Vol. 175, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1991 Pages 192-198

INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR IS PHOSPHORYLATED BY CYCLIC AMP-DEPENDENT PROTEIN KINASE AT SERINES 1755 AND 1589

Christopher D. Ferris, Andrew M. Cameron, David S. Bredt, Richard L. Huganir[+] and Solomon H. Snyder*

Departments of Neuroscience, Pharmacology and Molecular Sciences, Psychiatry and Behavioral Sciences, [+]Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205

Received December 19, 1990

IP_3 activates intracellular calcium release by binding to an intracellular ligand gated calcium permeable channel which has been shown to be regulated by protein kinase A phosphorylation. Two consensus sequences for protein kinase A phosphorylation are predicted by the recently isolated cDNA of the mouse and rat. In the present study we have isolated and sequenced the two peptides in the rat IP_3 receptor which are phosphorylated by protein kinase A and demonstrate protein kinase A phosphorylation on S-1755 and S-1589.

The release of calcium from endoplasmic reticulum stores (1,2) by inositol 1,4,5,-trisphosphate (IP_3) involves the binding of IP_3 to a membrane associated protein which has been purified (3), shown by functional reconstitution to include a calcium ion channel as well as an IP_3 recognition site (4), and molecularly cloned (5). The IP_3 receptor, a tetramer of four identical 260 kD subunits (3), is phosphorylated by cyclic AMP-dependent protein kinase (PKA) with an associated decrease in the potency of IP_3 in releasing calcium (6). This phosphorylation is stoichiometric with 1 mole of phosphate incorporated into each subunit (6,7). Molecular cloning of the IP_3 receptor revealed two consensus sequences for PKA phosphorylation, which in the moust are at amino acids 1588 and 1755, respectively. Stoichiometric phosphorylation of both of these sites would provide twice as many phosphates per receptor protein as we have previously observed (6,7). In the present study we have used higher levels of PKA to obtain IP_3 receptor phosphorylated to a stoichiometry as high as 2 moles phosphate per IP_3 receptor and have identified by direct

*To whom correspondence and reprint requests should be addressed.

0006-291X/91 $1.50

peptide sequencing these sites of phosphorylation of the IP_3 receptor by PKA.

MATERIALS AND METHODS

Materials. The catalytic subunit of PKA was purified as described (8). [^{32}P]ATP was purchased from DuPont/NEN (Boston, MA). Nitrocellulose was purchased from Schleicher and Schuell (Keene, NH). All other reagents were obtained from Sigma, unless otherwise indicated.

Methods. Phosphorylation and phosphopeptide mapping of purified IP_3 receptor: IP_3 receptor was purified as described (3) and as modified (4). For routine phosphorylation experiments, purified IP_3 receptor protein (1-5 μg) was incubated in 250 μM ATP (1000 cpm/pmol), 10 mM $MgCl_2$, 50 mM Tris.HCl (pH 7.4, 25°C), and various concentrations of purified catalytic subunit of PKA in a final volume of 100 μl for 60 min at 30°C. The reaction was stopped by the addition of 50 μl of 3X sample buffer for SDS-PAGE. Following SDS-PAGE, the IP_3 receptor protein was cut out of the gel, and cpm determined by Cerenkov counting. The protein was digested to peptides with thermolysin and used for phosphopeptide mapping and phosphoamino acid analysis as described (9).

Isolation and sequencing of phosphopeptides: For isolation and sequencing of the phosphorylated peptides, purified cerebellar type IP_3 receptor (500 pmol) was incubated with different amounts of PKA, as indicated, in the presence of 10 mM $MgCl_2$, 50 mM Tris.HCl (pH 7.4, 25°C) and 250 μM ATP (500 cpm/pmol) for 60 min at 30°C in a final volume of 1 ml. Again, the reaction was stopped with sample buffer (5X) for SDS-PAGE. Following SDS-PAGE the protein was transferred to nitrocellulose for 24-36 hr at 4°C in transfer buffer (20 mM Tris Base, 150 mM glycine and 20% MeOH). The blot was stained with Ponceau-S for visualization of protein. The IP_3 receptor was cut out, and the radioactivity was quantified by Cerenkov counting of the nitrocellulose pieces. About 50% of the receptor protein was recovered at this step (250 pmol). Trypsin digestion of the protein was carried out following the method of Aebersold et al. (10) with the following modifications. Before trypsin digestion, IP_3 receptor protein was denatured by incubation of the nitrocellulose pieces in 2 M urea and 100 mM NH_4HCO_3 for 15 min at room temperature. Then, dithiothreitol (DTT) was added to make the concentration of DTT 22.5 μM and reduction was carried out by incubation of the nitrocellulose pieces at 50°C for 15 min. Next, iodacetamide (IAA) was added to make the final IAA concentration 50 μM and alkylation was achieved by a 15 min incubation at room temperature. To prevent adsorption of the trypsin to nitrocellulose, the pieces were then treated with polyvinyl-pyrrolidone (PVP-40) as described (10) and digested with trypsin (1/20 wt./wt.) by incubation for 20 hr at 30°C in 50 mM NH_4HCO_3. Following digestion, over 90% of the radioactivity was recovered in the supernatant, the peptides were lyophilized to dryness, and resuspended in H_2O for injection on HPLC (Hewlett Packard HP 1090 liquid chromatograph). Trypsin fragments were loaded onto a C8 column (Aquapore RP-300/micron, 220 X 2.1 mM) and eluted by a gradient from 0-80% acetonitrile in 0.06% trifluoroacetic acid. The presence of eluting peptides was monitored as absorbance at 214 nM, and peaks were collected by hand. Radioactivity in all peaks was determined by Cerenkov counting of the samples. Peptide peaks containing radioactivity were lyophilized to near dryness and loaded onto a second HPLC column (Vydac, 219TP52 diphenyl, 2.1 X 250 mM). In all cases a single peptide peak containing all of the radioactivity was obtained. This sample was used for sequence analysis (Applied Biosystems Automated Sequencer) by Edman degradation.

RESULTS

Incubation of IP_3 receptor under phosphorylating conditions with low PKA concentrations (0.1 - 1 μg/ml) results in the incorporation of 1 mole of phosphate per mole of receptor. Phosphopeptide mapping of this phosphorylated receptor (1 μg/ml PKA) reveals phosphorylation at a single site (Fig. 1A). At a much higher concentration of PKA (500 μg/ml), we observe an increase in the stoichiometry of phosphorylation to 2 moles/mole receptor (Table 1). This increased phosphorylation occurs selectively at a second site. Phosphopeptide mapping of IP_3 receptor phosphorylated with 10 μg/ml PKA shows that the major basic peptide is again observed, while a new slightly less basic peptide becomes evident (Fig. 1B). To determine the peptide sequences which are phosphorylated we conducted experiments phosphorylating the IP_3 receptor with 20, 250 or 500 μg/ml of PKA and separated the resultant peptides by reverse phase chromatography on a HPLC using a C8 column (Table 1; Fig. 1). Two clearly separated peaks of radioactivity are apparent. Peak 2 contains the same amount of radioactivity at all three enzyme concentrations indicating that it is completely phosphorylated even at very low concentrations of PKA. Peak 1 contains only about a third as much radioactivity as peak 2 at 20 μg/ml PKA, 80% at 250 μg/ml, and the same amount as peak 2 at 500 μg/ml PKA. These experiments suggest that peak 2 corresponds to the major basic peptide observed in the phosphopeptide maps.

To insure the purity of the peptides separated by the C8 column, they were re-chromatographed on a second diphenyl HPLC column, and the purified

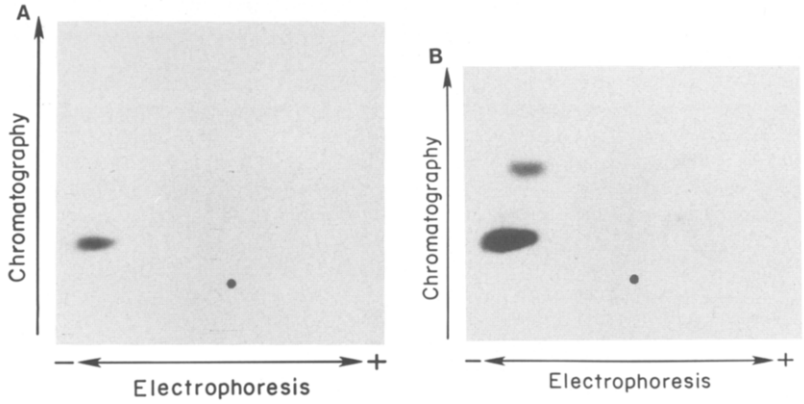

Figure 1

Phosphopeptide mapping of PKA phosphorylated IP_3R. Phosphopeptide mapping of IP_3 receptor was carried out following thermolysin digestion as described in Materials and Methods and reference 9. A) Phosphopeptide map obtained from phosphorylation in the presence of 1 μg/ml PKA. B) Phosphopeptide map from IP_3 receptor phosphorylated in the presence of 10 μg/ml PKA. This experiment has been repeated with the same result. In all cases radioactivity was incorporated only into serine as determined by phosphoamino acid analysis (data not shown) as described (9).

TABLE 1

PKA Phosphorylation of two distinct sites in the IP_3 receptor

[PKA] μg/ml	Peak 1 (S-1589)		Peak 2 (S-1754)	
	cpm	mol ^{32}P / mol peptide	cpm	mol ^{32}P / mol peptide
20	7,200	0.33	20,000	1.0
250	15,000	0.80	19,800	1.0
500	20,000	1.0	20,100	1.0

PKA phosphorylation of purified IP_3 receptor and HPLC purification of peptides were performed as described in Materials and Methods. Peak 1 and Peak 2 refer to the radioactive peptides having retention times of about 10 and 35 min respectively (Fig. 2). This experiment has been performed three times with the same results.

phosphorylated peptide was sequenced by Edman degradation (Table 2; Fig. 2). The sequencing of the peptide from peak 2 was stopped after 10 cycles, though this trypsin-derived peptide is somewhat longer than 10 amino acids. The peptide from peak 1 is only 9 amino acids long, terminating with an arginine. The sequences obtained fit exactly with the predicted amino acid sequence from the recently isolated rat cDNA (11). The results reveal that peak 2 is phosphorylated at serine 1755 while peak 1 is phosphorylated at serine 1589.

TABLE 2

Sequence analysis of PKA phosphorylated peptides

Peak 1				Peak 2			
cycle #	AA	pmol	rat IP_3R	cycle #	AA	pmol	rat IP_3R
1	X	--	R	1	X	--	R
2	X	--	D	2	E	39	E
3	S	18	S	3	S	52	S
4	V	10	V	4	L	42	L
5	L	12	L	5	T	35	T
6	A	10	A	6	S	21	S
7	A	16	A	7	F	28	F
8	S	5	S	8	G	28	G
9	R	7	R	9	N	13	N
				10	G	18	G

IP_3 receptor was purified, phosphorylated by PKA, digested with trypsin, and the phosphorylated peptides were purified and sequenced as described in Materials and Methods. Peak 1 and Peak 2 refer to the radioactive peptides having retention times of about 10 and 35 min respectively (Fig. 2). The phosphorylation and HPLC purification was repeated twice with the same results. Rat IP_3 receptor sequences were taken from published data (11). AA = amino acid.

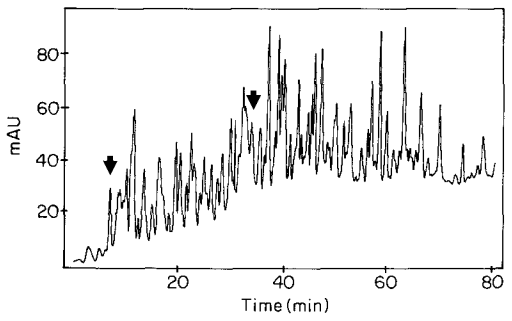

Figure 2
HPLC analysis of trypsin digested IP_3 receptor. IP_3 receptor was phosphorylated, digested, and the resulting peptides fractionated on a C8 column as described in Materials and Methods. Two peaks of radioactivity having retention times of approximately 10 (Peak 1) and 35 (Peak 2) min respectively were observed. These peaks were individually rechromatographed on a second column as described in Materials and Methods. In each case, the second column yielded a single radioactive peptide peak which was then sequenced. This experiment was repeated twice with the same results.

DISCUSSION

The main finding of this paper is that rat brain IP_3 receptor can be phosphorylated by PKA at two sites, serine's 1755 and 1589. In the mouse these sites are designated serines 1755 and 1588 (5). At low PKA concentrations, phosphorylation of the cerebellar type IP_3 receptor is exclusively at serine 1755. In our own previous studies of PKA phosphorylation of the IP_3 receptor (6) as well as those of Greengard's group studying protein PCPP-2600 (12,13), which we have shown to represent the IP_3 receptor (S. Supattapone and S.H. Snyder, unpublished), phosphorylation with a stoichiometry of one was always observed. The concentrations of PKA employed in those experiments would have favored selective phosphorylation at serine 1755. In this paper we have shown that at high concentrations of PKA another site is phosphorylated. However, it remains unclear whether physiologic conditions in intact cells will favor phosphorylation of one or both of these serines.

Recently, we have identified two separate forms of the IP_3 receptor derived by alternative splicing (14). One form occurring only in neuronal tissues, contains a 40 amino acid insert and corresponds to the brain IP_3 receptor as isolated (3) and previously cloned (5,11). In the second non-neuronal form there is a deletion from amino acids 1692 through 1732, which represents about one-third of the amino acids between serines 1589 and 1755, suggesting that PKA phosphorylation of the short form (non-neuronal) of the receptor might differ from the long form (neuronal). We examined PKA phosphorylation using purified "short" or "long" IP_3 receptor (14). Kinetic analysis reveals that the "short" or peripheral type IP_3 is phosphorylated with a 5-fold higher affinity (K_m = 3 nM) than the "long" or neuronal-type

IP_3 receptor (14). Thus, conditions for physiological phosphorylation will differ for the central neuronal and peripheral, non-neuronal forms of the IP_3 receptor. Stimulation of the cyclic AMP system may alter the phosphoinositide system via phosphorylation of the IP_3 receptor (6). This cross-talk between the two major second messenger systems may thus function differently for neuronal and non-neuronal tissues, having long and short forms of the IP_3 receptor, respectively (14).

In the model of IP_3 receptor structure described by Mikoshiba and associates (5) these PKA phosphorylation sites were postulated to be in a long cytoplasmic domain of the protein. Other investigators have suggested different transmembrane structures for the receptor (15). The direct demonstration that PKA phosphorylation occurs at serine 1589 and serine 1755 establishes that these two amino acids are in fact in the cytoplasmic domain of the IP_3 receptor protein.

Recently, we have also demonstrated other regulatory mechanisms for the IP_3 receptor which may interact with cAMP dependent regulation by PKA phosphorylation. For example, the IP_3 receptor is directly regulated by adenine nucleotide binding to the receptor protein, an effect which is selective for ATP (16). We have proposed that this allosteric mechanism may be important for controlling calcium oscillations (16). Also, the IP_3 receptor is specifically and stoichiometrically phosphorylated by both protein kinase C (PKC) and calcium-calmodulin dependent protein kinase II (7). Both of these protein kinases are activated in response to receptor mediated activation of the phosphatidylinositol (PI) system and may provide mechanisms of "feedback" regulation of the entire PI system itself, in contrast to the "cross-talk" mechanism of PKA phosphorylation.

ACKNOWLEDGMENTS

The authors with to thank Dr. Shengabamurthi for expert assistance in sequencing the peptides. Supported by USPHS grants MH-18501, Research Scientist Award DA-00074 to SHS, predoctoral fellowship MH-10018 to CDF, training grant GM-07309 to DSB and a gift from Bristol-Myers Squibb.

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