β2 Subunits of Sodium Channels from Vertebrate Brain

STUDIES WITH SUBUNIT-SPECIFIC ANTI-BODIES

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The sodium channel purified from rat brain is composed of three subunits: α (M, 260,000), β1 (M, 36,000), and β2 (M, 33,000). α and β2 subunits are linked through disulfide bonds. Procedures are described for preparative isolation of the β1 and β2 subunits under native conditions. Pure β2 subunits obtained by this procedure were used to prepare a specific anti-β2 subunit antiserum. Antibodies purified from this serum by antigen affinity chromatography recognize only disulfide-linked αβ2 complexes and β2 subunits in immunoblots, and immunoprecipitate 32P-labeled α subunits of purified sodium channels having intact disulfide bonds, but not those of sodium channels from which β2 subunits have been detached by reduction of disulfide bonds. These antibodies also immunoprecipitate 80% of the high affinity saxitoxin-binding sites from rat brain membranes, indicating that nearly all sodium channels in rat brain have disulfide-linked αβ2 subunits. Approximately 22% of β2 subunits in adult rat brain are not disulfide-linked to α subunits. Anti-β2 subunit antibodies are specific for sodium channels in the central nervous system and will not cross-react with sodium channels in skeletal muscle or sciatic nerve. The brains of a broad range of vertebrate species, including electric eel, are shown to express sodium channels with disulfide-linked αβ2 subunits.

The voltage-sensitive sodium channel mediates sodium influx during the rising phase of the action potential in electrically excitable cells. Sodium channels from rat brain, rat and rabbit skeletal muscle, and electric eel electroplax have been purified in functional form and characterized biochemically (reviewed in Refs. 1-4). The principal protein component of sodium channels from these three sources is an α subunit of Mr 260,000. In rat brain, α subunits are associated with β1 (Mr 36,000) and β2 (Mr 33,000) subunits. The β2 subunit is attached to α by disulfide bonds, whereas β1 is associated with α by noncovalent interactions (5-9). Sodium channels from skeletal muscle have a single β subunit which is noncovalently associated with α (10). Although we have consistently observed that α subunits of purified rat brain sodium channels are disulfide-linked to β2 subunits (5-8), β2 subunits were not observed in studies of rat brain sodium channels by two other groups (11, 12) and are not a component of purified sodium channels from skeletal muscle or electroplax. These results raise the possibility that a substantial fraction of sodium channels in adult rat brain might not contain β2 subunits.

The functional roles of the subunits of brain sodium channels have been studied by two approaches. Selective removal of the β1 subunits from purified sodium channels followed by reconstitution in phospholipid vesicles or bilayers results in inactive sodium channels (7, 8). In contrast, β2 subunits can be removed without effect (7, 8). Injection of mRNA encoding only the α subunits of the rat brain sodium channel into Xenopus oocytes leads to expression of functional channels (13-15), indicating that this mRNA contains all the information necessary to direct synthesis of functional sodium channels in the oocyte.

In this report, we describe a new procedure for preparative isolation of the β1 and β2 subunits under native conditions. Specific antibodies against pure β2 obtained by this method have been used to examine the association of α subunits with β2 subunits in rat brain and in neural tissues from a range of vertebrate species.

EXPERIMENTAL PROCEDURES

Materials—Protein A-Sepharose and bovine serum albumin were obtained from Sigma. Tetrodotoxin was from Behring Diagnostics. [γ-32P]ATP and [3H]labeled protein A were from Du Pont-New England Nuclear. Nitrocellulose was obtained from Bio-Rad. The catalytic subunit of cAMP-dependent protein kinase purified by the method of Bechtel et al. (16) was obtained from Dr. Sandra Rossi of this laboratory. Saxitoxin was obtained from the National Institutes of Health and [3H]saxitoxin was prepared, purified, and characterized as described previously (17). Sprague-Dawley rats were from Tyler Laboratories (Bellevue, WA). Rat optic nerves and sciatic nerves were obtained from Rockland Inc. Monkey brain (Macaca nemestrina) was obtained from the Regional Primate Center at the University of Washington. Electrophorus electricus was obtained from World Wide Scientific Tropical Fish, Apopka, FL.

Purification of the β1 and β2 Subunits by Elution from the α Subunit—Sodium channels purified through the step of chromatography on WGA-Sepharose or through the final step of sucrose gradient sedimentation as described by Hartshorne and Catterall (18) were adsorbed to hydroxylapatite resin in low phosphate buffer (50 mM NaP, pH 7.4, 68 mM Na2SO4, 0.2% Triton X-100, and 0.075% phosphatidylcholine), and 5 μM tetrodotoxin. Approximately 1 ml of settled resin was used per nanomole of partially purified sodium channel or 0.4 ml/nmol for highly purified preparations. After mixing for 30 min at 4 °C, the resin was sedimented in a clinical centrifuge and the supernatant was removed. One volume (1.0 ml) of 1M elution buffer (1.0 mM MgCl2 in low phosphate buffer) was added, the sample was mixed for 60 min, hydroxylapatite was sedi-

The abbreviations used are: WGA, wheat germ agglutinin; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Bis, N,N'-methylene bisacylamid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

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ment in a clinical centrifuge, and the supernatant containing α1 subunits was removed. The resin was washed with 4 volumes of β1 elution buffer, sedimented in the clinical centrifuge, resuspended with one volume (1.0 ml) of β2 elution buffer (0.1 mM histidine/Tris, 50 mM DTT in low phosphate buffer adjusted to pH 9.0), and incubated for 3 h. The sample was washed and the immunoprecipitated α1 subunits was removed. Subsequent incubation of the hydroxylapatite resin in 2 volumes of high phosphate buffer (0.4 M NaP, pH 7.4, 68 mM NaCl, 0.2% Triton X-100, and 0.075% phosphatidylcholine) for 30 min eluted the remaining proteins including α subunits.

**Immunoprecipitation of Anti-α and Anti-β2 Antibodies—** Approximately 300 pmol of α2 subunits were prepared as described above and injected into female New Zealand White rabbits with Freund's complete adjuvant. Subsequent booster injections contained Freund's incomplete adjuvant. Serum samples were collected and precipitated with saturated ammonium sulfate to prepare a purified IgG fraction. Approximately 300 pmol of purified sodium channels were resolved by nondenaturing PAGE as described by Hearing et al. (19) in the presence of 0.1% Triton X-100 and a linear gradient of acrylamide/Bis from 7%/0.04% to 20%/0.1%. Trace amounts of sodium channels were electrophoretically transferred to nitrocellulose sheets without saturation of the CY subunit by treatment of the sodium channel with 3ZP-labeled Bis from 7%/0.04% to 20%/0.1%. Trace amounts of sodium channels were recovered on a 2D gel blot as described previously (21). Anti-α subunit antibodies from antisera 7035 were purified by adsorption and elution from affinity absorbents prepared from α subunits resolved by SDS-PAGE as described previously (21). Solutions of affinity-purified antibodies were diluted approximately 10-fold compared to the starting antisera.

**Phosphorylation of Sodium Channels—** Sodium channels purified through the step of chromatography on WGA-Sepharose were phosphorylated with the catalytic subunit of cAMP-dependent protein kinase as previously described (23).

**Purification of Sodium Channels by Elution from the α Subunit Adsorbed to Hydroxylapatite—** The α2 subunit of the sodium channel is attached to the α subunit by disulfide bonds, whereas the β1 subunit is associated with the αβ2 complex by noncovalent interactions (1, 5–9). We have previously shown that the β1 and β2 subunits can be dissociated from the α subunit by treatment of the sodium channel complex with high ionic strength buffers to separate β1, or treatment with DTT to remove β2, followed by sedimentation on a sucrose density gradient (7). We have now developed a rapid and efficient preparative-scale procedure which takes advantage of the binding of α subunits to hydroxylapatite under conditions where the dissociated β1 and β2 subunits are not bound. Either purified sodium channel preparations (Fig. 1, lane 4) or partially purified sodium channels (Fig. 1, lane 1) were incubated with hydroxylapatite under conditions in which greater than 90% of the [3H]saxitoxin binding activity was bound to the resin. Washing with 1.0 M MgCl2 (Fig. 1, lane 2) released the β1 subunit in highly purified form. Subsequent washing with 50 mM DTT (Fig. 1, lane 3) eluted β2. The 3 subunits can be recovered by elution with a high phosphate buffer as described under "Experimental Procedures" (data not shown). They are obtained in highly purified form when fully purified sodium channels are used as the starting material, but not when partially purified channels are used.

**Immunoprecipitation of β2 Labeled Sodium Channel with Anti-β2 Antibodies—** Antibodies against β2 subunits were prepared and purified by adsorption to a native affinity matrix of immobilized β2 subunits after reduction of disulfide bonds or an affinity matrix of αβ2 complexes before reduction of disulfide bonds as described under "Experimental Procedures." The anti-β2 antibodies obtained from affinity matrices with reduced and nonreduced β2 subunits gave similar results in all assays tested.
Sodium channels are phosphorylated by cAMP-dependent protein kinase on the α subunit only (23). Increasing concentrations of anti-β2 subunit antibodies precipitate purified sodium channels containing 32P-labeled α subunits (Fig. 2, O). The binding of the antibodies is saturable with a maximum amount of approximately 10 fmol of sodium channels bound by 5 μl of the affinity-purified antibody solution. When the αβ2 disulfide bond is reduced by DTT, the anti-β2 antibodies are no longer able to immunoprecipitate the 32P-labeled α subunit (Fig. 2, O). Reduced and nonreduced sodium channel preparations react with the anti-β2 antibodies in a similar manner in radioimmunoassays (data not shown), and immunoprecipitation of nonreduced sodium channels containing 32P-labeled α subunits is not inhibited in the presence of reduced sodium channels (Fig. 2, O), indicating that reduction of internal disulfide bonds in the β2 subunit does not alter its immunoreactivity and that the reduced sodium channel preparation does not interfere with antibody binding to β2. Thus, we conclude that the immunoprecipitation of 32P-labeled α subunits by anti-β2 antibodies is due to the disulfide bond between β2 and α subunits in native sodium channels and that the anti-β2 antibodies are specific for β2 subunits alone. 

Immunoblot Analysis of Sodium Channels with Anti-β2 Antibodies—Sodium channels purified through the step of chromatography on WGA-Sepharose were analyzed by SDS-PAGE, and the resulting protein bands were electrophoretically transferred to nitrocellulose paper (21). The nitrocellulose was sequentially incubated with anti-β2 antibodies and 125I-protein A, and the immunoreactive protein bands were visualized by autoradiography. Under nonreducing conditions, one band of immunoreactivity is observed in the position of αβ2 (Fig. 3, lane 1). After reduction of disulfide bonds, this band disappears and a new band appears in the position of β2 (Fig. 3, lane 2). No immunoreactivity is observed at the migration position of the β2 subunit without reduction of disulfide bonds, indicating that there are no free β2 subunits in the purified preparation. No immunoreactivity is observed in the migration position of the β1 subunit or the free α subunit. These results provide further evidence that the antibodies are specific for β2 and do not cross-react with either the α or the β1 subunits.

β2 subunits in rat brain P3 membranes can also be detected with anti-β2 antibodies (Fig. 3, lanes 3 and 4). In this preparation, sodium channels account for less than 0.1% of the total protein present (18). Before reduction of disulfide bonds, two major bands of immunoreactivity are observed in the positions of αβ2 complexes and free β2 subunits (Fig. 3, lane 3). Upon reduction, the immunoreactive αβ2 band disappears and the counts per minute in the β2 band increases 4.4-fold (Fig. 3, lane 4). Thus, free β2 subunits accounted for 22.5 ± 2.5% of the β2 subunits in rat brain membranes. During purification, the free β2 subunits are separated from native sodium channels and only β2 subunits that are disulfide-linked to α subunits are found in the purified preparation (Fig. 3, lanes 1 and 2).

Immunoprecipitation of Saxitoxin Receptor Sites—Saxitoxin binds to the intact sodium channel and blocks ion transport. High affinity for saxitoxin is retained after solubilization. Since purified sodium channels from non-neuronal tissues do not contain β2 subunits, it was of interest to determine what fraction of sodium channels from rat brain contain a β2 subunit. Purified sodium channels were incubated with °H]saxitoxin and anti-β2 antibody-protein A-Sepharose complex, and the resulting complex was collected on GF/C filters by rapid filtration and washed to determine.
due to antibody reaction with β2 subunits, an equal amount of $^{32}$P-labeled sodium channels was immunoprecipitated under the same experimental conditions. Before reduction of disulfide bonds, greater than 90% of all the $^{32}$P-labeled sodium channels were immunoprecipitated under these conditions, whereas only background levels of $^{32}$P were immunoprecipitated after reduction of disulfide bonds, as observed under other conditions in Fig. 2 (data not shown). Thus, at least 89% of the sodium channels in purified preparations contain disulfide-linked β2 subunits.

A similar assay was performed on unpurified sodium channels. Rat brain P3 membranes, which contain approximately 80% of the sodium channels in the brain (27), were prepared and solubilized with 3% Triton X-100, 0.02% phosphatidylcholine. Immunoprecipitation of the sodium channels with high affinity for saxitoxin was measured by the same procedure as for purified channels (Fig. 4, O). As with purified sodium channels, 89% of the $[^3H]$saxitoxin binding activity was immunoprecipitated with the β2 antibodies, whereas 11% remained in the supernatant. These results show that at least 89% of sodium channels in purified preparations and in intact rat brain membranes contain disulfide-linked β2 subunits.

### Analysis of Cross-reactivity of Anti-β2 Antibodies with Sodium Channel Subtypes in Different Tissues

In previous studies, we have shown that antibodies against the α subunits of sodium channels from rat brain block immunoprecipitation of $^{32}$P-labeled sodium channels with antibodies to $\alpha$-subunits (24). To determine whether anti-β2 antibodies have similar specificity, we have measured their cross-reactivity by radioimmunoassay (Fig. 5). Sodium channels solubilized from rat brain block immunoprecipitation of $^{32}$P-labeled sodium channels without reduction; SDS-PAGE with or without reduction by β-mercaptoethanol and electrophoretically transferred to nitrocellulose (21). Transblots were incubated with anti-β2 antibodies followed by affinity-purified anti-β2 antibodies, protein A-Sepharose, and $[^3H]$saxitoxin; the sodium channels were immunoprecipitated under these conditions, whereas 11% remained in the supernatant. To verify that immunoprecipitation of 88% of the sodium channels was indeed

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**Fig. 3.** Immunoblot analysis of sodium channels with purified anti-β2 antibodies. Lysed P3 membranes were prepared from rat brain (27) and sodium channels were solubilized and purified through the step of chromatography on WGA-Sepharose (18). Samples were analyzed by 7–20%. SDS-PAGE with or without reduction by β-mercaptoethanol and electrophoretically transferred to nitrocellulose (21). Transblots were incubated with anti-β2 antibodies followed by $^{125}$I-protein A (21), and labeled protein bands were visualized by autoradiography. Lane 1, purified sodium channels analyzed without reduction; lane 2, purified sodium channels analyzed with reduction; lane 3, proteins solubilized from lysed P3 membranes and analyzed without reduction; lane 4, proteins solubilized from lysed P3 membranes and analyzed with reduction. The percentage of free β2 subunits in brain membranes was determined by excising the β2 bands from lanes 3 and 4 and measuring the bound $^{125}$I-protein A by gamma counting.

**Fig. 4.** Immunoprecipitation of saxitoxin receptor sites. Sodium channels solubilized from lysed P3 membranes (27) or purified sodium channels (O) were incubated with affinity-purified anti-β2 antibodies, protein A-Sepharose, and $[^3H]$saxitoxin; the sodium channels were immunoprecipitated; and the amounts of saxitoxin binding activity in the pellets and supernatants were determined as described under "Experimental Procedures." The results are plotted as a percentage of the total saxitoxin binding activity recovered in the pellet and supernatant. STXR represents saxitoxin receptor.

**Fig. 5.** Radioimmunoassay of the cross-reactivity of anti-β2 antibodies with sodium channels in various rat tissues. Tissue extracts were prepared and radioimmunoassays were performed as described under "Experimental Procedures." A, optic nerve; O, sciatic nerve; D, sciatic nerve extract containing 50 fmol of sodium channels plus brain extract containing 50 fmol of sodium channels. B, brain; O, skeletal muscle; D, skeletal muscle extract containing 50 fmol of sodium channels plus brain extract containing 50 fmol of sodium channel.
channels by anti-β2 antibodies completely, with half-maximal inhibition at 5 fmol of sodium channel/μl of antibody solution (Fig. 5B, •). In contrast, sodium channels solubilized from rat optic nerve, a central myelinated tract, inhibit immunoprecipitation of 32P-labeled brain sodium channels as effectively as sodium channels solubilized from rat brain (Fig. 5A, •). However, sodium channels in the sciatic nerve, a peripheral myelinated nerve, cross-react poorly with these anti-β2 antibodies (Fig. 5A, ○). Fifty fmol of sodium channels from sciatic nerve inhibit less than 20%, whereas an equivalent amount of sodium channels from brain or optic nerve completely blocks immunoprecipitation. It is possible that this small inhibition by sodium channels from sciatic nerve is nonspecific due to the large amounts of protein present in these samples. Mixtures of sodium channels from skeletal muscle and brain (Fig. 5B, □) or sciatic nerve and brain (Fig. 5A, □) inhibit immunoprecipitation as effectively as sodium channels from brain alone, indicating that there is nothing present in the tissue samples that prevents binding of the anti-β2 antibodies to the sodium channels in these tissues. Thus, these polyclonal anti-β2 antibodies are specific for sodium channels from the central nervous system. Sodium channels from skeletal muscle and sciatic nerve either have no β2 subunits or contain a different subtype of β2 subunits that cross-react poorly with these antibodies.

**Immunoprecipitation of Sodium Channels from Different Regions of the Rat Central Nervous System with Anti-β2 Antibodies**—The immunoreactivity of sodium channels from different regions of the rat central nervous system with anti-β2 antibodies was examined in immunoprecipitation experiments. The cerebral cortex, retina, and spinal cord, and the fiber tracts of the optic nerve, corpus callosum, cauda equina, and sciatic nerve were dissected, solubilized with Triton X-100, immunoprecipitated with anti-β2 antibodies, and phosphorylated with cAMP-dependent protein kinase. Ten fmol of sodium channels, as measured by high affinity saxitoxin binding, were analyzed by SDS-PAGE with or without reduction of disulfide bonds as indicated below each gel lane, and the 32P-labeled, immunoreactive protein bands were visualized by autoradiography. All the regions of the central nervous system that were studied contained sodium channel α subunits which were immunoprecipitated by anti-β2 antibodies (Fig. 6, samples 1–6). No immunoreactive α subunits were observed in samples from sciatic nerve (Fig. 6, sample 7). In each case, the apparent M, of the immunoprecipitated α subunits was reduced from 300,000 to 260,000 upon reduction of disulfide bonds, indicating that these immunoprecipitated α subunits are disulfide-linked to β2 subunits in these brain regions.

The immunoreactive α subunit bands from cerebral cortex, spinal cord, and retina, which contain neuronal cell bodies and primarily unmyelinated nerve fibers, and from corpus callosum, which contains myelinated nerve fibers, are labeled well by this method (Fig. 6, samples 1, 2, 4, and 5). In contrast, the labeling of the immunoreactive α subunit bands from optic nerve and cauda equina, which contain primarily myelinated nerve fibers, are significantly less intense (Fig. 6, samples 3 and 6). Since radioimmunoassays have shown that the anti-β2 antibodies bind sodium channels from optic nerve (Fig. 5) and cauda equina (data not shown) as well as those from the cortex, it seems likely that the lower intensity is due to reduced phosphorylation of the sodium channels immunoprecipitated from optic nerve and cauda equina. Sodium channels from the same regions of the central nervous system were examined using anti-α subunit antibodies with similar results (data not shown).

**Expression of Sodium Channels with Disulfide-linked αβ2 Subunits in the Brains of Higher Vertebrates**—Since the sodium channels in rat skeletal muscle and sciatic nerve differ in their immunoreactivity with anti-β2 antibodies, and β2 subunits have not been detected in sodium channels from electric eel electroplax, it was of interest to determine whether the expression of sodium channels having disulfide-linked α-β2 subunits is restricted to rat brain or is a general characteristic of the central nervous system of a broad range of species. The brains of representative higher vertebrates (Fig. 7, samples 1–6) including mammals (rat and monkey), amphibians (frog), avians (chicken), reptiles (gecko), bony fish (electric eel and salmon (Oncorhyncus kisutch, data not shown)) and the main electric organ of the electric eel (Fig. 7, sample 7) were dissected on ice, rapidly homogenized in the presence of protease inhibitors, solubilized, and immunoprecipitated with anti-α subunit antibodies (Fig. 7A) or anti-β2 subunit antibodies (Fig. 7B). The sodium channels in the immunoprecipitates were phosphorylated, resolved by SDS-PAGE, and visualized by autoradiography. Our anti-α subunit antibodies immunoprecipitated 32P-labeled sodium channel α subunits from the brains of all species tested (Fig. 7A, samples 1–6), although the band from samples of monkey brain was weak. In contrast, no sodium channel α subunits were immunoprecipitated from the electric eel electroplax (Fig. 7A, sample 7). The apparent M, values of the α subunits from the brain of each species were reduced by approximately 40,000 upon reduction of disulfide bonds (Fig. 7A), indicating that each sodium channel has a disulfide-linked subunit that is analogous to β2. The apparent M, values of the reduced α subunits were 260,000 for rat, monkey, and chicken, 243,000 for gecko and frog, and 230,000 for salmon and eel.

Anti-β2 subunit antibodies were also used to immunoprecipitate 32P-labeled sodium channel α subunits from these tissues. Immunoreactive sodium channels were observed in rat, monkey, and chicken (Fig. 7B, samples 1, 2, and 4). Reduction of disulfide bonds reduced the apparent M, of the immunoprecipitated α subunits from 300,000 to 260,000, indicating that they contained disulfide-linked β2 subunits. Sodium channels from frog, fish (both eel and salmon), and gecko were not recognized by this antibody (Fig. 7B, samples 3, 5–7). Evidently, the antigenic determinants recognized by
Our anti-β2 antibodies are not highly conserved in these species.

**DISCUSSION**

**Preparative Separation of the Subunits of the Sodium Channel from Rat Brain**—Previous studies in this laboratory have shown that the voltage-sensitive sodium channel from rat brain consists of a complex of α, β1, and β2 subunits with disulfide linkage between α and β2 (5–7). We have previously separated these three subunits analytically using gel filtration under denaturing conditions (6) or sucrose gradient sedimentation under native conditions (7, 8). Neither of these previous methods provides sufficient yield of native subunits for preparative work. The method for separation of these three subunits by adsorption to hydroxylapatite and sequential elution with high ionic strength buffer and then DTT that we describe here is rapid and efficient and provides a high yield of purified subunits. In contrast to denatured β2 subunits recovered from SDS gels,2 β2 subunits obtained in this manner are immunogenic and can be used to develop specific anti-β2 subunit antibodies.

**Nearly All Sodium Channels in Adult Rat Brain Contain Disulfide-linked αβ2 Subunits**—Anti-β2 subunit antibodies purified by antigen affinity chromatography recognize only the β2 subunit of the sodium channel in immunoblots. These antibodies immunoprecipitate 32P-labeled sodium channel α subunits from rat brain only when the disulfide bonds between α and β2 subunits are intact. They immunoprecipitate at least 89% of the sodium channels in rat brain membranes that have high affinity for saxitoxin. These results provide conclusive evidence that most, if not all, sodium channels solubilized from adult rat brain contain a disulfide-linked complex of α and β2 subunits. It is unlikely under the conditions of our experiments that this complex could arise by artifactual proteolytic cleavage of a pre-existing single polypeptide containing both subunits. Moreover, artifactual proteolytic cleavage is also ruled out by our unpublished results indicating that both of these subunits have blocked amino termini.

One report has appeared that confirms the presence of α, β1, and β2 subunits in purified preparations of rat brain sodium channels as we have previously described (9). In contrast, two other laboratories used similar procedures for purification of rat brain sodium channels but did not detect either β1 or β2 subunits (11, 12). These results are inconsistent with our present data, which show that nearly all of the sodium channels in rat brain membranes do indeed contain a disulfide-linked complex of α and β2 subunits. We cannot offer a definite explanation of this apparent discrepancy. However, we note that the β1 and β2 subunits comprise only approximately 8% of the mass of the purified sodium channel each and are variably visualized by different iodination and silver staining procedures used in analysis of the polypeptide composition of these purified preparations.

**β2 Subunits Are Present in Excess in Adult Rat Brain**—After reduction of disulfide bonds, affinity-purified anti-β2 antibodies recognize only the β2 subunit in immunoblot analyses of both purified sodium channels and sodium channels in brain membranes. Under nonreducing conditions, these antibodies recognize only disulfide-linked αβ2 complexes in purified sodium channel preparations, indicating that all the β2 subunits in these purified preparations are disulfide-linked to α subunits. In contrast, only 77.5% of the β2 subunits detected in brain membranes under nonreducing conditions are in αβ2 complexes, whereas 22.5% are not disulfide-linked to other proteins. Evidently, these free β2 subunits in brain membranes are separated from the sodium channel during purification. Our results do not establish whether they are bound to α subunits by noncovalent interactions before solubilization.

The function of this free pool of β2 subunits in the adult brain is uncertain. Neurons in the developing rat brain contain a large intracellular pool of α subunits that are not disulfide-linked to β2 subunits (25, 28). The β2 subunits are disulfide-linked to α subunits coincident with the appearance of the sodium channel complex in the cell surface membrane, and this process may be a rate-limiting step in assembly or membrane insertion of mature sodium channels. In the adult, free β2 subunits may insure that newly synthesized α subunits are inserted into the plasma membrane soon after synthesis. Alternatively, free β2 subunits may have an entirely different function in the adult brain.

**Immunoreactive β2 Subunits Are Detected in the Central Nervous System, but Not in Peripheral Nerve or Skeletal Muscle**—Previous work has shown that different sodium channel α subunit subtypes are present in various rat tissues. Sodium channels in brain and skeletal muscle are distinguished by μ-conotoxins which bind at the same receptor site as saxitoxin (29–32) and by polyclonal antibodies specific for α subunits (24). Sodium channels in the central and peripheral nervous systems are distinguished by polyclonal antibodies against the α subunits (24). Within the central nervous system, three distinct mRNAs for α subunits have been detected (33). In this report, we show by radioimmunoassay that anti-β2 subunit antibodies react with sodium channels from brain

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2 W. Downey, unpublished observations.
or from the myelinated fibers of the optic nerve, but not with sodium channels from skeletal muscle or from the myelinated fibers of the sciatic nerve. Similarly, these antibodies immunoprecipitate disulfide-linked αβ2 complexes from several different central nervous system sites but not sodium channels from sciatic nerve. Thus, we conclude that the β2 subunits of central nervous system sodium channels are not expressed in peripheral nerve or skeletal muscle. If sodium channels in peripheral nerve have disulfide-linked β2 subunits, they must be antigenically distinct from those of the sodium channels in the central nervous system. No disulfide-linked β subunit has been identified in purified preparations of sodium channels from skeletal muscle (2, 10) in agreement with our results indicating that there are no β2 subunits which cross-react with our antibodies.

Sodium Channels with Disulfide-linked αβ2 Subunits Are Expressed in the Central Nervous Systems of a Broad Range of Higher Vertebrates—Since sodium channels isolated from rat and rabbit tissues have one or two β subunits whereas those from electric eel electroplax do not (1, 4), it was of interest to examine sodium channels in the brains of a range of vertebrate species for the presence of disulfide-linked β2 subunits. Since our assay system involves immunoprecipitation and phosphorylation, both antigenic sites and CaM-dependent phosphorylation sites must be conserved in order for us to detect α subunits in these various species. In the brains of all species tested with anti-α subunit antibodies, a major immunoreactive protein band was detected with an apparent molecular weight of 270,000–300,000 before reduction of disulfide bonds and 230,000–260,000 after reduction. This electrophoretic behavior is characteristic of sodium channel αβ2 complexes and α subunits, indicating that sodium channels containing disulfide-linked αβ2 complexes are expressed in the brains of species representing each of the major groups of higher vertebrates.

When the same experiment was performed using anti-β subunit antibodies, αβ2 complexes and α subunits were observed in rat, monkey, and chicken brain, but not in the other species tested. Evidently, the β2 subunits of the other species examined do not have adequate immunological cross-reactivity with our antibodies to be detectable by this assay. The sodium channels of the eel electroplax were not recognized by α- or β2-specific antibodies in either immunoprecipitations or radioimmunoassays, indicating that they are antigenically distinct from central nervous system sodium channels from both rat and eel.

Our results demonstrate the expression of sodium channels with disulfide-linked αβ2 subunits in the central nervous system of a broad range of vertebrates. All α subunits recognized by our antibodies contain disulfide-linked β2 subunits in each of these species. In rat brain, we have shown that greater than 89% of all sodium channels have disulfide-linked αβ2 subunits. We consider it likely that this is true for central nervous system sodium channels in all the species we have studied. However, our results do not directly prove this point because the other species we have studied may have additional sodium channel subtypes that are not recognized by our antibodies. Nevertheless, our results confirm the presence of β2 subunits as a component of sodium channels from vertebrate brain and provide additional evidence for tissue-specific differences in the expression of sodium channel subtypes that differ in antigenic properties and subunit composition. Determination of the primary structural features that specify these differences in sodium channel properties and assessment of their physiological implications are important areas for further work.

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