Regulation of the Eel Electroplax Na Channel and Phosphorylation of Residues on Amino- and Carboxyl-Terminal Domains by cAMP-Dependent Protein Kinase

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ABSTRACT: Previous studies have shown that the short-motif electroplax Na channel is phosphorylated *in vitro* by cyclic AMP-dependent protein kinase (PKA) at serines 6 or 7 and 1776 and threonine 17 (Emerick & Agnew, 1989). We here show that phosphatase treatment of solubilized, purified Na channels enhanced subsequent PKA labeling of four of five tryptic phosphopeptides, indicating that these sites are phosphorylated *in vivo*. Microsequencing and analysis of PTH-amino acid products revealed endogenous labeling of serines 6, 444, 1680, and 1776. Serines 1680 and 1776 lie in the carboxyl-terminal cytoplasmic domain, while serine 6 lies in the amino terminus and serine 444 is in the cytoplasmic loop between domains I and II. Endogenous phosphorylation of serine 6 establishes experimentally that the Na channel amino terminus is cytoplasmic. In electrophysiological experiments, brief exposure of inside-out membrane patches excised from Sachs-organ cells to MgATP and purified PKA catalytic subunit produced rapid, sustained reduction of Na current amplitude by ~80% and a hyperpolarizing shift in the conductance/voltage relation by 10–12 mV. The effect was absent in controls omitting either PKA or MgATP. Serines 6 and 1776 and threonine 17 are labeled rapidly and extensively *in vitro*, and only threonine 17 appears to be unphosphorylated *in vivo*. We suggest that phosphorylation of the amino and carboxyl domains, perhaps especially at threonine 17, underlies the demonstrated downregulation of the electroplax Na channel.

Recent experiments have shown that voltage-sensitive Na channels in several types of electrically excitable cell are susceptible to neuromodulation through a variety of mechanisms. Protein kinase C activators have been reported to reduce detectably the amplitude of neuronal Na channels (Sigel & Baur, 1988; Linden & Routtenberg, 1989; Lotan *et al.*, 1990), prolong inactivation (Numann *et al.*, 1991), and alter the voltage dependence of activation (Dascal & Lotan, 1991). The first two effects were shown by site-directed mutagenesis to require a single conserved serine residue in the short cytoplasmic linker between domains III and IV which is associated with inactivation gating (West *et al.*, 1991). The activity of cardiac Na channels is reduced by β-adrenergic agonists through a membrane-associated G-protein-mediated mechanism which is likely to involve both a direct effect on the Na channel through interaction with G-proteins (Schubert *et al.*, 1989) and a relatively delayed effect of phosphorylation of the Na channel by cyclic AMP-dependent protein kinase (PKA)[1] on the channel (Ono *et al.*, 1989; Schubert *et al.*, 1990). Sorbera and Morad (1991) have reported modulation of cardiac Na channels linked to activation of a surface receptor for cyclic AMP; this modulation appeared to be mediated by GTP binding protein-dependent mechanisms rather than PKA-dependent phosphorylation. Other studies indicate that phosphorylation of cardiac Na channels by PKA reduced the amplitude and delayed the decay rate of ensemble macroscopic sodium currents (Sunami *et al.*, 1991).

All of the Na channel isoforms previously shown to be regulated exhibit a "long motif", possessing a ~200-residue putative regulatory segment in the cytoplasmic loop between subunit domains I and II [*cf.* Stephan and Agnew (1991)]. "Short form" channels, which include those from electroplax and adult skeletal muscle, have not been shown to be subject to neuromodulation and lack the putative regulatory segment. In a previous paper (Emerick & Agnew, 1989) we showed that the electroplax Na channel has at least three residues which serve as highly active phosphate acceptors for PKA phosphorylation *in vitro*: serines 6 or 7 (but not both) and 1776 and threonine 17; we argued further that because the maximum labeling stoichiometry was approximately 2 mol of ^{32}P/mol of Na channel peptide, the channel was likely to be phosphorylated on these residues in the electroplax.

We show in the present studies that the electroplax Na channel is highly phosphorylated *in vivo*. By direct microsequencing of the major phosphopeptides of complete tryptic phosphopeptide maps, we identify four residues which are subject to endogenous phosphorylation, serines 6, 444, 1680, and 1776; threonine 17 appears not to be phosphorylated *in vivo*. Direct phosphorylation by the purified catalytic subunit of PKA strongly inhibits sodium currents in inside-out patches excised from the excitable membrane of the eel electrocyte. This is the first demonstration that a Na channel may be simply "turned off" via phosphorylation and, furthermore, confirms that a "short form" Na channel may be modulated. Among the most active phosphate acceptor sites, three amino acid residues are identified whose phosphorylation is both rapid and extensive enough to implicate them in mediating the effect on channel activity. All three are on terminal domains. This provides the first direct demonstration that the amino acid terminus is cytoplasmic and the first indication

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[1] Abbreviations: PKA, cyclic AMP-dependent protein kinase; PTH, phenylthiohydantoin; TTX, tetrodotoxin; PAP, potato acid phosphatase; BTX, batrachotoxin.

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for the involvement of terminal cytoplasmic loops in regulating the gating properties of a Na channel.

MATERIALS AND METHODS

All reagents and equipment, unless otherwise specified, were from Sigma Chemical Co. or the sources listed previously (Emerick & Agnew, 1989). Calf intestinal alkaline phosphatase was from Boehringer-Mannheim. Protein assays were by the fluorescamine method of Udenfriend *et al.* (1972). SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was by the method of Maizel (1971). TTX binding activity was determined by the rapid gel filtration method of Levinson *et al.* (1979). The catalytic subunit of cyclic AMP-dependent protein kinase (PKA), isolated from adult bovine heart by the method of Reimann and Beham (1983), was estimated to be >90% pure on silver-stained SDS–polyacrylamide gels and had a specific activity of 8 units/mg. One unit of kinase transfers 1 μmol of phosphate to histone from ATP (at 120 μM) in 1 min at 30 °C. The kinase activity was assayed as described previously (Emerick & Agnew, 1989) using histone (Sigma, type VIIS) as reference. Na[γ-^{32}P]ATP (New England Nuclear), obtained at 30–40 Ci/mmol, 50–83 μM, in water, was diluted 20- to 50-fold with unlabeled carrier. The specific activity (in cpm/pmol) was determined by counting aliquots of the working dilution in the same aqueous volume and at the same time as the samples being analyzed.

Na Channel Purification. Electrophorus electricus were killed by hypothermia, and the main electric organ was excised rapidly at 4 °C and frozen immediately in liquid nitrogen. Solubilized Na channels, first partially purified by anion-exchange chromatography over DEAE-Sephadex (Rosenberg *et al.*, 1984), were highly purified by immunochromatography with horse anti-poly(sialic acid) IgM coupled to Sephadex (James *et al.*, 1989). All procedures were performed rapidly at ice–water temperature, except column elution, which was at 4 °C. Phosphatase inhibitors (100 mM NaF, 50 mM $NaPO_4$) were present in all solutions through adsorption to and washing of the IgM–Sepharose column. The column was then washed with phosphorylation buffer (below; omitting ATP), and elution was with this buffer containing 5 mM colominic acid.

In Vitro Na Channel Phosphorylation Reactions. The standard phosphorylation buffer contained 25 mM Hepes or Tris-HCl, pH 6.8, 30 mM Na_2SO_4, 5 mM $MgSO_4$, 1 mM EGTA, 0.03% Lubrol-PX, 0.055 mg/mL phosphatidylcholine, and 120 μM ATP. For phosphorylation in the presence of phosphastase inhibitors (100 mM NaF, 50 mM $NaPO_4$), magnesium was used at 0.5 mM to minimize the formation of MgF precipitate. The PKA activity was 0.5 milliunits/mL. Substrate concentrations and exceptions to the standard conditions are noted where appropriate. The sample volume was 25–50 μL per time point. [^{32}P]ATP was used at a specific activity of 500–2500 cpm/pmol. Reactants were preequilibrated in a water bath for 1–5 min (depending on the volume) to bring them to 30 °C, and the reaction was started with the addition of MgATP to the reaction tube. Incubation proceeded at 30 °C for 1–90 min, and labeling was stopped either by the addition of SDS–PAGE sample buffer and denaturing for 5–8 min at 70 °C or by adding ice-cold EDTA and unlabeled ATP (to 10 and 2 mM, respectively) and plunging into melting ice. ^{32}P incorporated into Na channel protein was assessed by separating the labeled proteins by SDS–PAGE, staining with either silver or Coomassie, drying, and exposing the dried gel to X-ray film. The radioactivity in individual protein bands was quantified by scintillation counting of the excised band which had been dissolved in H_2O_2 as described previously (Emerick & Agnew, 1989). Maximal phosphorylation of Na channels used in phosphopeptide mapping experiments was confirmed by quantitating aliquots removed from the incubation tube at intermediate times in the course of the labeling reaction. Under the conditions described, steady-state labeling could not be increased by further supplementation of kinase or [γ-^{32}P]ATP.

Potato Acid Phosphatase Incubations. The substrate (0.1–1 mL) and phosphatase (20–300 μL) were dialyzed in separate dialysis bags into phosphatase buffer (2.5 mM citric acid, pH 4.8, 100 mM NaCl, 0.5 mM $MgCl_2$) at 0 °C, with four changes of 45 min each. At zero time they were combined (typically 100–1500 pmol/mL TTX-binding sites, ~10–40 units/mL PAP; PAP was used at 1/12 dilution of the predialysis volume into the reaction mixture). The enzyme cannot be diluted directly into the incubation mixture at the concentration used here; the phosphatase is inhibited by the storage buffer, probably due to ammonium sulfate (see Figure 1A). Incubation was at 30 °C for 40–60 min. The reaction was stopped by denaturing the samples in PAGE sample buffer or by adding a 5×-concentrated Tris-HCl solution to bring the final solution to 50 mM Tris-HCl, pH 6.8, 100 mM NaF, 100 mM NaCl, and 0.5 mM $MgCl_2$.

Preparation of Limit Tryptic Phosphopeptides. A maximally labeled Na channel preparation, containing 300–500 μg of Na channel protein in ~1 mL, was reduced and denatured in SDS–PAGE sample buffer containing 0.25 M Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, and 4 mM DTT. The sample was carboxamidomethylated with 15 mM iodoacetamide at 21 °C for 15 min in the dark and resolved by SDS–PAGE. The Na channel band was electroeluted/dialyzed from the gel (Hunkapiller *et al.*, 1983), and the protein content and ^{32}P labeling stoichiometry of an aliquot were measured by quantitative amino acid analysis (Emerick & Agnew, 1989). The remainder was precipitated in acetone/1 mM HCl to remove SDS. The acetone pellet was washed twice with ice-cold acetone, air-dried, and stored at −20 °C. For digestion with trypsin, the pellet was resuspended in 50 μL of 8 M urea and 200 mM NH_4HCO_3, heated at 50 °C for 15 min, and dispersed with high-energy sonication for 10 s. The sample was collected at the bottom of the tube with brief centrifugation in an Eppendorf microcentrifuge and brought to 190 μL with water. Ten microliters of water containing trypsin (1 μg/50 mg of Na channel protein) was added, and the sample was incubated at 37 °C for 10 h. The sample was again sonicated and centrifuged, and another 10 μL containing the same amount of trypsin was added and incubation continued at 37 °C for an additional 10 h.

Purification of Phosphopeptides. Phosphopeptide purification was by the methods of Stone and Williams (1988) and Emerick and Agnew (1989). Buffers for reverse-phase HPLC were as follows: buffer A, 0.06% TFA; buffer B, 0.052% TFA, 80% acetonitrile, ± 0.002% TFA to equalize the absorbance of the two buffers at 210 nm. A 200-μL aliquot of the tryptic digest was subjected to HPLC on a C-18 reverse-phase column (experiment I: Vydac analytical 4.6 mm × 25 cm; experiment II: Vydac narrow-bore 2.1 mm × 25 cm). 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 at 210 nm was monitored with a Kratos 773 detector, and fractions were collected with a Foxy collector using an Isco peak separator. Aliquots (3.5 μL) of each fraction were counted for radioactivity by liquid scintillation

Table I: Range of Values Obtained for the Ratio x/x' for the PTH Derivatives of the "Unmodified" Residue (x) and the Dehydro Residue (x') in All Peptide Sequencer Cycles Where Detectable Levels of the Indicated Residues (x = Ser, Ser-P, Thr, or Thr-P) Occurred[a]

	x/x' (range)	n
Ser-P	0–0.15	12
Ser	0.31–∞	13
Thr-P	0–0.22	5
Thr	1.11–∞	16

[a] The number of such cycles (n) is given for each amino acid. Data include all sequencer runs from the present work, and from Emerick and Agnew (1989).

in 10 mL of scintillant. The total radioactivity in each labeled fraction was determined from the cpm in the aliquot and the fraction volume, determined gravimetrically. 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, peptide detection, and fraction collection were as above.

The peak radioactive C-8 column fractions were identified by two methods. Simple qualitative methods were used because of the large number of column fractions that were assayed. In experiment I, 3.5-μL aliquots of each fraction from several column runs were spotted onto 0.3-mm Whatman No. 1 paper disks glued to a sheet of bond paper which was exposed to preflashed X-ray film. The radioactivity in each fraction was assessed by quantitative densitometry of the exposed film. In experiment II, Cerenkov counts were measured directly from each fraction. This method is only qualitative because the counting efficiency depends strongly on sample volume and geometry. The first method is in principle very sensitive, but in this experiment it also gave only qualitative results because of a somewhat low signal and a variable background. Both methods were adequate to identify the principal labeled fraction in each column run. Although in experiment I this was best assessed by visual inspection of the exposed film, the densitometry profiles are plotted in Figure 4-I for comparison with the peptide elution profiles.

Peptide Sequencing. A C-8 column fraction containing a phosphopeptide was adsorbed to a Polybrene support. Sequence analysis was performed using an Applied Biosystems Model 470A gas-phase sequencer, and the resulting PTH-amino acids were analyzed with an on-line microbore HPLC. The recovery of each residue was calculated by scaling the UV absorbance peak area of that residue to that of norleucine or norvaline included as internal standard for each cycle.

Identification of the Phosphorylated Residue from the Amino Acid Sequencer Data. Phosphoserine (or phosphothreonine) is not recovered from a cycle of Edman degradation. This is because phosphate is cleaved from phosphoserine by two mechanisms: base-catalyzed β-elimination occurs during the first step to form dehydroserine (Ser'), and the remaining phosphate is cleaved by acid-catalyzed hydrolysis during the last two steps to form serine (Bylund & Huang, 1976; Samuel & Silver, 1963). The serine hydroxyl can also leave by β-elimination to form Ser' in a serine cycle, but resonance stabilization of the phosphate leaving group greatly increases the rate of β-elimination (March, 1977) and hence the Ser'/Ser ratio in phosphoserine cycles compared to serine cycles. Thus a skewed Ser/Ser' ratio can be used to identify a phosphoserine cycle. Analogously, phosphothreonine leads to an increased formation of dehydrothreonine and its DTT adducts compared to threonine. Table I gives, for each type of residue, the range of ratios of the dehydro to the unmodified form (x/x') which we have obtained in all sequencer runs performed in phosphopeptide mapping experiments to date [including those from this work and from Emerick and Agnew (1989)]. The distinction between phosphorylated and unmodified residues is clearly unambiguous. The actual recoveries of the unmodified and dehydro residues are shown in the results for the individual cases (Table II). Phosphorylated residues were found only in consensus PKA phosphorylation sites; these include some sites containing a single basic residue [see Emerick and Agnew (1989)].

Patch Clamp Experiments. Voltage clamp recordings were made on inside-out excised patches from slices of Sachs cell innervated membrane as described in Shenkel and Sigworth (1991). The pipet contained the "extracellular" solution: 200 mM NaCl, 3 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 5 mM Hepes (NaOH), pH 7.2. The bath contained the "intracellular" solution: 200 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 5 mM Hepes (KOH), pH 7.2. After excision and verification of patch stability by recording Na currents for 5–10 min the pipet was transferred through the solution from the chamber containing the tissue slice, over a partition (keeping the patch in solution) into a smaller (~0.40-mL) chamber containing the same "intracellular" solution. At this point the pipet was lowered and the fluid level reduced to separate the two chambers. Recordings were made at 1-s intervals from a holding potential of –90 mV to the indicated pulse potential (with respect to bath) using p/4 leak subtraction and were analyzed as previously described in detail (Shenkel & Sigworth, 1991).

The PKA catalytic subunit (~100 μL) was dialyzed into the bath solution (four changes of 250 mL for 1 h each). Na_2ATP was dissolved in bath solution, adjusted to pH 7.2 with NaOH, and stored at –80 °C. After transferring the patch to the small chamber and recording control traces, 10 μL of bath solution containing ~0.4 units/mL PKA and 2 mM ATP was added to the bath in the vicinity of the patch.

RESULTS

The Electroplax Na Channel Is a Phosphoprotein. Electroplax Na channels were purified in the presence of 100 mM NaF to inhibit endogenous phosphatase activity. These preparations were phosphorylated *in vitro* by PKA; ^{32}P incorporation plateaued at a stoichiometry of 0.05 mol of P/mol of TTX-binding sites (Figure 1A, open circles). This was far below the value of 1.7 obtained with channels purified in the absence of NaF (Emerick & Agnew, 1989) and indicates that the Na channels are heavily phosphorylated in the electroplax membrane. Treatment of such a preparation with potato acid phosphatase (PAP) at pH 4.8 after the PKA labeling reaction consistently removed 90–100% of the phosphate incorporated by PKA *in vitro* (Figure 1B). It was readily possible to block PAP action after dephosphorylation of the Na channels; the phosphatase reaction was 9.6-fold slower at pH 6.8 and was completely inhibited by sodium fluoride, with $IC_{50} < 1$ mM (not shown). When channels were isolated in the presence of phosphatase inhibitors and treated with PAP at low pH for 45 min before inhibiting phosphatase and labeling with PKA, the stoichiometry was enhanced 3.9-fold (Figure 1A, filled triangles). Doubling the duration of PAP pretreatment did not increase labeling significantly (filled circles). Note that the phosphatase was essentially completely inhibited under the conditions of phosphorylation (compare open circles and open triangles). Other phosphatases were not effective in removing phosphate

Table II: PTH-Amino Acid Recoveries from Automated Gas-Phase Sequencer[a]

4 - 13

		I A-	*I* C-	*II* C-	*II* D+
1	K	16.3			
2	F	19.8	6.0	24.4	22.3
3	**S**	**0 \ 5.9**	**0 \ 4.1**	**0 \ 0.6**	**0 \ 8.7**
4	S	2.4 \ 0	6.3	3.2 \ 0	2.1 \ 0
5	A	5.8	0 \ 0	17.6	14.4
6	R	1.5	2.6	4.2	13.4
7	P	14.1	4.1	19.4	14.5
8	E	10.8	2.4	17.8	13.2
9	M	2.1	0.5 \ 0	11.6	9.3
10	F	7.5	1.2	14.8	12.2
11	R	0.8	1.2	0.9	
12					
13					
14					
15					

1677 - 1691

	I D+	*I* D-
D		
N	13.7	15.7
P	14	16.3
S	**0.3 \ 2.1**	**0.3 \ 1.9**
P	7.2	11.2
T	2.0 \ 1.8	2.2 \ 1.3
F	5.7	8.4
F	0	4.4
E	4.4	6.7
P	5.2	8.7
V	1.4	2.1
V	0.7	0.8
T	0.5 \ 0	1.2 \ 0.7
T	0.5 \ 0	1.0 \ 0.6
L	1.2	2.2

14 - 30

		I E+	*I* E-	*II* I+	*II* I-
1	R				
2	F	8.3	1.5	4.7	10.4
3	**T**	**0.5 \ 2.2**	**0.6 \ 3.3**	**0 \ 0.7**	**1.0 \ 3.7**
4	P	5.8	15.0	4.4	13.1
5	D	3.0	6.7	2.6	6.9
6	S	1.6 \ 4.2	2.9 \ 7.6	0.5 \ 1.1	0.8 \ 2.6
7	L	3.0	5.8	3.1	6.3
8	E	4.4	10.8	1.7	7.4
9	E	1.4	5.0	1.5	1.6
10	I	2.6	5.5	2.2	5.3
11	E	0	4.0	5.5	3.2
12	A	2.1	3.4	3.6	3.7
13	F	1.4	5.3	2.6	5.3
14	T	1.7 \ 1.3	1.4 \ 1.0	0.8 \ 0.0	1.5 \ 1.1
15	E	1.4	3.5	1.6	4.1
16	L	0.5	1.3	2.9	3.1
17	K	0.9	1.1		2.2
18					
19					
20					

1774 - 1789

	II H+	*II* H-
M		36
P	26.4	52.5
S	**0 \ 9.2**	**0 \ 12.9**
L	19.7	25.9
S	4.1 \ 9.9	2.2 \ 5.0
V	7.0	9.7
P	17.4	22.1
E	10.4	14.8
T	2.8 \ 1.2	7.0
Y	4.8	6.9
P	8.2	13.6
I	5.6	6.7
Q	6.6	8.4
I	3.1	4.2
P	4.7	5.8
K	1.6	1.4

439 - 458

	II G+
K	
A	
S	
L	1.2
A	1.2
S	0.2 \ 0.1
Q	1.5
L	1.3
T	0.8 \ 0
Q	0.9
N	0.8
Q	1.0
E	0.4
A	0.5
E	0.5
I	0.5
T	0.5 \ 0
D	0.7
D	0.2
G	0.3

[a] The sequences are grouped by the parent tryptic peptide, which is indicated at the upper left of each group as the underlined range n_1–n_2, where n_1 is the frist and n_2 the last amino acid residue of the corresponding sequence in the Na channel protein. At the left of each peptide group is the residue recovered at the corresponding sequencer cycle. The cycle numbers are given at the extreme left. The PTH-amino acid recoveries are in picomoles. For serine and threonine residues they are listed as $x\backslash x'$, where x' is the total amount of the dehydro form recovered in the cycle, and x is the unmodified form. Phosphorylated residues are shown in bold. In cycles where recoveries are not given, the background levels of several amino acid residues were too high to allow identification of the proper residue. The experimental number and C-18 column peak are identified above the data set for each peptide: *e.g.*, "IA–" refers to pool A– from experiment I.

from PKA sites on the Na channel. These included calcineurin (Sigma) and alkaline phosphatase (Boehringer Mannheim). Several attempts to isolate an endogenous electroplax phosphatase [*cf.* Tung *et al.* (1985)] were also unsuccessful.

Although the labeling time course for the PAP-treated channels did not actually reach steady state in the experiment of Figure 1, the maximal stoichiometry was well below that obtained with channels purified in the absence of phosphatase inhibitors. This was borne out in other such experiments where steady state of the labeling reaction was achieved, and the maximum labeling stoichiometry following PAP treatment did not exceed 0.8. This is interesting given that PAP did remove essentially all phosphate added to such channels in the PKA labeling reaction. We did not discover an explanation for this, but it may be due to differences in effectiveness between PAP and the endogenous phosphatases. Because PAP did not appear to remove quantitatively the endogenous phosphate from Na channels purified in NaF, it was not used to assess the precise extent of endogenous phosphorylation through measurement of labeling stoichiometry alone. In phosphopeptide maps, however, we were able to resolve a number of distinct sites whose labeling was markedly enhanced by PAP pretreatment.

Phosphatase Unmasks Phosphate Acceptors on Multiple Sites. Na channels were isolated as above in ice-cold solutions containing NaF to inhibit serine/threonine phosphatases. For each experiment two samples were analyzed. Both were maximally labeled with ^{32}P by PKA following preincubation at pH 4.8 with or without PAP, as above. Each sample was purified electrophoretically and exhaustively digested with trypsin. The resulting tryptic phosphopeptides were resolved by two stages of reverse-phase HPLC and sequenced.

Figure 2 shows the first-stage (C-18 column) HPLC profiles for two experiments. Radioactivity eluted with peptides in 5–12 peaks. The enhancement by PAP of ^{32}P incorporated into the electrophoretically homogeneous Na channel protein before trypsin treatment was 2.8-fold for experiment I and 1.8-fold for experiment II. The stoichiometries in these experiments express moles of ^{32}P per mole of Na channel peptide, determined by quantitative amino acid analysis. In each experiment the fold enhancement induced by PAP pretreatment was identical to the ratio of combined counts recovered from the C-18 column in labeled fractions for the treated sample to that for the control sample, demonstrating that individual phosphopeptides were not selectively retained on the column. Compared to the control samples, those exposed to PAP showed both a greater number of peaks containing radioactivity and also a general increase in the amount of radioactivity eluting in individual peaks.

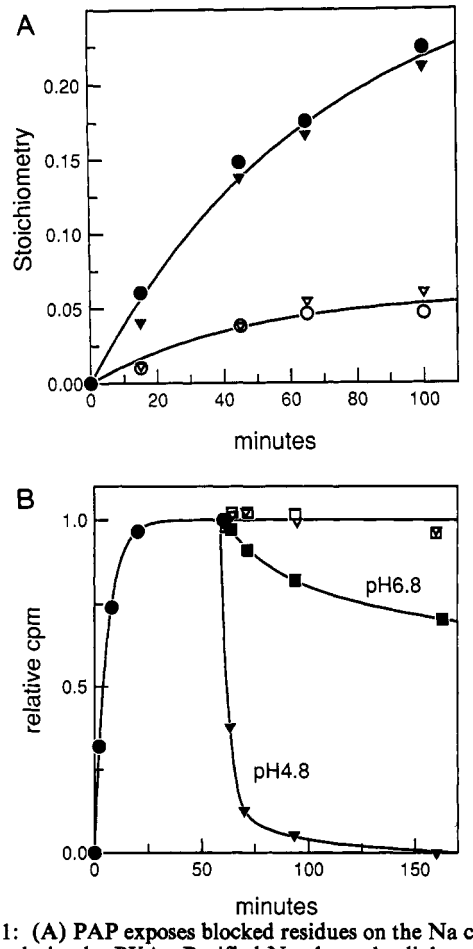

FIGURE 1: (A) PAP exposes blocked residues on the Na channel to phosphorylation by PKA. Purified Na channels, dialyzed into pH 4.8 phosphatase buffer, were preincubated at 30 °C in the presence (filled symbols) or absence (open symbols) of dialyzed PAP. 50 mM Tris and 100 mM NaF (final concentrations) were then added, and channels were labeled by PKA. The stoichiometries represent moles of ^{32}P per mole of active TTX-binding sites and may therefore overestimate the labeling stoichiometry for total Na channel peptide. Upper curve: The phosphatase preincubation was for 40 (triangles) or 90 min (circles). Incubation for more than 40 min did not enhance dephosphorylation significantly. Lower curve: Circles: Purified channels were both preincubated and labeled without PAP. Triangles: Channels were preincubated without phosphatase, but dialyzed PAP was added at the start of the labeling reaction. (B) Potato acid phosphatase removes phosphate added to the Na channel by PKA. Filled circles: Time course of phosphorylation of purified channels. At 60 min the sample was split in two and dialyzed into either (triangles) pH 4.8 phosphatase buffer or (squares) 50 mM tris, pH 6.8, 100 mM NaCl, and 0.5 mM $MgCl_2$. After dialysis both samples were brought to the same volume. Filled symbols: The phosphatase was dialyzed into the same buffers as the substrate and volumes were equalized after dialysis. Open symbols: Triangles: PAP [stored in a suspension of 3.2 M $(NH_4)_2SO_4$] was used straight from the storage vial without dialysis. The enzyme is inhibited by something in the storage buffer. Squares: PAP-free incubation.

Fractions were pooled for rechromatography somewhat differently in the two experiments. In experiment I the entire region of the elution profile containing the major labeled peptides (55–105 min) was partitioned into the five zones A–E, indicated by alternating dark/light shading in Figure 2-I. All of the fractions in each zone were combined and run on the second-stage (C-8) HPLC column. Figure 3-I compares the total counts recovered in each zone for the phosphatase-treated and control samples. In experiment II only the fraction(s) containing the bulk of the counts in each peak of radioactivity were pooled for rechromatography. This minimized contamination of the resulting chromatograph with unlabeled neighboring peptides from the first column (compare panels I and II of Figure 4). Twelve peaks of radioactivity were identifiable between 48 and 92 min in this experiment. Peak fractions from all 12 regions were rechromatogaphed and sequenced, but only the more pronounced ones (C, D, and G–I in Figure 3-II) resulted in identifiable sequence. The recovery of counts in each of the 12 peaks is given in Figure 3-II, which compares the control and PAP-treated samples peak-by-peak. All fractions comprising the peak were counted for every peak in this figure, including minor edge fractions not included in the second-stage chromatograph.

Four of Five Distinct Residues Bear Endogenous Phosphate. The second-stage HPLC profiles for both experiments are shown in Figure 4. In the following discussion a plus sign indicates that the sample was pretreated with PAP. The radioactivity traces in these profiles were noisy (see Materials and Methods); hence, they were not quantitative, but they were adequate to identify the major labeled peptide within a column run.

In experiment I, C-18 pools B and E were largely uniformative. Sequencing of the labeled fraction in pool B+ (Figure 4-I) and the corresponding fraction in pool B– yielded no sequence. The ^{32}P in these fractions may be inorganic phosphate not associated covalently with any peptide. The early labeled fractions in pool E also yielded no identifiable sequence. The principle fractions in both E– and E+ yielded sequence (at comparable PTH levels) from three coeluting peptides (Table II), only one of which contained a potential phosphorylated site. This peptide derives from residues 15–31 of the Na channel, confirming our earlier observation that this sequence, containing threonine 17, is a substrate for PKA *in vitro* (Emerick & Agnew, 1989). Because pool E did not show significant PAP enhancement, however, this experiment does not confirm that this is a site of endogenous phosphorylation. Sequencing of the earlier-eluting labeled peak in E+ was attempted, but no sequence was obtained. The three remaining pools did give useful information. The majority of the label in pools A– and A+ migrated in two peptide fractions in each C-8 column. The apparent reversal in mobility between the major labeled peptides in A+ and A– is intriguing and remains unexplained. It was observable again in experiment II. The fractions indicated by shading were sequenced. Only A– gave a sequence, deriving from Na channel residues 3–13 (Table II). The Ser/Ser′ ratio confirms serine 6 (cycle 3) as the phosphorylated residue. The correspondence of this peptide with the vast majority of counts in the PAP-enhanced pool A+ indicates serine 6 is a site of endogenous phosphorylation. Our previous studies indicated that *in vitro* phosphorylation of Na channels isolated in the absence of NaF could be labeled at either serine 6 or 7, but not both, while the present experiments suggest that serine 6 is the endogenous substrate. Of the labeled fractions from pool C, again, only C– yielded sequence (Table II). The peptide corresponds to Na channel residues 1774–1781. The Ser/Ser′ ratios indicate serine 1776 (cycle 3) is the phosphorylated residue. This is confirmed in experiment II, where the fragment was sequenced through to the end of the peptide. Sequencing of the strongly labeled peptides from pool D yielded the same peptide in both D– and D+ (Table II). This derives from Na channel residues 1677–1690, near the carboxyl terminus of the protein, with the Ser/Ser′ ratios indicating that the phosphorylated residue is serine 1680 (cycle 3). This residue is the only one that lies in a PKA consensus site in this peptide.

Pools C and D of experiment II were interesting (Figure 4). Fraction C–, which contained label in the C-18 peak, appeared very similar in peptide composition, in the rechro-

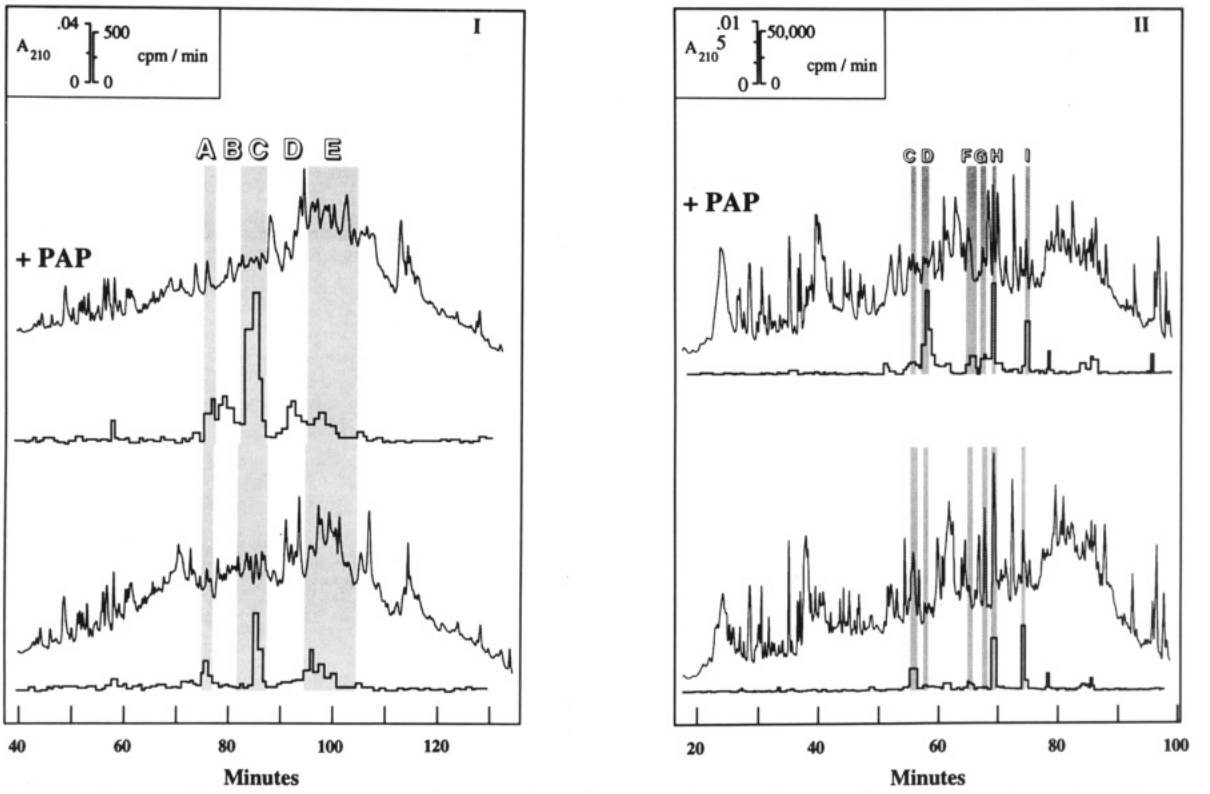

FIGURE 2: C-18 column profiles. (I) Two column profiles are shown for tryptic digests of maximally labeled Na channel peptide, prepared as described in the text, for experiment I. The upper pair of traces corresponds to channel protein pretreated with phosphatase. The lower pair was preincubated in phosphatase-free solution. The upper trace in each pair is the peptide elution profile. The lower trace is the radioactivity, determined from liquid scintillation counts of aliquots of each column fraction. ^{32}P eluted in about five broad peaks, delimited by shading and labeled A–E. The fractions within each of these peaks were pooled for rechromatography. (II) Profiles for experiment II. Maximally labeled, electrophoretically purified Na channel peptide was prepared essentially as in experiment I. Because of differences in yield from the electroelution step, 60 mg of PAP-treated and 90 mg of control tryptic digest were applied to the columns. Both the A_{210} and the radioactivity profiles of the control samples have been scaled by a factor of 67% in this and the following two figures to facilitate comparisons. The corresponding sequencer PTH recoveries of Table II have not, however. The six principle peaks of radioactivity are shaded and labeled C, D, and F–I. These were pooled individually and rechromatographed.

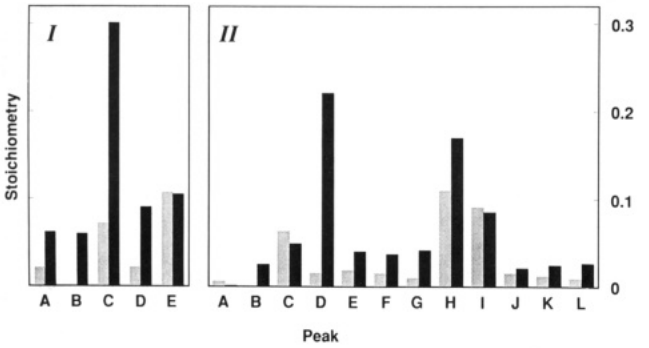

FIGURE 3: Total counts recovered in individual C-18 column peaks. (I) Total cpm above background are summed for all fractions constituting each C-18 pool for experiment I (delimited by the alternate shading in Figure 2-I). The stoichiometry represents the summed net cpm in each pool converted to moles of ^{32}P per mole of peptide, the latter being calculated from the moles of peptide applied to the column scaled by the recovery of total applied cpm. Darker shading corresponds to the phosphatase-treated samples in both panels I and II. (II) The same for experiment II. Note that whereas only the most highly labeled fractions of each peak were pooled for purification and sequencing (Figure 2-II, shaded bands), the entire peak (including unused edge fractions for each peak) was included for this figure. Also note that lesser peaks (*i.e.*, A, B, E, J, K, L) are included in addition to those subject to sequence analysis.

matography profile, to that of fraction D+, which was also a labeled peak in the C-18 column. The two unlabeled C-18 "peaks" (D– and C+) also looked similar to each other upon rechromatography. The labeled peptides from C– and D+ each yielded the same sequence (Table II) corresponding to the complete tryptic fragment from Na channel residues 4–14, with the Ser/Ser′ ratio at cycle 3 indicating that serine 6 is phosphorylated. The large stimulation of labeling by PAP indicates that this residue is subject to endogenous phosphorylation. Sequence was not obtained from C+ or D–. Note again the alteration in mobility, such that this peptide eluted at a later time in the PAP-treated sample than in the control, as in experiment I, pool A, with the same peptide. Rechromatography of G+ yielded a single phosphopeptide (Figure 4). The corresponding fraction was not labeled in the control column and was not sequenced. The labeled peptide in G+ yielded the sequence corresponding to Na channel residues 438–453 (Table II). Although the entire tryptic fragment was not sequenced, the Ser/Ser′ ratio in cycle 3 implicates this residue (serine 444) as the phosphorylated residue. This is the only serine or threonine in a PKA consensus site on the complete tryptic fragment predicted from the cDNA sequence, which strengthens the conclusion. Both H– and H+ gave a single strongly labeled peptide upon rechromatography (Figure 4), and both yielded the same sequence (Table II), corresponding to the entire tryptic fragment derived from Na channel residues 1774–1789. The Ser/Ser′ ratio of cycle 3 implicates this as the phosphorylated residue. This gives further evidence that serine 1776 is phosphorylated by an endogenous kinase. Rechromatography of peak I also gave a single labeled peptide in both cases. Sequencing of these peptides yielded fragments of the same Na channel peptide (residues 15–31, Table II). The Thr/Thr′ ratio of I+ once again indicates threonine 17 is the PKA phosphorylated

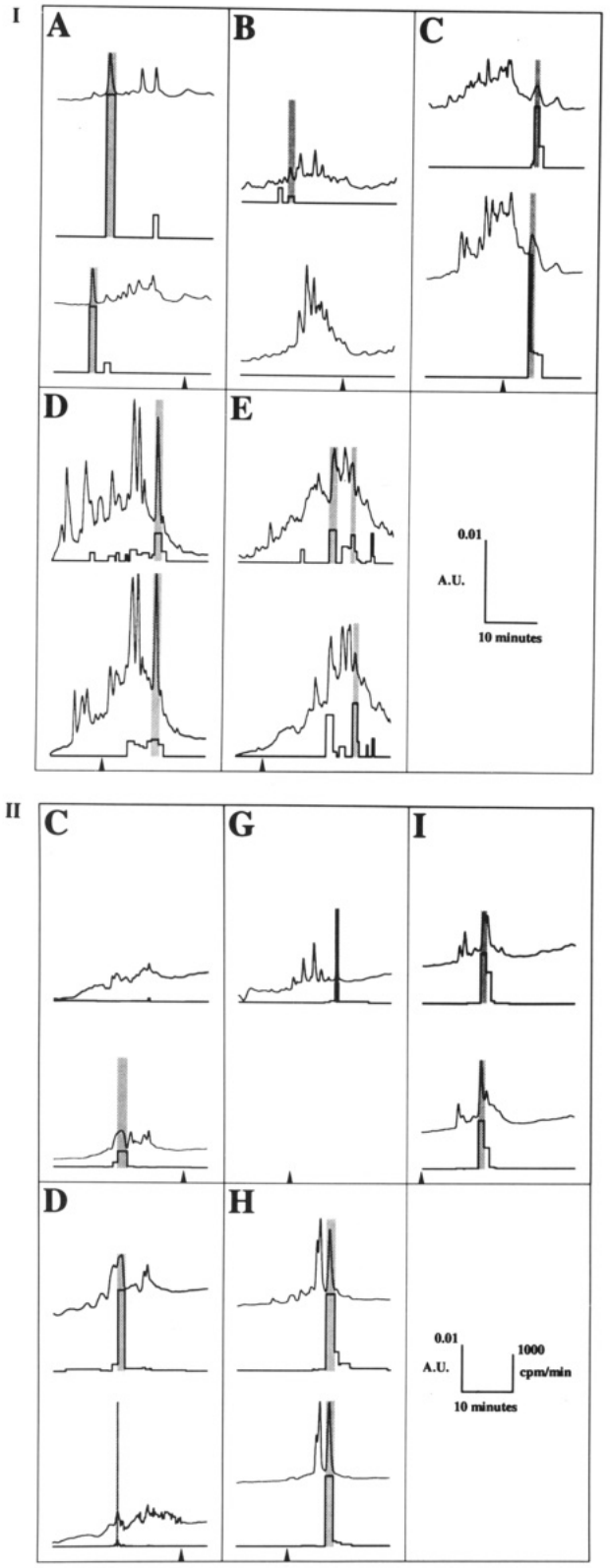

FIGURE 4: C-8 column profiles. (I) Rechromatography profiles are shown for the C-18 pools of experiment I (Figure 2-I). Only the portion of the trace that contains peptides deriving from the Na channel is shown. Each panel shows the two C-8 profiles for a single pool; the upper pair of traces is that for the phosphatase-treated sample. As usual, the upper trace in each pair is the peptide elution profile, and the lower trace is the radioactivity profile. The needle at the bottom of each panel indicates the 50-min elution time point. The radioactivity was determined by densitometry from an autoradiograph of aliquots of each column fraction spotted onto filter paper disks. (II) Rechromatography profiles for the C-18 radioactivity peaks of experiment II (Figure 2-II). The radioactivity profile was determined by Cerenkov counting of entire column fractions.

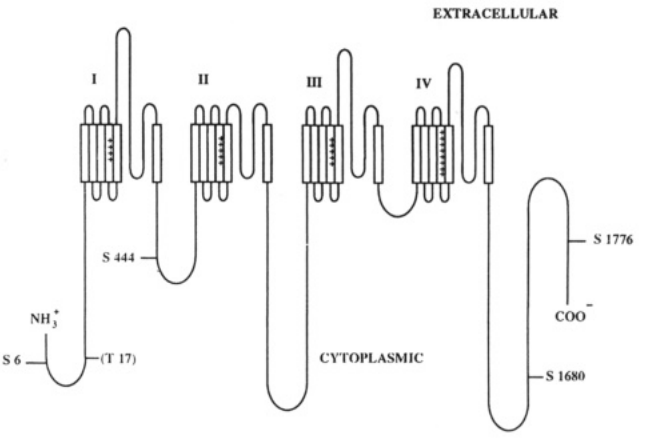

FIGURE 5: A map of the electroplax Na channel topology showing the location of phosphorylation sites identified here [cf. Guy and Conti (1990)]. Residues bearing endogenous phosphate are indicated by position in the primary structure. Threonine 17 is included in parenthesis to indicate that it was consistently labeled by PKA *in vitro*, but may not be phosphorylated *in vivo*.

residue, but the insignificant enhancement of labeling by PAP bars its designation as a site of endogenous phosphorylation.

The enhancement by PAP of radioactivity incorporated into all the labeled peptides, except the ones containing threonine 17, indicates that these sites are endogenously phosphorylated: serines 6, 444, 1680, and 1776. The positions of these sites are indicated in the topographical model of Figure 5.

PKA Phosphorylation Reduces the Amplitude of Voltage-Sensitive Sodium Currents in the Electrocyte Plasma Membrane. Na channels are present in high densities on the innervated face of the electrocyte [*cf.* Shenkel and Sigworth (1991)]. Electrophysiological recordings from cell-attached and excised patches exhibit up to several thousand channels, permitting measurement of "mini-macroscopic" currents. We performed patch-clamp recordings on membranes excised from native electrocytes to test whether there were functional consequences of exposure to PKA. Small slices of Sachs organ were excised as described, and a small area was exposed by microdissection following brief treatment with collagenase and hyaluronidase. Patches were excised, and a standard family of voltage–clamp recordings was performed. The patches were then exposed to bath-applied MgATP or to PKA or to MgATP + PKA. Application of PKA and MgATP to the patch rapidly and dramatically reduced the peak current amplitude by up to 80% of the control value, beginning within 30 s of application. The reduction did not reverse over a period of up to 15 min following the initial decrease. Figure 6A illustrates representative sodium currents before and after applications of PKA. Figure 6B shows peak current *versus* voltage derived from families of voltage-clamp recordings before and after addition of enzyme and substrate. The Na current amplitude was reduced at all voltages (Figure 6B). However, the extent of the reduction was voltage-dependent, being more pronounced at hyperpolarizing potentials. This results from a shift toward more positive potentials in the relative conductance *versus* voltage relation (Figure 6C). Repeated application of MgATP or kinase alone was without effect. Because of the restrictive incubations required for PAP activity, we were unable to test successfully whether the effects of PKA could be reversed enzymatically.

DISCUSSION

The Na channel of eel electroplax and adult mammalian muscle differs from other Na channel isoforms in lacking a

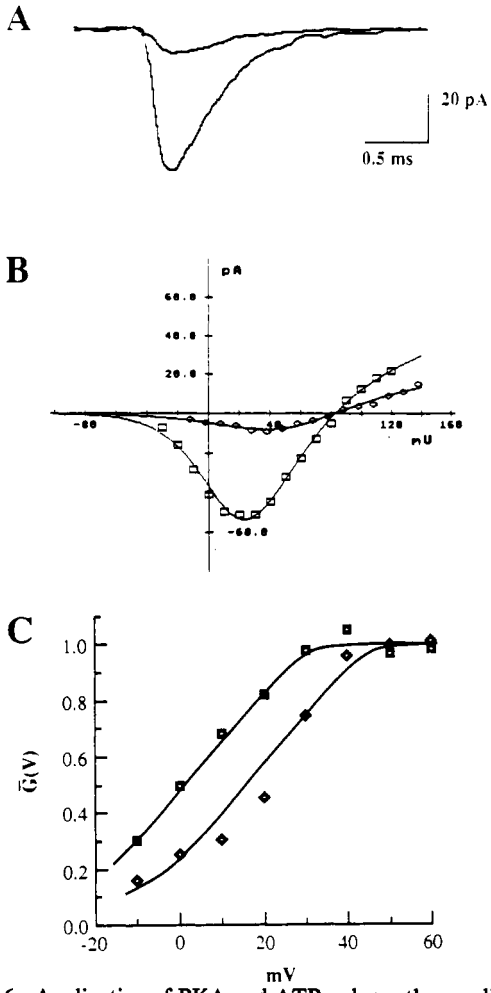

FIGURE 6: Application of PKA and ATP reduces the amplitude of electrocyte sodium currents. (A) Voltage clamp recordings were made on inside-out excised patches from slices of Sachs cell innervated membrane, as described in Materials and Methods. The large trace is a control trace taken after transferring the patch to the small chamber. The small trace was taken after addition of ATP + PKA catalytic subunit to the bath and waiting for the peak current to decline and stabilize. The test pulse was to +40 mV from a holding potential of −50 mV. The experiments in panels A and B were at room temperature (~22 °C). (B) The peak current *vs* test pulse potential for a patch taken from another animal. Squares: Control. Diamonds: Several minutes after addition of ATP + catalytic subunit to the bath. The peak currents declined within 30 s after addition of enzyme and substrate and had stabilized at the time these $I(V)$ curves were recorded. The conductance *vs* voltage relations for these data are given in panel C, where

$$\bar{G}(V) = [I(V)/I_{max}]/(V - V_{rev})$$

I_{max} is the amplitude of the sodium current at the voltage where the $I(V)$ curve shows the largest peak inward current, and V_{rev} is the reversal potential: +83.3 mV in both cases. The curve through the control data was fit by eye and translated by +10.8 mV to fit the data for the kinase-treated sample.

variable, putative regulatory segment between subunit domains I and II that provides a cluster of acceptor sites for protein phosphorylation. These isoforms have, furthermore, not been previously shown to exhibit modulation by activation of the protein kinase A second messenger pathway. Our previous studies demonstrated that the electroplax Na channel was phosphorylated in vitro by protein kinase A at four amino acid residues localized in the amino- and carboxyl-terminal segments. In contrast, neuronal α-subunits have been shown to be labeled *in vivo* at sites lying in the segments between I and II and at a single site between domains III and IV (Rossie & Catterall, 1989; West *et al*, 1991). Muscle channels have been reported to be labeled at a single site somewhere within domain I (Yang & Barchi, 1990).

The present studies show that the electroplax channel is phosphorylated endogenously to a high extent at the amino terminus at serine 6, at the carboxyl terminus at serines 1680 and 1776, and at a single site between homologous repeat domains I and II at serine 444. Threonine 17, near the amino terminus, is readily labeled *in vitro*, but apparently but not by endogenous kinases. Our studies also demonstrate that Na currents in membrane patches excised from the native electrocyte are rapidly and strongly reduced (up to 80%) by treatment with PKA, together with a shift in the peak current–voltage relationship in the depolarizing direction. The magnitude and rapid onset of the inhibition correlates with previously described kinetics of phosphorylation *in vitro*, of only a few of the amino- and carboxyl-terminal acceptor sites.

Back-Phosphorylation. Electroplax Na channels isolated in the presence of NaF were phosphorylated by PKA to a much lower stoichiometry than those isolated without phosphatase inhibitors, suggesting the presence of large amounts of endogenous phosphate on the Na channel glycopeptide. Treatment of such a preparation with PAP enhanced the subsequent PKA phosphorylation stoichiometry by 2- to 4-fold, confirming this conclusion. Tryptic phosphopoeptide mapping revealed four residues bearing endogenous phosphate: serines 6 on the amino terminus, 444 on the interdomain I–II and 1680 and 1776 on the carboxyl-terminal domain. Threonine 17 was rapidly and extensively phosphorylated in all labeling reactions, but no evidence was found for its phosphorylation *in vivo*. In previous studies, PKA appeared randomly to phosphorylate either serine 6 or 7, but labeling of serine 7 was not detected in the present studies.

Excluding the unlikely participation of an extracellular kinase, these results strongly indicate a cytoplasmic location for the domains bearing the phosphorylated residues; our data therefore support the prediction of topographical models in which the amino terminus of the Na channel is cytoplasmic.

We were concerned that PAP treatment did not permit labeling to the same high levels reproducibly obtained with peptide isolated in the absence of phosphatase inhibitors, even though (1) many of the same protein kinase sites were identified, (2) PAP has a broad specificity, and (3) PAP was able to remove all of the label incorporated *in vitro*. The consistently lower stoichiometry of incorporation suggests that endogenous phosphatases are more efficient that PAP; this could be due to partial denaturation of the purified protein, which would put PAP at a catalytic disadvantage over the endogenous enzymes which would have access even while the Na channel was in the initial membrane fraction.

The back-phosphorylation method used here has the advantage of identifying physiological sites where *in vivo* labeling is infeasible, as in the case of the electroplax which is enmeshed in connective tissue and difficult to use in metabolic labeling protocols. Potato acid phosphatase has broad specificity, reducing the likelihood that phosphorylated residues are missed. However, we note that although essentially all *in vitro* labeling was reversed by PAP, careful quantitation shows that we could not fully incorporate 5 equiv as predicted by the number of identified sites. This suggests that the sites were only partially dephosphorylated, perhaps due to some steric hindrance produced in partially denatured molecules. It is also possible that some sites are not dephosphorylated at all by PAP. Threonine 17 may be such a case, but this appears unlikely because in all labeling experiments PKA labeled the Na channel on threonine to about 23% of total phosphoamino

acids and, in all cases where the major phosphorylated residues were identified, phosphothreonine was found only at residue 17. If PAP could not dephosphorylate this residue, the dephosphorylation time course (Figure 1B) should plateau at about 20% of the initial labeling level. Instead, PAP consistently removed 90–100% of the phosphate added by PKA. Threonine 17 could be phosphorylated in the electrocyte given that it is not stoichiometrically modified by maximal phosphorylation by ^{32}P. An alternative explanation is that the electroplax kinase selectively avoids threonine 17 while bovine cardiac PKA does not.

Our assays do not allow firm identification of the actual cellular kinases involved, although the fact that all the identified phosphoamino acids were located within PKA consensus sequences and could be labeled with PKA *in vitro* is consistent with PKA being the cellular enzyme. We could not demonstrate labeling of the electroplax channel with C-kinase isoforms, even when isolated in the absence of phosphatase inhibitors.

Modulation of Na Currents for PKA. Exposure of inside-out patches of electrocyte membrane to PKA catalytic subunit and MgATP caused a dramatic and rapid reduction in the peak Na currents, typically up to 80%. This reduction was accompanied by a shift of the peak current–voltage relation of the residual current by about 11 mV in the depolarizing direction. These effects are opposite to that expected for simple electrostatic effects due to additional negative internal surface charge from the added phosphate groups (Perozo *et al.*, 1991). We observed no effect on the voltage dependence of inactivation nor on the kinetics of activation or inactivation (not shown), and the reversal potential of the Na current was not altered by PKA treatment (not shown), indicating that phosphorylation is not responsible for the previously reported variations in selectivity of the electroplax Na channel (Shenkel & Sigworth, 1991). It was not possible, in these initial studies, to provide a description of the effects of PKA in terms of the single-channel behavior because of the high channel surface density. These findings are the first indication of regulation of either electroplax or skeletal muscle short-form Na channels by phosphorylation.

Possible Site Responsible for Modulation. The current reduction on *in vitro* phosphorylation was reproducible, rapid, and extensive. The effect appeared within 1–2 min—sometimes within seconds—after applying the enzyme and ATP and was maximal within 1–5 min (at 21 °C). This time course is comparable to that of labeling in solution where maximum incorporation typically occurred by 20–40 min (at 30 °C), using 20-fold lower enzyme concentration ($Q_{10} = 1.9$ for PKA in this temperature range). Direct comparison of rates is complicated by the fact that the solutions were applied at 40 times their final concentration in the immediate vicinity of the patch and allowed to diffuse to their equilibrium concentrations. Only those residues which are rapidly and consistently phosphorylated seem likely to mediate the effect. Our previous kinetic experiments performed under limiting conditions revealed only three such residues, serine 6, threonine 17, and serine 1776 (Emerick & Agnew, 1990). Interestingly, all of these are on the amino- and carboxyl-terminal loops. The two remaining sites, serine 444 and 1680, were found only once to be phosphorylated and at low levels, following PAP treatment.

Serines 6 and 1776 are extensively phosphorylated *in vivo*; thus we speculate that these sites may not be linked to inhibiting channel opening. Threonine 17 appears not to be modified *in vivo*, but is rapidly labeled in vitro, and thus is the best candidate for mediating the effect. However, the magnitude of current reduction cannot be compared directly to the extent of phosphorylation of any particular site(s) because a substantial fraction of Na channels in the electrocyte membrane appear to be closed and unavailable for voltage activation (Shenkel & Sigworth, 1991), possibly reflecting substantial levels of endogenous phosphorylation.

Physiological Implications. Modulation of the electroplax Na channel, perhaps by a specific kinase, may serve to regulate the action potential firing threshold, or to regulate the power of the electric discharge. The electroplax resting potential arises from a dense population of inward rectifying K channels and lies near E_K. These channels close when the membrane is depolarized. The electroplax Na channels are nearly twice as selective for Na over K as any other isoform. Because of the high resting K permeability and the high peak Na permeability, the voltage transient is near the theoretical maximum of 150 mV and lasts for several milliseconds. Because of the near absence of classical delayed rectifier K channels, the action potential time course is controlled by the Na channel [*cf.* Shenkel and Sigworth (1991)]. Sharply inhibiting the peak Na current density could raise the threshold for discharge and would render the membrane entirely unexcitable, or weaken the magnitude of the discharge. The cumulative discharge of a medium-sized animal can exceed 1000 V. The power of the electroplax discharge may be regulated depending on electroplax recruitment by increased spinal electromotor neuron firing rate. In addition, however, large extracellular currents from the discharges of neighboring electromotor units might trigger spontaneous action potentials even without neurostimulation, unless excitability were inhibited.

The susceptibility of the electroplax Na channel to inactivation with phosphorylation by PKA may underlie the failure to express this channel from cloned cDNAs in frog oocytes. Our own studies demonstrate that cRNA injected into *Xenopus* oocytes results in synthesis of large amounts of glycosylated peptide but no functional channel expression (Ukomadu and Agnew, unpublished). Oocytes exhibit high levels of activated PKA, perhaps blocking channel expression (L. Kaczmarek, personal communication; M. White, personal communication; R. Huganir, personal communication). Kinases in the cell may be selective for different sites, with a specific enzyme involved in shutting channel activity on or off. This possibility may be tested by preparing mutant channels in which each phosphorylation site is modified by site-directed mutagenesis.

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