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A Protein Phosphatase 2cα–Ca^{2+} Channel Complex for Dephosphorylation of Neuronal Ca^{2+} Channels Phosphorylated by Protein Kinase C

Dongjun Li, Fushun Wang, Meizan Lai, Yuan Chen, and Ji-fang Zhang
Department of Physiology, Jefferson Medical College, Philadelphia, Pennsylvania 19107

Phosphorylation and dephosphorylation are primary means for rapid regulation of a variety of neuronal functions, such as membrane excitability, neurotransmitter release, and gene expression. Voltage-gated Ca^{2+} channels are targets for phosphorylation by a variety of second messengers through activation of different types of protein kinases (PKs). Protein phosphatases (PPs), like PKs, are equally important in regulating Ca^{2+} channels in neurons. However, much less is understood about whether and how a particular type of PP contributes to regulating neuronal Ca^{2+} channel activities. This is primarily because of the lack of specific inhibitors/activators for different types of PPs, particularly the PP2c family. The functional roles of PP2c and its substrates in the brain remain virtually unknown. During our yeast two-hybrid screening, PP2cα was pulled out by both N- and P/Q-type Ca^{2+} channel C termini. This raised the possibility that PP2cα might be associated with voltage-gated Ca^{2+} channels for regulation of the Ca^{2+} channel activity. Biochemical studies show that PP2cα binds directly to neuronal Ca^{2+} channels forming a functional protein complex *in vivo*. PP2cα, unlike PP1, PP2a and PP2b, is more effective in dephosphorylation of neuronal Ca^{2+} channels after their phosphorylation by PKC. In hippocampal neurons, disruption of the PP2cα–Ca^{2+} channel interaction significantly enhances the response of Ca^{2+} channels to modulation by PKC. Thus, the PP2cα–Ca^{2+} channel complex is responsible for rapid dephosphorylation of Ca^{2+} channels and may contribute to regulation of synaptic transmission in neurons.

Key words: **voltage-gated calcium channels; modulation; protein kinases; protein phosphatases; signal transduction; synaptic transmission**

Introduction

Phosphorylation and dephosphorylation are critical for the rapid regulation of a variety of neuronal functions, such as membrane excitability, neurotransmitter release, and gene expression (Catterall, 1998; Vaughan et al., 1998; Levitan, 1999; Dempsey et al., 2000). Voltage-gated Ca^{2+} channels (VGCCs) are targets for phosphorylation by second messengers through activation of different types of protein kinases (PKs), such as PKA and PKC (Hille, 1994; Dolphin, 1995; Catterall, 1997; Ikeda and Dunlap, 1999; Schiff et al., 2000). In neurons, many types of PKs and protein phosphatases (PPs) have been identified (Hille, 1994; Dolphin, 1995; Catterall, 1997; Cohen, 1997; Ikeda and Dunlap, 1999; Price and Mumby, 1999; Herzig and Neumann, 2000). Studies show that individual kinases are capable of mediating unique cellular functions (Pawson and Scott, 1997; Pawson and Nash, 2000, 2003). Formation of macromolecular signaling complexes is one of the mechanisms for targeting specific PKs to their selective targets (Pawson and Scott, 1997; Pawson and Nash, 2000, 2003; Bauman and Scott, 2002; Michel and Scott, 2002). As we have shown recently, PKCε, but not other PKCs, is brought to and modulate its specific target, N-type Ca^{2+} channels, through an adaptor protein, enigma homolog (ENH) (Maeno-Hikichi et al., 2003). One important advantage of PKCε regulation of Ca^{2+} channels is that PKCε activation is independent of Ca^{2+}, unlike conventional PKCs, such as PKCα (Tanaka and Nishizuka, 1994). Thus, modulation of the Ca^{2+} channel activity by PKCε is not affected by Ca^{2+} influx through VGCCs and can be precisely controlled by the second messenger pathways that activate PKCε.

Multiple families of serine/threonine (Ser/Thr) protein phosphatases have been identified in the brain, including PP1, PP2a, PP2b, and PP2c families (Cohen, 1997; Price and Mumby, 1999; Herzig and Neumann, 2000). Like PKs, PPs play an important role in regulating ion channel activities. However, in contrast to PKs, much less is understood about the particular type of PP involved in regulating Ca^{2+} channel activities in neurons. This is primarily because of the lack of specific inhibitors/activators for different types of PPs (Cohen, 1997; Price and Mumby, 1999; Herzig and Neumann, 2000). For instance, no specific inhibitors/activators are available for phosphatases of the PP2c family. Consequently, the functional roles of PP2cs in the brain remain vir-

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Correspondence should be addressed to Ji-fang Zhang, Department of Physiology, Jefferson Medical College, 1020 Locust Street, Philadelphia, PA 19107. E-mail: Ji-fang.Zhang@jefferson.edu.

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tually unknown. Only a few neuronal substrates for PP2cs have been identified, among which are CaMKII (calcium/calmodulin-dependent protein kinase II), mGluR3 (metabotropic glutamate receptor subtype 3) and DARPP-32 (dopamine- and cAMP-regulated phosphoprotein of 32 kDa) (Fukunaga et al., 1993; Desdouits et al., 1998; Flajolet et al., 2003). However, these results are based primarily on *in vitro* biochemical assays and have not yet been confirmed *in vivo*. Mechanisms for regulation of the PP2c activity and for targeting PP2c to its specific neuronal substrate or substrates remain unknown.

We show here, using biochemical assays, that PP2cα is a partner protein for VGCCs. *In vitro* dephosphorylation assays show that PP2cα, compared with PP1, PP2a, and PP2b, is more effective in dephosphorylation of VGCCs after their phosphorylation by PKCε. Finally, overexpression of a dominant-negative construct selectively disrupts the PP2cα–Ca^{2+} channel interaction and enhances the regulatory effects of phorbol dibutyrate (PDBu), a phorbol ester derivative, on the Ca^{2+} channel activity in neurons. Our results demonstrate that VGCCs are functional substrates for PP2cα in neurons and that formation of the PP2cα–Ca^{2+} channel complex allows rapid dephosphorylation of VGCCs after their phosphorylation by PKC.

Materials and Methods

Yeast two-hybrid screening

Details of screening have been published previously (Chen et al., 2003; Maeno-Hikichi et al., 2003). The C terminus of α_{1A} (CaV2.1, amino acid residues 1819–2422; GenBank accession number X57477), α_{1B} (CaV2.2, 1707–2339; GenBank accession number M94172), or α_{1C} (CaV1.2, 1505–2171; GenBank accession number X15539) subunit was used as bait to screen a rat brain cDNA library (OriGene, Rockville, MD). We screened $\sim 7 \times 10^6$ to 1×10^8 independent clones for each bait. A total of >200 clones were sequenced out of ~2000 positive clones.

Construction and expression of recombinant proteins

Reverse transcription-PCR was used to generate the full-length PP2cα (GenBank accession number J04503). PCR-based methods were used to generate all of the constructs used in this study. All constructs were verified by sequencing. To generate recombinant proteins, cDNA constructs were subcloned into pGEX-4T-1 (Amersham Biosciences, Arlington Heights, IL) for glutathione *S*-transferase (GST)-fused proteins or pQE-30 (Qiagen, Hilden, Germany) for polyhistidine-tagged (6×His) proteins. GST-fusion proteins and 6×His proteins were produced in *Escherichia coli*. Glutathione agarose beads (Pierce, Rockford, IL) were used to purify GST-fused proteins. Ni-NTA columns (Qiagen) were used for purification of 6×His proteins. To express the recombinant proteins in mammalian cells, cDNA constructs were subcloned into the following vectors: pCDNA3 (Invitrogen, San Diego, CA), HA-tagged pcDNA3, FLAG-tagged pcDNA3, or pEGFP-C2 (Clontech, Palo Alto, CA). The calcium phosphate method was used for transfection in human embryonic kidney 293 (HEK 293) cells or hippocampal neurons.

GST-fusion protein pulldown assays

Pulldown assays were performed as described previously (Chen et al., 2003; Maeno-Hikichi et al., 2003). Briefly, ^{35}S-labeled proteins were synthesized using an *in vitro* protein translation kit (Promega, Madison, WI). The assay buffer contained 100 mM NaCl, 20 mM Tris HCl, and 5% glycerol, pH 7.0, along with 1% Triton X-100 and a mixture of protease inhibitors (1 μM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 0.1 mg/ml benzamidine). Approximately 5 μg of GST-fused protein was incubated with 2 μl of ^{35}S-labeled protein with gentle rocking. The length of incubation varied from 2 h to overnight. All pulldown assays were performed at 4°C. The results were analyzed by SDS-PAGE and autoradiography. Nonspecific binding was insignificant.

In vitro *phosphorylation and dephosphorylation assays*

In vitro *phosphorylation.* 6×His-tagged Ca^{2+} channel I–II and II–III loops (α_{1B}, amino acid residues 357–463 for the I–II loop and amino acid residues 719–952 for the II–III loop) and 6×His-tagged myristoylated alanine-rich C-kinase substrate (MARCKS) were purified and quantified by the Bradford method. The purified proteins (2 μM) were phosphorylated by PKCε (0.01 μM; Oxford, Concord, MA) at 30°C for 10 min, in the buffer containing 50 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM DTT along with 0.05 mg/ml diacylglycerol, 0.5 mg/ml L-α-phosphatidylserine (Sigma, St. Louis, MO), 12.5 μM ATP, and 0.1–0.5 μCi [γ-^{32}P]ATP (Amersham Biosciences) for each reaction. The reaction was stopped by heat inactivation of PKCε (60°C for 10 min; our unpublished data).

In vitro *dephosphorylation.* The phosphorylated protein was aliquoted, in equal amounts, in a set of microtubes. Dephosphorylation was initiated by adding different PPs (0.02 μM), and the reaction was terminated at various time points by boiling in the SDS gel-loading buffer. For PP2b, Ca^{2+} (1 mM) and calmodulin (1 μM) were added for its activation. PP2cα was purified in the laboratory, and the others were purchased from Promega (PP2a and PP2b) and New England Biolabs (Beverly, MA) (PP1). All experiments were performed at 30°C. The results were analyzed by SDS-PAGE and quantified by a phosphoimager.

Coimmunoprecipitation and immunoblotting

Coimmunoprecipitation of PP2cα and calcium channels was performed using adult rat cortical extracts as described previously (Chen et al., 2003; Maeno-Hikichi et al., 2003). Briefly, the soluble membrane fraction of adult rat cortical extracts was incubated with anti-α_{1B} antibody (Alomone Laboratories, Jerusalem, Israel)-conjugated protein G-Sepharose beads overnight at 4°C. The immunoprecipitation buffer contained (in mM): 137 NaCl, 2.7 KCl, 4.3 Na_2HPO_4, 1.4 KH_2PO_4, 5 EGTA, and 5 EDTA, pH 7.5. Immune complexes were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies against PP2cα (Upstate Biotechnology, Lake Placid, NY) and α_{1B}.

Hippocampal neuron culture

Use of animals received University Institutional Animal Care and Use Committee approval. Briefly, hippocampal neurons were cultured from embryonic day 18 (E18) to E19 embryonic rats (Sprague Dawley). The neurobasal medium was supplemented with 10% fetal bovine serum and B-27 and supplied with 5% CO_2. Typically, cultured neurons were transfected with different cDNA constructs at 10 d *in vitro* (DIV), and physiology experiments were performed at 12 DIV.

Immunocytochemistry

Hippocampal neurons were fixed with 4% paraformaldehyde and 4% sucrose in PBS for 10 min, permeabilized with 0.25% Triton X-100 in PBS for 5 min, and quenched with 0.1 M glycine in PBS. An anti-synapsin I antibody (1:200; Oncogene Sciences, Uniondale, NY) was used for immunostaining. An Alexa 568-conjugated secondary antibody (1:4000; Molecular Probes, Eugene, OR) was used for visualization. Fluorescent images were collected with a Zeiss (Oberkochen, Germany) Axiovert 200 inverted microscope equipped with a Hamamatsu (Hamamatsu City, Japan) CCD camera.

Electrophysiology recordings

Details for electrophysiological recordings have been published previously (Chen et al., 2003; Maeno-Hikichi et al., 2003). Briefly, whole-cell recordings were performed using an Axopatch 200B amplifier. pClamp 8.0 software (Axon Instruments, Foster City, CA) was used for data acquisition and analysis. Leak currents and capacitive transients were subtracted on-line with a P/4 protocol. The bath solution was composed of the following (in mM): 128 NaCl, 5 KCl, 10 $BaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 TEA-Cl, and 10 glucose, pH 7.3. Tetrodotoxin (1 μM; Alomone Laboratories) and nifedipine (1 μM; Calbiochem, La Jolla, CA) were added in the bath solution to block Na^+ and L-type Ca^{2+} channels. Under this condition, the primary Ca^{2+} current component should be of N- and P/Q-type with a very small percentage of R-type Ca^{2+} current (Zhang et al., 1993). The recording pipette was filled with a solution containing the following (in mM): 110 CsCl, 5 $MgSO_4$, 10 EGTA, 25 HEPES, 2 ATP, and 0.2 GTP, pH 7.2. Current traces were digitized at 10 kHz and low-pass filtered at 1 kHz.

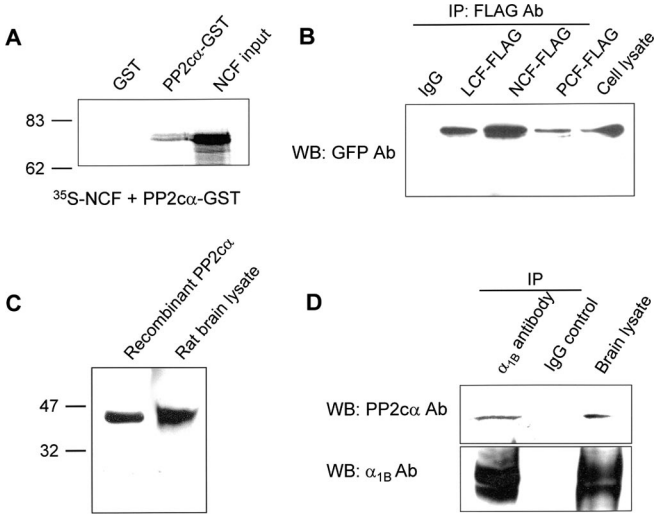

Figure 1. Association of PP2cα with neuronal voltage-gated Ca^{2+} channels. ***A***, GST-fusion protein pulldown assays. Experiments were performed in the presence of 1 mM EGTA. ***B***, PP2cα interacts with all three types of Ca^{2+} channels. GFP-tagged PP2cα and FLAG-tagged channel C termini were coexpressed in HEK 293 cells. CoIP assays were performed 2 d after transfection. ***C***, Presence of PP2cα in the brain. Recombinant PP2cα (in pcDNA3) was expressed in HEK 293 cells. The calculated molecular mass for PP2cα is 42.2 kDa. ***D***, Formation of the PP2cα–Ca^{2+} channel complex *in vivo*. Brain lysates were immunoprecipitated with an anti-α_{1B} antibody, and blots were detected with antibodies against PP2cα and N-type Ca^{2+} channels (α_{1B}). WB, Western blot.

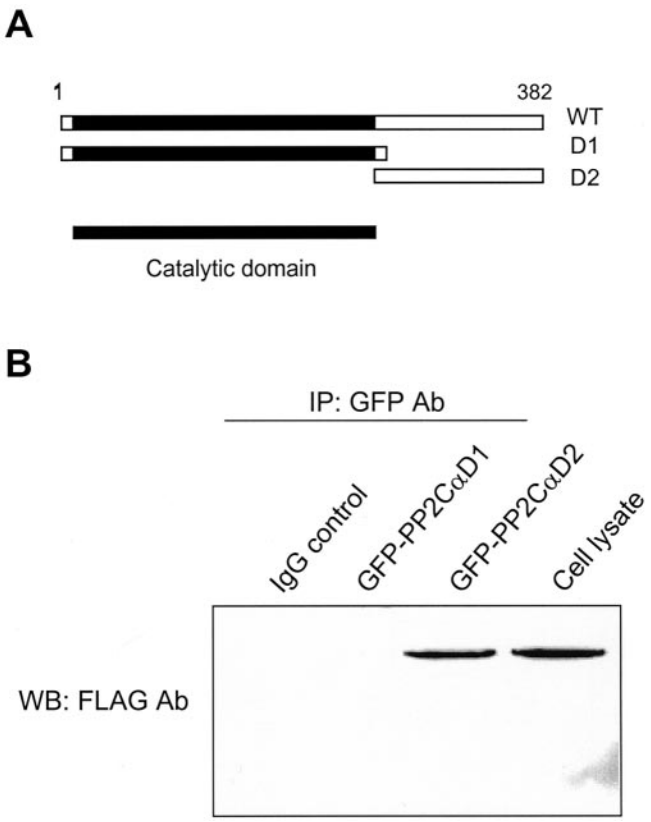

Figure 2. Identification of the Ca^{2+} channel binding domain in PP2cα. ***A***, Schematic representation of the wild-type PP2cα and its deletion mutants. The catalytic domain is from amino acid residues 22–284. D1 covers amino acid residues 1–291, and D2 covers 283–382. ***B***, Effects of deletion mutations on the interaction between PP2cα and the N-type Ca^{2+} channel C terminus.

Results

Formation of a PP2cα–Ca^{2+} channel complex

To identify proteins that might interact with Ca^{2+} channels and play a role in Ca^{2+}-dependent signal transduction and/or regulating the Ca^{2+} channel activity, we performed yeast two-hybrid screening of a rat brain cDNA library using as bait the full-length C termini of three different Ca^{2+} channel α_1 subunits, α_{1A} (CaV2.1, P/Q-type), α_{1B} (CaV2.2, N-type), and α_{1C} (CaV1.2, L-type) (Chen et al., 2003; Maeno-Hikichi et al., 2003). One clone, pulled out by both the N- and P/Q-type Ca^{2+} channel C termini (NCF and PCF), encoded a partial sequence of PP2cα, a member of PP2c family (Price and Mumby, 1999; Herzig and Neumann, 2000; Sim et al., 2003). Of the 200 plus clones we have sequenced, PP2cα is the only phosphatase identified during screening (our unpublished data). The interaction between PP2cα and the channel C termini was rigorously evaluated by several different approaches. In yeast, the interaction was established by both *LacZ* reporter gene assay and galactose-dependent growth in leucine-deficient medium. Binding of PP2cα to NCF was verified by GST-fusion protein pulldown assays (Fig. 1*A*). The interaction was further confirmed by coexpressing PP2cα and Ca^{2+} channel C termini in HEK 293 cells and coimmunoprecipitation (CoIP) assays (Fig. 1*B*). Reciprocal CoIP yielded the same results (Fig. 2*B*).

Multiple types of Ca^{2+} channels coexist in neurons, including L-, N-, and P/Q-types (Zhang et al., 1993; Dunlap et al., 1995; Catterall, 1998). In addition to the N-type, both L- and P/Q-type Ca^{2+} channel C termini could also bind to PP2cα (Fig. 1*B*). This pattern differs from what we reported for the formation of a PKC-N-type Ca^{2+} signaling complex, in which ENH binds to N- but not P/Q-type Ca^{2+} channels (Maeno-Hikichi et al., 2003). The results indicated that the PP2cα binding domain might be located at a segment with a high degree of sequence homology among all three types of Ca^{2+} channels (see below). In the rest of the paper, we refer to the interaction between PP2cα and Ca^{2+} channels, although most of the data were collected using the N-type Ca^{2+} channel (α_{1B}) and its mutants. Immunoblotting showed PP2cα was present in the brain (Fig. 1*C*), in agreement with previous reports (Price and Mumby, 1999; Herzig and Neumann, 2000). To directly demonstrate the existence of the PP2cα–Ca^{2+} channel complex, CoIP was performed using lysates prepared from the adult rat brain. Consistent with the *in vitro* data using expressed Ca^{2+} channels or their C termini, anti-α_{1B} antibody was able to immunoprecipitate PP2cα from the crude membrane fraction of the brain preparation (Fig. 1*D*) (also see Fig. 6*A*). These results demonstrate that PP2cα and Ca^{2+} channels form a complex *in vivo*. This conclusion is further substantiated by results of mutagenesis and functional studies (see Figs. 2, 6–8),

As an enzyme, PP2cα could, in principle, bind to its substrate, Ca^{2+} channels, through its catalytic domain. Such an interaction, common to all enzyme-mediated catalysis, would be less interesting in terms of cellular signal transduction and would not be the basis for formation of a specific signaling protein complex. We sought to locate the Ca^{2+} channel binding domain within PP2cα through deletion mutations. The phosphatase catalytic domain is located in the N-terminal two-thirds of PP2cα (Fig. 2*A*) (Price and Mumby, 1999; Herzig and Neumann, 2000). Mutational analysis showed that the channel binding domain was mapped to the C-terminal end of PP2cα, outside of the catalytic domain (PP2cα-D2) (Fig. 2*B*). The PP2cα binding domain was narrowed to a fragment of 156 aa residues right after IVS6 of the α_{1B} subunit (amino acid residues 1708–1864; data not shown), where all

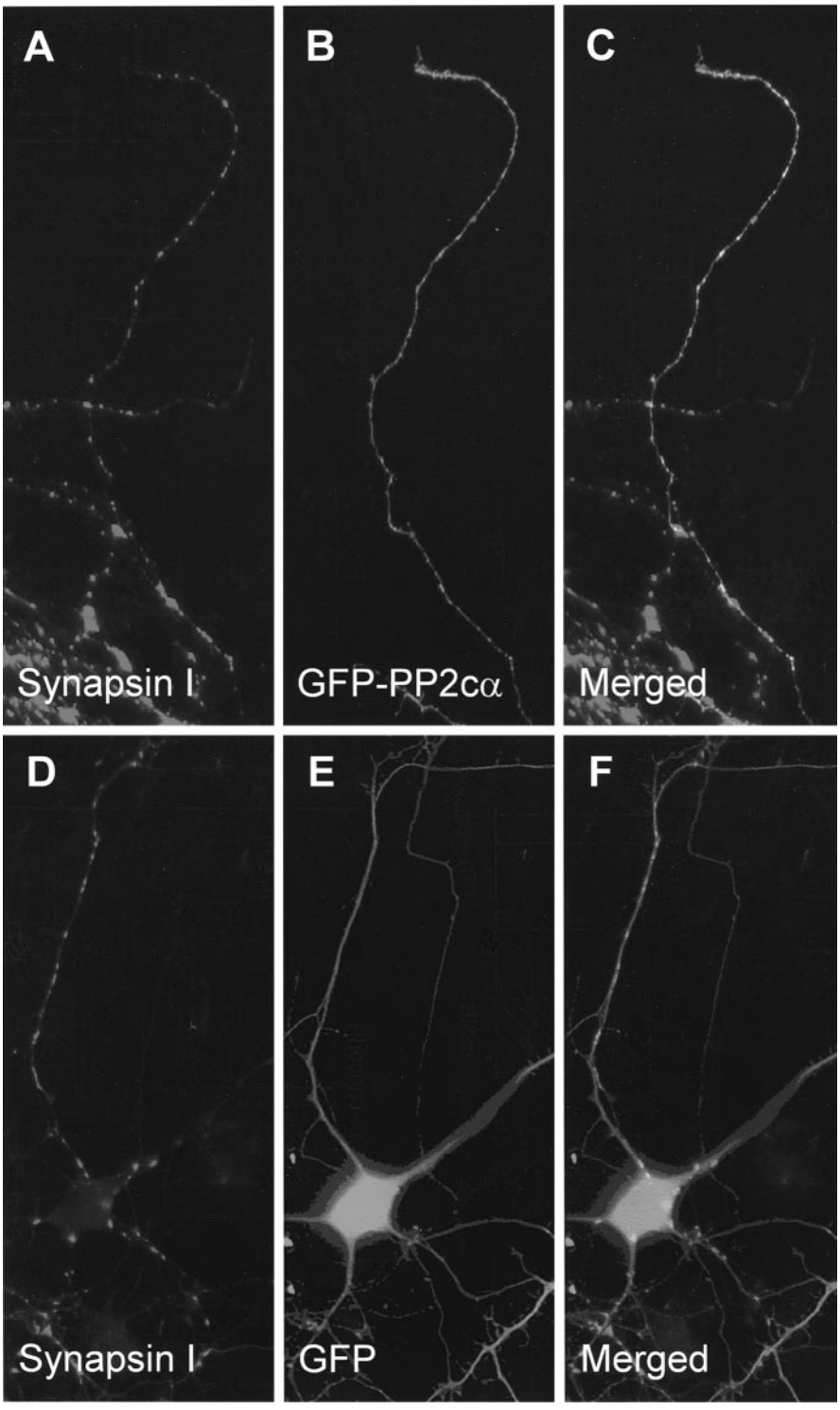

Figure 3. Presence of PP2cα in presynaptic boutons in hippocampal neurons. ***A***, Immunostaining of a presynaptic protein, synapsin I, in GFP-PP2cα-transfected neurons. ***B***, Green fluorescence of the same neuron, exhibiting punctate distribution of the GFP-tagged PP2cα. ***C***, Overlay of the red (synapsin I) and green fluorescent (GFP-PP2cα) images. ***D***, Synapsin I staining of a neuron transfected with GFP alone. ***E***, Green fluorescence of the same neuron, exhibiting uniform distribution of GFP. The fluorescence on the cell body was saturated. ***F***, Overlay of the red (synapsin I) and green fluorescent (GFP) images.

three types of Ca^{2+} channels share a high degree of homology in their primary amino acid sequences [see references listed in the study by Catterall (1998)]. Existence of a unique binding domain in PP2cα as well as in the channel C terminus provides another piece of evidence strongly supporting the specific interaction between PP2cα and the Ca^{2+} channel. In addition to understanding the molecular nature of the interaction, the deletion mutant, PP2cα-D2, which encompasses the channel binding domain (Fig. 2*A*), provided a useful tool for functional studies (see Figs. 7, 8).

Next, we sought to determine the subcellular localization of PP2cα in neurons using immunofluorescence microscopy. Its subcellular localization may provide a clue whether PP2cα is located in the same subcellular compartments as VGCCs. Hippocampal neurons were transfected with green fluorescent protein (GFP)-tagged PP2cα and stained with an antibody against synapsin I, a synaptic vesicle protein known to be present in presynaptic boutons (Sudhof et al., 1989). Neurons transfected with GFP–PP2cα displayed punctate green fluorescence along the neurites (Fig. 3*B*). This is in sharp contrast to neurons transfected with GFP alone, which showed uniform distribution of the green fluorescence throughout the neuron (Fig. 3*E*). These green fluorescent puncta could be simultaneously stained with the synapsin antibody (Fig. 3*A*,*C*), suggesting that these puncta are in fact presynaptic buttons and that PP2cα is enriched in presynaptic nerve terminals where VGCCs are known to be present for release of neurotransmitters (Catterall, 1998).

PP2cα is more effective in dephosphorylation of Ca^{2+} channels phosphorylated by PKCε

Could VGCCs, once phosphorylated by PKC, be a substrate for PP2cα? Furthermore, among different types of PPs, which one might play a major role in dephosphorylation of Ca^{2+} channels? To address these questions, we performed *in vitro* dephosphorylation assays using I–II and II–III loops of the Ca^{2+} channel α_{1B} subunit (N-type) and four different types of PPs: PP1, PP2a, PP2b, and PP2cα. Functional PKC phosphorylation sites have been identified in both I–II and II–III loops of the Ca^{2+} channel α_1 subunit (Fig. 4*A*) (Yokoyama et al., 1997; Zamponi et al., 1997). Phosphorylation of Ca^{2+} channels by PKC potentiates the channel activity and regulates interactions between Ca^{2+} channels and other signaling proteins (Yang and Tsien, 1993; Yokoyama et al., 1997; Zamponi et al., 1997). For instance, whereas phosphorylation of the I–II loop by PKC results in relief of the inhibitory effects by G-proteins, phosphorylation of the II–III loop promotes dissociation of N-type Ca^{2+} channels from syntaxin, a synaptic vesicle protein for release of neurotransmitters.

Both I–II and II–III loop fragments could be phosphorylated

by PKCε, similar to previous reports using a mixture of multiple PKCs (Yokoyama et al., 1997; Zamponi et al., 1997). Once dephosphorylation was initiated, the four PPs exhibited remarkably different capabilities in their dephosphorylation of the phosphorylated I–II and II–III loops (Fig. 4). Most notably, for the phospho-II–III loop, dephosphorylation by PP2cα developed much faster than the rest of PPs, and removal of ^{32}P was nearly complete after 30 min, which is significantly higher than that by other PPs (Fig. 4*B*,*C*) ($p < 0.001$; $n = 3$). Although all PPs are able to dephosphorylate the phosphorylated II–III loop, PP2cα is the most effective. The relative effectiveness for dephosphorylation of the phospho-II–III loop by these PPs is PP2cα ≫ PP2a > PP1 > PP2b. Dephosphorylation of the phospho-II–III loop by PP1, PP2a and PP2b was incomplete at their plateaus, leaving significant amount of the II–III loop remaining phosphorylated, 37.6 ± 1.3% for PP1, 26.4 ± 4.6% for PP2a, and 48.9 ± 4.7% for PP2b (compared with 5.4 ± 2.1% for PP2cα).

In contrast, for the phospho-I–II loop, none of the four PPs was very effective in removing the phospho-groups (Fig. 4*C*). After 60 min, a substantial amount of the I–II loop remained phosphorylated (69.0 ± 2.0% for PP1, 45.1 ± 3.1% for PP2a, 71.3 ± 3.2% for PP2b, and 56.9 ± 5.3% for PP2cα). At least for both PP2a and PP2cα, dephosphorylation had not reached the plateau after 60 min. If different PPs have distinct intrinsic activities but no preferences over different substrates, we expected to see that these four PPs should be equally effective in dephosphorylation of both I–II and II–III loops, when the assays were performed under the identical experimental conditions.

To further test whether different PPs had their preferred substrates, we turned to a different neuronal protein, MARCKS, which is a specific substrate for PKC (Aderem, 1992, 1995; Seki et al., 1995). Phospho-MARCKS is dephosphorylated by PPs, with PP1 being most effective, followed by PP2a and PP2b (Seki et al., 1995). Thus, MARCKS was used as control for the *in vitro* dephosphorylation assays. Polyhistidine-tagged MARCKS was purified and phosphorylated by PKCε, followed by dephosphorylation by PPs. The rank order for dephosphorylation of MARCKS is PP1 > PP2a > PP2b ≥ PP2cα, which is entirely different from that for the phosphorylated I–II and II–III loops (Fig. 4*D*). The effectiveness of PP1, PP2a, and PP2b is the same as reported previously (Seki et al., 1995). In contrast to what we saw for the phosphorylated II–III loop, PP2cα was least effective in dephosphorylation of MARCKS, with >50% of the protein remaining in the phosphorylated state 60 min after dephosphorylation was started (53.0 ± 2.3%, compared with 22.6 ± 3.2% for PP1; $p < 0.002$; $n = 3$). These results demonstrate that PPs indeed have their preferred substrates or even preferred phospho-groups. This conclusion is further supported by the observation that dephosphorylation of the I–II loop becomes more effective when all four PPs are present (Fig. 4*D*, filled square).

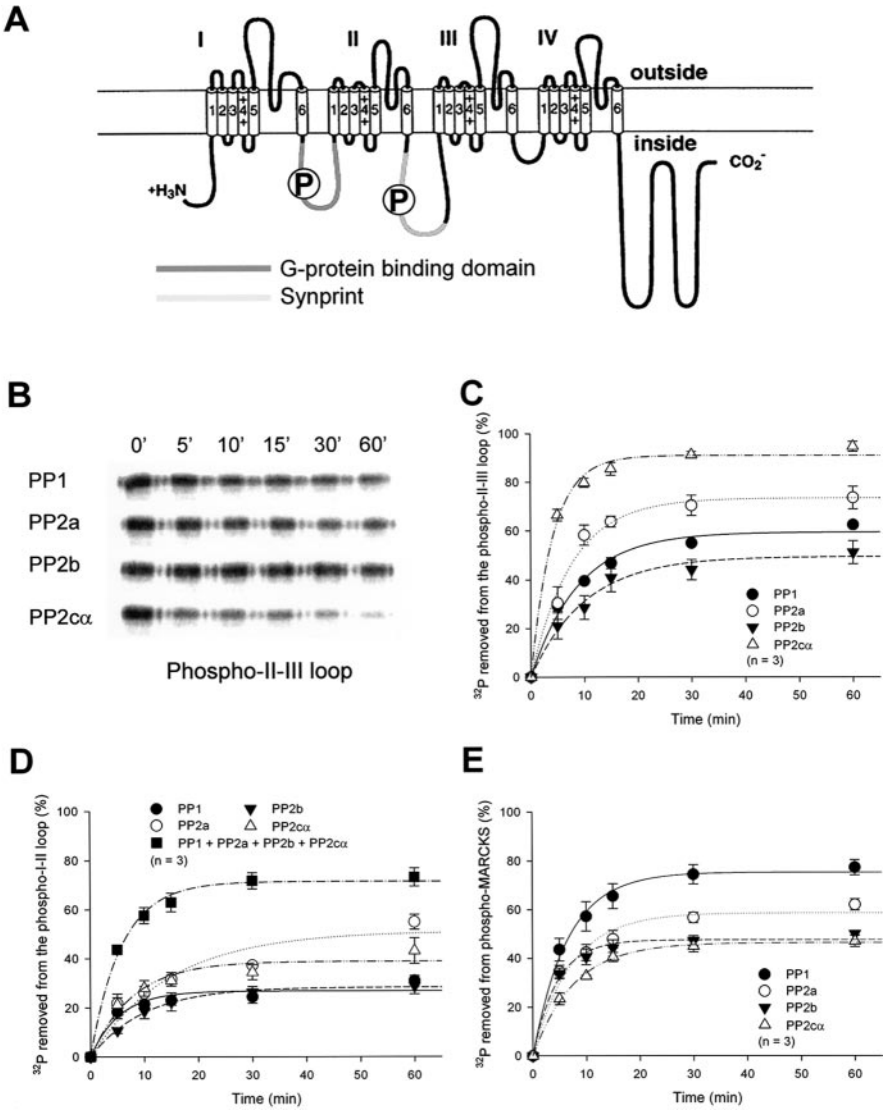

Figure 4. The Ca^{2+} channel II–III loop is a preferred substrate for PP2cα. ***A***, Schematic representation showing the functional PKC phosphorylation sites in the Ca^{2+} channel α_1 subunit. Phosphorylation of the I–II or II–III loop by PKC regulates the interactions between Ca^{2+} channels and G-proteins or syntaxin, respectively (synprint: the syntaxin binding domain). ***B***, Autoradiogram showing dephosphorylation of the phospho-II–III loop by these four different PPs. The II–III loop was phosphorylated by PKCε. Purified PP2cα was constitutively active. ***C***, Time course of dephosphorylation of the II–III loop by four PPs. The intensity of each band was quantified by a phosphoimager and normalized to that of time 0. ***D***, Time course of dephosphorylation of the I–II loop by four PPs. ***E***, Time course of dephosphorylation of MARCKS by four PPs. All experiments were performed under identical conditions, and data are mean ± SEM.

Quantitative analysis of the dephosphorylation data revealed that the apparent rate constant for dephosphorylation by PP2cα was significantly larger than that for other PPs (Fig. 5*A*). The preference of PP2cα for the phospho-II–III loop and the rapid rate of dephosphorylation by PP2cα should result in rapid removal of the phospho-groups from Ca^{2+} channels. For instance, >60% of ^{32}P was removed by PP2cα within 5 min after initiation of dephosphorylation (compared with <30% for the rest of PPs) (Fig. 5*B*). In contrast, for the I–II loop, fitting the data with a single exponential function was inadequate (Fig. 4*D*). The apparent rate constant for PP2cα is not significantly different from the

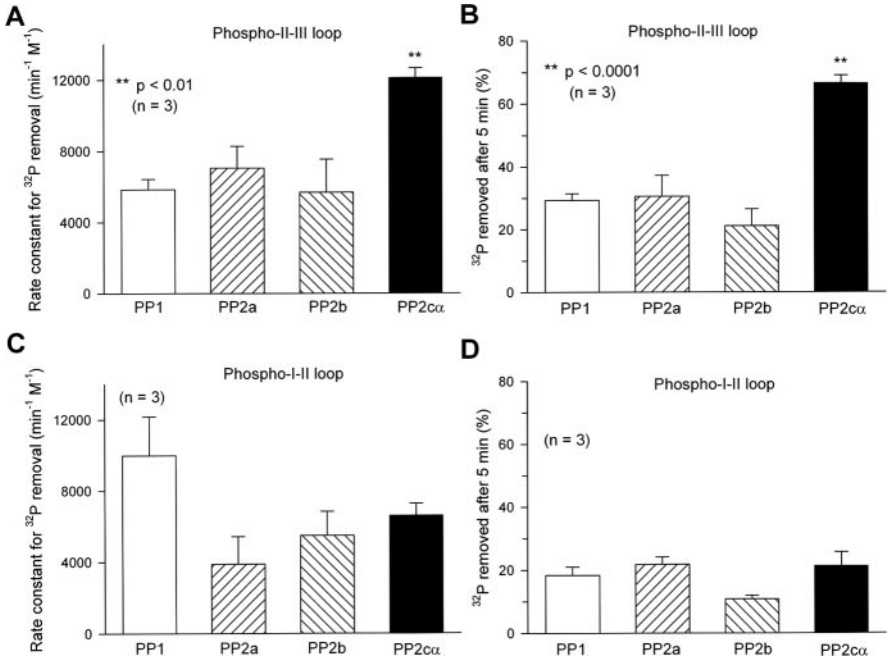

Figure 5. Quantitative analysis of dephosphorylation of the I–II and II–III loops by PP1, PP2a, PP2b, and PP2cα. ***A***, Apparent rate constants for dephosphorylation of the II–III loop by PPs. The rate constants were derived from fit of the time course to a simple exponential function. ***B***, Removal of ^{32}P from the phospho-II–III loop 5 min after initiation of the dephosphorylation reaction. ***C***, Apparent rate constants for dephosphorylation of the I–II loop by PPs, which displayed a distinct rank order for these PPs. ***D***, Removal of ^{32}P from the phospho-I–II loop 5 min after initiation of the dephosphorylation reaction.

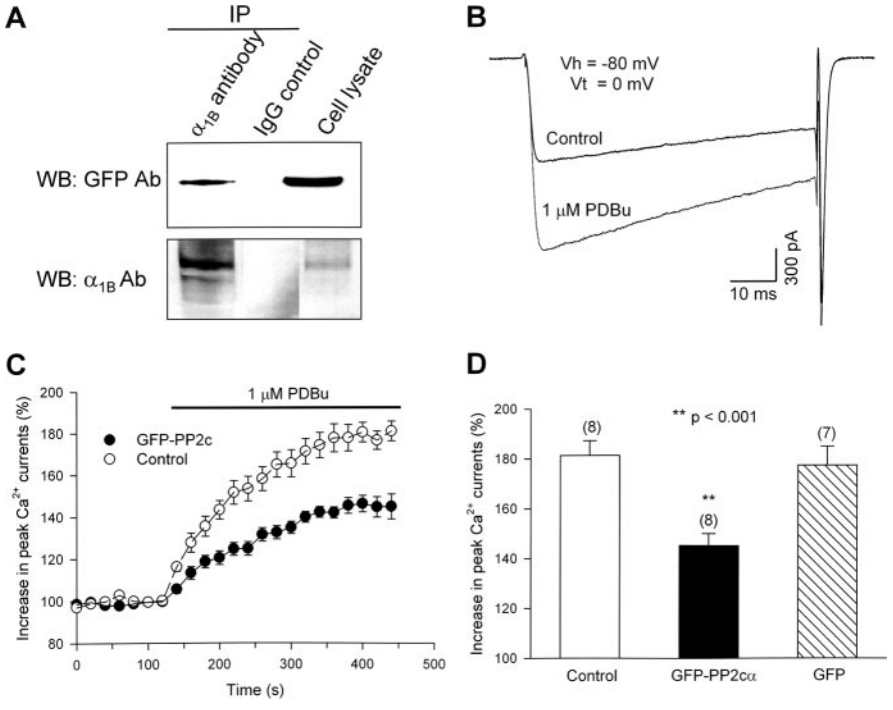

Figure 6. VGCCs are substrates for PP2cα *in vivo*. ***A***, Association of PP2cα and N-type Ca^{2+} channels expressed in tsA cells. CoIP assays were performed 48 h after transfection of GFP-tagged PP2cα. ***B***, Exemplar current traces recorded from a cell before and after application of PDBu. ***C***, Expression of PP2cα decreases potentiation of the Ca^{2+} channel activity by PKC. $V_t = 0$ mV, and $V_h = -80$ mV. Peak Ca^{2+} currents were normalized to that recorded before application of PDBu and plotted as a function of time ($n = 8$). ***D***, Summary of the effects of PDBu on the channel activity with different constructs expressed in tsA cells.

others (Fig. 5*C*). Overall, ~20% of the phospho-groups were removed from the phospho-I–II loop 5 min after start of dephosphorylation by these four PPs (Fig. 5*D*). Analysis of the dephosphorylation data from MARCKS yielded a different profile (data not shown). Collectively, these data show that among these four major PPs, PP2cα is more effective in dephosphorylation of neuronal Ca^{2+} channels after their phosphorylation by PKCε, at least in the *in vitro* settings. The results also illustrate that dephosphorylation of VGCCs by Ser/Thr protein phosphatases is more complicated than previously thought.

VGCCs are a substrate for PP2cα *in vivo*

Are full-length Ca^{2+} channels, once phosphorylated by PKC, a substrate for PP2cα *in vivo*, as suggested by our *in vitro* assays using the channel fragments? We approached this question by expressing PP2cα in a tsA (temperature-sensitive allele of simian virus 40 large T antigen) cell line that stably expresses N-type Ca^{2+} channels (α_{1B} plus α_2-δ plus β_3 subunits; a generous gift from Dr. D. Lipscombe, Brown University, Providence, RI) (Lin et al., 1997). Immunoblotting showed the presence of the α_{1B} subunit (Fig. 6*A*) and PKCε (data not shown). Ca^{2+} currents could be recorded from this tsA cell line using Ba^{2+} as the charge carrier (Fig. 6*B*). Activation of PKC by PDBu (1 μM) resulted in acute potentiation of the Ca^{2+} currents (Fig. 6*B*,*C*), a response similar to that of native N-type Ca^{2+} channels in neurons (Swartz et al., 1993; Yang and Tsien, 1993).

When expressed in this tsA cell line, GFP-PP2cα was associated with the N-type Ca^{2+} channels as demonstrated by CoIP assays (Fig. 6*A*). Introduction of PP2cα significantly reduced the response of N-type Ca^{2+} channels to modulation by PKC (Fig. 6*C*). The maximal response to 1 μM PDBu was decreased from 181.3 ± 6.0 to 145.2 ± 4.7% ($p < 0.001$; $n = 8$) (Fig. 6*D*). Transfection of the cell line with GFP alone did not produce any significant impact on potentiation of the channel activity by PKC (177.3 ± 7.6%; $n = 7$) (Fig. 6*D*). These results suggest that the full-length N-type Ca^{2+} channels, when phosphorylated by PKC, are a substrate for PP2cα. This conclusion is further strengthened by the results of expressing a dominant-negative construct in hippocampal neurons (Fig. 7).

PP2cα is functionally associated with VGCCs in hippocampal neurons

We next asked whether a functional PP2cα–Ca^{2+} channel complex existed in neurons, as suggested by the results of CoIP assays (Figs. 1*D*, 6*D*). We sought to test whether overexpression of a dominant-negative construct could interfere with the PP2cα channel interaction and consequently the response of Ca^{2+} channels to mod-

ulation by PKC in neurons. The rationale is that overexpression of the channel binding domain from PP2cα will compete with and therefore displace endogenous PP2cα for binding to Ca^{2+} channels and effectively reduce the local PP2cα concentration near Ca^{2+} channels. As a result, more channels will become phosphorylated when PKC is activated (Fig. 7*A*).

Mutagenesis studies showed that the channel binding domain in PP2cα was located downstream of the catalytic domain, within a fragment of 100 aa residues (Fig. 2, PP2cα-D2). Blast of the GenBank database with this fragment did not pick up any other proteins except PP2cα itself, indicating that overexpression of PP2cα-D2 is less likely to interfere with other signaling processes in neurons. As expected, when GFP-tagged PP2cα-D2 was overexpressed in hippocampal neurons, modulation of Ca^{2+} currents by PDBu developed much faster and became more effective, compared with nontransfected control neurons (Fig. 7). The time required for modulation to develop from 10 to 90% of the maximal response (T_{10-90}) was reduced from 270.5 ± 44.9 s for control ($n = 12$) to 147.9 ± 17.1 s for PP2cα-D2-transfected neurons ($n = 9$; $p < 0.02$). Transfection of GFP-PP2cα-D2 did not affect voltage-dependent activation of the Ca^{2+} currents (Fig. 7*D*,*E*). Effects of GFP-PP2cα-D2 on potentiation of Ca^{2+} currents by PDBu were observed at all the test voltages (Fig. 7*E*). On average, potentiation of the Ca^{2+} currents by PDBu was increased from 163.7 ± 6.4% ($n = 12$) to 211.0 ± 6.6% ($n = 9$) as a result of overexpression of GFP-PP2cα-D2 ($p < 0.001$; $V_t = 0$ mV) (Fig. 8). Expression of GFP alone had no impact on modulation of Ca^{2+} currents by PDBu (154.0 ± 8.5%; $p > 0.3$) (Fig. 8).

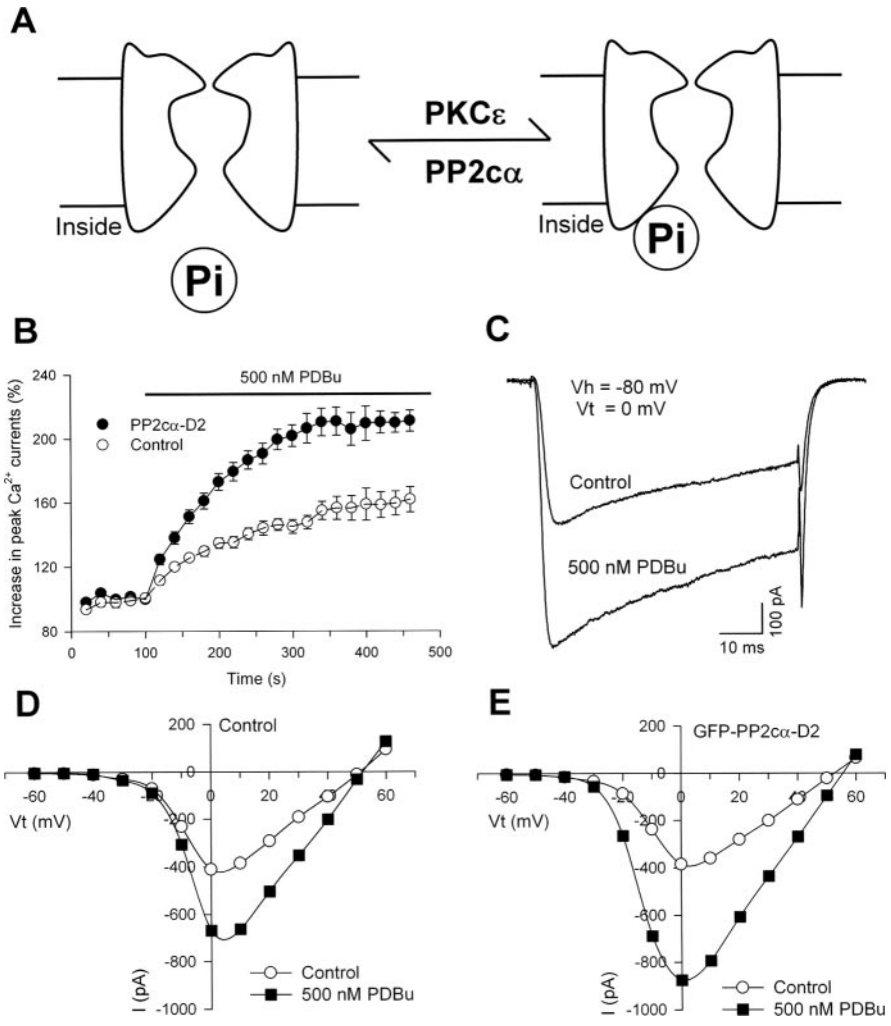

Figure 7. Disruption of the PP2cα–Ca^{2+} channel interaction enhances modulation of the channel activity by PDBu in hippocampal neurons. ***A***, A simplified model of phosphorylation and dephosphorylation of Ca^{2+} channels by PKC and PP2cα. ***B***, A dominant-negative construct, PP2cα-D2, enhances the modulatory effects of PDBu on the Ca^{2+} channel activity. Neurons were depolarized from a holding potential of −80 to 0 mV. Data are mean ± SEM. ***C***, Exemplar current traces obtained before and after application of PDBu from a nontransfected neuron. ***D***, *I–V* curves obtained before and after application of PDBu from a nontransfected control neuron. ***E***, *I–V* curves obtained before and after application of PDBu from a neuron transfected with GFP-PP2cα-D2. Recordings were performed 48 h after transfection.

To further test the specificity of the effects by GFP-PP2cα-D2, we transfected neurons with GFP-tagged endophilin, another Ca^{2+} channel partner protein. As we reported recently, endophilin binds to the C terminus of the Ca^{2+} channel α_1 subunit, and the channel-endophilin interaction serves to bring the endocytotic machinery into the presynaptic nerve terminal (Chen et al., 2003). On the channel C terminus, the endophilin binding site is further downstream of the PP2cα binding site, as revealed by mutagenesis studies, and endophilin does not interfere with the PP2cα channel interaction (our unpublished data). Overexpression of GFP–endophilin did not have any significant impact on the effects by PDBu (148.0 ± 8.9%; $p > 0.15$) (Fig. 8). Together, these data suggest that the effects of PP2cα-D2 are caused specifically by blockade of the interaction between endogenous PP2cα and VGCCs.

Discussion

Consensus has emerged that formation of macromolecular signaling complexes is an important mechanism for targeting specific PKs and/or PPs to ion channels (Pawson and Scott, 1997; Pawson and Nash, 2000, 2003; Bauman and Scott, 2002; Michel and Scott, 2002). Our results demonstrate that VGCCs and PP2cα form a functional signaling complex in neurons. This complex is responsible for rapid dephosphorylation of Ca^{2+} channels after their phosphorylation by PKC (Fig. 7*B*). The results also extend our previous study, which shows that through interactions with a common adaptor protein, ENH, a specific PKC isozyme, PKCε, is brought to its specific substrate, N-type Ca^{2+} channels (Maeno-Hikichi et al., 2003). Thus, at least for the N-type Ca^{2+} channels, modulation of the channel activity by PKC is achieved through a macromolecular complex, which includes ENH, PKCε, and PP2cα. This complex allows rapid modulation of the channel activity by second messengers that activate/regulate the activities of PKCε and/or PP2cα. Whereas PKCε is brought to the N-type Ca^{2+} channel via an adaptor protein, ENH, PP2cα is targeted to the N-type Ca^{2+} channel through their direct interactions. This PKC signaling complex differs from that of the PKA complex, in which AKAP (A-kinase anchor pro-

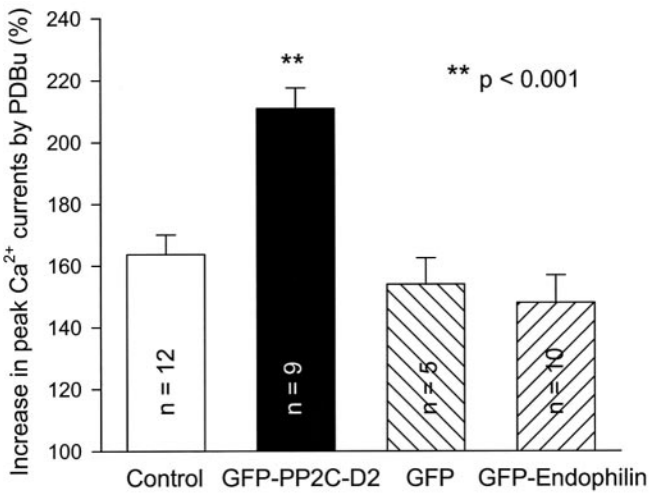

Figure 8. PP2cα is functionally associated with VGCCs in neurons. Effects of PP2cα-D2 are caused by its selective disruption of the PP2cα–Ca^{2+} channel interaction *in vivo*. Hippocampal neurons were transfected with different constructs as indicated, and their responses to modulation by PKC were recorded 48 h after transfection.

tein) brings both PKA and PPs to their substrate, such as cardiac L-type Ca^{2+} channels (Bauman and Scott, 2002).

Because of the lack of selective inhibitors/activators for different PPs, particularly the PP2c family, it is not clear whether a particular type of PP is involved in dephosphorylation of VGCCs, although PP1, PP2a, and PP2b have been implicated (Cohen, 1997; Price and Mumby, 1999; Herzig and Neumann, 2000). We have demonstrated, for the first time, that PP2cα, at least in the *in vitro* settings, is more effective in dephosphorylation of VGCCs after their phosphorylation by PKC and that VGCCs are a functional substrate for PP2cα in the mammalian CNS. Although all four major PP families, PP1, PP2a, PP2b, and PP2c, are capable of dephosphorylating VGCCs, PP2cα is clearly the most efficient one in removing the phospho-groups from the phosphorylated II–III loop. Selective association with and preferential dephosphorylation of VGCCs by PP2cα suggest that PP2cα may play a major role in dephosphorylation of neuronal VGCCs after their phosphorylation by PKC. Lack of specific inhibitors/activators, particularly for PP2cα, and the differential capabilities of different PPs in dephosphorylation of VGCCs are among the primary reasons why there has been no consensus in the literature on which PPs may play a major role in dephosphorylation of neuronal VGCCs after their phosphorylation by PKC. It remains to be determined the effectiveness of PP2cα in dephosphorylation of VGCCs after their phosphorylation by PKA, which is also known to modulate VGCCs (Hille, 1994; Dolphin, 1995; Catterall, 1997; Ikeda and Dunlap, 1999).

Compared with PP1, PP2a, and PP2b families, members of the PP2c family belong to an entirely different gene family and have their own unique properties. Unlike other PPs that have both regulatory and catalytic subunits, PP2cs are monomers (Fig. 2) (Cohen, 1997; Price and Mumby, 1999; Herzig and Neumann, 2000). Unlike PP2b, PP2cs are constitutively active, and their activity does not require activation by second messengers (Fig. 4). No specific inhibitors/activators are available for PP2cs. Thus, their functional roles in the mammalian CNS are not clear, although in plant cells and other mammalian cell lines they are implicated in the stress-associated processes (Cohen, 1997; Price and Mumby, 1999; Herzig and Neumann, 2000).

Regulation of the PP2c activity remains virtually unknown, although Mg^{2+} is essential for its activity. Structural data reveal that at least two Mg^{2+} ions are associated with one PP2c molecule (Das et al., 1996). Because the intracellular free Mg^{2+} concentration is ~0.5–1 mM in neurons (Brocard et al., 1993), PP2cs should always be active. Our results seem to support this notion, because overexpression of the dominant-negative construct, PP2cα-D2, significantly enhanced the effects of PKC on the Ca^{2+} channel activity (Fig. 7). If activation of PP2cα were necessary, expression of the dominant-negative construct itself would not have produced such significant effects. Therefore, PP2cα may act as housekeeping to rapidly dephosphorylate the target protein, and the degree of phosphorylation of the target protein is primarily determined by the regulated activity of the kinases. Such rapid dephosphorylation of VGCCs ensures precise and dynamic modulation of the Ca^{2+} channel activity by PKC. Other phosphatases, such as PP1, PP2a, or PP2b, although much less efficient in dephosphorylation of VGCCs, may play a role of fine-tuning the modulation of the Ca^{2+} channel activity, especially in response to activation by different second messenger pathways. It is likely that activation of certain second messenger pathways may promote the role of these other PPs in dephosphorylation of VGCCs. Identification of a functional substrate, VGCCs, will allow us to explore whether and how the PP2cα activity can be regulated by second messengers in neurons.

Dephosphorylation of VGCCs by Ser/Thr protein phosphatases is more complicated than previously expected. Different PPs have distinct capability of removing the phospho-groups from VGCCs (Figs. 4, 5). Unlike PP2cα, dephosphorylation of the II–III loop by other PPs is primarily incomplete. Furthermore, none of the four major PPs is able to remove the phospho-groups completely from the I–II loop. Such differences are not attributable to the distinct intrinsic activities of these PPs and are similar to results by recent reports of PP2c and other PPs on other proteins (Desdouits et al., 1998; Flajolet et al., 2003). One likely scenario is that the phospho-groups on the channel I–II and II–III loops are nonhomogenous to these PPs, and not all of them can be accessed and/or dephosphorylated. Sequence examination shows that both the I–II and II–III loop fragments that we used in this study have three consensus sites for phosphorylation by PKC. Therefore, individual PPs are not able to remove some of these phospho-groups effectively, although PP2cα can remove all the phospho-groups in the II–III loop. This notion is supported by the observation that dephosphorylation of the I–II loop becomes more effective when all four PPs are mixed together (Fig. 4*D*).

Indeed, previous studies have shown that different PPs may prefer distinct phospho-groups within a particular substrate. For instance, MARCKS has multiple PKC phosphorylation sites (Aderem, 1992, 1995; Seki et al., 1995). Dephosphorylation of MARCKS by different PPs is also incomplete. Detailed analysis revealed that PP2b preferred Ser155, whereas PP2a was selective of Ser162. Neither of them could remove the phospho-group from Ser151 effectively (Seki et al., 1995). Thus, preferential removal of the phospho-groups from different phosphorylation sites by distinct PPs adds another layer of complexity of modulation of the Ca^{2+} channel activity by PPs. It remains to be determined whether any individual phosphorylation site in the I–II and II–III loops is preferred by a particular type of PP and how much phosphorylation of a particular site by PKC will contribute to the potentiation of the Ca^{2+} channel activity by PKC. Our results also raise the possibility that in neurons dephosphorylation of the N-type Ca^{2+} channel I–II loop is either incomplete or mediated by a different PP yet to be identified. Furthermore, our results indicate that phosphorylation of the channel II–III loop

may contribute to potentiation of the Ca^{2+} channel activity by PKC. As shown by a recent study, the role of the I–II and II–III loops in potentiation of the Ca^{2+} channel activity by PKC is more complicated than previously expected (Kamatchi et al., 2004).

VGCCs are known to interact directly with several signaling molecules for modulation of the channel activities. This growing list includes, but is not limited to, G-proteins, calmodulin, PP2a for dephosphorylation of L-type Ca^{2+} channels, Ca^{2+} binding protein 1, ENH, and PP2cα (Zhang et al., 1996; De Waard et al., 1997; Herlitze et al., 1997; Qin et al., 1997; Zamponi et al., 1997; Lee et al., 1999, 2002; Peterson et al., 1999; Zuhlke et al., 1999; Davare et al., 2001; Simen et al., 2001; Maeno-Hikichi et al., 2003). Some of these signaling molecules, such as PKC and G-proteins, exert opposing modulatory effects on Ca^{2+} channels. Although PKC enhances the channel activity, activation of G-protein inhibits the channel activity (Dolphin, 1995; Catterall, 1997). Binding of G-protein to Ca^{2+} channels can be abolished by phosphorylation of the channel I–II loop by PKC (Zamponi et al., 1997). Furthermore, association of syntaxin 1A with the Ca^{2+} channel II–III loop enhances the inhibitory effects by G-proteins (Jarvis and Zamponi, 2001; Lu et al., 2001). Thus, dephosphorylation of Ca^{2+} channels by PP2cα, particularly the II–III loop, may regulate the inhibition of the Ca^{2+} activity by G-proteins. It will be interesting to test whether some of the signaling pathways converge on the Ca^{2+} channel partner proteins instead of directly on Ca^{2+} channels themselves and regulate their activities.

The presence of PP2cα in presynaptic boutons (Fig. 3) and association of PP2cα with VGCCs raise the possibility that PP2cα may be involved in dephosphorylation of synaptic vesicle proteins and thus contribute to regulation of synaptic transmission and synaptic plasticity. Indeed, synaptic vesicle proteins, such as SNAP-25 and syntaxin, can be phosphorylated by PKC (Yokoyama et al., 1997). The channel II–III loop fragment includes the syntaxin binding domain (Fig. 4*A*, synprint), through which VGCCs bind to syntaxin and become part of the SNAP receptor (SNARE) complex for release of neurotransmitters (Sheng et al., 1994; Catterall, 1998). Phosphorylation of the II–III loop by PKC promotes dissociation of the SNARE complex from VGCCs (Yokoyama et al., 1997). Therefore, phosphorylation by PKC and dephosphorylation by PP2cα of the Ca^{2+} channel II–III loop will determine how much of the SNARE complex is associated with VGCCs. Activation of PKC increases synaptic transmission and contributes to synaptic plasticity, at least partially as a result of increased Ca^{2+} channel activity (Malenka et al., 1986; Swartz et al., 1993; Wheeler et al., 1994).

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