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Competitive binding of α-actinin and calmodulin to the NMDA receptor

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The mechanisms by which neurotransmitter receptors are immobilized at postsynaptic sites in neurons are largely unknown. The activity of NMDA (*N*-methyl-D-aspartate) receptors is mechanosensitive[1] and dependent on the integrity of actin[2], suggesting a functionally important interaction between NMDA receptors and the postsynaptic cytoskeleton. α-Actinin-2, a member of the spectrin/dystrophin family of actin-binding proteins, is identified here as a brain postsynaptic density protein that colocalizes in dendritic spines with NMDA receptors and the putative NMDA receptor-clustering molecule PSD-95. α-Actinin-2 binds by its central rod domain to the cytoplasmic tail of both NR1 and NR2B subunits of the NMDA receptor, and can be immunoprecipitated with NMDA receptors and PSD-95 from rat brain. Intriguingly, NR1-α-actinin binding is directly antagonized by Ca^{2+}/calmodulin. Thus α-actinin may play a role in both the localization of NMDA receptors and their modulation by Ca^{2+}.

NMDA receptors exist *in vivo* as heteromultimeric complexes consisting of the essential NR1 subunit coassembled with various subunits of the NR2 subfamily[3–8]. An association between NR2 subunits and the PSD-95/SAP90 family of synaptic proteins has been identified[9,10], but the nature of specific proteins that mediate NMDA receptor interaction with the postsynaptic actin cytoskeleton is not known. The carboxy-terminal intracellular domain of NR1 (Fig. 1) represents a potential target for intracellular anchoring molecules, especially as alternative splicing within this C-terminal region can affect the subcellular distribution of NR1(ref. 11).

A yeast two-hybrid screen of a human brain complementary DNA library was performed using as bait the C-terminal tail from the most abundant NR1 splice variant (NR1A)[12] (Fig. 1a, b). This C-terminal splice variant contains both C-terminal exon cassettes (termed C1 and C2) as well as the membrane-proximal region (here termed C0), and is thus referred to as C0-C1-C2 (Fig. 1, inset). The two-hybrid screen yielded multiple isolates of two distinct clones (A1.7 and A2.10) which interacted specifically with the C-terminal intracellular tail of NR1, but not with Shaker-type K^+ channel subunits or the AMPA-receptor subunit GluR1. Clone A1.7 interacted with the C0-C1-C2 but not with the C0-C2 C-terminal variant and encoded a new protein with weak homology to muscle myosin heavy chain (M.W. and J.L., unpublished observations). Clone A2.10 interacted with both the C0-C1-C2 and C0-C2 variants of NR1 and contained a 2.2-kilobase (kb) cDNA encoding the C-terminal two thirds of the human α-actinin-2 gene (Fig. 1a)[13]. The

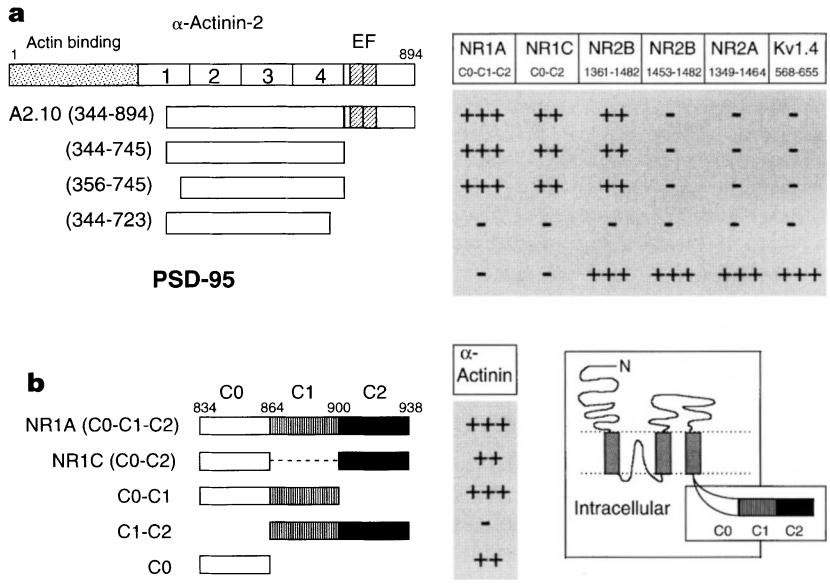

Figure 1 a, α-actinin-2 binds to the cytoplasmic tails of NR1 and NR2B through its central rod domain. Clone A2.10, and further deletion variants, are shown aligned under full-length α-actinin-2, in which the actin-binding domain, repeats 1–4 of the central rod domain, and EF hands are indicated. The interaction of α-actinin-2 constructs with the cytoplasmic tails of NR1, NR2B, NR2A and Kv1.4 are shown, as measured by the yeast two-hybrid assay. Numbers refer to amino-acid residues at the boundaries of each construct. Yeast two-hybrid interactions were semi-quantified on the basis of induction of the reporter genes *HIS3* and β-galactosidase[21]. **b**, The C0 segment of the NR1 C-terminal tail is necessary and sufficient for binding to α-actinin-2. Deletion constructs of the NR1 C-terminal tail were tested for interaction with α-actinin-2 (A2.10) by yeast two-hybrid assay. Numbers refer to the amino-acid residues at the boundaries of C0, C1 and C2 segments[25]. Inset, membrane topology of NR1. The intracellular tail consists of the membrane proximal segment C0, and C-terminal exon-segments C1 and C2.

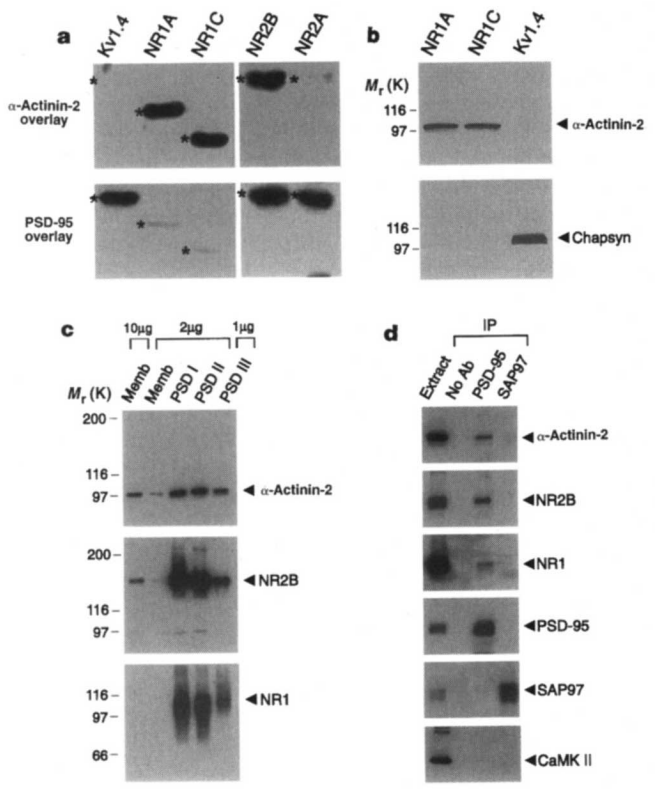

Figure 2 Biochemical association of α-actinin-2 and NMDA receptor subunits *in vitro* and in rat brain. **a**, Filter overlay assay showing specific *in vitro* binding of α-actinin-2 to NR1A, NR1C or NR2B C-terminal tails. GST-fusion proteins of the C-terminal tails of NR1A, NR1C NR2A, NR2B or Kv1.4[10] were probed with a hexahistidine-tagged fusion protein of α-actinin-2 (top) or PSD-95 (bottom)[21]. Asterisks indicate the positions of the fusion proteins. **b**, The C-terminal tail of NR1 selectively 'pulls-down' α-actinin-2 from rat brain extracts. Detergent extracts of rat brain were incubated with GST-fusion proteins of the C termini of NR1A, NR1C or Kv1.4 coupled to glutathione–Sepharose beads. Bound proteins were immunoblotted for α-actinin-2 and chapsyn-110 (ref. 17). Positions of molecular size markers are shown. **c**, Copurification of α-actinin-2 and NMDA receptor subunits into the postsynaptic density (PSD) fraction. Lanes were loaded with rat brain fractions (10, 2 or 1 μg, as indicated): crude synaptosomal membrane fraction (Memb); purified postsynaptic density fractions extracted with Triton X-100 once (PSD I), twice (PSD II), or with Triton X-100 followed by Sarkosyl (PSD III)[26]. Immunoblots were probed for α-actinin-2, NR1 and NR2B (a major component of the PSD[27]). **d**, Coimmunoprecipitation of α-actinin-2 with NMDA receptors and PSD-95 from rat brain. Detergent extracts of rat cerebral cortex synaptosomal membranes were immunoprecipitated with PSD-95 or SAP97 antibodies or with no primary antibodies. Immunoprecipitates were then immunoblotted for α-actinin-2, NR2B, NR1, PSD-95, SAP97 and CaM kinase II α-subunit. First lane (extract) contains 5% of input used for the immunoprecipitation.

α-actinins belong to the spectrin/dystrophin family of proteins[14,15], which is characterized by an N-terminal actin-binding domain and a central rod domain consisting of multiple spectrin-like repeats.

Clone A2.10 lacks the actin-binding domain of α-actinin-2, but extends from a region within the first spectrin-like repeat to the 3′ untranslated region (UTR) of α-actinin-2 (Fig. 1a). Deletion of the C-terminal region of α-actinin-2, including the EF hands, did not affect its interaction with NR1 C-terminal tail (C0-C1-C2 or C0-C2), indicating that the central rod domain of α-actinin-2 is sufficient for binding to NR1 (Fig. 1a). A deletion extending into repeat 4 of the rod domain abolished the interaction, but whether this deletion disrupted the NR1 binding site or affected the antiparallel dimerization of the rod domain remains to be determined[14,15]. NR1 showed no detectable binding to α-actinin-3.

The C0 segment of the NR1 C-terminal tail was both necessary and sufficient for interaction with α-actinin-2 (Fig. 1b), suggesting that the C0 segment contains the α-actinin-2 binding site. The membrane-proximal C0 region is present in all known C-terminal splice variants of NR1 (refs 12, 16), so the interaction of α-actinin-2 with NR1 presumably applies to all splice forms of NR1. Two-hybrid assays revealed that α-actinin-2 also bound to the C-terminal 122 residues, but not to the last 30 residues, of the cytoplasmic tail of the NMDA receptor subunit NR2B (Fig. 1a). In contrast, there was no detectable binding of α-actinin-2 to the cytoplasmic tail of NR2A (Fig. 1a). As expected, however, all NR2B and NR2A tail constructs interacted with PSD-95, which recognizes the very C-terminal ESDV sequence motif of NR2 subunits[9,10]. Thus, α-actinin binds to NR2B at a site distinct from that recognized by the PDZ domains of PSD-95.

A direct association between α-actinin-2 and NMDA receptor subunits was confirmed by *in vitro* binding of recombinant fusion proteins in a filter overlay assay (Fig. 2a). α-Actinin-2 binds to glutathione-*S*-transferase (GST) fusion proteins of the cytoplasmic tails of NR1 (C0-C1-C2 and C0-C2) and of NR2B, but not to the cytoplasmic tail of Kv1.4 or NR2A. By contrast, PSD-95, binds to Kv1.4 and NR2 subunits but not to NR1 (Fig. 2a).

A rabbit polyclonal anti-peptide antibody specific to α-actinin-2 (termed 4B2) recognized a band of M_r~100K on immunoblots of rat brain membranes, consistent with the predicted size of α-actinin-2 (ref. 13) (Fig. 2b, c). When coupled to beads, GST fusion proteins of the cytoplasmic tails of NR1A and NR1C were able specifically to affinity-purify ('pull-down') α-actinin-2 protein from brain extracts, but not chapsyn-110 (ref. 17) or other members of the PSD-95 family of proteins (Fig. 2b). Conversely, the Kv1.4 C-terminal tail selectively 'pulled-down' members of the PSD-95 family, including chapsyn-110 (Fig. 2b) and PSD-95 (not shown), but not α-actinin-2.

Upon subcellular fractionation, α-actinin-2 and NMDA receptor subunits copurify into the postsynaptic density fraction where they are both resistant to extraction with Triton X-100 and Sarkosyl detergents (Fig. 2c). Thus, like NMDA receptors and PSD-95, α-actinin-2 appears to be tightly associated with the postsynaptic density. Moreover, α-actinin-2 could be coimmunoprecipitated with NR1 and NR2B proteins by PSD-95-specific antibodies (Fig. 2d), consistent with a direct or indirect association of these proteins in brain membranes. The relative specificity of this coimmunoprecipitation is shown by first, the lack of NMDA receptor/α-actinin-2 precipitation with antibodies against SAP97, a presynaptic relative of PSD-95 (refs 17, 18); and second, by the absence of coimmunoprecipitated calmodulin (CaM) kinase II α-subunit, an abundant component of the postsynaptic density[19] (Fig. 2d).

α-Actinin-2 was localized by fluorescence immunohistochemistry in rat brain sections using rabbit 4B2 antibodies and a mouse monoclonal antibody (EA-53) specific for α-actinin-2 and α-actinin-3. α-actinin-3 is not expressed in the rat brain (M.W. and A.H.B., unpublished observations), therefore EA-53 should recognize only α-actinin-2 in central neurons. Immunostaining with both these independent antibodies revealed that α-actinin-2 was highly concentrated in dendritic spines in rat brain neurons (Fig. 3a–c).

In cultured hippocampal neurons, α-actinin-2 colocalized closely with NR1 (Fig. 3d–f) and with PSD-95 in dendritic spines (Fig. 3g–i), at presumed synaptic sites (that is, these puncta were apposed to the presynaptic markers synaptophysin and SV2 (not shown)). In addition, F-actin was itself highly concentrated in spines and colocalized with α-actinin-2 (Fig. 3j–l). The colocalization of α-actinin-2, NMDA receptors and PSD-95 in dendritic spines is consistent with the association of these proteins in a coimmunoprecipitable complex at postsynaptic sites (Fig. 2d).

As α-actinin is a known actin-binding protein, a direct interaction between α-actinin-2 and NR1/NR2B would link NMDA

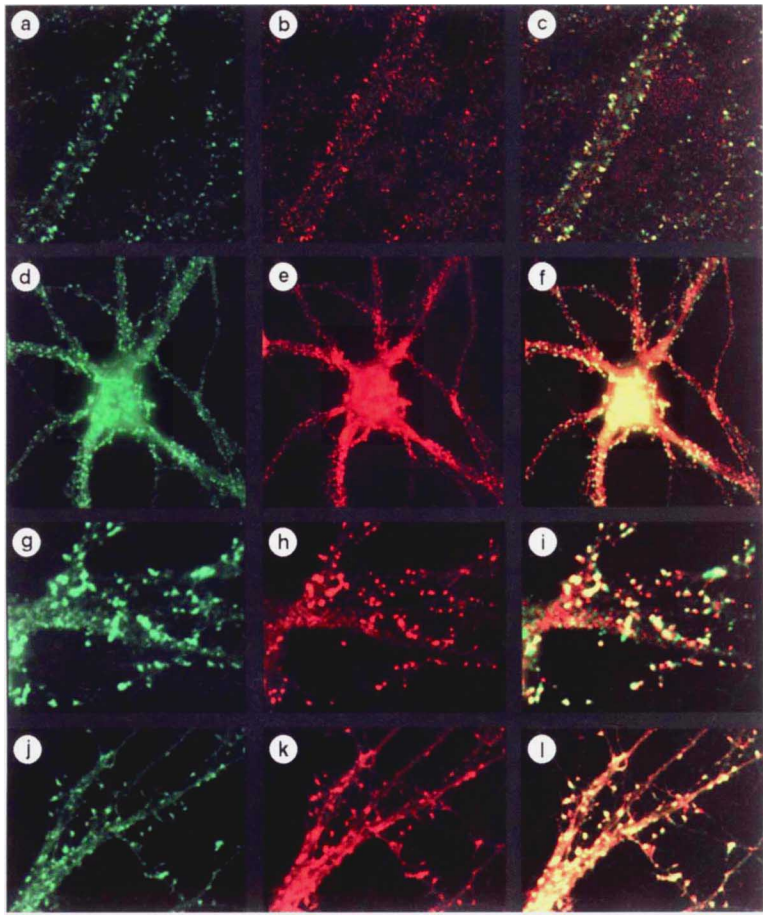

Figure 3 Colocalization of α-actinin-2 with NR1, PSD-95 and F-actin in dendritic spines. **a–c**, α-Actinin-2 in apical dendrites of cortical layer-5 pyramidal neurons of rat brain visualized using two independent α-actinin-2 antibodies: mouse monoclonal EA-53 (green; **a**), and rabbit polyclonal 4B2 (red; **b**). **d–l**, Colocalization of α-actinin-2 with NR1, PSD-95 and F-actin in cultured hippocampal neurons. **d–f**, Colocalization of α-actinin-2 (green; **d**) and NR1 (red; **e**). **g–i**, Colocalization of α-actinin-2 (green; **g**) and PSD-95 (red; **h**). **j–l**, Colocalization of α-actinin-2 (green; **j**) and F-actin (stained with rhodamine-conjugated phalloidin; **k**). Composite images show colocalization of signals (yellow; **c**, **f**, **i** and **l**).

receptors to postsynaptic actin and could provide a molecular basis for the dependence of NMDA receptor function on the integrity of the actin cytoskeleton[2]. Ca^{2+}/CaM can bind to the C0 and C1 segments of the NR1 cytoplasmic tail and inhibit the activity of recombinant NR1/NR2A NMDA receptors[20]. α-actinin-2 binds to NR1 and NR2B with a K_D of 30–100 nM (M.W. and J.L., unpublished observations), compared with a K_D of ~90 nM reported for CaM binding to the C0 segment of NR1 (ref. 20). α-Actinin-2 binding to NR1 is directly antagonized by calmodulin in a Ca^{2+}-dependent fashion: half-maximal inhibition of α-actinin-2 binding was seen at ~1–3 μM Ca^{2+}/CaM for either the C0-C1-C2 or the C0-C2NR1 cytoplasmic tail variant (Fig. 4, and data not shown). α-Actinin-2 binding to NR1 was antagonized by calmodulin at 100 μM but not at 100 nM free Ca^{2+} (data not shown), suggesting that α-actinin-2 may bind to NR1 at resting intracellular Ca^{2+} levels *in vivo*. As calmodulin is abundant in brain and can be coimmunoprecipitated with NMDA receptors[20], our results indicate that Ca^{2+}/CaM may displace α-actinin-2 from NR1 subunits in response to postsynaptic Ca^{2+} influx, perhaps through activated NMDA receptors themselves. Such a mechanism could contribute not only to Ca^{2+}-dependent inactivation and rundown of NMDA receptors[2], but may also lead to Ca^{2+}-dependent detachment of NMDA receptors from the actin cytoskeleton and their redistribution during synaptic activity.

Ca^{2+}/calmodulin does not bind to the NR2B cytoplasmic tail and hence has no direct effect on NR2B interaction with α-actinin-2 (Fig. 4). Thus, NMDA receptors with distinct NR2 subunit compositions may be differentially regulated by Ca^{2+}/calmodulin and the cytoskeleton. □

Methods

Yeast two-hybrid screening. Yeast two-hybrid screening and assays were done as described[21]. About 2×10^6 clones were screened using a human brain cDNA library (Clontech) constructed in the Gal4-activation domain vector pGAD10. The C-terminal tail constructs of NR1 splice variants, NR2B, NR2A and Kv1.4, were generated by PCR with specific primers and subcloned into pBHA to obtain LexA fusions[10]. Deletion constructs of α-actinin-2 were made by PCR using specific primers and subcloned into pGAD10 to generate Gal4-activation-domain fusions.

Antibodies. α-Actinin-2-specific rabbit polyclonal antibodies were raised against a unique peptide sequence within the N terminus (NQIEPGVQYNYVYDEDE) of α-actinin-2 and affinity-purified. These antibodies do not crossreact with any of the other known α-actinin isoforms (M.W. and A.H.B., unpublished results). Antibodies against chapsyn-110 (ref. 17), SAP97 (ref. 17), PSD-95 (ref. 21) and NR2B[8] have been described. Anti-T7.Tag antibodies (Novagen), monoclonal anti-calmodulin antibody (UBI), monoclonal anti-Ca^{2+}/calmodulin-dependent protein kinase II antibody (Chemicon), and anti-NR1 monoclonal antibodies 54.1 (PharMingen) were used for immunoblotting at 1 μg ml^{-1}.

Filter overlay, 'pull-down' assays, immunoblotting and immunoprecipitation. Filter overlay binding was assayed as described[10,21,22]. For affinity-purification ('pull-down') of α-actinin-2 from rat brain, 1% Triton

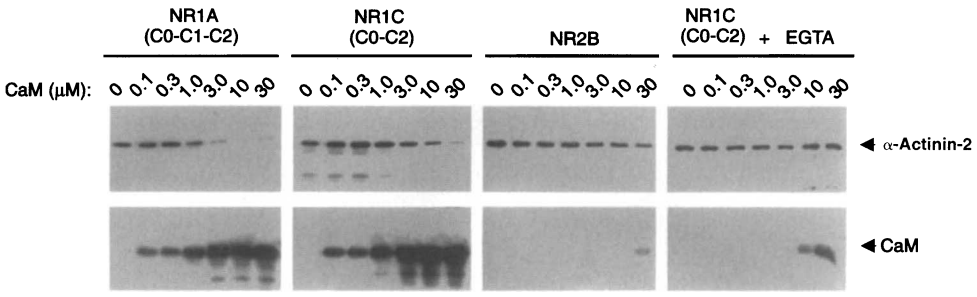

Figure 4 Competition between α-actinin-2 and Ca^{2+}/calmodulin for binding to the C-terminal tails of NR1A and NR1C. GST-fusion proteins (2 μg) of the C-terminal tails of NR1A, NR1C or NR2B bound to glutathione-Sepharose beads were incubated with a fixed concentration of hexahistidine-tagged fusion protein of α-actinin-2 (150 nM) and increasing concentrations of calmodulin (CaM). Bound proteins were eluted and immunoblotted for α-actinin-2 using anti-T7.Tag antibodies, and for calmodulin with a monoclonal anti-calmodulin antibody. Binding reactions containing 5 mM EGTA (+EGTA) are indicated. All other binding reactions contained 2 mM $CaCl_2$.

X-100 extracts of rat brain (200 μg protein) were incubated with glutathione–Sepharose beads (Pharmacia) bound to GST-fusion proteins (100 μg) at 4 °C for 1 h. After washing with phosphate-buffered saline containing 0.1% Triton X-100, bound proteins were eluted with SDS-sample buffer and detected by immunoblotting. Coimmunoprecipitation of α-actinin-2, NR2B, NR1 and PSD-95 complexes from rat brain was done on SDS-extracted rat brain membranes as described[23]. For α-actinin-2/calmodulin competition, purified hexahistidine-tagged α-actinin-2 and glutathione–Sepharose bound GST-fusion proteins were incubated with purified calmodulin (Calbiochem) in 25 mM HEPES, 120 mM KCl, 1 mM EDTA, 0.2% Triton X-100, pH 7.6, and either 2 mM $CaCl_2$ or 5 mM EGTA at 4 °C for 1 h. All proteins were visualized using peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL, Amersham).

Immunocytochemistry. Brain immunohistochemistry was done as described[17]. Hippocampal neuronal cultures were prepared as described[24]. Neurons were fixed and permeabilized 18–35 d after plating, either with cold methanol or with 4% paraformaldehyde and 0.25% Triton X-100. Rhodamine-conjugated phalloidin was used at a concentration of 100 ng ml^{-1}. Primary antibodies were used at the indicated concentrations: 4B2 (1 μg ml^{-1}), EA-53 (1:20,000; Sigma), anti-NR1 monoclonal antibodies 54.1 (2 μg ml^{-1}; PharMingen) and were visualized with FITC-, Cy3-, and biotin-conjugated secondary antibodies (2.5 μg ml^{-1}; Jackson ImmunoResearch) and Texas red-conjugated streptavidin (500 ng ml^{-1}; Vector Labs). Fluorescent images of cells were captured on a Photometrics cooled CCD camera mounted on a Zeiss Axioskop microscope (cultured hippocampal neurons), or on a Biorad Confocal MRC 1000 (rat brain sections).

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Crosstalk between G proteins and protein kinase C mediated by the calcium channel α_1 subunit

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The modulation of voltage-dependent Ca^{2+} channels at presynaptic nerve terminals is an important factor in the control of neurotransmitter release and synaptic efficacy. Some terminals contain multiple Ca^{2+}-channel subtypes (N and P/Q)[1–3], which are differentially regulated by G-protein activation[4–8] and by protein kinase C (PKC)-dependent phosphorylation[9–11]. Regulation of channel activity by crosstalk between second messenger pathways has been reported[12,13], although the molecular mechanisms underlying crosstalk have not been described. Here we show that crosstalk occurs at the level of the presynaptic Ca^{2+}-channel complex. The α_1 subunit domain I–II linker, which connects the first and second transmembrane domains, contributes to the PKC-dependent upregulation of channel activity, while G-protein-dependent inhibition occurs through binding of Gβγ to two sites in the I–II linker. Crosstalk results from the PKC-dependent phosphorylation of one of the Gβγ binding sites which antagonizes Gβγ-induced inhibition. The results provide a mechanism for the highly regulated and dynamic control of neurotransmitter release that depends on the integration of multiple presynaptic signals.

Coexpression of a Ca^{2+}-channel β subunit antagonizes the G-protein-dependent inhibition of neuronal Ca^{2+} currents[8,14]. Because the Ca^{2+}-channel β subunit binds to the α_1 subunit domain I–II cytoplasmic linker[15], we tested whether this region also contributes to G-protein modulation. Expression of α_{1A} and α_{1B} subunits (with α_2 and β_{1b}) in HEK cells results in P/Q-type and N-type currents