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Topical Review

The Inositol 1,4,5-Trisphosphate ($InsP_3$) Receptor

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Introduction

Inositol 1,4,5-trisphosphate ($InsP_3$), a compound generated by enzymatic breakdown of the lipid precursor phosphatidylinositol 4,5-bisphosphate after activation of phospholipase C, is a second messenger used by many cell types to stimulate the release of calcium (Ca) from intracellular Ca stores into the cytoplasm via activation of $InsP_3$ receptors/Ca channels ($InsP_3R$) (Berridge, 1993). This mechanism is used by many cell types in response to a variety of stimuli and thereby make $InsP_3R$ vital for cell function. In the last several years, our knowledge about structural and functional properties of $InsP_3R$ was increased dramatically thanks to the combined efforts of biochemists, molecular biologists, and biophysicists. Several recent reviews have covered the properties of $InsP_3R$ in depth (Berridge & Irvine, 1989; Taylor & Richardson, 1991; Berridge, 1993; Furuichi et al., 1994). In this review, we will focus on the most recent data with emphasis on the functional properties and regulation of $InsP_3R$ activity. When appropriate, the properties of the $InsP_3R$ will be compared with the properties of another type of intracellular Ca release channel, the ryanodine receptor (RyR).

Key words: Intracellular calcium release channel — Calcium signaling — Second messenger — Ryanodine receptor

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Molecular Structure of the $InsP_3R$

After the role of $InsP_3$ as a Ca mobilizing agent was discovered (Streb et al., 1983) several laboratories started to use radiolabelled $InsP_3$ in binding assays in order to find the molecular target of this messenger. Specific $InsP_3$ binding sites with an affinity in the nanomolar range were first found in liver and adrenal cortex (Baukal et al., 1985; Spat et al., 1986), but the density of these receptors was low. A detailed biochemical characterization of the $InsP_3$-binding sites became possible after an unusually high density of these sites was found in cerebellum (Worley et al., 1987). The binding of $InsP_3$ to its receptor was dramatically increased by alkalinization and inhibited by submicromolar concentrations of free Ca (Worley et al., 1987). The use of heparin-agarose and concanavalin A-sepharose chromatography of Triton-solubilized cerebellar extracts yielded the first successful purification of the $InsP_3R$ (Supattapone et al., 1988). $InsP_3R$ were also purified from aortic smooth muscle (Chadwick et al., 1990) and vas deferens (Mourey et al., 1990) using a similar approach. Extensive purification of $InsP_3R$ from cerebellum in a single step was recently demonstrated using a wheat germ agglutinin column (Hingorani & Agnew, 1992) and by immunoaffinity chromatography (Nakade et al., 1994). Reconstitution of purified $InsP_3R$ into proteoliposomes established that a single protein mediates both recognition of $InsP_3$ and efflux of Ca (Ferris et al., 1989), suggesting that the protein is both a receptor for $InsP_3$ and a Ca channel.

Electrophoretic analysis of the purified $InsP_3R$ preparation revealed a single protein band with an M_r of 260 kDa (Supattapone et al., 1988). If solubilized cerebellar membrane proteins were fractionated on sucrose density

gradients, the $InsP_3R$ migrated to a density compatible with an apparent molecular mass of over 1,000 kDa (Mignery et al., 1989), suggesting a tetrameric structure for the channel complex. Support for the hypothesis that the $InsP_3R$ is a tetramer was obtained later in experiments where the purified cerebellar $InsP_3R$ was cross-linked with bis(sulfosuccinimidyl)suberate (Maeda et al., 1991) and in electron microscopic analysis of the purified receptor showing that the complex has the appearance of a pinwheel with four spokes radiating from a common center (Chadwick et al., 1990).

Use of antibodies specific for the $InsP_3R$ revealed that the Purkinje cells of cerebellum contained a very high density of these receptors (Ross et al., 1989) and antibodies against one of the proteins expressed specifically in Purkinje cell (PCD6, (Nordquist et al., 1988)) cross reacted with the purified $InsP_3R$ (Mignery et al., 1989). These observations allowed the determination of the sequence coding the carboxyl terminus of $InsP_3R$ (Mignery et al., 1989) and then the complete primary structure of the receptor from rat cerebellum (Mignery et al., 1990). At the same time the coding sequence of P_{400}, a Purkinje cell specific protein from mouse, was determined by another laboratory and from the primary structure of this protein it was proposed to be an $InsP_3R$ (Furuichi et al., 1989). The $InsP_3R$ from both species contained 2749 amino acids, corresponding to an estimated molecular weight of 313 kDa. A very high degree of conservation between rat and mouse forms of the receptor was observed (Furuichi et al., 1989; Mignery et al., 1990).

Soon after the first clone was obtained ($InsP_3R$-I), additional isoforms of $InsP_3R$ (type II and type III) were cloned from rodent and human cDNA libraries using PCR techniques (Sudhof et al., 1991; Ross et al., 1992; Blondel et al., 1993; Maranto, 1994; Yamamoto-Hino et al., 1994). All three isoforms of $InsP_3R$ are 60–70% homologous to each other and differ in tissue distribution. Type I and II are the most abundant in the central nervous system and especially in cerebellum, and type III is specific for pancreatic islets, kidney and gastrointestinal tract. It also was found that the $InsP_3R$ from *Drosophila melanogaster* (Yoshikawa et al., 1992) and *Xenopus* oocytes (Kume et al., 1993) have extensive sequence similarity to the mouse, rat, and human isoforms of the receptor. Thus, only one closely related family of $InsP_3R$ coding genes was discovered so far despite the use of a large number of tissues and organisms. Alternative splicing of RNA coding the $InsP_3R$ was also revealed (Mignery et al., 1990). Interestingly, different splice variants of the receptor were expressed preferentially in specific regions of the brain and at different stages of neuronal development (Nakagawa et al., 1991a; Nakagawa et al., 1991b). PCR analysis also revealed that a 39 amino acid sequence was present in the brain RNA coding $InsP_3R$-I but these 39 amino acids were

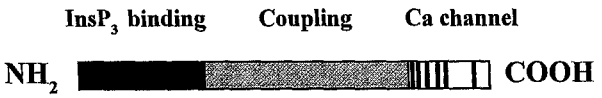

Fig. 1. Domain structure of the $InsP_3$ receptor. Modified from (Sudhof et al., 1991) with permission from the copyright holder. The coupling domain contains consensus sequences for phosphorylation by protein kinases, putative binding sites for Ca and ATP, and alternative splicing sites. Information on the location of regulatory sites in the $InsP_3R$ was recently reviewed (Furuichi et al., 1994).

spliced out in the RNA coding $InsP_3R$-I in all peripheral tissues tested (Danoff et al., 1991), thus providing a potential marker for neuronal and nonneuronal forms of the type I $InsP_3R$.

Cloning of the $InsP_3R$ has allowed the possibility of probing the link between structure and function of this protein. In an elegant series of experiments, COS cells were transfected with expression vectors containing truncated constructs of the $InsP_3R$ gene and the biochemical properties of the expressed proteins were evaluated (Mignery & Sudhof, 1990). From the results of these experiments, a domain structure of the $InsP_3R$ has been proposed (Fig. 1) (Mignery & Sudhof, 1990). In this model the $InsP_3R$ consists of three domains, each with a distinct functional role; the amino terminal is the $InsP_3$ binding domain, the carboxyl terminal domain forms the Ca channel and is important for tetramerization of the receptor complex, and there is a very long coupling domain between the ligand binding and channel-forming domains (Fig. 1). A prediction from this model is that binding of ligand to the $InsP_3$ binding domain will cause a dramatic change in the conformation of the coupling domain which will, in turn, induce Ca channel opening. Indeed, a large-scale conformational change of the coupling domain upon binding of $InsP_3$ was directly observed (Mignery & Sudhof, 1990). This model was later independently supported using truncated constructs of the mouse $InsP_3R$ expressed in NG 108-15 cells (Miyawaki et al., 1991).

Despite some initial discrepancies (Furuichi et al., 1989; Mignery et al., 1990), it is now generally accepted that there are an even number of transmembrane spanning regions in the channel-forming domain. Thus, both the amino and carboxyl termini of the $InsP_3R$ reside in the cytoplasm (Mignery et al., 1989). It is believed that there are six membrane-spanning regions with a relatively large intraluminal loop between the fifth (M5) and sixth (M6) transmembrane segments (Mikoshiba, 1993). This model of $InsP_3R$ topology was recently confirmed using antibodies directed against the M5-M6 loop and by localizing *N*-glycosylation sites on the $InsP_3R$ (Michikawa et al., 1994; Takei et al., 1994). Thus, the $InsP_3R$ channel complex, composed of four subunits each containing six membrane-spanning segments, belongs to the superfamily of voltage-gated (Catterall, 1988) and second messenger-gated channels (Jan & Jan, 1992).

Analysis of the $InsP_3R$-I sequence revealed the presence of several putative regulatory sites in the coupling domain of the receptor (Furuichi et al., 1989; Mignery et al., 1990). These sites include, but are not limited to, two consensus sequences for phosphorylation by cAMP-dependent protein kinase, two putative ATP binding sites, and a potential Ca binding region (Taylor & Richardson, 1991; Ferris et al., 1992; Mignery et al., 1992; Mikoshiba, 1993). The alternative splicing event that creates neuronal and nonneuronal forms of $InsP_3R$-I (*see above*) also occurs in the coupling domain. Type II and type III receptors have a consensus Gly-rich sequence indicative for a nucleotide binding site and a conserved Ca binding region in the coupling domain (Sudhof et al., 1991; Blondel et al., 1993; Furuichi et al., 1994; Maranto, 1994; Yamamoto-Hino et al., 1994) suggesting that all three $InsP_3R$ isoforms are modulated by ATP and Ca. As interaction with the coupling domain appears to be an ideal way to modulate $InsP_3$-gated channel activity, more sites of regulation will probably be discovered in this region of the protein.

Because there is no experimental evidence to support the formation of heterotetrameric receptors from subunits coded by different isoforms or splice variants of $InsP_3R$, it is assumed that the $InsP_3R$/Ca channel is formed by four identical subunits ~2,750 amino acids each with total molecular weight of the complex of about 1,200 kDa. Despite some variations between different isoforms of $InsP_3R$ these genes have similar overall design (domain structure; Fig. 1) and extensive sequence similarities (60–70% homology). Another intracellular Ca release channel, the RyR, is also homotetramer but it is about twice as large as the $InsP_3R$ (the molecular weight of the RyR complex is more than 2,000 kDa (Fleischer & Inui, 1989; McPherson & Campbell, 1993; Sorrentino & Volpe, 1993; Furuichi et al., 1994). The members of the RyR gene family, skeletal (Takeshima et al., 1989; Zorzato et al., 1990), cardiac (Nakai et al., 1990; Otsu et al., 1990), and brain (Hakamata et al., 1992) isoforms of the RyR are very different in sequence from the $InsP_3R$ genes. The only region with some sequence similarity (~40%) between RyR and $InsP_3R$ is the transmembrane domain that forms the Ca channel pore and is responsible for the tetramerization (Mignery et al., 1989). Thus, $InsP_3R$ and RyR are two structurally distinct families of intracellular Ca channels.

Conduction Properties of the $InsP_3R$

The main function of the $InsP_3R$ is to allow the flow of Ca from intracellular stores to the cytoplasm in response to $InsP_3$ binding. Evidence that the receptor and the ion channel reside in the same protein has been obtained from reconstitution of purified receptor into lipid vesicles (Ferris et al., 1989). In this manner the $InsP_3R$ is analogous to other receptor-operated ion channels, except that the $InsP_3R$ is primarily associated with intracellular membranes. Because intracellular membranes are relatively inaccessible for patch clamping, the information about conduction properties of $InsP_3R$ was obtained from single channel current measurements of $InsP_3R$ incorporated into planar lipid bilayers. $InsP_3$-gated Ca channels were first recorded in bilayers using aortic smooth muscle SR vesicles (Ehrlich & Watras, 1988) but a more extensive characterization of $InsP_3R$ functional properties was obtained when reconstitution experiments were performed using cerebellar microsomes (Bezprozvanny et al., 1991; Watras et al., 1991; Bezprozvanny & Ehrlich, 1993; Bezprozvanny & Ehrlich, 1994). Although several subconductance states of $InsP_3R$ were observed in these experiments (Watras et al., 1991), in optimal recording conditions (*defined below*) the $InsP_3R$ spends more than 90% of the open time in the main subconductance state. Only openings to this state will be considered in the following discussion.

Recently the conduction properties of $InsP_3R$ for divalent cations were studied in detail using channels reconstituted into planar lipid bilayers (Bezprozvanny & Ehrlich, 1994). All four alkaline earth cations tested were able to pass through the $InsP_3R$ with single channel conductances that fall in the sequence Ba > Sr > Ca > Mg. The same order of conductances was reported for the RyR (Tinker & Williams, 1992) although the absolute values of the single channel conductance are approximately twice as large for the RyR. The ability of Mg ions to carry substantial currents through these channels is especially striking when the very high hydration energy and extremely slow substitution rate of water molecules in the inner hydration shell of Mg ions (Hille, 1992) is taken into consideration. One possible explanation of this observation is that when Mg ions pass through the selectivity filters of both intracellular Ca channels they are able to keep the inner shell of water molecules. This suggestion implies that the narrowest portion of the channel pore should be at least 10 $Å^2$ for both channels. An even larger estimate of the pore size (40 $Å^2$) was obtained for the RyR (Lindsay et al., 1991) based on the ability of large organic cations like $Tris^+$ and TEA^+ to permeate through these channels. The idea of a fairly wide pore fits well with the observed order of conductances of these channels for divalent cations which corresponds to the order of mobilities of these ions in water. Both channels have a similar selectivity for divalent *vs.* monovalent cations: the estimated P_{Ba}/P_K permeability ratio is 6.3 for $InsP_3R$ (Bezprozvanny & Ehrlich, 1994), whereas reported values of $P_{Divalent}/P_K$ permeability ratios for the RyR range between 4 and 8 (Smith et al., 1988; Liu et al., 1989; Lindsay & Williams, 1991; Tinker & Williams, 1992). When channel permeation was studied with mixtures of divalent cations, the "anomalous mole fraction effect" was not observed in

the experiments with $InsP_3R$ (Bezprozvanny & Ehrlich, 1994) and RyR (Lindsay et al., 1991; Tinker & Williams, 1992) suggesting that no more than one ion is present in the permeation pathway of these channels at the same time. The similarity in the conduction and selectivity properties of $InsP_3R$ and RyR is not surprising because the transmembrane domains of these proteins are 40% homologous (Mignery et al., 1989) and both channels are organized as homotetramers (Anderson et al., 1989; Chadwick et al., 1990).

It could be concluded from the studies of $InsP_3R$ (Bezprozvanny & Ehrlich, 1994) and RyR (Lindsay et al., 1991; Tinker & Williams, 1992) permeation that both channels are rather nonspecific cation selective channels, permeable to Ca and monovalent cations. Under physiological conditions, there is no gradient for K and Na across the reticular membrane (Somlyo et al., 1977) and the main current through RyR and $InsP_3R$ is carried by Ca due to the large electrochemical gradient for Ca across the reticular membrane. As an aside, it follows from this discussion that if plasma membrane $InsP_3$-gated Ca-selective channels do exist (Kuno & Gardner, 1987; Fujimoto et al., 1992) they must be much more selective for divalent cations than intracellular $InsP_3R$. Thus, plasma membrane $InsP_3$-gated channels are not "ordinary" $InsP_3R$ that happen to be located in the plasma membrane due to a nonspecificity in the sorting mechanism, but rather they must be an entirely different species with distinct functional properties.

In contrast to the intracellular Ca release channels, the voltage-gated Ca channels (for example, L-type) must discriminate very well against Na and K ions in order to avoid large fluxes of monovalent cations at every opening of a channel. It is notable that the order of conductances for divalent cations, selectivity against monovalent cations, and the mechanism of ion permeation for $InsP_3R$ and RyR differ dramatically from those obtained in the studies of plasma membrane L-type Ca channels. L-type Ca channels are extremely selective against monovalent cations ($P_{Divalent}/P_{Monovalent} > 1000$ (Lee & Tsien, 1982; Lee & Tsien, 1984)) and the current amplitude follows the sequence $Ba > Ca = Sr \gg Mg$ with Mg being practically impermeable (Hess et al., 1986). The "anomalous mole fraction effect" in the presence of a mixture of divalent cations is observed with L-type Ca channels (Almers & McClesky, 1984; Hess & Tsien, 1984; Tsien et al., 1987) suggesting a multi-ion mechanism of permeation in these channels. Indeed, a "two-site model" was proposed (Almers & McClesky, 1984; Hess & Tsien, 1984; Tsien et al., 1987) to describe Ca permeation through these channels as a result of electrical repulsion between Ca ions bound in the two intrapore binding sites. Recently, both of these sites were localized to a ring of four glutamates in the SS1-SS2 homologous repeats of the $\alpha 1$ subunit of the L-type cardiac channel (Yang et al., 1993). From structure/functional analyses it was proposed that these four glutamates form a "fuzzy" Ca binding site that is able to bind one Ca ion with high affinity or two ions with low affinity (Yang et al., 1993). Some inequality in the properties of these four glutamates seems to be crucial for this model to work (Yang et al., 1993).

The selectivity filters of intracellular Ca release channels ($InsP_3R$ and RyR) are formed by amino acid residues from the four identical subunits of the $InsP_3R$ or RyR homotetramers and therefore, the pore is most likely symmetrical. One can speculate that to create the asymmetrical "fuzzy" Ca binding site that plays a critical role in the voltage-gated Ca channels selectivity process (Yang et al., 1993) the homotetrameric structure of intracellular Ca channels, composed of independent subunits, had to be replaced by four homologous, but not identical, repeats in a single peptide chain ($\alpha 1$ subunit) to form the asymmetric pore proposed for L-type Ca channels and probably present in other types of voltage-gated Ca channels.

Indirect evidence in support of this hypothesis could be obtained from the studies of the transient receptor potential (*trp*) mutant of *Drosophila* that was shown to be defective in Ca signalling (Minke & Selinger, 1992). Analysis of *trp* (Montell & Rubin, 1989; Wong et al., 1989) and *trp*-like (*trpl*) (Phillips et al., 1992) clones revealed sequence similarities between putative transmembrane regions of these proteins and the membrane-spanning segments at each of the four homologous repeats of the brain isoform of the L-type Ca channel (Hui et al., 1991). Based on this comparison it was suggested that the plasma membrane Ca-permeable channel could be formed by tetramerization of *trp* or *trpl* proteins (Phillips et al., 1992). Indeed, measurements of light-induced currents in the wild-type and *trp* mutant Drosophila photoreceptors suggested that the *trp* gene product is a Ca-permeable plasma membrane channel (Hardie & Minke, 1992). Interestingly, the selectivity and permeability properties of the channel formed by *trp* gene products are much more like the properties of $InsP_3R$ and RyR than the properties of voltage-gated Ca channels. The P_{Ca}/P_{Cs} permeability ratio is 40 for the channel formed by *trp* proteins (Hardie & Minke, 1992) and >1,000 for L-type Ca channels (Lee & Tsien, 1982; Lee & Tsien, 1984). Substantial current through the channels formed by *trp* gene products was detected with Mg as a current carrier (Hardie & Minke, 1992) whereas Mg is practically impermeable through L-type channels (Hess et al., 1986). It should be noted that in the *trp* gene products the glutamates conserved in all four SS1-SS2 repeats of voltage-gated Ca channels shown to play a critical role in Ca selectivity process (Yang et al., 1993) are replaced in equivalent positions by nonpolar residues methionine (*trpl*) or leucine (*trp*) based on sequence alignment from Phillips et al., 1992. Therefore, the low Ca selectivity of the channels formed by the *trp* gene

product (Hardie & Minke, 1992) may result from the removal of the "glutamate ring" found in the L-type Ca channel (Yang et al., 1993) as well as from the symmetrical homotetrameric structure of this channel. Thus, despite some sequence similarities with the L-type Ca channel (Phillips et al., 1992) the plasma membrane channel formed by *trp* proteins is much closer to the intracellular Ca release channels than to the voltage-gated Ca channels in terms of its conduction and selectivity properties.

The main function of the $InsP_3R$ is to conduct the flux of Ca ions from intracellular stores into the cytosol. How many Ca ions are released into the cytosol every time an $InsP_3R$ opens? To answer this question it is necessary to know the size of the unitary Ca current through an $InsP_3R$ under physiological conditions and the mean open time of the channels. The unitary Ca current through the $InsP_3R$ with the ionic conditions expected in the physiological situation was estimated to be about 0.5 pA (Bezprozvanny & Ehrlich, 1994) from the recordings of $InsP_3R$ activity in the presence of symmetrical 110 mM K and assuming that intraluminal free Ca concentration is equal to 2.5 mM. Notably, this value is 4-fold less than the unitary Ca current through the cardiac RyR under exactly the same conditions (Tinker et al., 1992; Tinker et al., 1993). The mean open time of the $InsP_3R$ under physiological conditions is 3.7 msec (Bezprozvanny & Ehrlich, 1994). Taking this number and the size of the Ca current of 0.5 pA estimated above, a value of 5,400 Ca ions per $InsP_3R$ opening is obtained (Bezprozvanny & Ehrlich, 1994). It is notable that not only is the size of the Ca current through the RyR 4-fold higher, but the mean open time of RyR type channels is approximately 20 msec (Smith et al., 1986), 5 times longer than for the $InsP_3R$. As a result, at every channel opening ~20 times more Ca ions are released through the RyR than through the $InsP_3R$. One can speculate that the coexistence of two intracellular Ca channel types activated via different mechanisms allows the cell to choose between two modes of Ca release—rapid dumping of accumulated Ca through the RyR channel (as in skeletal or cardiac muscle) or slow leakage through the $InsP_3R$ (as in smooth muscle). Additional complexity in neuronal Ca signaling is suggested by the presence of both receptor types in the brain.

Bell-shaped Ca Dependence of the $InsP_3R$

Cytoplasmic Ca was shown to inhibit $InsP_3$ binding to the $InsP_3R$ with half inhibition at 300 nM Ca (Supattapone et al., 1988). From these data, one may expect a monotonic inhibition of $InsP_3$-gated channels with increases in the cytoplasmic Ca concentration in the submicromolar range. However, when the Ca dependence of $InsP_3$-induced Ca release was determined in permeabilized smooth muscle, a biphasic effect of Ca was observed with a maximum rate of release at 300 nM free Ca (Iino, 1990). The mechanism for the activation by Ca below 300 nM in these experiments could occur by a Ca dependent alteration in $InsP_3$ metabolism or by a direct effect on the channel's gating mechanism. Although it was suggested that the latter possibility was responsible for the biphasic Ca effect (Iino, 1990), use of permeabilized cells did not allow elimination of the other possibilities.

Use of a rapid superfusion system allowed studies that focused on analysis of the subsecond kinetics of $InsP_3$-induced Ca release from brain synaptosomal vesicles (Finch et al., 1991). It was found that extravesicular Ca acted as a coagonist of $InsP_3$-induced release with a maximum amount of Ca released at 500 nM free Ca. Further increases in extravesicular Ca inhibited $InsP_3$-induced Ca release. Although Ca potentiation of $InsP_3$-induced release occurred faster than the detection limit of the rapid superfusion system, a more slowly developing Ca-induced inhibition was measured with a time constant of 580 msec at 10 μM free Ca (Finch et al., 1991). In a recent report, use of caged Ca further improved the time resolution of the experimental system and experiments on permeabilized smooth muscle cells showed that both activating and inhibitory effects of Ca on the $InsP_3R$ were extremely rapid in the presence of $InsP_3$, which would allow for immediate feedback control of $InsP_3$-induced Ca release (Iino & Endo, 1992). When the ability of cytosolic Ca to modulate $InsP_3$-induced Ca release was studied in *Xenopus* oocytes using light-flash photolysis of caged $InsP_3$, both inhibition of $InsP_3R$ at high Ca levels (Parker & Ivorra, 1990) and facilitation at low Ca levels (Yao & Parker, 1992) were observed.

To test whether there is a direct effect of Ca on the gating of the $InsP_3R$, the effects of cytoplasmic Ca on $InsP_3R$ activity were monitored in planar lipid bilayers (Bezprozvanny et al., 1991). The open probability of the channel increased as the free Ca concentration was elevated from 10 nM to 250 nM and decreased at Ca concentrations above 250 nM (Fig. 2*a*). Both activating and inhibitory effects of Ca on channel activity were reversible, ruling out the possibility of Ca-induced chemical modification of the receptors. A bell-shaped Ca dependence curve was obtained with the maximum open probability at 200–300 nM free Ca (Fig. 2*b*). The single channel open probability decreased sharply on both sides of the maximum with the entire curve falling within the physiological range of Ca concentrations.

Despite some differences among the reports, a general conclusion can be made based on the experimental data described above. It is evident that the function of the $InsP_3R$ is regulated by cytoplasmic Ca in a biphasic manner with the maximum activity of the channels at Ca levels of 200–300 nM. This regulation does not seem to be mediated via some sort of enzymatic modification, because it occurs in an in vitro reconstitution system, it is very fast, and it is reversible. It is not clear whether these

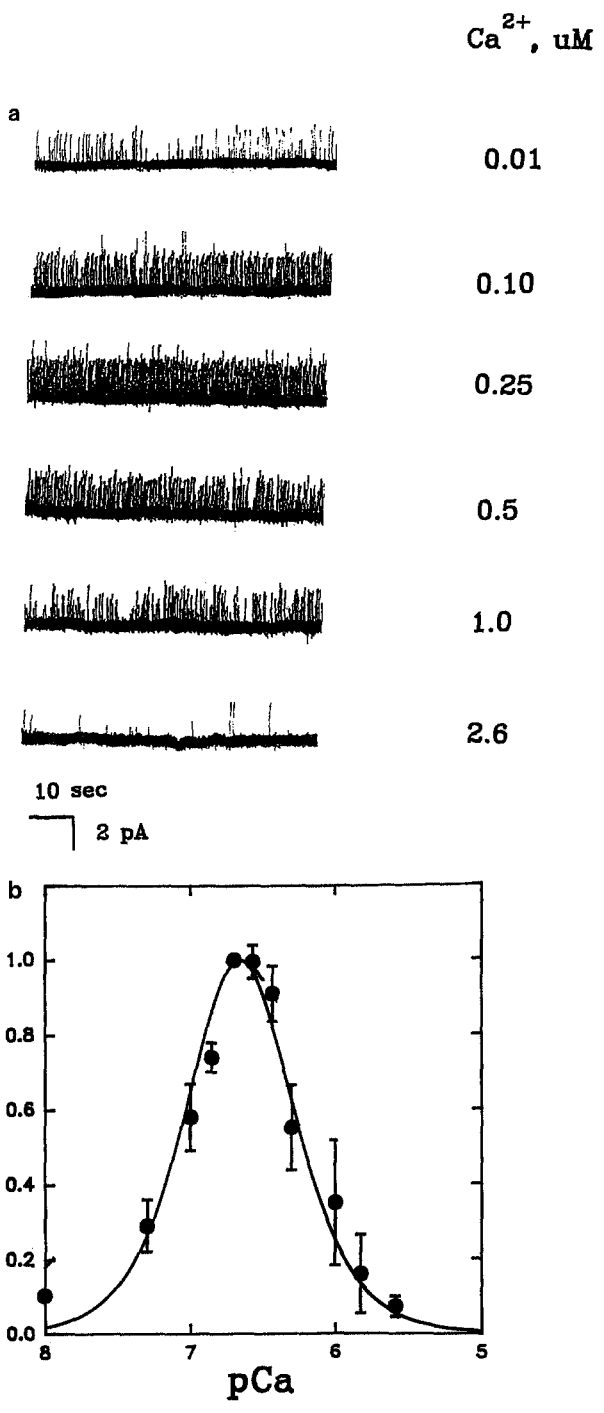

Fig. 2. Ca dependence of the $InsP_3R$ in planar lipid bilayers. (*a*) Channel activity was monitored in the presence of 2 μM $InsP_3$ and 330 μM AMP-PCP. Various amounts of a calibrated 20 mM $CaCl_2$ solution were added to a mixture of 1 mM EGTA and 1 mM HEEDTA and the free Ca concentration calculated after each addition is indicated in μM at the right side of each record. The effect of Ca was reversible. (*b*) Open probability of the $InsP_3$-gated channel versus the free Ca concentration. To compare four different experiments, values were normalized to the maximum channel activity observed in each experiment, which never exceeded 15% assuming one channel in the bilayer. Modified from (Bezprozvanny et al., 1991) with permission from the copyright holder.

changes in channel function are mediated by Ca interactions with the $InsP_3R$ itself or with Ca binding proteins associated with the receptor. The purified $InsP_3R$ binds Ca (Danoff et al., 1988) and at least one Ca-binding site has been found in the coupling domain of the receptor (Mignery et al., 1992). These observations provide evidence in favor of a direct effect of Ca on $InsP_3R$ function. The existence of a Ca-binding protein, "calmedin," was proposed (Danoff et al., 1988) to explain Ca-induced inhibition of $InsP_3$ binding to the cerebellar $InsP_3R$. Although the observed Ca-induced inhibition of $InsP_3$ binding could be attributed to displacement of radiolabeled $InsP_3$ from the $InsP_3R$ due to $InsP_3$ generation by a Ca-sensitive phospholipase C in the course of the binding assay (Mignery et al., 1992), it was recently demonstrated that inhibition of $InsP_3$ binding by submicromolar Ca can be independent from phospholipase C activation (Van Delden et al., 1993; Benevolensky et al., 1994). These later observations revive the existence of "calmedin" despite the fact that this protein has not been identified so far. It is also possible that inhibition of $InsP_3R$ function by high Ca develops from Ca-induced densensitization of the receptor, rather than from a decrease in $InsP_3$ binding. For example, Ca-mediated interconversion between functional and nonfunctional states of the $InsP_3R$ in liver was reported (Pietri et al., 1990; Pietri Rouxel et al., 1992); a similar mechanism might be important for Ca-induced inactivation of the $InsP_3R$ in the other tissues as well. Although the exact location of the "activating" and "inhibitory" Ca-binding sites is not yet known, recent experiments with the $InsP_3R$ purified from *Xenopus* oocytes suggested that some associated protein or factor mediates the Ca-induced inhibition of $InsP_3R$ but the "activating" site is on the $InsP_3R$ itself (Callamaras & Parker, 1994).

Although a bell-shaped Ca dependence of RyR on cytosolic Ca was also reported (Meissner et al., 1986; Bezprozvanny et al., 1991), the descending part of the curve is in the millimolar range of Ca concentrations, much higher than the physiological range. Based on steady-state measurements of channel activity within the physiological range of cytosolic Ca concentrations, the RyR displays solely Ca-dependent activation whereas the $InsP_3R$ displays both Ca-dependent activation and inhibition. In experiments using caged Ca to rapidly alter the Ca concentration, the RyR demonstrated adaptation (Gyorke & Fill, 1993), suggesting a potential role for Ca-induced inactivation of the RyR as well. The Ca-dependent enhancement of $InsP_3R$ activity implies that the $InsP_3R$ can also act as a Ca-gated Ca channel in the presence of a fixed concentration of $InsP_3$ if the cytoplasmic Ca is less than 300 nM. Traditionally, Ca-induced Ca release was considered to be the functional marker for the presence of RyR in a cell, but this postulate has to be reconsidered in view of the bell-shaped Ca dependence of $InsP_3R$. Indeed, when the time course

of cytosolic free Ca changes in smooth muscle was studied, it was found that a slow rise in Ca in agonist-stimulated cells was followed by a wave of rapid regenerative Ca release from $InsP_3$-sensitive stores as the local Ca concentration reached a critical level of about 160 nM (Iino et al., 1993), a behavior characteristic for Ca-induced Ca release.

Ca imaging techniques revealed that in many instances stimulation of the $InsP_3$ signaling pathway caused repetitive Ca waves propagating through the cytoplasm of the cell or periodic oscillations in the cytosolic Ca level (Tsien & Tsien, 1990; Meyer, 1991; Meyer and Stryer, 1991; Rooney & Thomas, 1993). It is generally recognized that the bell-shaped Ca dependence of the $InsP_3R$ (Fig. 2) (Iino, 1990; Parker & Ivorra, 1990; Bezprozvanny et al., 1991; Finch et al., 1991; Iino & Endo, 1992; Yao & Parker, 1992) is the key characteristic of the $InsP_3R$ that allows creation of models of Ca waves and oscillations based solely on the properties of the $InsP_3R$ (Parker & Yao, 1991; DeLisle & Welsh, 1992; DeYoung & Keizer, 1992; Lechleiter & Clapham, 1992; Atri et al., 1993; Othmer & Tang, 1993). These models are especially useful for understanding of Ca waves and oscillations in *Xenopus* oocytes, the most extensively studied system (Parker & Ivorra, 1990; Lechleiter et al., 1991; Parker & Yao, 1991; DeLisle & Welsh, 1992; Lechleiter & Clapham, 1992; Parker & Ivorra, 1993). Indeed, *Xenopus* oocytes have only $InsP_3$-sensitive Ca store (Parys et al., 1992) and changes in $InsP_3$ concentration are not essential for generation of Ca waves and oscillations in this system as concluded from the experiments with nonhydrolyzable $InsP_3$ analogs (DeLisle & Welsh, 1992; Lechleiter & Clapham, 1992).

The cytoplasm of *Xenopus* oocytes can be viewed as an excitable medium composed of Ca release processes ($InsP_3R$), coupled by a common stimulatory signal (Ca) through diffusion (Lechleiter & Clapham, 1992). When numerical simulations of Ca wave propagation and Ca oscillations were performed based on this hypothesis, good agreement with experimental results was obtained (DeYoung & Keizer, 1992; Atri et al., 1993; Othmer & Tang, 1993). Recently, theoretical analysis of Ca wave propagation based on $InsP_3R$ functional properties allowed the derivation of analytical expressions for Ca wave amplitude and the velocity of the wave propagation (Bezprozvanny, 1994). In this analysis the bell-shaped Ca dependence of $InsP_3R$ was accounted for by using a simplified four-state dynamic model of $InsP_3R$ regulation by cytosolic Ca with one "activating" and one "inhibitory" site (Fig. 3). The amplitude of the Ca wave was determined by a combination of cell-specific parameters and the functional properties of single $InsP_3R$ obtained experimentally. The velocity of wave propagation was also determined from the Luther equation for diffusion-driven autocatalytic reactions (Jaffe, 1991) using the model of $InsP_3R$ gating shown in Fig. 3 (Bezprozvanny, 1994). Both equations provided reasonable estimations for Ca wave amplitude (1.3 μM free Ca) and the velocity of Ca wave propagation (21 μm/s) in *Xenopus* oocytes when numerical values for parameters were used (Bezprozvanny, 1994). Thus, the presentation of the bell-shaped Ca dependence of $InsP_3R$ in the form of the four-state model shown in Fig. 3 can be used in the modeling and analysis of complex spatiotemporal characteristics of intracellular Ca signaling.

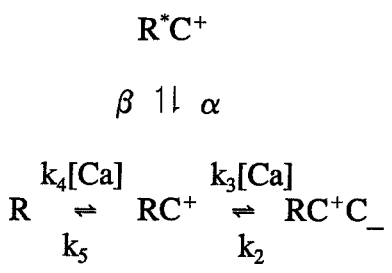

Fig. 3. Simplified four-state model of $InsP_3R$ regulation by cytosolic Ca (Bezprozvanny, 1994). State R is the $InsP_3R$ without Ca bound, state RC^+ is the $InsP_3R$ with Ca bound to the activating site, state $RC^+C\_$ is the $InsP_3R$ with Ca bound to both activating and inhibitory sites and R^*C^+ is the only open state of the channel. It is assumed that the channel can open only when Ca is bound to the activating site and cannot open when Ca is bound to the inhibitory site. $InsP_3$ is bound to the $InsP_3R$ in all four states of the model. The model is based on the bell-shaped Ca dependence of the $InsP_3R$ (Bezprozvanny et al., 1991). It was assumed that binding of Ca to "inhibitory" site happens only after Ca is bound in "activating" site. This requirement could be achieved because of conformational changes of $InsP_3$ receptor complex or because Ca binding in "activating" site happens much faster than in the "inhibitory" site (Finch et al., 1991). The model is simplified because most probably multiple activating and inhibitory sites are present in the $InsP_3R$ complex (Bezprozvanny et al., 1991). However, this model adequately reflects the essence of $InsP_3R$ regulation by cytosolic Ca (Iino, 1990; Bezprozvanny et al. 1991; Finch et al., 1991).

$InsP_3R$ are Potentiated by ATP

The initial studies of $InsP_3$-mediated signal transduction mechanisms demonstrated that the presence of ATP or nonhydrolyzable ATP analogs was essential for $InsP_3$-induced Ca release (Smith et al., 1985). These authors came to the conclusion that ATP was a necessary cofactor for the activation of what was then a hypothetical $InsP_3R$ (Smith et al., 1985). The role of ATP as an allosteric activator of the $InsP_3R$ was proposed later based on experiments with receptor that was purified and reconstituted into liposomes (Ferris et al., 1990). It was found that 10 μM ATP or nonhydrolyzable ATP analogues dramatically potentiated $InsP_3$-mediated Ca flux into vesicles containing purified $InsP_3R$. The existence of a specific ATP-binding site on the $InsP_3R$ was also demonstrated in the same report (Ferris et al., 1990). In agreement with the earlier data (Smith et al., 1985)

AMP and GTP could not substitute for ATP in the activation of the $InsP_3R$, but nonhydrolyzable ATP analogs were equally potent (Ferris et al., 1990), ruling out the possibility that channel phosphorylation is involved. The effects of adenine nucleotides on the characteristics of $InsP_3$-induced Ca release were studied in detail using permeabilized vascular smooth muscle cells (Iino, 1991). In accordance with the earlier data, potentiation of $InsP_3$-induced Ca release by submillimolar concentrations of ATP and nonhydrolyzable ATP analogs was observed in these experiments. AMP was also able to potentiate the release, but much larger concentrations were needed.

Despite the general agreement that submillimolar ATP acts as an allosteric activator of $InsP_3R$ function, the mechanism of ATP action was not yet known. Addition of ATP was reported to cause an increase in the open probability of $InsP_3$-gated Ca channels from aortic smooth muscle or cerebellar microsomes reconstituted into planar lipid bilayers (Ehrlich & Watras, 1988; Bezprozvanny et al., 1991). In experiments with the purified and reconstituted $InsP_3R$ from cerebellum both the open probability and the channel conductance were affected by ATP (Maeda et al., 1991). Recently, the modulation of the $InsP_3R$ by ATP was investigated in detail at the single channel level (Bezprozvanny & Ehrlich, 1993). In these experiments, addition of 2 μM $InsP_3$ to the cytoplasmic side of the membrane activated the channels even in the absence of ATP (Fig. 4, second trace), but the open probability was low despite the use of a high $InsP_3$ concentration (2 μM). Subsequent addition of ATP in the presence of $InsP_3$ increased the probability of finding the channel open (Fig. 4, traces 3–7), although ATP in the absence of $InsP_3$ did not activate the channels (Bezprozvanny et al., 1991). Channel openings were completely abolished by the addition of 10 μg/ml heparin (Fig. 4, bottom trace). Inhibition of the channel activity by heparin shows that the ATP-enhanced channel activity is not due to opening of the cerebellar RyR (Ashley, 1989; Bezprozvanny et al., 1991), but is indeed enhancing the activity of the $InsP_3R$. Although heparin is known to inhibit the $InsP_3R$ (Ghosh et al., 1988; Kobayashi et al., 1988), it activates the RyR (Ritov et al., 1985; Bezprozvanny et al., 1993). Single channel data analysis revealed that ATP activated the $InsP_3R$ by increases in both the average duration of channel opening and the frequency of opening (Bezprozvanny & Ehrlich, 1993); the evidence for a nucleotide-dependent alteration in current amplitude (Maeda et al., 1991) was lacking. Similar effects on $InsP_3R$ gating were observed if nonhydrolyzable ATP analogues were used in the experiments (Bezprozvanny & Ehrlich, 1993).

Where does ATP have to bind in order to cause these effects? Biochemical studies have identified a specific binding site for ATP on each subunit of the $InsP_3R$ with an affinity of 17 μM (Maeda et al., 1991). This site was

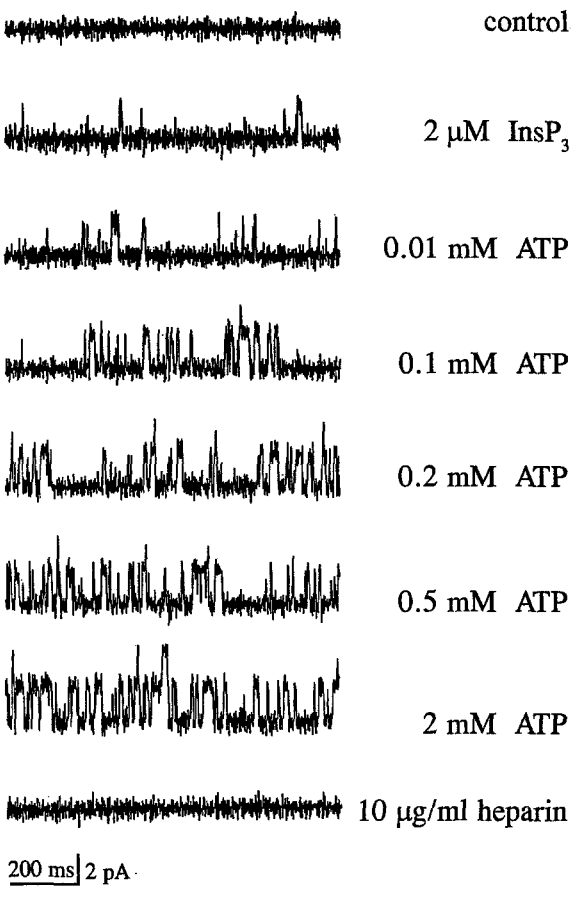

Fig. 4. Potentiation of $InsP_3R$ by ATP. In the absence of ATP and $InsP_3$ channel openings were not observed (top trace). Addition of 2 μM $InsP_3$ induced channel openings, but the open probability was submaximal (second trace). Subsequent addition of ATP to the cytoplasmic side of the membrane increased the channel open probability (traces 3–7). Inhibition of the channel activity by heparin (10 μg/ml) showed that ATP indeed enhanced the activity of the $InsP_3R$ (last trace). Reproduced from (Bezprozvanny & Ehrlich, 1993) with permission from the copyright holder.

found to be quite selective for ATP in comparison to AMP and GTP. Interestingly, the predicted amino acid sequence of the $InsP_3R$-I contains two consensus sequences identified at ATP-binding sites (Furuichi et al., 1989; Mignery et al., 1990; Mignery & Sudhof, 1990); both sequences are located in the coupling domain of the receptor (Fig. 1). The other isoforms of the $InsP_3R$ (type II and type III) also contain a consensus sequence for nucleotide binding in the coupling domain (Sudhof et al., 1991; Blondel et al., 1993; Maranto, 1994; Yamamoto-Hino et al., 1994). It seems reasonable to suggest that ATP binding to this region may affect the coupling between $InsP_3$ binding and channel gating, causing the functional effects described above. It does not seem likely that ATP produces its effect by increasing the affinity of $InsP_3R$ for $InsP_3$ because the affinity of $InsP_3$ for its receptor was not increased by adenine nucleotides

(Worley et al., 1987). More recently, stimulation of $InsP_3$ binding by ATP was observed (Spat et al., 1992), but the degree of potentiation was small (30%) in comparison to the dramatic increase in channel activity caused by addition of ATP. It was suggested (Bezprozvanny & Ehrlich, 1993) that ATP binding to the coupling domain of the $InsP_3R$ increases the intrinsic efficacy of $InsP_3$ severalfold. That is, ATP improves the ability of $InsP_3$ to open the channel when both compounds are bound to the receptor. The modulation of $InsP_3R$ activity by ATP could be formally described using Monod-Wyman-Changeux model (Monod et al., 1965) of allosteric regulation (Bezprozvanny & Ehrlich, 1993).

The activity of the RyR is also dramatically increased by submillimolar levels of ATP (Smith et al., 1986) with both the mean open time and the frequency of openings affected. Is there any physiological relevance of ATP effects on $InsP_3R$ and RyR activity? Although the precise physiological significance of adenine nucleotide regulation of the Ca release channels in intact cells is unclear, allosteric regulation by ATP is a common motif used by the cells to maintain viability (Katz, 1992). This form of regulation can play an important role in the preservation of the cell under conditions of energy starvation which occurs in diseases such as heart failure and occlusive stroke. For example, after several minutes of ischemia, levels of ATP in the brain fall below 0.1 mM (Abe et al., 1987), a concentration where changes in ATP alter $InsP_3R$ and RyR gating. Decreased levels of ATP will reduce Ca release into the cytoplasm from both types of intracellular Ca stores which ultimately will avoid Ca overload.

Phosphorylation of the $InsP_3R$

It was originally shown that P_{400} protein (Yamamoto et al., 1989) or PCPP-260 protein (Walaas et al., 1986) (names given to the $InsP_3R$ protein before its final identification) is one of the best substrates for both endogenous and exogenous cAMP-dependent protein kinase (PKA) in the cerebellum. After biochemical purification of the $InsP_3R$ was achieved (Supattapone et al., 1988), a more detailed and quantitative analysis of $InsP_3R$ phosphorylation became possible. It was initially thought that purified $InsP_3R$ was phosphorylated exclusively by PKA (Supattapone et al., 1988), but later experiments revealed that the $InsP_3R$ is stoichiometrically (one phosphate per receptor subunit) phosphorylated by PKA, PKC and Ca/calmodulin-dependent protein kinase II (CAM-K-II) (Ferris et al., 1991). Phosphorylation by these three enzymes was additive (Ferris et al., 1991), indicating that they act at different sites. Furthermore, serine residues are phosphorylated by all three enzymes, but two-dimensional phosphopeptide maps of $InsP_3R$ trypsin digest products showed that in each case different sites on the $InsP_3R$ peptide were phosphorylated (Ferris et al., 1991).

Analysis of the $InsP_3R$-I primary structure revealed the presence of two putative PKA phosphorylation sites (Furuichi et al., 1989; Mignery et al., 1990), both in the coupling domain of the receptor (*see* Fig. 1). If low PKA concentrations were used in the experiment, then only one of these sites in the cerebellar $InsP_3R$ (serine-1756) was phosphorylated. However, at much higher PKA concentrations another site (serine-1589) was also phosphorylated, resulting in transfer of two phosphate residues to each subunit of the $InsP_3R$ (Ferris et al., 1991). It is interesting to note that the amino acid sequence between these two phosphorylation sites is alternatively spliced, with a stretch of 39 amino acid residues deleted in the form of the receptor expressed in non-neuronal tissues (Danoff et al., 1991). What effect, if any, does this splicing event have on the phosphorylation of $InsP_3R$ by PKA? To address this question the $InsP_3R$ was purified to homogeneity from cerebellum (the long, neuronal form) and from vas deferens (the short, non-neuronal form) and the phosphorylation of these proteins by PKA was monitored. It was found that the $InsP_3R$ from cerebellum was phosphorylated by low concentrations of PKA primarily on serine-1756, whereas the receptor from vas deferens was phosphorylated almost exclusively on serine-1589 (Danoff et al., 1991). Potentially, this difference may form the basis for tissue specific regulation of $InsP_3R$ function by cAMP-mediated signaling pathways.

The functional consequences of cAMP-dependent phosphorylation of $InsP_3R$ were studied using cerebellar microsomes (Supattapone et al., 1988; Volpe & Alderson-Lang, 1990) and permeabilized hepatocytes (Burgess et al., 1991). Phosphorylation of the $InsP_3R$ from cerebellum by the catalytic subunit of PKA caused a 10-fold (Supattapone et al., 1988) or 2-fold (Volpe & Alderson-Lang, 1990) shift to the right in the concentration dependence of $InsP_3$-induced Ca release without any effect on $InsP_3$ binding to the receptor. That is, higher concentrations of $InsP_3$ are needed to achieve the same response as that obtained before phosphorylation. Thus, the intrinsic efficacy of $InsP_3$ is affected by $InsP_3R$ phosphorylation as can be predicted from the location of the putative PKA phosphorylation sites in the coupling domain of the $InsP_3R$-I (*see* Fig. 1). Interestingly, phosphorylation of liver $InsP_3R$ by PKA caused a 4-fold shift to the left in the concentration dependence of $InsP_3$-induced Ca release in permeabilized hepatocytes (an increase in the intrinsic efficacy of $InsP_3$) (Burgess et al., 1991). This observation may be the functional manifestation of the difference in PKA phosphorylation sites between neuronal and non-neuronal splice forms of $InsP_3R$-I discussed above.

Notably, type II and type III $InsP_3R$ do not have corresponding consensus sequences for phosphorylation

by PKA (Sudhof et al., 1991; Blondel et al., 1993; Maranto, 1994; Yamamoto-Hino et al., 1994). Thus, these isoforms are not expected to be regulated by cAMP-dependent phosphorylation. The interpretation of the results obtained testing the effects of PKA on cerebellar microsomes are obscured because these microsomes contain a mixture of $InsP_3R$ isoforms. Recently, affinity purified $InsP_3R$-I from cerebellum was incorporated into proteoliposomes and $InsP_3$-dependent Ca flux was measured (Nakade et al., 1994). Both the rate and extent of Ca influx into the proteoliposomes increased 20% after phosphorylation of the $InsP_3R$ by PKA (Nakade et al., 1994). Because different isoforms of the $InsP_3R$ are expressed in different tissues, type-specific regulation of $InsP_3R$ function may lead to cell-specific cross-talk between $InsP_3$ and cAMP signaling systems.

Phosphorylation of the $InsP_3R$ by PKA provides a means whereby the hormones that affect adenylate cyclase activity could also regulate the function of the inositol phospholipid pathway and Ca mobilization. It also seems attractive to suggest that phosphorylation of the $InsP_3R$ by PKC and CAM-K-II (Ferris et al., 1991) provides additional pathways for feedback regulation of the phosphoinositide transduction mechanism. Activation of PLC in the phosphoinositide cycle signaling pathway generates hydrophobic DAG and the cytoplasmic diffusible messenger $InsP_3$. DAG stimulates the activity of PKC. Production of $InsP_3$ causes an increase in the intracellular Ca level due to $InsP_3$-induced Ca release; this elevation in the intracellular Ca provides additional activation of PKC and turns on CAM-K-II. If the activity of the $InsP_3R$ is indeed regulated by PKC and/or CAM-K-II mediated phosphorylation, then this aspect of receptor phosphorylation may function as a slow feedback mechanism, which operates on a time scale different from the immediate and direct regulation of $InsP_3R$ function by cytosolic Ca described in the previous section. Interestingly, immunohistochemical experiments demonstrated that the cerebellar-specific form of CAM-K-II is highly concentrated in Purkinje cells (Ouimet et al., 1984; Fukunaga et al., 1988) where the density of $InsP_3R$ is the highest (Ross et al., 1989). A variable stoichiometry between PKC and $InsP_3R$ in different parts of the brain was reported (Worley et al., 1987), suggesting the possibility of regional specificity in this form of regulation. Despite the fact that all these suggestions seem to be very plausible, serious consideration is not possible until the functional consequences of $InsP_3R$ phosphorylation by PKC and/or CAM-K-II and the ability of these enzymes to phosphorylate $InsP_3R$ in vivo are clearly demonstrated.

The cardiac, brain, and skeletal RyR are substrates for phosphorylation by CAM-K-II, but the effect of phosphorylation on the channel isoforms differs (Witcher et al., 1991; Wang & Best, 1992; Witcher et al., 1992). The cardiac isoform is activated by phosphorylation by CAM-K-II (Witcher et al., 1991; Witcher et al., 1992), whereas the skeletal isoform is inhibited by the same kinase (Wang & Best, 1992) suggesting tissue specific regulation of Ca release. The RyR also contains consensus sequences for cAMP-dependent phosphorylation (Otsu et al., 1990) but functional effects of phosphorylation by PKA remain to be determined. Thus, the activity of both intracellular Ca release channels is probably modulated by a number of second messenger systems through protein phosphorylation.

Conclusion

In this review, we have described the functional properties and regulation of the $InsP_3R$. Not all aspects of $InsP_3R$ function and regulation were covered, the main focus was on the most recent and physiologically important data. Information about the structure, heterogeneity, functional properties, and regulation of the $InsP_3R$ is useful for understanding the spatiotemporal aspects of Ca signaling. The combination of biochemical, biophysical and molecular biological techniques has revealed the intricacies of the $InsP_3R$ over the past decade. However, questions about the functional differences between various isoforms and splice variants of the $InsP_3R$, the structural determinants responsible for regulation of $InsP_3R$ by Ca and ATP, the functional effects of $InsP_3R$ phosphorylation and many others remain to be elucidated. Future investigations can be expected to provide answers to these important questions.

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Note added in proof: In two recent reports (Mak & Foskett, 1994; Stehno-Bittel et al., 1995) isolated *Xenopus* oocyte nuclei were patch-clamped to measure the activity of the $InsP_3R$ *in situ*. The functional properties of the channels observed in these studies were very similar to the properties of cerebellar InsP3-gated channels reconstituted into planar lipid bilayers.

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