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29. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
30. In order to compare the topologies of the combining sites of EcorL, Con A, and PL, the Mn^{2+}, Ca^{2+}, and 16 atoms of conserved residues and water molecules in the coordination spheres of the metals in the three lectins were superimposed [from EcorL: Oδ1 and Oδ2 of Asp^{89}, Asp^{129}, and Asp^{136}, Oε1 and Oε2 of Glu^{127}, O of Phe^{131}, Oδ1 and Nδ2 of Asn^{133}, Nε2 of His^{142}, and water molecules 801, 807, 808, and 809; the equivalent residues in Con A (*17*) are Asp^{208}, Asp^{10}, Asp^{19}, Glu^{8}, Tyr^{12}, Asn^{14}, His^{24}, and the four water molecules in W3.239 and in PL (entry 2LTN in the Brookhaven Protein Data Bank) are Asp^{81}, Asp^{121}, Asp^{129}, Glu^{119}, Phe^{123}, Asn^{125}, His^{136}, and water molecules A417, A422, A456, and A657]. The rms values for the superposition of the EcorL atoms on those of Con A and PL were 0.34 and 0.27 Å, respectively.
31. Thanks are due to D. R. Davies for interest in the project and support, E. A. Padlan, G. H. Cohen, and C. C. Hyde for useful suggestions, K. D. Hardman for coordinates of Con A, D. Ashford and C. Edge for the initial model of the N-linked heptasaccharide, R. Adar for the primary sequence, and S. Haalman and M. Laschver for technical assistance. Supported in part by grants to N.S. from BSF (3727), from BARD (IS-1636-89), and the Leo and Julia Forchheimer Center for Molecular Genetics at The Weizmann Institute. The coordinates of the current model have been deposited in the Brookhaven Protein Data Bank (entry 1LTE). The model on the cover was generated by the program Ribbon 2.0 [M. Carson, *J. Mol. Graphics* **5**, 103 (1987)] and rendered by AVS software (Stardent Computer Inc.).

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A Phosphorylation Site in the Na^+ Channel Required for Modulation by Protein Kinase C

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Voltage-gated sodium channels are responsible for generation of action potentials in excitable cells. Activation of protein kinase C slows inactivation of sodium channels and reduces peak sodium currents. Phosphorylation of a single residue, serine 1506, that is located in the conserved intracellular loop between domains III and IV and is involved in inactivation of the sodium channel, is required for both modulatory effects. Mutant sodium channels lacking this phosphorylation site have normal functional properties in unstimulated cells but do not respond to activation of protein kinase C. Phosphorylation of this conserved site in sodium channel α subunits may regulate electrical activity in a wide range of excitable cells.

VOLTAGE-SENSITIVE Na^+ CHANnels are responsible for the initiation and propagation of the action potential and therefore play a crucial role in neuronal excitability (*1*). The Na^+ channel isolated from rat brain is a heterotrimeric protein consisting of a 260-kD α subunit and 30- to 40-kD β_1 and β_2 subunits (*2*). Expression of the α subunit is sufficient for formation of functional Na^+ channels in *Xenopus* oocytes (*3*) or mammalian cells (*4*). Four rat brain Na^+ channel subtypes have been defined by cloning and sequencing (*5–7*). Types II and IIA are the predominant Na^+ channel α-subunit subtypes expressed in adult rat forebrain (*8, 9*). Physiological modulation of these Na^+ channels would lead to significant changes in neuronal excitability in the brain. Protein kinase C (PKC) modulates the activity of many ion channels (*10*) and is known to phosphorylate the α subunit of the Na^+ channel in rat brain (*11*). Activation of PKC in *Xenopus* oocytes expressing brain Na^+ channels decreases peak Na^+ current (*12*). We have recently found that activation of PKC in rat brain neurons or in transfected Chinese hamster ovary (CHO) cells expressing type IIA Na^+ channels (CNaIIA-1) (*13*) causes rapid and reversible slowing of Na^+ channel inactivation and reduction of peak Na^+ current (*14*).

Both slowing of inactivation and reduction of peak Na^+ current were recorded in every cell-attached patch (*15*) from CNaIIA-1 cells (*16*) treated with 40 to 75 μM 1-oleoyl-2-acetyl-*sn*-glycerol (OAG). To quantify the slowing of inactivation without fitting to a specific model, we normalized the peak Na^+ currents, measured their integral, and expressed it as percent of untreated controls. Treating the cells with 40 to 50 μM OAG reduced the peak Na^+ current to 33 ± 7% of control and increased the normalized peak current integral to 206 ± 68% of control (SEM, n = 16); treating the cells with 65 to 75 μM OAG further reduced the peak current to 6 ± 0.3% of control (SEM, n = 6). PKC phosphorylation can almost completely inhibit Na^+ currents in these CNaIIA-1 cells.

The intracellular loop between domains III and IV (Fig. 1) plays a critical role in Na^+ channel inactivation (*17, 18*). The presence of a consensus PKC phosphorylation site (*19*) centered at Ser^{1506} in this intracellular loop led us to consider whether phosphorylation of Ser^{1506} might be involved in modulation of Na^+ channel activity by PKC. The synthetic peptide SP19 (*17*) (Fig. 1) represents a portion of the intracellular loop between homologous domains III and IV and contains the putative PKC site Lys-Lys-Leu-Gly-Ser-Lys-Lys. Peptide SP19 was rapidly phosphorylated by purified PKC (*20*) in vitro in the presence of diolein and phosphatidylserine (240 nmol/min per milligram of enzyme) but not by adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (<2 nmol/min per milligram

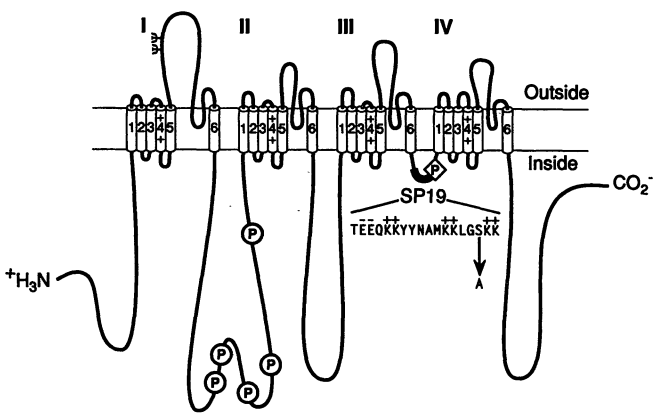

Fig. 1. The transmembrane folding model of the Na^+ channel α subunit with experimentally determined cAMP-dependent protein kinase phosphorylation sites (P enclosed in a circle) and the putative PKC phosphorylation site (P enclosed in a diamond). The single-letter amino acid code of the primary sequence of the SP19 peptide in the intracellular loop between homologous domains III and IV of the type IIA Na^+ channel α subunit (*6*) is illustrated. The arrow indicates the position of the oligonucleotide-directed mutation of Ser^{1506} to alanine. Abbreviations for the amino acid residues are as follows: A, Ala; E, Glu; M, Met; N, Asn; Q, Gln; T, Thr; and Y, Tyr.

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of enzyme), consistent with a role for phosphorylation of this site in modulation of Na^+ channels by PKC.

To test this hypothesis directly, we used oligonucleotide-directed mutagenesis (*21*) to construct the mutant Na^+ channel α-subunit gene S1506A in which Ser^{1506} was replaced with alanine (Fig. 1). CHO-K1 cells were transfected (*22*) with the expression vector ZemRVSP6–S1506A (*23*) containing the S1506A Na^+ channel α-subunit cDNA. Two independently isolated clonal cell lines, CNaS1506A-9 and CNaS1506A-10, expressing S1506A Na^+ channel α-subunit mRNA, were isolated from a pool of G418-resistant cell lines. The PKC activity of these two cell lines was equivalent to that of CNaIIA-1. Na^+ currents were recorded in nearly every cell examined from both cell lines by whole-cell voltage clamp (*15*) (Fig. 2). Na^+ current magnitude varied from 0.9 to 11 nA (mean = 4.63 ± 2.79 nA, 227 ± 164 pA/pF). Expressed as channel density, this Na^+ current corresponds to 100 to 300 active channels per picofarad or 1 to 3 active channels per square micrometer. Na^+ currents recorded in both cell lines displayed kinetics identical to those of type IIA Na^+ channels in CNaIIA-1 cells over a physiological range of depolarization steps (Fig. 2A). The voltage dependence of activation (Fig. 2B) of the Na^+ channel in CNaS1506A-9 ($V_{1/2}$ = −24 mV, k = −6.62, n = 9; see legend to Fig. 2B for equation and definition of parameters) is unaltered with respect to wild-type IIA Na^+ channels expressed in CNaIIA-1 cells ($V_{1/2}$ = −22 mV, k = −7.21, n = 22). The voltage dependence of steady-state inactivation of mutant S1506A channel (Fig. 2B) is slightly shifted in the depolarizing direction (CNaIIA-1, $V_{1/2}$ = −59 mV, k = 5.42, n = 12; CNaS1506A-9, $V_{1/2}$ = −54 mV, k = 5.3, n = 6), but the time course of inactivation is unchanged (Fig. 2A). Thus, the functional properties of the Na^+ channel expressed in unstimulated CHO cells are not significantly altered by mutation of Ser^{1506}.

The requirement for phosphorylation of Ser^{1506} in the action of PKC was tested in parallel recordings in CNaIIA-1 cells and in the CNaS1506A cell lines (Fig. 3). In cell-attached patches, addition of 20 μM OAG to the bathing solution caused a rapid decrease in peak current and a slowing of inactivation in CNaIIA-1 cells (Fig. 3A), but 75 μM OAG had no effect on Na^+ currents in CNaS1506A cells (Fig. 3C) (n = 17). In excised membrane patches, addition of PKC to the bathing medium caused a rapid decrease in peak current and a slowing of inactivation for CNaIIA-1 cells (Fig. 3B) but had no effect on Na^+ currents in CNaS1506A cells (Fig. 3D) (n = 5). Because strong modulatory effects have been observed with all CNaIIA-1 cells treated with 75 μM OAG, these results demonstrate that the single mutation S1506A blocks the reduction of Na^+ current and the slowing of inactivation caused by PKC. Thus, phosphorylation of this residue is required for both effects of PKC.

These results begin to define the molecu-

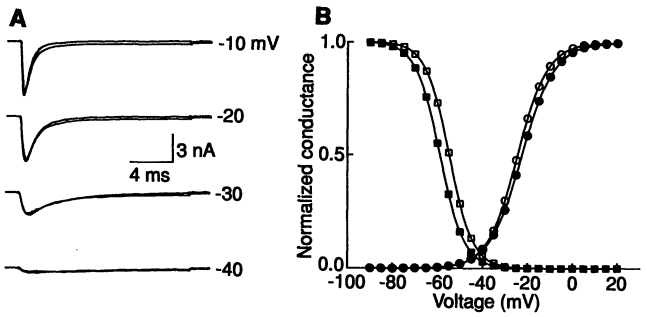

Fig. 2. Comparison of Na^+ currents in CNaIIA-1 and CNaS1506A-9 cells. Ionic currents were measured in the whole-cell configuration of the patch clamp technique by standard procedures. Pipettes were formed from soda lime glass (Scientific Products) and had resistances ranging from 0.4 to 1 megohms. Whole-cell capacitance was compensated by using the internal analog circuitry of the patch clamp (List EPC-7), and about 70% of the series resistance was compensated. The bathing solution contained 130 mM NaCl, 4 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes, pH 7.4. The pipette solution contained 90 mM CsF, 60 mM CsCl, and 10 mM NaCl, pH 7.4. (**A**) Whole-cell Na^+ currents recorded at the indicated voltages from a control and an S1506A cell. Control and mutant cells compared were chosen to have equal $V_{1/2}$ values to avoid differences in current time course caused by the degree of channel activation. Currents from the mutant cell were scaled by a factor of 1.4 at each potential to match the current magnitude in the control cell. The small differences in current time course that remain are within the variability observed when two cells of the same type are compared by use of this procedure. (**B**) Comparison of mean activation (circles) and inactivation (squares) curves in control (filled symbols) and mutant cells (open symbols). The curves and symbols were calculated from the mean values of $V_{1/2}$ and k determined in the two cell types. Activation curves were derived from current-voltage relations, which we measured by using 16-ms test pulses to voltages ranging from −60 to +100 mV in 5- or 10-mV increments. Holding potential was −100 mV. Reversal potential (V_{rev}) was measured and conductance (G) was calculated as follows: $G = I/(V - V_{rev})$, where I is the peak current and V is the test-pulse voltage. We determined inactivation curves using 200-ms prepulses to a variable voltage followed by a 16-ms test pulse to 0 mV. Peak test pulse current was measured as a function of prepulse potential and normalized to the maximum test pulse current obtained at negative potentials giving normalized G (G_{norm}). Activation and inactivation curves for individual experiments were fit according to the following: $G_{norm} = 1/\{1 + \exp[(V - V_{1/2})/k]\}$.

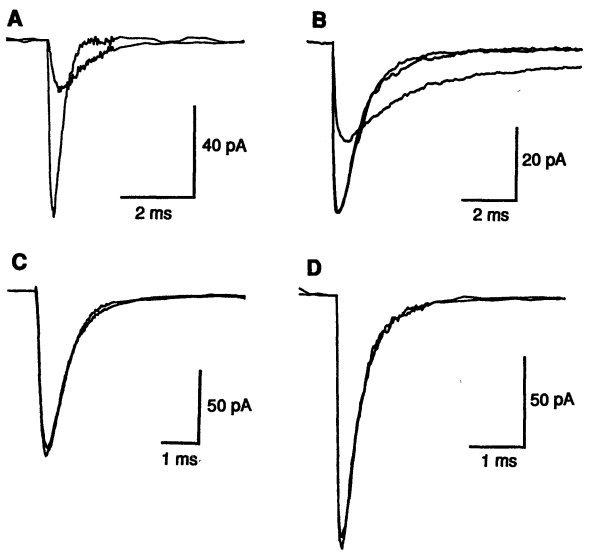

Fig. 3. Comparison of effects of PKC on Na^+ currents recorded from CNaIIA-1 and CNaS1506A cells. Currents were recorded from either cell-attached or excised inside-out membrane patches (*15, 16*). PKC was activated in cell-attached patches by addition of OAG to the recording chamber. Inside-out membrane patches were treated with 1 μM diolein, 1 μM phosphatidylserine, and 1 mM ATP alone or in the presence of PKC (*20*) by addition to the recording chamber. (**A**) Ensemble currents were evoked in a cell-attached patch from CNaIIA-1 cells by depolarization to −20 mV from a holding potential of −110 mV. The larger current was recorded under control conditions; the smaller current was recorded from the same patch after exposure to 20 μM OAG. (**B**) Ensemble average of 50 current traces was recorded from a single membrane patch excised from a CNaIIA-1 cell. Control Na^+ currents were evoked by depolarization to −30 mV from a holding potential of −110 mV before and after treatment of the cytoplasmic surface of the patch with 1 μM diolein, 1 μM phosphatidylserine, and 1 mM ATP. PKC (10 nM) was added and Na^+ currents were recorded in response to the same test pulse. The two superimposed larger current records are control recordings in the presence and absence of diolein, phosphatidylserine, and ATP; the smaller current record was recorded after addition of PKC. (**C**) Ensemble currents were evoked from a CNaS1506A-9 cell-attached patch by depolarization to −30 mV from a holding potential of −110 mV. Na^+ current traces recorded in the presence or absence of 75 μM OAG are superimposed. (**D**) Ensemble average of 50 current traces was recorded from a membrane patch excised from a CNaS1506A-9 cell as in (B). Current records measured in the same membrane patch before and after treatment of the cytoplasmic surface with diolein, phosphatidylserine, ATP, and PKC are superimposed.

lar basis for modulation of Na^+ channel function by PKC. The effects of PKC on Na^+ channel activity result directly from phosphorylation of the Na^+ channel α subunit and are not secondary to phosphorylation of another cellular constituent that might be present in excised membrane patches. Phosphorylation of a single residue, Ser^{1506}, is required for both regulatory effects of PKC. This serine residue is a good substrate for phosphorylation by PKC but not by cAMP-dependent protein kinase. It is likely that this site is not significantly phosphorylated in unstimulated CHO cells because wild-type and mutant S1506A Na^+ channels have similar functional properties if PKC is not activated. The location of Ser^{1506} in an intracellular loop that is important in Na^+ channel inactivation (*17, 18*) suggests that the slowing of inactivation may be caused by a direct effect of phosphorylation on the function of this protein segment in inactivation gating.

Ser^{1506} and the adjacent amino acids of the PKC phosphorylation site are conserved in Na^+ channel α subunits from brain, heart, and skeletal muscle (*5–7, 24*), suggesting a common modulatory mechanism in all three tissues. The slowing of Na^+ channel inactivation by PKC would modulate membrane depolarization and action potential duration and thereby regulate electrical activity, neurotransmitter release, and muscle contraction. The reduction in peak Na^+ current by PKC could limit electrical activity and protect nerve, cardiac, and skeletal muscle cells from excitotoxicity. Our results show that phosphorylation of Ser^{1506} in the intracellular peptide loop between homologous domains III and IV of the Na^+ channel α subunit is necessary for slowing and reducing of Na^+ current in response to activation of PKC and may be involved in modulation of Na^+ channel function by hormones and neurotransmitters in a wide range of excitable tissues.

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16. Cells were cultured at 37°C in RPMI1640 (Difco), 5% CO_2, 10% fetal bovine serum (HyClone), geneticin (200 μg/ml) (Gibco-Bethesda Research Laboratories). In the patch clamp experiments, we used the Axopatch 1C voltage clamp. The 500-megohm headstage resistor was used unless otherwise noted. Currents were digitally sampled at 40 kHz. Linear leakage and capacitance have been corrected by subtraction of null traces with no channel activity and scaled pulses recorded in voltage ranges where channel activity was absent. Current traces used for leakage subtraction were sometimes fit with a series of exponentials and the resulting fit trace used for subtraction to avoid addition of noise to the records. The bathing solution for both excised and cell-attached patch clamp recording was 150 mM KCl, 10 mM NaCl, 1.5 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM EGTA, 5 mM Hepes, KOH to pH 7.4. The pipette solution for both excised and cell-attached patch clamp recording was 150 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4.
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