Biochemical Properties of Sodium Channels in a Wide Range of Excitable Tissues Studied with Site-Directed Antibodies†

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ABSTRACT: Antibodies against a peptide (SP19) corresponding to a highly conserved, predicted intracellular region of the sodium channel α subunit bind rat brain sodium channels with a similar affinity as the peptide antigen, indicating that the corresponding segment of the α subunit is fully accessible in the intact channel structure. These antibodies recognize sodium channel α subunits from rat or eel brain, rat skeletal muscle, rat heart, eel electroplax, and locust nervous system. α subunits from all these tissues except rat skeletal muscle are substrates for phosphorylation by cAMP-dependent protein kinase. Disulfide linkage of α and $\beta 2$ subunits was observed for both the R_I and R_{II} subtypes of rat brain sodium channels and for sodium channels from eel brain but not for sodium channels from rat heart, eel electroplax, or locust nerve cord. Treatment with neuraminidase reduced the apparent molecular weight of sodium channel α subunits from rat and eel brain and eel electroplax by 22 000–58 000, those from heart by 8000, and those from locust nerve cord by less than 4000. Our results provide the first identification of sodium channel α subunits from rat heart and locust brain and nerve cord and show that sodium channel α subunits are expressed with different subunit associations and posttranslational modifications in different excitable tissues.

The voltage-sensitive sodium channel mediates the increase in sodium permeability that is responsible for the rising phase of the action potential in nerve, skeletal muscle, and heart. Sodium channels isolated in functional form contain a large α subunit with apparent M_r of 260 000 associated with a variable number of small subunits (Agnew, 1984; Barchi, 1984; Catterall, 1984, 1986). In rat brain, α subunits are associated noncovalently with a $\beta 1$ subunit with M_r of 36 000 and are disulfide linked to a $\beta 2$ subunit with M_r of 33 000. In rat and rabbit skeletal muscle, α subunits are associated noncovalently with a single subunit with M_r of 38 000. In eel electroplax, smaller subunits have not been detected. $\beta 2$, but not $\beta 1$, subunits can be removed from the purified rat brain sodium channel without loss of functional activity (Messner & Catterall, 1986; Messner et al., 1986), while the purified electroplax sodium channel is functionally active as a single α subunit (Tomiko et al., 1986; Recino-Pinto et al., 1987).

cDNA clones encoding the α subunits of eel electroplax (Noda et al., 1984), three forms of α subunits of rat brain sodium channels (Auld et al., 1985; Mandell et al., 1986; Noda et al., 1986a), and α subunits of one sodium channel subtype from *Drosophila* (Salkoff et al., 1987) have been isolated and characterized, and the complete primary structures of the α subunits of the sodium channel from eel electroplax and two sodium channel subtypes from rat brain (R_I and R_{II}) have been deduced (Noda et al., 1984, 1986a). Consistent with a primary functional role for the α subunit, high molecular weight mRNA from brain (Sumikawa et al., 1984), α subunit mRNA isolated by hybrid selection (Goldin et al., 1986), and α subunit mRNA made from cloned cDNA (Noda et al., 1986b) can all direct the synthesis of functional sodium channels in *Xenopus* oocytes.

Studies with site-directed antibodies which specifically recognize the R_I and R_{II} sodium channel subtypes show that they are the primary sodium channel subtypes expressed in spinal cord and brain, respectively, but are not measurably expressed in skeletal muscle, heart, and several sites in the peripheral nervous system (Gordon et al., 1987). In order to study the conserved features of sodium channels in a wide range of excitable tissues, we have developed site-directed antibodies against highly conserved segments of the α subunits. In this paper, we describe experiments with antibodies against a predicted intracellular segment that is conserved in sodium channels from vertebrate brain, skeletal muscle, heart, electroplax, and insect nervous system.

Experimental Procedures

Materials. The catalytic subunit of cAMP-dependent protein kinase (Beavo et al., 1974), saxitoxin (Ritchie et al., 1976; Waechter et al., 1983), purified sodium channels (Hartshorne & Catterall, 1984), and ^{32}P-labeled sodium channels (Costa & Catterall, 1984) were prepared as described previously. Neuraminidase (type X from *Clostridium perfringens*) was obtained from Sigma.

Synthetic Peptides. Peptide SP19 (KTEEQKKYYNA-[norleucine]KKLGSKK) corresponding to residues 1501–1518 of the α subunit of rat brain sodium channel R_I, residues 1491–1508 of the α subunit of rat brain sodium channel R_{II}, or residues 1282–1299 of the electroplax sodium channel with an N-terminal Lys extension and substitution of norleucine for methionine was synthesized by the solid-phase method (Merrifield, 1983) and purified by reversed-phase HPLC on a Vydac 218TP10 column. The identity of the purified peptide was verified by amino acid composition and determination of the amino acid sequence.

Preparation of Antibodies. The purified peptide was coupled through amino groups to bovine serum albumin with glutaraldehyde (Orth, 1979), dialyzed against phosphate-buffered saline, emulsified in an equal volume of Freund's

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complete (initial injection) or incomplete adjuvant, and injected in multiple subcutaneous sites on New Zealand white rabbits at 3-week intervals. Antisera were collected after the second injection and tested by radioimmune assay (Costa & Catterall, 1984). IgG fractions were isolated by precipitation with 50% saturated $(NH_4)_2SO_4$ and dialyzed against 0.15 M NaCl–10 mM sodium phosphate, pH 7.4.

Antibodies against peptides $SP11_I$ and $SP11_{II}$ which recognize the R_I and R_{II} sodium channels, respectively, and antibodies against peptide SP1 which recognize central nervous system sodium channels have been described previously (Gordon et al., 1987). Antibodies were purified by antigen affinity chromatography (Olmsted, 1981; Wollner & Catterall, 1987).

Preparation of Tissue Fractions. A partially purified synaptosomal fraction of rat brain (lysed P3), a light surface membrane fraction from rat skeletal muscle, and a total membrane fraction from eel brain were prepared as described previously (Gordon et al., 1987) in the presence of the protease inhibitors phenylmethanesulfonyl fluoride (50 μg/mL), iodoacetamide (1 mM), and pepstatin A (1 μM). The ventricles of freshly dissected rat hearts were weighed, minced on ice, and homogenized in 0.1 M choline chloride, 10 mM EDTA, 10 mM EGTA, and 50 mM potassium phosphate, pH 7.4, in the presence of the same protease inhibitors. A light surface membrane fraction was then prepared as described for skeletal muscle (Gordon et al., 1987). The electroplax of *Electrophorus electricus* was freshly dissected, minced on ice, and homogenized in 10 mL/g wet weight 0.25 M sucrose, 10 mM EDTA, 10 mM EGTA, and 50 mM potassium phosphate, pH 7.4, in the presence of the same protease inhibitors as above. Cellular debris was removed by centrifugation at 800*g* for 10 min, and the membranes were collected by centrifugation at 100000*g* for 60 min and resuspended in the same buffer. Locust (*Schistocerca americana*) brains and ventral nerve cords were freshly dissected, homogenized in 10 mL/g wet weight 0.25 M sucrose, 10 mM EDTA, 10 mM EGTA, and 50 mM potassium phosphate, pH 7.4, in the presence of the same protease inhibitors as above, and P2 membranes were prepared as described by Gordon et al. (1985). The concentration of sodium channels with high affinity for saxitoxin in each membrane preparation was measured by determination of specific binding of 20 nM [^{3}H]saxitoxin at 0 °C with a rapid filtration assay as described previously (Catterall et al., 1979).

Partial Purification of Sodium Channels by Chromatography on WGA–Sepharose. WGA–Sepharose was prepared as described previously (Hartshorne & Catterall, 1984) and equilibrated with 350 mM choline chloride, 10 mM EDTA, 10 mM EGTA, and 50 mM potassium phosphate, pH 7.4, and decanted from excess buffer. Membrane fractions prepared as described above were adjusted to 350 mM choline chloride and 5% Triton X-100, and membrane extract containing 25 pmol of sodium channels, as assessed from the saxitoxin binding activity of the membranes, was mixed with 0.8 mL of packed WGA–Sepharose. After being mixed for 2.5 h at 4 °C, bound glycoproteins were washed eight times with 1 mL of 0.1% Triton X-100, 350 mM choline chloride, 10 mM EDTA, 10 mM EGTA, and 50 mM potassium phosphate, pH 7.4, twice with 1 mL of 10 mM EDTA, 10 mM EGTA, and 0.1% Triton X-100, and twice with 1 mL of H_2O. The bound glycoproteins were eluted twice by denaturation in 320 μL of SDS sample buffer (see below), and the WGA–Sepharose was removed by sedimentation.

Solubilization and Immunoprecipitation of Sodium Channels. Membrane fractions were diluted to 1 nM sodium channels (100 fmol per sample), as assessed by saxitoxin binding activity, in 100 mM choline chloride, 10 mM EDTA, 10 mM EGTA, 50 mM potassium phosphate, pH 7.4, and 5% Triton X-100 containing the protease inhibitors described above. After being mixed for 30 min at 4 °C, the residual membranes were sedimented at 8000*g* for 15 min. The supernatants were incubated for 16 h with antibodies at 4 °C. The antigen–antibody complexes were isolated by adsorption to 10 mg of protein A–Sepharose and centrifugation at 8000*g* for 1 min.

Phosphorylation of Immunoprecipitated Sodium Channels. The sodium channels in protein A–Sepharose immunoprecipitates were washed twice with phosphorylation buffer (Schmidt et al., 1985) and radiolabeled by phosphorylation with 500 ng of cAMP-dependent protein kinase and 10 μCi of [γ^{32}P]ATP for 1 min at 36 °C (Schmidt et al., 1985).

SDS–PAGE. Immunoprecipitated samples were suspended in 3% SDS, 30 mM Tris (adjusted to pH 8.6 with HCl), 2 mM EDTA, 5% sucrose, and 5% 2-mercaptoethanol and were boiled for 5 min. The pH was adjusted to 7.4, and the proteins were resolved by electrophoresis through a stacking gel of 3% acrylamide and a running gel with a 3–10% gradient of acrylamide as previously described (Maizel, 1971; Schmidt et al., 1985). Radiolabeled bands were visualized by autoradiography. When samples were compared before and after reduction of disulfide bonds, both reduced samples prepared as described above in the presence of 15 mM 2-mercaptoethanol and unreduced samples prepared without 2-mercaptoethanol were incubated with 60 mM iodoacetamide at room temperature for 45 min.

Immunoblotting. Membranes containing 5 pmol of sodium channels as assessed by saxitoxin binding were solubilized, the residual membranes were removed by centrifugation, and the sodium channels were immunoprecipitated as described above with 40 μL of antibody solution and 40 mg of protein A–Sepharose. Alternatively, 5 pmol of sodium channels as assessed by saxitoxin binding activity was taken from preparations of sodium channels solubilized and partially purified by chromatography on WGA–Sepharose as described above. The proteins in these samples were resolved by SDS–PAGE and electrophoretically transferred to a nitrocellulose filter. The nitrocellulose filter was incubated in 1% (v/v) anti-SP19 antibodies in 0.05% (v/v) Tween 20, 150 mM NaCl, 10 mM Tris-HCl, and 10% nonfat milk (w/v), pH 7.5, for 16 h. After five washes with the same buffer without antibody and nonfat milk, the bound antibodies were visualized by ^{125}I-protein A binding and autoradiography.

RESULTS

Immunoprecipitation of Rat Brain Sodium Channels by Antibodies against a Conserved Segment. The primary structure of the sodium channel α subunit contains four repeated domains with approximately 50% sequence identity (Noda et al., 1984, 1986a). In general, the most highly conserved segments of the sequence are the proposed transmembrane segments within these four homologous domains. However, these sequences are likely to be inaccessible to antibodies in the intact sodium channel protein. Figure 1 illustrates the homology of the predicted intracellular domains of the α subunits from electroplax and rat brain (Noda et al., 1984, 1986a) as a function of amino acid residue number. Inspection of the homology plot reveals a highly conserved segment between residues 1538 and 1557 (consensus sequence numbers) in the predicted intracellular loop connecting the third and fourth homologous domains (Figure 1). This segment contains the longest predicted intracellular amino acid

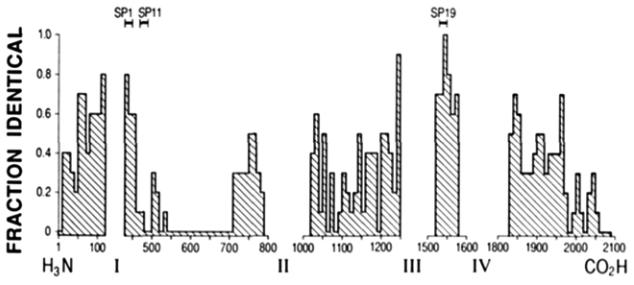

FIGURE 1: Homology of predicted intracellular regions of sodium channel α subunits. The fraction of identical amino acid residues in 10-residue segments of the amino acid sequences of sodium channels from electroplax and rat brain was calculated from the data of Noda et al. (1986a) and plotted as a function of residue number for the five main predicted intracellular domains of the α subunit. The abscissa corresponds to the consensus sequence numbering system of Noda et al. (1986a).

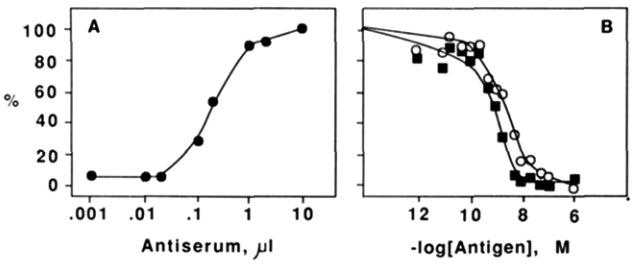

FIGURE 2: Immunoprecipitation of sodium channels by anti-SP19 antibodies. (A) Purified sodium channels were radioactively labeled on the α subunit by phosphorylation with cAMP-dependent protein kinase (Costa & Catterall, 1984). Fifty femtomoles of sodium channels was incubated with anti-SP19 antibodies equivalent to the indicated volumes of antiserum for 16 h at 4 °C in a final volume of 100 μL of NET buffer (75 mM NaCl, 2.5 mM EDTA, 25 mM Tris, 1% Triton, 20 mM KF, 50 mM NaH_2PO_4, 0.01% NaN_3, adjusted to pH 7.4 with NaOH) containing 1 mg/mL bovine serum albumin. Ten milligrams of protein A–Sepharose was added, and the incubation was continued for 0.5 h. The samples were diluted with 1 mL of NET buffer, and the protein A–Sepharose and bound antibodies were sedimented by centrifugation in a microfuge for 30 s. Immunoprecipitated ^{32}P-labeled sodium channels were determined by liquid scintillation counting. The ordinate represents the percent of ^{32}P-labeled sodium channels immunoprecipitated. (B) Immunoprecipitation of ^{32}P-labeled sodium channels by 1-μL equivalent of anti-SP19 antibodies was carried out as described in panel A in the presence of the indicated concentrations of unlabeled sodium channels (○) or SP19 (■). The ordinate represents percent of the ^{32}P-labeled sodium channels immunoprecipitated under control conditions with no added peptide.

sequence that is identical in the three sodium channels from rat brain and eel electroplax whose primary structures are known. A peptide, designated SP19 (see Experimental Procedures), corresponding to residues 1282–1289 of the electroplax sodium channel, 1501–1518 of R_I, or 1491–1508 of R_{II} was synthesized, coupled to a protein carrier, and used to immunize rabbits as described under Experimental Procedures. The resulting antibodies efficiently immunoprecipitate purified rat brain sodium channels (Figure 2A). Immunoprecipitation is completely blocked by purified sodium channels and by the peptide antigen with half-maximal inhibition at concentrations of 3 and 1.5 nM, respectively (Figure 2B). Since the affinities of the antibody for the sodium channel and the free peptide are comparable, the sodium channel segment corresponding to SP19 must be freely available to antibody in the purified sodium channel and must assume similar conformations to peptide SP19 in solution.

Identification of Sodium Channel α Subunits in Muscle-Derived Tissues by Immunoblotting. Membrane preparations

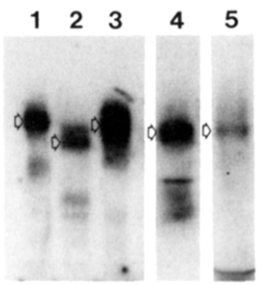

FIGURE 3: Identification of sodium channel α subunits by immunoprecipitation and immunoblotting. (Lanes 1–3) Lysed P3 membranes from rat brain (lane 1), a light surface membrane fraction from rat skeletal muscle (lane 2), and a total membrane fraction from eel electroplax (lane 3) were prepared, and sodium channels were solubilized as described as described under Experimental Procedures. Five picomoles of sodium channels, as assessed by saxitoxin binding activity of the corresponding membrane fractions, was immunoprecipitated by anti-SP19 antibodies, resolved by SDS–PAGE, and visualized by immunoblotting with anti-SP19 antibodies as described under Experimental Procedures. (Lane 4) Sodium channels from the transverse tubule membrane fraction of rat skeletal muscle were partially purified by WGA–Sepharose chromatography, resolved by SDS–PAGE, and visualized by immunoblotting with anti-SP19 antibodies. (Lane 5) A light surface membrane fraction was prepared from rat heart as described under Experimental Procedures. Sodium channels were solubilized, immunoprecipitated with anti-SP19 antibodies, resolved by SDS–PAGE, and visualized by immunoblotting with anti-SP19 antibodies. Arrows mark the migration position of the main α subunit band in each sample.

Table I: Apparent Molecular Weight of Sodium Channel α Subunit

tissue	apparent molecular weight ($\times 10^{-3}$) unreduced	reduced	desialylated
rat brain	300	260	238
rat skeletal muscle		255	
rat heart	230	230	222
eel brain	290	240	214
eel electroplax		260–290	202
locust nervous system	280	280	280

from rat brain, rat skeletal muscle plasma membrane and transverse tubules, eel electroplax, and rat heart containing 5 pmol of saxitoxin binding sites were solubilized in Triton X-100, the sodium channels were purified by immunoprecipitation with anti-SP19 antibodies or by chromatography on WGA–Sepharose, polypeptides were resolved by SDS–PAGE, and α subunits were visualized by electrophoretic transfer to nitrocellulose and immunoblotting (Figure 3). α subunits with apparent molecular weight values of 230 000–280 000 (Table I) are observed in each sample indicating that anti-SP19 antibodies recognize sodium channels in each of these tissues. Immunoprecipitation of the sodium channel α subunits from skeletal muscle was inhibited by prior incubation of the antibodies with excess peptide SP19, confirming their identity (data not shown). Similar experiments confirmed the identity of sodium channel α subunits from electroplax and heart using phosphorylation by cAMP-dependent protein kinase to detect the α subunits as described below. These results indicate that the segment of the sodium channel corresponding to SP19 is highly conserved in sodium channels in heart and in both the plasma membrane and transverse tubules of skeletal muscle.

Phosphorylation of Sodium Channel α Subunits in Different Tissues by cAMP-Dependent Protein Kinase. The α subunit of the sodium channel from rat brain is an unusually good substrate for cAMP-dependent protein kinase and incorporates 3–4 mol of phosphate in situ (Costa & Catterall, 1984; Rossie & Catterall, 1987). Sodium channels in other tissues have not been shown to be substrates for this enzyme. To examine phosphorylation of sodium channels in a range

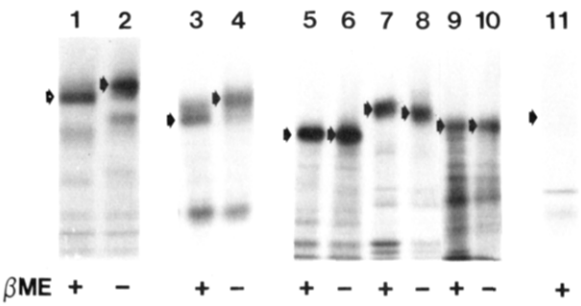

FIGURE 4: Immunoprecipitation and phosphorylation of sodium channel α subunits. Sodium channels were solubilized from membrane preparations, immunoprecipitated with anti-SP19 antibodies, radioactively labeled by reaction with cAMP-dependent protein kinase and [γ-^{32}P]ATP, resolved by SDS–PAGE with (lanes 1, 3, 5, 7, 9, and 11) or without (lanes 2, 4, 6, 8, and 10) reduction of disulfide bonds with 5 mM 2-mercaptoethanol (βME), and visualized by autoradiography as described under Experimental Procedures. For each tissue sample, antibodies blocked by prior incubation with SP19 peptide were shown not to immunoprecipitate α subunits. (Lanes 1 and 2) Rat brain; (lanes 3 and 4) eel brain; (lanes 5 and 6) rat heart; (lanes 7 and 8) eel electroplax; (lanes 9 and 10) locust brain and nerve cord; (lane 11) rat skeletal muscle.

of tissues, sodium channels were solubilized with Triton X-100 and immunoprecipitated with anti-SP19 antibodies. The immunoprecipitates were then incubated with [γ-^{32}P]ATP and cAMP-dependent protein kinase under conditions appropriate for phosphorylation of sodium channels from brain, and the immunoprecipitated polypeptides were analyzed by SDS–PAGE. As illustrated in Figure 4, sodium channel α subunits from rat cerebral cortex (lane 1), rat heart (lane 5), and eel electroplax (lane 7) are phosphorylated by cAMP-dependent protein kinase. Immunoprecipitation of sodium channel α subunits from electroplax and heart was blocked by prior incubation of the antibodies with excess SP19 peptide, confirming their identification (data not shown). In contrast, α subunits from skeletal muscle sodium channels are not phosphorylated under these conditions, suggesting that they are not substrates for cAMP-dependent protein kinase (lane 11).

Anti-SP19 antibodies also recognize sodium channel α subunits in the central nervous systems of a wide range of species. Sodium channels from eel brain have α subunits that are phosphorylated by cAMP-dependent protein kinase (Figure 4, lane 3). Similarly, sodium channel α subunits from locust brain and nerve cord are also detected by immunoprecipitation and phosphorylation by cAMP-dependent protein kinase (Figure 4, lane 9). In each case, prior incubation of the antibodies with excess SP19 peptide blocked immunoprecipitation of the sodium channel α subunits, confirming their identity (data not shown). These results indicate that fish and insect central nervous system sodium channels contain cAMP-dependent phosphorylation sites.

Disulfide Linkage of α Subunits with β2 Subunits. Sodium channels purified from rat brain contain α subunits which are disulfide linked to β2 subunits (Catterall, 1984, 1986), and antibodies directed against the β2 subunits immunoprecipitate greater than 89% of sodium channels solubilized from rat brain membranes (Wollner et al., 1987). When purified rat brain sodium channels are immunoprecipitated, phosphorylated, and analyzed by SDS–PAGE, the phosphorylated α subunits have an apparent M_r of 300 000 before reduction of disulfide bonds (Figure 4, lane 2) characteristic of the disulfide-linked $\alpha\beta$2 complex, whereas they have an apparent M_r of 260 000 after reduction of disulfide bonds characteristic of the free α subunit (Figure 4, lane 1). This change in apparent molecular weight

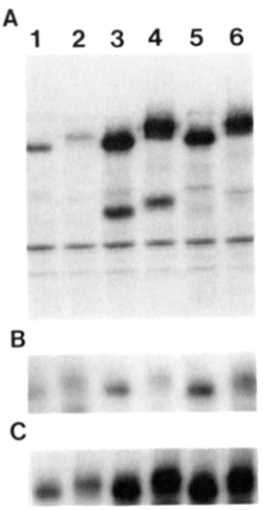

FIGURE 5: Disulfide-linked α and β2 subunits of R_I and R_{II} sodium channel subtypes. (A) Sodium channels (100 fmol) were solubilized from lysed P3 membranes from rat cerebral cortex, immunoprecipitated with anti-SP11$_I$ antibodies (lanes 1 and 2), anti-SP11$_{II}$ antibodies (lanes 3 and 4), or polyclonal antiserum that recognizes all rat brain sodium channels (lanes 5 and 6), radiolabeled by phosphorylation with cAMP-dependent protein kinase and [γ-^{32}P]ATP, analyzed by SDS–PAGE with (lanes 1, 3, and 5) or without (lanes 2, 4, and 6) reduction of disulfide bonds by 5 mM 2-mercaptoethanol (βME), and visualized by autoradiography as described under Experimental Procedures. (B) A similar experiment was carried out with sodium channels solubilized from spinal cord membranes. Only the α subunit bands are shown. (C) A similar experiment was carried out with purified sodium channels from rat brain. Only the α subunit band is shown.

of the α subunit has been shown directly to be due to release of the β2 subunit (Hartshorne et al., 1982; Messner & Catterall, 1985). A shift of the apparent molecular weight of α subunits to values that are lower by 30 000–40 000 upon reduction of disulfide bonds therefore provides evidence for the presence of disulfide-linked β2 subunits in sodium channels from other sources in tissue extracts.

Two different mRNAs encoding α subunits with 87% primary structural identity have been cloned and sequenced from rat brain (Noda et al., 1986a). We have prepared antibodies directed against a segment of the α subunit (SP11) whose amino acid sequence differs between the R_I and R_{II} subtypes. These antibodies recognize R_I and R_{II} specifically (Gordon et al., 1987). Analysis of the tissue-specific expression of these two α subunit subtypes showed that R_{II} is preferentially expressed in brain, R_I is preferentially expressed in spinal cord, and neither subtype is expressed detectably outside the central nervous system (Gordon et al., 1987). Since nearly all sodium channel α subunits in brain are disulfide linked to β2 subunits (Wollner et al., 1987), it was of interest to determine whether both the R_I and R_{II} subtypes are present in this form. In cerebral cortex, approximately 80% of sodium channels are the R_{II} subtype (Gordon et al., 1987). Analysis of the apparent molecular weight of total sodium channels from cerebral cortex (Figure 5A, lanes 5 and 6), R_{II} subtype from cerebral cortex (Figure 5A, lanes 3 and 4), and R_I subtype from cerebral cortex (Figure 5A, lanes 1 and 2) by SDS–PAGE with and without reduction of disulfide bonds reveals that both R_I and R_{II} subtypes in the cortex are disulfide linked to β2 subunits. A similar analysis of sodium channels in rat spinal cord, where approximately 39% of sodium channels are the R_I subtype and 18% are R_{II} (Gordon et al., 1987), shows that both R_I and R_{II} subtypes are disulfide linked to β2 subunits in this region of the central nervous system as well. In different purified sodium channel preparations from rat brain, from 18% to 27% of

sodium channels are immunoprecipitated by anti-SP11$_I$ and the apparent molecular weight of all of these R_I α subunits is decreased approximately 30 000 by reduction of disulfide bonds (Figure 5C, lanes 1 and 2). The remainder of the sodium channels are immunoprecipitated by anti-SP11$_{II}$, and the apparent molecular weight of all of these R_{II} α subunits is correspondingly decreased by reduction of disulfide bonds (Figure 5C, lanes 3 and 4). The apparent molecular weight of the R_I subtype is smaller than that of the R_{II} subtype (compare lanes 1 and 3 of each panel) verifying that a complete separation is achieved by immunoprecipitation. These results show that the R_I and R_{II} α subunit subtypes both in purified preparations and in tissue extracts are linked to β2 subunits by disulfide bonds.

We have analyzed total α subunits of sodium channels from the central nervous systems of two additional species using similar procedures. α subunits from eel brain migrate with an apparent molecular weight that is lower by 30 000 after reduction of disulfide bonds (Figure 4, lanes 3 and 4), while α subunits of sodium channels from locust nervous system do not have altered apparent molecular weights after disulfide bond reduction (Figure 4, lanes 9 and 10).

Sodium channels purified from electroplax also do not contain disulfide-linked β2 subunits (Agnew, 1984). As expected from these results, we find that the apparent molecular weight of the α subunit of the electroplax sodium channel increases substantially upon reduction of disulfide bonds (Figure 4, lanes 7 and 8). This behavior is common among proteins containing internal disulfide bonds which restrict unfolding of the polypeptide in SDS and is consistent with the conclusion that the α subunit from electroplax is not disulfide linked to smaller polypeptides. The apparent molecular weight of sodium channel α subunits from rat heart is not significantly decreased after reduction of disulfide bonds (Figure 4, lanes 5 and 6), indicating that they are not disulfide linked to β2 subunits. The results of these experiments in which sodium channel α subunits are examined by immunoprecipitation and phosphorylation lead to the conclusion that sodium channels in vertebrate central nervous systems have disulfide-linked α and β2 subunits while those in insect nervous system and in heart and electroplax do not.

Sialylation of Sodium Channel α Subunits in Different Tissues. Biochemical analyses of sodium channel α subunits purified from brain, skeletal muscle, and electroplax have shown that approximately 15–30% of the apparent mass of these polypeptides is due to N-linked carbohydrate, half of which is sialic acid (Miller et al., 1983; Grishin et al., 1984; Elmer et al., 1985; Messner & Catterall, 1985; Roberts & Barchi et al., 1987). Therefore, glycosidases reduce the apparent molecular weight of these α subunits substantially (Miller et al., 1983; Messner & Catterall, 1985; Schmidt & Catterall, 1987), providing a method to examine the extent of glycosylation of α subunits in tissue extracts. Since the large amount of sialic acid on sodium channel α subunits may have functional significance in providing a large negative charge on the extracellular surface of the protein, it is of interest to determine whether sodium channels in all tissues have α subunits with such a large amount of sialic acid. Sodium channel α subunits were solubilized with Triton X-100, immunoprecipitated, phosphorylated, incubated with neuraminidase, and analyzed by SDS–PAGE after reduction of disulfide bonds. The apparent molecular weight values of the α subunits from rat brain and eel electroplax were reduced 22 000 and 58 000, respectively, as expected from previous biochemical data (Figure 6, samples 1 and 3; Table I). α subunits from eel brain gave similar results to rat brain (Figure 6, sample 2; Table I). In contrast, the apparent molecular weight of α subunits of sodium channels from rat heart was reduced only 8000 (Figure 6, sample 4; Table I), and the apparent molecular weight of sodium channels from locust nerve cord was not reduced detectably by neuraminidase treatment (Figure 6, sample 5). These results indicate that the extent of sialylation of sodium channels from heart and insect nervous system is substantially smaller than that for sodium channels from vertebrate brain and eel electroplax.

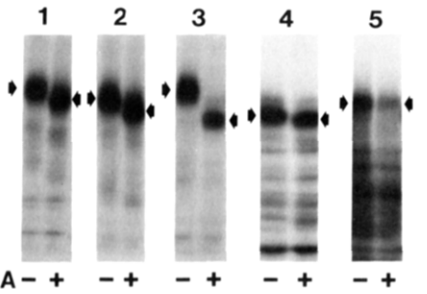

FIGURE 6: Effects of neuraminidase treatment on the apparent molecular weight of sodium channel α subunits. Sodium channels (100 fmol) solubilized from membrane preparations were immunoprecipitated with anti-SP19 antibodies and incubated with or without 0.5 unit of neuraminidase (NA) in 50 mM Tris-HCl, pH 5.5, containing 25 μg/mL phenylmethanesulfonyl fluoride and 1 μM pepstatin A for 16 h at 4 °C. Under these conditions, rat brain sodium channels are completely desialylated in 30 min (E. McHugh and W. A. Catterall, unpublished observations). Each pair of tissue samples was then analyzed by SDS–PAGE after reduction of disulfide bonds and visualized by autoradiography as described under Experimental Procedures. (Sample 1) Rat brain; (sample 2) eel brain; (sample 3) eel electroplax; (sample 4) rat heart; (sample 5) locust brain and nerve cord.

Discussion

Biochemical Properties of Sodium Channel α Subunits in Different Excitable Tissues. The similarities in biochemical properties of sodium channel α subunits purified from brain, skeletal muscle, and electroplax (Agnew, 1984; Barchi, 1984; Catterall, 1984, 1986) suggest that sodium channels have similar, but not identical, α subunits in all excitable tissues. The substantial primary structural homology among the rat brain, eel electroplax, and *Drosophila* sodium channels also supports this view (Noda et al., 1986a; Salkoff et al., 1987). Our present results provide the first identification of the α subunit polypeptide of sodium channels in eel brain, rat heart, and insect nervous system. In each case, the α subunit has an apparent M_r of 230 000–290 000. However, several differences among these α subunits were noted. Both the R_I and R_{II} sodium channels from rat brain contain disulfide-linked α and β2 subunits. Sodium channels from eel brain, like brains from several vertebrates (Wollner et al., 1987), contain disulfide-linked α and β2 subunits. However, no evidence of smaller subunits disulfide linked to α subunits was found for sodium channels from heart, electroplax, or insect nervous system. All sodium channel α subunits examined except those from rat skeletal muscle were good substrates for phosphorylation by cAMP-dependent protein kinase. However, comparison of the known sites of cAMP-dependent phosphorylation in the intracellular loop between the first and second homologous domains of the rat brain sodium channel (Rossie et al., 1987) with the amino acid sequence of the electroplax sodium channel (Noda et al., 1984) shows that, at least in the case of these two α subunits, the location of the sites of phosphorylation must be different since the region which contains the cAMP-dependent phosphorylation sites of the rat

brain sodium channel is not present in the electroplax channel. Finally, our results indicate that the degree of sialylation of sodium channel α subunits differs substantially among different tissues. Sialylation makes the largest contribution to apparent molecular weight of sodium channels from electroplax, an intermediate contribution to apparent molecular weight of α subunits of sodium channels from rat and eel brain, only a slight contribution to apparent molecular weight of α subunits of sodium channels from rat heart, and no detectable contribution to apparent molecular weight of sodium channel α subunits from locust nervous system (Table I). The lack of effect of neuraminidase treatment on the apparent molecular weight of sodium channel α subunits is consistent with previous work showing that insect glycoproteins generally do not have complex carbohydrate chains or sialic acid (Hsieh & Robbins, 1984). Considered together, the results show that sodium channels from all these sources contain an α subunit with apparent M_r of 230000–290000 as a principal component but that the biochemical properties and posttranslational modification of these α subunits vary in significant respects among different tissues and species.

Inhibition of core glycosylation of newly synthesized proteins prevents synthesis of functional sodium channels (Bar-Sagi & Prives, 1983; Waechter et al., 1983) because the newly synthesized α subunit polypeptide is unstable and is degraded before it can be assembled into a functional channel and inserted into the cell surface (Schmidt & Catterall, 1986). In contrast, incorporation of 80% of the sialic acid into rat brain sodium channels can be inhibited without effect on biosynthesis and assembly of rat brain sodium channels or on their saxitoxin binding activity (Schmidt & Catterall, 1987). These previous results indicate that cotranslational glycosylation of sodium channels is essential for their biosynthesis but that extensive posttranslational sialylation is not required for either biosynthesis or basic function. Our present data provide independent support for this conclusion by showing that sodium channels in rat heart and insect nervous system are not extensively sialylated.

A Highly Conserved, Predicted Intracellular Domain of the Sodium Channel α Subunit. Our results show that the amino acid sequence corresponding to the SP19 peptide is identical, or at least highly conserved, in sodium channels from rat brain, heart, and skeletal muscle, eel brain and electroplax, and insect nervous system. This high degree of conservation suggests an important structural or functional role for this segment of the protein. Voltage-dependent activation of sodium channels and transport of permeant cations by the activated channel are thought to involve primarily the transmembrane regions of the four homologous domains of the sodium channel, which are the most highly conserved regions in the brain and electroplax channels. What channel functions are thought to take place at the intracellular surface of the channel protein to which the highly conserved domain recognized by the anti-SP19 antibodies might contribute? Inactivation of sodium channels during a maintained depolarization is a relatively voltage-independent process (Armstrong, 1981; Aldrich et al., 1983) which is prevented by treatment of the intracellular surface of the membrane with a wide variety of proteolytic enzymes in a broad range of tissues and species [Armstrong et al., 1973; Gonoi and Hille (1987) and references cited therein] and reagents specific for tyrosine, histidine, arginine, and tryptophan (Eaton et al., 1978; Oxford et al., 1978). It has been proposed that these treatments prevent inactivation by cleavage or damage of specific components of the sodium channel that are present at the intracellular surface and are required for inactivation. The highly conserved segment recognized by anti-SP19 antibodies contains the appropriate amino acid targets for these proteases and reagents and is part of a predicted intracellular loop (Noda et al., 1986a). Our results demonstrate that this segment is indeed accessible to macromolecular reagents and that its primary structure is conserved in a broad range of tissues and species. Recent electrophysiological studies show that SP19 antibodies slow inactivation of sodium channels (Vassilev, Scheuer, and Catterall, unpublished results). These results are consistent with a direct role for this segment of the channel in inactivation.

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Registry No. Peptide SP19, 115464-51-2; protein kinase, 9026-43-1.

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Factors Modulating Filament Formation by Bovine Glial Fibrillary Acidic Protein, the Intermediate Filament Component of Astroglial Cells†

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ABSTRACT: Glial fibrillary acidic protein (GFAP) is soluble in low ionic strength solutions but shows a strong tendency toward assembly with increasing ionic strength as revealed by electron microscopy and turbidity measurements. Increasing K^+, Na^+, and Li^+ concentrations cause an increase followed by a decrease in GFAP turbidity with a maximum at 200 mM, but their effects are much weaker than effects of divalent cations at the same ionic strength. Ca^{2+}, Mg^{2+}, Mn^{2+}, and Ba^{2+} promote assembly at millimolar concentrations, and 10 μM Cu^{2+} causes rapid aggregation. The critical concentration for GFAP assembly was 0.08 ± 0.04 mg/mL in 2 mM Tris-HCl, 60 mM KCl, and 1 mM $CaCl_2$, pH 6.8. The M_r 38 000 rod domain of GFAP obtained by limited chymotryptic digestion is more soluble in 100 mM imidazole hydrochloride buffer, pH 6.8, than the intact molecule, and removal of the end pieces greatly reduces the ability of GFAP to form filaments. BNPS-skatole (2-[(2-nitrophenyl)sulfenyl]-3-methyl-3-bromoindolenine) treatment releases a M_r 30 000 N-terminus and a M_r 20 000 C-terminus. The M_r 30 000 polypeptide shows a higher affinity than the M_r 20 000 fragment for intact GFAP. Arginine and lysine at low concentrations slightly accelerate GFAP assembly, but above 100 mM both amino acids inhibit assembly. ATP, GTP, CTP, and UTP do not show significant effects on GFAP assembly. Dephosphorylation by alkaline phosphatase slightly reduces the assembly ability of GFAP, but phosphatase-treated GFAP still is assembly competent.

Intermediate filaments, distinguished by their 7–11-nm diameter and low solubility, are a major component of the cytoskeleton. On the basis of their immunological and biochemical properties, intermediate filaments are grouped into five subclasses: keratin, desmin, vimentin, neurofilaments, and glial filaments (Lazarides, 1980). These subclasses are distinct in their composition and cellular origins. Their polypeptide subunits, however, share a common structure: a conserved rod domain covering about 310 amino acid residues and flanking N-terminal and C-terminal domains (Geisler & Weber, 1982; Geisler et al., 1982). The rod domain contains extended α-helical regions which are able to form coiled coils and probably are responsible for the formation of the filament backbone (Steinert, 1978; McLachlan, 1978; Steinert et al., 1980).

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