The voltage-sensitive sodium channel is regulated by cAMP-dependent protein kinase (PKA) phosphorylation. Using purified preparations of rat brain sodium channels, we have shown that the α subunit was phosphorylated by a co-purifying protein kinase. The co-purifying kinase was stimulated by cAMP and phosphorylated PKA substrate peptides. Both the regulatory and catalytic subunits of PKA were detected by immunoblotting in purified sodium channel preparations. Bound PKA was immunoprecipitated with anti-SP19 antibodies directed against the sodium channel α subunit. PKA bound to sodium channels phosphorylated the sodium channel α subunit on the same four serine residues as observed with exogenously added PKA, indicating that association with the sodium channel does not restrict the sites of phosphorylation. Analysis of proteins with high affinity for the type II α regulatory subunit of PKA in a gel overlay assay identified a 15-kDa cAMP-dependent protein kinase-anchoring protein (AKAP) in these preparations. Determination of its amino acid sequence by mass spectrometry revealed two peptides identical to AKAP15, a recently described AKAP that targets PKA to skeletal muscle calcium channels. The co-purifying AKAP was also immunoreactive with antibodies generated against AKAP15, and antibodies directed against AKAP15 co-immunoprecipitated the sodium channel. Our results indicate that PKA is bound to brain sodium channels through interaction with AKAP15. Association of AKAP15 with both skeletal muscle calcium channels and brain sodium channels suggests that it may have broad specificity in targeting PKA to ion channels for regulation.

Voltage-sensitive sodium channels are responsible for the generation of action potentials in nerve cells. The sodium channel purified from adult rat brain is composed of three glycoprotein subunits: α (280 kDa), β1 (36 kDa), and β2 (33 kDa) (1). The α subunit is sufficient to form voltage-sensitive sodium channels when expressed in mammalian cells or *Xenopus* oocytes (2–5). The brain sodium channel is rapidly phosphorylated by PKA in purified preparations (6–8), in synaptosomes (9), and in intact neurons or transfected mammalian cells expressing the type IIA sodium channel (8, 10) on four serine residues in the intracellular loop connecting homologous domains I and II of the α subunit (8). In neurons and transfected cells expressing type IIA brain sodium channels, phosphorylation of the α subunit by PKA reduces peak sodium current by 20–50%, with little change in the voltage dependence of activation or inactivation (11). This effect is blocked by a specific peptide inhibitor of PKA (PKI) and reversed by a mixture of catalytic subunits of phosphatases 1 and 2A (11). Similarly, activation of D1-like dopamine receptors in acutely isolated hippocampal neurons reversely reduces peak sodium current by activation of the cAMP pathway (12). The intracellular loop between domains I and II is necessary for regulation of sodium channels expressed in *Xenopus* oocytes (13), and phosphorylation of serine 573 in this loop is both necessary and sufficient for modulation of sodium channels expressed in *Xenopus* oocytes and transfected cells (12, 14).

The PKA holoenzyme is a tetramer composed of two regulatory subunits and two catalytic subunits (15, 16). When bound to the regulatory subunits, the catalytic subunits are maintained in an inactive state. The binding of two cAMP molecules/ regulatory subunit causes the dissociation of the catalytic subunits. Type II PKA is localized to specific subcellular compartments by association of the RII subunits with cAMP-dependent protein kinase-anchoring proteins (AKAPs) (17, 18). AKAPs are localized in many different compartments of a wide range of cell types and range in size from 15 to 420 kDa (18). Although AKAPs were originally defined by their ability to bind RII, a new class of dual-specificity AKAPs has recently been described that can also bind the type I regulatory subunit (19). AKAPs contain an RII-binding domain and a targeting domain that tethers the PKA holoenzyme near specific substrates (17, 18). Although AKAPs do not share significant primary sequence homology, the RII-binding domains each contain a conserved secondary structure motif consisting of an amphipathic helix structure (20, 21). A peptide derived from the amphipathic helix of human thyroid ARAP (Ht31) selectively disrupts RII-AKAP interactions (20, 21). Insertion of a proline residue into this peptide disrupts the helical structure and causes the loss of its RII-binding ability (20). The Ht31 anchoring inhibitor peptide has been used to determine the functional importance of PKA anchoring in intact cells.

PKA anchoring to specific subcellular locations is required for modulation of ion channels. In hippocampal neurons, basal α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor currents require anchored PKA and can be inhibited by the Ht31 inhibitor peptide (22). Kinase anchoring is also required for PKA-dependent potentiation of skeletal kinase-anchoring protein; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; Tricine, N(-2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; LCMS/MSMS, liquid chromatography mass spectrometry/mass spectrometry mass spectrometry.
AKAP15 Associated with Brain Sodium Channels

muscle L-type calcium channels, which is blocked by the H31 inhibitor peptide (23). PKA co-purifies and co-immunoprecipitates with skeletal muscle L-type calcium channels, demonstrating a physical link between the PKA holoenzyme and the channel complex (24). Association of PKA with skeletal muscle calcium channels is mediated by a 15-kDa AKAP (AKAP15) that is targeted to the membrane by N-terminal myristoyl and palmitoyl anchors (25). A PKA-binding peptide containing an amphipathic helix derived from AKAP15 blocks voltage-dependent potentiation of calcium channel activity (25).

Previous studies have shown that partially purified rat brain sodium channel preparations contain a cAMP-stimulatable protein kinase that phosphorylates the α subunit (6). In this study, we confirm that the co-purifying protein kinase is PKA and that it phosphorylates the α subunit on the same sites that are phosphorylated by PKA in vitro and in intact cells in response to cAMP. Both the RII and catalytic subunits of PKA are co-immunoprecipitated with the brain sodium channel. AKAP15 is present in purified sodium channel preparations and is also co-immunoprecipitated with the channel complex. Our results show that AKAP15 targets PKA to voltage-gated brain sodium channels as well as voltage-gated skeletal muscle calcium channels.

EXPERIMENTAL PROCEDURES

Materials—The H31, H31-P (20, 21), and SP48 (LYTEKRF-SSSHQSSLIR) (8) peptides were synthesized and purified by the University of Washington Molecular Pharmacology Protein Core Facility.

Purification of Rat Brain Sodium Channels and PKA—Rat brain sodium channels were purified as described by Hartshorne and Catterall (26) sequentially utilizing ion-exchange, hydroxylapatite, and wheat germ agglutinin-Sepharose chromatography. Quantitation of the sodium channel was based on 3H]saxitoxin binding as described previously (27). The catalytic subunit of PKA was purified from bovine heart as described previously (28).

Phosphorylation of Sodium Channel and Synthetic Peptides by an Endogenous Protein Kinase—0.5–1 pmol of purified sodium channel was incubated at 37 °C in 50 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 10 mM MgCl₂, 1 mM EDTA, 0.1% Triton X-100, and 0.1 mM [γ-32P]ATP (0.005 M cpm/μmol) in the presence or absence of 0.25 mM PKA or 5 μM cAMP. Phosphorylation reactions were terminated by heating at 65 °C for 3 min in 80 mM Tris-HCl (pH 6.8), 10% glycerol, 10 mM dithiothreitol, and 2% SDS (SDS sample buffer) and subsequently analyzed by SDS-PAGE on Novex 6% Tris/glycine gels (28). In some cases, individual phosphoprotein bands were excised from the gels, and 32P incorportation was quantified by liquid scintillation counting.

Synthetic peptides were phosphorylated by incubation at 37 °C in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, 0.1% Triton X-100, and 1 mM [γ-32P]ATP (0.005 M cpm/μmol) for 10 min in the presence of 0.5–1.0 pmol of purified sodium channel. Phosphorylation reactions were terminated by acidification to 16% acetic acid, spotted on P-81 paper, and washed twice for 10 min each with 1% phosphoric acid. Incorporation of 32P into peptides was quantified by liquid scintillation counting.

Two-dimensional Tryptic Phosphopeptide Analysis—Two-dimensional tryptic phosphopeptide mapping was carried out essentially as outlined previously (29, 30) in 1% (NH₄)₂CO₃ (pH 8.9), except that digestion was carried out with 1.2 μg of sequencing-grade trypsin.

Expression and Purification of RIIAs—Expression, purification, and biotinylation of RIIAs were carried out as outlined previously (24, 31).

RII Overlay and Immunoblotting—Conditions for SDS-PAGE, electrophoresis, and protein detection via antibody or RII-biotin overlay were the same as described previously (24). For RII overlays, nitrocellulose membranes were blocked in 10% normal horse serum, washed three times for 10 min each with Tris-buffered saline containing 0.05% Tween 20 (TBST), and then incubated for 2 h with 5 μg RIIAs in TBST. Where indicated, RIIAs was first preincubated with 0.4 μg H31 or H31-P peptide for 30 min at room temperature in TBST prior to incubation with goat anti-RII antibody (kindly provided by Dr. John Scott, Vollum Institute, Oregon Health Sciences University).

Co-immunoprecipitation of Endogenous Protein Kinase Activity, AKAP, and the Sodium Channel—Approximately 10 pmol of partially purified sodium channel were incubated at 0 °C with 20 μg of affinity-purified anti-SP19 antibody (32) or control antibody for 2 h at 4 °C in a final volume of 0.5 ml in 50 mM Tris-HCl (pH 7.4), 75 mM NaCl, 2.5 mM EDTA, and 0.1% Triton X-100 (buffer D). 5 μg of pre-swell protein A-Sepharose beads were added to each 0.5-ml reaction and incubated 0.5 h at 4 °C. Protein A-Sepharose beads containing precipitated complexes were sedimented by centrifugation, washed three times with 1 ml of buffer D and twice with 50 mM Tris-HCl (pH 7.5), 10 mM magnesium chloride, 1 mM EGTA, and 0.1% Triton X-100 (phosphorylation buffer). Assicated proteins were used in phosphorylation experiments as described above or eluted by incubation in SDS sample buffer for 10 min at 65 °C. SDS-eluted proteins were detected by SDS-PAGE as described above.

Co-immunoprecipitation of the Sodium Channel Using Anti-AP1 Antibodies—Approximately 10 pmol of partially purified rat brain sodium channel were incubated with 20 μg of anti-AP1 antibody in the presence or absence of 2 μg of AP1 peptide for 2 h at 0 °C in a final volume of 0.5 ml of buffer D. 2–5 μg of protein A-Sepharose beads were added to each 0.5-ml reaction and incubated for 0.5 h at 4 °C. Protein A-Sepharose beads containing precipitated complexes were washed twice with 1 ml of buffer D and three times with phosphorylation buffer. Precipitated sodium channel was detected by back-phosphorylation. Immunoprecipitated samples were incubated for 15 min at 37 °C in 0.05 ml of phosphorylation buffer containing 0.25 μg PKA and 0.1 mM [γ-32P]ATP (0.005 M cpm/μmol). The precipitated complexes were then washed three times with 1 ml of phosphorylation buffer, and proteins were eluted by incubation in SDS sample buffer at 65 °C for 10 min and separated on 6% Tris/glycine-polyacrylamide gels. γ-32P-labeled phosphoproteins were detected by autoradiography.

Isolation and Amino Acid Sequence Determination of Rat Brain Sodium Channel-associated AKAP—Rat brain membranes, prepared as described by Hartshorne and Catterall (26), were washed at high pH by dilution (1:25) in 10 mM CAPS at pH 12. Diluted membranes were incubated by rotation at 4 °C for 45 min and pelleted by centrifugation at 100,000 × g for 15 min. Washed membranes were resuspended in 2 mM Hepes/Tris (pH 7.6) and solubilized in 3% Triton X-100 and 0.025% egg phosphatidylycholine containing aprotinin (10 μg/ml), leupeptin (10 μg/ml), pepstatin A (1 μM), benzamidine (15.7 μg/ml), and 4-(aminomethyl)benzenesulfonfoly fluoride (1 mM). The sodium channel-associated AKAP was isolated from solubilized rat brain membranes based on the method of Gray et al. (25). Briefly, DEAE-Sephadex A-25 (16-ml packed bed volume) was equilibrated with 10 mM Hepes/Tris (pH 7.6), 0.1% Triton X-100, and 0.025% phosphatidylcholine containing aprotinin (10 μg/ml), leupeptin (10 μg/ml), pepstatin A (1 μM), benzamidine (15.7 μg/ml), and 4-(aminomethyl)benzenesulfonfoly fluoride (1 mM). The sodium channel-associated AKAP was excised from the polyvinylidene difluoride membrane was then washed extensively in 3% Triton X-100 (phosphorylation buffer). Associated proteins were used in phosphorylation experiments as described above, and fractions containing the 15-kDa RII-binding protein were pooled for further purification. Pooled fractions were incubated overnight at 4 °C with biotinylated RIIAs bound to cross-linked streptavidin-agarose as described by Gray et al. (25). The column was then sequentially washed with 30 ml of Buffer A, 30 ml of 1 M NaCl in Buffer A, and 30 ml of Tris-buffered saline. Proteins were eluted in 4-ml fractions by incubation with 0.1% SDS and 25 mM Tris-HCl (pH 7.4) for 10 min at room temperature. Eluted proteins were separated by SDS-PAGE, electrotransferred to nitrocellulose, and tested for RII binding using RII-biotin as a probe. Affinity-purified samples containing RII-binding activity were pooled, concentrated by ultrafiltration, and separated by SDS-PAGE. Proteins transferred to polyvinylidene difluoride membranes were visualized by staining with 0.1% Coomassie Blue in 50% (v/v) methanol for 1 min. The polyvinylidine diflouride membrane was then washed extensively in H₂O and destained in 50% (v/v) methanol and 5% (v/v) acetic acid for 2 min, followed by three 15-min H₂O washes. A 15-kDa band containing ~15 pmol of affinity-purified AKAP was excised from the polyvinylidene difluoride membrane and digested with 10 μg of sequencing-grade trypsin (33). Sequence data were obtained by mass spectrometry (LCMS/MSMS) coupled with on-line data base searching using the computer program SEQUEST (34). Using this approach, peptides were identified that corresponded to AKAP15 sequences.

Amino Acid Composition of the Sodium Channel-associated AKAP with AKAP15 Anti-peptide Antibodies—The isolated sodium channel-associated AKAP was analyzed by SDS-PAGE and electrotransferred to nitrocellulose. Immunoblotting and washing were carried out essentially as described above. Briefly, blots were blocked overnight in 5% milk in Tris-buffered saline and then incubated with affinity-purified rabbit polyclonal antibody directed against a 13-amino acid peptide (anti-AP1 antibody) derived from the amino acid sequence of AKAP15 (25) for
RESULTS

PKA Co-purifies with the Rat Brain Sodium Channel—We examined partially purified brain sodium channel preparations for endogenous protein kinase activity. Brain sodium channels were prepared using the method of Hartshorne and Catterall (26) through the wheat germ agglutinin-Sepharose chromatography step. These preparations were then incubated under phosphorylating conditions in the presence of \( \gamma^{32}\)PATP as described under “Experimental Procedures,” separated by SDS-PAGE, and visualized by autoradiography. A single major high molecular mass phosphoprotein of 250 kDa was detected (Fig. 1, first lane). This protein was also phosphorylated by exogenous PKA (third lane), and it was recognized in immunoblots by the anti-SP19 antibody (32) against the loop connecting domains III and IV of the sodium channel α subunit (fourth lane). These results show that the endogenous protein kinase specifically phosphorylates the sodium channel α subunit.

Inclusion of 5 \( \mu\)M cAMP resulted in a 156 ± 27% increase in \( \gamma^{32}\)PATP incorporation into the sodium channel α subunit (Fig. 1, second lane). The known PKA peptide substrate, sodium channel peptide SP48 (8), is a substrate for the endogenous kinase, and addition of cAMP consistently stimulated the phosphorylation of SP48 by 339 ± 57% (n = 8). In addition, the basal phosphorylation of SP48 was inhibited 60% by the specific PKA inhibitor peptide PKI-(5–24) (data not shown). These results indicate that the endogenous kinase is PKA.

Immunoblot analyses were performed using antibodies directed against both the catalytic and regulatory subunits of PKA to directly determine if they are present in sodium channel preparations. Sodium channel preparations were separated by SDS-PAGE, electrophoresed into nitrocellulose membranes, and probed with antibodies as described under “Experimental Procedures.” A single polypeptide of 55 kDa was identified using an anti-RIα antibody, corresponding to the expected SDS-PAGE mobility of RIIα in our gel system (Fig. 2). When blots were probed with the anti-catalytic subunit antibody, a band of 45 kDa was identified, demonstrating the presence of the catalytic subunit of PKA (Fig. 2). The presence of the RII and catalytic subunits in purified sodium channel preparations indicates that the PKA holoenzyme co-purifies with the sodium channel through several chromatographic purification steps and provides evidence for a physical association between the channel and kinase.

PKA Co-immunoprecipitates with the Sodium Channel—Co-immunoprecipitation experiments were used to further test whether PKA is physically associated with the sodium channel complex. The sodium channel was first immunoprecipitated from partially purified sodium channel preparations with the α subunit-specific anti-SP19 antibody (32) and then incubated under phosphorylating conditions in the presence of \( \gamma^{32}\)PATP and 5 \( \mu\)M cAMP as described under “Experimental Procedures.” Under these conditions, the sodium channel was phosphorylated by the co-precipitating PKA (Fig. 3). The co-precipitation of the kinase activity was specific since no kinase activity was detected when a control nonimmune IgG antibody was used (Fig. 3). These co-immunoprecipitation experiments demonstrate a physical association between the sodium channel complex and PKA.

Two-dimensional Tryptic Phosphopeptide Analysis—To determine which residues on the sodium channel are phosphorylated by the endogenous co-purifying PKA bound to the sodium channel, two-dimensional tryptic phosphopeptide maps of the phosphorylated α subunit were generated. Purified sodium channel was incubated under phosphorylating conditions in the presence or absence of exogenous PKA and \( \gamma^{32}\)PATP, and the phosphorylated α subunit was subjected to tryptic digestion and two-dimensional phosphopeptide mapping as described under “Experimental Procedures.” Fig. 4 shows that the bound PKA phosphorylated the same seven major tryptic phosphopeptides generated by exogenous PKA phosphorylation of the rat brain sodium channel in previous experiments with purified sodium channels, transfected cells, and neurons (7, 8). Phosphopeptide 4 is a minor variable tryptic phosphopeptide (8) and is not clearly visible in this exposure of the autoradiograph. An identical tryptic phosphopeptide map was also obtained when the endogenous kinase was stimulated by cAMP (data not shown). These results indicate that bound PKA phosphorylates serines 573, 610, 623, and 687 in the α subunit of the sodium channel, as observed previously in purified sodium channel preparations.
channels, transfected cells, and intact neurons (7, 8).

Detection of a Low Molecular Mass AKAP in Rat Brain Sodium Channel Preparations—We next determined whether AKAPs are present in partially purified sodium channel preparations. AKAPs were detected using a modified kinase overlay assay with RIIα as outlined under “Experimental Procedures.” Using this assay system, we detected a low molecular mass RII-binding protein of ~15 kDa (Fig. 5A). Preincubation of RIIα with Ht31, an anchoring inhibitor peptide, prevented recognition of the 15-kDa AKAP (Fig. 5A). Preincubation of RIIα with an inactive control peptide (Ht31-P) had no effect on RII binding to AKAP (Fig. 5A). These findings indicate that RIIα binding to the sodium channel-associated AKAP is a specific interaction. An RIIα-binding protein of ~80 kDa was also detected; however, the binding of RII to this protein was not consistently blocked by Ht31 (Fig. 5A). The ~55-kDa protein detected in all lanes was identified as RIIα using RII-specific antibodies alone (Fig. 5A).

In recent experiments, Gray et al. (24, 25) employed a RII-biotin overlay assay to detect AKAPs. The low molecular mass AKAP detected in partially purified sodium channel preparations was also detected using the RII-biotin overlay system (Fig. 5B) (24, 25). Labeling of this AKAP was selectively blocked by Ht31, whereas Ht31-P had no effect (Fig. 5B). Thus, two different AKAP overlay systems detected a 15-kDa AKAP present in rat brain sodium channel preparations.

Isolation and Identification of the Low Molecular Mass AKAP Present in Rat Brain Sodium Channel Preparations—The low molecular mass AKAP was isolated from rat brain membrane preparations by affinity chromatography on immobilized RIIα, based on the purification scheme used to isolate skeletal muscle AKAP15 (see “Experimental Procedures”) (25). During each purification step, the RII overlay assay was used to detect RII-binding proteins. Approximately 15 pmol of RII affinity-purified AKAP were separated by SDS-PAGE, electrotansferred to a polyvinylidene difluoride membrane, excised from

Fig. 3. Co-immunoprecipitation of the endogenous protein kinase activity with the sodium channel. Purified sodium channel was subjected to immunoprecipitation using the anti-SP19 antibody or nonimmune IgG as described under “Experimental Procedures.” Immunoprecipitated samples were incubated under phosphorylating conditions (25) containing [γ-32P]ATP and 5.0 μM cAMP and analyzed by SDS-PAGE on a 6% Tris/glycine gel. 32P-Labeled α subunits were visualized by autoradiography. Molecular mass markers are indicated in kilodaltons.

Fig. 4. Two-dimensional tryptic phosphopeptide analysis of the sodium channel. Purified sodium channel (NaCh) was incubated under phosphorylating conditions in the presence (A) or absence (B) of the catalytic subunit of PKA and processed for tryptic phosphopeptide analysis as described under “Experimental Procedures.” Tryptic phosphopeptides were separated in two dimensions by high voltage electrophoresis (pH 8.9) followed by thin-layer chromatography. Arrows designate the direction of electrophoresis (+) and chromatography (C). ENDOG, endogenous.

Fig. 5. Identification of a 15-kDa AKAP in partially purified brain sodium channel preparations. A, purified sodium channel was separated by SDS-PAGE, electrotansferred to nitrocellulose, and probed for AKAPs with RII as described under “Experimental Procedures.” The blotted channel was probed with RII alone, with RII preincubated with the Ht31 peptide, or with RII preincubated with the Ht31-P peptide. B, purified sodium channel was analyzed by SDS-PAGE, electrotansferred to nitrocellulose, and detected following an RII-biotin overlay assay as described (25). RII-biotin was employed alone or after preincubation with either the anchoring inhibitor peptide Ht31 or the proline-substituted Ht31-P peptide. Molecular mass markers are indicated in kilodaltons.
the blot, digested with trypsin, and subjected to mass spectrometry analysis (LC/MS/MS) coupled with data base searching (33). Two distinct peptide sequences that both match AKAP15 sequences (AVQQYLEETQNKK and DRKEPEDAELVR) were identified in these AKAP samples, indicating that the sodium channel-associated AKAP is AKAP15.

AKAP15 Associated with Brain Sodium Channels

To determine if AKAP15 and the rat brain sodium channel are physically associated, we performed co-immunoprecipitation studies. Polyclonal antibodies directed against the AP1 peptide sequence derived from AKAP15 (25) were used to probe blots containing isolated skeletal muscle AKAP15 and the co-purifying brain sodium channel AKAP (Fig. 6). The low molecular mass AKAP in brain sodium channel preparations was immunoreactive with polyclonal anti-AP1 antibodies (Fig. 6). Fig. 6 also shows that the apparent molecular masses of the brain and skeletal muscle AKAPs are the same. Together, these results show that skeletal muscle AKAP15 and the co-purifying brain AKAP are likely the same protein.

The Brain Sodium Channel Co-immunoprecipitates with AKAP15—To determine if AKAP15 and the rat brain sodium channel are physically associated, we performed co-immunoprecipitation studies. Polyclonal antibodies directed against the AP1 peptide were used to immunoprecipitate AKAP15 from partially purified sodium channel preparations. The presence of co-precipitating sodium channel was assessed by phosphorylation of the α subunit by PKA in the presence of \([\gamma^{32}\text{P}]\text{ATP}\) as described under "Experimental Procedures." The precipitated phosphoproteins were then separated by SDS-PAGE and subjected to autoradiography. Fig. 7 shows that the brain sodium channel α subunit co-precipitated with AKAP15 present in purified sodium channel preparations. To test the specificity of this co-precipitation, the AP1 antibody was preincubated with the AP1 peptide prior to immunoprecipitation. Co-immunoprecipitation of the sodium channel α subunit by the anti-AP1 antibody was substantially diminished by the preincubation with the AP1 peptide (Fig. 7). Furthermore, preimmune IgG did not immunoprecipitate the sodium channel α subunit (Fig. 7). These results further demonstrate a physical association of AKAP15 with the rat brain sodium channel complex.

**DISCUSSION**

**Phosphorylation of Sodium Channels by Associated PKA**—We have found that a protein kinase activity co-purifies with rat brain sodium channels through multiple chromatography steps and specifically co-immunoprecipitates with sodium channels. This kinase activity phosphorylates the α subunit of the sodium channel as well as specific PKA peptide substrates and is stimulated by addition of cAMP. This result is consistent with the findings of Costa et al. (6), who reported the presence of a cAMP-stimulated protein kinase in purified rat brain sodium channel preparations. Our immunoblots show that both the catalytic and regulatory subunits of PKA are present in purified sodium channel preparations, directly demonstrating the association of the PKA holoenzyme in brain sodium channel preparations.

Phosphorylation by the endogenous co-purifying protein kinase generated the same tryptic phosphopeptides as exogenous PKA, demonstrating phosphorylation of serines 573, 610, 623, and 687. Thus, association of PKA with the sodium channel does not alter the sites that it phosphorylates. Phosphorylation of Ser-573 is both necessary and sufficient for sodium channel regulation under the conditions studied to date (12, 14). However, phosphorylation of Ser-610, Ser-623, and Ser-687 by PKA specifically bound to sodium channels suggests that phosphorylation of these sites may also be important for PKA regulation of sodium channels.

**AKAP15 and PKA Are Associated with Sodium Channels**—Recent studies have shown that PKA activity also co-purifies with L-type calcium channels isolated from skeletal muscle, due to association with a 15-kDa RII-binding protein designated AKAP15 (24, 25). In this study, we demonstrate that AKAP15 co-purifies with rat brain sodium channels and likely mediates the association of the sodium channel and PKA. Three lines of evidence indicate that the AKAP associated with sodium channels is identical to skeletal muscle AKAP15. The co-purifying sodium channel AKAP is immunoreactive with antibodies generated against AKAP15. Antibodies directed against AKAP15 co-immunoprecipitate the brain sodium channel from partially purified sodium channel preparations, sug-
gesting that the brain sodium channel and AKAP15 are physically associated. Two peptides isolated from the AKAP associated with brain sodium channels are identical to sequences found within AKAP15 (25). The association of AKAP15 with both skeletal muscle calcium channels and brain sodium channels indicates that it is likely to be involved in a broad range of ion channel regulation by PKA.

**Physiological Role of AKAP15**—Brain sodium channels are regulated by PKA when expressed in non-neuronal cells or Xenopus oocytes without coexpression of AKAP15 (11–14). Thus, it is likely that the forms of sodium channel regulation studied to date can occur without AKAP15. For skeletal muscle calcium channels, the effect of AKAP15 anchoring of PKA is most striking for rapid, voltage-dependent potentiation on the millisecond time scale (23, 25). Presumably, immediate access of the kinase to its substrate is required for this rapid form of regulation. It will be of interest to search for rapid modes of sodium channel modulation in which anchored kinase may have a unique role in the channel regulatory process.

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