Protein Phosphatase 2A Is Associated with Class C L-type Calcium Channels (Ca\textsubscript{\textit{a}},1.2) and Antagonizes Channel Phosphorylation by cAMP-dependent Protein Kinase*

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Phosphorylation by cAMP-dependent protein kinase (PKA) regulates a vast number of cellular functions. An important target for PKA in brain and heart is the class C L-type Ca\textsuperscript{2+} channel (Ca\textsubscript{\textit{a}},1.2). PKA phosphorylates serine 1928 in the central, pore-forming α\textsubscript{1C} subunit of this channel. Regulation of channel activity by PKA requires a proper balance between phosphorylation and dephosphorylation. For fast and specific signaling, PKA is recruited to this channel by an protein kinase A anchor protein (Davare, M. A., Dong, F., Rubin, C. S., and Hell, J. W. (1999) \textit{J. Biol. Chem.} 274, 30280–30287). A phosphatase may be associated with the channel to effectively balance serine 1928 phosphorylation by channel-bound PKA. Dephosphorylation of this site is mediated by a serine/threonine phosphatase that is inhibited by okadaic acid and microcystin. We show that immunoprecipitation of the channel complex from rat brain results in coprecipitation of PP2A. Stoichiometric analysis indicates that about 80% of the channel complexes contain PP2A. PP2A directly and stably binds to the C-terminal 557 amino acids of α\textsubscript{1C}. This interaction does not depend on serine 1928 phosphorylation and is not altered by PP2A catalytic site inhibitors. These results indicate that the PP2A-α\textsubscript{1C} interaction constitutively recruits PP2A to the channel complex rather than being a transient substrate-catalytic site interaction. Functional assays with the immunoisolated class C channel complex showed that channel-associated PP2A effectively reverses serine 1928 phosphorylation by endogenous PKA. Our findings demonstrate that both PKA and PP2A are integral components of the class C L-type Ca\textsuperscript{2+} channel that determine the phosphorylation level of serine 1928 and thereby channel activity.

Voltage-gated Ca\textsuperscript{2+} channels consist of several subunits including α\textsubscript{1}, α\textsubscript{2}δ, and β (1). The central subunit is α\textsubscript{1}, which constitutes the ion-conducting pore (1). Based on pharmacological and physiological criteria, high-threshold voltage-gated Ca\textsuperscript{2+} channels are classified as L-type and non-L type channels. Class C (Ca\textsubscript{\textit{a}},1.2) and class D (Ca\textsubscript{\textit{a}},1.3) Ca\textsuperscript{2+} channels, containing α\textsubscript{1C} and α\textsubscript{1D}, respectively, constitute most neuronal L-type channels in the brain (2). Ca\textsuperscript{2+} influx through L-type channels is involved in regulation of membrane excitability, synaptic plasticity, and gene expression (3–7). In the heart, Ca\textsuperscript{2+} influx through the class C channel is the critical first step that triggers myocardial contraction.

A crucial signaling pathway that regulates the heart beat is β-adrenergic stimulation which results in PKA\textsuperscript{1}-mediated phosphorylation of this channel (8), thereby increasing the channel activity (9, 10). Only α\textsubscript{1C} is required for stimulation of channel activity by PKA (11), although phosphorylation of the β subunit also contributes to the up-regulation of channel activity by PKA (12). PKA phosphorylates α\textsubscript{1C} on serine 1928 \textit{in vitro} and \textit{in vivo} (8, 13–15). Mutation of serine 1928 to alanine eliminates PKA-mediated phosphorylation of α\textsubscript{1C} and inhibits up-regulation of the channel activity (16). This site is only present in the full-length, 220-kDa form of α\textsubscript{1C}. It is deleted by calpain-mediated proteolytic cleavage upon Ca\textsuperscript{2+} influx through NMDA-type glutamate receptors in neurons, which results in the 180-kDa short form of α\textsubscript{1C} (17, 18). C-terminal truncation increases the activity of this channel about 4-fold (19). This modification is permanent in contrast to that by PKA-mediated phosphorylation, which is readily reversed by protein phosphatases. The phosphatase inhibitor okadaic acid increased the PKA-stimulated activity of class C channels ectopically expressed in Chinese hamster ovary cells (11). In addition, forskolin did not elevate phosphorylation of α\textsubscript{1C} ectopically expressed in HEK293 cells unless cells were preincubated with phosphatase inhibitors (16).

Protein kinase A anchor proteins or AKAPs target PKA to various substrates (20–22) including class C channels (Ca\textsubscript{\textit{a}},1.2) (15). Disruption of PKA binding to AKAPs prevents PKA-mediated regulation of AMPA-type glutamate receptors (23) and skeletal muscle L-type channels (Ca\textsubscript{\textit{a}},1.1) (24). Phosphorylation of α\textsubscript{1C} by PKA is observed when wt AKAP79 but not when a PKA binding-deficient mutant of AKAP79 is co-expressed with the class C channel in HEK293 cells (16). Furthermore, PKA is associated with α\textsubscript{1C} in the brain and this interaction may be mediated by the microtubule-associated protein MAP2B (15), the first AKAP to be recognized as such (25). Collectively, these data indicate that PKA anchoring at α\textsubscript{1C} is essential for efficient phosphorylation of the channel. Previous studies suggest

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†The abbreviations used are: PKA, cAMP-dependent protein kinase; AKAP, protein kinase A anchor protein; AMPA, \textit{N}-methyl-D-aspartate-4-protonic acid; MAP, microtubule-associated protein; NMDA, \textit{N}-methyl-D-aspartate; PKC, protein kinase C; GST, glutathione S-transferase; wt, wild type; aa, amino acid(s); HA, hemagglutinin; PKI, AMP-dependent protein kinase inhibitor.
that phosphorylation and dephosphorylation of α1C counteract each other in a dynamic way (11, 26). We hypothesized that a phophatase has to be localized at or near the channel for effective reversal of channel phosphorylation. In fact, run-down of class C channel activity in inside-out patches excised from ventricular myocytes was greatly slowed by okadaic acid (26). These results indicate that a phosphatase was attached to the patch, possibly through a direct interaction with the channel complex. We found that the serine/threonine phosphatase PP2A is associated with the class C L-type channel, reversing phosphorylation of α1C by PKA.

**EXPERIMENTAL PROCEDURES**

**Materials**—The PKA inhibitory peptide PTKI (5–24) was a gift from Dr. L. M. Graves (University of North Carolina, Chapel Hill, NC). The ECL detection kit and protein G-Sepharose were purchased from Amersham Pharmacia Biotech, microcystin LR and okadaic acid from Calbiochem (San Diego, CA), and purified catalytic subunit of PKA and protein A-Sepharose from Sigma. [3H]PN200-100 (2948 GBq/mmol) was from PerkinElmer Life Sciences and digitonin from Gallard-Schlesinger (Carle Place, NY). Other reagents were obtained from commercial suppliers and were of standard biochemical quality.

**Expression of Class C Channels in HEK293 Cells**—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum and antibiotics. Cells were transfected with plasmid DNA using Lipofectamine (Gibco BRL, Gaithersburg, MD) according to the manufacturer’s instructions.

**Expression of Class C Channels in HEK293 Cells**—HEK293 cells were transfected with plasmid DNA using Lipofectamine according to the manufacturer’s instructions.

**Production of GST and Poly-His Fusion Proteins and in Vitro Binding Assays—α1C, and PP2A/C-encoding cDNA templates (31, 35) were amplified by PCR with oligonucleotides containing engineered endonuclease restriction sites for subcloning in frame with a 5’ nucleotide sequence encoding glutathione S-transferase (GST) or a polyhistidine tag and T7 epitope by standard methods (36). Forward primers (containing a BamHI and a SalI site) and reverse primers (containing an EcoRI site), respectively, were used for the following α1C fusion proteins: AGT GGA TCC GTC AAT GAA AAG AGG AGG ATG and AGC TAG TCG TAA CAA (loop II; aa 754–901), AGT GGA TCC ATG GTC ACC TGT CAC GAG TGT CAT G and AGC TAG TCG TAA CAA (loop III; aa 1165–1219), AGT GGA TCC GAT GTC ACC TGT CAT G and AGC TAG TCG TAA CAC TCT TGT GCT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’ GC GGA TCC GC TCT TGT G (for HA tag) and 5’.
and okadaic acid (2 nM) were added. Samples were incubated for 2.5 or 40 min as before, washed with radiomunounassay buffer, extracted with SDS sample buffer, and analyzed by immunoblotting with anti-CH1923–1932P and subsequently with anti-α1C. For phosphorylation of GST-CT1584–2140, this fusion protein was immobilized on glutathione-Sepharose and incubated in phosphorylation buffer containing 0.5–1 μg of the purified catalytic subunit of PKA and 50 μM ATP. After washing with 150 mM NaCl, 10 mM Tris-Cl, pH 7.4, it was used in parallel with unphosphorylated GST-CT1584–2140 for pull-down experiments of PP2A out of brain cytosol.

RESULTS

Okadaic Acid and Microcystin Inhibit Dephosphorylation of α1C in HEK293 Cells and Brain Extract—An HEK293 cell line stably expressing the wt β2-adrenergic receptor (34) was used for transient expression of class C channels (Ca,1.2) consisting of α1C, α2δ, and β2δ. Cells were co-transfected with AKAP75, the bovine homolog of human AKAP79 (20). Like class C channels (18), this AKAP may be present at postsynaptic sites (40) and had been used to reconstitute PKA-mediated phosphorylation of α1C in HEK293 cells (16). For the following experiments we used anti-α1C, an antibody against an epitope in the central part of α1C (14, 17), and anti-CH1923–1932P, an antibody that specifically recognizes α1C when phosphorylated at serine 1928 (15). Serine 1928 is the main if not exclusively phosphorylation site for PKA on α1C. Phosphorylation of this site was determined after immunoprecipitation of class C channels with anti-α1C by immunoblotting with anti-CH1923–1932P (15). Treatment of the cultures with 100 nM okadaic acid significantly increased the level of serine 1928 phosphorylation (Fig. 1A). The extent of this increase was comparable to that induced by stimulation of the β2-adrenergic receptor with 10 μM isoproterenol. These data suggest that phosphorylation by PKA and dephosphorylation by an okadaic acid-sensitive phosphatase of α1C are in a dynamic equilibrium that is influenced by inhibition of the phosphatase or stimulation of PKA.

Okadaic inhibits the serine/threonine phosphatases PP2A (IC50: 0.1 nM), PP1 (IC50: 10 nM) and also, though much less potently, PP2B (calcinerein; IC50: < 5 μM) (41, 42). Microcystin inhibits these phosphatases with similar potencies (43-45). Immunoblotting with anti-CH1923–1932P shows that serine 1928 is completely dephosphorylated when α1C is immunosolated from Triton X-100 extracts of rat brain membranes lacking phosphatase inhibitors; however, if microcystin is present, phosphorylation is preserved (Fig. 1B). This result demonstrates that a microcystin-sensitive phosphatase in the brain extract is responsible for the dephosphorylation of α1C. Therefore, we investigated whether PP1, PP2A, or, although less likely, PP2B is associated with the class C channel.

PP2A Is Associated with the Class C Channel—Immunoprecipitation of this channel from Triton X-100 extracts of rat cerebral cortex resulted in specific coprecipitation of PP2A (Fig. 2A) but not of PP1 or PP2B (Fig. 2, B and C). The specificity of this coprecipitation is indicated by the absence of any PP2A immunoreactivity in precipitations with non-immune antibody and in AMPA-type glutamate receptor complexes isolated with antibodies against the GluR1 subunit of this receptor. PP2A is a heterotrimeric holoenzyme that exists in multiple forms composed of a core structure, which interacts with regulatory B subunits. Usually the core enzyme complex consists of the 36-kDa catalytic C subunit (PP2A/C) and the 65-kDa scaffolding A subunit (PP2A/A) and interacts with a B subunit, although the C subunit has recently been found in complexes lacking an A or B subunit (46). PP2A/C and PP2A/A are ubiquitously expressed; each subunit is encoded by two highly related genes (97% and 87% identity, respectively) (47). Both

![Image](367x457 to 495x729)

Fig. 1. Okadaic acid and microcystin inhibit dephosphorylation of serine 1928 of α1C, the class C channel subunits α1C, α2δ, and β2δ, and AKAP75 were transiently expressed in HEK293 cells stably transfected with the wt β2-adrenergic receptor (34). Cells were treated with control medium or medium containing 100 nM okadaic acid (OA) or 10 μM isoproterenol (ISO) for 5 min before lysis, immunoprecipitation (IP) with anti-α1C, and immunoblotting with anti-CH1923–1932P and subsequently anti-α1C. Immunosignals for anti-CH1923–1932P and α1C long form were quantified by densitometry and the former signals corrected for differences in relative amounts of α1C long and short form, the latter of which is missing the serine 1928 phosphorylation site. Cont., control.

To determine the stoichiometry of the class C channel-PP2A interaction, channels were labeled with saturating amounts of the tritiated form of the L-type-specific ligand isradipine, [3H]PN200-110. After solubilization with digitonin, which affords purification of L-type channels with the ligand bound, and immunoprecipitation of class C channels, the amount of channel in each immunoprecipitate was determined by scintillation counting (see “Experimental Procedures” for more details). An equal portion of each sample was analyzed by immunoblotting with the antibody against PP2A/C. Defined amounts of affinity-purified GST-PP2A/C were loaded onto the same gel to provide a standard curve for quantification of the PP2A/C immunosignal. Our quantitative analysis indicates that, on average, 423 fmol of class C channel were specifically immunoprecipitated with the anti-α1C antibody and that 337 fmol of PP2A/C coprecipitated. Accordingly, 79.7 ± 11.1% (average ± S.E. of three experiments) of the class C channel complexes contain PP2A/C. Because GST-PP2A/C was produced using human cDNA as a template and immunoprecipitations were performed from rat brain, it is important to emphasize that the PP2A/C antibody was made against a sequence (resides 153–
were employed for immunoprecipitations of class C channel complexes (anti-a1C), AMPA-type glutamate receptor complexes (anti-GluR1), and nonspecific control IgG from rabbit. Immunoblotting with antibodies against PP2A/A and C (nonspecific control IgG from rabbit. Immunoblotting with antibodies against GST (Fig. 3A) but not with those against the γ isofrom of PP1 (B) or PP2B (C) yielded positive results showing the specific coprecipitation of these two PP2A subunits with a1C. Molecular mass markers are indicated on the right (in kDa). IP, immunoprecipitation; Cont., control.

**Fig. 2. Co-immunoprecipitation of PP2A/A and PP2A/C with class C channels.** Triton X-100 extracts of brain membrane fractions were employed for immunoprecipitations of class C channel complexes (anti-a1C), AMPA-type glutamate receptor complexes (anti-GluR1), and nonspecific control IgG from rabbit. Immunoblotting with antibodies against PP2A/A and C (A) but not with those against the γ isofrom of PP1 (B) or PP2B (C) yielded positive results showing the specific coprecipitation of these two PP2A subunits with a1C. Molecular mass markers are indicated on the right (in kDa). IP, immunoprecipitation; Cont., control.

309) that is 100% conserved in the human α and β and rat α and β isoforms and, therefore, permits a direct comparison of all the PP2A/C immunosignals on a quantitative level.

**PP2A/C Binds Directly to the C-Terminal Region of a1C**

Voltage-gated Ca\(^{2+}\) channels consist of multiple subunits, and various interaction partner proteins have been identified (1, 48). For example, a1C binds MAP2B (15). MAP2B is an AKAP (25) and may recruit PKA to the channel complex (15). Therefore, we investigated whether PP2A can directly associate with a1C or whether this association might require an adapter protein. a1C consists of four domains, which are homologous to each other. The loops between these domains as well as the N and C terminus are intracellular (Fig. 3A) and available for interaction with intracellular proteins (1, 48). GST fusion proteins covering these domains were immobilized on glutathione-Sepharose and incubated with cytosolic rat brain extracts as a source of native PP2A, PP2A/A and PP2A/C associated exclusively with the fusion protein that carries the C-terminal 557 residues of a1C (Fig. 3A). Loading of the glutathione-Sepharose was comparable for all fusion proteins, as indicated by immunoblotting with antibodies against GST (Fig. 3B).

Because a protein in the cytosolic brain extract could mediate such an interaction, we expressed the last 557 residues of a1C as poly-His-tagged fusion protein and full-length PP2A/C as a GST fusion protein in two different versions (one contained as additional N-terminal tag an HA epitope). The GST-PP2A/C fusion proteins were adsorbed onto glutathione-Sepharose and the resins incubated with lysates of bacterial cultures expressing the poly-His-tagged C terminus of a1C. The C terminus effectively bound to both PP2A/C fusion proteins but not GST

(Fig. 3C, top panel), indicating that PP2A/C can directly attach to the C terminus of a1C. Equal amounts of the two PP2A/C fusion proteins and of GST itself, the negative control, were present on the glutathione resins (Fig. 3C, bottom panel). To further corroborate that the PP2A/C-a1C interaction is direct, GST-PP2A/C and, as a negative control, GST was affinity-purified on glutathione-Sepharose and eluted with glutathione. In parallel, poly-His-tagged C terminus, N terminus, and loop II of a1C were purified on a nickel resin. GST-PP2A/C and GST were added to the poly-His fusion proteins, which were left on the affinity resins to allow subsequent washing steps. The resulting interaction of poly-His-C terminus with GST-PP2A/C was detected by immunoblotting with an antibody against GST (Fig. 3D, left panel). It was specific with respect to the C terminus because GST-PP2A/C did not bind to the other two poly-His fusion proteins and with respect to GST-PP2A/C because GST by itself did not interact with any of the three poly-His fusion proteins including the C terminus of a1C. The presence of similar quantities of GST and GST-PP2A/C in these experiments was indicated by direct immunoblotting of an aliquot of each of these two proteins (Fig. 3D, right panel). The amount of poly-His-C terminus in these experiments was somewhat smaller than that of the other two poly-His fusion proteins, as determined in parallel by immunoblotting with an antibody against the T7 epitope that is part of the N-terminal poly-His tag expressed from pTrcHis vectors (Fig. 3E). The use of quantitatively more control poly-His-N terminus and loop II than poly-His-C terminus in these experiments further bolsters the argument for a specific interaction of PP2A/C with the C terminus.

**PP2A/C Binding to a1C Is Not Driven by a Catalytic Site Interaction—**A typical enzyme-substrate interaction is short-lived and ends with the usually fast release of the product. In contrast, the PP2A-a1C association is stable arguing that this association recruits PP2A more permanently to the channel complex and has, therefore, the character of an adapter function for PP2A rather than that of a transient catalytic interaction. Furthermore, PP2A/C directly binds to the C-terminal GST fusion protein of a1C, which is not phosphorylated at serine 1928 unless pretreated with PKA and ATP (Fig. 4A, upper panel). Because the dephosphorylated form constitutes a phosphatase product, which would be expected to be released from the catalytic site of PP2A, we would not at all expect that PP2A binding to the non-phosphorylated C terminus of a1C would be strong and long-lasting if the interaction were purely catalytic. In contrast, the pull-down assays of PP2A with the unphosphorylated C-terminal fusion proteins suggest a constitutive interaction (Figs. 3A and 4A). Phosphorylation of the fusion protein with PKA on serine 1928 did not at all increase PP2A binding (Fig. 4A, middle and bottom panels; compare lanes 2 and 4). Such an increase in PP2A binding would have been expected if PP2A would only interact with this region in a catalytic manner.

We also evaluated the effect of microcystin on the PP2A-a1C interaction. The crystal structure of the microcystin-PP1 complex shows that microcystin binding overlaps with the catalytic site of PP1, and evidence exists that indicates that the same is true for PP2A (45, 49). Inclusion of microcystin did not at all alter binding of cytosolic PP2A to the C-terminal a1C fusion protein (Fig. 4A, middle and bottom panels; compare lanes 2 and 3). In addition, coprecipitation of PP2A with the class C channel from Triton X-100 brain extracts was not reduced by the presence of microcystin (Fig. 4B), although microcystin has full access to the channel-associated PP2A, as indicated by its ability to block dephosphorylation of serine 1928 (Fig. 1B). Collectively, these data indicate that the PP2A-a1C association is not merely a catalytic site interaction. It is rather stable and...
allows the constitutive recruitment of PP2A to the channel complex.

Association of a B′ Subunit with the PP2A-α1C Complex in a Heterologous Expression System—The PP2A A-C core complex usually associates with one subunit of a heterogeneous class of regulatory B subunits. More than twenty B subunits are now known, many of which are expressed in a tissue and cell-specific manner. B subunits have been divided into the B, B′, and B″ classes, although some of the more recently identified B subunits do not belong to any of these three classes (32, 50–52). These subunits are thought to be involved in determining the substrate specificity of the PP2A holoenzyme (50, 53, 54). As a first attempt to identify whether a B subunit might be present in the α1C-associated PP2A complex, class C channel immunoprecipitates were analyzed by immunoblotting with antibodies against various B type B subunits including Bo, B3I, and Bγ, with negative results (data not shown).

Because expression of B′γ (B568 in Ref. 51) determined by Northern analysis is most prominent in the brain (32), we investigated whether this B subunit may associate with the channel complex. An HA-tagged form of B′γ (32) was ectopically co-expressed with class C channels in HEK293 cells. Immunoprecipitation of α1C resulted in specific coprecipitation of Bγ (Fig. 5A). The anti-HA immunosignal associated with the channel was comparable to that obtained with 5% of the input. Accordingly, about 5% of the total amount of HA-tagged B′γ subunit is associated with α1C. A similar estimate is obtained by comparing the anti-HA signal in the channel complex with that resulting from immunoprecipitation of HA-tagged B′γ with the anti-HA antibody. The HA-B′γ immunosignal on the blot obtained after immunoprecipitation with anti-HA is more than 10-fold higher than that in the 5% input lane, showing that the anti-HA antibody precipitate most of the HA-tagged B′γ (Fig. 5A).

To get an impression of how much PP2A/C subunit relative to B′γ might be present in the channel complex, HA-tagged PP2A/C and untagged PP2A/A was coexpressed with the channel in HEK293 cells. Although overexpression of the PP2A A and C subunits cannot usually be up-regulated by more than 1.5–2-fold because PP2A is toxic at higher levels (31, 55, 56), the presence of the HA tag in PP2A/C affords immunoprecipitation of HA-PP2A with high efficacy as shown above for HA-B′γ (see Fig. 5A). Immunoprecipitation of α1C resulted in a weak immunosignal for the PP2A/C HA tag, which was 1% or less of the HA tag signal obtained by immunoprecipitation of HA-PP2A/C with the anti-HA antibody (Fig. 5B). Despite the low level of the immunoreactivity in the channel precipitation, the signal was clearly specific because immunoprecipitation with non-immune control antibody from the same cell extracts did not result in any HA immunosignal. Furthermore, the HA signal was also absent when the ectopically expressed channel was immunosolated from HEK293 cells, which had been co-transfected with green fluorescent protein instead of HA-PP2A/C. Similar results were obtained when PP2A/A levels in
tion buffer and used for immunoprecipitation (IP). GST fusion proteins of the αC C terminus (C-term; aa 1584–2140) and (as negative control) N terminus were adsorbed onto glutathione-Sepharose. Some samples were preincubated with PKA under phosphorylation conditions before the pull-down assay resulting in phosphorylation of serine 1928 as shown by immunoblotting with anti-CH1923–1932P (upper panel, lane 4). Resin samples were incubated with brain cytosol supplemented with 1 mM microcystin where indicated (Mc, lane 3) before immunoblotting with antibodies against PP2A/A and C (middle and lower panels). IP, immunoprecipitations with anti-αC, was performed from Triton X-100 membrane extracts, followed by immunoblotting with anti-αC (upper panel) and anti-PP2A/C. The presence of 2 μM microcystin during the brain extraction and immunoprecipitation did not reduce PP2A/C binding to αC (right lane).

**FIG. 4.** PP2A binding to αC is not Influenced by the phosphorylation state of serine 1928 or by microcystin. A, GST fusion proteins of the αC C terminus (C-term; aa 1584–2140) and (as negative control) N terminus were adsorbed onto glutathione-Sepharose. Some samples were preincubated with PKA under phosphorylation conditions before the pull-down assay resulting in phosphorylation of serine 1928 as shown by immunoblotting with anti-CH1923–1932P (upper panel, lane 4). Resin samples were incubated with brain cytosol supplemented with 1 mM microcystin where indicated (Mc, lane 3) before immunoblotting with antibodies against PP2A/A and C (middle and lower panels). B, immunoprecipitations with anti-αC, was performed from Triton X-100 membrane extracts, followed by immunoblotting with anti-αC (upper panel) and anti-PP2A/C. The presence of 2 μM microcystin during the brain extraction and immunoprecipitation did not reduce PP2A/C binding to αC (right lane).

**FIG. 5.** Class 3 channels interact with PP2A C, A, and B′γ B subunit in HEK293 cells. Parental HEK293 cells were transfected with expression constructs encoding the class C channel subunits αC, αC, αC, or βB, and either PP2A/B′γ (A), or green fluorescent protein (GFP) as a negative control, or a combination of PP2A/A and HA-tagged PP2A/C subunits (B and C) as given at the bottom of each panel. Cells were extracted 24 h after transfection with 1% Triton X-100 solubilization buffer and used for immunoprecipitation (IP) with antibodies against αC, the HA tag of recombinant PP2A/B′γ or PP2A/C, or PP2A/A or with nonspecific control IgG from rabbit as indicated on top of each panel. The positions of the HA-tagged B′γ (A) and C (B) subunits and of the untagged A subunit (C) are indicated by arrowheads on the right side together with the position of the light chain (LC) of the immunoprecipitating mouse anti-HA tag antibody in B.

immunoprecipitates with antibodies against αC, and PP2A/A were compared; only a small portion of PP2A/A was associated with the channel compared with the amount precipitated with the anti-PP2A/A antibody (Fig. 5C).

**Channel-associated PP2A Antagonizes Phosphorylation of αC by PKA**—To evaluate the physiological relevance of the constitutive interaction of PP2A with the class C channel, we tested whether channel-associated PP2A can effectively dephosphorylate serine 1928. Class C channels were solubilized and immunoprecipitated in the absence of phosphatase inhibitors, which may block PP2A activity in the subsequent steps. The channel immunocomplex containing PKA (15) was incubated for 15 min with ATP and cAMP. Under these conditions the channel-associated PKA phosphorylates serine 1928 to near completion (15). Further phosphorylation was then stopped by adding the PKI (5–24) peptide, which effectively blocks the channel-associated PKA (15). In parallel Mn2+ was added to increase the phosphatase activity (57, 58). Dephosphorylation was not very effective under these conditions (Fig. 6A). However, when protamine was added, substantial dephosphorylation occurred within 2–3 min (Fig. 6A). Protamine is a polyamine that selectively activates PP2A (58–61). Although protamine is selectively expressed in sperm cells, it has been speculated that it may be able to mimic endogenous as yet unidentified PP2A activating factors. Our results indicate that PP2A stably bound to the class C channel effectively dephosphorylates serine 1928 after its activation. Protamine-stimulated dephosphorylation of serine 1928 was highly sensitive to okadaic acid. This toxin blocked the dephosphorylation at a concentration of 1 nM over an extended incubation time of 40 min (Fig. 6B). Because PP2A/A and C are coprecipitating with αC in both control (Fig. 6A) and because only PP2A (and its close relatives PP4 and PP5) are blocked by low concentrations of okadaic acid (41, 42, 45, 54), these functional studies argue that the phosphatase that dephosphorylates serine 1928 is PP2A.

**DISCUSSION**

Our results indicate that about 80% of the class C channel (Ca1.2) in rat forebrain are associated with PP2A. The rest of the channel complexes that lacked PP2A may represent an intracellular channel pool that had not yet bound PP2A. It is also possible that some PP2A dissociated from the channel during the whole immunoprecipitation procedure. The channel interacts with a PP2A holoenzyme that consists of the A and C subunit and likely a B′ type B subunit. A comparison of the PP2A/A and PP2A/C immunosignals present in αC immuno-
precipitates from brain with those from samples of the load suggest that 1% or less of total PP2A might be associated with α1C in brain (Fig. 2A). This result is not unexpected because PP2A is an abundant ubiquitous enzyme with multiple substrates, α1C being only one of many targets. We were not able to detect a specific B subunit in the channel complex isolated from rat brain due, in part, to a lack of availability of antibodies with high affinity for most B subunit types. However, in HEK293 cells, the coexpressed HA-tagged B′γ subunit interacted with the channel. Of note, a significantly larger portion of the total B′ than of the PP2A/A and C pool coprecipitated with the channel, probably as much as 5% of the total amount of B′γ present in the cell lysates. The fact that a larger fraction of the pool of ectopically expressed B′γ than of the ectopically expressed PP2A/C and PP2A/A pool is associated with the channel suggests that the PP2A holoenzyme containing B′γ targets only a subset of all PP2A substrates in these cells. It has been hypothesized that B subunits regulate targeting and substrate specificity of the trimeric PP2A holoenzyme (54).

Overexpression in mammalian cell lines can promote the interaction of proteins, which are usually not associated with each other in vivo. However, ectopic expression of PP2A/A and C does not increase their level of interaction with α1C as compared with brain; it appears that a significantly smaller portion of the total PP2A/A and C pool coprecipitated with α1C from HEK293 cells than from brain (compare the ratios of PP2A immunosignals of load or PP2A immunoprecipitates versus α1C immunoprecipitates of Figs. 2A and 5 (B and C)). These results validate our experimental approach of co-expression of B′γ as a first step in demonstrating that this or a structurally related B′ type subunit may be the B subunit present in the class C channel complex in brain. This notion is further supported by the observation that a significantly larger fraction of the B′γ pool is associated with α1C than of overexpressed PP2A/A or PP2A/C. Because B′ type subunits are very similar to each other (amino acid identity is usually higher than 50–60% (Refs. 32 and 51)), it is quite possible that another B′ type subunit is associated with class C channels in vivo but that the overexpression promoted the interaction with B′γ. It is important to note that of the B′ type subunits described to data, B′γ is most abundantly expressed in brain. However, confirmation of the presence of B′γ or another B′ type subunit has to await the production of antibodies with higher affinity that can specifically identify B′γ or its isoform in channel immunoprecipitates.

PKA has to be anchored by AKAPs in close proximity to substrates such as AMPA-type glutamate receptors (23) and L-type channels (16, 24) for their efficient phosphorylation. Some AKAPs can directly bind to these PKA targets. Examples include the interaction of MAP2B with the class C channel (15), of AKAP79/150 and AKAP250 with the β2-adrenergic receptor (62, 63), and of AKAP350/ytiao with the NMDA receptor (64). Phosphorylation is only an efficient way for regulating the activity of ion channels or enzymes if it is counteracted by dephosphorylation. Because PKA is stably bound to the class C channel (15), we hypothesized that a phosphatase may also have to be constitutively associated with the channel to effectively balance PKA-mediated phosphorylation. We show that the phosphatase PP2A not only forms a stable complex with α1C but also reverses its phosphorylation by PKA.

It has been known for a while that AKAP79/150 can act as a scaffolding molecule for PKA, protein kinase C, and PP2B/calcineurin, suggesting that kinase-phosphatase complexes are anchored next to their target proteins to ensure fast phosphorylation and dephosphorylation (22). However, only a few examples have been described, which demonstrate that anchoring of phosphatases in close proximity to their targets is as important for dephosphorylation as kinase anchoring is for phosphorylation. These include docking of PP1 by spinophilin near AMPA-type glutamate receptors (65) and by AKAP250/ytiao at NMDA-type glutamate receptors (64) and binding of PP2A to the β2-adrenergic receptor (60), and Ca2+− and calmodulin-dependent kinase IV (66).

The functional relevance of an anchored okadaic acid-sensitive phosphatase regulating the class C channel activity had already been suggested by earlier experiments in heart tissue, where the class C channel induces and regulates myocardial contraction. Okadaic acid inhibited run-down of channel activity in inside-out patches of ventricular myocytes (26). These results suggest that a phosphatase was attached to the patch at or near the channel complex. Class C channels exist in three different modes (67). They are not available for activation in mode 0, show brief openings in mode 1, and long-lasting openings and brief closings in mode 2 (67). Transition from mode 0 to mode 1 or 2 is induced by the β-adrenergic receptor-PKA signaling pathway (10). Okadaic acid inhibited while application of PP2A/C to excised inside-out membrane patches promoted the reversal of mode 2 and 1 in cardiac and smooth muscle cells (68–70). These electrophysiological findings together with our biochemical evidence demonstrating that PP2A reverses phosphorylation of serine 1928 in α1C, raise the possibility that phosphorylation of this site by PKA and its dephosphorylation by PP2A is involved in controlling the transition between mode 1 and 2. Switching from mode 1 to mode 0 is less sensitive to okadaic acid but more susceptible to inhibition by calcineurin A than the transition from mode 2 to mode 1 (68, 69). Calcinulin A blocks PKP with higher potency than PP2A. These results indicate that PP1 may play a larger role in reversing mode 1 to mode 0 than PP2A. PKA phosphorylates not only serine 1928 in α1C but also serine 478 and serine 479 in the β2a isoform of the Ca2+-channel β subunit (12). Phosphorylation of serine 478 and serine 479 in β2a contributes to up-regulation of the channel activity by PKA (12). We do not know whether phosphorylation of serine 1928 in α1C, or serine 478 or 479 in the β2a subunit are at all regulating the transitions between modes 0, 1, and 2 and whether PKP dephosphorylates the latter two sites. However, it is tempting to speculate that phosphorylation of serine 478 or 479 in β2a is involved in the transition from mode 0 to mode 1 and that these sites are dephosphorylated by PP1, whereas phosphorylation of serine 1928 in α1C, which is dephosphorylated by PP2A, may play a role in the transition from mode 1 to mode 2. In any case, our findings indicate that precise localization PP2A and regulation of its activity in brain and heart is likely to be crucial for the correct physiological function of class C L-type Ca2+ channels.

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