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Feature Article

Inositol Phosphate Receptors and Calcium Disposition in the Brain

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In recent years it has become increasingly apparent that the phosphoinositide (PI) cycle represents a major second messenger system, comparable in importance to cAMP for actions of hormones, neurotransmitters, and other regulatory molecules. The PI cycle responds to the actions of an agonist at a membrane receptor that, via coupling to a G-protein, triggers the hydrolysis of phosphatidylinositol bisophosphate, resulting in the generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG enhances the activity of protein kinase C (PKC) by rendering it more sensitive to stimulation by Ca^{2+} (Nishizuka, 1988), while IP_3 stimulates the release of Ca^{2+} from endoplasmic reticulum (ER) stores (Berridge, 1987; Berridge and Irvine, 1989). The Ca^{2+} released by IP_3 stimulation activates many calcium-dependent processes. For instance, it enhances the phosphorylation of diverse substrates by PKC. IP_3 can be further phosphorylated to form inositol 1,3,4,5-tetrakisphosphate (IP_4), inositol 1,3,4,5,6-pentakisphosphate (IP_5), and inositol hexakisphosphate (IP_6). These higher inositol phosphates are present in the brain and other tissues in concentrations comparable to those of IP_3, and so they may have comparably important functions, which have not been established, however.

Clarification of physiological functions for the PI cycle has emerged mainly from studies of PI metabolism or turnover in response to various stimuli. In brain tissue, PI turnover had usually been monitored by labeling the inositol-containing phospholipids with the precursor ^{3}H-inositol (Berridge et al., 1982). At the steady state, ^{3}H-inositol is present in the various inositol phosphates, whose levels can be monitored in various ways. It is also possible to monitor PI turnover by using ^{3}H-cytidine as an IP precursor. Cytidine feeds into the PI cycle via cytidine diphosphate diacylglycerol (CDP-DAG), whose levels increase with the rate of PI turnover (Godfrey, 1989). Since CDP-DAG is membrane bound, ^{3}H-CDP-DAG localization by autoradiography can monitor PI turnover at the cellular level (Hwang et al., 1990). Such autoradiographic localization in the cerebellum reveals selective glutamatergic stimulation of PI turnover in Purkinje cells and their processes. In the hippocampus, both muscarinic cholinergic stimulation and glutamatergic stimulation increase PI turnover, but their sites of action differ, with cholinergic stimulation affecting CA1, CA3, and CA4, and the subiculum, whereas glutamatergic stimulation is restricted to CA3 and the subiculum (Hwang et al., 1990). While many studies have monitored PI turnover, few measured endogenous levels of the inositol phosphates, largely because of technical difficulties. The high affinity and specificity of IP_3 receptors for IP_3 have facilitated development of a simple and specific radioreceptor assay for IP_3 (Bredt et al., 1989; Challiss et al., 1990). In this assay, endogenous IP_3 in protein-free tissue extracts competes with added exogenous ^{3}H-IP_3 for binding to receptors in crude preparations of cerebellar or adrenal membranes, providing selectivity for the physiologic form of IP_3 and detection of low nanomolar concentrations. Similar procedures allow radioreceptor assays for IP_4 (Donie and Reiser, 1989; Challiss and Nahorski, 1990).

Inositol phosphate receptor characterization and isolation

The first attempts to label IP_3 receptors by ligand binding utilized peripheral tissues such as neutrophils, liver, and adrenal membranes (Baukal et al., 1985; Spat et al., 1986; Guillemette et al., 1987). The relatively low levels of specific IP_3 binding in these tissues precluded their direct biochemical characterization. By utilizing autoradiography to detect receptor bound ^{3}H-IP_3, much higher levels of IP_3 receptors were found in the CNS, where they are present at a very high density in the Purkinje cells of the cerebellum, the tissue source presently utilized for most biochemical studies of IP_3 receptors (Worley et al., 1987a, 1989). The inositol phosphate specificity of the cerebellar IP_3 receptor is essentially the same as that of peripheral tissues (Berridge, 1987; Berridge and Irvine, 1989).

Several properties of IP_3 receptors appear to reflect their physiologically important regulatory function. For instance, physiologic levels of Ca^{2+} inhibit IP_3 binding to its receptor with an IC_{50} of about 300 nM (Worley et al., 1987b; Danoff et al., 1988). This finding implies that Ca^{2+} released by IP_3 feeds back to inhibit further stimulation of Ca^{2+} release by IP_3. Increases in intracellular pH from 7.5 to 8.5 cause a tripling in IP_3 binding to its receptor (Worley et al., 1987b). Increases in intracellular pH also augment the ability of IP_3 to release Ca^{2+} (Joseph et al., 1989), which presumably reflects the effect of pH on binding of IP_3 to its receptor.

The very high density of IP_3 receptors present in the cerebellum facilitated purification of the receptor to apparent homogeneity (Supattapone et al., 1988b). The potent enhancement by heparin of IP_3 binding to its receptor (Worley et al., 1987b) and of Ca^{2+} release (Hill et al., 1987; Ghosh et al., 1988; Guille-

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mette et al., 1989; Joseph and Rice, 1989) allowed the purification of the IP_3 receptor on heparin-agarose affinity columns. A second purification step resorted to concanavalin-A Sepharose chromatography, which took advantage of the glycosylation of the IP_3 receptor. Together, these purification steps allowed a thousandfold purification of the receptor. Meanwhile, the same degree of purification can be achieved also by use of an IP_3 affinity column (Prestwich et al., 1991). When analyzed by PAGE, the purified IP_3 receptor protein moved as a single 260 kDa band. However, the molecular weight of the native receptor is about 1000 kDa, reflecting a homotetramer (Supattapone et al., 1988b) structure. This structure has been confirmed recently by chemical cross-linking studies (Maeda et al., 1991). The release of Ca^{2+} stimulation by IP_3 may be positively cooperative (Meyer et al., 1988, 1990), suggesting that the four subunits interact such that activation of one subunit increases the probability that another subunit will be activated. However, ^{3}H-IP_3 binding to the pure receptor shows no evidence of cooperativity, with a Hill coefficient of approximately unity (Supattapone et al., 1988b).

The stimulation by Ca^{2+} of IP_3 binding to its receptor largely disappears with purification of the receptor. In crude detergent-solubilized receptor preparations, as well as in intact membranes, Ca^{2+} in the 100–300 nM range inhibits IP_3 binding, while Ca^{2+} has no influence on IP_3 binding to the purified receptor protein at a thousandfold higher concentration (Supattapone et al., 1988b). Adding detergent-solubilized brain extracts to purified IP_3 receptors restores the ability of Ca^{2+} to inhibit IP_3 binding, indicating that a Ca^{2+}-binding protein, designated calmedin, confers on Ca^{2+} the ability to influence IP_3 binding (Danoff et al., 1988). Recently, calmedin has been isolated by taking advantage of its adhesion to the IP_3 receptor so that under appropriate circumstances calmedin copurifies with the IP_3 receptor on an IP_3 affinity chromatography column (J. Steiner and S. H. Snyder, unpublished observations). Purified calmedin is a monomer with a molecular weight of about 15 kDa. In the presence of nanomolar concentrations of calmedin, Ca^{2+} substantially inhibits IP_3 receptor binding. Calmedin levels in the brain do not parallel the high density of the IP_3 receptor in cerebellum, suggesting that calmedin may influence other systems besides the IP_3 receptor. Overall, calmedin comprises about 1% of membrane protein in the brain, similar to tissue levels of calmodulin.

IP_3 receptors have also been purified to homogeneity from the aorta (Chadwick et al., 1990) and the vas deferens (Mourey et al., 1990) revealing properties closely similar to those of IP_3 receptors in the brain (Marks et al., 1990). However, in the vas deferens Ca^{2+} does not inhibit IP_3 binding to its receptor (Mourey et al., 1990), as it does not inhibit IP_3 binding in neutrophils, liver cells, and adrenal membranes (Baukal et al., 1985; Spat et al., 1986; Guillemette et al., 1987). Experiments in which partially purified calmedin is mixed with purified IP_3 receptors from cerebellar and vas deferens tissue indicate that the IP_3 receptor of the vas deferens can respond to calmedin, so its lack of response to Ca^{2+} *in situ* reflects the absence of calmedin protein in that tissue (Mourey et al., 1990).

Purified IP_3 receptors can be phosphorylated by cAMP-dependent protein kinase (PKA) (Supattapone et al., 1988a; Ferris et al., 1991b), PKC (Ferris et al., 1991b), and Ca^{2+}/calmodulin-dependent protein kinase (CAM-K-II) (Ferris et al., 1991b). Phosphorylation by any one of these enzymes proceeds stoichiometrically with one molecule of phosphate incorporated per receptor subunit. Phosphorylation by the three enzymes is additive, indicating that they act at different sites. Phosphopeptide maps and phosphoamino acid determination show that, while all three enzymes phosphorylate serine residues, they phosphorylate residues in different domains of the polypeptide chain (Ferris et al., 1991b). Isolation and direct sequencing of the peptides phosphorylated by PKA reveal that two serine residues, namely 1589 and 1755, are phosphorylated (Ferris et al., 1991a). Serine 1755 is phosphorylated at low concentrations of PKA, while much higher concentrations of PKA are needed to phosphorylate serine 1589.

We have found recently that IP_3 receptors can autophosphorylate (Ferris et al., 1992a). This autophosphorylation is magnesium dependent and reaches a final stoichiometry of 0.3–0.4 mol ^{32}P/mol IP_3 receptor at 4°C; possibly only a subpopulation of the receptors have this activity. Attribution of this phosphorylation to contaminating kinases is ruled out by several lines of evidence, including renaturation experiments in which autophosphorylation of the 260 kDa IP_3 receptor is observed on nitrocellulose membranes following SDS-PAGE (Ferris et al., 1992a). Additionally, the IP_3 receptor phosphorylates a synthetic peptide substrate, showing that the IP_3 receptor possesses protein kinase activity.

How can phoshorylation of the IP_3 receptor regulate its function? In cerebellar cell membranes, receptor phosphorylation by PKA decreases the potency of IP_3 to release Ca^{2+} (Supattapone et al., 1988a). Since PKA phosphorylation also stimulates the Ca^{2+} pump, total ER Ca^{2+} levels are increased so that the absolute amount of Ca^{2+} released by IP_3 is enhanced by PKA phosphorylation (Supattapone et al., 1988a). Recent studies indicate that cAMP-dependent hormones increase IP_3-induced release of Ca^{2+} in liver cells (Burgess et al., 1991). It is conceivable that phosphorylation of the IP_3 receptor by PKC and CAM-K-II provides feedback regulation of the PI cycle following the generation of DAG, which stimulates the action of PKC, and the release of Ca^{2+} by IP_3, which can activate both PKC and Cam-K-II. A cerebellar-specific form of CAM-K-II is highly concentrated in Purkinje cells (Ouimet et al., 1984; Fukunaga et al., 1988; Walaas et al., 1988) and has a similar subcellular localization as the IP_3 receptor.

The biological function of IP_4, IP_5, and IP_6 has not yet been identified despite their levels in the brain being as high as those of IP_3. Some studies suggest a role for IP_4 in regulating the movement of Ca^{2+} into the cell and/or in maintaining levels of IP_3-sensitive Ca^{2+} pools (Morris et al., 1987; Irvine, 1989; Cullen et al., 1990). IP_5 and IP_6 can increase Ca^{2+} accumulation (Nicoletti et al., 1989) and decrease blood pressure, heart rate (Vallejo et al., 1987; Barraco et al., 1989), and respiratory rate (Barraco et al., 1989). However, there is no evidence indicating whether any of these effects represent normal roles for these substances. Characterizing and localizing receptor proteins for these higher inositol phosphates might clarify their functions. IP_4 binding sites have been identified in brain cell membranes (Theibert et al., 1987; Donie and Reiser, 1989), and IP_6 binding sites have also been characterized (Hawkins et al., 1990). High-affinity IP_4- and IP_6/IP_5-binding proteins have recently been isolated from the brain (Theibert et al., 1991), which possess selectivity and high affinity for their respective ligands. The putative IP_4 receptor binds IP_4 with a K_D of about 3 nM, while the putative IP_6/IP_5 receptor binds IP_5 and IP_6 with a K_D of about 6–12 nM. The putative IP_5/IP_6 receptor comprises two 115 and 105 kDa doublets and a 50 kDa singlet. Tryptic peptides

from the 105 kDa doublet have been sequenced recently, revealing that the IP_6 receptor complex is identical to the clathrin-associated assembly protein AP-2 (A. B. Theibert, S. Voglmaier, C. D. Ferris, J. Keen, and S. H. Snyder, unpublished observations). Three prominent IP_4 receptor subunits of molecular weight about 182, 174, and 84 kDa can be distinguished. Another IP_4 receptor protein has been isolated independently with similar properties except that it is of lower molecular weight (Donie and Reiser, 1991). This difference may reflect proteolytic cleavage of the latter isolate. To ascertain which subunits of the IP_4 and IP_5/IP_6 receptor proteins carry recognition sites for their ligands, a radiolabeled photoaffinity ligand was employed, permitting analysis of the pH dependence and other properties of these proteins and their subunits (Theibert et al., 1992). The inositol phosphate binding specificity, pH dependence, and influence of Ca^{2+} were found to be identical for the 105 and 115 kDa IP_6 proteins. By contrast, the IP_4 receptor subunits differ in various respects. The pH dependence for ligand binding by the 174 and 182 kDa proteins is similar, with an optimum at about pH 7, while the 85 kDa protein displays substantial binding at lower pH levels, similar to the IP_4 receptor protein isolated by Donie and Reiser (1991). Ca^{2+} markedly enhances IP_4 binding to the 182 and 174 kDa proteins, unlike the marked Ca^{2+} stimulation of binding observed for the 84 kDa protein.

Reconstitution of IP_3-activated Ca^{2+} channels and their regulation

Does the IP_3-binding protein also contain the Ca^{2+} channel that responds to IP_3, or is the Ca^{2+} channel a distinct protein? To address this question, experiments were carried out in lipid vesicles containing only IP_3 receptor protein in the reconstituted membrane. This permitted measurement of the IP_3 influence on $^{45}Ca^{2+}$ release as well as ^{3}H-IP_3 binding in the same vesicles. In such vesicles, IP_3 was found to stimulate Ca^{2+} release (Ferris et al., 1989). The relative potencies of IP_3 and other inositol phosphates to influence Ca^{2+} release correspond closely with their affinities for IP_3 binding sites. Moreover, heparin, a known IP_3 antagonist, blocks the actions of IP_3 on Ca^{2+} release in the reconstituted system. These experiments established that the purified IP_3-binding protein contains both the IP_3 recognition site and the Ca^{2+} channel (Ferris et al., 1989).

Use of reconstituted vesicles containing IP_3 receptors has permitted demonstration of an allosteric regulation of the receptor by adenine nucleotides (Ferris et al., 1990). At concentrations between 1 and 10 μM, ATP markedly and cooperatively enhances the ability of IP_3 to stimulate Ca^{2+} release. This effect is biphasic, since the enhancing effects on IP_3-stimulated Ca^{2+} release diminish between 0.1 and 1 mM until, at the physiological concentration of 1 mM, ATP no longer enhances IP_3-stimulated Ca^{2+} release at all. This enhancement by ATP is readily distinguished from phosphorylation by use of nonhydrolyzable ATP analogs and is specific for ATP, in comparison with other adenine or guanine nucleotides. It is mediated via a high-affinity ATP recognition site on the IP_3 receptor, which can be labeled by its binding of ^{32}P-ATP or ^{35}S-ADP-β-S (Ferris et al., 1990) or by its reaction with an azido derivative of ATP (Maeda et al., 1991). The predicted amino acid sequence of the cerebellar IP_3 receptor contains two possible consensus sequences (GXGXXG) for ATP binding at amino acid residues 1773 and 2016 (Furuichi et al., 1989).

How might ATP influence the action of IP_3? At physiological concentrations (about 1 mM) of ATP, one would expect no obvious influence. However, once IP_3 stimulates Ca^{2+} release, the Ca^{2+}-dependent ATPase would be activated to replenish the Ca^{2+} stores, which can deplete ATP in the vicinity of the IP_3 receptor. ATP bound with low affinity will then dissociate, and IP_3-stimulated Ca^{2+} release would be augmented in feedforward manner. If ATP levels fall even further, then the ATP bound with high affinity would dissociate, and IP_3-stimulated release of Ca^{2+} would diminish, perhaps protecting the cells from noxious Ca^{2+} concentrations. This model might contribute to the precipitous, spikelike changes in intracellular Ca^{2+} concentrations associated with Ca^{2+} oscillations (Smith et al., 1986; Berridge, 1990; Petersen and Wakui, 1990; Meyer and Stryer, 1991) and help account for the marked cooperativity of IP_3-induced Ca^{2+} release (Meyer et al., 1988, 1990). A similar augmentation of Ca^{2+} release induced by ATP in reconstituted IP_3 receptors in planar lipid bilayers has been reported (Maeda et al., 1991). ATP also enhances IP_3 stimulation of Ca^{2+} channels in crude preparations of ER from the aorta (Ferris et al., 1989) and permeabilized cells (Smith et al., 1985).

In permeabilized pancreatic acinar cells, IP_3-stimulated Ca^{2+} release is a noncontinuous process, such that submaximal concentrations of IP_3 release submaximal portions of IP_3-sensitive Ca^{2+} stores (Muallem et al., 1989) even under conditions where the IP_3 is not degraded. This process was described as "quantal" since subfractions of IP_3-sensitive Ca^{2+} stores are activated by IP_3. In permeabilized basophils, Meyer and Stryer (1990) described the same phenomenon as "incremental" release. Sequential additions of IP_3 evoke a transient release of Ca^{2+}, monitored with Ca^{2+}-sensitive dyes, such that the final free Ca^{2+} level for two sequential IP_3 applications is the same as a single application of the sum (Meyer and Stryer, 1990). Incremental stimulation of Ca^{2+} release by IP_3 could be attributable to many factors. The quantal Ca^{2+} release in purified, reconstituted IP_3 receptors indicates that the phenomenon is a fundamental property of the IP_3 receptor (Ferris et al., 1992b). Submaximal concentrations of IP_3 fail to activate all of the Ca^{2+} channels in the reconstituted system (Ferris et al., 1992b). Moreover, successive additions of IP_3 provide an arithmetic increase in Ca^{2+} release over the range of 10–200 nM IP_3 (Fig. 1) (Ferris et al., 1992b). Since Ca^{2+} signaling in cells is known to be spatially and temporally complex (Alkon and Rasmussen, 1988), involving Ca^{2+} oscillations and waves (Berridge, 1990; Meyer and Stryer, 1991), it is likely that the sequential release properties of the IP_3 receptor are critical for this precise subcellular regulation of Ca^{2+} levels in cells.

Molecularly cloned IP_3 receptors in multiple forms

The first cloning of the IP_3 receptor gene arose from efforts to characterize a cerebellar-specific protein designated P-400 (Furuichi et al., 1989), originally identified as a 400 kDa cerebellar protein localized to Purkinje cells (Mallet et al., 1976). The characteristics of P-400 (Mikoshiba and Changeux, 1978; Mikoshiba et al., 1979; Maeda et al., 1990) were recognized to be those of the previously isolated IP_3 receptor (Supattapone et al., 1988b). Based on the partial sequence of the cDNA of a Purkinje cell–specific protein (Nordquist et al., 1988) antibodies were raised, and resultant immunohistochemical maps revealed that the protein encoded in the cloned P-400 gene was the IP_3 receptor (Mignery et al., 1989, 1990). The human IP_3 receptor was cloned and sequenced from brain libraries (Ross et al., 1991). Comprising more than 2700 amino acid residues, the IP_3 receptor is one of the largest proteins to have been cloned and

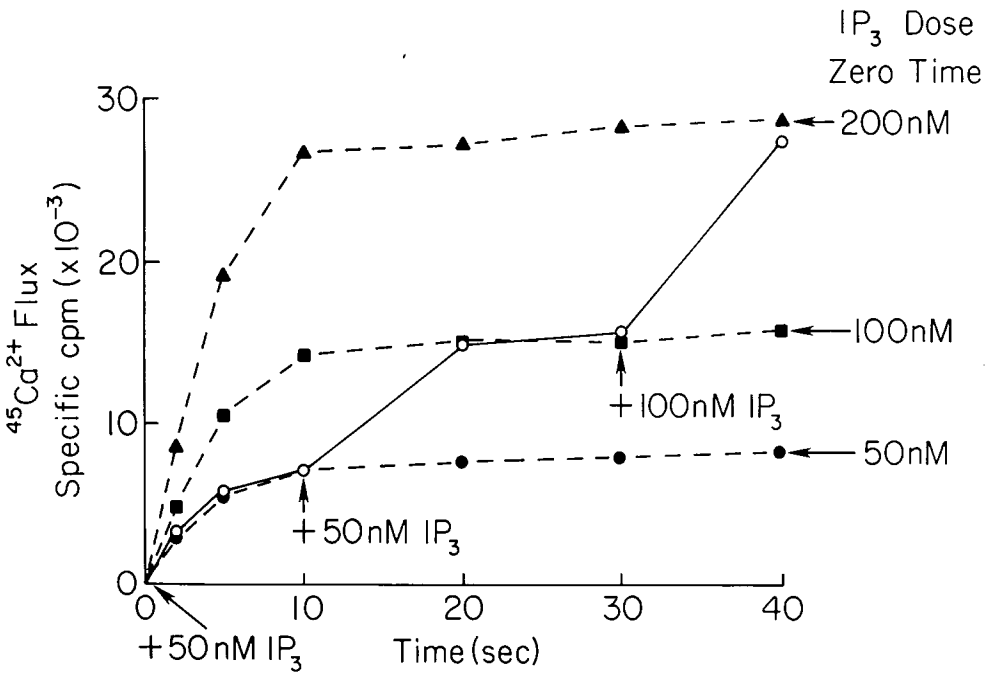

Figure 1. Incremental activation of IP_3 channels in reconstituted IP_3 receptor by successive additions of IP_3. IP_3-induced $^{45}Ca^{2+}$ release was measured as previously described (Ferris et al., 1989). In this preparation, IP_3 activates Ca^{2+} channels, which allows the rapid equilibration of $^{45}Ca^{2+}$ into vesicles with activated channels in the absence of an actual Ca^{2+} concentration gradient. Specific cpm refers to the difference between the presence and absence of IP_3. Addition of 50 nM IP_3 to vesicles following 10 sec incubation with 50 nM IP_3 results in enhanced $^{45}Ca^{2+}$ entry to the same level as an initial addition of 100 nM IP_3. Likewise, successive 10 sec incubations with final IP_3 concentrations of 50 nM, 100 nM, and 200 nM result in quantitative detection of changes in IP_3 concentration. Thus, unlike other known ligand activated ion channels which either desensitize (e.g., the nicotinic ACh receptor) or allow continuous ion transport, the IP_3 receptor exhibits quantal release properties. Adapted from Ferris et al. (1992b).

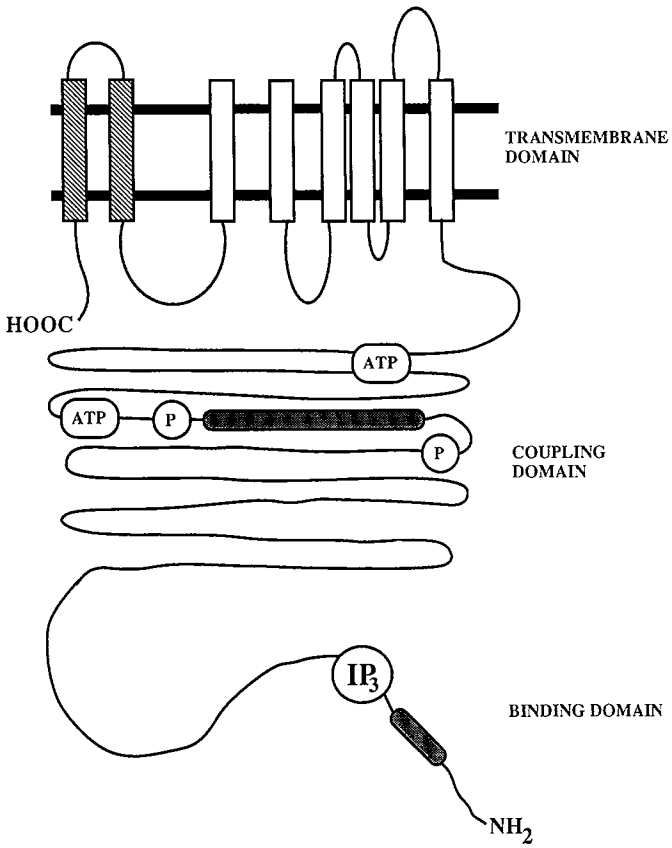

Figure 2. Topology of the IP_3 receptor. The IP_3 receptor is a large transmembrane protein with eight hydrophobic putative transmembrane domains at the carboxyl terminus (*hatched*), a domain of 300–400 amino acid residues at the amino terminus that are sufficient for minimal ligand binding (Mignery and Sudhof, 1990), and a large hydrophobic and cytosolic coupling domain between which are regulatory sites for phosphorylation, ATP binding, and alternative splicing in the mRNA. In the binding and coupling domains, known alternative splicing sites are *shaded*. The putative sites for ligand (*IP_3* and *ATP*) binding are labeled, as are sites known to be phosphorylated by PKA (*P*; Ferris et al., 1991a). The sites phosphorylated by PKC and CAM-K-II (Ferris et al., 1991b) are not yet known.

sequenced. It is highly conserved among the mammals, as the sequences in rodents and humans differ by less than 10% and only by 1% in rat and mouse.

Several very similar molecular models for the IP_3 receptor have been suggested. A consensual model (Fig. 2) of an individual subunit comprises eight transmembrane domains located at the carboxy terminus of the molecule, with both the amino and carboxy termini on the cytoplasmic surface. Among the eight transmembrane domains, the first four are flanked by net positive charges and the last four by net negative charges (Mignery and Sudhof, 1990). The last four transmembrane domains show the closest homology with the ryanodine receptor (Furuichi et al., 1989; Mignery et al., 1989, 1990), the Ca^{2+}-stimulated Ca^{2+} release channel of muscle, and so are likely to comprise the Ca^{2+}-permeable pore.

Mutagenesis studies suggest that the IP_3 binding site lies in the amino-terminal 400 amino acid residue since receptors with this domain deleted fail to bind IP_3 (Mignery and Sudhof, 1990; Miyawaki et al., 1991). Moreover, truncated forms of the receptor retaining only the N-terminal quarter of the molecule provide soluble peptides that are monomeric but retain IP_3-binding capacity (Mignery and Sudhof, 1990; Miyawaki et al., 1991). The N-terminal peptide binds IP_3 with substantially less affinity than does the full protein, so the overall conformation of the intact protein may be important for physiologic IP_3 binding. Because the IP_3 binding site is at the extreme N-terminal part of the protein, with the putative Ca^{2+} channel at the extreme C-terminal, IP_3 binding must elicit a conformational change over a span of virtually 1400 amino acid residues. This fits with findings that a substantial decrease in apparent molecular weight on gel filtration occurs consequent to IP_3 binding (Mignery and Sudhof, 1990). Both sites at which PKA catalyzes serine phosphorylation (Ferris et al., 1991a), as well as the ATP consensus binding sites (Ferris and Snyder, 1991), are located in the "coupling region" between domains for IP_3 binding and the Ca^{2+} channel, where these regulatory sites may affect the ability of IP_3 binding to activate the Ca^{2+} channel.

The evolutionary conservation of the primary structure of the IP_3 receptor suggests that there might be only a single type of IP_3 receptor. However, multiple forms of IP_3 receptors derived by alternative mRNA splicing have been observed (Mignery et al., 1990; Danoff et al., 1991; Nakagawa et al., 1991). One of these forms has been found in rats and mice, with a deletion of 15 amino acids close to the N-terminus (Mignery et al., 1990; Nakagawa et al., 1991). Alternatively spliced forms with a deletion of 40 amino acids in the N-terminal third of the molecule, but not as close to the N-terminus as the 15 amino acid deletion, have also been observed (Danoff et al., 1991; Nakagawa et al., 1991). Several forms of the receptor with different portions of the 40 amino acid sequence deleted have been detected, suggesting that the 40 amino acids derive from two separate exons (Nakagawa et al., 1991). By use of the PCR to monitor relative levels of the receptor with and without the 40 amino acid deletion, it was observed that the long form is exclusively neuronal while the short form appears to be predominantly in non-neuronal tissues (Danoff et al., 1991), although the "non-neuronal" form has also been detected in the spinal cord (Nakagawa et al., 1991). Substantial differences in levels of the alternatively spliced form of the receptor occur throughout the brain (Nakagawa et al., 1991) For instance, the form with the 15 amino acid deletion accounts for 75–85% of IP_3 receptors in the spinal cord and cerebellum but only for 12% of receptors in the cerebral cortex. The proportion of receptors with different extents of loss of the 40 amino acid sequence varies substantially in mouse brain regions as well (Nakagawa et al., 1991). While all peripheral tissues lack the 40 amino acid sequence, the presence or absence of the 15 amino acid sequence varies substantially among different peripheral organs.

The 40 amino acids deleted in the non-neuronal form lie between two serines at positions 1589 and 1756, which are phosphorylated by PKA (Ferris et al., 1991a). To examine the influence of the deletion upon phosphorylation, we compared phosphorylation by PKA of the neuronal (cerebellum) and non-neuronal (vas deferens) receptors. While both are stoichiometrically phosphorylated by PKA, the pattern of phosphorylation differs. The brain receptor is preferentially phosphorylated on serine 1756, while the vas deferens receptor is phosphorylated on serine 1589. The 40 amino acid sequence also lies in the area of two consensus sequences for ATP binding, and removal of the 40 amino acid sequence creates a new consensus sequence for ATP binding (Ferris and Snyder, 1991).

Evidence has recently been obtained for the existence of other members of the IP_3 receptor family derived from distinct genes (Ross et al., 1991; Sudhof et al., 1991). These other putative members of the IP_3 receptor group are less abundant in brain than the "parent" IP_3 receptor, and their functions are unclear. The most striking "cousin" of the IP_3 receptor is the ryanodine receptor (Furuichi et al., 1989; Mignery et al., 1989; Takeshima et al., 1989). Ryanodine is an alkaloid that binds to a protein that is responsible for Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum of striated muscle as a key component of excitation–contraction coupling (Endo, 1977; Fleischer and Inui, 1989). Thus, both IP_3 and ryanodine receptors have Ca^{2+} channels to promote release of intracellular Ca^{2+}, with the ligand for one being IP_3 and for the other being Ca^{2+} itself. Ryanodine and IP_3 receptors are both large homotetrameric proteins that, when examined under the electron microscope, display fourfold symmetry, with the IP_3 receptor having forms that appear more mobile than the ryanodine receptor. ATP directly induces Ca^{2+} release at the ryanodine receptor, whereas at the IP_3 receptor, ATP enhances the stimulation of Ca^{2+} release by IP_3. While IP_3 receptors are concentrated in the brain and smooth muscle, ryanodine receptors are at their highest levels in skeletal and cardiac muscle, with much lower levels in smooth muscle and brain. A limited capacity to bind ^{3}H-ryanodine in the brain has been reported (McPherson and Campbell, 1990), and high-resolution immunohistochemistry has revealed both ryanodine and IP_3 receptors within the same Purkinje cells in the cerebellum (Ellisman et al., 1990).

Localization of IP_3 and ryanodine receptors in the brain in relation to Ca^{2+} pools

IP_3 receptors have been localized by autoradiography (Worley et al., 1987a, 1989) and immunohistochemistry (Ross et al., 1989), and their mRNA has been detected by *in situ* hybridization with cloned DNA probes (C. A. Ross, C. Glatt, T. Dawson, and S. H. Snyder, unpublished observations). IP_3 receptors are at their highest density in Purkinje cells of the cerebellum and at their second highest density in the hippocampus, especially in the CA1 region. Receptors have been observed throughout the processes of neurons, with substantial densities being present in terminals of Purkinje cells in the deep nuclei of the cerebellum. In the retina, IP_3 receptors are at high density in presynaptic nerve terminals of photoreceptors and bipolar cells, as well as in synaptic specializations of amacrine cells (Peng et al., 1991). The association of these receptors with nerve terminals may reflect a role for them in neurotransmitter release, perhaps modulating the influx of Ca^{2+} through voltage-gated channels that trigger exocytosis of synaptic vesicles. The first studies of IP_3-stimulated release of Ca^{2+} indicated a nonmitochondrial source, which might reflect ER (Streb et al., 1983). Electron microscopic immunohistochemistry (Mignery et al., 1989; Ross et al., 1989; Otsu et al., 1990; Satoh et al., 1990) confirms an association of IP_3 receptors with subcomponents of the ER. Both rough and smooth ER are labeled, but with considerably heterogeneous distribution: some elements that show high densities of the receptor label may lie adjacent to morphologically similar but unlabeled elements. In some studies on Purkinje cells, substantial densities of receptor were evident in the ER close to the nuclear membrane and in the nuclear membrane itself (Ross et al., 1989; Satoh et al., 1990). A role for IP_3 in nuclear function is further strengthened by observations of nuclear ATP-dependent Ca^{2+} uptake (Nicotera et al., 1989), IP_3-stimulated release of Ca^{2+} (Malviya et al., 1990; Nicotera et al., 1990), as well as IP_3 binding (Malviya et al., 1990) in liver nuclei. Cocco et al. (1987) reported polyphosphoinositide synthesis in nuclei of erythroleukemia cells with changes during differentiation.

Immunohistochemical studies of IP_3 localization in Purkinje cells in cerebellum have failed to detect its presence in mitochondria, Golgi cisternae, or the plasma membrane. However, IP_3-activated Ca^{2+} channels may be present in plasma membranes of lymphocytes (Kuno and Gardner, 1987). IP_3 activation of dihydropyridine-sensitive Ca^{2+} channels has also been detected in preparations of skeletal muscle transverse tubules, which presumably represent plasma membrane channels (Vilven and Coronado, 1988). Direct evidence has recently been obtained for the existence of IP_3 receptors in the plasma membrane of olfactory cilia and lymphocytes. Immunohistochemical studies resorting to confocal microscopy reveal a selective association of IP_3 receptors with olfactory cilia, which lack ER (A. Cunningham, G. Ronnett, R. Reed, and S. H. Snyder, unpublished observations). Moreover, IP_3 receptors are selectively localized

in the plasma membrane of intact Jurkat T lymphocytes under conditions in which the antibody does not penetrate into cells (A. Khan, J. Steiner, and S. H. Snyder, unpublished observations). Moreover, in these cells capping induced by concanavalin A is associated with a concentration of IP_3 receptor at one pole of the cell, a behavior manifested only by plasma membrane proteins.

IP_3 receptors in brain slices colocalize with IP_3-sensitive Ca^{2+} pools in the ER labeled with $^{45}Ca^{2+}$ in the presence of ATP (Verma et al., 1990). The ER stores of Ca^{2+} labeled in this way display discrete localizations. IP_3 can stimulate the release of most of the Ca^{2+} from some brain regions, while other brain regions are relatively resistant to the effects of IP_3. Recently, it has been possible to identify Ca^{2+}-sensitive Ca^{2+} stores in these preparations (A. Verma, Hirsch, and S. H. Snyder, unpublished observations). In both brain ER and slices, Ca^{2+} accumulation increases with increasing concentrations of added Ca^{2+}, but then begins to decrease. The decline in accumulated Ca^{2+} reflects CICR, since the decline is prevented by local anesthetics such as tetracaine and Mg^{2+}, which block CICR. Moreover, this decline is accentuated by caffeine and ATP, which enhance CICR. Ca^{2+} release stimulated by IP_3 occurs at lower free Ca^{2+} concentrations than the CICR process. Thus, as IP_3 induces release of Ca^{2+} to provide concentrations greater than 0.5 μM, the released Ca^{2+} inhibits further actions of IP_3 and also triggers initiation of the CICR process. The coordinated effects of IP_3 and CICR on Ca^{2+} release provide an enhancement of Ca^{2+} release that may influence Ca^{2+} oscillations.

IP_3-sensitive and CICR pools of Ca^{2+} are differentially localized. For instance, in the hippocampus CA1 is enriched in the IP_3 pool, while CA3 has higher densities of CICR. The corpus striatum is enriched in IP_3-stimulated Ca^{2+} channels, while the closely adjacent medial septum has higher densities of CICR. Cerebellar Purkinje cells are greatly enriched in the IP_3 pool, while the CICR pool predominates in the olfactory bulb. It will be a challenging task to differentiate physiological functions associated with IP_3 and CICR, respectively.

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