Phosphorylation of the Inositol 1,4,5-Trisphosphate Receptor by Cyclic Nucleotide-dependent Kinases in Vitro and in Rat Cerebellar Slices in Situ

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We have examined cyclic nucleotide-regulated phosphorylation of the neuronal type I inositol 1,4,5-trisphosphate (IP₃) receptor immunopurified from rat cerebellar membranes in vitro and in rat cerebellar slices in situ. The isolated IP₃ receptor protein was phosphorylated by both cAMP- and cGMP-dependent protein kinases on two distinct sites as determined by thermolytic phosphopeptide mapping, phosphopeptide 1, representing Ser-1589, and phosphopeptide 2, representing Ser-1756 in the rat protein (Ferris, C. D., Cameron, A. M., Bredt, D. S., Huganir, R. L., and Snyder, S. H. (1991) Biochem. Biophys. Res. Commun. 175, 192–198). Phosphopeptide maps show that cAMP-dependent protein kinase (PKA) labeled both sites with the same time course and same stoichiometry, whereas cGMP-dependent protein kinase (PKG) phosphorylated Ser-1756 with a higher velocity and a higher stoichiometry than Ser-1589. Synthetic decapeptides corresponding to the two phosphorylation sites (peptide 1, AARRDSVLAA (Ser-1589), and peptide 2, SGRRESSLTSF (Ser-1756)) were used to determine kinetic constants for the phosphorylation by PKG and PKA, and the catalytic efficiencies were in agreement with the results obtained by in vitro phosphorylation of the intact protein. In cerebellar slices prelabeled with [³²P]orthophosphate, activation of endogenous kinases by incubation in the presence of cAMP/cGMP analogues and specific inhibitors of PKG and PKA induced in both cases a 3-fold increase in phosphorylation of the IP₃ receptor. Thermolytic phosphopeptide mapping of in situ labeled IP₃ receptor by PKA showed labeling on the same sites (Ser-1589 and Ser-1756) as in vitro. In contrast to the findings in vitro, PKG preferentially phosphorylated Ser-1589 in situ. Because both PKG and the IP₃ receptor are specifically enriched in cerebellar Purkinje cells, PKG may be an important IP₃ receptor regulator in vivo.

Activation of intracellular signal transduction cascades frequently involves increased phosphoinositide hydrolysis following stimulation of phospholipase C. Inositol 1,4,5-trisphosphate (IP₃), a second messenger produced by phosphoinositide hydrolysis, mediates Ca²⁺ release from intracellular stores by binding to IP₃-sensitive Ca²⁺ channels, thereby increasing their “open” probability (1). IP₃ receptors derive from at least three different genes, constituting types I, II, and III, which are approximately 70% identical at the amino acid level but differ in distribution and regulation (reviewed in Refs. 1–3). An assembly of four 260-kDa subunits forms the receptor. Each subunit consists of a cytoplasmic, amino-terminal IP₃ binding domain, a coupling domain, and a Ca²⁺ channel pore of six transmembrane segments (2–4). Type I is further diversified by alternative RNA splicing, resulting in two main forms, of which the longest (SII⁺, containing the 40 amino acid residues 1693–1732) is specifically expressed in neurons (2). One or more IP₃ receptor forms have been found in virtually all cell types examined (reviewed in Ref. 2), but particularly high amounts of type I IP₃ receptor are seen in smooth muscle cells and in cerebellar Purkinje neurons. Calcium release mediated by IP₃ receptors appears to be an essential step for the induction of long term depression (LTD) in Purkinje cells (5).

A number of different mechanisms modulates IP₃ receptor function, including binding of ATP, fatty acids, and calcium (reviewed in Ref. 2); a number of neurodegenerative processes (6, 7); and phosphorylation of the IP₃ receptor by specific protein kinases. cAMP-dependent protein kinase (PKA) phosphorylates the type I IP₃ receptor both in vitro and in vivo (8–11) and has also been reported to phosphorylate type II and III in intact cells (12). Ca²⁺/calmodulin-dependent protein kinase II, protein kinase C and the tyrosine kinase Fyn have also been reported to phosphorylate the type I IP₃ receptor (13–17). In addition, the receptor may undergo autophosphorylation (18). Early work indicating that the neuronal IP₃ receptor (SII⁺) can be phosphorylated by cGMP-dependent protein kinase (PKG) (8) was later confirmed by in vitro experiments (19–21). Likewise, the nonneuronal type I IP₃ receptor (SII−) found in smooth muscle cells, also termed the G₀ protein (22, 23), is a substrate for phosphorylation by both PKA (10) and PKG (19, 20, 23–25). Recent reports of IP₃ receptor phosphorylation by PKA and PKG in hepatocytes (26–28), kidney cells (11), and platelets (29, 30) support these observations.

Phosphorylation of the IP₃ receptor by PKA and PKG represents a possible mechanism for cross-talk whereby cyclic nucleotides can modulate IP₃-mediated regulation of Ca²⁺ levels (20, 31). Because cAMP and cGMP levels in most cells are regulated by various extracellular signals, identification of phosphorylation sites labeled by these kinases is of interest. Amino acid sequencing indicated that PKA phosphorylates the cerebrospinal fluid; 8-pCPT, 8-((4-chlorophenylthio); DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; EPPS, N₂- hydroxyethylpiperazine-N’-3-propanesulfonic acid; LTD, long term depression.
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rat neuronal receptor on Ser-1756 and, less efficiently, on Ser-1589 (9). Similarly, PKG appears to phosphorylate mainly Ser-1756 in vitro (19). In contrast, the smooth muscle IP₃ receptor is preferentially phosphorylated by PKA on Ser-1589 in vitro (10), whereas Ser-1756 is more prominently phosphorylated by PKA in kidney cells in vivo (11). Hence, the preferred substrate sites for PKA-mediated phosphorylation of the IP₃ receptor differ according to receptor isoforms and tissues, and the factors determining these responses remain unclear. Moreover, IP₃ receptor phosphorylation by PKG in neuronal cells has not been characterized in detail.

In the present study, we have examined in vitro phosphorylation catalyzed by the cyclic nucleotide-dependent protein kinases both of the immunopurified cerebellar IP₃ receptor and of synthetic peptides corresponding to the phosphorylation sites. We have further characterized the in situ phosphorylation of the IP₃ receptor in cerebellar slices stimulated with cyclic nucleotide analogues. The results confirm earlier reports stating that the cerebellar IP₃ receptor can be efficiently phosphorylated by PKA and PKG on two distinct sites in vitro. In addition, we show that both PKA and PKG can phosphorylate the cerebellar IP₃ receptor in situ on the same two sites, albeit with distinct time courses and kinetics.

Some of these data have been reported in abstract form (21, 32).

EXPERIMENTAL PROCEDURES

Materials—[γ-³²P]ATP was from ICN (Irvine, CA). [³²P]Orthophosphate, 10 mCi/ml, was from Amersham Pharmacia Biotech. 8-(4-para-chlorophenylthio)-guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP) and 8-pCPT-cAMP were from BioLog Life Science Institute (Bremen, Germany). KT 5720 and KT 5823 were from Calbiochem-Novabiochem Corp. Protein A-Sepharose beads were from Amersham Pharmacia Biotech. Wistar rats were from Melgaard Breeding Center (Ejby, Denmark). cGMP-dependent protein kinase, purified from bovine lung, was a gift from Dr. Suzanne Lothman (University of Würzburg, Würzburg, Germany). Thermolysin, the catalytic subunit of PKA, the heat-stable inhibitor of PKA (Walsh inhibitor), and EPPS were from Sigma. Other reagents, of analytical grade or better, were from standard commercial suppliers.

Preparation of Peptides—A synthetic peptide comprising the carboxyl-terminal 18 amino acid residues of the mouse IP₃ receptor (33), used to raise antibodies to the IP₃ receptor (6, 7, 34), and two 10-amino acid peptides (peptide 1, with the sequence AARRDSVLAA, corresponding to residues 1751–1760) were synthesized at the Biotechnology Center, University of Oslo. A 30-amino acid synthetic peptide, termed D32 peptide (residues 1584–1593), and peptide 2, SGRRESLTSF, corresponding to residues 1751–1760) were incubated in a buffer containing 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 1 mM EGTA, 10 µM leupeptin, and the phosphatase inhibitors (including 6 mM 0.02 mM sodium vanadate). The two synthetic decapeptides (peptide 1, residues 1584–1593, and peptide 2, residues 1751–1760) were incubated in a buffer containing 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 0.2 mM [γ-³²P]ATP (35 mCi/mmol), bovine serum albumin (0.5 mg/ml), and 10–20 µM peptide dissolved in water. Phosphorylation was initiated by addition either of the catalytic subunit of PKA (final concentration, 12 nM) or PKG holozyme (final concentration, 4 nM) plus 10 µM 8-Br-CGMP. Incubations were carried out for 5 min at 30 °C, and the reactions were terminated by spotting aliquots onto P81 phosphocellulose paper (Whatman) and washing in phosphoric acid (75 mM). Phosphorylations were quantified by Cerenkov counting of the filter papers (39), and kinetic constants were derived from purified Hanes-Woolf plots.

Preparation and Incubation of Rat Cerebellar Slices—Wistar rats (100–200 g) were sacrificed by halothane, and the cerebellum was quickly removed and cooled in artificial cerebrospinal fluid (ACSF) at 0–4 °C of the following composition: NaCl, 124 mM; KCl, 2 mM; KH₂PO₄, 1.25 mM; MgSO₄, 2 mM; NaHCO₃, 26 mM; glucose, 10 mM; and 95% O₂/5% CO₂ (pH 7.4). The hemispheres were glued to a mounting block, and sagittal slices (400 µm) were cut with a vibrisolde in cold oxygenated ACSF. The slices were placed in an interface chamber exposed to humidified gas and maintained in ACSF at a temperature of 25–27 °C for at least 2 h. After equilibration, the slices were carefully transferred to wells (12-well cell culture cluster, Costar) containing 500 µl of ACSF, where incubations with different reagents were performed in an O₂-enriched atmosphere at room temperature. The slices were continuously kept in calcium-free solutions to diminish disturbances induced by calcium influx, e.g. proteolysis by calpains (34), protein kinase C or Ca²⁺/calmodulin-dependent protein kinase II phosphorylation (12–15, 30), and protein phosphatase 2B dephosphorylation (40). Incubations were terminated by removal of the reaction buffer and addition of 400 µl of ice-cold buffer (containing 0.25 M sucrose, 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and the phosphatase inhibitors β-glycerophosphate (60 mM), sodium vanadate (0.1 mM) and sodium fluoride (0.1 mM)) and homogenized by sonication. Following centrifugation at 1,000 x g for 15 min at 4 °C, deoxycholate was added to the supernatant (1% w/v, final concentration), and extraction on ice for 30–45 min was followed by centrifugation for 30 min at 27,000 x g. The resulting supernatant was incubated with protein A-Sepharose beads preincubated with anti-serum to immunoisolate the IP₃ receptor as described above.

The analysis of sequential addition of the two cyclic nucleotide-dependent protein kinases, initial phosphorylation of the immunopurified IP₃ receptor was performed as described above, but employing nonradioactive ATP (8.5 µM) for 20 min. Following washing of the beads (twice in TBS-Tween, the second incubation was initiated by addition of exogenous kinase and [γ-³²P]ATP (8.5 µM, 12 Ci/mmol). The reaction was terminated after 5 min by EDTA, and the samples were washed and analyzed by SDS-PAGE as described above.

Phosphorylation of Synthetic IP₃ Receptor Peptides—The two synthetic decapeptides (peptide 1, residues 1584–1593, and peptide 2, residues 1751–1760) were incubated in a buffer containing 20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 0.2 mM [γ-³²P]ATP (35 mCi/mmol), bovine serum albumin (0.5 mg/ml), and 10–20 µM peptide dissolved in water. Phosphorylation was initiated by addition either of the catalytic subunit of PKA (final concentration, 12 nM) or PKG holozyme (final concentration, 4 nM) plus 10 µM 8-Br-CGMP. Incubations were carried out for 5 min at 30 °C, and the reactions were terminated by spotting aliquots onto P81 phosphocellulose paper (Whatman) and washing in phosphoric acid (75 mM). Phosphorylations were quantified by Cerenkov counting of the filter papers (39), and kinetic constants were derived from purified Hanes-Woolf plots.
was visualized by autoradiography and quantified by densitometry and Cerenkov counting. The phosphoprotein was further characterized by thermolytic peptide mapping as described above.

For the back-phosphorylation experiments, immunopurified samples from cerebellar slices that had been incubated with protein kinase inhibitors and cyclic nucleotide analogues in phosphate-containing ACSF as described above were in vitro phosphorylated at room temperature for 20 min using exogenous PKA and [γ-32P]ATP. The reactions were terminated by EDTA and washing in TBS-Tween, followed by SDS-PAGE, as described above.

Miscellaneous Methods—SDS-PAGE was performed using the buffers of Laemmli (37). Protein content was analyzed by the bicinchoninic acid method (41). When phosphorylation stoichiometry was examined, the amount of IP₃ receptor in the sample was quantified by densitometry of the gels following staining with Coomassie Blue, using standard amounts of bovine serum albumin in the same gels to construct standard curves. Images of autoradiograms were prepared using a Hewlett-Packard ScanJet IIC/ADF scanner, and Desk Scan II (version 1.0, Hewlett-Packard) together with Adobe Photoshop 2.5 or Corel 4.0 software. Statistical analysis and Hanes-Woolf-plots were obtained using Graph-Pad Prism 2.01.

RESULTS

Phosphorylation of the Cerebellar IP₃ Receptor in Vitro—Previous work has shown that the ~260 kDa (apparent from SDS-PAGE) protein band phosphorylated by PKA in cerebellum mostly represents the IP₃ receptor (8, 34, 42). When the IP₃ receptor, extracted from rat cerebellar membranes and immunoprecipitated with rabbit antibodies raised against the carboxyl-terminal end of the brain type I IP₃ receptor, was used as substrate for PKA and PKG in vitro (Fig. 1A), we found similar phosphorylation reactions. PKA rapidly phosphorylated the immunopurified IP₃ receptor during 5-min incubations (Fig. 1B); longer incubation produced only marginal additional effects (not shown). PKG phosphorylated the immunopurified IP₃ receptor more slowly and to a lower maximum level (Fig. 1B).

To estimate the extent of phosphorylation, 0.60 μg of IP₃ receptor was phosphorylated for 5 min. Employing the predicted molecular mass of 313 kDa for the immunopurified receptor subunit (33), our results indicated that PKA phosphorylated the immunopurified IP₃ receptor to a stoichiometry of 1.02 mol of phosphate per mol of protein, whereas PKG phosphorylated the IP₃ receptor to a stoichiometry of 0.48 mol of phosphate per mol of protein under these conditions (see also Fig. 4, a and b).

Site-specific Phosphorylation of the Immunopurified IP₃ Receptor—The phosphorylated domains of the IP₃ receptor were characterized by phosphopeptide mapping. When the phosphorylated protein was subjected to extensive thermolytic digestion and peptide separation in two dimensions on silica plates, two major (phosphopeptides 1 and 2) and two minor phosphopeptides were seen following incubation with PKA (Fig. 2A). In contrast, incubation with PKG resulted in one major and one minor phosphopeptide only; the former comigrated with phosphopeptide 2, and the latter comigrated with phosphopeptide 1 (Fig. 2B). Quantitation of the [32P]-labeled phosphopeptides showed that PKA induced a rapid phosphorylation of both the major phosphorylation sites and that these two phosphorylation sites were phosphorylated to the same extent during the different incubation times (not shown). PKG induced a rapid phosphorylation of site 2 but a more protracted and less complete phosphorylation of site 1 as a function of time (Fig. 3). This is in accordance with the time course for the phosphorylation of the isolated protein, shown in Fig. 1B.

Additive Phosphorylation of the Immunopurified IP₃ Receptor—The relation between the serine residues phosphorylated by PKA and PKG was further examined by phosphorylating the isolated IP₃ receptor with sequential addition of the two kinases. Incubation with nonradioactive ATP in the presence of PKA for 20 min prevented the protein from subsequent phosphorylation with [γ-32P]ATP in the presence of PKG (Fig. 4). In contrast, incubation of the IP₃ receptor with nonradioactive ATP and PKG for 20 min allowed the protein to become further phosphorylated by subsequent addition of [γ-32P]ATP in the presence of PKA, with the final 32P labeling representing approximately half of that obtained by PKA alone (Fig. 4). Thermolytic phosphopeptide mapping of this sample showed that incubation with nonradioactive ATP and PKG led to a considerable decrease in the subsequent PKA-catalyzed 32P labeling of phosphorylation site 2, but not site 1 (Fig. 2C).

Taken together, the phosphopeptide mapping of the IP₃ receptor indicates that PKA catalyzed in vitro phosphorylation of at least two residues within 5 min, whereas PKG catalyzed phosphorylation of one residue within the first minute, with a subsequent and slower phosphorylation of the second residue (Fig. 3). Moreover, the residues phosphorylated by PKA and PKG are identical.

Phosphorylation of Synthetic IP₃ Receptor Peptides—PKA has been shown to phosphorylate Ser-1756 and, less efficiently, Ser-1589, in the neuronal IP₃ receptor (9). To confirm that the same two serines are phosphorylated by PKG and to study the kinetics and phosphorylation efficiency of these in vitro phosphorylation reactions, we used two synthetic decapeptides as substrates for PKA and PKG: peptide 1, encompassing Ser-1589 (AARRDSVLAA), and peptide 2, encompassing Ser-1756 (SGRRESLTSF) (10, 19). The results were compared with those obtained with a 30-amino acid peptide derived from DARPP-32, D32-(Ser-34)8–38, which is known to represent a good substrate for both cyclic nucleotide-depend-
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**Fig. 2.** Two-dimensional phosphopeptide maps of in vitro phosphorylated IP₃ receptor. Immunoisolated IP₃ receptor was phosphorylated for 5 min with [γ-³²P]ATP and PKA (A), [γ-³²P]ATP and PKG (B), or PKG alone for 20 min with nonradioactive ATP followed by PKA with [γ-³²P]ATP for 5 min (C). After separation by SDS-PAGE, the bands corresponding to the IP₃ receptor were excised from the gel and subjected to complete proteolytic digestion with thermolysin. The phosphopeptides generated were separated by electrophoresis (pH 3.5) in the first dimension and chromatography in the second dimension and visualized by autoradiography. Phosphopeptides (1 and 2) and application points (●) are indicated.

**Fig. 3.** Time course of site-specific IP₃ receptor phosphorylation by PKG. Immunoisolated IP₃ receptor was phosphorylated by PKG plus 8-Br-cGMP and [γ-³²P]ATP for 1 (A), 2 (B), and 5 (C) min. After separation by SDS-PAGE, the IP₃ receptor was visualized by autoradiography, excised from the gel, and subjected to proteolytic digestion with thermolysin. The phosphopeptides generated were separated and visualized as described in the legend to Fig. 2. Phosphopeptides (1 and 2) and application points (●) are indicated.

**Fig. 4.** Additive phosphorylation of the IP₃ receptor by PKA and PKG. Immunoisolated IP₃ receptor was phosphorylated for 5 min in the presence of [γ-³²P]ATP by the catalytic subunit of PKA (a) and PKG plus 8-Br-cGMP (b) as described. In c, the IP₃ receptor was phosphorylated for 20 min with nonradioactive ATP in the presence of PKA, followed by phosphorylation for 5 min with [γ-³²P]ATP in the presence of PKG plus 8-Br-cGMP. In d, the IP₃ receptor was phosphorylated for 20 min with nonradioactive ATP in the presence of PKG plus 8-Br-cGMP, followed by phosphorylation for 5 min with [γ-³²P]ATP in the presence of PKA. Following incubations and separation by SDS-PAGE, the amount of IP₃ receptor was quantified by densitometry of the Coomassie-stained gels, and the amount of ³²P-labeled IP₃ receptor was measured by Cerenkov counting of the excised bands. The bars represent mol of incorporated phosphate per mol of IP₃ receptor protein, calculated on basis of a predicted molecular mass of 313 kDa per subunit. In repeated experiments, essentially identical results were obtained.

**TABLE I**

Kinetic data concerning the phosphorylation, catalyzed by PKA and PKG, of synthetic peptides 1 and 2 from the IP₃ receptor and of the D32 peptide (D32-D(Ser-34)8–38) from DARPP-32

| Substrate | Enzyme | Vₘₐₓ | Kᵣᵣ | Kᵣᵣ/Kₘₐₓ |
|-----------|--------|------|-----|-----------|
| Peptide 1 | PKA    | 1.2  | 17.1| 0.77      |
| Peptide 2 | PKG    | 1.1  | 69.7| 2.7       |
| Peptide 2 | PKA    | 0.74 | 11.6| 0.48      |
| Peptide 2 | PKG    | 0.45 | 17.3| 1.2       |
| D32-peptide PKA | 0.85 | 27.0| 0.42 |
| D32-peptide PKG | 0.14 | 11.3| 0.37 |

the synthetic peptide 1 (Table I). The kinetic data were thus in agreement with the in vitro phosphorylation of the intact IP₃ receptor. The DARPP-32 peptide showed kinetic properties similar to those of peptide 2 for both PKA and PKG (Table I).

**Phosphorylation of IP₃ Receptor in Situ in Rat Cerebellar Slices—**It was of interest to ascertain that these kinases are able to phosphorylate the IP₃ receptor in neurons in situ. Because the IP₃ receptor is specifically abundant in Purkinje neurons of the rat cerebellum (2), we utilized cerebellar slice preparations to study these neurons. The slices were incubated for 2–2.5 h in ACSF before stimulation to ensure equilibration of physiological processes (43). Preparation and incubation of slices were in accordance with previously published techniques for electrophysiological slice experiments (44).

**Incorporation of [³²P]Orthophosphate into Stimulated Slices from Rat Cerebellum—**Rat cerebellar slices were preincubated in [³²P]orthophosphate, followed by addition of 8-pCPT-cGMP or 8-pCPT-cAMP together with the specific PKA and PKG inhibitors, KT 5720 and KT 5823, respectively. Immunopurification of the IP₃ receptor and quantitation of the radioactive
Phosphorylation of the IP$_3$ receptor has been observed in different cell types, involving different signaling systems and protein kinases. In the present work, we show that both PKA and PKG mediate in vitro phosphorylation of at least two sites in the neuronal IP$_3$ receptor immunopurified from rat cerebellum. When the intact neuronal IP$_3$ receptor is phosphorylated in vitro, low concentrations of PKA label Ser-1756, whereas high levels of PKA label both Ser-1756 and Ser-1589 (9). In vitro phosphorylation by PKG appears to mimic the effect of low PKA, because a strong preference for Ser-1756 is observed (20). In contrast, the smooth muscle IP$_3$ receptor is phosphorylated by PKA in vitro only on Ser-1589 (10). The smooth muscle G$_o$ protein, later reported to represent an IP$_3$ receptor (20), is preferentially phosphorylated by endogenous PKG in vitro on a phosphopeptide comigrating on phosphopeptide maps with the IP$_3$ receptor phosphopeptide containing Ser-1589 (25). A proposed cause for the differential phosphorylation between neuronal and smooth muscle IP$_3$ receptors is the 40-amino acid stretch comprising the alternatively spliced residues 1693–1732, which are not found in the nonneuronal IP$_3$ receptor subtype from smooth muscle (SII–).

Under the experimental conditions used in this work, we have found that the two phosphorylation sites showed distinct kinetic features when their in vitro phosphorylation was studied by the use of synthetic peptide substrates. Phosphorylation of synthetic decapetides containing the putative phosphorylation sites for PKA and PKG showed that both enzymes display higher values for $V_{\text{max}}$ and $K_{\text{cat}}$ for the Ser-1589-containing peptide 1 than for the Ser-1756-containing peptide 2 (Table I).
Despite the lower maximal rate of phosphorylation, PKG had approximately 4-fold higher affinity for peptide 2 than peptide 1, whereas PKA exhibited 1.5-fold higher affinity for peptide 2 compared with peptide 1. Hence, the catalytic efficiency (Kcat/Km) of PKG was higher for peptide 2, indicating a preference for Ser-1756 by this kinase (Table I), which is in agreement with earlier results (19). However, the differences in catalytic efficiency were small, and both IP3 receptor-derived peptides appeared to be phosphorylated by the two kinases with a higher catalytic efficiency than D32-(Ser-34)8-38 (Table I). The latter peptide represents the phosphorylation domain of DARPP-32, a protein that is a substrate for both PKA (35) and PKG (45) in vitro. Thus, from these data we cannot exclude any of these IP3 receptor peptides as possible targets for cyclic nucleotide-activated protein kinases in vivo.

In in situ data appear to contradict the report from Komalavilas and Lincoln (19), in which Ser-1589 was proposed not to be phosphorylated in nonradioactive, phosphate-containing ACSF for 2.5 h, followed by incubation with the PKG inhibitor KT 5823 (control) (a), KT 5720 for 20 min and 8-pCPT-cAMP for 10 min (b), the PKA inhibitor KT 5720 (control) (c), KT 5720 for 20 min and 8-pCPT-cGMP for 10 min (d). Homogenization of the slices and immunopurification of the IP3 receptor was followed by in vitro phosphorylation with exogenous PKA and [γ-32P]ATP for 20 min. The conclusion of the reaction, SDS-PAGE, and quantitation of the IP3 receptor was performed as described. The bars represent mean ± S.D. of four slices; the experiment was performed three times with similar results. * significantly different from control, p < 0.05 (Student’s t test; † p < 0.05 (Mann-Whitney U test).

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