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Inositol trisphosphate receptor: Phosphorylation by protein kinase C and calcium calmodulin-dependent protein kinases in reconstituted lipid vesicles

(cAMP/inositol phospholipid turnover/diacylglycerol/serine/threonine)

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ABSTRACT **We have previously demonstrated that the inositol 1,4,5-trisphosphate (IP_3) receptor is phosphorylated by cyclic AMP-dependent protein kinase (PKA). In the present study, phosphorylation of IP_3 receptors has been examined with purified receptor protein reconstituted in liposomes to remove detergent that can inhibit protein kinases. The IP_3 receptor is stoichiometrically phosphorylated by protein kinase C (PKC) and Ca^{2+} calmodulin-dependent protein kinase II (CaM kinase II) as well as by PKA. Phosphorylation by the three enzymes is additive and involves different peptide sequences. Phosphorylation by PKC, which is stimulated by Ca^{2+} and diacylglycerol, and by CaM kinase II, which requires Ca^{2+}, provides means whereby Ca^{2+} and diacylglycerol, formed during inositol phospholipid turnover, may regulate IP_3 receptor physiology.**

The inositol phospholipid second messenger system acts through the formation of inositol 1,4,5-trisphosphate (IP_3), which triggers the release of calcium from portions of the endoplasmic reticulum (1, 2). The purified IP_3 receptor protein (3), reconstituted into lipid vesicles, mediates IP_3 alterations of Ca^{2+} flux, indicating that the protein contains both the IP_3 recognition site and the Ca^{2+} ion channel (4). The IP_3 receptor is stoichiometrically phosphorylated by cAMP-dependent protein kinase (PKA), resulting in diminished potency of IP_3 in releasing Ca^{2+} from brain membranes (5). This provides a mechanism for "cross-talk" between the cAMP and inositol phospholipid systems. Utilizing reconstituted lipid vesicles, we now demonstrate specific, selective, and stoichiometric phosphorylation of the IP_3 receptor by protein kinase C (PKC) and Ca^{2+} calmodulin-dependent protein kinase II (CaM kinase II).

MATERIALS AND METHODS

Materials. [^{32}P]ATP was purchased from Du Pont/NEN. CaM kinase II was the generous gift of Mary Kennedy and Bruce Patton (California Institute of Technology, Pasadena, CA). Trypsin and thermolysin were obtained from Boehringer Mannheim. All other chemicals were purchased from Sigma. IP_3 receptor was purified (3, 4) and reconstituted into lipid vesicles (4) as described. Catalytic subunit of protein kinase A was purified from bovine heart as described (6). PKC was purified from rat brain as described (7).

Phosphorylation of Purified and Reconstituted IP_3 Receptor. Reconstituted proteoliposomes (50 μl unless otherwise indicated) were incubated at 30°C for 60 min or as indicated in a final incubation vol of 100 μl. In all experiments, final concentrations were 10 mM for $MgCl_2$ and 50 μM for ATP. The final concentrations of phosphorylating enzymes were 2 μg/ml, 3 μg/ml, and 1 μg/ml for PKC, CaM kinase II, and catalytic subunit of PKA, respectively. For both CaM kinase II and PKC phosphorylation, 1 mM $CaCl_2$ was added, while a final calmodulin concentration of 0.05 mg/ml was present for CaM kinase II phosphorylation. Phosphorylated amino acids and phosphopeptide maps were determined as described (8).

RESULTS

In a previous study of the phosphorylation of the IP_3 receptor by cAMP-dependent protein kinase, we reported that the purified IP_3 receptor protein was not phosphorylated by PKC or CaM kinase II (5). In addition, before identification of the IP_3 receptor protein, Greengard and coworkers (9, 10) had described a phosphorylated protein in Purkinje cells of the cerebellum, designated PCPP-260, which was phosphorylated by PKA and which we have shown to be identical to the IP_3 receptor (S. Supattapone and S.H.S., unpublished data). PCPP-260 did not appear to be phosphorylated by CaM kinase II (9, 10). In these earlier studies, the proteins were purified in the presence of detergents, which were not removed from the purified preparation for phosphorylation experiments. Many detergents inhibit protein kinases and may also denature substrates. Recently, we have reconstituted the IP_3 receptor protein into lipid vesicles (4) in which the detergent is completely removed, and the receptor is restored to a lipid environment. Accordingly, we have reexamined the phosphorylation of IP_3 receptors by various protein kinase in these vesicles (Fig. 1, Table 1).

Purified and reconstituted IP_3 receptor protein is phosphorylated by PKC, CaM kinase II, and PKA. SDS/PAGE analysis reveals a single phosphorylated protein band of 260 kDa with all three phosphorylating enzymes (data not shown). All three enzymes phosphorylate the IP_3 receptor stoichiometrically (Table 1). At 30°C, the time course of phosphorylation appears somewhat different for the three enzymes. CaM kinase II produces about half-maximal phosphorylation at 10 min, slightly more rapid than PKA, while PKC requires 30 min for half-maximal phosphorylation (Fig. 1).

We wondered whether the three phosphorylating enzymes act at the same or different sites. To examine this question, we measured the stoichiometry of phosphorylation in the presence of mixtures of the enzymes. Phosphorylation appears to be additive for the three enzymes (Table 1); in the

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Abbreviations: IP_3, inositol 1,4,5-trisphosphate; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; CaM kinase II, calcium calmodulin-dependent protein kinase II.

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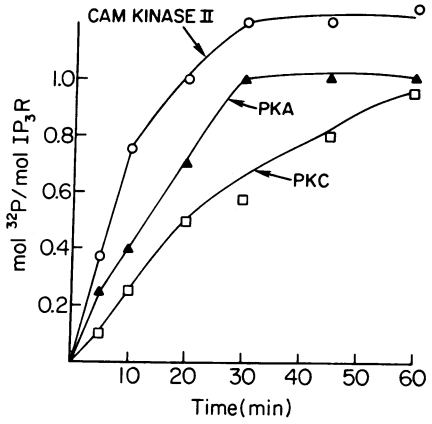

FIG. 1. Time course of phosphorylation of purified and reconstituted IP_3 receptor by PKA (catalytic subunit), PKC, and CaM kinase II. Phosphorylation reactions were performed as described. At the indicated times, phosphorylation was stopped by the addition of 50 μl of 3× sample buffer for SDS/PAGE. All samples were electrophoresed on 7.5% polyacrylamide gels. The gels were stained with Coomassie brilliant blue, the IP_3 receptor band was cut out, and radioactivity was determined by Cerenkov counting of the gel bands. These experiments have been repeated three times with essentially the same results.

presence of all three enzymes, the extent of phosphorylation is ≈3 times greater than with any of the enzymes alone. The specificity of the three enzymes is ensured by experiments showing that PKC activity is eliminated in the absence of Ca^{2+}, while CaM kinase II activity is dependent on both Ca^{2+} and calmodulin. Confirming our earlier observations, PKA phosphorylation of the IP_3 receptor is blocked by the Walsh peptide inhibitor of this enzyme (5).

PKC, PKA, and CaM kinase II can phosphorylate on either serine or threonine. To distinguish these alternatives, we analyzed by electrophoresis phosphorylated amino acids after acid hydrolysis of phosphopeptides (Fig. 2). With all three enzymes, a single major amino acid is phosphorylated, which corresponds to phosphoserine. In the case of CaM kinase II phosphorylation, some phosphothreonine is present, although it represents <5% of the total incorporated phosphate.

To identify phosphorylation sites of these enzymes, we performed two-dimensional phosphopeptide analysis (Figs. 3 and 4). The IP_3 receptor, phosphorylated by PKA, PKC, or CaM kinase II, was digested with thermolysin or trypsin and the resultant phosphopeptides were analyzed by electrophoresis and thin-layer chromatography in two dimensions. With PKA, we observe a single basic phosphopeptide that migrates

Table 1. Additive phosphorylation of IP_3 receptor by PKA, PKC, and CaM kinase II

Phosphorylating condition	^{32}P/IP_3 receptor, mol/mol
PKA	1.0
+ Walsh inhibitor	<0.1
PKC	0.9
− Ca^{2+}	<0.1
CaM kinase II	1.25
− Ca^{2+}	<0.1
− Calmodulin	<0.1
PKA + PKC + CaM kinase II	2.7

Specific and stoichiometric phosphorylation of purified and reconstituted IP_3 receptor by PKA (catalytic subunit), PKC, and CaM kinase II. Phosphorylation was performed as described. The incubation was for 60 min at 30°C. This experiment was replicated with <10% variation.

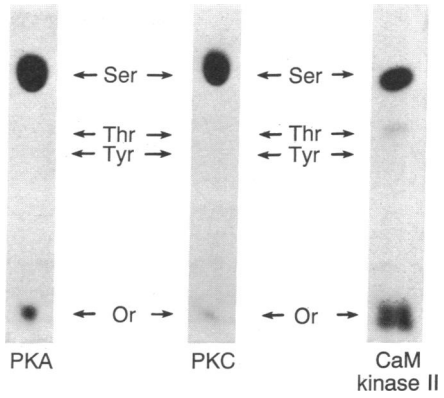

FIG. 2. Determination of phosphorylated amino acids for PKA, PKC, and CaM kinase II phosphorylation of purified and reconstituted IP_3 receptor. Phosphorylation of IP_3 receptor was performed as described. After SDS/PAGE, the IP_3 receptor was cut out of the gel and digested by thermolysin as described in the legend to Fig. 3 and ref. 7, and the peptides were hydrolyzed to amino acids by incubation in 6 M HCl at 105°C as described (7). This experiment was repeated with the same result. Or, origin.

toward the cathode slightly ahead of the marker basic fuchsin (Fig. 3*A*). PKC produces one major phosphopeptide that migrates partially to the anode (Fig. 3*B*). A minor product of

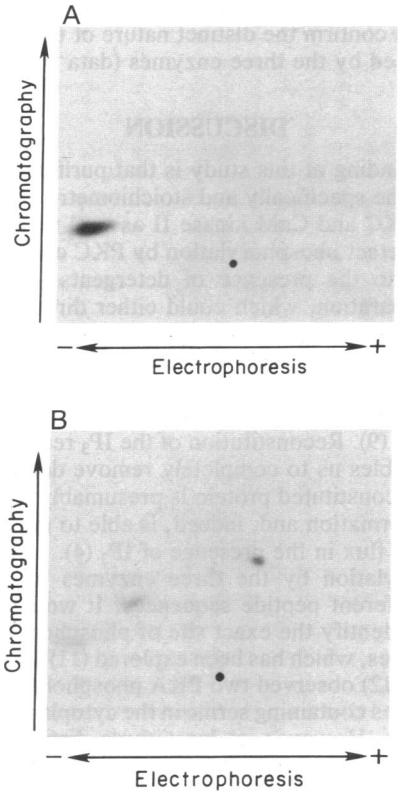

FIG. 3. Two-dimensional phosphopeptide map of IP_3 receptor phosphorylated by PKA (*A*) and PKC (*B*). IP_3 receptor was phosphorylated as described. After SDS/PAGE, the IP_3 receptor band was cut out of the gel and digested by incubating the gel band at 30°C for 20 hr in a final vol of 1 ml in the presence of thermolysin (300 μg/ml) and 50 mM NH_4HCO_3. After lyophilization, the peptides were separated by electrophoresis and chromatography as described (7). Phenol red and basic fuschin were used as negative and positive markers, respectively. Electrophoresis was carried out at 500 V until the markers had migrated 6 cm from the origin. Chromatography was done as described (7) and was stopped when the markers had migrated 6 cm. These experiments have been repeated three times with the same results.

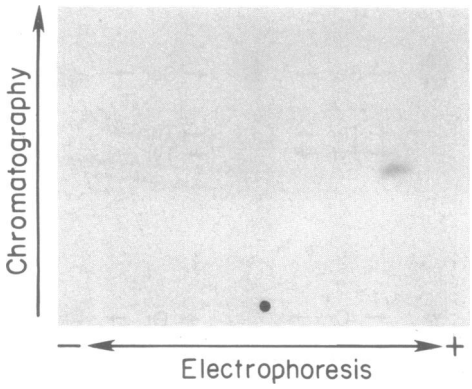

FIG. 4. Two-dimensional phosphopeptide map of CaM kinase II-phosphorylated IP_3 receptor. Phosphopeptide mapping was performed as described for Fig. 3 except that trypsin (150 μg/ml) was used to digest the phosphorylated IP_3 receptor and electrophoresis was stopped when the markers had migrated only 2 cm. This experiment has been repeated twice with the same result.

PKC is a peptide that appears identical to that produced by PKA, while a second minor phosphopeptide is also apparent with PKC. CaM kinase II produces a single highly acidic phosphopeptide whose migration differs from any of the products of PKA or PKC (Fig. 4). This peptide migrates about twice as far as the marker phenol red. Experiments in which phosphopeptides are produced by various mixtures of the enzymes confirm the distinct nature of the phosphopeptides produced by the three enzymes (data not shown).

DISCUSSION

The major finding of this study is that purified IP_3 receptor protein can be specifically and stoichiometrically phosphorylated by PKC and CaM kinase II as well as PKA. Earlier failures to detect phosphorylation by PKC or CaM kinase II may relate to the presence of detergents in the purified protein preparation, which could either directly inhibit the phosphorylating enzymes or provide a denatured form of the substrate protein that might not be a satisfactory substrate for phosphorylation by certain protein kinases. Studies of PCPP-260 indicate that detergents do inhibit phosphorylation of the IP_3 receptor (9). Reconstitution of the IP_3 receptor into lipid vesicles enables us to completely remove detergent. Moreover, the reconstituted protein is presumably restored to its native conformation and, indeed, is able to mediate physiological Ca^{2+} flux in the presence of IP_3 (4).

Phosphorylation by the three enzymes is additive and involves different peptide sequences. It would be of great interest to identify the exact site of phosphorylation by the three enzymes, which has been explored (11). Mikoshiba and coworkers (12) observed two PKA phosphorylation consensus sequences containing serine in the cytoplasmic domain of the receptor. However, at least three distinct serines are phosphorylated by these enzymes. In studies of the protein P_{400}, before its identification as the IP_3 receptor, weak phosphorylation of P_{400} by endogenous and exogenous CaM kinase II was observed (13). In our own examination of the predicted amino acid sequence for the IP_3 receptor, we have found several sites that could serve as substrates for PKC or CaM kinase II.

Our earlier observation of phosphorylation of the IP_3 receptor by PKA (5), confirmed here, suggested the existence of cross-talk between the two second messenger systems. Thus, PKA phosphorylation results in increased Ca^{2+} accumulation by the endoplasmic reticulum, providing a larger pool for IP_3 to release (5), but it also decreases the potency of IP_3 10-fold in releasing Ca^{2+} from cerebellar microsomes (5). Studies of the effects of phosphorylation by PKC and CaM kinase II on IP_3-stimulated Ca^{2+} flux in reconstituted IP_3 receptors and in brain membranes as well as electrophysiologic investigations of the effects of phosphorylation may clarify mechanisms by which these distinct phosphorylations regulate the function of the IP_3 receptor.

IP_3 receptor phosphorylation by PKC and CaM kinase II provides avenues for physiological regulation of the entire inositol phospholipid system. Agonist stimulation of phospholipase C gives rise to diacylglycerol as well as IP_3. In the presence of the Ca^{2+} released by IP_3, diacylglycerol stimulates PKC activity, which would be expected to phosphorylate the IP_3 receptor. PKC colocalizes in the central nervous system with the IP_3 receptor with variable stoichiometries, suggesting differential regulation of the IP_3 receptor by PKC phosphorylation in various brain regions (14). IP_3 releases Ca^{2+}, which activates CaM kinase II, causing another "feedback" phosphorylation of the IP_3 receptor. Immunohistochemical localization of CaM kinase II (15–17) demonstrates selective enrichment of this enzyme in the Purkinje cells of the cerebellum where IP_3 receptors are most highly concentrated. The Purkinje cells appear to have a specific isoform of CaM kinase II (16) with a unique subunit structure and intracellular distribution that matches nearly perfectly that of the IP_3 receptor (18, 19).

Phosphorylation may also interact with other regulatory influences of the IP_3 receptor. Thus, declining ATP concentrations enhance the ability of IP_3 to stimulate Ca^{2+} flux when ATP levels are lowered from their physiologic millimolar concentrations (20). This effect of ATP is independent of any phosphorylation event and may contribute to Ca^{2+} oscillations in a variety of cells (20). At physiologic intracellular concentrations of ≈500 nM, Ca^{2+} inhibits IP_3 receptor binding (21, 22). This action of Ca^{2+} on the IP_3 receptor is independent of influences on CaM kinase II. These multiple mechanisms for regulating IP_3 receptor phosphorylation and function may "fine tune" intracellular free Ca^{2+} and thereby multiple aspects of cellular physiology, including calcium oscillations.

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