsP_3$ concentration on the regulation of the $InsP_3$ receptor. We found that the peak of channel activity shifted to higher Ca concentrations as the $InsP_3$ concentration was increased from 20 nM to 2 μM and that elevating the $InsP_3$ concentration above 2 μM leads to persistent activation of the $InsP_3$-gated channel. To explain this unexpected response, we measured $InsP_3$ binding at $InsP_3$ concentrations from 0.4 nM to 30 μM. We found the well-characterized high affinity $InsP_3$ binding sites (with $K_d$'s $< 1$ and 50 nM) (Mignery et al., 1989; Ross et al., 1989; Maeda et al., 1990) and a low affinity $InsP_3$ binding site ($K_d$ = 10 μM). We then developed a new model that accounts for both the channel activity and the $InsP_3$ binding properties over the entire range of $InsP_3$ and Ca concentrations tested.

We measured a large increase in steady state channel activity by elevating the $InsP_3$ concentration (Fig. 1). It is interesting to note that these changes occurred in the absence of other cellular processes that have been implicated in the regulation of $InsP_3$-gated channel activity. For example, it has been proposed that the activating phase of the calcium dependence curve relies upon phosphorylation of the channel by protein kinase C and that the inhibitory phase of the calcium-dependence curve reflects the dephosphorylation of the channel by calcineurin (Cameron et al., 1995). In this series of experiments, the increase in channel activity is unlikely to be attributed to phosphorylation because Mg-ATP was not present (Na-ATP was used in the experimental protocol) and no kinase was added to the system. Similarly, it is unlikely that the decrease in channel activity measured in these experiments is the consequence of calcineurin activity because, in preliminary experiments, we were unable to detect calcineurin in our microsomal preparation. The ability to reverse the effects of elevated Ca in the absence of added kinase or Mg-ATP in our experiments also argues against an absolute requirement for phosphorylation/dephosphorylation in the Ca-dependent regulation of $InsP_3$-gated channel activity.

The mechanism underlying the Ca-dependent inhibition of $InsP_3$ binding to the 50-nM $InsP_3$ binding site is unclear, but may involve the presence of an accessory protein associated with the $InsP_3$ receptor (Danoff et al., 1988; Ferris and Snyder, 1992; Benevolensky et al., 1994). Our experiments suggest that the 10-μM site cannot reside on the associated protein that confers Ca sensitivity to the $InsP_3$ receptor. The $InsP_3$ receptor purified with heparin affinity chromatography is calculated to be 94% pure and it lacks Ca sensitivity. The heparin affinity purified channel, however, does retain the 10-μM site in an approximately one-to-one stoichiometry with the 50-nM $InsP_3$ binding site. Other proteins thought to associate with the $InsP_3$ receptor do not appear to bind $InsP_3$ (e.g., FKBP12, calcineurin), strongly implying that the 10-μM site resides on the $InsP_3$ receptor.

Two properties of the 10-μM site are crucial for $InsP_3$-gated channel function. First, binding of $InsP_3$ to the 10-μM site is not Ca dependent (Fig. 3). This allows $InsP_3$ to remain bound to the receptor even in the presence of high Ca. Indeed, even in the presence of 150 μM Ca it was not possible to remove all of the $InsP_3$ (Benevolensky et al., 1994). With this site, the channel can remain open even when cytoplasmic Ca is at micromolar concentrations. Second, the existence of a binding site with affinity orders of magnitude different from previously identified binding sites provides a wider dynamic range over which the channel can function.

Three models have been proposed for the regulation of the $InsP_3$-gated Ca channel (De Young and Keizer, 1992; Atri et al., 1993; Othmer and Tang, 1993; Bezprozvanny and Ehrlich, 1994). Only one of these models (De Young and Keizer, 1992), however, predicts a rightward shift in the Ca dependence as the $InsP_3$ concentration is increased from 0 to 2 μM. This model included only one binding site for $InsP_3$ and it gave reasonable fits of our single channel data over the range of $InsP_3$ concentrations from 0 to 2 μM. However, this

model was unable to fit our $InsP_3$ binding data over the same range of $InsP_3$ concentrations with the parameters used to fit the single channel data. Moreover, the one $InsP_3$ binding site model cannot explain the persistence of channel activity at very high concentrations of $InsP_3$ and high levels of Ca.

When $InsP_3$-induced Ca release was measured in Purkinje cells of rat cerebellar slices, the cytoplasmic free Ca was elevated to 26 μM (Khodakhah and Ogden, 1995), well beyond the Ca concentration one would have initially expected for $InsP_3$-mediated Ca release. Indeed, it was thought that this could never happen because the $InsP_3$-gated channel would be closed by 5 μM cytoplasmic Ca. The data in the present paper show that intracellular Ca in the tens of micromolar range could be achieved by an $InsP_3$-dependent pathway. Moreover, the ability of the channel to remain open at high intracellular Ca occurs when $InsP_3$ concentrations are elevated, which is consistent with the need for $InsP_3$ concentrations of 9 μM and higher to induce Ca release in intact Purkinje cells (Khodakhah and Ogden, 1993, 1995). Thus, the values for intracellular Ca predicted by the model presented here (see Fig. 5) are within the range found in intact Purkinje cells.

The interaction between $InsP_3$ and Ca in the regulation of the $InsP_3$-gated channel (Fig. 2) also may explain the pattern of cytoplasmic Ca oscillations evoked by $InsP_3$ in pancreatic acinar cells. These cells generate Ca oscillations in the continued presence of low concentrations of $InsP_3$. The oscillations are a consequence, at least in part, of the Ca-dependent inhibition of Ca release through the $InsP_3$-gated channel. When $InsP_3$ levels are ≥50 μM, sustained elevations in cytoplasmic Ca are observed (Wakui et al., 1989; Petersen et al., 1991). The ability to sustain an elevation in intracellular Ca in the presence of high concentrations of $InsP_3$ is consistent with the response of the $InsP_3$-gated channel seen in the presence of high concentrations of $InsP_3$ (Fig. 2 *C*).

High concentrations of $InsP_3$ normally exist in a number of cell types under basal (0.1–3 μM $InsP_3$) and agonist-induced (1–20 μM $InsP_3$) conditions (Putney, 1990). These values for intracellular $InsP_3$ concentrations may actually be underestimates when confined portions of the cell, such as dendrites, are considered. That many cell types have resting and stimulated concentrations of $InsP_3$ thought to be super saturating now has a purpose, to provide a prolonged elevation of intracellular Ca and to provide a larger dynamic range for $InsP_3$-mediated Ca signaling.

In summary, we show that the interactions between Ca and $InsP_3$ with the $InsP_3$-gated channel are complex (Fig. 5). The ability of Ca to regulate the activity of the $InsP_3$ receptor within the expected range of intracellular Ca concentrations by interacting directly with the channel complex has been useful for understanding Ca signaling, waves, and oscillations (Allbritton and Meyer, 1993; Berridge, 1993; Bezprozvanny, 1994; Clapham, 1995). The presence of the newly identified $InsP_3$ binding site on the purified $InsP_3$ receptor provides physiological relevance for the seemingly high levels of basal (0.1–3 μM) and agonist-induced $InsP_3$ concentrations (1–20 μM; Putney, 1990). Our results and model show that more complex interactions among the regulatory ligands are needed to explain $InsP_3$-gated channel function. The expanded relationship between $InsP_3$ and Ca demonstrated in the present paper is of great functional importance as a cell is able to overcome Ca-induced channel inhibition during sustained stimulation by producing more $InsP_3$.

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