Phosphorylation by cAMP-dependent protein kinase (PKA) increases the activity of class C L-type Ca\(^{2+}\) channels which are clustered at postsynaptic sites and are important regulators of neuronal functions. We investigated a possible mechanism that could ensure rapid and efficient phosphorylation of these channels by PKA upon stimulation of cAMP-mediated signaling pathways. A kinase anchor proteins (AKAPs) bind to the regulatory R subunits of PKA and target the holoenzyme to defined subcellular compartments and substrates. Class C channels isolated from rat brain extracts by immunoprecipitation contain an endogenous kinase that phosphorylates kemptide, a classic PKA substrate peptide, and also the main phosphorylation site for PKA that phosphorylates kemptide, a classic PKA substrate peptide, and also the main phosphorylation site for PKA with the immunoisolated class C channel complex was confirmed by immunoblotting. A direct protein overlay binding assay performed with \(^{32}\)P-labeled RI\(\beta\) revealed a prominent AKAP with an \(M_r\) of 280,000 in class C channel complexes. The protein was identified by immunoblotting as the microtubule-associated protein MAP2B, a well established AKAP. Class C channels did not contain tubulin and MAP2B association was not disrupted by dilution or addition of nocodazole, two treatments that cause dissociation of microtubules. 

In vitro experiments show that MAP2B can directly bind to the \(\alpha_1\) subunit of the class C channel. Our findings indicate that PKA is an integral part of neuronal class C L-type Ca\(^{2+}\) channels and suggest that the AKAP MAP2B may mediate this interaction. Neither PKA nor MAP2B were detected in immunoprecipitates of \(\alpha\)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid-type glutamate receptors or class B N-type Ca\(^{2+}\) channels. Accordingly, MAP2B docked at class C Ca\(^{2+}\) channels may be important for recruiting PKA to postsynaptic sites. 

Ca\(^{2+}\) influx through voltage-gated L-type Ca\(^{2+}\) channels controls a variety of neuronal functions including synaptic plasticity, membrane excitability, and gene expression (1–5). Like other voltage-gated Ca\(^{2+}\) channels, neuronal L-type channels consist of several subunits including the \(\alpha_1, \alpha_2\delta, \beta, \gamma, \alpha_6\), and \(\beta\) polypeptides (6). \(\alpha_1\) is the critical subunit which constitutes the ion-conducting pore (6). Most if not all neuronal L-type channels in the brain are formed from either \(\alpha_{1C}\) or \(\alpha_{1D}\) (7). Two sizes of \(\alpha_{1C}\) and \(\alpha_{1D}\) polypeptides are evident in situ (7). The shorter forms (180–190 kDa) are derived from their longer counterparts (200–220 kDa) by C-terminal truncation (8, 9). In hippocampal slices, this modification of the C terminus of \(\alpha_{1C}\) is induced by Ca\(^{2+}\) influx through N-methyl-D-aspartate receptors and mediated by the Ca\(^{2+}\)-dependent cytosolic protease calpain (10). C-terminal truncation increases the activity of this channel about 4-fold (11). In contrast to \(\alpha_{1D}\), \(\alpha_{1C}\) immunoreactivity has a punctate pattern consistent with a synaptic location (7). This pattern of \(\alpha_{1C}\) immunoreactivity is detected at neuronal somata and proximal dendrites in most areas of the brain and in the hippocampus throughout the dentritic regions. Localization of \(\alpha_{1C}\) was further established by immunoelectron microscopy which disclosed that class C L-type channels are clustered at postsynaptic sites of excitatory synapses in the hippocampus (10). 

PKA increases the activity of L-type channels in neurons (12, 13) and in the heart which possesses only class C L-type channels (14, 15). Myocardial contraction is induced by Ca\(^{2+}\) influx through class C channels and up-regulated by \(\beta\)-adrenergic signaling resulting in PKA-mediated stimulation of these channels (14, 15). Only \(\alpha_{1C}\) and none of the auxiliary subunits is required for this effect (16). PKA phosphorylates the long form of neuronal and cardiac \(\alpha_{1C}\) on serine 1928 in vitro and in vivo (8, 17, 18). The C-terminal truncation deletes this site and the shorter channel protein is not phosphorylated by PKA (8, 18). Mutation of serine 1928 to alanine prevents PKA-mediated phosphorylation and functional up-regulation of the class C channel (19). 

In its inactive form, PKA is a tetramer consisting of two regulatory (R) and two catalytic (C) subunits (20). Four R subunits (RI\(\alpha, \beta, \gamma, \delta\)) and three C subunits (C\(\alpha, \beta, \gamma\)) are encoded by different genes. Binding of 4 cAMP molecules to homodimeric R subunits induces a conformational change, drastically reduces the affinity for C subunits, and promotes dissociation of C subunits which phosphorylate target substrates, thereby modifying their functions (20). The PKA holoenzyme is directed to a variety of substrates and intracellular
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locations by adapter proteins called kinase A anchor proteins or AKAPs (21, 22). The RII subunits bind tightly with a large hydrophobic surface along one side of an amphipathic helix in AKAPs. The RII tethering site is conserved among the otherwise structurally divergent AKAPs (21, 22). Peptides corresponding to the RII-binding site disrupt PKA-mediated regulation of AMPA-type glutamate receptors in neurons (23) and of L-type channels in skeletal muscle cells (24). Thus it is essential that PKA is anchored at or near certain substrates for their efficient phosphorylation and regulation. Both neurotransmitter-activated adenyl cyclase and the class C Ca\(^{2+}\) channel accumulate in the postsynaptic plasma membrane of dendrites. One potential mechanism that could facilitate rapid and precisely focused transmission of signals borne by cAMP to the channel involves the direct physical interaction between a PKA-AKAP complex and the target channel. We discovered that the neuronal class C L-type Ca\(^{2+}\) channel complex contains PKA as well as MAP2B, a neuronal AKAP (25, 26). Other neuronal AKAPs including AKAP15, AKAP150, or AKAP220 were not evident in the channel complex.

**EXPERIMENTAL PROCEDURES**

**Materials—**The ECL detection kit and protein G-Sepharose were purchased from Amersham Pharmacia Biotech. (γ-32P)ATP (111 TBq/mmol) was obtained from NEN Life Science Products, protein A-Sepharose from Sigma, microcin Lr from CalBiochem (San Diego, CA), and Pefabloc (4-[&-aminoethyl]-benzensulfonyl Fluoro) from Roche Molecular Biochemicals. PKA and PKC isoforms purified by established procedures (27–29) were obtained from Sigma, and Dr. P. J. Bertics, Department of Biomolecular Chemistry, University of Wisconsin (Madison, WI, respectively). The PKA inhibitory peptide PKI(5–24) and NH\(_{2}\)-LRRASLG-COOH (Remptide) were gifts from Dr. L. M. Graves, University of North Carolina (Chapel Hill, NC). All other reagents were purchased from commercial suppliers and were of standard biochemical quality. When indicated, the following protease inhibitors were present: pepstatin A (1 μg/ml), leupeptin (10 μg/ml), aprotonin (20 μg/ml), phenylmethylsulfonyl fluoride (200 nM), and calpain inhibitor I and II (8 μg/ml each).

**Antibodies—**The anti-CNC1 and anti-CNB1 antibodies were produced against peptides corresponding to residues 921–938 and 851–867, respectively. The latter contained epitopes two and three of α\(_{1C}\) and α\(_{1D}\), respectively, and characterized as described (7, 8, 30, 31). To produce phosphospecific antibodies for the PKA phosphorylation site at serine 1928 in MAP2B, 20 mM EDTA, 10 mM Tris-HCl, pH 7.4, and protease inhibitors (see “Materials”). Homogenates were centrifuged at 5,000 rpm for 2 min at 4 °C (JA-18 rotor) to remove larger cell fragments including nuclei. Membranes were collected by ultracentrifugation for 30 min (50,000 rpm at 4 °C) and solubilized on ice in 10 ml of Triton X-100 solubilization buffer and insoluble material removed by ultracentrifugation as described above. For the isolation of MAP2B by double-immunoprecipitation, membranes from one rat forebrain were solubilized in 2 ml of 1% SDS, 20 mM EDTA, 10 mM EGTA, 10 mM Tris-HCl, pH 7.4, and protease inhibitors for 20 min at 60 °C. SDS was neutralized by addition of 8 ml of Triton X-100 solubilization buffer and insoluble material removed by ultracentrifugation.

**Immunoprecipitation—**Proteins which nonspecifically bind to Sepharose beads were removed by preincubation with Sepharose CL-4B (75 μl of Sepharose/0.5 ml of extract) for 30 min and subsequent centrifugation. After washing the beads were incubated on ice with either 10 μg of affinity purified anti-CN1 antibody, 10 μg of control rabbit IgG, 2 μl of anti-GluR1 antiserum, 10 μg of affinity purified anti-CN1, or 0.5 μg of anti-NR-1 antisera. After 1.5 h, 3–5 μg of protein A-Sepharose, preswollen and washed three times with TBS (150 mM NaCl, 10 mM Tris-HCl, pH 7.4) containing 1% Triton X-100, were added and the samples were placed on a tilting mixer for 2.5 h at 4 °C. The immunocomplexes were sedimented by centrifugation and washed three times with 1% Triton X-100 in TBS and once with 50 mM Tris-HCl, pH 7.4. Samples were then either extracted with 20 μl of SDS sample buffer (5% SDS, 20 mM dithiothreitol, 10% sucrose, 2 mM EDTA, and 125 mM Tris-Cl, pH 6.8) for 20 min at 60 °C and subjected to SDS-PAGE (7) or incubated as described below for kinase assays or MAP2B association experiments.

For testing whether endogenous MAP2B binds directly to α\(_{1C}\), double immunoprecipitations were performed with anti-CN1C (8, 17) out of heart extracts. Initial immunocomplexes were treated with 30 μl of dissociation buffer (1.5% SDS, 50 mM Tris-Cl, pH 8, 5 mM dithiothreitol, 10 mM EDTA, protease inhibitors) for 20 min at 60 °C in a thermomixer, diluted with 450 μl of dilution buffer (1% Triton X-100, 10 mM EDTA and inhibitors) and spun. Supernatants were incubated with anti-CN1C or control rabbit IgG and protein A-Sepharose as described above. Immunocomplexes were washed, incubated for 2 h on a tilting mixer with 1 ml of the cytosolic brain fraction (see above), washed three times with 1% Triton X-100 in TBS and once with 10 mM Tris-HCl, pH 7.4, and analyzed by immunoblotting. In some MAP2B binding experiments, MAP2B was first purified to homogeneity by double immunoprecipitation from SDS solubilized brain membrane extracts with anti-MAP2B and protein G-Sepharose by the same procedure described for α\(_{1C}\). After the second immunoprecipitation MAP2B was eluted with dissociation buffer and, after addition of a 10-fold excess of dilution buffer, incubated with protein A-Sepharose-α\(_{1C}\) immunocomplexes (or control IgG immunocomplexes) obtained from cardiac tissue by double immunoprecipitation (see above). The purity of MAP2B isolated by double immunoprecipitation was analyzed by SDS-PAGE and silver staining and immunoblotting.

**Immunoblotting—**After SDS-PAGE using gels polymerized from 6% acrylamide and 0.16% bisacrylamide unless indicated otherwise, proteins were electrotransferred to nitrocellulose (0.2 μm pore diameter). Blots were blocked for 2 h with 10% skim milk powder in TBS (TBS-milk) and probed for 2 h with anti-CH1923–1932P (1:10 in TBS-milk), anti-CN1C (1:100 in TBS-milk), anti-PKA C subunit (1:50 in TBS-milk), anti-MAP2B (1:1,000 in TBS-milk), anti-AKAP150 (1:500 in TBS-milk), anti-AKAP220 (1:1,000 in TBS-milk), or anti-α-tubulin (1:1,000 in TBS-milk). Blots were washed five times with TBS-milk, incubated for 1 h with horseradish peroxidase-labeled sheep anti-mouse IgG or horseradish peroxidase-labeled protein A, diluted 2,000-fold and 1,100-fold in TBS-milk. Membrane was washed twice with 0.1% TBS/0.05% Triton X-100 in TBS with at least 8 changes, and developed with ECL reagent.
dipeptide, and 2.5 mg of phosphatidylserine for phosphorylation in the presence of PKC. Samples were incubated with 0.5–1 μg of PKA or PKC and 50 μM unlabeled ATP (for immunoblotting with anti-CH1923–1932P) or 0.2 μM [γ-32P]ATP (for autoradiography) in a thermomixer for 30 min at 32°C, washed three times with radiomimetic washing buffer (10 mM Tris-HCl, pH 7.4, 75 mM NaCl, 20 mM EDTA, 10 mM EGTA, 20 mM sodium pyrophosphate, 50 mM NaF, 20 mM 2-glycerol phosphate, 1 mM p-nitrophenyl phosphate), and once in 10 mM Tris-HCl, pH 7.4. Phosphorylation of α1C by endogenous kinase was carried out as above without any exogenous kinase added in the absence or presence of 5 μM cAMP. The pellets were extracted with SDS sample buffer (see above) before SDS-PAGE and immunoblotting with anti-CH1923–1932P or autoradiography.

For pretreatment with cAMP, immunocomplexes were incubated with various concentrations of cAMP in buffer containing 50 mM HEPES-NaOH, pH 7.4, 75 mM NaCl, 1 mM EGTA, for 10 min at 32°C in a thermomixer, and washed three times with 1% Triton X-100 in TBS and once in 10 mM Tris-HCl, pH 7.4. Samples were then phosphorylated by endogenous kinase with 50 μM unlabeled ATP in the absence or presence of 5 μM cAMP and 1 μM PKI for 25 min at 32°C before immunoblotting with anti-CH1923–1932P.

Kemptide Assay—Immunocomplexes were resuspended in phosphorylation buffer in the absence or presence of 1 μM PKI, 30 μM kemptide (NH2-L-RLASLG-COOH) and 10 μM [γ-32P]ATP were added and samples incubated in a thermomixer for 25 min at 32°C. The reaction was stopped by addition of 16% (final concentration) acetic acid. The supernatants were spotted onto P81 cation exchange paper (Whatman). The P81 paper was washed four times for 15 min with 0.1% phosphoric acid, and incorporation of 32P measured by Cerenkov counting. To ensure that PKC was active and capable of phosphorylation, 210-kDa long form of α1C was phosphorylated with purified PKC and analyzed by autoradiography as described previously (37, 38).

RESULTS
Serine 1928, which is included only in the 210-kDa form of α1C, has been identified as the main if not only phosphorylation site for PKA in this subunit (18). To produce a phosphospecific antibody that specifically recognizes α1C when phosphorylated at serine 1928, we immunized rabbits with a PKA-phosphorylated peptide covering residues 1923–1932 of α1C. The resulting antibody anti-CH1923–1932P detected a single band when total brain homogenate in the presence of phosphatase inhibitors was used for immunoblotting. The band migrated with an apparent molecular mass of 210 kDa in a gel made from 5% acrylamide (Fig. 1A, lane 2) and hardly entered the separating phase of a gel polymerized from 10% acrylamide, a classic property of the larger form of α1C (Fig. 1A, lane 1). These data indicate that anti-CH1923–1932P recognizes the long form of α1C with high specificity. To test whether α1C has to be phosphorylated by PKA for anti-CH1923–1932P binding, class C channels were solubilized with Triton X-100 in the absence of phosphatase inhibitors and immunoprecipitated with anti-NC1 which is directed against a different epitope in the central part of α1C. Under these conditions, α1C will be dephosphorylated by phosphatases present in the tissue and is not recognized by anti-CH1923–1932P (Fig. 1A, lane 3). However, if the immunocomplex containing α1C was phosphorylated with purified PKA before immunoblotting, anti-CH1923–1932P detected α1C (Fig. 1A, lane 4). This phosphorylation was mediated by PKA because it was completely blocked by the PKI (5–24) peptidomimetic (39). A highly specific PKA inhibitory peptide derived from the endogenous PKA inhibitor PKI (Fig. 1B, lanes 1–2) and [γ-32P]ATP (Fig. 2A, lanes 1–5) also resulted in phosphorylation of serine 1928; however, the phosphorylation was blocked by the PKI peptide (Fig. 1B, lanes 3 and 4). Thus, phosphorylation of serine 1928 was catalyzed by endogenous PKA that copurified with the class C channel. To ensure that PKC was active and capable of phosphorylating α1C, as described earlier (8), the immunocomplex was incubated with [γ-32P]ATP and PKC and analyzed by SDS-PAGE and autoradiography (Fig. 1C). The short (190 kDa) form of α1C, which is not a PKA substrate, was phosphorylated as efficiently as the long form (Fig. 1C, lane 2). In addition, in several experiments a third, phosphorylated polypeptide was observed migrating above the long form of α1C. No signals were detected in immunoprecipitates isolated with nonspecific antibody, demonstrating that immunosaturation of class C channels was specific (Fig. 1C, lane 1).

Channel complexes were immunoprecipitated in the absence of phosphatase inhibitors to promote dephosphorylation via endogenous protein phosphatases. Subsequent incubation with ATP and immunoblotting with anti-CH1923–1932P demonstrated that serine 1928 in α1C (Fig. 2A, lanes 1–3) was phosphorylated by an endogenous kinase during the in vitro incubation. Phosphorylation was blocked by the PKI peptide, suggesting that PKA is the endogenous kinase (Fig. 2A, lanes 4 and 5). In the PKA holoenzyme, activity of the C subunit is inhibited by the R subunit unless cAMP is added. As shown in Fig. 2B, cAMP markedly increased the phosphorylation rate suggesting that the PKA holoenzyme is tightly associated with
Class C channels were immunoprecipitated with anti-CNIC1 from Triton X-100-solubilized extracts of brain membranes prepared without phosphatase inhibitors and incubated under various conditions before SDS-PAGE and immunoblotting with anti-CH1923–1932P. Immunocomplexes were treated under phosphorylation conditions with 50 μM unlabeled ATP, as indicated. Immunoblotting revealed that cAMP substantially accelerates phosphorylation of α2C, C, lanes 1–4, D, lanes 4 and 5). Mock-pretreated samples retained cAMP-stimulated, PKI-sensitive kinase activity (C, lanes 5–10). Pretreatment with 1–5 μM PKI was added to samples analyzed in C, lanes 9 and 10. Pretreatment with 1–5 μM cAMP nearly completely eliminated phosphorylation of α2C (C, lanes 1–4, D, lanes 4 and 5). Mock-pretreated channel complexes and AMPA receptors were immunoprecipitated from Triton X-100 extracts of rat brain with anti-CNIC1 and anti-GluR1 antibodies, respectively. Precipitation was also performed with nonspecific control IgG. C subunit was only detected in the class C channel (Fig. 3). The specificity of the co-immunoprecipitation of PKA activity with class C channels was further confirmed by a peptide phosphorylation assay (Fig. 4). Immunocomplexes were incubated under phosphorylation conditions with [γ-32P]ATP and kemptide, a substrate peptide for PKA (39). Phosphorylation of kemptide was performed in the presence and absence of the PKI inhibitory peptide. PKI-insensitive phosphorylation was usually less than 20% and may reflect background activity of contaminating kinases. Shown is the difference between the total and PKI-insensitive phosphorylated, reflecting the PKI-sensitive portion which is mediated by PKA. PKA activity was severalfold higher in class C channel than AMPA receptor complexes or nonspecific immunoprecipitates (Fig. 4). Collectively, these data show that the presence of PKA in class C channel immunocomplexes is not due to nonspecific interactions between PKA and either immunoprecipitating antibodies or protein A-Sepharose. Furthermore, PKA does not bind to other integral membrane proteins such as AMPA receptors in an indiscriminatory fashion.

To investigate which AKAP is present in the class C channel complex and may mediate anchoring of PKA, protein overlay assays with 32P-labeled RIβ subunits were performed (37, 42). Two high molecular weight RI-binding proteins were evident in Triton X-100 extracts of brain membranes (Fig. 5, lane 4). The apparent Mr values of the RI tethering polypeptides suggested that they might correspond to two principal brain AKAPs, MAP2B (280,000) (25, 26) and AKAP150 (150,000) (34, 37). The larger AKAP was recovered in class C channel complexes isolated by immunoprecipitation (Fig. 5, lane 1). This interaction was specific for the class C channel because no signal was observed for immunoprecipitated AMPA receptor complexes or control antibodies (Fig. 5, lanes 2 and 3). The identity of the larger RII-binding protein in membrane extract was established as MAP2B by immunoblotting (Fig. 6A, upper panel, lane 4). MAP2B was selectively associated with the class C channel complexes isolated by immunoprecipitation (Fig. 6A, upper panel, lane 4). The interaction between MAP2B and the Ca2+ channel was specific because MAP2B was excluded from precipitates obtained with control or anti-GluR1 antibodies (Fig. 6A, upper panel, lanes 2 and 3). Although AKAP150 and AKAP220 (43) were evident in total membrane extracts (Fig. 6A, middle and lower panels, lane 4), these anchor proteins were not co-isolated with class C channels or AMPA receptors (Fig. 6A, middle and lower panels, lanes 1–3).
MAP2B and PKA Are Associated with Class C L-type Ca\textsuperscript{2+} Channels

Class C L-type channels are specifically localized and clustered in dendritic spines, the postsynaptic sites of excitatory synapses (10). MAP2B is a major microtubule-associated protein in the brain but microtubules are usually not detectable in postsynaptic sites (44). However, MAP2B has been shown to be present in dendritic spines by immunohistochemical methods (45). Accordingly, MAP2B may not only associate with microtubules but may also bind to other proteins independent of tubulin. These observations suggest that MAP2B and class C channels are present in the same subcellular compartment where they could associate with each other.

To exclude the possibility that MAP2B binding to class C channels occurred via microtubules formed after extraction of the particulate protein fraction with Triton X-100, nocodazole was added to the homogenization buffer. This agent disassembles microtubules and prevents polymerization of free tubulin which is present in the Triton X-100 extracts (Fig. 6B, lane 5). After preparation of Triton X-100 extracts in the presence of nocodazole, MAP2B was still associated with class C channel immunocomplexes (Fig. 6B, lane 2). The proportion of MAP2B recovered with the class C channel complex increased relative to total membrane extract upon addition of nocodazole (compare Fig. 6, A and B, upper panels). No MAP2B immunoreactivity was observed in immunocomplexes of class B N-type Ca\textsuperscript{2+} channels (Fig. 6B, lane 1) which share a high degree of sequence similarity with class C channels but are localized at presynaptic sites (46) or in other control precipitations (Fig. 6B, lanes 3 and 4). Of note, although tubulin was present in the Triton X-100 extracts, it was not detectable in class C channel complexes (Fig. 6B, lower panel). These data indicate that the interaction between class C channels and MAP2B does not require microtubules and is not mediated by tubulin.

To scrutinize the possibility that MAP2B binding to class C channels occurred during homogenization or after solubilization, brain tissue was homogenized at high dilution (500 ml of buffer/g of brain) in the presence of nocodazole. Membranes and nocodazole-resistant cytoskeleton were collected by ultracentrifugation and extracted with 1 ml of Triton X-100 solubilization buffer. Samples were cleared by ultracentrifugation. Immunoprecipitations were performed with anti-CNB1 (B, lane 1), anti-CNC1 (B, lane 2, C, lane 1), anti-GluR1 (B and C, lane 3), or control antibody (B, lane 4 and C, lane 2). 20 μl of brain extract was also directed loaded (lane 4). B and C, 100-mg samples of cerebral cortex were homogenized in 1 ml (B) or 50 ml (C) homogenization buffer supplemented with 10 μM nocodazole to disrupt microtubules. Membranes and cytoskeleton were collected by ultracentrifugation and extracted with 1 ml of Triton X-100 solubilization buffer. Samples were cleared by ultracentrifugation. Immunoprecipitations were performed with anti-CNB1 (B, lane 1), anti-CNC1 (B, lane 2, C, lane 1), anti-GluR1 (B and C, lane 3), or control antibody (B, lane 4 and C, lane 2). No MAP2B immunoreactivity was observed in immunocomplexes of class B N-type Ca\textsuperscript{2+} channels (Fig. 6B, lane 1) which share a high degree of sequence similarity with class C channels but are localized at presynaptic sites (46) or in other control precipitations (Fig. 6B, lanes 3 and 4). Of note, although tubulin was present in the Triton X-100 extracts, it was not detectable in class C channel complexes (Fig. 6B, lower panel). These data indicate that the interaction between class C channels and MAP2B does not require microtubules and is not mediated by tubulin.

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immunoblotting with anti-MAP2B (in the presence of dithiothreitol were run in duplicate on the same gel; half of the gel was used for silver staining and the other half for absence (lane 1). Blots were stripped with SDS and re-probed with anti-CNC1 to monitor the presence of αC, isoforms (upper panel). In some samples, EDTA, EGTA, and calpain inhibitors were omitted during the extraction of heart tissue resulting in the complete conversion of the long form of αC into its short form (upper lane, lane 3). MAP2B still associated with the immunoisolated αC, short form (lower lane, lane 3). As expected, control IgG immunoprecipitates (“Cont.”) did not result in MAP2B association (lower lane, lanes 2 and 4). B, MAP2B was extracted with SDS from a forebrain membrane fraction and purified to homogeneity by double immunoprecipitation with anti-MAP2B antibodies (see “Experimental Procedures”). After the second immunoprecipitation, MAP2B immunocomplexes were extracted with SDS sample buffer and analyzed after SDS-PAGE in the absence (left panel) and presence (middle panel) of dithiothreitol (DTT) by silver staining. Immunoprecipitates extracted with SDS sample buffer in the presence of dithiothreitol were run in duplicate on the same gel; half of the gel was used for silver staining and the other half for immunoblotting with anti-MAP2B (upper half of right panel) and anti-tubulin (lower half of right panel; the blots were aligned with the silver-stained lanes using the M, markers which were present on the different gel pieces as indicated between the images). Control precipitations were performed in parallel with control IgG (Cont.). The position of MAP2B in the silver-stained gel is indicated by arrows. Cytosolic extract was also directly loaded for immunoblotting as positive control for tubulin which is not present in the purified MAP2B fraction (B, lower half of right panel). C, to test for direct interaction between MAP2B and αC, MAP2B was eluted after double immunoprecipitation (see B) with dissociation buffer, SDS was neutralized with an excess of Triton X-100 and the extract added to the αC immunocomplex isolated from heart by double immunoprecipitation (see A). After incubation and washing, the immunocomplexes were analyzed by immunoblotting. MAP2B did associate with αC but not with control IgG (lanes 2 and 3). No endogenous MAP2B was detectable in the αC immunocomplex (lane 1). Results similar to those shown in A-C were obtained in two other experiments.

for the glutamate receptor and Ca\(^{2+}\) channel protein in the immunocomplexes.\(^{2}\)

Class C channel complexes consist of several subunits including αC, αδ, and β (6). To investigate whether MAP2B binds directly to αC, we took advantage of the fact that class C channels but not MAP2B are expressed in the heart. Class C channels were immunoprecipitated from cardiac extracts. Immunocomplexes were then dissociated with SDS at 60 °C and αC was re-immunoprecipitated. This protocol enables isolation of αC in the absence of other channel subunits or associated proteins (8, 10, 17). αC-IgG complexes or control IgG complexes were then incubated in the absence or presence of brain cytosol which is a source of native, soluble MAP2B. The cytosolic fraction had been cleared by ultracentrifugation to remove microtubules. MAP2B bound to immunocomplexes containing both size forms of αC but not to immunoprecipitates prepared with control IgG (Fig. 7A, lanes 1 and 2). No MAP2B was detectable in αC precipitates from heart when brain cytosol was omitted (Fig. 7C, middle panel, lane 1). Accordingly, αC alone is sufficient for MAP2B binding.

To test whether the C-terminal region present in the long but not short form of αC is necessary for MAP2B binding, heart extracts were prepared in the absence of EDTA, EGTA, and calpain inhibitors. Under these conditions, the long form of αC is completely converted into its short form (Fig. 7A, upper panel, lane 3). MAP2B still bound to the re-precipitated short form (Fig. 7A, lower panel, lane 3) arguing that the C terminus of the long form is not required for MAP2B binding.

It is possible that MAP2B binding to αC involves another protein present in the cytosolic fraction that served as source of native MAP2B. To address this point, MAP2B was purified by double immunoprecipitation with anti-MAP2B. When the resulting immunocomplexes were analyzed by SDS-PAGE and subsequent silver staining only two bands were visible with apparent molecular masses in the range of 280 and 50 kDa (Fig. 7B, middle panel). The 280-kDa polypeptide is most likely the expected MAP2B because this polypeptide comigrated with MAP2B immunoreactivity as determined by immunoblotting of another part of the same gel (Fig. 7B, right panel); the gel and nitrocellulose pieces have been aligned according to corresponding marker proteins present on the different pieces). The other polypeptide at 50 kDa is presumably the heavy chain of the antibody used in the immunoprecipitation because it was (in contrast to the 280-kDa polypeptide) also present in the control IgG precipitate (Fig. 7B, middle panel). To further address the identity of the IgG band at 50 kDa and to exclude the presence of an additional polypeptide in the MAP2B immunocomplex that might comigrate with the heavy chain of the antibody, the MAP2B and control IgG immunocomplexes were also analyzed by SDS-PAGE in the absence of dithiothreitol (Fig. 7B, left panel). Under these nonreducing conditions the 280-kDa polypeptide band was still present (arrow) but the 50-kDa band completely disappeared and two novel bands migrating around 150 and 100 kDa became apparent. These two bands likely correspond to the full antibody complex consisting of two heavy and two light chains and to the heavy chain core dimer, respectively. This analysis indicates the purity of MAP2B after double-immunoprecipitation.

After purification by this double immunoprecipitation procedure, MAP2B was eluted from the resin with SDS which was neutralized by an excess of Triton X-100. The extract was added to αC immunocomplexes which had been prepared in

\(^{2}\) M. A. Davare and J. W. Hell, unpublished data.
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FIG. 8. Phosphorylation of \(\alpha_{1C}\) by PKA does not regulate MAP2B binding. Crude rat brain membrane fractions were extracted with 1% Triton X-100 and class C channel complexes immunoprecipitated with anti-CNC1 (lanes 1–3, 5, and 6) or control IgG (lane 4) before incubation under phosphorylation conditions in the absence and presence of ATP, PKI, or exogenous PKA, as indicated at the bottom of the blots. Samples were washed and used for immunoblotting with anti-MAP2B (middle panel). Blots were stripped with SDS, reprobed with anti-Chi1923–1932P to monitor the phosphorylation of \(\alpha_{1C}\) (upper panel), and stripped and reprobed with anti-CNC1 to test for equal loading of channel complexes (lower panel). Phosphorylation by endogenous or exogenous PKA did not change association of MAP2B with \(\alpha_{1C}\). Of note, phosphorylation in the presence of exogenous PKA under our conditions results in near stoichiometric phosphorylation of the PKA sites of the \(\alpha_{1C}\) subunits (8).

Parallel from heart extracts by double immunoprecipitation. Under these conditions, the immunopurified MAP2B bound to the \(\alpha_{1C}\) complex (Fig. 7C, middle panel, lane 2) but not to a control IgG (lane 3). As expected, \(\alpha_{1C}\) immunocomplexes did not contain endogenous MAP2B (lane 1) and tubulin was not detectable in any of these samples (Fig. 7C, lower panel) indicating that neither free nor polymerized tubulin is necessary for MAP2B binding. Taken together, these data show that MAP2B can directly associate with \(\alpha_{1C}\).

\(\alpha_{1C}\) is an excellent substrate for PKA. Therefore, we tested whether phosphorylation by PKA regulates the association of MAP2B with the class C channel. Class C channel complexes were immunosolubilized and incubated under phosphorylation conditions before further washing and immunoblotting with anti-Chi1923–1932P, anti-MAP2B, and anti-CNC1 (Fig. 8). MAP2B association with the channel was not significantly altered upon phosphorylation by either endogenous or exogenous PKA as observed in the presence of ATP (Fig. 8, middle panel). Of note, the phosphorylation conditions had earlier been optimized to obtain near stoichiometric phosphorylation of \(\alpha_{1C}\) during the in vitro incubation with purified PKA under our conditions at least 0.85 mol of phosphate/mol of phosphorylation sites are incorporated (8). These findings suggest that phosphorylation of \(\alpha_{1C}\) by PKA does not regulate the interaction between MAP2B and \(\alpha_{1C}\).

**DISCUSSION**

AKAP-mediated targeting of PKA appears to be essential for efficient phosphorylation and regulation of ligand- and voltage-gated ion channels, including AMPA receptors and L-type channels (19, 23, 24). However, it is largely unclear how AKAPs themselves are recruited toward these PKA substrates. Our results suggest that the class C L-type Ca\(^{2+}\) channel complex contains a docking site for an AKAP-PKA complex. We demonstrate that the previously characterized AKAP MAP2B associates with the neuronal class C channels. MAP2B was the only detectable AKAP present in immunoprecipitated class C channel complexes. Neither AKAP150 nor AKAP220 were detected in class C channel complexes by RII overlay assays or immunoblotting. AKAP15, a recently discovered anchor protein is involved in recruiting PKA to the muscle L-type channel (47–49). AKAP15 is also expressed in heart and brain. The RII overlay binding procedure did not detect AKAP15 in class C L-type channel complexes isolated by immunoprecipitation from heart or brain\(^3\) under our conditions. Thus AKAP15 may not mediate PKA association with neuronal or cardiac class C channels. A recent report revealed that AKAP15 is associated with Na\(^+\) channels in brain (50). AKAP15 may route PKA to subcellular locations enriched Na\(^+\) channels rather than class C channels. Collectively, our data show that MAP2B association with the class C channel is specific with respect to both the channel and the AKAP. MAP2B did not bind AMPA receptors or class B N-type channels and no other AKAPs were coisolated with class C channels.

Class C L-type channels are specifically clustered in dendritic spines (10). In contrast, a high proportion of MAP2B is associated with microtubules which are absent in dendritic spines (44). However, MAP2B immunoreactivity is evident in dendritic spines and postsynaptic densities (45). Accordingly, MAP2B may bind to protein complexes other than microtubules at postsynaptic sites. The \(\alpha_{1C}\) subunit of neuronal L-type channel appears to be a docking partner for MAP2B at postsynaptic sites.

Like L-type channels (19, 24), AMPA receptors require anchored PKA for efficient phosphorylation and physiological regulation (23). However, two different approaches, immunoblotting with anti-C subunit antibodies (Fig. 3) and the kemptide phosphorylation assay (Fig. 4), failed to detect PKA in a complex with AMPA receptors. It cannot be ruled out that association of PKA with AMPA receptors is mediated by interactions that are not resistant to our extraction methods. However, our extraction and immunoprecipitation conditions were rather mild and did not involve high salt concentrations or ionic detergents as is necessary for solubilization of N-methyl-D-aspartate receptor protein complexes (51, 52). Furthermore, under the same conditions we were able to show by immunoblotting as well as the kemptide phosphorylation assay that PKA is associated with class C channel complexes, suggesting that those conditions are appropriate to study the interactions of complexes involving PKA, AKAPs, and ion channels. It is, therefore, possible that PKA anchoring in the vicinity of AMPA receptors involves another postsynaptic protein that serves as attachment point for the AKAP-PKA complex. Because immunoelectron microscopy of class C channels reveals a postsynaptic staining pattern very similar to that of AMPA receptors (10, 53), it is tempting to speculate that recruitment of MAP2B to postsynaptic sites by class C channels may facilitate phosphorylation of neighboring AMPA receptors.

Our work identifies the first specific interaction between an AKAP, MAP2B, and a protein, the class C L-type Ca\(^{2+}\) channel, that is an integral and specific component of the postsynaptic structure of excitatory synapses. The class C channel apparently plays a key role in recruiting PKA to the postsynaptic channel complex by providing a docking site for MAP2B. Accordingly, class C channels may be crucial for effective postsynaptic targeting of PKA which is an important player in multiple aspects of synaptic function including synaptic plasticity and neurotransmission.

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\(^3\) M. A. Davare, F. Dong, C. S. Rubin, and J. W. Hell, unpublished data.
