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Topical Review

Regulation of Voltage-Dependent Sodium Channels

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Introduction

Voltage-dependent Na^+ channels are transmembrane proteins present in different electrically excitable cells. Na^+ channels open and inactivate in response to a depolarization of the resting membrane potential. When these channels open, an influx of Na^+ occurs, depolarizing the membrane potential, and generating the upstroke phase of the action potential. Inactivation of Na^+ channels stops Na^+ influx, preventing a permanent depolarization of the resting membrane potential. Recovery from inactivation allows the cell membrane to regain its resting excitable properties. Therefore, voltage-dependent Na^+ channels are essentially responsible for the generation of action potentials in different nerve and muscle fibers. Because opening and inactivation of voltage-dependent Na^+ channels proceed within a few msec after the depolarizing stimulus, action potentials propagate very fast. In the canine heart, for example, the high propagation speed of action potentials ensures that ventricles are depolarized in approximately 20 msec. This enables the ventricles to contract in a physiologically synchronous manner, thus generating enough internal pressure to eject blood in the circulation. Na^+ channels do not trigger muscle contraction or synaptic transmission *per se*, but they ensure that these essential physiological events occur with a proper timing.

One important question concerns the regulation of voltage-dependent Na^+ channels. Protein kinase activation, G-proteins, and hormones can modify the gating (opening-inactivation-closing) kinetics of Na^+ channels. However, it is not clear whether these are significant phenomena in vivo. While the physiological meaning of voltage-dependent Na^+ channel regulation is not firmly established, the study of Na^+ channel modulation offers an excellent opportunity to gain biophysical insights to how Na^+ channels work, and to structure-function relationships. The main objective of this review is to critically evaluate information and hypothesis concerning regulation of Na^+ channels by different protein kinases and related phenomena. Only short term alterations in Na^+ currents will be discussed. Short term effects are alterations in the electrophysiological properties of Na^+ currents or channels that occur within minutes after a change in an experimental condition. It must be noticed however, that several studies investigating long-term (hours-days) effects of growth factors and protein kinase involvement on induction of Na^+ channels have recently appeared (Kalman et al., 1990; Ginty et al., 1992; D'Arcangelo et al., 1993). While this review is focused on a limited topic, excellent and more extensive reviews on Na^+ channels have recently appeared (Catterall, 1992; Goldin, 1995). Also of interest are several reviews on modulation of ion channels in general by protein phosphorylation (*see* Levitan, 1994).

Structure and Biochemistry of Na^+ Channel Phosphorylation by Protein Kinases

Brain Na^+ Channels

A discussion on different aspects of Na^+ channel structure can be found in Goldin (1995). Functional effects of protein kinase activation were studied on Na^+ channels from rat brain, eel, skeletal muscle, and heart. A brief comparison of structural information will be given in relation to these channels. Na^+ channels consist of a main α subunit (260 kDa) and two additional smaller subunits β_1 and β_2 with molecular weights of 36 and 33 kDa, respectively (Hartshorne et al., 1982; Hartshorne & Catterall, 1984; Messner & Catterall, 1985). The β_1 subunit is not covalently attached to the α subunit. The β_2 subunit is attached to the α subunit by a disulfide bond.

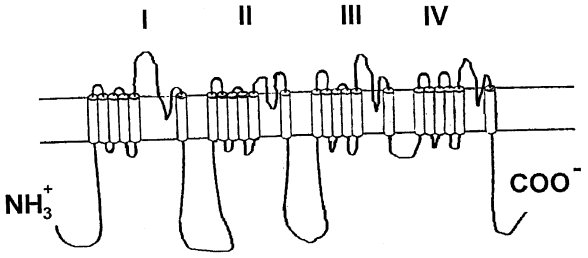


Fig. 1. Hypothetical organization of the α subunit of Na^+ channels. This subunit is comprised of four different homologous domains. Each domain is comprised of six transmembrane α helices which are represented by cylinders. The channel's pore is assumed to be formed by extension of residues located between segments 5 and 6 in different domains. Several potential phosphorylation residues are located on cytoplasmic loops of the α subunit (*see text*).

Three different cDNA clones encode for different rat brain α subunits (Noda et al., 1986). These are rat brain types I, II, and III Na^+ channels. An alternative spliced variant of rat brain Na^+ channel II was identified (type IIA) with similar electrophysiological properties (Auld et al., 1988). A schematic diagram of the proposed membrane topology for the α subunit of Na^+ channel is shown in Fig. 1. In this model, the α subunit consists of four homologous membrane domains (I–IV). Each domain is comprised of six putative α helices (S1–S6). The channel's pore is assumed to extend between segments S5 and S6 in analogy to what had been proposed for K^+ channels (Yellen et al., 1991). Amino and carboxyl termini, and peptide linkers connecting the different domains are on the intracellular side of channel. Relatively short peptide linkers connecting adjacent segments in the same domain are located either on the external or internal side of the channel (Noda et al., 1984; Guy & Seetharamulu, 1986). The voltage-dependence and permeation properties of Na^+ channels are essentially given by the α subunit (Goldin et al., 1986; Trimmer et al., 1989, Cribbs et al., 1990). Interestingly, both β subunits (expressed individually or in conjunction) modulate Na^+ channel function (Isom et al., 1992, 1995). However, and at least in relation to rat heart Na^+ channels (SkM2), coexpression of β_1 subunits did not modify PKA-induced modulation of Na^+ currents in α subunits (Schreibmayer et al., 1994). This preliminary conclusion must be taken with caution in view of the complexity of pathways related to modulation of Na^+ currents.

Several PKA or PKC consensus phosphorylation sites are present on the intracellular linkers of α subunits of rat brain IIA Na^+ channels. The existence of these sites does not imply that they are necessarily phosphroylated. In addition, other serine or threonine residues which do not reside in typical consensus phosphorylation sites could be phorphorylated by protein kinases (Kennely & Krebs, 1991). Therefore, it is essential to measure the incorporation of phosphate groups in Na^+ channels induced by protein kinase stimulation. Few studies however, have provided this essential information. Initial work from Catterall's laboratory has determined that 3–4 mol of phosphate are incorporated in 1 mol of purified rat brain Na^+ channel α subunit following activation of PKA under basal conditions (Rossie & Catterall, 1987). Phosphate incorporation occurred in the cytoplasmic linker connecting domains I and II (Rossie, Gordon, & Catterall, 1987; Rossie & Catterall, 1989). It is known that four serine residues located in that linker can be phosphorylated by PKA in vitro (Murphy et al., 1993). A fifth serine residue located at position 554 in linker I–II might be endogenously phosphorylated.

The effects of phosphatases on phorphoyrlated rat brain Na^+ channels have also been studied. Two different types of phosphatases, similar to phosphatase 2A and calcineurin, removed phosphate groups from brain Na^+ channels (Chen et al., 1995). The inhibition of phosphatases in rat brain synaptosomes enhanced significantly the level of cAMP-induced phosphorylation of Na^+ channels (Murphy et al., 1993; Chen et al., 1995), indicating that these channels are endogenously phosphorylated.

PKC activated the incorporation of 2.5 mol of phosphate per mol of purified α subunit of rat brain Na^+ channel (Murphy & Catterall, 1992). Because mapping of PKC phorphorylated α subunits generated three major tryptic phosphopeptides, a minimum of three different residues can be phosphorylated by PKC in vitro. One of these residues, probably S^{1506}, rests between domains III and IV of the α subunit. In addition to other effects that will be discussed later, PKC phosphorylation of this residue primes Na^+ channels for functional modulation by PKC phosphorylation. A second residue, which can also be phosphorylated by PKC, is located in the linker connecting domains I and II. Alpha subunits of rat brain Na^+ channels can be additively phosphorylated by PKA and PKC. Additional work may be required to define other possible PKC phosphorylation sites (Murphy & Catterall, 1992).

Eel Na^+ Channels

Significant structural differences exist between Na^+ channels from rat brain and eel electroplax; (i) Eel Na^+ channels contain the α subunit only; (ii) The linker connecting domains I–II in eel Na^+ channels is approximately 200 amino acid residues shorter than in rat brain IIA Na^+ channels. Thus, several PKA consensus phosphorylation sites present in brain Na^+ channels are absent in α subunits of eel channels. While PKC could not phorphorylate eel Na^+ channels, these channels are considered excellent substrates for PKA phosphorylation (Emerick & Agnew, 1989). Four serine residues are endogenously phosphorylated by PKA (Emerick & Agnew, 1989; Emerick, Shenkel & Agnew, 1993). Two of them

are located in the carboxyl terminal, one lies in the amino terminus, and one in the linker connecting domains I and II. A threonine residue located in the amino terminus does not seem to be phosphorylated in vivo, but can be phosphorylated by PKA in vitro. Treatment of purified eel Na^+ channels with potato acid phosphatase removed almost all phosphate groups added by PKA. In contrast with rat brain Na^+ channels, calcineurin and alkaline phosphatase did not remove phosphate groups in eel Na^+ channels. Significant differences in phosphorylation pattern, and in the biochemistry of phosphorylation exist between Na^+ channels from eel and rat brain. In spite of these differences, PKA activation attenuated macroscopic currents in both eel and rat brain. Na^+ channels.

Skeletal Muscle and Heart Na^+ Channels

As with eel Na^+ channels, the linker connecting domains I and II in adult rat skeletal muscle Na^+ channels is considerably shorter than in rat brain channels (Trimmer et al., 1989). Each α subunit of rat skeletal muscle Na^+ channel can incorporate one phosphate via PKA activation (Yang & Barchi, 1990). This is considerably less than in eel or in rat brain Na^+ channels, and may be a consequence of a significant endogenous phosphorylation.

In spite of a significant number of studies on functional effects of PKA on heart Na^+ channels, structural and biochemical information on phosphorylation of these channels is not available. The cardiac Na^+ channel SkM1 (or rH1) has one PKA consensus site located in the amino terminus, and two in each of the I–II and II–III linkers (Schreibmayer et al., 1994). Recently, incorporation of phosphate via PKA was demonstrated in α subunits of rat heart Na^+ channels (Cohen & Levitt, 1993).

Functional Effects of PKA on Na^+ Channels

Brain IIA Na^+ Channels

The effects of PKA activation were studied on α subunits of rat brain IIA Na^+ channels expressed either in Chinese hamster ovary (CHO) cells (Li et al., 1992, 1993), or in *Xenopus* oocytes (Gershon et al., 1992; Smith & Goldin, 1992, 1996; Hebert et al., 1994). Additionally, Li et al. (1992) have also studied the influence of PKA activation on single Na^+ channels recorded from embryonic rat brain neurons in culture. When the intracellular side of excised inside-out membrane patches from CHO cells was exposed to the catalytic subunit of PKA and ATP, peak macroscopic Na^+ currents was reduced by approximately 40% (Li et al., 1992). This effect was not accompanied by significant changes in the position of peak Na^+ current-voltage curves in relation to control conditions. Also, the steady-state inactivation curve (peak Na^+ conductance at a given potential *vs.* membrane holding potential), and the macroscopic decay of Na^+ currents were not modified by (PKA + ATP). In single channel recordings from rat brain neurons in culture, it was shown that the open probability of Na^+ channels (P_o) was reduced by the catalytic subunit of PKA and ATP while the single channel conductance was not changed (Li et al., 1992).

In a subsequent study, Li et al. (1993) reported that while the catalytic subunit of PKA attenuated Na^+ currents in *excised* inside-out membrane patches from CHO cells (Li et al., 1992), PKA activators did not modify Na^+ currents that were being recorded in the *cell-attached* mode. However, if the cell had been previously treated with a PKC activator (OAG, 1-oleoyl,2-acetylglycerol), the external addition of a PKA activator resulted in attenuation of Na^+ currents in the cell-attached patch configuraion. These different experimental findings led to the remarkable discovery that a serine residue (S^{1506}) located in the linker connecting domains III–IV of the Na^+ channel needs to be phosphorylated by PKC in order for Na^+ currents to be attenuate by PKA phosphorylation. Replacement of S^{1506} by a negatively charged amino acid enabled PKA to attenuate Na^+ currents. This strongly suggests that the modulatory role of PKC phosphorylation on the attenuation of Na^+ currents by PKA is, at least in part, a consequence of electrostatic effects. What causes Na^+ channels in an excised membrane patch to be responsive to the catalytic subunit of PKA remains an intriguing question. One possibility is that once the membrane patch is excised, Na^+ channel changes its configuration in such a way as to make S^{1506} available to the catalytic subunit of PKA. Another possibility is that the mechanical stress caused by forming and excising a membrane patch somehow triggers phosphorylation of S^{1506}. Responses to these questions will allow a better understanding of the well known differences in single ion channel behavior in excised *vs.* cell-attached modes.

By contrast, the effects of PKA activation on the α subunit of rat brain IIA Na^+ channels expressed in frog oocytes seem quite complex. Extracellular application of forskolin, or intracellular injection of cAMP (or PKA catalytic subunit) reduced peak Na^+ currents by approximately 25% in frog oocytes (Gershon et al., 1992). This reduction could be partially prevented by pretreating oocytes with a peptide inhibitor of PKA suggesting the involvement of PKA on Na^+ current attenuation. As with previous results in CHO cells (Li et al., 1992, 1993), attenuation of Na^+ currents in frog oocytes occurred in the absence of other alterations of macroscopic Na^+ currents, and of voltage shifts in the peak Na^+ conductance-voltage relationship or in the voltage-dependence of steady-state inactivation. It should be remarked however, that due to technical difficulties, an accurate de-

scription of macroscopic Na^+ current kinetics is not possible with conventional membrane voltage clamp of *Xenopus* oocytes. Intracellular injection of protein phosphatase 2A caused, on average, a moderate increase of peak Na^+ currents (Gershon et al., 1992). Qualitatively similar effects of PKA activation on rat brain Na^+ currents in frog oocytes were obtained by Hebert et al. (1994). In addition, Hebert et al. (1994) showed that insertion of four amino acids disrupting a typical PKA consensus phosphorylation site at the end of the loop connecting domains I–II (residues 684 and 685) attenuated (but did not completely abolish) the PKA-induced decrease of Na^+ currents.

Smith and Goldin (1992) reported that activation of PKA via stimulation of β_2 adrenergic receptors coexpressed in *Xenopus* oocytes enhanced rat brain IIA Na^+ currents by approximately 80%. This effect was mimicked by external application of dibutyryl-cAMP, and was prevented by PKA inhibitors. Consistent with this finding was the observation that oocytes injected with alkaline phosphatases had, on average, a decreased Na^+ current density in relation to noninjected oocytes. These results suggested that PKA phosphorylation enhanced rat brain IIA Na^+ currents in frog oocytes, which is in disagreement with studies from other laboratories employing comparable methods. More recently, Smith and Goldin (1996) have reexamined the effects of PKA activation on Na^+ currents. The essential conclusion of this latter study is that PKA activation can either enhance or attenuate macroscopic rat brain IIA Na^+ currents depending on the physiological (or biophysical) status of Na^+ channels. Smith and Goldin (1996) have coexpressed β_2 adrenergic receptors at a level 100-fold less than in their previous study. Under these conditions, receptor activation with extracellular isoproterenol attenuated Na^+ currents by approximately 20%, which is a result in concert with previous observations from other laboratories using similar methods and techniques. It was reasoned that in their previous study (Smith and Goldin, 1992), overexpression of adrenergic receptors triggered an endogenous stimulation of PKA which was independent of extracellular agonists. This had presumably caused phosphorylation of residues located in linker connecting domains I–II of the α subunit. Further activation of receptors with isoproterenol or direct activation of PKA, caused enhancement of Na^+ currents. Smith and Goldin (1996) demonstrated that enhancement of Na^+ currents by PKA activation occurred in each of the following conditions: (i) when an appreciable portion of linker connecting domains I–II containing PKA consensus phosphorylation sites was deleted; (ii) when serine residues in PKA consensus phosphorylation sites in the linker were replaced by alanines; (iii) when serine residues in PKA consensus phosphorylation sites were replaced by aspartates. Interestingly, the latter manipulation by itself attenuated averaged Na^+ current density thus mimicking the effects of PKA activation, and suggesting that these phosphorylation effects on Na^+ channels may be triggered by electrostatic phenomena. Therefore, enhancement of Na^+ currents by PKA activation is independent of PKA consensus phosphorylation sites in linker I–II. What causes PKA to enhance macroscopic Na^+ currents remains an open subject. It is conceivable that Na^+ current enhancement by PKA may be caused by phosphorylation of channel at a different site and/or interaction with a phosphorylated protein that could modulate the channel.

An apparently unexplored experimental difference persists between results from Li et al. (1992, 1993) obtained in CHO cells with the cell-attached mode, and those obtained by different investigators with the same rat brain Na^+ channel α subunit expressed in *Xenopus* oocytes using the two microelectrode voltage-clamp method of recording. PKA activation in intact *Xenopus* oocytes attenuated rat brain IIA Na^+ currents. In contrast with CHO cells, pretreatment of frog oocytes with PKC activators was not necessary for PKA activation to attenuate Na^+ currents. Following results with CHO cells, this is expected to occur if S^{1506} had been previously phosphorylated by PKC. Is this residue phosphorylated in basal conditions in *Xenopus* oocytes but not in CHO cells?

Figure 2 shows previously unpublished observations on PKA-induced phosphorylation of rat brain Na^+ channels reconstituted in planar lipid bilayers. The experimental findings contain two additional features in relation to previous results on PKA modulation of rat brain Na^+ currents. Firstly, the effects of PKA on the opening-closing events of Na^+ channels were studied in the absence of inactivation. Na^+ channels reconstituted in planar lipid membranes had their inactivation removed with batrachotoxin (for experimental details see legend to Fig. 1, and Cukierman, 1991). Secondly, reconstitution protocols presumably involve the loss of soluble factors that could exert some modulatory role on Na^+ channels. The upper two panels in Fig. 2 show single Na^+ channel currents at −90 mV in control conditions, and at −75 mV after treatment of channel with the catalytic subunit of PKA and 1 mM ATP. Notice that the gating characteristics of the channel are about the same in the two recordings indicating that after (PKA + ATP), a depolarizing shift in the P_o-V relationship had occurred. This effect was not observed with ATP or with the catalytic subunit alone, or when these substances were applied to the external side of the channel. The lower left panel shows that the P_o-V relationships shifted to more depolarized voltages (the midpoint of the control curve shifted from −96 to −77 mV) after treatment with (PKA + ATP). It was also found that the decrease in P_o at a given voltage, is a consequence of a decrease in the open rate constant and increase in the closing rate constant of

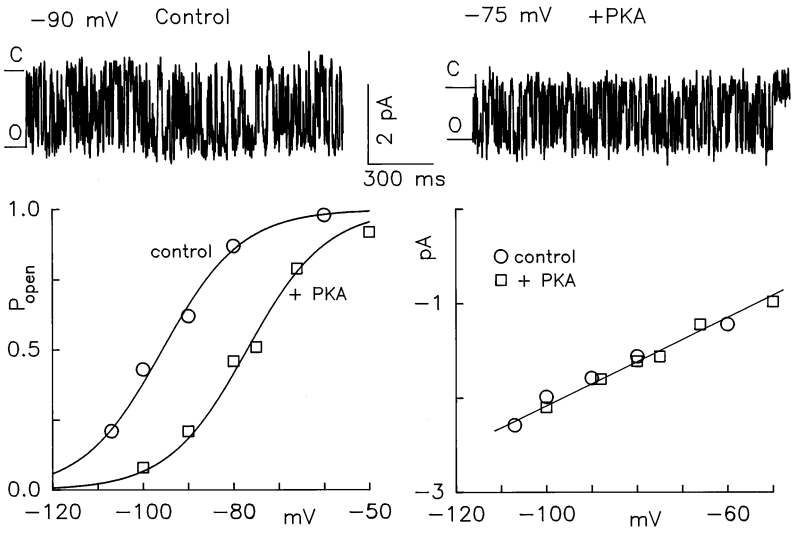
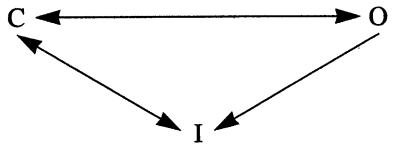


Fig. 2. A single rat brain Na^+ channel whose fast inactivation had been removed with batrachotoxin was reconstituted in a neutral phospholipid bilayer composed of 80% phosphatidylethanolamine and 20% phosphatidylcholine in decane. The channel was exposed to symmetrical solutions of 150 mM NaCl and 10 mM HEPES (pH = 7.00). The upper row shows recordings of a single channel at −90 mV (cell convention) in control conditions. The recording at −75 mV was obtained after addition of 30 units of catalytic subunit of PKA/ml (Sigma) and 1 mM ATP to the intracellular side of the channel. *C* and *O* indicate the full closed and open states of the channel. P_o was calculated by [(time channel spent in the open state)/(total recording time)] at different voltages. $P_o \times V_m$ plots are shown on the bottom left panel. Curves were drawn according to a simple Boltzmann distribution between one open and one closed state. Single-channel current voltage relationships are shown on the bottom right panel. See text for discussion, and Cukierman (1991) for more information on experimental protocols and analysis. The result shown in this figure was seen in three out of six different channels. No effects were observed following addition of (PKA + ATP) in three different single Na^+ channels.

the Na^+ channel by approximately *the same proportion* (*not shown* in Fig. 2). The direction of voltage shift and those alterations in rat constants caused by (PKA + ATP) are consistent with an electrostatic effect arising from an increased intracellular negative surface charge density on the channel (Cukierman, 1991). Notice that even in the absence of fast inactivation, PKA decreased Na^+ channel P_o between −110 and −60 mV. This shift in the voltage dependence of channel gating was not observed previously in rat brain channels but was present in eel Na^+ channels after PKA treatment (*see below*). It is possible that PKA phosphorylation affects different gating parameters of rat brain Na^+ channels, and that a shift of the gating curve could have been masked by effects on channel inactivation which is known to be coupled to Na^+ channel activation. Alternatively, this PKA-induced shift on channel gating may have been caused by modification of the rat brain Na^+ channel with batrachotoxin. On the other hand, single channel current-voltage relationship was not changed by PKA in the voltage range of gating (lower right panel), which is in agreement with other results (Li et al., 1992). The experimental results in Fig. 2 also provide a mechanistic interpretation for what may well be the first demonstration of functional effects of PKA activation on voltage-dependent Na^+ channels. Costa and Catterall (1984) showed that Na^+ channels in rat brain synaptosomes are phosphorylated by PKA activation. When synaptosomes were treated with batrachotoxin (as well as with other neurotoxins that remove fast inactivation) a reduction of ^{22}Na influx in rat brain synaptosomal fraction was observed with PKA activation. The observed decrease in ^{22}Na influx was probably a consequence of a reduction of P_o as shown in Fig. 2 in Na^+ channels from a similar preparation.

The effects of PKA at the single-channel level are not completely understood. Li et al. (1992) have suggested that the reduction in macroscopic Na^+ currents could be caused by PKA-induced attenuation of P_o. Even in a relatively simple kinetic scheme like



where *C*, *O*, and *I* represent the Na^+ channel in the closed, open, or inactivated state, respectively, a decrease in P_o can be caused by alterations of different kinetic rate constants. A decrease (or increase) of the opening (or closing) rate constant, and/or an increase of the inactivation rate constants (either from open or/and closed states) reduce the open probability of Na^+ chan-

nels. Li et al. (1992) noticed a relative increase in the number of current traces showing no single channel opening in response to voltage clamp pulses after PKA treatment. This can effectively decrease macroscopic Na^+ currents. Because the decay of macroscopic Na^+ currents (macroscopic inactivation) was not significantly affected by the catalytic subunit of PKA and ATP, Na^+ channels may be inactivating faster directly from the closed state after PKA phosphorylation. However, the P_o of batrachotoxin-treated Na^+ channels (no fast inactivation present) is still modified by PKA (*see* Fig. 2). It is possible that PKA-induced phosphorylation stabilizes the closed state of Na^+ channels and/or leads Na^+ channels into different gating modes. Results from a patch containing a single Na^+ channel revealed that in addition to an increase in the number of null traces (single channel current traces containing no channel openings), there was a noticeable decrease in the mean open time of Na^+ channels after (PKA + ATP) treatment (Li et al., 1992). This information *per se* predicts that PKA should accelerate macroscopic Na^+ current inactivation, thus decreasing the duration of Na^+ currents. This effect was not observed in several different studies. It is likely that PKA phosphorylation may simultaneously modify more than one microscopic property of Na^+ channels, and more single-channel studies are necessary to clarify this issue.

Eel Na^+ Channels

Unfortunately, eel Na^+ channels cannot be functionally expressed in *Xenopus* oocytes. This is a serious technical limitation for the functional study of these channels. PKA attenuated Na^+ currents and shifted the activation curve of Na^+ channels by 10 mV in the depolarizing direction in macroscopic current recordings from excised inside-out patches from eel electrocytes. As discussed above in relation to Fig. 2, this depolarizing shift is consistent with an increased intracellular negative surface charge density on Na^+ channels.

Skeletal Muscle Na^+ Channels

The linker connecting domains I and II in adult rat skeletal muscle (SkM1) Na^+ channels, is significantly shorter than in α subunits of rat brain IIA channels. The absence of PKA consensus sites in this linker contributes to the lack of effects of PKA stimulation on currents from SkM1 channels expressed in oocytes (Smith and Goldin, 1992). Recently, the I–II linker containing PKA consensus sites from rat brain IIA channels was inserted into SkM1 channels. When these mutant channels were expressed in *Xenopus* oocytes, activation of PKA did attenuate Na^+ currents (Smith and Goldin, 1996). Even though enhancement of Na^+ currents via PKA stimulation in rat brain IIA Na^+ channels does not depend on the I–II linker (Smith and Goldin, 1996), such a response is not observed in wild type SkM1 channels, which lack significant portion of said linker. The mechanism by which PKA enhances Na^+ currents in rat brain as well as in heart remains obscure (see below).

Heart Na^+ Channels

Alpha subunits from rat heart Na^+ channels (SkM2 or rH1) were coexpressed in *Xenopus* oocytes with β_2 adrenergic receptors (Schreibmayer et al., 1994). Isoproterenol enhanced Na^+ currents in SkM2 channels via PKA activation. It should re remarked that PKA attenuated Na^+ currents in rat brain channels and augmented these currents in SkM2 channels independently of coexpression of β_2 receptors (Schreibmayer et al., 1994). The effects of PKA activation on SkM2 currents seem to be limited to enhancement of macroscopic Na^+ currents. Na^+ current enhancement caused by isoproterenol increased with the amount of β_2 receptor cRNA injected into oocytes. Five threonine or serine residues residing in PKA consensus sites located in the amino terminus, I–II, and II–III linkers of the α subunit of SkM2 Na^+ channels were replaced by different amino acids (Schreibmayer et al., 1994). When this mutant channel with disrupted PKA consensus sites was expressed in *Xenopus* oocytes, intracellular injection of cAMP could still enhance Na^+ currents. As with PKA-induced potentiation of Na^+ currents in rat brain IIA channels, it is not known what causes the same effects in SkM2 Na^+ currents. Either an unknown phosphorylated protein is modulating Na^+ channels and/or phosphorylation of Na^+ channels is occurring at other threonine or/and serine residues (Schreibmayer et al., 1994).

A variety of effects of PKA activation on native Na^+ channels from the same or different cardiac cells has been reported (*see* references below). The application of the catalytic subunit of PKA to excised inside-out membrane patches from guinea-pig ventricular myocytes decreased P_o, and increased the first opening latency of single Na^+ channels (Sunami et al., 1991). Attenuation of peak currents, and a slowing of macroscopic inactivation were present on ensemble average currents from single-channel recordings. Mean open times and single-channel conductances were not modified by PKA. These effects were dependent on the holding membrane potential with depolarizing voltages enhancing attenuation of Na^+ currents by PKA. The voltage-dependent effects of PKA activation on Na^+ channels in cell attached membrane patches from ventricular cells of different species were carefully analyzed by Ono, Fozzard and Hanck (1993). While single-channels' conductances were not modified by PKA activation, consistent hyperpolarizing voltage shifts of the activation, and steady-state inacti-

vation curves were observed. Therefore, activation of PKA could either attenuate or potentiate Na^+ currents depending on the holding membrane potential (Ono et al., 1993). However, this cannot be the sole explanation for the variability of responses of cardiac Na^+ currents to PKA activation (Ono et al., 1993). In single-channel recordings from excised membrane patches from rabbit cardiac myocytes, the catalytic subunit of PKA enhanced ensemble Na^+ currents and accelerated the rate of macroscopic inactivation. A subsequent exposure of PKA-treated channels to calcineurin reversed these effects (Matsuda, Lee & Shibata, 1992).

Functional Effects of PKC on Na^+ Channels

Brain Na^+ Channels

At relatively high concentrations (20–75 μM), the diacylglycerol OAG has two different effects on rat brain IIA Na^+ channels expressed in CHO cells: it attenuates macroscopic Na^+ conductance, and delays the inactivation of macroscopic currents (Numann, Catterall & Scheuer, 1991). These effects are modulated by PKC activation. Following PKC activation, (re)openings of single Na^+ channels were observed as late as 200 msec after opening the depolarizing voltage clamp pulse. This delayed single channel activity contributes to the slower macroscopic inactivation of Na^+ currents (Numann et al., 1991). When S^{1506} (which resides in an ideal PKC consensus site) in the III–IV linker was replaced by an alanine, OAG failed to attenuate currents and retard macroscopic inactivation. Therefore, PKC phosphorylation of S^{1506} must be involved in both OAG effects (West et al., 1991). PKC activation did not modify the steady-state inactivation and activation curves of Na^+ currents (*see below* for different results). However, when S^{1506} was replaced by a negatively charged amino acid (either aspartate or glutamate), the voltage dependence of Na^+ channel activation shifted by 20 mV in the depolarizing direction (Li et al., 1993). This result is intriguing because depolarizing voltage shifts in activation curves did not occur following a presumed PKC phosphorylation of S^{1506}. It would be instructive to investigate the effects of adding two negatively charged amino acids to that specific region in the III–IV linker on Na^+ current activation. Also of interest would be to verify whether mutant channels possessing a negatively charged residue at position 1506 are able to display late single channel (re)-openings, as observed after PKC activation. One possibility that cannot be eliminated is that PKC phosphorylation occurring at a different site might allosterically interfere with III–IV linker causing current attenuation and retarding macroscopic inactivation.

Slowing of inactivation in the absence of Na^+ current attenuation was observed at low (<10 μM) concentrations of OAG (Numann et al., 1992, 1994). This was taken as evidence that more than one PKC phosphorylation site is involved with OAG effects on Na^+ channels. S^{1506} is essential for both phenomena to occur (West, 1991). Additionally, a serine residue in the I–II linker seems to be necessary for OAG to induce attenuation of Na^+ currents, but not for the delay of macroscopic inactivation (Numann et al., 1992). Therefore, phosphorylation of S^{1506} by PKC is a necessary but not sufficient condition for OAG to attenuate Na^+ currents, while being necessary and sufficient for retarding macroscopic inactivation of Na^+ currents.

Activation of PKC by phorbol ester in *Xenopus* oocytes expressing type IIA Na^+ channels caused qualitatively different effects from those described above with the same channels in CHO cells. Although Na^+ currents in frog oocytes were attenuated by PKC activation, this effect was assumed to be a consequence of a 10 mV depolarizing shift in the activation curve of Na^+ channels (Dascal & Lotan, 1991), which was not observed in CHO cells. The effect of phorbol ester on Na^+ current activation was supported by single channel recordings (Schreibmayer et al., 1991). A voltage-dependent increase in the latency to first single-channel opening was observed after phorbol ester treatment. This predicts that macroscopic Na^+ current inactivation should be retarded, an effect that was indeed observed (Dascal & Lotan, 1991; Schreibmayer et al., 1991). Another prediction is a slowing of Na^+ current activation. However, the temporal resolution of Na^+ current activation in *Xenopus* oocytes is not reliable enough with conventional voltage clamp techniques. In contrast with results in CHO cells, the mean open time of Na^+ channels were not modified by PKC activation. Also, late (re)openings of channels were not induced by PKC as occurred with Na^+ channels expressed in CHO cells.

The effects of *different* activators of PKC on Na^+ currents in a mouse neuroblastoma cell line (N1E115) were characterized with the nystatin-based perforated patch method. Under these conditions, cells have an intracellular milieu close to normal physiological conditions (Godoy & Cukierman, 1994*a*,*b*). Several different phorbol esters in a wide range of concentrations did not modify Na^+ currents (*see* Rorsman, Arkhammar & Berggren, 1986 for lack of acute phorbol ester effects on Na^+ currents in an insulin-secreting cell line, and Linden and Routtenberg (1989) for similar results in N1E115 cells). However, two different diacylglycerols (OAG or DOG (1,2-dioctanolyglycerol); concentration range: 4–75 μM) had the following effects on Na^+ currents (i) Currents were attenuated in a voltage-dependent manner with depolarizing voltages enhancing attenuation; (ii) The steady-state inactivation curve was shifted by 22 mV in the hyperpolarizing direction; (iii) The rate of Na^+ chan-

nel inactivation, as measured by double-pulse voltage clamp protocols was increased in a voltage-dependent manner, with positive membrane potentials increasing this rate. Neither the activation curve nor the waveform of macroscopic Na^+ currents were significantly modified by diacylglycerols (DAGs) (Godoy & Cukierman, 1994*a*,*b*). Single-channel recordings in the cell-attached mode revealed that neither single channel conductance nor open times of Na^+ channels were modified by DAG. However, the percentage of traces containing no single-channel opening in response to a series of depolarizing voltage-clamp pulses was increased by DAG. Overall, these observations suggested that DAG attenuation of Na^+ currents occurred by increasing the rate of inactivation of Na^+ channels directly from closed state(s) (Godoy & Cukierman, 1994*b*). Interestingly, the alkaloid batrachotoxin, which eliminates fast inactivation of Na^+ channels, prevented the effects of DAG on macroscopic Na^+ currents, consistent with proposed hypothesis. When N1E115 cells were pretreated with different pharmacological inhibitors of PKC, Na^+ currents were not attenuated by DAG.

A different category of PKC activators is comprised of *cis*-unsaturated fatty acids (CUFAs). These compounds activate several PKC isoforms including atypical isoforms which are not activated by DAG (Nishizuka, 1995). Oleic acid (4 μM), a monounsaturated C18 fatty acid, *enhanced* Na^+ currents in N1E115 cells by approximately 70% in relation to control conditions (Godoy & Cukierman, 1994*a*). This enhancement was not accompanied by changes in kinetic or steady-state properties of Na^+ currents. Interestingly, inward Na^+ currents were consistently more enhanced by oleate than outward Na^+ currents, a result which has also been obtained with other CUFAs (*see below*). Na^+ current enhancement induced by oleate was prevented by pretreating N1E115 cells with PKC inhibitors. In addition, the optical isomer of oleate (elaidic acid), or the fully saturated C18 (stearic acid), which do not activate PKCs, did not enhance Na^+ currents. This suggests that PKC activation is involved with Na^+ current enhancement. By contrast, C18 CUFAs with more than one *cis* double bond (linoleic, linolenic, and arachidonic) *attenuated* macroscopic Na^+ currents and shifted the steady-state inactivation curve to more negative voltages (Godoy & Cukierman, 1994*a*). These effects were significantly more pronounced on inward than on outward Na^+ currents. Thus, the main qualitative difference between attenuation of Na^+ currents by DAG or CUFA is that the latter affected predominantly inward Na^+ currents while the former compounds actually increased the attenuation of currents at voltages where outward Na^+ currents were present. Thus, four different PKC activators had qualitative different effects on Na^+ currents in N1E115 cells: phorbol esters did not affect Na^+ currents, and DAG attenuated Na^+ currents by what seems to be an acceleration of inactivation from closed state(s). Oleic acid, and polyunsaturated C18 fatty acids enhanced and attenuated, respectively, inward Na^+ currents predominantly. These effects seem to be mediated by PKC activation. It should be remarked that qualitatively different results were observed by Linden and Routtenberg (1989) on N1E115 cells using the conventional whole-cell voltage-clamp method. PKC activators modified Na^+ currents independently of PKC in N1E115 cells under the conventional whole cell voltage-clamp method (*see* Renganathan, Godoy and Cukierman, (1995) for a more complete discussion). An idea that is being currently examined concerns the possibility that different PKC activators mobilize different sets of PKC isoforms. Each set of activated PKCs would phosphorylate Na^+ channels (and/or a modulatory protein) in a distinctive pattern. This, in turn, would determine a specific behavior of Na^+ channels. Along this line of reasoning, it is worth mentioning that intracellular injection of different PKC subtypes in *Xenopus* oocytes expressing chick brain Na^+ channels resulted in different modulations of Na^+ currents (Lotan et al., 1991). While the purified subtype I did not modulate Na^+ currents, subtype II or III attenuated Na^+ currents. Interestingly, intracellular injection of a mixture of three subtypes did not affect Na^+ currents (Lotan et al., 1991).

Skeletal Muscle Na^+ Channels

Numann et al. (1994) have analyzed the effects of OAG (or phorbol ester) on skeletal muscle Na^+ channels in a mouse satellite cell line (MM14) and in primary cultures of rat skeletal muscle. Na^+ currents in MM14 cells have electrophysiological and pharmacological properties similar to type SkM1 or (μ1) Na^+ channels. The effects of OAG on Na^+ currents in MM14 cells were similar to those previously described in rat brain IIA channels in CHO cells (Numann et al., 1991): slowing of macroscopic inactivation and attenuation of macroscopic currents. With low concentrations of OAG (1-10 μM), only the slowing of macroscopic inactivation was observed. At higher concentrations, both effects were observed in steady-state conditions. However, each effect developed with a typical time course. Based on the relative independence of these effects, and in analogy to what was observed in rat brain channels (*see above*), it was proposed that modulation of Na^+ channels by PKC occurs via phosphorylation of two different sites. One of these sites could well be the conserved serine residue in linker III–IV (analogous to S^{1506} in brain IIA Na^+ channel). The other, which also needs to be identified, is not the same as in rat brain IIA Na^+ channels because of a short I–II linker in SkM1 channels (Numann et al., 1994).

HEART Na^+ CHANNELS

Phorbol esters or DOG attenuated ensemble average Na^+ currents, and increased the rate of their macroscopic inactivation in neonatal rat cardiac myocytes (Moorman et al., 1989). Benz, Herzig and Kohlhardt (1992) also found that OAG attenuated Na^+ currents in cultured neonatal rat cardiac myocytes. This attenuation was a consequence of reduced P_o. Because mean open times, burst activity, and first channel opening latencies were not affected by OAG, it is possible that channels may be inactivating directly from the closed state.

Recently, a Chinese hamster lung cell line was stably transfected with rat heart Na^+ channels (rH1 or SkM2, Qu et al., 1994). OAG (10 μM) attenuated Na^+ currents and caused an approximately 10 mV hyperpolarizing voltage shift of the steady-state inactivation curve of Na^+ channels. OAG did not induce delayed openings of single Na^+ channels. These effects are similar to those caused by DAGs or Na^+ channels in N1E115 cells (Godoy and Cukierman, 1994*a*,*b*), but different from those observed in rat brain IIA or SkM1 Na^+ channels (*see above*).

In closing this section, it is important to note that different PKC activators have ''direct'' (independent of different metabolic pathways) effects on Na^+ channels (Wieland, Fletcher & Gong, 1992; Fraser et al., 1993; Renganathan et al., 1995). The possibility that some of the effects related to PKC activation on Na^+ currents may be caused by ''direct'' interaction of the PKC activator with Na^+ channels should be addressed experimentally.

Na^+ Channel Modulation by Extracellular Activation of Different Membrane Receptors

Under physiological conditions, protein kinase activation is one of the last steps of a sequence of events starting with activation of a membrane receptor. Can Na^+ channels be modulated by extracellular activation of membrane receptors? Studies of isoproterenol modulation of Na^+ channels were already analyzed in relation to PKA (section 3). In addition, Na^+ channels can also be modulated by direct interaction with different G-proteins.

Isoproterenol activation of β-adrenergic receptors in neonatal rat heart myocytes attenuated Na^+ currents and shifted their steady-state inactivation curve to more negative voltages. These effects were mediated by G-proteins acting through the intracellular cAMP system (indirect pathway), and via a membrane delimited (direct) pathway (Schubert et al., 1989). Attenuation of Na^+ currents via direct pathways was more efficient than via cAMP activation. The presence of two distinct pathways in Na^+ current modulation by isoproterenol was confirmed by Matsuda et al. (1992). However, in contrast with results obtained by Schubert et al. (1989), Na^+ currents and the decay of macroscopic inactivation were *enhanced* by isoproterenol in rabbit heart cells (Matsuda et al., 1992). In the latter study, the acceleration of macroscopic inactivation was accounted by an indirect effect via cAMP activation.

External cAMP attenuated Na^+ currents in heart cells from many different species (Sorbera & Morad, 1991). This attenuation was accompanied by a negative voltage shift of the steady-state inactivation curve. Both effects were prevented by intracellular GDPβS or long-term incubation of cells with pertussis toxin, suggesting involvement of G-proteins.

Cardiac Na^+ channels also responded to extracellular angiotensin II (AII). Nilius, Tytgat and Albitz (1989) showed that All (between 0.05 and 1 μM) increased the P_o of Na^+ channels by decreasing the percentage of current traces containing no single channel openings in cell-attached mode. A delayed macroscopic inactivation, due to an increased number of late single-channel (re)openings, was also observed. Moorman et al. (1989) have also observed current enhancement with AII. In their experiments however, the inactivation rate was enhanced. These authors suggested that AII modulates Na^+ currents via PKC activation, a conclusion that was not corroborated by Benz et al. (1992).

Contrasting results were observed with G-protein modulation of rat brain Na^+ channels. G-protein activation by GTP-γS-attenuated currents carried by brain Na^+ channels expressed in *Xenopus* oocytes (Cohen-Armon, Sokolovsky & Dascal, 1989). This attenuation occurred in the absence of alterations in current kinetics. By contrast, GTPγS increased Na^+ currents in acutely dissociated rat hippocampal neurons as well as in CHO cells expressing rat brain IIA Na^+ channels (Ma et al., 1994). This enhancement was caused by a 10 mV hyperpolarizing shift of the activation curve of Na^+ currents. Similar voltage shifts were also noticed for steady-state inactivation curves. Pertussis toxin or GDPβS prevented these effects in CHO cells or in dissociated neurons from hippocampus (Ma et al., 1994), but not in *Xenopus* oocytes (Cohen-Armon et al., 1989). G protein enhancement of Na^+ currents seems to occur via a membrane delimited pathway (Ma et al., 1994).

The postsynaptic actions of dopamine are mediated by a family of G-protein coupled receptors. The effects of dopamine agonists were evaluated in acutely dissociated dopaminergic striatonigral neurons from rat (Surmeier et al., 1992). SKF38393 (a D1 dopamine agonist) attenuated Na^+ currents in both the whole cell and cell-attached patch recording modes. By contrast, D2 agonists (quinpirole or bromocriptine) could attenuate or enhance Na^+ currents (Surmeier et al., 1992). Bath application of dopamine to neostriatal cells reversibly inhibited Na^+-dependent action potentials (Calabresi et

al., 1987). The inhibitory action of dopamine on the electrical excitability of those neurons seem to be caused by a decrease in voltage-dependent Na^+ currents (*see* Fraser et al., 1993).

Concluding Remarks

The regulation of voltage-dependent Na^+ channels by protein kinases is a new field of study which has grown considerably in recent years. As with any field in its infancy, it presents novel experimental and conceptual problems.

Protein kinase activation did not modify the conductance of different single Na^+ channels. This is probably the only common point between different studies on protein kinase modulation at the single channel level. It would be instructive to determine whether this remains true in low ionic strength conditions. The negative electric field generated by the addition of phosphate groups to Na^+ channels should be enhanced in low ionic strength conditions (Cukierman, 1991). Single channel conductance under these conditions can be increased if intracellular linkers containing negatively charged groups are sufficiently close to the internal mouth of the channel's pore. Such experiments should provide information on the spatial relationship between intracellular linkers and the channel's pore.

In contrast with the conductance of single Na^+ channels, different effects of protein kinase activation on macroscopic Na^+ currents were observed. Either Na^+ current enhancement or attenuation was observed following activation of PKA or PKC or G-proteins. In several studies, the same channel expressed in different cells responded differently to similar experimental manipulations. It seems that an essential experimental variable was not being properly controlled. One of these variables is related to posttranslational modifications of Na^+ channels. Channel phosphorylation is one of these modifications, and results from a complex balance between activities of protein kinases, different kinase isoforms, and phosphatases. Different cells express different protein kinases and phosphatases. The same Na^+ channel may have different phosphorylation patterns in different cells, or even in the same cell at different times. It was shown that additive phosphorylation can occur in Na^+ channels leading to alterations in Na^+ channel function. Therefore, it is not surprising that protein kinase activation caused a prolific repertoire of effects on Na^+ currents in different cells. To understand how Na^+ channels are modulated by protein kinase, it is necessary to determine the initial state of these channels, i.e., how they were modified by the cell under basal conditions. Equally important is to determine how a given protein kinase activator modifies the complex balance between different protein kinases, their isoforms, and different phosphatases. Without this information, it is not possible to understand the different results obtained with the same or different Na^+ channels in various preparations. The enormous technical difficulties in determining these experimental variables in vivo make the understanding of ion channel regulation by phosphorylation a serious challenge. The increasing availability of different isoforms of protein kinases and phosphatases will allow their study on reconstituted Na^+ channels in artificial membranes or in excised inside-out patches. These simplified experimental conditions will expand our knowledge of Na^+ channel regulation.

Because protein kinase activation did not modify single Na^+ channel conductance and the number of channels in membrane patches remained apparently unchanged, the effects of kinase activation on macroscopic currents must be explained via alterations in channel's P_o. Complete studies of kinase effects at the single channel level are not available. However, the presence of a variety of effects of kinase activation on macroscopic Na^+ currents suggests that more than one single channel kinetic property can be altered by phosphorylation.

Finally, another exciting problem concerns the coupling between structure and function of protein kinase modulation of Na^+ channels. The incorporation of phosphate in Na^+ channels is often determined in experimental conditions different from those followed in functional studies. Because protein phosphorylation is in general sensitive to experimental conditions, the coupling of currently available structural information on channel phosphorylation to modulation of channel function is not straightforward. On the other hand, while mutagenesis experiments have been an important tool in the study of phosphorylation effects on Na^+ channels, our lack of knowledge of channel's structure adds considerable uncertainty to the interpretation of experimental results.

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