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Identification of Phosphorylation Sites for Adenosine 3′,5′-Cyclic Phosphate Dependent Protein Kinase on the Voltage-Sensitive Sodium Channel from *Electrophorus electricus*†

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ABSTRACT: The voltage-sensitive sodium channel from the electroplax of *Electrophorus electricus* is selectively phosphorylated by the catalytic subunit of cyclic-AMP-dependent protein kinase (protein kinase A) but not by protein kinase C. Under identical limiting conditions, the protein was phosphorylated 20% as rapidly as the synthetic model substrate kemptamide. A maximum of 1.7 ± 0.6 equiv of phosphate is incorporated per mole. Phosphoamino acid analysis revealed labeled phosphoserine and phosphothreonine at a constant ratio of 3.3:1. Seven distinct phosphopeptides were identified among tryptic fragments prepared from radiolabeled, affinity-purified protein and resolved by HPLC. The three most rapidly labeled fragments were further purified and sequenced. Four phosphorylated amino acids were identified deriving from three consensus phosphorylation sites. These were serine 6, serine 7, and threonine 17 from the amino terminus and a residue within 47 amino acids of the carboxyl terminus, apparently serine 1776. The α-subunits of brain sodium channels, like the electroplax protein, are readily phosphorylated by protein kinase A. However, these are also phosphorylated by protein kinase C and exhibit a markedly different pattern of incorporation. Each of three brain α-subunits displays an ~200 amino acid segment between homologous repeat domains I and II, which is missing from the electroplax and skeletal muscle proteins [Noda et al. (1986) *Nature (London) 320*, 188; Kayano et al. (1988) *FEBS Lett. 228*, 1878; Trimmer et al. (1989) *Neuron 3*, 33]. Most of the phosphorylation of the brain proteins occurs on a cluster of consensus phosphorylation sites located in this segment. This contrasts with the pattern of highly active sites on the amino and carboxyl termini of the electroplax protein. The detection of seven labeled tryptic phosphopeptides compared to the maximal labeling stoichiometry of ~2 suggests that many of the acceptor sites on the protein may be blocked by endogenous phosphorylation.

Second messenger systems involving protein phosphorylation play prominent roles in regulating many aspects of excitable cell physiology (Walaas & Greengard, 1987; Levitan, 1985). Several lines of evidence now demonstrate that the α-subunits of neuronal voltage-sensitive sodium channels are subject to enzymatic phosphorylation and suggest that this modification may play a role in modulating sodium channel gating or permeability (Rossie & Catterall, 1988b). The α-subunits of brain sodium channels differ from those of mammalian skeletal muscle or eel electroplax in possessing a specialized cytoplasmic segment which is enriched in consensus phosphorylation sites. This domain, which is the major site of phosphorylation both in vitro and in vivo, has been proposed to play a regulatory role unique to neuronal sodium channels. In this paper we examine the enzymatic phosphorylation of the muscle-type electroplax sodium channel, which lacks this segment and which displays a markedly different pattern of consensus

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phosphorylation sites for the cyclic-AMP-dependent kinase (protein kinase A).[1]

Sodium channel proteins have been purified from several sources (Agnew, 1978; Barchi et al., 1980; Hartshorne & Catterall, 1981; Kraner et al., 1985). Common to all of these preparations is a large (260 000–285 000 Da) glycopeptide or α-subunit. This is the only constituent of the sodium channel from eel electroplax. The mammalian brain proteins also exhibit two smaller peptides (β-1 and β-2) of 33 000 and 36 000 Da (Messner & Catterall, 1981), while that from mammalian muscle has a single β-subunit of 39 000 Da (Roberts & Barchi, 1987). Apparently most, if not all, of the mechanisms responsible for sodium channel biophysical and pharmacological behavior reside in the larger peptides. This is indicated by the functional reconstitution of the single peptide electroplax protein (Rosenberg et al., 1984; Tomiko et al., 1986; Correa et al., 1989), and the expression in *Xenopus* oocytes of functional sodium channels from mRNA encoding only the α-subunits from brain (Noda et al., 1986b; Goldin et al., 1986; Suzuki et al., 1988; Auld et al., 1988) and muscle (Trimmer et al., 1989).

From the nucleic acid sequences of cloned cDNAs the primary structures of the α-subunits of the electroplax sodium channel, three brain proteins, and a skeletal muscle sodium channel have been deduced (Noda et al., 1984, 1986a; Kayano et al., 1987; Auld et al., 1988; Trimmer et al., 1989). In each case the peptide exhibits four internally homologous domains, which contain all of the putative membrane-spanning regions. These are thought to be arranged in a tetrameric arrangement, penetrating the membrane to form the ion pathway, the voltage-sensing elements, and sites of action for externally acting neurotoxins (Noda et al., 1984; Greenblatt et al., 1985; Guy & Seetharamulu, 1986; Guy, 1988). The amino and carboxyl termini, and the segments lying between the homologous domains, are hydrophilic and thought to lie on the cytoplasmic side of the membrane.

All of these proteins are strongly homologous, with the notable exception that the three brain peptides have a segment of 166–175 amino acids, between homologous domains I and II, which is deleted from the electroplax protein. These segments differ from one another, but all contain four to five optimal and several less than optimal consensus phosphorylation sites for protein kinase A. Like the electroplax protein, the skeletal muscle protein lacks a 219 amino acid segment in this region.

Biochemical studies have shown that purified brain sodium channel α-subunits can be phosphorylated in vitro by protein kinase A at a rate comparable to those of the best established substrates (Costa et al., 1982) and by protein kinase C (Costa & Catterall, 1984b). Subsequent studies demonstrated phosphorylation of this protein in situ (Costa & Catterall, 1984a) and in vivo (Rossie & Catterall, 1987a), which could be stimulated by cyclic-AMP agonists. Peptide maps from such metabolically labeled α-subunits show that up to five phosphopeptides derive from the I–II interdomain segment (Rossie et al., 1987). These investigators concluded that the presence of an active cluster of cyclic-AMP-dependent phosphorylation sites within this insertion singles out this segment as a potential regulatory domain unique to sodium channels of neuronal origin.

We have now examined the enzymatic phosphorylation of the electroplax sodium channel in vitro. We find that this channel is readily phosphorylated, both in detergent solution and in reconstituted form, by protein kinase A, but not by protein kinase C. Initial velocity studies indicate that the sodium channel is phosphorylated at a rate comparable to those of well-established physiological substrates for protein kinase A, and similar to that of the brain proteins. Quantitative analysis indicates that approximately 1.7 equiv of phosphate is incorporated per mole of the purified protein. Phosphoserine and phosphothreonine are observed as the labeled products in the reproducible ratio of 3.3:1. HPLC separation of tryptic fragments of the radiolabeled protein reveals seven phosphopeptides. We have identified the specific sequences surrounding four of the labeled amino acids. The identified residues include three serines and one threonine, lying in consensus phosphorylation sites near the amino and carboxyl termini of the protein. According to current models of peptide folding, these are likely to lie near one another in the native molecule. We compare the pattern of consensus phosphorylation sites in each of the known sodium channel sequences and discuss evidence that the protein is likely to be phosphorylated by cellular kinases in vivo.

Materials and Methods

Materials. Protein kinase C was the gift of J. Smallwood. The catalytic subunit of bovine brain type I protein kinase A was prepared by the method of Reimann and Beham (1983). We obtained kinase activities of 1–8 units/mg. The electroelution apparatus was from CBS Scientific, Del Mar, CA. The bath sonicator was from Laboratory Supplies, Hicksville, NY. Cellulose thin-layer sheets were Eastman Kodak No. 13255. *Limax flavus* agglutinin (LFA) from EY labs, San Mateo, CA, was coupled to CNBR-activated Sepharose (Sigma) as described in James et al. (1989). Phosphocellulose paper was Whatman P-81. Kemptamide was from Peninsula Laboratories, Belmont, CA; 1,2-dioleoylglycerol and bovine brain phosphatidylserine were from Avanti Polar Lipids, Birmingham, AL. [γ-^{32}P]ATP (30–40 Ci/mmol) was from New England Nuclear. Unlabeled ATP was from Boehringer Mannheim. Horse anti-(polysialic) acid IgM was the generous gift of Dr. J. N. Robbins of NIH. Affinity-purified IgM was coupled to CNBr-activated Sepharose following the procedure provided by the manufacturer, to a final level of 7 mg of IgM/mL of packed resin. Other reagents were from Sigma or the sources listed in Rosenberg et al. (1984a).

Sodium Channel Purification. The purification procedure for the experiment of Figure 1A was as described in Rosenberg et al. (1984a) through the Sepharose 6B chromatography step. [^{3}H]TTX-binding was measured by the Sephadex G-50 assay of Agnew et al. (1978). The trailing edge of the TTX-binding peak, chosen to contain significant amounts of contaminating proteins, was pooled, concentrated 7-fold by pressure dialysis on an Amicon XM-50 membrane, and dialyzed against 25 mM Hepes (pH 7.2), 75 mM Na_2SO_4, 5 mM $MgSO_4$, and 1 mM EGTA. The final solution also contained ~488 nM TTX-binding sites (697 pmol of TTX-binding activity/mg of protein), 0.67% Lubrol PX, and 1.22 mg/mL PC.

For the remaining experiments, the purification was by the lectin affinity method described in James et al. (1989). The pooled TTX-binding fractions (5.0 mL, 0.5 mg of protein/mL) from the DEAE desorbate [as in Rosenberg et al. (1984a)] were adsorbed to an LFA–Sepharose column (5.0-mL bed

[1] Abbreviations: ATP, adenosine 5′-triphosphate; cyclic-AMP, adenosine 3′,5′-cyclic phosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; LFA, *Limax flavus* agglutinin; PAGE, polyacrylamide gel electrophoresis; protein kinase A, cyclic-AMP-dependent protein kinase; protein kinase C, calcium/phospholipid-dependent protein kinase; SD, standard deviation; SDS, sodium dodecyl sulfate; STX, saxitoxin; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane; TTX, tetrodotoxin.

volume, 6.2 mg of LFA/mL of hydrated resin) for 90 min, washed with standard Lubrol buffer, constiting of 50 mM Na_2HPO_4 (pH 7.4), 0.1% Lubrol PX, 0.183 mg/mL PC, 5 mM $MgSO_4$, and 200 mM KCl, and eluted with the same buffer containing 10 mM *N*-acetylneuraminic acid. The peak TTX-binding fraction (containing 400–600 nM TTX-binding sites, ~2200 pmol/mg) was dialyzed against the dialysis buffer of the previous paragraph. For the experiment of Figure 3 the protein was reconstituted into phospholipid vesicles [10 mg/mL egg phosphatidylcholine (Sigma)] by lipid supplementation and removal of detergent with Bio-Beads SM2 as described (Rosenberg et al., 1984a).

Kinase Assays and Units. Kinase activities were determined in phosphorylation assays as described below with as substrate Sigma type VIIS histone (for protein kinase A) or Sigma type IIIS histone (for protein kinase C). The protein kinase A assay was by the phosphocellulose method described below. The protein kinase C reactions were in 50 μL. They were stopped by the addition of 1 mL of ice-cold 20% TCA. This solution was run through a Millipore filter (0.45 μm) and washed with 4 mL of ice-cold 5% TCA. Filter papers were counted in 10 mL of Optifluor. Background counts, from enzyme-free samples, were subtracted in each case. One unit of kinase transfers 1 μmol of phosphate to histone in 1 min.

Phosphorylation Reactions. The standard phosphorylation buffer contained 25 mM Hepes (pH 6.8 for protein kinase A, pH 7.2 for protein kinase C), 30 mM Na_2SO_4, 15 mM $MgSO_4$, 1 mM EGTA, and 120 μM ATP, with enzyme and protein substrate concentrations noted in the legends. Kinase activities ranged from 0.5 to 260 milliunits/mL (~0.26–135 nM catalytic subunit). The sodium channel phosphorylation buffer contained 10 μL of purified solubilized or reconstituted sodium channel in a final volume of 25 μL. Reactions were at 30 °C, started by addition of 5 μL of [γ-^{32}P]ATP, and stopped by addition of 8.3 μL of 4× SDS–PAGE sample buffer (0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 20% 2-mercaptoethanol) and heating at 70 °C for 8 min. Samples were electrophoresed on polyacrylamide–SDS gels (Figure 1, 5.5% 10-cm tube gel; Figures 2–5, 5.5-cm slab minigels, 4.5–15% gradient; Figure 6, standard 13-cm slab gel, 4.5–15% gradient) by the method of Laemmli (1970). After being run, the gels were stained with Coomassie blue R-250 and destained or silver stained by the method of Merril et al. (1981) with Bio-Rad reagents. Slab gels were dried under heat and vacuum and exposed to Kodak X-Omat AR film at −70 °C with a Du Pont Cronex Lightning Plus intensifying screen for from 30 min to 3 days. To quantify ^{32}P bound to sodium channel, the diffuse 260-kDa glycopeptide band was excised from the gel and dissolved overnight in 1 mL of 30% H_2O_2 at 80 °C in uncovered tubes. The residue was resuspended in 1 mL of water and counted by liquid scintillation in 10 mL of Optifluor. Stained tube gels were scanned by densitometry and sliced into 1-mm slices. The slices were dried and counted directly in Optifluor.

Kemptamide phosphorylation was in a volume of 30 μL. The reaction was started by addition of [γ-^{32}P]ATP and stopped with 10 μL of ice-cold 8 mM ATP and 40 mM EDTA and plunged into melting ice. The assay was by the phosphocellulose paper method as described in Reimann and Beham (1983). The papers were counted in 10 mL of Optifluor. Background counts from kemptamide-free controls were subtracted from each time point.

Quantitative Amino Acid Analysis. Purified, phosphorylated sodium channel glycopeptide was electrophoresed in gradient SDS slab gels as described, stained for 5 min with Coomassie blue R-250, and destained for 90 min at 4 °C. The peptide band was excised from the gel and the protein electroeluted by the method of Hunkapillar et al. (1983). Aliquots of electroeluted material were counted by liquid scintillation, and aliquots (~2 μg) were hydrolyzed (6 N HCl, 0.5% phenol, 115 °C, 16 h under vacuum) and run on a Beckman 121 M amino acid analyzer. Amino acid recoveries were quantified by normalizing to the recovery of 1.0 nmol of norleucine included as internal standard. The amount of sodium channel peptide in the original aliquot was derived from the recoveries of the five most stable amino acids (Asx, Glu, Ala, Phe, Arg) scaled by the amino acid composition of the electroplax sodium channel (Miller et al., 1983; Noda et al., 1984). Background signals from control samples taken from a protein-free lane of the gel were subtracted.

Phosphoamino Acid Analysis. An aliquot (1 μg) of the electroeluted material, obtained as above, was hydrolyzed as described for 2 h and transferred to a Pierce Reactivial. The HCl was evaporated in a Speed Vac, and the material was resuspended in 2 μL of water plus 10 μg each of unlabeled phosphoserine and phosphothreonine. This sample was spotted onto a cellulose thin-layer sheet and electrophoresed in 7% formic acid (pH 1.6) at 1250 V for 30 min. The sheet was dried in a stream of warm air, sprayed with fluorescamine and triethylamine, and visualized under long-wave UV illumination as described by Gracy (1977). The fluorescent spots were outlined in pencil, and the sheet was exposed to X-ray film with an intensifying screen as above. The amount of radioactivity in the spots was determined by scraping the cellulose powder into a scintillation vial and counting in 10 mL of Optifluor.

Preparation of Tryptic Phosphopeptides. Sodium channel protein (562 pmol of TTX-binding sites) purified through the LFA step was phosphorylation for 5 min at 30 °C in the standard phosphorylation medium with protein kinase A (0.2 unit/mL) and 4 μM [γ-^{32}P]ATP (56 000 cpm/pmol) in a volume of 1 mL. Next, 50 μL of 100 mM ATP was added and phosphorylation continued for 1 h with additions of protein kinase A at 20-min intervals: three additions, totaling 100 μL, brought the final protein kinase A level to 0.5 unit/mL. Then, 50 μL of 0.2 M EDTA was added, and free ATP and orthophosphate were removed by passing the sample through a 1.4 × 15 cm column of Sephadex G-50 equilibrated with standard Lubrol buffer. Elution was with the same buffer. The counts eluting in the void volume were pooled. To remove contaminating proteins, including those added with the kinase, this material was adsorbed to 12.5 mL of packed anti-(polysialic) acid IgM–Sepharose (James et al., 1989) with constant swirling on ice for 90 min. The resin was loaded into a 1 cm diameter column, washed with 30 mL of standard Lubrol buffer, and bound sodium channel protein was eluted at 4 °C with the same buffer containing 5 mg/mL colominic acid, at a flow rate of 0.15 mL/min. The labeled sodium channel peak was pooled, and colominic acid was removed by Sephadex G-50 chromatography. This material was repurified and concentrated by readsorption to the IgM column and elution with colominic acid. The IgM eluate (2 mL total) was pooled and delipidated by adsorption, with frequent mixing at room temperature, to 0.3 mL of DEAE-Sepharose CL-6B equilibrated with standard Lubrol buffer. After 2.5 h counts remaining in the supernatant were negligible. The resin was then loaded into a 1-mL column and washed with 10 mL of ammonium bicarbonate buffer (0.05 M) with 8 M urea. Bound protein was eluted with the same buffer plus 0.5 M NaCl, in 50-μL fractions. The peak fractions (150 μL, containing 50%

of the radioactivity) were pooled. The sample was reduced with 15 μL of 45 mM DTT at 50 °C for 15 min; 15 μL of 100 mM iodoacetamide was then added and incubation continued for 15 min at room temperature in the dark. The sample was dialyzed against 50 mM ammonium bicarbonate and 2 M urea. The final sample of 344 μL contained 39 μg of protein (~188 pmol of TTX-binding protein) and 45 pmol of ^{32}P. Trypsin (0.78 μg) was added, and the sample was proteolyzed for 12 h at 37 °C followed by addition of another 0.78 μg of trypsin and incubation for another 8 h. The sample was concentrated in a Speed Vac and brought up to a final volume of 210 μL with water.

Purification of Tryptic Peptides. Buffers for reverse-phase HPLC were as follows: (buffer A) 0.06% TFA; (buffer B) 0.052% TFA and 80% acetonitrile ± 0.002% TFA to balance the pH. The tryptic digest (200 μL) was subjected to reverse-phase HPLC on a Vydac narrow-bore (2.1 mm × 25 cm) C-18 column. Elution was at a flow rate of 0.15 mL/min with the following profile (buffer A + buffer B = 100%): 0–60 min, 2–37.5% B; 60–90 min, 37.5–75% B; 90–105 min, 75–98% B. Peptide bond absorbance was monitored at 210 nm with a Kratos 773 detector, and fractions were collected with a Foxy collector using an Isco peak separator; 3.5-μL aliquots of each fraction were counted for radioactivity by liquid scintillation in 10 mL of Optifluor. Fractions containing peak radioactivity were brought up to 205 μL, and 200 μL was rechromatographed on a 1 mm × 25 cm Aquapore C-8 column. Elution conditions and collection and counting of fractions were the same as above. The HPLC was performed by the Yale University Protein Chemistry Facility.

Amino Acid Sequence Determination. Fractions from the second HPLC run, which contained 50–180 pmol of phosphopeptide, were sequenced by automated Edman degradation on an Applied Biosystems gas-phase sequencer at the Yale University Protein Chemistry Facility. The PTH derivatives eluted from each cycle were separated and quantified by reverse-phase HPLC.

Identification of the Phosphorylated Amino Acid in a Phosphopeptide. The basis for our determinations is the fact that PTH-serine or PTH-threonine is not released in an Edman cycle in which phosphoserine or phosphothreonine is the terminal residue. [^{32}P]Orthophosphate is released but remains undetected it because binds tightly to the polybrene filter [cf. Wang et al. (1987)]. Thus, a cycle with phosphoserine or phosphothreonine is characterized by an absence of an unmodified amino acid. Some PTH-amino acid derivatives arise from both the unmodified and phosphorylated amino acids, however. For example, dehydroserine derives from both serine and phosphoserine, and dehydrothreonine derives from both threonine and phosphothreonine. In a cycle where serine was not recovered, therefore, the presence of dehydroserine confirms that serine or phosphoserine was present in the peptide at that position. Also several DTT adducts of threonine and dehydrothreonine are produced in the Edman degradation from both threonine and phosphothreonine residues. The recovery of one of these, which we have denoted DT_3, is plotted in the figure provided in supplementary material, to affirm the presence of threonine at that position. In a phosphopeptide containing serine or threonine residues, therefore, to identify unambiguously one of them as the phosphoamino acid, it is necessary and sufficient that (1) no serine or threonine is detected at the cycle corresponding to the phosphorylated amino acid and (2) unmodified serine or threonine is recovered at all other serine and threonine cycles.

Classification of Consensus Phosphorylation Sites for Protein Kinase A. The substrate specificity of protein kinase A is determined largely by the primary structure of the substrate in the immediate vicinity of the phosphorylated amino acid, although determinants of higher order structure can have considerable influence on the reactivity of a given target amino acid (Dale & Carnegie, 1974; Krebs & Beavo, 1979; Small et al., 1977). Among biological substrates for protein kinase A, the most prevalent sequences at the phosphorylation sites fall into two classes: sites of the form RRx[S/T] and those of the form KRxx[S/T] (Krebs & Beavo, 1979), where x can be any amino acid and [S/T] represents the target amino acid, which can be either serine or threonine. We refer to these as "optimal" consensus protein kinase A phosphorylation sequences. A significant number of substrates, however, do not fit one of these patterns, but all known phosphorylation sites are of the form

$$[R/K]_n\text{-}(x)_m\text{-}[S/T] \qquad n \geq 1 \qquad (1)$$

Studies with synthetic peptides show that by far the most effective substrates are those for which $n = 2$ and $m = 1$, which have serine as the target amino acid and have at least one arginine on the amino side (Kemp et al., 1977; Faramisco et al., 1980). Deviations from this pattern tend to raise K_m and/or lower V_{max} for the reaction. In the following we have designated as a "high-specificity" consensus phosphorylation site any region of primary structure of the form shown in eq 1, for which $n = 2$ and $m = 1$ or 2. Those sites for which $n = 1$ are referred to as "lower specificity" consensus sites. Phosphorylation sites for which $m = 0$ or $m > 2$ have been found but are relatively rare [cf. Carlson et al. (1979)].

Results

Reactions with Protein Kinases A and C. As was previously done by others with purified brain sodium channels (Costa et al., 1982, 1984), we tested whether the purified electroplax peptide serves as an active substrate for protein kinase A and protein kinase C in vitro. Figure 1A shows a brief incubation of a partially purified sodium channel preparation with a low level of protein kinase A catalytic subunit (12.5 nM) and Mg[γ-^{32}P]ATP (4 μM). Although the sodium channel glycopeptide (~280 000 Da) accounts for less than half of the protein in the sample, it is the only band with significant incorporation of ^{32}P. Most notably, the contaminant of 95 kD, which is present at about the same level as the sodium channel protein, is not significantly labeled here. As a further indication of the relative specificity of the kinase for the sodium channel, Figure 1B shows phosphorylation of a more highly purified sodium channel preparation with a very high catalytic subunit concentration. This experiment shows that minor impurities can also be phosphorylated with higher exposure to the enzyme. Note the substantial level of labeling of the 95-kD protein (lane c), which in this case was present in only trace amounts in the sodium channel preparation (lane a). Figure 1B (lane d) also shows that the kinase preparation, even at the high levels of this experiment, contributes no background labeling in the region of the sodium channel protein.

Figure 2 illustrates experiments with the calcium/phospholipid-dependent protein kinase (protein kinase C). A purified preparation was incubated with a high level of this enzyme for an extended time. This resulted in negligible lipid-stimulated incorporation of ^{32}P into the sodium channel band (Figure 2, left and center lanes). In contrast, protein kinase C autophosphorylation and the phosphorylation of a small amount of histone H1 included in the incubation were markedly stimulated by lipid. The kinase preparation used

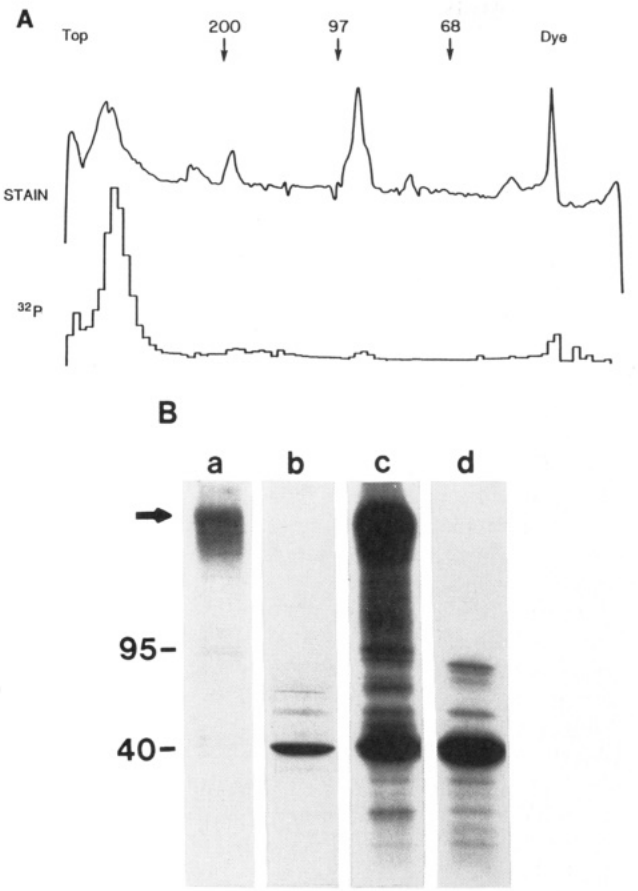

FIGURE 1: Selective phosphorylation of the sodium channel by protein kinase A. (A) Phosphorylation of impure sodium channel. Pooled, concentrated trailing-edge fractions from a Sepharose 6B column were incubated for 5 min at 30 °C in the standard phosphorylation buffer including 244 nM TTX-binding sites (697 pmol/mg), 4.0 μM [γ-^{32}P]ATP (44 400 cpm/pmol), and 0.5 milliunit/mL (12.5 nM) protein kinase A catalytic subunit, and the products were separated by SDS–PAGE on a 5% tube gel. The upper trace is a densitometer scan of the gel stained with Coomassie. The lower trace is the radioactivity profile of the same gel (10 cm long) cut into 1-mm sections and counted by liquid scintillation. The peak slice in the lower trace contained 7710 cpm. (B) Phosphorylation of LFA-purified sodium channel. The TTX-binding fraction from the LFA column was incubated for 8 min at 30 °C in the standard phosphorylation buffer containing 75 nM TTX-binding sites (1440 pmol/mg), 83 μM [γ-^{32}P]ATP (55 000 cpm/pmol), and 24.0 milliunits/mL protein kinase A catalytic subunit and separated on a 4.5–15% acrylamide–SDS slab gel. Lanes a and b: Silver stain showing peptide composition of (a) the purified sodium channel preparation and (b) the catalytic subunit preparation. Lanes c and d: Autoradiographs of material from parallel phosphorylation reactions with (c) and without (d) the sodium channel preparation (2-h exposure). The arrow indicates the position of migration of the sodium channel glycopeptide. Molecular masses are at the left in kilodaltons. The catalytic subunit is the major band at 40 kDa in lanes b–d.

for this assay was only partially pure, but there was no evidence of proteolysis in these samples, on the basis of the silver stain intensity of protein bands in parallel incubations with and without protein kinase C (not shown). The small amount of phosphate that is apparent in the sodium channel band in the center lane of this figure was not reproducible, and the illustrated experiment represents the highest level of incorporation that we encountered. In this experiment, the sodium channel labeling, stimulated by addition of diacylglycerol and phosphatidylserine (DG/PS) amounted to 7 fmol in a 20-min incubation with protein kinase C. In contrast, under identical conditions, protein kinase A, at three-fourths of the activity, incorporated 145 fmol of ^{32}P into the sodium channel band in only 5 min.

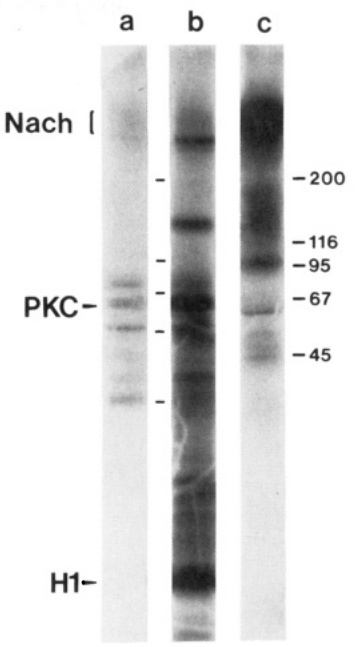

FIGURE 2: Protein kinase C does not phosphorylate the sodium channel glycopeptide. Lanes a and b: protein kinase C incubation. The phosphorylation reaction was as described under Materials and Methods. The incubation was for 20 min in the standard phosphorylation mixture, except for the following: The final mixture contained 20 μM [γ-^{32}P]ATP, 13 mM $CaCl_2$, 0.5 μg/mL histone (Sigma type VIIS), and 0.68 milliunit/mL protein kinase C, without (a) or with (b) 2 μg/mL DG and 200 μg/mL PS (denoted DG/PS). The samples were run on gels which were dried down and exposed to X-ray film as described. The autoradiographs are shown. Lane c: protein kinase A incubation. An autoradiograph from the same gel of the material incubated under identical conditions except that 0.52 milliunit/mL protein kinase A catalytic subunit replaced DG, PS, and protein kinase C. The incubation was for 5 min. The positions of histone H1 and autophosphorylated protein kinase C are marked on the left. The acrylamide gradient shifted gradually between the C kinase and A kinase lanes in this gel. The positions of migration of molecular mass markers from a lane adjacent to lane c are shown on the right. The positions of the same molecular mass markers taken from a lane adjacent to lane a are shown between lanes a and b.

Protein Kinase A Labeling of Reconstituted Sodium Channel. The above experiments were conducted with detergent-solubilized preparations of the protein. We further tested whether the protein, reconstituted into phospholipid vesicles, was susceptible to phosphorylation by protein kinase A. Sodium channels were reconstituted into liposomes of egg phosphatidylcholine and phosphorylated (i) under conditions where all reagents were accessible to both internal and external space (via sonication) or (ii) in the presence of detergent (Lubrol PX), to solubilize the protein (Figure 3). Specific incorporation into the sodium channel band was determined as described under Materials and Methods. It was clear from these results that under both conditions the reactions proceeded to the same final level of labeling over approximately the same time course. These results are consistent with the simple interpretation that the same sites are phosphorylated in each case and that solubilization with this mild detergent does not create conformational changes exposing otherwise inaccessible sites. The slight difference in the time courses may be attributed to some restriction in the access of the enzyme, perhaps due to multilamellar vesicles in the sample. Detergent concentrations used in the solubilized samples were at a detergent to phospholipid weight ratio of 1.5:1. Higher proportions of detergent did not alter the time course, and these concentrations of detergent had no effect on kinase activity with the synthetic substrate kemptamide (data not shown).

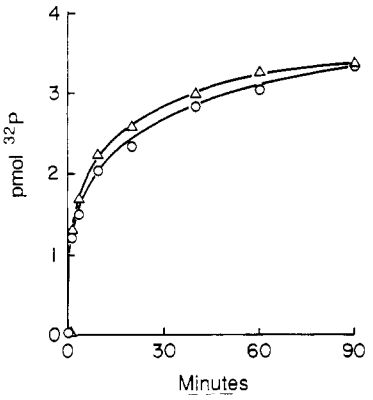

FIGURE 3: Phosphorylation of the membrane-associated sodium channel by protein kinase A. (Circles) Sodium channels purified and reconstituted as described under Materials and Methods were phosphorylated at 30 °C in the standard buffer with 83 nM TTX-binding sites (2100 pmol/mg), 120 μM [γ-^{32}P]ATP (1380 cpm/pmol), 0.26 milliunit/mL protein kinase A catalytic subunit, and 3.3 mg/mL phosphatidylcholine. The reaction was started by addition of Mg-[^{32}P]ATP (starting reaction volume = 270 μL) and sonication for 2 s in a bath sonicator. At the indicated times 30-μL samples were withdrawn and denatured immediately in SDS–PAGE sample buffer. The zero-time sample was denatured before Mg[^{32}P]ATP was added. At 47 min the mixture was supplemented with an additional 230 units/μL catalytic subunit (in 10% of the remaining volume) and sonicated for 2 s. The level of labeling in the latter time points was adjusted for the dilution factor. (Triangles) All conditions are the same as above except that the mixture contained 0.6% Lubrol PX at the start of the reaction.

Specificity of the Sodium Channel as Substrate for Protein Kinase A. Although the sodium channel appears to be more readily phosphorylated than other peptides in a partially purified sample, a more relevant measure of specificity is its reactivity with protein kinase A as compared to that of proteins which are known to be physiologically regulated by this kinase. To estimate this, the initial rate of phosphorylation of the sodium channel was compared to that of the artificial substrate kemptamide. This peptide, LRRASLG-amide, is a model of the protein kinase A phosphorylation site on pig liver pyruvate kinase (Kemp, 1979) and is among the most specific of known substrates. A highly specific substrate is one with a high maximum velocity (V_{max}, proportional to catalytic turnover rate, k_{cat}) and a low K_m; k_{cat}/K_m is called the "specificity constant" (Ferscht, 1985). By incubating the enzyme with protein substrates, either the sodium channel or kemptamide, at identical concentrations well below the K_m, at constant total enzyme concentration with saturating MgATP, the observed rate of phosphorylation of each (V_{obs}) will be proportional to its specificity constant:

$$V_{obs} \sim (k_{cat}/K_m)[\text{acceptor site}] \quad (2)$$

$$[\text{acceptor site}] \ll K_m$$

Because the K_m for kemptamide under physiological conditions is approximately 2.5 μM (Kemp, 1979), and only better substrates will have a lower K_m, this expression should be valid when submicromolar concentrations of phosphate acceptor sites are used. For these experiments, the total concentration of sodium channel phosphate acceptor sites in a sample of purified protein was determined as in Figure 4A. This shows an extended time course of phosphorylation with high levels of kinase and ATP. Control experiments (not shown) indicated that the plateau at long times was stable, because no change in the level of labeling was produced by further addition of kinase and [^{32}P]ATP after the plateau was reached. Parallel reactions were then conducted under identical incubation conditions with identical concentrations of kemptamide and sodium channel phosphate-acceptor sites (290 nM). In these incubations the initial rate of sodium channel phosphorylation was about 5-fold lower than that for kemptamide. Figure 4B shows a time course of kemptamide phosphorylation superimposed on a time course of sodium channel phosphorylation in which the vertical axis has been multiplied by 5.2. In this and other similar experiments we noted that the sodium channel time course was nonlinear at very early times, even in the first minute when the reaction was only ~8% complete.

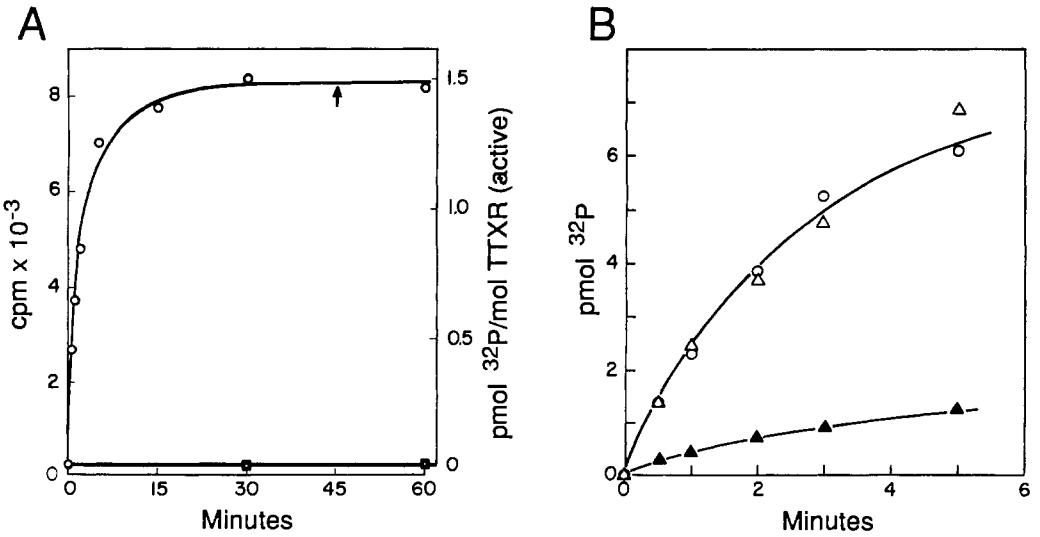

FIGURE 4: Comparison of the rate of phosphorylation of sodium channel with that of kemptamide. Electroplax sodium channels were purified over LFA and dialyzed into the standard phosphorylation buffer as described. (A) Maximal phosphorylation of sodium channels. (Circles) The phosphorylation was at 30 °C in the standard buffer including 211 nM TTX-binding sites (1317 pmol/mg), 120 μM [γ-^{32}P]ATP (1308 cpm/pmol), 0.2 milliunit/mL protein kinase A catalytic subunit, 0.04% Lubrol PX, and 0.7 mg/mL phosphatidylcholine. (Squares) Enzyme-free incubation, otherwise the same as above. The mechanics of the assays were as described in Figure 3. The bulk of the material from this time course was withdrawn at 45 min (arrow) and used for phosphoamino acid determination and quantitative amino acid analysis. (B) Time courses of phosphorylation of kemptamide and sodium channel with identical concentrations of reactants and buffer components. The conditions were the same as in (A) except that 20 units/μL catalytic subunit was used, and the substrate concentrations were 290 nM sodium channel phosphate-acceptor sites/193 nM TTX-binding sites (closed triangles) and 290 nM kemptamide (circles). The ordinate value for the sodium channel have been multiplied by 5.2 and replotted (open triangles). The sodium channel time course was performed as described in (A), and the kemptamide assay was as described under Materials and Methods.

This may be explained by the presence on the sodium channel of a mixture of phosphorylation sites which are labeled at varying rates. Although not rigorously quantitative, this experiment shows the initial phosphorylation rate to be approximately 20% that of the model substrate.

Stoichiometry of Phosphorylation by Protein Kinase A. The possible presence of multiple phosphorylation sites is supported by experiments such as that in Figure 4A, which shows that the sodium channel can be phosphorylated to a level of 1.5 equiv of phosphate per active TTX-binding site. This determination is subject to error, however. Although control experiments have shown the phosphorylation sites to be quite stable to relatively harsh treatments (such as freezing and thawing prior to phosphorylation), the TTX-binding activity is much more labile and subject to variable losses during purification steps (Agnew & Raftery, 1979). For a more accurate determination of phosphorylation stoichiometry, total sodium channel peptide was measured by quantitative amino acid analysis. A phosphorylation time course was carried out as in Figure 4A to demonstrate that incorporation had gone to completion (45 min). The incubation mixture was denatured and separated by SDS–PAGE. The sodium channel glycopeptide was excised and electroeluted from the gel. A fraction of this material was counted for ^{32}P. A second fraction was hydrolyzed and the amino acid content determined. The concentration of the peptide in the original sample was calculated as described under Materials and Methods. Three determinations gave a stoichiometry of 1.7 ± 0.6 (SD) equiv of ^{32}P per sodium channel peptide. The individual values were 1.22 (0.17), 2.26 (0.29), and 1.61 (0.09), where the values in parentheses give the error (SD) in the individual measurement resulting from pipet error and variation in amino acid recoveries. These data alone suggest a minimum of two sites on the sodium channel peptide capable of being phosphorylated by protein kinase A. Because we have no estimate of the amount of phosphate attached to the protein by endogenous kinases, however, this may represent a substantial underestimate.

Phosphoamino Acid Analysis. Of the eight high-specificity consensus protein kinase A phosphorylation sites in the electroplax sodium channel sequence, four involve threonine as the target amino acid (cf. Figure 6). This is interesting in light of the fact that sites involving threonine are relatively rare among physiological substrates for protein kinase A (Carlson et al., 1979). Also, phosphoamino acid determination with neuronal sodium channels phosphorylated by protein kinase A in vitro revealed only phosphoserine (Rossie et al., 1987). None of the other four sodium channel species for which complete amino acid sequence data are available have high-specificity consensus protein kinase A phosphorylation sites containing threonine as the target amino acid (Figure 6).

The phosphoamino acid composition was determined with sodium channel glycopeptide that was maximally labeled as in Figure 4A, purified electrophoretically by SDS–PAGE, and electroeluted from the gel. The sample was partially hydrolyzed and the solvent evaporated, supplemented with nonradioactive carrier phosphoamino acids, and separated by thin-layer electrophoresis (Figure 5). The label distributed among four spots. Free phosphate migrated farthest toward the anode and, in the experiment illustrated, comprised 55% of the counts. A small, variable fraction of the counts, perhaps associated with small amounts of unhydrolyzed protein, remained at the origin. The remaining counts comigrated with the carrier phosphothreonine and phosphoserine. The fraction of total counts recovered in these two compounds was variable,

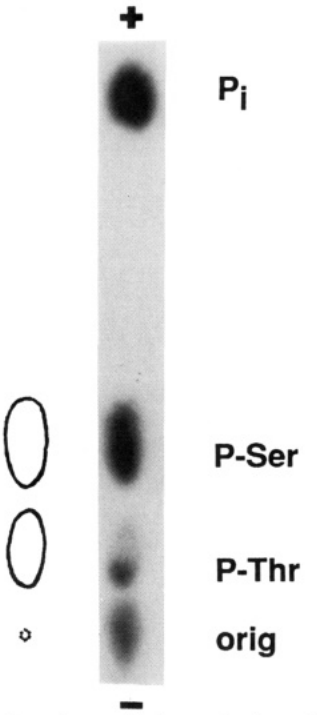

FIGURE 5: Phosphoamino acid determination. Sodium channel glycopeptide, maximally phosphorylated as in Figure 4A (arrow) and electroeluted from an SDS gel, was briefly hydrolyzed, supplemented with unlabeled carrier phosphoamino acids, and subjected to thin-layer electrophoresis as described under Materials and Methods. Left: Traces outline the position of migration of the indicated carrier phosphoamino acids, visualized with fluorescamine under UV illumination. Right: An autoradiograph of the same thin-layer sheet.

but the ratio of phosphoserine to phosphothreonine was constant. Phosphoserine comprised 77% of the recovered phosphoamino acids. Employing the stoichiometry of 1.7 equiv of phosphate/mol of protein, this suggested at least 1.3 mol of phosphate is attached to serine. Because there must be at least two serine-containing sites, this analysis shows that the sodium channel presents at least three sites for phosphate attachment, including two on serine and one on threonine.

Protein Kinase A Consensus Phosphorylation Sites. The demonstration that protein kinase A rapidly labels multiple sites on the electroplax sodium channel is similar to observations made with rat brain sodium channel α-subunits. This is somewhat surprising, as most of the brain peptide phosphorylation occurs in the insertion between homology domains I and II, which is not present in the electroplax protein. To compare the distribution of consensus sites in the brain- and muscle-derived sodium channels, Figure 6 illustrates a map of high- and low-specificity sites. This map shows the positions of consensus sites in the electroplax peptide (ϵ), in the muscle peptide (μI), and in three brain peptides (rI, rII, and rIII). Sodium channel structural models [e.g., Greenblatt et al. (1985)] typically designate as consensus phosphorylation sites only those which we call optimal or high specificity. This is useful for modeling because it focuses on the most likely targets for the enzyme, but it minimizes the fact that less optimal phosphorylation sites may be physiologically relevant. We include these in our discussion.

In the primary structures of the brain peptides, 7–10 high-specificity sites are found, all containing serine as the potential acceptor (Figure 6). Among these three sequences, seven such sites are completely conserved. Five conserved high-specificity sites are located in the hydrophilic region between domains I and II, and four of those are in the insertion (Δ) which is absent in the electroplax and muscle α-peptides.

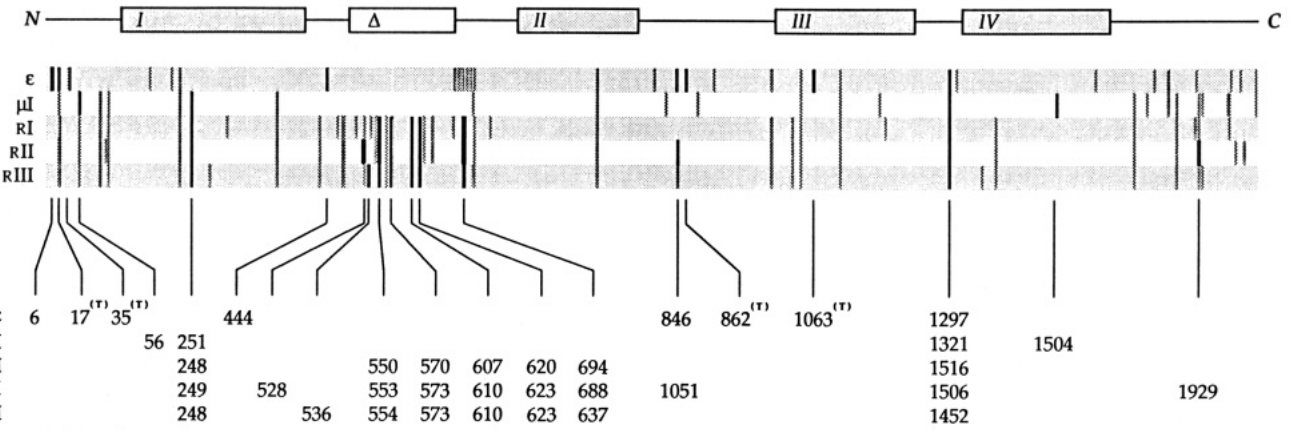

FIGURE 6: Locations of consensus protein kinase A phosphorylation sites in the amino acid sequences of sodium channels from *Electrophorus* electroplax (ϵ), a rat skeletal muscle clone (μ1), and three distinct rat brain clones (RI, RII, and RIII). The upper illustration depicts the linear arrangement of amino acids along a sodium channel backbone. The shaded boxes, labeled I–IV, denote the four internally homologous domains predicted to contain the transmembrane segments. The open box, labeled Δ, corresponds to the heavily phosphorylated insertion which is present only in the brain sequences. N and C are the amino and carboxyl termini, respectively. The lengths of the segments are proportional to the number of amino acids in each, a five-way alignment obtained by the method of Needleman and Wunsch (1970) being used. The middle illustration is a plot of consensus phosphorylation sites for each of the five sequences, as indicated at the left of the figure. Dark vertical bars correspond to high-specificity consensus sites defined according to eq 1 (see Materials and Methods) with $n = 2$ and $m = 1$ or 2. Light vertical bars correspond to lower specificity sites defined according to eq 1 with $n = 1$ and $m = 1$ or 2. The thickness of a bar is proportional to the number of phosphate-acceptor residues in that site, ranging from 1 to 3. The table at bottom lists the position(s) of the target amino acid(s) for each high-specificity consensus site in the sodium channel sequences indicated on the left. (T) denotes that the target amino acid is threonine; all others are serine. Consensus sites that are conserved between sodium channel subtypes fall in the same column of the table. The lines between the cartoon and the table connect each site listed in the table to its appropriate position along the sequence.

The one not located in the insertion is the very high probability consensus site RKRRSSS. The electroplax sequence contains a total of eight high-specificity sites, four containing serine and four containing threonine as acceptor amino acids. The electroplax sequence also exhibits a tight cluster of six lower specificity sites in the interdomain I–II segment overlapping the region of the high-specificity brain site mentioned above. The electroplax sequence also contains a high-specificity site in the region between domains I and II. A second feature of the electroplax sequence is the grouping of three high-specificity sites on the amino terminus. The muscle sequence has only four high-specificity sites, all involving serine as the phosphate acceptor. One of these is located near the amino terminus. None are found in the interdomain I–II segment; this region contains but a single lower specificity site, located in the same position as the cluster of lower specificity sites in the electroplax sequence. In fact, all five peptide sequences have a consensus site in this position. All five sequences also share a single conserved high-specificity site in the span between domains III and IV.

Identification of the specific amino acids which are susceptible to phosphorylation by protein kinase A will provide insights into the accessibility of local domains for enzymatic modification. Also, in future studies aimed at testing channel functions which may be altered by phosphorylation, we may be able to associate these domains with particular physiological behavior.

Amino Acid Sequences Phosphorylated by Protein Kinase A. Inspection of the complete amino acid sequence of the electroplax sodium channel reveals that, with the exception of a segment containing nine consecutive glutamates in interdomain II–III, no sequence of four specific amino acids occurs more than once. Hence, identification of four consecutive amino acids is sufficient to identify the origin of any tryptic peptide. With recent improvements in the sensitivity of PTH-amino acid detection using microbore HPLC (Stone & Williams, 1988) four or more cycles can be obtained easily with tens of picomoles of starting material. We were able to detect reliably PTH-amino acid derivatives present at 0.1 pmol, but no recoveries below 0.3 pmol were observed. In every case the sequencing was carried several cycles beyond the last detectable amino acid. For our experiments, we used protein purified nearly to homogeneity by lectin-affinity and immunoaffinity chromatography (James et al., 1989).

The mobility of small tryptic peptides on reverse-phase HPLC can be influenced strongly by the presence or absence of a phosphate group under the conditions we used. Thus, to maximize the yield of phosphopeptides for sequence analysis, it was important to phosphorylate the protein extensively. Our objective in this experiment, however, was to phosphorylate only briefly with ^{32}P in order to label selectively the most active phosphate acceptor sites on the molecule. Also, as we have mentioned, low-level phosphorylation labels the sodium channel in preference to any contaminants in the preparation. Hence, we incubated LFA-purified sodium channel briefly with low levels of protein kinase A catalytic subunit and [γ-^{32}P]ATP. The sample was then augmented with excess catyalytic subunit and unlabeled ATP, and the reaction was continued for an extended time. The sodium channel was separated from kinase components by immunoaffinity chromatography; 39 μg (188 pmol) of homogeneous glycopeptide was recovered (Figure 7A). This material was labeled to only 14% of maximum in this procedure (not shown), still on the steeply rising portion of the phosphorylation time course (cf. Figure 5A).

The immunoaffinity-purified material was delipidated and denatured in urea, reduced, carboxyamidomethylated, and subjected to exhaustive trypsin proteolysis, and the peptide fragments were separated and purified by two steps of reverse-phase HPLC. In Figure 7B is shown the chromatogram from the first HPLC dimension (C-18 column). All of the applied ^{32}P was recovered from the column. The majority of the counts (76%) was recovered in seven peaks, labeled I–VII in Figure 7B. Three major peaks (II, IV, and V) contained 29, 17, and 11% of the total counts (Table I), respectively. These were chosen for sequencing.

The first peptides to be studied were those of C-18 fractions 55 and 56 of peak II. To obtain material sufficiently pure for microsequencing, fraction 55 was rechromatographed on a

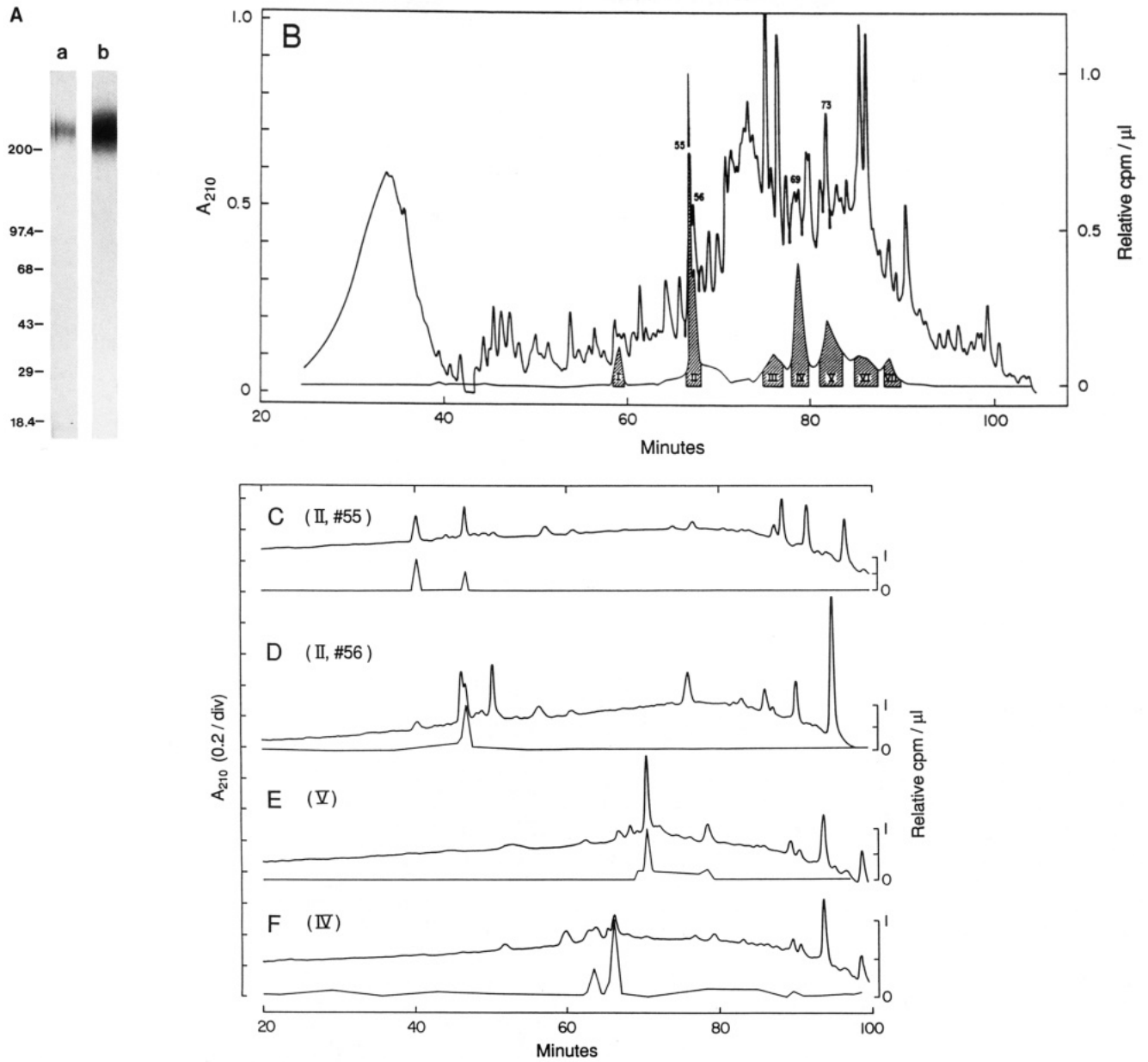

FIGURE 7: (A) Silver stain (a) and autoradiograph (b) of lectin-purified sodium channel peptide which was phosphorylated and repurified as described in the text. (B) C-18 column profile. Chromatogram of material in (A) which was delipidated, denatured, carboxyamidomethylated, exhaustively digested with trypsin, and separated by reverse-phase HPLC on a narrow-bore C-18 column. Upper trace: absorbance profile. Fraction numbers are indicated above selected peaks. Thick vertical dashes on the tracing at those peaks denote where the fractions were separated. Lower trace: radioactivity profile. Aliquots of each fraction were counted by liquid scintillation. Shading delimits peak labeled fractions. Each peak of radioactivity is referred to in the text by the indicated Roman numeral. (C–F). C-8 column profile. Selected fractions off the C-18 column were rechromatographed by reverse-phase HPLC on a microbore C-8 column. The upper trace in each panel is the absorbance profile, and the lower trace is the radioactivity profile from the same chromatography run, as in (A). The C-18 column fraction used for each run is given in parentheses in the appropriate panel. Peak II resolved into two fractions, 55 and 56, which were run separately in (C) and (D), respectively. C-18 fraction 73 contained most of the counts in peak V. This fraction was run in (E). Fraction 69 contained all of the counts in peak IV. This fraction was run in (F). For details refer to the text.

Table I: ^{32}P Recovery from the C-18 Column[a]

peak	cpm (% total)	rel cpm	peak	cpm (% total)	rel cpm
I	4.1	0.14	V	11.0	0.38
II	28.8	1.00	VI	6.6	0.23
III	5.9	0.20	VII	3.0	0.10
IV	17.0	0.59			

[a] The peaks listed in the left column are designated in Figure 7B. The middle column lists the ^{32}P recovered in each peak as a percent of the total applied to the column. The column on the right expresses these numbers relative to peak 2.

reverse-phase column with different separation properties (C-8). The radioactivity emerged in two peaks (Figure 7C). The major peak, eluting at 40.2 min, was sequenced directly. PTH-amino acids were recovered through 10 cycles, yielding the sequence KFSS′ARPEMF, corresponding to amino acid residues 4–13 in the electroplax amino acid sequence (see Figure 8). Recovery of terminal arginines (i.e., residues 14 and/or 15) is often low because they are poorly retained on the polybrene during washing of the filter between the reactions of the Edman cycle (K. Stone and K. Williams, personal communication). This accounts for the failure of this peptide to yield amino acids past phenylalanine. In general, a site containing contiguous arginine or lysine residues is a strong trypsin cleavage site, especially in the absence of neighboring acidic amino acids. The double arginine following phenylalanine 13 is such a site, and the sequence subsequently ob-

tained from peptide V (below) confirms that this site was cleaved efficiently. Dehydroserine (S′) was recovered in both cycles 3 and 4, confirming the identity of these residues as serine. The sequence RKFSS corresponds to a high-specificity consensus protein kinase A phosphorylation site, and the two adjacent serines are the only possible sites of phosphate attachment in this tryptic peptide. Unmodified serine was recovered in cycle 4 but was not detectable in cycle 3, indicating that the first serine (corresponding to serine 6 in the electroplax sodium channel) is the phosphorylated residue (cf. Materials and Methods).

Sequencing of the minor C-8 column fraction (46.6 min in Figure 7C) from the rechromatography of C-18 fraction 55 yielded a double sequence (Figure 8B), indicating the coelution of two peptides in this fraction. The major sequence (about two-thirds of the total recovery) GNIINRFNAER, which derives from amino acids 90–100 of the sodium channel sequence, does not contain a serine or threonine to which a phosphate could be bound. The minor sequence KFxS′ARPEMF (x denotes a blank cycle) derives from amino acids 4–13 and is the same as that obtained above. Dehydroserine was recovered in cycle 3 only, and no unmodified serine was recovered in either cycle 3 or cycle 4, making the identification of the phosphorylated residue impossible in this case. There are two reasons why this phosphopeptide could have a substantially different mobility than the first. It is possible that serine 7 or both serines 6 and 7 are phosphorylated on this peptide, or it could be that trypsin cleavage at the double arginine at positions 14 and 15 produced similar peptides ending in one or two arginines. To pursue this, we rechromatographed C-18 column fraction 56, because this contained a substantial fraction of the total peak II counts but showed slightly greater retention on the column, suggesting that it might contain the same phosphopeptide as in the peak derived from fraction 55. The rechromatography profile is shown in Figure 7D. Sequencing of the major radioactive fraction again yielded a double sequence, revealing the same two comigrating species obtained above (this time in equal amounts), including the sequence KFSS′ARPEMF. In this case, unmodified serine was recovered in cycle 3 and was absent in cycle 4, whereas dehydroserine was present in both. This indicates that phosphate is attached to the second of the two serines in this peptide, corresponding to serine 7 in the electroplax channel sequence (see Figure 8).

We also attempted to identify the labeled peptide in peak V. Rechromatography of C-18 column fraction 73 yielded a single radioactive peak (Figure 7E). Sequencing revealed this to be a peptide of 18 amino acids (RFT′PDSLEEIEAFTELKK) derived from positions 15–32 of the sodium channel primary sequence (see Figure 8). This peptide contains one serine (cycle 6) and two threonines (cycles 3 and 14). Only threonine 17 (cycle 3) is within a consensus phosphorylation site, however, making it the likely phosphate acceptor in this peptide. Dehydrothreonine (T′) and DTT adducts of dehydrothreonine produced during Edman degradation were present in cycle 3, but no unmodified threonine was recovered, suggesting that threonine 17 is indeed the phosphoamino acid. This is confirmed by the recovery of unmodified serine and threonine in cycles 6 and 14, respectively.

To analyze peak IV, C-18 column fraction 69 was rechromatographed on the C-8 column, yielding two radiolabeled peaks (Figure 7F). Sequencing of the major peak yielded a single sequence (MPxLS′VPE), with low recoveries, corresponding to sodium channel residues 1774–1781, near the C-terminus (Figure 8). The terminal glutamate of this fragment does not adjoin a trypsin cleavage site, however. The primary structure of the protein shows that the 45 residues between serine 1776 and the carboxyl terminus of the sodium channel contain two other possible sites of phosphorylation, i.e., threonine 1792 and serine 1818. Because the terminal glutamate does not correspond to the end of a predicted tryptic fragment, it is possible a site other than serine 1776 is labeled. However we discuss below arguments that implicate this residue as the most likely candidate.

Discussion

We report here that the sodium channel from eel electroplax is an excellent substrate for the catalytic subunit of cyclic-AMP-dependent protein kinase. The electroplax protein differs from the α-subunits of brain sodium channels in the distribution of consensus phosphorylation sites, in the pattern of phosphorylation observed in vitro, and in that it is not a substrate for phosphorylation by protein kinase C.

In several experiments, the electroplax protein was not phosphorylated by protein kinase C but was readily labeled by protein kinase A. In contrast, the α-subunit of brain sodium channels is labeled by protein kinase C to the extent of 3–4 equiv/mol of STX-binding sites (Costa & Catterall, 1984b). The failure of the electroplax protein to be labeled could be due to the absence of suitable acceptor sites or alternatively to complete block by endogenous phosphorylation. The latter seems unlikely because no effort was made to control endogenous phosphatase activity.

The electroplax protein, either reconstituted into membranes or in detergent solution, was rapidly phosphorylated by protein kinase A. The initial velocity of labeling was 20% of that for the model substrate kemptamide under identical conditions, where the concentration of sodium channel phosphate acceptor sites was equal to that of kemptamide. Kemptamide (LRRASLY-amide), the analogue of the phosphorylation site of pig liver pyruvate kinase, is among the best known substrates for the enzyme. The rate of labeling observed for the sodium channel is in the range encountered for known physiological protein kinase A substrates. For comparison, the α-subunit of phosphorylase kinase is phosphorylated at $\sim$20% of the rate of the synthetic peptide LRRASVA (Zetterqvist et al., 1976), the analogue of the phosphorylation site of rat liver pyruvate kinase. Individual sites may represent even better substrates than the measured rates suggest. The time course reflects only the average rate of labeling of several sites. Thus, the initial rate of labeling of the peptide of peak II in Figure 7B is more than twice the average rate for the seven fragments, or 40% of the rate of kemptamide. Thus, the specificity constant for this site may substantially exceed 40% if we were to take into account the lower concentration of "site II" relative to the total concentration of sites (cf. eq 2). This suggests that the sodium channel may well serve as a site for this enzyme in vivo. Consistent with this conclusion is the observation that maximal labeling resulted in incorporation of 1.2–2.3 mol of phosphate/mol of sodium channel, despite the fact that exhaustive trypsin digestion produced seven well-resolved phosphopeptides. This suggests that a substantial fraction of the acceptor sites on the electroplax protein may be blocked by endogenous phosphate, as found for neuronal sodium channel α-subunits (Rossie & Catterall, 1987a).

Phosphoamino acid analysis of limited acid hydrolysates of maximally labeled material showed that 77% of the phosphate was bound to serine and 23% to threonine. This analysis involves a competition between hydrolytic release of amino acids from the peptide and the acid-catalyzed cleavage of the phosphoryl bond (Bylund & Huang, 1976). Longer hydrolysis,

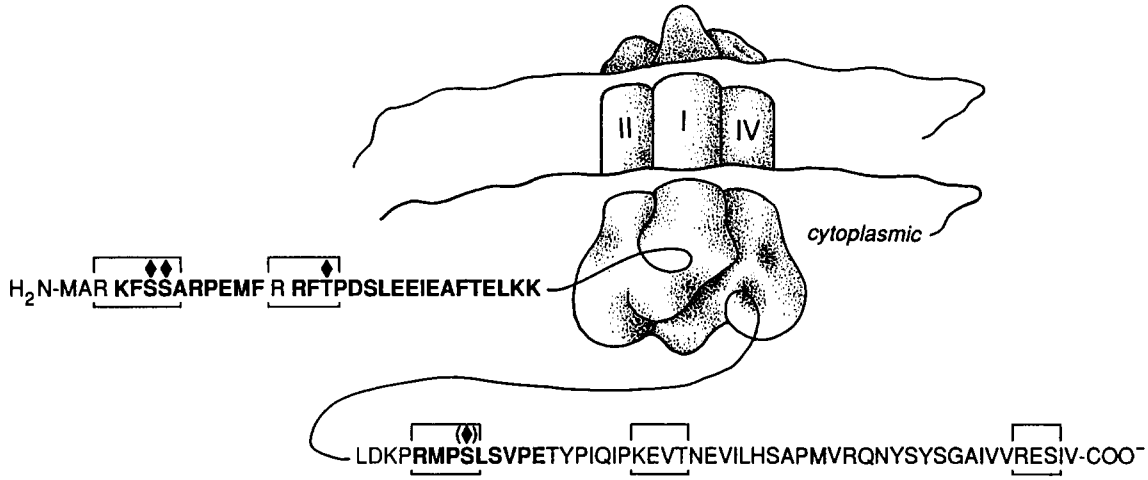

FIGURE 8: Cartoon depicting the predicted folding of the sodium channel in the electroplax membrane. Roman numerals refer to the four pseudosubunits, or domains of intrachain homology (three are visible here). The primary sequences at the amino and carboxyl termini are shown. The three tryptic peptides that were isolated and identified in this work are shown in boldface. The boxes on the primary sequence indicate consensus protein kinase A phosphorylation sites. Diamonds label amino acids which have been identified as sites of phosphate incorporation. The last diamond is in parentheses to emphasize that this fragment does not end at a trypsin cleavage site.

which would produce more complete recovery of amino acids, exposes them to harsh conditions under which the phosphoamino acids decay at varying rates. Because radioactivity in the incompletely hydrolyzed protein core was present at only low levels (while some fluorescamine-positive material remained at the origin in all cases), we concluded that longer hydrolysis times would be counterproductive. The rate of phosphoryl bond cleavage depends significantly on the phosphoamino acid and the local peptide environment. In general, however, phosphoserine is degraded more rapidly than phosphothreonine (Bylund & Huang, 1976). Thus, 77% probably represents a low estimate of the fraction of phosphoserine, suggesting that the electroplax protein contains at least two sites for phosphorylation on serine and one on threonine.

When the purified sodium channel was phosphorylated briefly, to label preferentially the most active sites, and then digested with trypsin and the fragments separated by HPLC, seven major radioactive peaks were recovered, containing 79% of the total radioactivity. The most heavily labeled peak (peak II), containing 29% of the total counts, was a doublet of radioactive peptides. Both were shown to contain the same high-specificity consensus phosphorylation site (RKFSS) derived from the amino terminus of the sodium channel. Either serine (serines 6 and 7 of the sodium channel sequence) could serve as a phosphate acceptor. On the basis of the recovery of radioactivity, however, the form with phosphate on serine 6 was more abundant, consistent with the observation that a spacing of one amino acid ($m = 1$) between the basic and target amino acids is optimal and that increasing this spacing tends to decrease substrate specificity. The next most heavily labeled peak (V), with 17% of the counts, also contained a high-specificity consensus site (RRFT), also from the amino terminus, with threonine 17 as the target amino acid. We showed this threonine to be the phosphate acceptor (lower specificity sites at, or very near this residue are found in the four mammalian peptides as well, including the protein from muscle).

Sequencing the major phosphopeptide in peak IV yielded 8 residues, beginning 47 amino acids from the carboxyl terminus of the sodium channel. The fact that the recovered sequence ends on a glutamate, which is not a trypsin cleavage site and is not followed immediately by such a site, raised the possibility that we may not have sequenced to the end of the fragment. We can say conclusively, therefore, only that the sodium channel is phosphorylated at a site within 47 residues of the carboxyl terminus. There are two strong arguments, however, implicating serine 1776 (tagged with a diamond in Figure 8) as the site of phosphate attachment. First, of three possible protein kinase A phosphorylation sites in this 47-residue region (Figure 8), that containing serine 1776 is predicted to have the highest specificity: (1) This site contains arginine as the basic amino acid, which improves specificity markedly over a similar site with lysine at that position (Kemp et al., 1976, 1977), and is consistent with the observation that most physiological protein kinase A sites contain arginine. (2) This site contains serine as the acceptor, which likewise improves specificity over a similar site with threonine (Kemp et al., 1977). (3) This target serine is followed by a hydrophobic amino acid, which also has been shown to improve substrate specificity (Meggio et al., 1981). (4) The second and third consensus sites contain negatively charged glutamate residues on the immediate carboxyl site of the basic amino acid, which has been shown to decrease substrate specificity (increases the K_m) for the substrate [Table VI of Kemp et al. (1977)]. The second consensus site fails on all four of these points, rendering it a much less likely candidate than the first. Finally, (5) the explanation that the peptide contains the last consensus site rests on the tenuous supposition that trypsin failed to cleave at each of three lysine or arginine residues between the first and last sites, an unlikely occurrence in an exhaustive proteolysis with such a hydrophilic peptide. The second argument is that it is quite possible that we have sequenced to the end of the peptide. This seems likely because the recovery of PTH-amino acids dropped abruptly to zero after the eighth cycle, without any decline in previous cycles (supplementary material). Further, we note that this same tryptic peptide has been sequenced by others (Noda et al., 1984) on a larger scale, yielding a sequence ending with the same residue. Hence, we believe it is likely that the sodium channel is sensitive to cleavage by endogenous proteases during the initial stages of purification or perhaps is modified in the cell by posttranslation proteolysis after glutamate 1781. Such cleavage would produce only a 4.4-kDa reduction in molecular mass, which would not be apparent on SDS gels. If cleavage occurs during purification, it may be functionally silent in the reconstituted protein.

We have thus demonstrated four phosphate acceptor sites for protein kinase A on the electroplax sodium channel, three

in the amino terminus of the protein and one in the carboxyl-terminal segment. This pattern of labeling is markedly different from that observed with the brain α-subunits.

Although brain α-subunits differ from those of electroplax and muscle by having a $\sim$200 amino acid insert between domains I and II, there are two interesting similarities between these classes of channels. First, the electroplax clone has a tight cluster of six lower specificity consensus sites in the interdomain I–II segment, in a region which in the brain sequences contain a very high probability site along with a lower specificity site which is conserved in all sodium channel α-peptides. Thus, while we have not detected labeling of this site in vitro, phosphorylation of the interdomain I–II region may have a physiological role in muscle-derived as well as neuronal cells. The second feature is the presence in all five proteins of a high-specificity site in the region between domains III and IV. This highly conserved segment has been hypothesized to play a role in inactivation gating (Guy, 1989). Mutations introduced at this location of rII result in loss of fast-inactivation gating (Stuhmer et al., 1989). Also, antibodies directed against an 18-residue segment containing this consensus site (KKLGS) slow the inactivation rate of sodium currents in rat muscle cells (Vassilev et al., 1988). It is an interesting possibility, therefore, that phosphorylation at this site may also markedly alter inactivation kinetics in both neuronal and muscle-derived sodium channels.

One notable difference between muscle-type and neuronal α-subunits is the occurrence of high-specificity sites on the amino terminus of the muscle-derived peptides. This is intersting especially in light of our observation that the three most active sites for phosphorylation of the purified electroplax protein are on the terminal segments of the molecule, one on the carboxyl and two on the amino terminus. If, as proposed from theoretical models of peptide folding (Noda et al., 1984; Greenblatt et al., 1985; Guy & Seetharamulu, 1986; Guy, 1988), the protein forms a tetrameric rosette in the plane of the bilayer, these regions, while at opposite ends of the peptide sequence, may nonetheless be in close proximity to one another in the membrane-bound channel (cf. Figure 8). Because these segments are the first and last portions of the peptide to be synthesized, it is conceivable that phosphorylation or dephosphorylation of these sites may play a role in the positioning of the terminal domains in the final stages of channel assembly, analogous to the role proposed for phosphorylation in the assembly of nicotinic acetylcholine receptors (Ross et al., 1987).

Recently, two studies have provided evidence suggestive of neuromodulation of sodium channel function. In one case activators of protein kinase C were found to cause a reduction in amplitude of sodium currents expressed in *Xenopus* oocytes from chick forebrain mRNA; this effect was prevented in cells also treated with tamoxifen, an inhibitor of protein kinase C. No effects on the kinetics or voltage dependence of the currents was observed (Sigel & Baur, 1988). This result is consistent with the observed ability of protein kinase C to phosphorylate neuronal sodium channels in vitro, but there is no evidence that the result obtained in this study was caused directly by phosphorylation of the sodium channel α-subunit. In another case, Auld et al. (1988) report that while sodium currents expressed in *Xenopus* oocytes from rIIA cDNA inactivate 10-fold more slowly than sodium currents in native membranes, coinjection of a low molecular weight brain mRNA fraction increased inactivation kinetics and maximal activity levels 10- and 5-fold, respectively. This effect may be due to the inclusion of mRNA encoding one or more small sodium channel subunits. However, it is also plausible that these effects are due to expression of a modulatory enzyme, such as a specific protein kinase or phosphatase. Added in proof: We have found that poly(A+) mRNA from brain or muscle exerts similar effects on the inactivation gating of the muscle sodium channel (Zhou et al., 1989). Other attempts to correlate phosphorylation of the brain α-subunit with effects on channel function have yielded marginal results. Stimulation of brain synaptosomes with 8-bromo-cyclic-AMP resulted in a slight decrease in BTX-stimulated sodium flux (Costa & Catterall, 1984a). The absence of dramatic functional effects of phosphorylation may be due a high level of basal phosphorylation of neuronal sodium channels (Rossie & Catterall, 1987a) such that kinase stimulation does not increase phosphorylation appreciably. Our results also suggest that the electroplax sodium channel has a high level of endogenous phosphorylation.

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Supplementary Material Available

One figure depicting PTH-amino acid recoveries from automated Edman degradation of tryptic phosphopeptides purified by reverse-phase HPLC (6 pages). Ordering information is given on any current masthead page.

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Diversity of Oligosaccharide Structures Linked to Asparagines of the Scrapie Prion Protein†

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ABSTRACT: Prion proteins from humans and rodents contain two consensus sites for asparagine-linked glycosylation near their C-termini. The asparagine-linked oligosaccharides of the scrapie isoform of the hamster prion protein (PrP 27-30) were released quantitatively from the purified molecule by hydrazinolysis followed by N-acetylation and NaB^3H_4 reduction. The radioactive oligosaccharides were fractionated into one neutral and three acidic oligosaccharide fractions by anion-exchange column chromatography. All oligosaccharides in the acidic fractions could be converted to neutral oligosaccharides by sialidase digestion. Structural studies on these oligosaccharides including sequential exoglycosidase digestion in combination with methylation analysis revealed that PrP 27-30 contains a mixture of bi-, tri-, and tetraantennary complex-type sugar chains with Manα1→6(GlcNAcβ1→4)(Manα1→3)Manβ1→4GlcNAcβ1→4-(Fucα1→6)GlcNAc as their core. Variation is produced by the different combination of the oligosaccharides Galβ1→4GlcNAcβ1→, Galβ1→4(Fucα1→3)GlcNAcβ1→, GlcNAcβ1→, Siaα2→3Galβ1→4GlcNAcβ1→, and Siaα2→6Galβ1→4GlcNAcβ1→ in their outer chain moieties. When both asparagine-linked consensus sites are glycosylated, the diversity of oligosaccharide structures yields over 400 different forms of the scrapie prion protein. Whether these diverse asparagine-linked oligosaccharides participate in scrapie prion infectivity or modify the function of the cellular prion protein remains to be established.

Scrapie is a degenerative neurological disease of sheep and goats, which can be transmitted to laboratory rodents (Prusiner, 1987). Because the unusual properties of the scrapie agent distinguish it from viruses, the term prion was introduced (Prusiner, 1982). Three human diseases may also be caused by prions: Creutzfeldt-Jakob disease, kuru, and Gerstmann-Sträussler syndrome (Gajdusek, 1977; Masters et al., 1981). Purification of scrapie prion infectivity led to isolation of a protein with relative molecular weight of 27000-30000 (Bolton et al., 1982; Prusiner et al., 1982). This protein was designated prion protein (PrP) 27-30.[1]

PrP cDNA clones were isolated from libraries that were constructed by using poly(A+)RNA from scrapie-infected brains of hamsters and mice (Chesebro et al., 1985; Oesch et al., 1985). Southern blotting revealed that PrP 27-30 is encoded by a cellular gene and not by the infectious prion particles (Oesch et al., 1985). Unexpectedly, Northern blotting indicated that normal and scrapie-infected brains contain similar levels of PrP mRNA (Chesebro et al., 1985; Oesch et al., 1985). The presence of PrP mRNA in normal brain led to the discovery of a normal cellular isoform of PrP, termed PrP^C, and the demonstration that PrP 27-30 is derived by limited proteolysis from a larger protein, designated PrP^{Sc} (Oesch et al., 1985). Sensitivity to proteinase K and solubility after detergent extraction differentiate PrP^{Sc} from PrP^C (Oesch et al., 1985; Meyer et al., 1986). Because the entire PrP open reading frame (ORF) is contained within a single exon, the differences between PrP^C and PrP^{Sc} are not due to alternative splicing of exons but probably result from a posttranslational event (Basler et al., 1986). To date, there is evidence for at least six posttranslational modifications of both PrP isoforms including glycosylation (Prusiner, 1987; Stahl et al., 1987; Turk et al., 1988; Haraguchi et al., unpublished observations). Since earlier studies suggested that PrP 27-30 is a sialoglycoprotein and the amino acid sequence of PrP contains two potential asparagine-linked glycosylation sites (Bolton et al., 1985; Oesch et al., 1985), we undertook a structural study of the sugar chains of PrP 27-30 with a view toward eventually determining whether the different properties of two PrP isoforms arise from differences in their asparagine-linked oligosaccharides. Recently, we demonstrated the presence of asparagine-linked oligosaccharides in both PrP isoforms, which were resistant to endoglycosidase H digestion but sensitive to *N*-glycanase

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[1] Abbreviations: PrP, prion protein; PrP^{Sc}, scrapie isoform of the prion protein; PrP^C, cellular isoform of the prion protein; PrP 27-30, scrapie prion protein derived from PrP^{Sc} by limited proteolysis. Subscript OT is used in this paper to indicate NaB^3H_4-reduced sugars. All sugars mentioned in this paper were of the D configuration, except for fucose, which had an L configuration.

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