Phosphorylation of Protein Phosphatase Inhibitor-1 by Protein Kinase C

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Inhibitor-1 becomes a potent inhibitor of protein phosphatase 1 when phosphorylated by cAMP-dependent protein kinase at Thr35. Moreover, Ser67 of inhibitor-1 serves as a substrate for cyclin-dependent kinase 5 in the brain. Here, we report that dephosphoinhibitor-1 but not phospho-Ser67 inhibitor-1 was efficiently phosphorylated by protein kinase C at Ser66 in vitro. In contrast, Ser67 phosphorylation by cyclin-dependent kinase 5 was unaffected by phospho-Ser65. Protein kinase C activation in striatal tissue resulted in the concomitant phosphorylation of inhibitor-1 at Ser65 and Ser67, but not Ser65 alone. Selective pharmacological inhibition of protein phosphatase activity suggested that phospho-Ser65 inhibitor-1 is dephosphorylated by protein phosphatase 1 in the striatum. In vitro studies confirmed these findings and suggested that phospho-Ser67 protects phospho-Ser65 inhibitor-1 from dephosphorylation by protein phosphatase 1 in vivo. Activation of group I metabotropic glutamate receptors resulted in the up-regulation of diphasopho-Ser65/Ser67 inhibitor-1 in this tissue. In contrast, the activation of N-methyl-D-aspartate-type ionotropic glutamate receptors opposed increases in striatal diphasopho-Ser65/Ser67 inhibitor-1 levels. Phosphomimetic mutation of Ser65 and/or Ser67 did not convert inhibitor-1 into a protein phosphatase 1 inhibitor. On the other hand, in vitro and in vivo studies suggested that diphasopho-Ser65/Ser67 inhibitor-1 is a poor substrate for cAMP-dependent protein kinase. These observations extend earlier studies regarding the function of phospho-Ser67 and underscore the possibility that phosphorylation in this region of inhibitor-1 by multiple protein kinases may serve as an integrative signaling mechanism that governs the responsiveness of inhibitor-1 to cAMP-dependent protein kinase activation.

Intracellular signal transduction in eukaryotic organisms relies on the precise regulation of protein phosphorylation by protein kinases and protein phosphatases. These enzymes are broadly classified based on their specificity for serine, threonine, or tyrosine residues. Approximately equal numbers of protein-tyrosine kinases and phosphatases are encoded by most eukaryotic genomes (1, 2). In contrast, merely a handful of protein serine/threonine phosphatases appear to be required for reversing the actions of a much larger cohort of protein serine/threonine kinases (3–5), raising the question of how protein serine/threonine phosphatase specificity is achieved. To counter this numerical disparity, protein serine/threonine phosphatases rely on a rich array of regulatory subunits that control the localization, activity, and substrate specificity of protein phosphatase catalytic subunits. In the case of protein phosphatase 1 (PP-1), 2 one of the major eukaryotic protein serine/threonine phosphatases, nearly 60 actual and putative regulator proteins have been identified to date (6, 7). Most of these regulators are involved in the targeting of PP-1 to specific subcellular locations, whereas several modulate its catalytic activity.

Historically, protein phosphatase inhibitor-1 (inhibitor-1, or I-1) was the first such endogenous molecule found to regulate protein phosphatase activity in vivo (8). This 19-kDa protein has a highly conserved primary sequence in vertebrates ranging from fish to mammals (9, 10). It largely lacks elements of secondary structure (11), possibly explaining why it is unusually flexible at the N-terminal region, allowing it to interact with many different phosphatase catalytic subunits, some of which exhibit substrate specificity.

2 The abbreviations used are: PP, protein phosphatase; PKA, cAMP-dependent protein kinase; Cdk, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; PKC, Ca2+/phospholipid-dependent protein kinase, or protein kinase C; JNK, (protein phosphatase) inhibitor-1; DARPP-32, dopamine- and cAMP-regulated phosphoprotein, M, 32,000; GBPI, gut and brain phosphatase inhibitor; GluR1, glutamate receptor 1; CBB, Coomassie Brilliant Blue; MOPS, 3-(N-morpholino)propanesulfonic acid; MS, mass spectrometry; MS/MS, tandem mass spectrometry; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PDBu, phorbol-12,13-dibutrylate; Ad I-1 and Ad PKC-α, adenovirus encoding inhibitor-1 and PKC-α, respectively; DHPG, (S)-3,5-dihydroxyphenylglycine; NMDA, N-methyl-D-aspartate.

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stable to heat, acid, detergents, and organic solvents (12). Phosphorylation at Thr35 by cAMP-dependent protein kinase (PKA) converts the inactive protein into a selective and highly potent inhibitor of the catalytic subunit of PP-1 (IC50 ~ 1 nM) (8, 13). This site is dephosphorylated by the type-2 protein serine/threonine phosphatases, PP-2A and PP-2B (Ca2+/calmodulin-dependent protein phosphatase, or calcineurin) (14–16). PP-2B activity predominates in the presence of high intracellular Ca2+, placing inhibitor-1 regulation under the opposing influences of cAMP and Ca2+ signaling. Downstream from inhibitor-1, a mechanism for signal amplification is provided by the substrate specificity of PP-1. PP-1 dephosphorylates a broad spectrum of phosphoproteins targeted by an array of protein serine/threonine kinases, including PKA as well as others (14). Thus, the inhibition of PP-1 by phospho-Thr35 inhibitor-1 results in the amplification of PKA-dependent signaling cascades and has the potential to impose CAMP regulation upon the phosphorylation state of cellular substrates phosphorylated by other protein kinases.

Inhibitor-1 is highly expressed in the brain, adipose tissue, kidney, and skeletal muscle (17), with lower levels occurring in the heart and lung (9). It has been shown to play a particularly important role as a PP-1 inhibitor in excitable tissues like brain and cardiac muscle, where it has emerged as a key player in models of synaptic plasticity (16, 18) and cardiomyocyte contractility (19–21), respectively. Within the brain, inhibitor-1 is especially enriched in the cerebral cortex, striatum, and dentate gyrus of the hippocampal formation (22). In the heart, nearly 80% of inhibitor-1 is found in the sarcoplasmic reticulum (23), consistent with a role for inhibitor-1 in the regulation of PP-1 substrates associated with this compartment in cardiomyocytes.

Protein phosphatase regulatory subunits like inhibitor-1 can be regulated by multiple protein kinases and phosphatases, thus serving as potential points of integration for disparate signal transduction cascades. For instance, DARPP-32, a homologous PP-1 inhibitor highly enriched in the striatum, is phosphorylated at different serine/threonine residues by PKA (24), casein kinases 1 and 2 (25, 26), and cyclin-dependent kinase 5 (Cdk5) (27). Consistent with this notion, inhibitor-1 isolated from rabbit skeletal muscle in earlier studies was found to be heavily phosphorylated at Ser67 (28). This residue was later characterized as a site of phosphorylation by mitogen-activated protein kinase (MAPK), cyclin-dependent kinase 1 (Cdk1), and Cdk5 in vitro, with Cdk5 accounting for all phosphorylation in the brain (29). The same study found that phospho-Ser67 inhibitor-1 does not inhibit PP-1 but rather serves as a slightly less efficient substrate for PKA at Thr35. More recently, it was suggested that inhibitor-1 may be phosphorylated by protein kinase C (PKC) in cardiac muscle, where phospho-Ser67 inhibitor-1 was implicated in modulating the contractile response by regulating the activity of the SERCA-2 pump in a PKC-dependent fashion (30). However, the precise interaction between PKC and inhibitor-1 in this novel signaling pathway has not yet been delineated and is the focus of the present study.

In this report, we show that inhibitor-1 is phosphorylated by PKC at Ser65 in vitro and in intact striatal brain tissue. We further demonstrate that under basal conditions in the stria-

**EXPERIMENTAL PROCEDURES**

**Chemicals and Enzymes**—All chemicals were from Sigma, except where indicated. Trypsin, shrimp alkaline phosphatase, and endoprotease Lys-C were from Promega. PKC (a mixture of the Ca2+-dependent conventional isoforms, α, β, and γ) was purified from rat brain (31). The catalytic subunit of PKA was purified from bovine heart (32). MAPK and PP-1 were from Upstate Biotechnology, Inc. (Lake Placid, NY). Phosphorylase b was from Calzyme. Cdk5 and p25-His6 were co-expressed in insect SF9 cultures using baculovirus vectors and affinity-purified (33). Cell culture reagents were from Invitrogen. Deoxyoligonucleotides were from Integrated DNA Technologies. Protease inhibitors, dithiothreitol, isopropyl-β-d-thiogalactopyranoside, and ATP were from Roche Applied Science. [γ-32P]ATP was from PerkinElmer Life Sciences. Peptides and phosphopeptides were synthesized at the Protein Chemistry Technology Center of the University of Texas Southwestern Medical Center. Phorbol-12,13-dibutyrate (PDBu), Ro-32-0432, and fostriecin were from Calbiochem. Roscovitine was from Laurent Meijer (Centre National de la Recherche Scientifique, Roscoff, France). Cyclosporin A, calyculin A, and forskolin were from LC Laboratories. Okadaic acid was from Alexis. (S)-3,5-dihydroxyphenylglycine (DHPG) and NMDA were from Tocris.

**Site-directed Mutagenesis**—The pET-15b expression vector incorporating the cDNA for rat inhibitor-1-His6 (29) served as a template for site-directed mutagenesis using the polymerase chain reaction. Mutants containing single or multiple amino acid substitutions were generated using the QuikChange kit (Stratagene) and following the manufacturer’s recommendations for mutagenic primer design. Mutations were confirmed by DNA sequencing along both strands, using primers specific for the T7 promoter and T7 terminator.

**Purification of Inhibitor-1**—Recombinant inhibitor-1 was generated as previously described (29). Chemically competent BL21 (DE3) cells were transformed with pET-15b expression vectors encoding wild-type or mutant rat inhibitor-1. Cultures were induced with isopropyl-β-D-thiogalactopyranoside and lysed by French press. Cleared lysates were incubated with a nickel-nitrilotriacetic acid-agarose resin (Qiagen). Bound protein was eluted from the resin using a linear gradient of 0–500 mM imidazole. All forms of inhibitor-1 eluted at ~150 mM.
Inhibitor-1 Phosphorylation by PKC

imidazole. Samples were dialyzed overnight in 10 mM HEPES, pH 7.4, with two changes of buffer. Dialyzed protein (10 μg) was analyzed for purity by SDS-15% PAGE and Coomassie Brilliant Blue (CBB) staining.

In Vitro Protein Phosphorylation and Dephosphorylation Reactions—All protein phosphorylation reactions were performed at 30 °C in a final volume of at least 30 μl containing 10 mM substrate (unless otherwise specified). 100 μM ATP, and 0.2 mCi/ml [γ-32P]ATP. The PKC reaction solution included 20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 1 mM dithiothreitol, 1 mM CaCl2, 10 mM MgCl2, 0.1 mg/ml phosphotidylserine, and 0.01 mg/ml diacylglycerol, where the final two components were added to the reaction mixture following sonication on ice for 1 min. PKA reactions were conducted in 50 mM HEPES, pH 7.4, 1 mM EGTA, 10 mM magnesium acetate, and 0.2 mg/ml bovine serum albumin; MAPK reactions were in 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, and 20 mM EGTA; and Cdk5 reactions were in 30 mM MOPS, pH 7.2, and 5 mM MgCl2. Time course reactions were performed by removing 10–μl aliquots from the reaction solution at various time points and adding an equal volume of 5× SDS protein sample buffer to stop the reaction. Kinetic parameters and initial reaction rates were determined from the results of at least four experiments performed in duplicate with 1.5–20 μM substrate under empirically defined linear steady-state conditions. In all cases, [32P]phosphate incorporation was assessed by SDS-PAGE and PhosphorImager analysis. To calculate reaction stoichiometries, radiolabeled reaction products and radioactive standards were quantified by densitometry using ImageQuant software (Amersham Biosciences). Standards consisted of spotted aliquots from serial dilutions of each reaction mixture (1:100, 1:500, 1:1000), with the moles of phosphate per unit volume of each dilution defined using the ATP concentration of the original reaction. Division of the signal per mole of substrate by the signal per mole of phosphate yielded the stoichiometry of phosphate incorporation (mol of phosphate/mol of substrate).

In some studies, preparative phosphorylation of inhibitor-1 by a protein kinase was followed by repurification of phoshoinhibitor-1 prior to use in a subsequent assay. For these experiments, phosphoinhibitor-1 was repurified from in vitro protein phosphorylation reaction mixtures with trichloroacetic acid precipitation and dialysis as previously described (29). Protein dephosphorylation reactions were conducted at 30 °C in a final volume of 30 μl containing 10 μM substrate, 50 mM Tris-HCl, pH 7.4, 0.01% Brij, 0.1% β-mercaptoethanol, 0.3 mg/ml bovine serum albumin, 0.1 mM EGTA, and 5 mM caffeine, using the catalytic subunit of PP-1 in the absence or presence of 25 nm phospho-Thr35 inhibitor-1 repurified from preparative phosphorylation reactions conducted with PKA. Phosphoinhibitor-1 forms evaluated as PP-1 substrates in these studies were generated by preparative phosphorylation of inhibitor-1 with PKC in the presence of [γ-32P]ATP, followed by repurification and preparative phosphorylation with or without Cdk5. Reactions conducted with PKC and Cdk5 for these studies achieved final stoichiometries of 0.90 and 0.92 mol/mol, respectively. Following 30 min of incubation, protein dephosphorylation reactions were stopped by the addition of an equal volume of 5× SDS protein sample buffer and quantified by SDS-PAGE and PhosphorImager analysis.

PP-1 inhibition assays were performed under linear steady-state conditions as previously described (24), using 1 mg/ml [γ-32P]-labeled phospholipase a as a PP-1 substrate in the absence or presence of wild-type and site-directed mutant forms of recombinant inhibitor-1 repurified from preparative phospho-rylation reactions conducted with and without PKA. These protein dephosphorylation reactions were stopped by the addition of 100 μl of 10% trichloroacetic acid. Following centrifugation at 15,000 × g for 3 min, supernatants were analyzed by liquid scintillation counting for the amount of free [32P]phosphate released into solution.

Phosphorylation Site Identification by Mass Spectrometry (MS)—In one set of experiments, inhibitor-1 from in vitro protein phosphorylation reaction mixtures with and without PKC was subjected to SDS-PAGE and in-gel digestion with trypsin. Dried digest mixtures were resuspended in 20 μl of 5% formic acid and loaded onto a POROS R2 reversed-phase column (Applied Biosystems) for purification. After being washed with 5% formic acid, peptides were eluted directly into a nanoelectrospray needle (Proxeon Biosystems) with 1 μl of either 50% methanol, 1% NH4H2O for precursor ion scanning in negative ion mode or 50% methanol, 1% acetic acid for tandem mass spectrometry (MS/MS) in positive ion mode. MS analysis was performed on a QSTAR Pulsar I quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems/MD SCIEX) equipped with a nanoelectrospray ion source (MDS Proteomics, Odense, Denmark). For precursor ion scanning, the instrument was set in negative ion mode to detect the PO3− fragment ion at m/z −79. After data acquisition, the instrument was switched to positive ion mode, and the phosphopeptide sequence and site of phosphorylation were determined by nanoelectrospray-QSTAR Pulsar I quadrupole time-of-flight MS/MS. In the MS/MS scan mode, the precursor ion was selected in the quadrupole (Q1) and fragmented in the collision cell (Q2), using argon as the collision gas.

In parallel studies, [32P]-labeled phosphoinhibitor-1, resulting from phosphorylation by PKC in the presence of [γ-32P]ATP, was subjected to SDS-PAGE and digested with endoproteinase Lys-C. The digest mixture was fractionated by reversed-phase HPLC on a C18 column (1.0-mm inner diameter × 150 mm; Vydac), and collected fractions were screened for radioactivity. The fraction containing the radiolabel was subjected to MALDI-TOF analysis. To confirm phosphorylation, a small aliquot of the fraction was treated with shrimp alkaline phosphatase in 50 mM NH4HCO3 at 37 °C for 30 min and analyzed by MALDI-TOF. The remainder of the fraction was dried, and the phosphorylation site was modified with 1-propanethiol to produce 3-propylcysteine after β-elimination of the phosphate. This modification results in a net mass shift of ~21 Da, generating a derivative that is stable to Edman degradation chemistry and elutes after phenylthiohydantoin-leucine. The reaction was performed by incubating the phosphopeptide with 10 μl of 1 M NaOH, 3 μl of saturated Ba(OH)2, and 15 μl of 0.5 M 1-propanethiol at 37 °C for 2 h. Edman degradation was performed after the modified peptide was analyzed by MALDI-TOF to confirm the completion of the reaction.
Generation of Phosphorylation State-specific Antibodies—Polyclonal phosphorylation state-specific antibodies for phospho-Ser\(^{65}\) and diphospho-Ser\(^{65}/Ser^{67}\) inhibitor-1 were generated and affinity-purified as previously described (34), using synthetic phosphopeptides encompassing the local amino acid sequence around Ser\(^{65}\) of inhibitor-1. Briefly, New Zealand White rabbits were immunized with one of two inhibitor-1 phosphopeptides, incorporating either phospho-Ser\(^{65}\) alone or phospho-Ser\(^{65}\) and phospho-Ser\(^{67}\). Phosphorylation state-specific antibodies were purified from the resulting antisera using a SulfoLink resin (Pierce) conjugated to the corresponding phosphopeptide antigens. Purified antibodies were evaluated for specificity by immunoblot analysis of dephospho- and phosphorynhibitor-1 standards (50 ng). Three forms of phosphoinhibitor-1 were generated by preparative phosphorylation of inhibitor-1 at Ser\(^{65}\), Ser\(^{67}\), or Ser\(^{65}\) and Ser\(^{67}\) using PKC and/or Cdk5. The reactions with PKC achieved a final stoichiometry of 0.35 mol/mol at Ser\(^{65}\), whereas the Cdk5 reactions reached a stoichiometry of 1 mol/mol at Ser\(^{67}\). Diphospho-Ser\(^{65}/Ser^{67}\) inhibitor-1 was generated by phosphorylating inhibitor-1 first with PKC and then with Cdk5.

Infection of Cultured Cells with Replication-deficient Adenoviruses—The adenoviruses encoding rat inhibitor-1 (20) and constitutively active PKC-\(\alpha\) (35) were previously described. PC12 rat pheochromocytoma cells, courtesy of Eric Nestler (University of Texas Southwestern Medical Center), were cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 10% horse serum in 60-mm plates. After 24 h, the culture medium was changed, and cells were infected with a selected adenovirus at a multiplicity of infection of 100 plaque-forming units for 2 h at 37 °C in a humidified 5% CO\(_2\) incubator. Infected cells were cultured for an additional 24 h before being harvested for analysis. N2a mouse neuroblastoma cells, a gift from Susanne Mumby (University of Texas Southwestern Medical Center), were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and infected with the adenovirus encoding inhibitor-1. Subsequently, cells were cultured for 24 h and treated with 1 \(\mu\)M PDBu for 10 min following incubation in the presence or absence of 1 \(\mu\)M Ro-32-0432 for 1 h. After drug treatment, cells were collected in phosphate-buffered saline using a cell scraper. Following centrifugation, cell pellets were rapidly frozen on dry ice and stored at −80 °C until assayed.

Preparation and Incubation of Acute Striatal Slices—Male C57BL/6 mice (8–10 weeks old) were killed by decapitation. The brains were rapidly removed and placed in cold, oxygenated Krebs buffer (124 mM NaCl, 4 mM KCl, 26 mM NaHCO\(_3\), 1.5 mM CaCl\(_2\), 1.25 mM KH\(_2\)PO\(_4\), 1.5 mM MgSO\(_4\), and 10 mM D-glucose, pH 7.4). 350-\(\mu\)m coronal sections were prepared using a vibrating blade microtome. Striatal slices were dissected from these sections in cold, oxygenated Krebs buffer using a dissecting light microscope. Each slice was transferred to a polypropylene incubation tube containing 2 ml of Krebs buffer and allowed to recover at 30 °C under constant oxygenation with 95% O\(_2\)/5% CO\(_2\) for 60 min, with a change of buffer following the first 30 min of recovery. Slices were subsequently treated with drugs as specified for each experiment, transferred to microcentrifuge tubes, snap-frozen on dry ice, and stored at −80 °C until further analysis.

RESULTS

Identification of Ser\(^{65}\) as the Inhibitor-1 Site Phosphorylated by PKC—Inhibitor-1 serves as an efficient substrate for PKC \(in vitro\) (30). This phosphorylation reaction was further characterized by time course analysis conducted over 120 min, using recombinant inhibitor-1 in the presence of excess enzyme (Fig. S1). The reaction displayed linear conversion of substrate to product in the first 10 min and reached near saturation by 60 min, achieving a maximal stoichiometry of \(\sim 0.65\) mol/mol. This result suggests the phosphorylation of a single inhibitor-1 site by PKC. In other experiments, the maximal stoichiometry of PKC-dependent inhibitor-1 phosphorylation \(in vitro\) reached 0.90 mol/mol but did not exceed 1.0 mol/mol (see below).

Our previous studies had implicated Ser\(^{67}\) of inhibitor-1 as a possible PKC target (30). Indeed, mutation of this serine residue to alanine resulted in a partial reduction (\(\sim 30\%\)) in the ability of PKC to phosphorylate inhibitor-1 \(in vitro\) (Fig. 1A). Since this mutation alone was insufficient to eliminate PKC-dependent phosphorylation entirely, we concluded that Ser\(^{67}\) might be one of at least two inhibitor-1 sites phosphorylated by PKC. How-
Inhibitor-1 Phosphorylation by PKC

**A**

![Graph showing phosphorylation levels over time for wild-type and S67A inhibitor-1](image)

**B**

![Graph showing phosphorylation levels over time for PKC and MAPK](image)

**C**

![Graph showing phosphorylation levels over time for wild-type and S65A inhibitor-1](image)

**FIGURE 1. Identification of Ser65 as the PKC site of inhibitor-1.**

A, evaluation of Ser65 as the PKC site of inhibitor-1 using site-directed mutagenesis. The panels show SDS-PAGE analysis of 32P-labeled (top) and CBB-stained (bottom) wild-type and Ser67 -> Ala (S67A) inhibitor-1 phosphorylated by PKC in vitro. Reaction times are indicated along the top. The plot represents phosphate incorporation over time. B, evaluation of Ser65 as the PKC site of inhibitor-1 using a phosphorylation state-specific antibody. Immunoblot analysis is shown for PKC- and MAPK-dependent inhibitor-1 phosphorylation in vitro, using an antibody for phospho-Ser65 (P-S65) (top panels) or total inhibitor-1 (bottom panels). Reaction times are indicated along the top. 50 ng of protein are loaded per lane. (MAPK was used for these studies because this readily available reagent efficiently phosphorylates inhibitor-1 at Ser67 in vitro, as do Cdk1 and Cdk5 (29).) C, confirmation of Ser65 as the PKC site of inhibitor-1. The panels show SDS-PAGE analysis of 32P-labeled (top) and CBB-stained (bottom) wild-type and Ser65 -> Ala (S65A) inhibitor-1 phosphorylated by PKC in vitro. Reaction times are indicated along the top. The plot represents phosphate incorporation as a function of time. (The physical identification of Ser65 as the PKC site of inhibitor-1 is shown in Fig. S2.)

However, a phosphorylation state-specific antibody for phospho-Ser67 inhibitor-1 failed to detect any phosphorylation of this residue by PKC in vitro (Fig. 1B). In contrast, the phosphorylation of inhibitor-1 at Ser67 by a protein kinase known to target this site in vitro was efficiently detected by the same antibody. These findings suggest that Ser67 of inhibitor-1 is not a PKC substrate, although mutation of this serine residue to alanine adversely affects phosphorylation of another site by PKC.

Two different approaches were used to directly identify the inhibitor-1 site phosphorylated by PKC. First, inhibitor-1 from in vitro phosphorylation reactions conducted with and without PKC was digested with trypsin and analyzed by nanoelectrospray-QSTAR Pulsar 1 quadrupole time-of-flight MS/MS. Based on the generation of a PO3- fragment ion at m/z -79, a single phosphopeptide candidate was isolated by the quadrupole and fragmented in the collision cell. Spectral analysis of the collision fragments identified the phosphopeptide as STLpSMSPR, phosphorylated at the second serine residue (Fig. S2A), which corresponds to Ser65 of inhibitor-1.

This finding was reproduced by a second approach, in which inhibitor-1 pre phosphorylated by PKC in the presence of [γ-32P]ATP was digested with endoproteinase Lys-C, and proteolytic fragments were subjected to MALDI-TOF MS analysis and Edman degradation microsequencing. Specifically, the digest mixture was fractionated by reversed-phase HPLC, and collected fractions were screened for radioactivity. Counts were found primarily in a single fraction, which produced a predominant mass of 1371.2, corresponding to the sequence STLMSMSPRKR + 80 Da (Fig. S2B, top) and indicating the presence of a phosphorylation site. A small portion of the fraction was treated with shrimp alkaline phosphatase, and a mass of 1291.6 was observed (Fig. S2B, middle), confirming that the peptide was phosphorylated. The remainder of the fraction was dried, and the phosphorylation site was modified with 1-propanethiol to produce a stable derivative to Edman degradation chemistry. A mass of 1349.4 was observed after MALDI-TOF MS analysis (Fig. S2B, bottom), corresponding to the expected mass shift of -21 Da and indicating a complete reaction. Edman degradation was performed on the derivatized fraction. Phenylthiohydantoin-S-propylcysteine was observed in cycle 4 (data not shown), indicating that the fourth N-terminal residue, corresponding to Ser65 of inhibitor-1, is the site of phosphorylation.

These findings were confirmed by site-directed mutagenesis of Ser65 to alanine (Fig. 1C). Unlike wild-type inhibitor-1 or the Ser67 -> Ala mutant, Ser65 -> Ala inhibitor-1 did not serve as a PKC substrate to any detectable extent, consistent with the
versus dephospho plots

The phosphorylation of inhibitor-1 at Ser65. Similar results were obtained in the maximal stoichiometry achieved in the PKC-dependent phosphorylation reactions conducted in the absence or presence of inhibitor-1 repurified from preparative scale reactions. The Cdk5-dependent phosphorylation of inhibitor-1 at Ser67 was also evaluated. Phospho-Ser67 inhibitor-1 was phosphorylated by PKC with an initial reaction rate that was 8.7 ± 0.4-fold lower than that of the PKC-dependent phosphorylation of dephosphoinhibitor-1 (Fig. 2C), indicating that under linear steady-state conditions, phospho-Ser67 inhibitor-1 does not serve as an efficient PKC substrate. Thus, the generation of diphospho-Ser65/Ser67 inhibitor-1 in vitro requires the sequential phosphorylation of inhibitor-1 first by PKC and then by Cdk5.

These in vitro protein phosphorylation reactions were conducted in the presence of excess enzyme over the course of 90 min. Thus, the maximal stoichiometries achieved in these reactions provide only an indirect measure of reaction efficiency or physiological likelihood. In contrast, kinetic parameters, such as initial in vitro reaction rates, can be readily invoked as more accurate indicators of the possible PKC-dependent phosphorylation of different inhibitor-1 forms in vivo. Therefore, the effect of phospho-Ser67 on the initial rate of inhibitor-1 phosphorylation by PKC was also evaluated. Phospho-Ser67 inhibitor-1 was phosphorylated by PKC with an initial reaction rate that was 8.7 ± 0.4-fold lower than that of the PKC-dependent phosphorylation of diphosphoinhibitor-1 (Fig. 2C), indicating that under linear steady-state conditions, phospho-Ser67 inhibitor-1 does not serve as an efficient PKC substrate in vitro.

Generation of Phosphorylation State-specific Antibodies against Phospho-Ser65 Inhibitor-1—To further study the phosphorylation of inhibitor-1 by PKC in vitro and in vivo, two phosphorylation state-specific antibodies were generated for the purpose of detecting inhibitor-1 when it is either singly phosphorylated at Ser65 or doubly phosphorylated at Ser65 and Ser67 (Fig. 3). Immunoblot analysis of recombinant inhibitor-1 preparatively phosphorylated using purified PKC and/or Cdk5 showed that the two novel antibodies and the previously characterized antibody for phospho-Ser67 inhibitor-1 do not detect dephosphoinhibitor-1. Moreover, each antibody is highly selective for the form of phosphoinhibitor-1 against which it was raised.

The antibody for phospho-Ser65 inhibitor-1 detected inhibitor-1 only when it was phosphorylated by PKC (Fig. 3, top panel). Interestingly, the Cdk5-dependent phosphorylation of phospho-Ser65 inhibitor-1 eliminated the ability of the antibody to detect phospho-Ser65, presumably due to steric hindrance from the additional phosphate group transferred to the nearby Ser65 residue by Cdk5. Thus, this antibody was found to

FIGURE 2. Effect of phospho-Ser65 and phospho-Ser67 on inhibitor-1 phosphorylation by Cdk5 and PKC, respectively. A, comparison of the Cdk5-dependent phosphorylation of dephosphoinhibitor-1 (deP) versus phospho-Ser65 (P-S65) inhibitor-1 (left) and the PKC-dependent phosphorylation of dephospho- versus phospho-Ser65 (P-S65) inhibitor-1 (right) in vitro. The final reaction stoichiometry is shown for each 90-min reaction as the mean ± S.E. of 4–5 experiments conducted in duplicate. *, p < 0.01, Student’s unpaired t test. B, time course analysis of the Cdk5-dependent phosphorylation of wild-type versus Ser65 → Asp (S65D) inhibitor-1 (left) and the PKC-dependent phosphorylation of wild-type versus Ser65 → Asp (S65D) inhibitor-1 (right) in vitro. The plots represent phosphate incorporation over time. C, phosphorylation of dephospho- versus phospho-Ser65 inhibitor-1 by PKC under linear steady-state conditions in vitro. The panels show SDS-PAGE analysis of 32P-labeled (top) and CBB-stained (bottom) dephospho- and phospho-Ser65 inhibitor-1 from one of four experiments conducted in duplicate. The average initial rate of phosphate incorporation is plotted for each substrate. *, p < 0.001, Student’s unpaired t test.

Conclusion that this residue is the only site of phosphorylation by PKC. The proximity of this PKC site to Ser65 (within two positions in the N-terminal direction) may help explain the adverse effect of the Ser67 → Ala mutation on the ability of PKC to phosphorylate inhibitor-1 at the nearby Ser65 residue.

Analysis of the Effect of Phospho-Ser65 and Phospho-Ser67 on the Ability of Inhibitor-1 to Serve as a Cdk5 and PKC Substrate, Respectively—The proximity of Ser65 to Ser67 and the reduced ability of PKC to phosphorylate Ser65 → Ala inhibitor-1 raise the possibility that Ser65 phosphorylation may affect Ser67 phosphorylation and vice versa. The effect of phospho-Ser65 on the Cdk5-dependent phosphorylation of inhibitor-1 at Ser67 was assessed by evaluating the ability of Cdk5 to phosphorylate inhibitor-1 repurified from preparative in vitro protein phosphorylation reactions conducted in the absence or presence of PKC (Fig. 2A, left). Similarly, PKC was evaluated for its ability to phosphorylate inhibitor-1 from in vitro protein phosphorylation reactions conducted with or without Cdk5 (Fig. 2A, right).

Prior phosphorylation of Ser65 by PKC (0.70 mol/mg) did not have a significant effect on the Cdk5-dependent phosphorylation of inhibitor-1 at Ser67. In contrast, prior phosphorylation of Ser67 by Cdk5 (0.90 mol/mg) resulted in a 91 ± 6% decrease in the maximal stoichiometry achieved in the PKC-dependent phosphorylation of inhibitor-1 at Ser65. Similar results were obtained when Cdk5 and PKC were used to phosphorylate the phosphomimetic inhibitor-1 mutants, Ser65 → Asp and Ser67 → Asp, respectively (Fig. 2B). These findings indicate that

dephospho- and phospho-Ser65 inhibitor-1 are equally efficient substrates for Cdk5, whereas relative to dephosphoinhibitor-1, phospho-Ser65 inhibitor-1 is greatly attenuated in its ability to serve as a PKC substrate. Thus, the generation of diphospho-Ser65/Ser67 inhibitor-1 in vitro requires the sequential phosphorylation of inhibitor-1 first by PKC and then by Cdk5.
have a high degree of specificity for inhibitor-1 singly phosphorylated at Ser<sup>65</sup>.

The antibody for diphospho-Ser<sup>65</sup>/Ser<sup>67</sup> inhibitor-1 selectively detected inhibitor-1 phosphorylated first by PKC and then by Cdk5 (Fig. 3, third panel). Cdk5-dependent phosphorylation alone was not detectable with this antibody. On the other hand, the antibody did detect inhibitor-1 phosphorylated by PKC alone, although 6-fold less efficiently than it detected inhibitor-1 serially phosphorylated by PKC and Cdk5. Hence, this antibody was found to be highly selective for diphospho-Ser<sup>65</sup>/Ser<sup>67</sup> inhibitor-1, with little (phospho-Ser<sup>65</sup>) or no (phospho-Ser<sup>67</sup>) detection of phosphoinhibitor-1 species phosphorylated only at one of these two serine residues.

The previously characterized antibody for phospho-Ser<sup>67</sup> inhibitor-1 was also highly selective for its target (Fig. 3, second panel). It did not detect dephosphoinhibitor-1 or inhibitor-1 phosphorylated by PKC. The apparent ability of the antibody to partially detect inhibitor-1 serially phosphorylated by PKC and Cdk5 was likely attributable to the incomplete phosphorylation of Ser<sup>65</sup> in the diphospho-Ser<sup>65</sup>/Ser<sup>67</sup> inhibitor-1 standard used in this screen. During the generation of this doubly phosphorylated form of inhibitor-1, a final stoichiometry of 0.35 mol/mol was achieved in the preparative phosphorylation reaction with PKC, whereas the subsequent reaction with Cdk5 reached a stoichiometry of 1.0 mol/mol. As a result, a heterogeneous product mixture was produced, containing 35% diphospho-Ser<sup>65</sup>/Ser<sup>67</sup> and 65% phospho-Ser<sup>67</sup> inhibitor-1. The antibody for phospho-Ser<sup>67</sup> inhibitor-1 detected this mixture 63% as efficiently as it detected inhibitor-1 phosphorylated by Cdk5 alone. Therefore, the relative signal intensity of 63% can be explained by the abundance of phospho-Ser<sup>67</sup> inhibitor-1 (65%) in the sample and the inability of the antibody for phospho-Ser<sup>67</sup> inhibitor-1 to detect inhibitor-1 doubly phosphorylated at Ser<sup>65</sup> and Ser<sup>67</sup>. It is reasonable to conclude from these findings that the antibody for phospho-Ser<sup>67</sup> inhibitor-1 is highly specific for inhibitor-1 phosphorylated at Ser<sup>67</sup> and does not detect its target when the nearby Ser<sup>65</sup> residue is also phosphorylated, possibly due to the same steric factors mentioned above for the antibody for phospho-Ser<sup>65</sup> inhibitor-1.

In addition to illustrating the specificity of these antibodies for the forms of inhibitor-1 they were generated to detect, these results further confirm that PKC phosphorylates inhibitor-1 at Ser<sup>65</sup> in vitro.

**Phosphorylation of Inhibitor-1 by Exogenous PKC-α in PC12 Cells**—To determine whether inhibitor-1 can be phosphorylated by PKC in living cells, recombinant inhibitor-1 and a constitutively active form of PKC-α were overexpressed in PC12 rat pheochromocytoma cells using the adenoviral vectors, Ad I-1 (20) and Ad PKC-α (35), respectively. Acute infection of PC12 cells with Ad PKC-α resulted in a 10-fold increase in the expression of PKC-α in these cells (Fig. 4A, top panel). Infection with Ad I-1 led to a 2.5-fold increase in the level of inhibitor-1 protein expression (Fig. 4A, second panel).

No phosphorylation of inhibitor-1 at Ser<sup>65</sup> was detectable with the antibody for phospho-Ser<sup>65</sup> inhibitor-1 in cells infected with Ad I-1 or co-infected with Ad I-1 and Ad PKC-α (Fig. 4A, third panel). However, when the same blot was reprobed with the antibody for diphospho-Ser<sup>65</sup>/Ser<sup>67</sup> inhibitor-1, dual phosphorylation of inhibitor-1 at Ser<sup>65</sup> and Ser<sup>67</sup> was readily detected in cells co-infected with Ad I-1 and Ad PKC-α (Fig. 4A, fourth panel). Basal levels of diphospho-Ser<sup>65</sup>/Ser<sup>67</sup> inhibitor-1 remained undetectable in cells infected with Ad I-1 alone. This result illustrates that PKC-α is able to phosphorylate inhibitor-1 at Ser<sup>65</sup> in living cells and further suggests that this phosphorylation occurs in the context of phosphorylation at the nearby Ser<sup>67</sup> residue.

In these experiments, levels of phospho-Ser<sup>67</sup> inhibitor-1 increased in cells as a result of infection with Ad I-1 but remained unaltered by co-infection with Ad PKC-α (Fig. 4A, fifth panel).

**Regulation of Diphospho-Ser<sup>65</sup>/Ser<sup>67</sup> Inhibitor-1 by a Phorbol Ester and a Specific PKC Inhibitor in N2a Cells and Acute Striatal Slices**—To assess the phosphorylation of inhibitor-1 by endogenous PKC activity in another cell line, N2a mouse neu-
robustoma cells acutely infected with Ad I-1 were treated with the PKC-activating phorbol ester, PDBu, in the absence or presence of the specific PKC inhibitor, Ro-32-0432. Treatment of infected N2a cells with PDBu resulted in an increase in the phosphorylation of inhibitor-1 at Ser65 that was detected by the antibody for diphospho-Ser65/Ser67 inhibitor-1 (Fig. 4B, second panel), but not the antibody for phospho-Ser65 inhibitor-1 (Fig. 4B, first panel). This increase in phosphorylation was reversed by pretreatment of the cells with Ro-32-0432, suggesting that the effect of PDBu is PKC-dependent. Treatment with PDBu or Ro-32-0432 did not alter the levels of phospho-Ser67 inhibitor-1 in these cells (Fig. 4B, third panel). These results demonstrate that activation of endogenous PKCs in living cells results in the phosphorylation of inhibitor-1 at Ser65, also in the context of phosphorylation at Ser67.

Phosphorylation of inhibitor-1 by PKC was next assessed in mouse striatal tissue, where inhibitor-1 is highly expressed. As in PC12 cells infected with Ad PKC-α and N2a cells treated with PDBu, phosphorylation of inhibitor-1 at Ser65 was detectable in PDBu-treated acute striatal slices using the antibody for diphospho-Ser65/Ser67 inhibitor-1 (Fig. 4C), but not the antibody for phospho-Ser65 inhibitor-1 (data not shown). Diphospho-Ser65/Ser67 inhibitor-1 was virtually undetected in striatal slices under basal conditions (Fig. 4C, 0 min and 0 μM PDBu). However, treatment with PDBu resulted in a time-dependent (Fig. 4C, top) and dose-dependent (Fig. 4C, bottom) increase in diphospho-Ser65/Ser67 inhibitor-1 levels in this tissue. The optimal PDBu concentration and incubation time for maximal phosphorylation were defined as 5 μM PDBu for 15 min (n = 4). Pretreatment of striatal slices with the PKC inhibitor, Ro-32-0432, reduced the level of maximal phosphorylation by 88 ± 3% (n = 5) (Fig. 4D, left), indicating that the PDBu-induced increase in diphospho-Ser65/Ser67 inhibitor-1 levels in the striatum is dependent on PKC activity. A similar reduction (73 ± 6%, n = 5) was observed with a known PKC target, Ser831 of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-type glutamate receptor subunit, GluR1 (Fig. 4D, right).

Evaluation of the Mechanism by Which PKC Activation Results in the Detectable Generation of Only Diphospho-Ser65/Ser67 Inhibitor-1 in Vivo—As described above, the activation or overexpression of PKC in vivo results in an increase in the levels of diphospho-Ser65/Ser67 inhibitor-1, with no detectable generation of inhibitor-1 phosphorylated at Ser65 alone. This raises the possibility that either 1) PKC only phosphorylates phospho-Ser67 inhibitor-1 in vivo, or 2) phospho-Ser65 inhibitor-1 does not persist long enough for detection due to the rapid dephosphorylation of this form of inhibitor-1 by Cdk5 at Ser67 and its consequent conversion into diphospho-Ser65/Ser67 inhibitor-1. The first possibility appears unlikely, given the greatly reduced ability of PKC to phosphorylate phospho-Ser67 or Ser67 → Asp inhibitor-1 in vitro (Fig. 2). Therefore, the results are more consistent with a sequence of events in which PKC activation in vivo results in the phosphorylation of diphosphoinhibitor-1 at Ser65, followed by the rapid Cdk5-dependent phosphorylation of phospho-Ser65 inhibitor-1 at Ser67, generating diphospho-Ser65/Ser67 inhibitor-1 in the process and precluding detection of Ser65 phosphorylation by itself. This mechanism predicts that phospho-Ser65 inhibitor-1 may be detected in brain tissue under conditions of reduced Cdk5 activity. In order to capture this potentially transient phosphoinhibitor-1 species in vivo, acute striatal slices were treated with the Cdk5 inhibitor, roscovitine, in the absence or presence of PDBu and assessed for phospho-Ser65 inhibitor-1 levels (Fig. 5A). Pretreatment with roscovitine attenuated the PDBu-induced increase in the levels of diphospho-Ser65/Ser67 inhibitor-1 by 68 ± 2% (p < 0.001, Student’s unpaired t test, n = 4) (Fig. 5A, second panel), confirming that this form of inhibitor-1 is jointly regulated by PKC and Cdk5 activities in vivo. In contrast, roscovitine had no effect on the PDBu-induced increase in the level of phosphorylation of the control PKC substrate, Ser831 of GluR1 (Fig. 5A, bottom panel), indicating that the roscovitine-dependent decrease in the levels of diphospho-Ser65/Ser67 inhibitor-1 in striatal slices treated with PDBu is most likely a result of Cdk5 inhibition and not due to a nonspecific effect of roscovitine on PKC activity. Surprisingly, however, no generation of phospho-Ser65 inhibitor-1 was detected in these striatal tissue samples (Fig. 5A, top panel). These findings suggest that phospho-Ser65 inhibitor-1, conceivably generated as a result of simultaneous PKC activation and Cdk5 inhibition in acute striatal slices, may have nonetheless escaped immunodetection due to rapid dephosphorylation by a basal protein serine/threonine phosphatase activity in vivo.

Regulation of Diphospho-Ser65/Ser67 Inhibitor-1 Levels by Protein Phosphatase Inhibitors in Acute Striatal Slices—The in vitro and in vivo observations described above raise the possibility that phospho-Ser65 inhibitor-1 is a labile inhibitor-1 form that is highly susceptible to dephosphorylation in vivo unless it occurs in the context of phospho-Ser67. Phospho-Ser67 inhibitor-1 is dephosphorylated by PP-2A and PP-2B in the striatum (29). To characterize the dephosphorylation of phospho-Ser65 inhibitor-1 in this tissue, striatal slices were treated with a panel of selective protein serine/threonine phosphatase inhibitors (Fig. 5B). Treatment with inhibitors of PP-1, PP-2A, or PP-2B activity did not result in the generation of detectable levels of phospho-Ser65 inhibitor-1 in acute striatal slices (data not shown). The PP-2A inhibitor, fostriecin, and the PP-2B inhibitor, cyclosporin A, also had no significant effect on diphospho-Ser65/Ser67 inhibitor-1 levels. In striatal slices, okadaic acid inhibits PP-1 and PP-2A differentially in a dose-dependent manner (38). At a concentration of 0.2 μM, it inhibits 80% of PP-2A activity and 5% of PP-1 activity, whereas at 1 μM, it inhibits 95% of PP-2A activity and 35% of PP-1 activity. Okadaic acid (0.2 μM), which predominantly inhibits PP-2A, caused a small, but significant, increase in the levels of diphospho-Ser65/Ser67 inhibitor-1 in striatal slices. On the other hand, treatment with 1 μM okadaic acid resulted in a more substantial increase that was comparable with the maximal effect of PDBu and was (4.6 ± 0.3)-fold larger than the increase caused by the lower dose of okadaic acid. This difference, together with the failure of fostriecin to cause a change in diphospho-Ser65/Ser67 inhibitor-1 levels, suggests that PP-1 is the major protein serine/threonine phosphatase responsible for dephosphorylating phospho-Ser65 inhibitor-1 in the striatum. Treatment with calyculin A, which equally inhibits most PP-1 and PP-2A activity at 1 μM (38), caused an increase in diphospho-Ser65/Ser67 inhibitor-1 levels that was comparable with the increase due to
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A

|           | PDBu | Roscovitine | +   | +   | +   | +   | def-P | P-S65/S67 | P-S65/S67 | Total I-1 | P-S831 GluR1 |
|-----------|------|-------------|-----|-----|-----|-----|-------|-----------|-----------|-----------|-------------|
| control   | -    | -           | -   | +   | +   | +   | -     | -         | -         | -         | -           |
| P-S65 I-1 | -    | -           | -   | -   | -   | +   | -     | +         | +         | +         | -           |
| P-S65/S67 I-1 | -    | -           | -   | -   | +   | +   | -     | +         | +         | +         | -           |
| Total I-1 | -    | -           | -   | -   | +   | +   | -     | +         | +         | +         | -           |
| P-S831 GluR1 | -    | -           | -   | -   | +   | +   | -     | +         | +         | +         | -           |

B

|           | control | PDBu | FST | OKA | 0.2 μM | 1 μM | Ro | CalyA | CalyA | P-S65/S67 I-1 level |
|-----------|---------|------|-----|-----|--------|------|-----|-------|-------|----------------------|
|           | 0.0     | 0.5  | 1.0 | 0.3  | 1.0    | 0.3  | 0.3 | 1.0   | 0.3   | 1.0                  |

C

|           | PP-1 | P-T35 | P-S65 I-1 | P-S65/S67 I-1 |
|-----------|------|-------|------------|---------------|
|           | -    | -     | -          | -             |
|           | -    | -     | +          | +             |
|           | +    | +     | +          | +             |
|           | +    | +     | +          | +             |

FIGURE 5. Functional significance of phospho-Ser67 for the phosphorylation state of inhibitor-1 at Ser65. A, immunoblot analysis of the levels of phospho-Ser65 (P-S65), diphospho-Ser65/Ser67 (P-S65/S67), and total inhibitor-1 and phospho-Ser65 (P-S65) GluR1 in acute striatal slices incubated in the absence or presence of roscovitine (a Cdk5 inhibitor, 50 μM, 60 min) before being treated with or without PDBu (5 μM, 15 min). The recombinant dephosphoinhibitor-1 (dep) and phospho-Ser65 inhibitor-1 (5 ng) standards used in Fig. 3 are also included. The panels show representative results from one of four experiments. B, regulation of diphospho-Ser65/Ser67 inhibitor-1 by protein phosphatase inhibitors in the striatum. Quantification is shown for diphospho-Ser65/Ser67 inhibitor-1 levels in acute striatal slices incubated in the absence or presence of roscovitine (5 μM, 15 min), cyclosporin A (CycA, a PP-2B inhibitor, 2.5 μM, 60 min), fosfostatin (FST, a PP-2A inhibitor, 0.5 μM, 60 min), 0.2 μM okadaic acid (OKA, predominantly a PP-2A inhibitor, 60 min), 1.0 μM okadaic acid (a PP-1 and PP-2A inhibitor, 60 min), calyculin A (CalyA, a PP-1 and PP-2A inhibitor, 1.0 μM, 60 min), and Ro-32-0432 (Ro, a PKC inhibitor, 5 μM, 60 min). Slices co-incubated with Ro-32-0432 and a protein phosphatase inhibitor were pretreated with Ro-32-0432 for 60 min before being incubated for an additional 60 min with okadaic acid (1 μM) or calyculin A in the presence of Ro-32-0432. Data represent means ± S.E. for four experiments. Diphospho-Ser65/Ser67 inhibitor-1 levels are normalized to the mean value obtained from slices treated with PDBu. *, p < 0.01 compared with untreated controls, Student’s unpaired t test. **, p < 0.001 compared with untreated controls, Student’s unpaired t test. †, p < 0.01, Student’s unpaired t test. C, SDS-PAGE analysis and autoradiographic quantification of phospho-Ser65 (left) versus diphospho-Ser65/Ser67 (right) inhibitor-1–32P-labeled at phospho-Ser65 and diphosphorylated by PP-1 in the absence or presence of phospho-Thr35 (P-T35) inhibitor-1 in vitro. Data represent means ± S.E. for four experiments conducted in duplicate. *, p < 0.01 compared with control, Student’s unpaired t test. **, p < 0.001 compared with results with PP-1 and without phospho-Thr35 inhibitor-1, Student’s unpaired t test.

The high dose of okadaic acid, consistent with a role for PP-1 in the dephosphorylation of phospho-Ser65 inhibitor-1. Pretreatment of striatal slices with the PKC inhibitor, Ro-32-0432, attenuated the effects of okadaic acid (1 μM) and calyculin A by 64 ± 2% and 71 ± 3%, respectively, indicating that PKC activity is required for the increase in diphospho-Ser65/Ser67 levels as a result of PP-1 inhibition.

These results suggest that under basal conditions, strong PP-1 activity maintains inhibitor-1 in a dephosphorylated state at Ser65 in vivo. On the other hand, under conditions of PP-1 inhibition, basal PKC activity in the striatum may be sufficiently high to generate phospho-Ser65 inhibitor-1. However, this phosphoinhibitor-1 species appears to be phosphorylated rapidly by Cdk5 to yield diphospho-Ser65/Ser67 inhibitor-1.

Analysis of the Effect of Phospho-Ser67 on the Ability of PP-1 to Dephosphorylate Phospho-Ser65 Inhibitor-1—These findings indicate that phospho-Ser65 inhibitor-1 may be dephosphorylated rapidly by PP-1 in the striatum, whereas diphospho-Ser65/Ser67 inhibitor-1 is resistant to PP-1 activity. The possibility that phospho-Ser67 protects phospho-Ser65 inhibitor-1 from dephosphorylation by PP-1 was tested in vivo (Fig. 5C). Consistent with the in vivo pharmacological findings depicted in Fig. 5B, PP-1 efficiently dephosphorylated 32P-labeled inhibitor-1 preparatively phosphorylated to a stoichiometry of 0.90 mol/mol by PKC at Ser67 (Fig. 5C, left). Moreover, the effect of PP-1 was blocked by the inclusion of phospho-Thr35 inhibitor-1 in the reaction mixture, confirming that this dephosphorylation reaction is PP-1-dependent. In contrast, PP-1 had no significant activity toward diphospho-Ser65/Ser67 inhibitor-1 (Fig. 5C, right), which was similarly 32P-labeled at Ser67 by PKC and subsequently phosphorylated to a stoichiometry of 0.92 mol/mol by Cdk5 at Ser67 in the absence of [γ-32P]ATP. These results suggest that phospho-Ser65 inhibitor-1 is a short lived, transitional inhibitor-1 form that requires rapid conversion into diphospho-Ser65/Ser67 inhibitor-1 by Cdk5 to be protected from PP-1 activity in vivo. Interestingly, cis-acting phospho-Ser67 was sufficient to fully protect phospho-Ser65 from PP-1-dependent dephosphorylation in vitro. As a result, the inclusion of phospho-Thr35 inhibitor-1 in the reactions with diphospho-Ser65/Ser67 inhibitor-1 did not provide phospho-Ser65 any additional protection against PP-1 (Fig. 5C, right), suggesting that once PKC phosphorylates Ser65, subsequent Ser67 phosphorylation by Cdk5 may enable Ser67 to remain phosphorylated regardless of the level of PP-1 activity in the cell.

Analysis of the Effect of Phospho-Ser65 upon the Ability of Inhibitor-1 to Serve as a PP-1 Inhibitor—Unlike phospho-Thr35 inhibitor-1, phospho-Ser65 inhibitor-1 does not serve as an inhibitor of PP-1; however, phosphorylation at Ser65 results in a modest decrease in the ability of inhibitor-1 to become phosphorylated by PKA and get converted into a PP-1 inhibitor (29). To assess the effect of phosphorylation at Ser65 on the ability of inhibitor-1 to inhibit PP-1 or serve as a PKA substrate, we used the phosphomimetic inhibitor-1 mutants, Ser65→Asp, Ser67→Asp, and Ser65/
Ser$^{67} \rightarrow$ Asp, in order to simulate constitutive phosphorylation of inhibitor-1 at Ser$^{65}$ and/or Ser$^{67}$ in vitro.

Wild-type and Ser$^{65}$/Ser$^{67} \rightarrow$ Asp inhibitor-1 from in vitro protein phosphorylation reactions conducted with and without PKA were evaluated for their ability to inhibit PP-1 activity (Fig. 6A). In the absence of phosphorylation at Thr$^{35}$, wild-type or Ser$^{65}$/Ser$^{67} \rightarrow$ Asp inhibitor-1 did not inhibit the activity of PP-1 at any of the concentrations tested (0–1 μM). On the other hand, phospho-Thr$^{35}$ forms of wild-type and Ser$^{65}$/Ser$^{67} \rightarrow$ Asp inhibitor-1 inhibited PP-1 activity with equal potency (IC$_{50}$ = 6.0 nM). Identical results were derived using dephospho- and phospho-Thr$^{35}$ forms of Ser$^{65} \rightarrow$ Asp and Ser$^{67} \rightarrow$ Asp inhibitor-1 (data not shown). These results suggest that phosphorylation of Ser$^{65}$, alone or together with Ser$^{67}$, neither converts inhibitor-1 into a PP-1 inhibitor nor exerts a significant effect on the inhibitor activity of phospho-Thr$^{35}$ inhibitor-1 toward PP-1.

Analysis of the Effect of Phospho-Ser$^{65}$ upon the Ability of Inhibitor-1 to Serve as a PKA Substrate—We next assessed the ability of phosphomimetic inhibitor-1 mutants to serve as a substrate for PKA under linear conditions (Fig. 6B). Wild-type and Ser$^{65} \rightarrow$ Asp inhibitor-1 were phosphorylated by PKA with similar efficiency. However, mutation of Ser$^{65}$ to aspartate resulted in a 42.3 ± 4.1% reduction in the initial rate of inhibitor-1 phosphorylation by PKA. The initial reaction rate was further reduced (55.6 ± 2.5%) by mutation of both Ser$^{65}$ and Ser$^{67}$ to aspartate. Thus, whereas the Ser$^{67} \rightarrow$ Asp mutation by itself was not sufficient to alter the ability of inhibitor-1 to serve as a substrate, it had a small, but significant, inhibitory effect when Ser$^{65}$ was also mutated to aspartate.

To better understand the mechanism by which these phosphomimetic mutations reduce the ability of inhibitor-1 to be phosphorylated by PKA, apparent $K_m$ and $k_{cat}$ values were determined for the PKA-dependent phosphorylation of wild-type and Ser$^{65}$/Ser$^{67} \rightarrow$ Asp inhibitor-1. The mutation of both Ser$^{65}$ and Ser$^{67}$ to aspartate did not alter the apparent $K_m$ of the reaction but decreased the $k_{cat}$ value from 0.45 ± 0.07 to 0.20 ± 0.01 s$^{-1}$ (n = 4), suggesting that phosphomimetic mutation of these serine residues does not alter the binding affinity of inhibitor-1 for PKA but reduces the turnover number, resulting in a significant decrease in catalytic efficiency ($k_{cat}/K_m$) from (7.6 ± 0.2) × 10$^4$ M$^{-1}$ s$^{-1}$ to (3.5 ± 0.1) × 10$^3$ M$^{-1}$ s$^{-1}$ (p < 0.001, Student’s unpaired t test, n = 4) (Fig. 6C).

The possibility that the PKC-dependent phosphorylation of inhibitor-1 at Ser$^{65}$ reduces the ability of inhibitor-1 to serve as a PKA substrate at Thr$^{35}$ was evaluated in vivo by treatment of striatal slices with the adenylyl cyclase activator, forskolin, following incubation in the absence or presence of PDBu. Pretreatment with PDBu attenuated the increase in the levels of phospho-Thr$^{35}$ inhibitor-1 in striatal slices treated with forskolin (Fig. 6D, left). In contrast, PDBu had no significant effect on the elevation of phospho-Thr$^{35}$ DARPP-32 levels resulting from forskolin treatment of the same slices (Fig. 6D, right), suggesting that the activation of PKC in striatal slices specifically alters the ability of inhibitor-1 to be phosphorylated by PKA.

Based on the results described above, this probably occurs through the PKC- and Cdk5-dependent generation of diphospho-Ser$^{65}$/Ser$^{67}$ inhibitor-1, an inhibitor-1 form not readily phosphorylated by PKA.
Inhibitor-1 Phosphorylation by PKC

![Diagram](Image)

**FIGURE 7. Effect of metabotropic versus ionotropic glutamate receptor activation on diphospho-Ser65/Ser67 inhibitor-1 levels in the striatum.** A, quantitative immunoblot analysis of diphospho-Ser65/Ser67 (P-S65/S67) inhibitor-1 levels in striatal slices incubated in the absence or presence of DHPG (a group I metabotropic glutamate receptor agonist, 50 μM, 5 min), following treatment with calcineurin A (a PK-1 inhibitor, 0.5 μM, 30 min). *, p < 0.01. Student’s unpaired t test, n = 4. B, quantitative immunoblot analysis of diphospho-Ser65/Ser67 inhibitor-1 levels in acute striatal slices treated with or without PDBu (5 μM, 15 min), NMDA (100 μM, 5 min), or cyclosporin A (CycA, a PP-2B inhibitor, 2.5 μM, 60 min). **, p < 0.001 compared with results with PDBu. Student’s unpaired t test, n = 4. **, p < 0.01 compared with results with PDBu and NMDA. Student’s unpaired t test, n = 4.

from the substantia nigra, accounts for the majority of afferent inputs to the striatum (39). To determine whether the PKC-dependent phosphorylation of inhibitor-1 in this structure is regulated by Gα1-coupled glutamate receptors, diphospho-Ser65/Ser67 inhibitor-1 levels were assessed in acute striatal slices treated with DHPG, a group I metabotropic glutamate receptor agonist. DHPG treatment alone had no effect on the levels of diphospho-Ser65/Ser67 inhibitor-1 (data not shown). As discussed above, diphospho-Ser65/Ser67 inhibitor-1 levels are virtually undetectable in unstimulated striatal slices (Fig. 4C). This is probably a result of tonic PP-1 activity at Ser65. To improve the probability of detecting the regulation of this site by group I metabotropic glutamate receptors, acute striatal slices were briefly incubated with a suboptimal dose of the PP-1 inhibitor, calyculin A. Subsequent treatment with DHPG resulted in a 1.7 ± 0.2-fold increase in the levels of diphospho-Ser65/Ser67 inhibitor-1 relative to slices treated with calyculin A alone (Fig. 7A), suggesting that under conditions of reduced PP-1 activity, the phosphorylation state of inhibitor-1 at Ser65 may be regulated by glutamate signaling through Gα1-coupled glutamate receptors in the striatum.

It has been shown that the activation of NMDA-type glutamate receptors reduces phospho-Ser67 inhibitor-1 levels in the striatum and may down-regulate global Cdk5 activity (29, 40). Thus, like the Cdk5 inhibitor, roscovitine, NMDA may be expected to attenuate the PDBu-induced increase in diphospho-Ser65/Ser67 inhibitor-1 levels in striatal tissue. To assess the possible role of this class of ionotropic glutamate receptors in the regulation of diphospho-Ser65/Ser67 inhibitor-1 in the striatum, acute striatal slices were treated with PDBu, NMDA, and/or cyclosporin A and assessed for levels of diphospho-Ser65/Ser67 inhibitor-1 (Fig. 7B). As expected, PDBu caused an increase in diphospho-Ser65/Ser67 inhibitor-1 relative to untreated controls (Fig. 7B, first and second columns). Whereas NMDA had no effect in the absence of PDBu (data not shown), co-incubation of striatal slices with PDBu and NMDA attenuated the PDBu-dependent increase in diphospho-Ser65/Ser67 inhibitor-1 levels by 81 ± 3% (Fig. 7B, third column). In this regard, NMDA was more potent than roscovitine, which caused a 68 ± 2% decrease in diphospho-Ser65/Ser67 inhibitor-1 levels in striatal slices co-treated with PDBu (Fig. 7B, second panel).

Phospho-Ser65 inhibitor-1 is dephosphorylated by PP-1 in vitro and in intact striatal tissue (Fig. 5, B and C). In contrast, phospho-Ser67 inhibitor-1 is dephosphorylated by PP-2A and PP-2B, with the contribution from PP-2B activity increasing under conditions that elevate intracellular Ca2+ (29), such as NMDA-type glutamate receptor activation (29). To determine whether the dephosphorylation of phospho-Ser67 by PP-2B mediates part of the NMDA-induced decrease in diphospho-Ser65/Ser67 inhibitor-1 levels in striatal tissue treated with PDBu, striatal slices were pretreated with the PP-2B inhibitor, cyclosporin A, before further incubation in the absence or presence of PDBu and NMDA. Consistent with the results shown in Fig. 5B, cyclosporin A had no effect on diphospho-Ser65/Ser67 levels in the absence of PDBu (data not shown). However, treatment with cyclosporin A partially reversed the effect of NMDA in PDBu-treated tissue samples, increasing diphospho-Ser65/Ser67 inhibitor-1 levels 2.1 ± 0.4-fold relative to striatal slices treated with PDBu and NMDA in the absence of cyclosporin A (Fig. 7B, third and fourth columns). These results suggest that the increased dephosphorylation of phospho-Ser67 by PP-2B is partly responsible for the NMDA-induced decrease in diphospho-Ser65/Ser67 inhibitor-1 levels in striatal slices treated with PDBu.

The recovery with cyclosporin A was incomplete and represented a net decrease of 60 ± 8% relative to the levels of diphospho-Ser65/Ser67 inhibitor-1 achieved with PDBu treatment alone (Fig. 7B, second and fourth columns). Interestingly, this value is comparable with the roscovitine-induced reduction of 68 ± 2% in striatal diphospho-Ser65/Ser67 inhibitor-1 levels (Fig. 5A, second panel). These observations indicate that mechanisms in addition to PP-2B activation, such as an NMDA-induced decrease in tissue Cdk5 activity (40), may mediate a substantial portion of the NMDA effect on diphospho-Ser65/Ser67 inhibitor-1 levels in the striatum, where NMDA-dependent signaling may activate PP-2B and down-regulate Cdk5 at the same time.

Finally, the effects of PDBu and cyclosporin A on the levels of diphospho-Ser65/Ser67 inhibitor-1 were additive in the absence of NMDA (133 ± 8% of PDBu alone) (Fig. 7B, second and fifth columns), suggesting that PP-2B inhibition may enhance PKC-mediated increases in diphospho-Ser65/Ser67 inhibitor-1 levels by allowing more Cdk5-dependent phosphorylation of phospho-Ser65 inhibitor-1. The ability of the PP-2B inhibitor, cyclosporin A, to both potentiate the effect of PDBu and attenuate the effect of NMDA on diphospho-Ser65/Ser67 inhibitor-1 levels suggests that PP-2B can readily dephosphorylate phospho-Ser67 of diphospho-Ser65/Ser67 inhibitor-1. Thus, in contrast to the complete block imposed by phospho-Ser67 on the dephosphorylation of phospho-Ser65 by PP-1, phospho-Ser65 does not protect phospho-Ser67 from PP-2B-dependent dephosphorylation in vivo.
DISCUSSION

We report here that Ser\(^{65}\) of protein phosphatase inhibitor-1 is phosphorylated by PKC in vitro, in cultured cells, and in striatal brain tissue. To monitor inhibitor-1 phosphorylation by PKC, we generated antibodies that detect inhibitor-1 when it is either singly phosphorylated at Ser\(^{65}\) or doubly phosphorylated at Ser\(^{65}\) and Ser\(^{67}\). Interestingly, only the diphospho-form was detectable in vivo. Pharmacological studies showed that phospho-Ser\(^{65}\) inhibitor-1 is probably dephosphorylated by PP-1 in the striatum. Furthermore, in vitro protein phosphorylation and dephosphorylation assays indicated that Ser\(^{67}\) phosphorylation probably follows Ser\(^{65}\) phosphorylation in vivo and protects phospho-Ser\(^{65}\) from dephosphorylation by PP-1. Consistent with our earlier studies of Cdk5-dependent phosphorylation in this region of inhibitor-1 (29) and its homolog, DARPP-32 (27), in vitro PP-1 inhibition assays showed that phosphorylation of Ser\(^{65}\) and/or Ser\(^{67}\) probably has no effect on the inhibitory activity of phospho-Thr\(^{35}\) inhibitor-1. Further in vitro experiments suggested that phosphorylation of Ser\(^{65}\), either alone or together with Ser\(^{67}\), reduces the efficiency of inhibitor-1 phosphorylation by PKA at Thr\(^{35}\). This effect was also observed in intact striatal tissue, where PKC activation specifically attenuated the increase in phospho-Thr\(^{35}\) inhibitor-1 levels in response to the activation of adenylyl cyclase. Thus, the ability of inhibitor-1 to be converted into a PP-1 inhibitor may be regulated by the PKC-dependent phosphorylation of Ser\(^{65}\).

Previously, we reported that the Cdk5-dependent phosphorylation of inhibitor-1 at Ser\(^{65}\) has a modest negative effect on the ability of PKA to phosphorylate Thr\(^{35}\) of inhibitor-1 (29). This was in contrast to the inhibitor-1 homolog, DARPP-32, in which the Cdk5-dependent phosphorylation of Thr\(^{175}\) results in a complete block of DARPP-32 phosphorylation by PKA at the homologous Thr\(^{234}\) residue (27). This functional difference between inhibitor-1 and DARPP-32 with respect to Cdk5-dependent phosphorylation left the significance of inhibitor-1 phosphorylation at Ser\(^{67}\) somewhat unclear. The present study shows that phospho-Ser\(^{65}\) and diphospho-Ser\(^{65}/Ser^{67}\)-mimetic inhibitor-1 mutations have a much greater effect on the PKA-dependent phosphorylation of Thr\(^{35}\) than the phospho-Ser\(^{65}\)-mimetic mutation alone. As stated previously, phospho-Ser\(^{65}\) inhibitor-1 occurs only in the context of phospho-Ser\(^{67}\) in cultured cells and intact brain tissue. We present in vitro and in vivo evidence that in the absence of subsequent Ser\(^{67}\) phosphorylation, the PKC-dependent phosphorylation of inhibitor-1 at Ser\(^{65}\) is probably subject to rapid reversal by PP-1 activity in striatal neurons. Thus, one function of phospho-Ser\(^{65}\) may be to stabilize the PKC-dependent phosphorylation of inhibitor-1 at Ser\(^{65}\), a pathway that counters the activation of inhibitor-1 by PKA in the striatum.

Pretreatment with low concentrations of a pharmacological PP-1 inhibitor revealed the up-regulation of diphospho-Ser\(^{65}/Ser^{67}\) inhibitor-1 levels in striatal tissue in response to the activation of group I metabotropic glutamate receptors. Therefore, under physiological conditions in the striatum, the generation of phospho-Ser\(^{65}\) inhibitor-1 that can serve as a substrate for Cdk5 at Ser\(^{67}\) may require reduced PP-1 activity, as may occur in the presence of high phospho-Thr\(^{35}\) inhibitor-1 levels. Once diphospho-Ser\(^{65}/Ser^{67}\) inhibitor-1 is generated by the sequential actions of PKC and Cdk5, the need for reduced PP-1 activity is eliminated by phospho-Ser\(^{67}\), which serves as a potent cis-acting PP-1 blocker. This intramolecular context, protecting its PP-1-sensitive partner, phospho-Ser\(^{65}\), through a mechanism that does not rely on the actual inhibition of PP-1 activity by a bona fide PP-1 inhibitor like phospho-Thr\(^{35}\) inhibitor-1. Thus, under initial conditions of PP-1 inhibition and basal levels of Cdk5 activity, PKC activation by G\(_{o}\)-coupled guanine receptors in the striatum may restrict the further inhibition of PP-1 by limiting the availability of diphosphoinhibitor-1 that can serve as a PKA substrate at Thr\(^{35}\).

Taken together, our findings support a model in which inhibitor-1 pools readily phosphorylated by PKA are differentially regulated by competing signaling events arising from the activation of group I metabotropic and NMDA-type ionotropic glutamate receptors in the striatum (Fig. 8). PKA activation due to signaling through G\(_{o}\)-coupled receptors, such as dopamine D$_{1}$ and adenosine A$_{2A}$ receptors in striatal neurons, results in the phosphorylation of inhibitor-1 at Thr\(^{35}\) and the inhibition of PP-1. (For simplicity, only dopamine D$_{1}$ receptors are included in the schematic.) The activation of group I metabotropic glutamate receptors under these conditions limits the further PKA-dependent conversion of inhibitor-1 into a PP-1 inhibitor through the PKC-dependent phosphorylation of Ser\(^{65}\). Phospho-Ser\(^{65}\) inhibitor-1 is subsequently phosphorylated at Ser\(^{67}\) by Cdk5, generating diphospho-Ser\(^{65}/Ser^{67}\) inhibitor-1, which is resistant to PP-1 and not readily phosphorylated by PKA. An initial estimate in acute striatal slices treated with PDBu places the maximal in vivo stoichiometry of diphospho-Ser\(^{65}/Ser^{67}\) inhibitor-1 at 0.58 mol/mol (data not shown), which is nearly twice the basal in vivo stoichiometry of 0.34 mol/mol reported for phospho-Ser\(^{67}\) inhibitor-1 in this tissue (29). Together, these two forms of inhibitor-1 (phospho-Ser\(^{67}\) and diphospho-Ser\(^{65}/Ser^{67}\)) may therefore represent the majority of all inhibitor-1 species in the striatum under conditions of high PKC activity. Thus, PKC activation in the striatum has the potential to result in the conversion of almost all available inhibitor-1 pools into forms not readily targeted by PKA.

Our model further posits that these actions of PKC and Cdk5 are reversed by NMDA-type ionotropic glutamate receptor...
activation. NMDA-mediated signaling reduces Ser\textsuperscript{67} phosphorylation (29, 40), possibly through a combination of PP-2B activation and Cdk5 down-regulation. These effects leave phospho-Ser\textsuperscript{65} susceptible to dephosphorylation by PP-1, resulting in reduced diphospho-Ser\textsuperscript{65}/Ser\textsuperscript{67} inhibitor-1 levels and increasing the availability of inhibitor-1 that can be activated as a PP-1 inhibitor through the phosphorylation of Thr\textsuperscript{25} by PKA. This novel signaling mechanism, involving the first endogenous inhibitor found to regulate protein phosphatase activity \textit{in vivo}, exemplifies the diversity of protein phosphatase regulation pathways arising from the differential modulation of protein phosphatase regulatory subunits.

Opposing actions of PKA and PKC have been reported to regulate a number of physiological processes in excitable tissues, including cardiac contraction, cellular tolerance to ethanol, synaptic transmission, neurite outgrowth, and Hebbian synaptic plasticity (41–45). These cases of opposite regulation often involve the phosphorylation of distinct serine/threonine residues by PKA and PKC on a single type of effector molecule, such as nicotinic acetylcholine receptors at the neuromuscular junction (46–48) and \gamma-aminobutyric acid type A receptors (49, 50) and NMDA-type glutamate receptors in the central nervous system (51). Recently, we showed that PKA and PKC conversely regulate inhibitor-1 function in cardiac muscle, where PKC-dependent phosphorylation of inhibitor-1 may render it unable to associate with, and therefore inhibit, PP-1 (30). Here we demonstrate the differential regulation of inhibitor-1 by PKA and PKC in the brain, where inhibitor-1 may be less readily activated by PKA to become a PP-1 inhibitor if it is already phosphorylated by PKC. Both of these PKC-mediated mechanisms of inhibitor-1 regulation would be predicted to result in the net disinhibition of PP-1 activity and may represent synergistic components of the same general process by which PKA and PKC oppositely regulate PP-1 activity through inhibitor-1 phosphorylation.

Inhibitor-1 is the second PP-1 inhibitor shown to be under the opposing influence of PKA and PKC activities, after the recently discovered gut and brain phosphatase inhibitor (GBPI) (52). GBPI belongs to a growing family of PKC-activated PP-1 inhibitors (53), but unlike other members of this family, it is also regulated by PKA. Interestingly, the PKA-dependent phosphorylation of GBPI reverses the activating effect of phosphorylation by PKC. Based on the present study, inhibitor-1 regulation by these two protein kinases represents the converse scenario involving the PKA-mediated activation and PKC-mediated inactivation of a PP-1 inhibitor. Thus, inhibitor-1 and GBPI may be well positioned biochemically to play an integrative role at junctions between the PKA- and PKC-mediated signaling pathways.

Counterregulation of PP-1 inhibitors by PKA and PKC is not a universal property of these small, heat-stable molecules. Like inhibitor-1, DARPP-32 is activated by PKA in the striatum to become a potent PP-1 inhibitor, but in our studies, it was not directly phosphorylated by PKC \textit{in vitro} (data not shown). Consistent with this result, PKC activation failed to attenuate the PKA-dependent phosphorylation of DARPP-32 in striatal slices. These observations suggest a role for PKC in regulating access by PKA to inhibitor-1 but not to other PP-1 inhibitors, such as DARPP-32, further demonstrating the complexity of the network of signaling cascades regulating PP-1 activity \textit{in vivo}.

In the present study, we focused on the regulation of inhibitor-1 by PKC in the striatum, where inhibitor-1 has been implicated in the mediation of cocaine reward (54). We also observed PKC-dependent phosphorylation of inhibitor-1 in acute hippocampal slices (data not shown). Inhibitor-1 is expressed in certain regions of the hippocampus (22). Moreover, several of the demonstrable functions of inhibitor-1 in the central nervous system are associated with this brain region (16, 18, 55). With the notable exception of the liver in some species, inhibitor-1 is also expressed to various levels in many peripheral tissues, such as heart, kidney, and skeletal muscle (9, 17). As noted previously, in the heart, levels of inhibitor-1 expression and phosphorylation regulate cardiac contractility and predisposition to heart failure (19, 20, 23, 30). It will be interesting to learn how PKC-dependent phosphorylation affects the function of inhibitor-1 in brain regions like the cortex and hippocampus and peripheral tissues outside cardiac muscle.

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