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Effects of phosphorylation on ion channel function

ISKANDER I. ISMAILOV and DALE J. BENOS

Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, Alabama, USA

There is considerable evidence suggesting that intracellular second messengers can modulate the activity of ion channels, and that protein phosphorylation by the different protein kinases is a frequent intermediary in these modulatory effects. This conclusion, namely, that ion channel proteins are indeed substrates for phosphorylation, has been verified in numerous biochemical studies [reviewed in 1–6].

The functional correlates of channel phosphorylation are known to involve a change in channel open probability and, in the case of voltage-sensitive ion channels, a shift in the voltage dependence of channel activation. The voltage dependence of ion channel gating appears to be governed by movement of charge in the voltage-sensing moiety. Analogous to alterations in enzyme activities following biochemical modification, phosphorylation of ion channel proteins may lead to conformational changes that subsequently alter their gating and/or conductive properties, giving rise to the observed changes in electrical activity. However, in many cases, it is not yet clear whether it is the ion channels themselves that are directly modified, or whether phosphorylation is simply an early step in a cascade of events that leads ultimately to modulation of channel activity. The development and application of single-channel recording techniques in membrane patches and in artificial planar lipid bilayers has provided a means to investigate the effects of phosphorylation on the kinetic properties of ion channels. Moreover, the recent application of site directed mutagenesis to cloned ion channels has pinpointed specific amino acid residues critical for the specific kinase effects.

Siegelbaum and Tsien [7] and Tsien [8] considered how macroscopic currents flow through ion channels may be modulated by physiological or biochemical effectors. Their analysis starts from the equation for macroscopic membrane current (I):

$$I = N P_o i \qquad \text{(Eq. 1)}$$

where i is the unitary current, P_o is the channel's open probability, and N is the number of functional channels. The number of functional channels is given by the product of the total number of available channels in a pool (N_T) and a probability factor (P_f):

$$N = N_T P_f \qquad \text{(Eq. 2)}$$

$$\text{Substituting: } I = N_T P_f P_o \qquad \text{(Eq. 3)}$$

It is apparent that changes in the total current flowing through a population of active channels can arise from alterations in any of the terms on the right hand side of equation 3. Figure 1 depicts the manner in which any of these changes could be detected with single channel recording techniques. Figure 1A illustrates the effects predicted by an increase in single channel current (i), Figure 1B an increase in channel open probability (P_o), Figure 1C, an increase in the number of functional channels ($N_T P_f$), and Figure 1D, an increase in the total number of channels (N_T). These examples, although simple, apply equally well to all ion channels.

In this brief review we will summarize the known experimental findings defining the intimate intramolecular events producing changes in single channel activity that could result from phosphorylation events. We will first discuss experiments involving voltage sensitive Na^+, Ca^{2+}, and K^+ channels because most of our current information concerning phosphorylation effects on ion channel function have been derived from these systems. For each channel our discussion is limited to the effects of protein kinase A and protein kinase C. A summary of much of this information is provided in Table 1. We conclude with a consideration of how phosphorylation and G-proteins influence renal amiloride-sensitive Na^+ channel function, integrating the knowledge derived from voltage-sensitive ion channels in the hope of providing a comprehensive paradigm of how the renal cell modulates Na^+ reabsorption through phosphorylation reactions. Because of space limitations, we have not attempted to provide a comprehensive review of the literature, and we apologize to those authors whose excellent work we have omitted.

Voltage-sensitive Na^+ channels

Protein kinase A

The Na^+ channel protein from mammalian brain consists of three subunits of molecular mass 260 kD (α-subunit), 36 kD (β_1-subunit, and 33 kD (β_2-subunit). The Na^+ channel from mammalian skeletal muscle is composed of only 260 and 38 kD polypeptides. The Na^+ channel from eel electroplax contains only a single 260 kD polypeptide. Reconstitution of the eel Na^+ channel into planar lipid bilayers and expression of the RNA encoding the rat brain α-subunit have verified that the 260 kD polypeptide is the one containing all of the conduction, toxin binding, and regulatory sites.

Investigations concerning the role of protein kinase A- (PKA) mediated phosphorylation on voltage-sensitive Na^+ channel function have led to conflicting conclusions. Costa and Catterall [9] reported substantially reduced Na^+ influx into rat brain synaptosomal membrane vesicles following phosphorylation. In agreement with these early tracer flux studies, it was shown that phosphorylation by PKA reduced peak Na^+ currents by 50% in

Fig. 1. *Possible routes of modulation of ion channels.* For each cartoon, a schematized channel and, immediately below, an idealized current record is shown. Only increases in channel activity are depicted: (**A**) increases in single channel current (Δi); (**B**) increases in single channel open probability (P_o); (**C**) increases in functional channel number (ΔN); and (**D**) increases in the total number of available channels (ΔN_T). Adapted from [7, 8].

whole cell and membrane patches excised from cultured rat embryonic brain neurons or from Chinese hamster ovary (CHO) cells expressing type IIA Na^+ channels [10, 45]. Because neuronal Na^+ channels in primary culture are constituitively phosphorylated in their basal state [46], Li et al [10] transfected CHO cells with a plasmid encoding a dominant negative mutant form of the regulatory subunit of PKA that cannot bind ATP, but which still retains the ability to bind the catalytic subunit. Na^+ currents measured in whole cell patches from the resulting functional catalytic subunit deficient cells were inhibited by 80% upon phosphorylation with PKA and ATP. These results indicate that basal phosphorylation of these Na^+ channels results in a down-regulation of their activity. The specificity of these effects was additionally substantiated by the observation that these effects could be blocked by a specific peptide inhibitor of PKA and reversed by phosphoprotein phosphatases.

One limitation of whole cell or excised membrane patch clamping is the so-called "run-down" phenomenon, usually attributed to washout of intracellular components that may participate in channel modulation. Expression of ion channels in oocytes with subsequent measurement by two electrode voltage clamp allows more stable recording and easy intracellular injections without the necessity of dialyzing the cell's interior. Using this technique, Gershon et al [12] found an inhibition of voltage-dependent Na^+ channel activity (rat brain type IIA α-subunit) expressed in *Xenopus* oocytes after the intracellular injection of cAMP or catalytic subunit of PKA, or after forskolin was applied extracellularly. The inhibition by PKA showed little voltage dependence, and the voltage dependence of the processes of activation and inactivation was not altered by cAMP. Comparable effects were elicited by forskolin and cAMP on Na^+ channels expressed in *Xenopus* oocytes after the injection of total RNA from rat brain. Similarly, exposure of inside-out membrane patches excised from cells of the eel electroplax to the purified catalytic subunit of PKA in the presence of MgATP produced a rapid, sustained reduction of Na^+ current amplitude with a hyperpolarizing shift in the current/voltage relation by 10 to 12 mV [13]. The addition of PKA or MgATP alone were without effect.

In contrast to these results, Smith and Goldin [11] reported an enhancement rather than a depression of rat brain Na^+ channel currents expressed in *Xenopus* oocytes following isoproterenol stimulation of the α2-adrenergic receptor, or following treatment with dibutyryl cAMP. Sodium current amplitude in these experiments was increased without affecting the voltage dependence of channel activation or inactivation. These increases in channel activity were also phosphatase sensitive and could be blocked completely by preinjection of protein kinase inhibitor, indicating that phosphorylation was directly involved in modulating Na^+ channel activity. The PKA enhancement of activity was specific for the rat brain IIA sodium channel, because currents expressed from the rat muscle μI sodium channel were not enhanced by the same procedures. The authors proposed that Na^+ currents were stimulated either because phosphorylation promoted the membrane insertion of new functional channels, or that phosphorylation decreased the degree of inactivation. The authors argue that the most likely explanation for their results is altered channel gating, resulting from direct phosphorylation of the channel protein, although no experimental evidence was presented. Moreover, no explanation was offered (nor is any apparent) for the discrepancy between their results and those of Gershon et al [12].

Using intact rabbit cardiac myocytes, Matsuda et al [16] showed that maximum whole-cell sodium currents were enhanced by isoproterenol. This enhancement of current was potential dependent (that is, significant stimulation of Na^+ current was observed only when the membrane potential was held at depolarizing values) and was associated with an increase in the rate of Na^+ channel inactivation by some 30%. Application of forskolin or dibutyryl cAMP produced similar effects. In order to confirm these whole cell measurements, inside-out patch clamp experiments were performed. Addition of the catalytic subunit of protein kinase A plus ATP to the solution bathing the cytoplasmic face of the patch increased Na^+ channel activity as well as the rate of channel inactivation. However, the authors contended that the whole-cell current stimulation was not entirely due to PKA-induced phosphorylation because isoproterenol still increased Na^+ currents even in the presence of a protein kinase A inhibitor. Moreover, application of either GTP γS or Gsα to the cytoplasmic surface of inside-out patches increased maximal ensemble Na^+ channel current by 36%, but did not alter the inactivation time constant. They proposed that these channels were directly stimulated by isoproterenol through direct activation of the G-protein, Gsα, and indirectly by cAMP-dependent phosphorylation.

Thus PKA-mediated phosphorylation of voltage gated Na^+ channels can result in either an increase or decrease in channel activity, depending upon the system. Unfortunately, there is as yet no consensus as to the causes underlying these contrary observations. If G-proteins are involved in modulating Na^+ channel function, perhaps some of these discrepancies can be accounted for by a differential influence of G-proteins versus phosphorylation.

Protein kinase C

Activation of PKC by phorbol esters or diacylglycerol reduces the whole-cell sodium current in rat brain neurons [17], neuroblastoma cells [47], and in rat brain type IIA sodium channel α-subunits expressed in CHO cells [17, 48]. The reduction of peak current was accompanied by a substantial slowing of channel inactivation, an increased channel open time, and an increased probability of channel reopening due to depolarization. The voltage dependence of activation and inactivation and the single channel conductance were not significantly altered. These effects were specific for PKC because they could be blocked by specific peptide inhibitors of PKC, and could be reproduced by direct application of PKC to the cytoplasmic surface of excised inside-out membrane patches.

Similar observations concerning the inhibitory effects of PKC on activity of the chick brain Na^+ channel were made in *Xenopus* oocyte expression studies [23, 49, 50]. However, only small

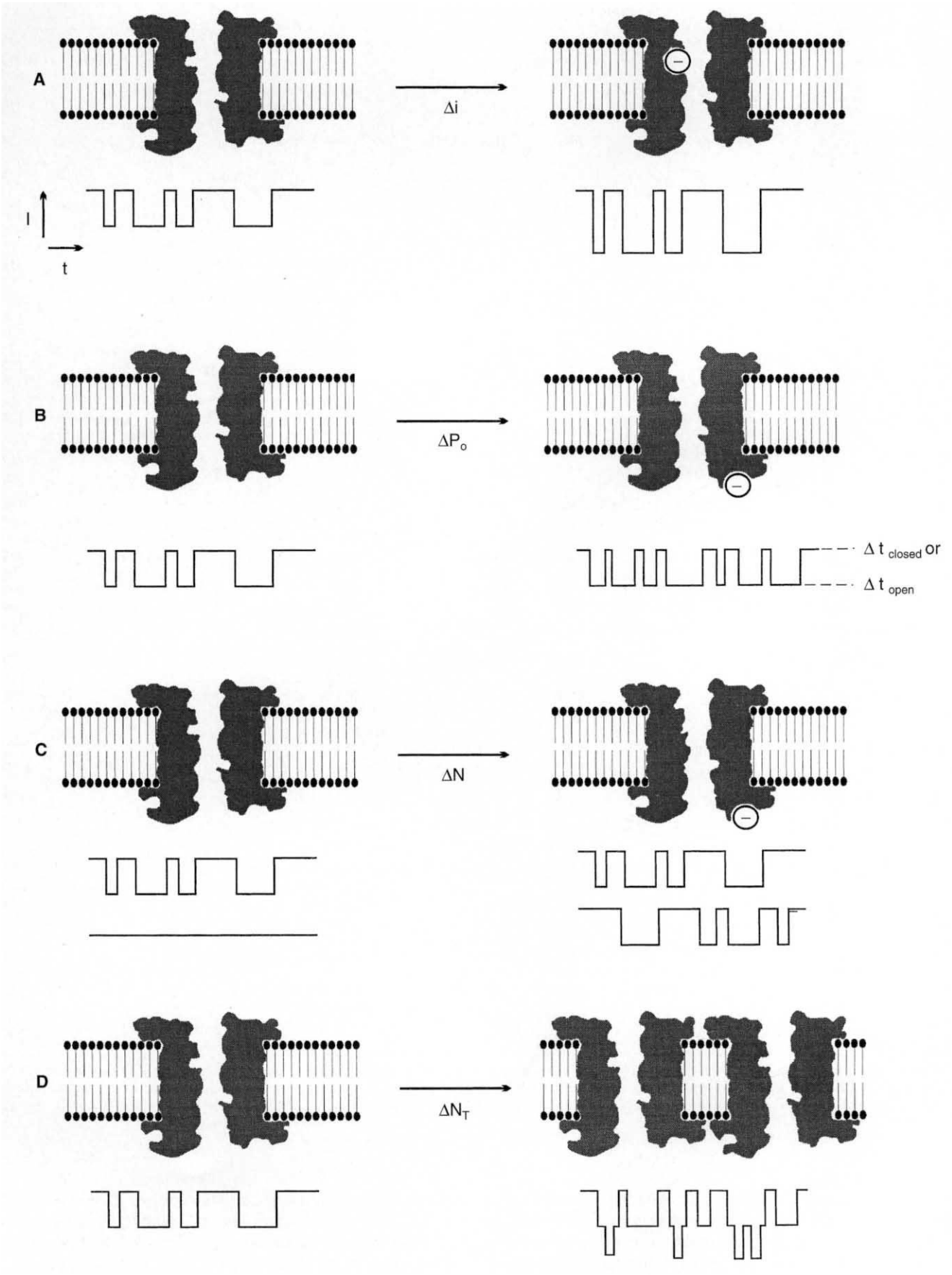
A
Δi
I
t
B
ΔP_o
Δt_{closed} or
Δt_{open}
C
ΔN
D
ΔN_T

Table 1. Properties of phosphorylated ion channels

Channel	Source	Kinase	Modulating agent	Experimental environment	Effect	Reference
Na Ch	Rat brain	PKA	PKA-cs	Synaptosomes	Na^{22} influx ↓	[9]
Na Ch Type IIA	Rat brain	PKA	PKA-cs	Inside-out patch	^{I}Na ↓ P_o ↓	[10]
Na Ch Type IIA	Rat brain	PKA	IISO cAMP	Two electrode voltage clamp	i ↑ I_{Na} ↑	[11]
Na Ch Type IIA	Rat brain	PKA	PKA-cs cAMP	Two electrode voltage clamp	I_{Na} ↓	[12]
Na Ch Type	Electrophorus Electricus Electroplax	PKA	PKA-cs	Inside-out patch	I_{Na} ↓ I/V → shift	[13]
Na Ch	Land snail neurons	PKA	PKA-cs	Two electrode voltage clamp	I_{Na} ↑	[14]
Na Ch	Rat cardiomyocytes	PKA	ISO	Whole cell patch clamp	I_{Na} ↓	[15]
Na Ch	Rabbit cardiomyocytes	PKA	ISO Forskolin	Whole-cell patch clamp, Inside-out patch	I_{Na} ↑ I/V shift Inactivation rate ↑	[16]
Nc Ch Type IIA	Rat brain	PKC	DAG OAG	Whole cell patch clamp	I_{Na} ↓	[17, 18]
Ca Ch P-type	Rat cerebellum	PKA	Isoprenaline cAMP	Two electrode voltage clamp	I_{Ca} ↑	[19]
Ca Ch L-type	Chick cardiomyocytes	PKA	ISO	Whole cell patch clamp	I_{Ca} ↑ V-activation shift P_o ↑ ; t_o ↑	[20]
Ca Ch L-type	Rabbit cardiomyocytes	PKA	PKA-cs	Inside-out patch	I_{Ca} ↑	[21]
Ca Ch L-type	Rabbit cardiomyocytes	PKA	PKA-cs	Whole cell patch clamp	I_{Ca} ↑ V-activation shift	[22, 23]
Ca Ch DHP-receptor	Rabbit skeletal muscle	PKA	PKA-cs	PLB	P_o ↑ V-activation shift Inactivation rate ↓	[24]
Ca Ch DHP-receptor	Chick skeletal muscle	PKA	PKA-cs	Proteoliposomes	Ca influx ↑	[25]
Ca Ch DHP-receptor	Rabbit skeletal muscle	PKA	PKA-cs	Proteoliposomes	Ca influx ↑	[26]
Ca Ch DHP-receptor	Rabbit skeletal muscle	PKC	PKC	PLB	P_o ↑	[27]
Ca Ch DHP-receptor	Rabbit skeletal muscle	PKC	PKC	Proteoliposomes	Ca influx ↑	[26]
Ca Ch RY-receptor	Canine cardiac muscle	CaM-K	CaM-K	PLB	I_{Ca} ↑ t_o ↑	[28]
K Ch delayed rectifier	Squid axon	PKA	PKA-cs	Cell-attached patch Outside-out patch	I_K ↑ I/V shift	[29–33]
K Ch Muscarinic gated	Rat cardiac muscle	PKA	A-Ch	Whole cell patch clamp Cell attached patch Inside-out patch	I_K ↑ t_o ↑ ↑ t_c ↑	[34, 35]
K Ch Ca^{2+}-activated	Land snail neurons	PKA	PKA-cs	Inside-out patch PLB	I_K ↑ Np/V shift P_o ↑	[36]
K Ch Ca^{2+}-activated	Canine color smooth muscle	PKA	PKA-cs	Inside-out patch	I_K ↑ P_o ↑ V-activation ← shift	[37]
K Ch Ca^{2+}-activated	Rabbit trachea smooth muscle	PKA	Iosprenaline PKA	Cell attached patch Inside-out patch	P_o ↑ i ↑	[38]
K Ch ATP activated	Porcine artery smooth muscle	PKA	ISO Forskolin PKA-cs	Cell attached patch Inside-out patch	I_K ↑ P_o ↑	[39]
K Ch N-type	T-lymphocytes	PKA	cAMP	Whole cell patch clamp	I_K ↓	[40]
K Ch dendrotoxin binding	Rat brain	PKA	PKA-cs	PLB	Np ↑	[41]
K Ch	Mollusc photoreceptor	PKC	PKC	Two electrode voltage clamp	I_K ↑	[42]
K Ch N-type	T-lymphocytes	PKC	PKC	Whole cell patch clamp	I_K ↓	[40]
K Ch Ca^{2+}-activated	Land snail neurons	CaM-K	CaM-K	Whole cell patch clamp	I_K ↑	[43, 44]

Abbreviations are: PKA-cs, catalytic subunit of protein kinase A; P_o, channel open probability; i, single channel current; ISO, isoproterenol; NaCh, Na^+ channel; CaCh, Ca^{2+} channel; KCh, K^+ channel; t_o, single channel mean open time; t_c, single channel mean closed time; PLB, planar lipid bilayer; CaM-K, multifunctional Ca^{2+}/calmodulin-dependent protein kinase. Arrows are: ↑ up-regulation; ↓ down-regulation; → rightward shift; ← leftward shift.

differences in channel kinetics were observed. Modulation of expressed Na^+ channels by phorbol ester was reported to shift the voltage dependence of channel activation to more depolarizing voltages without any change in the time course of current inactivation or conductance [51]. The α subunit of the Na^+ channel is thus a substrate for PKC phosphorylation. Although the differences in the voltage dependence of the PKC effects on activation between these different studies is not apparent, it is

nonetheless clear that PKC can modulate Na^+ channel function in a predictable fashion, that is, a depression of macroscopic current flow presumably by decreasing channel open probability as well as slowing the time course of channel inactivation [17].

Voltage-sensitive Ca^{2+} channels

Protein kinase A

Early proposals for specific mechanisms that regulate Ca^{2+} channels by PKA induced phosphorylation were based on the idea that gating of these channels is both voltage and phosphorylation dependent [52, 53]. These authors proposed that phosphorylation by PKA plays a permissive role making a given channel available for opening in response to membrane depolarization. Because phosphorylation by protein kinase can be reversed by dephosphorylation through protein phosphatase, a steady-state balance between phosphorylated (that is, functionally available) and dephosphorylated (that is, functionally unavailable) pools may be achieved for a given physiological condition.

Hymel et al [54] examined changes in properties of single purified rat skeletal muscle 1,4-dihydropyridine (DHP) receptor Ca^{2+} channels incorporated into planar lipid bilayer membranes before and after phosphorylation. These channels are comprised of at least four associated subunits. cAMP-dependent phosphorylation occurred exclusively on the α_1 polypeptide (170 kD), with a stoichiometry of incorporation of 0.7 mole ^{32}P/mole subunit. Nonphosphorylated channels had a single channel conductance of 0.9 pS and displayed no voltage-dependent gating. After phosphorylation, both voltage-dependent gating and higher current levels were observed. Stabilized current levels post-phosphorylation assumed values of even multiples of 0.9 pS, predominately 7.5 and 15 pS, as well as multiples of these values even up to 60 pS. Because of this multiplication of conductances, the authors deduced that individual channel complexes became functionally coupled, that is, they displayed synchronous openings and closings. One possibility for this behavior may be the physical association of channel molecules within the membrane post phosphorylation. Yue, Herzig and Marban [55] inferred that the basis for the increase in cardiac Ca^{2+} current observed following β-adrenergic receptor stimulation was cAMP-dependent phosphorylation. These authors showed that L-type Ca^{2+} channel gating was shifted to a mode characterized by long openings subsequent to exposure to isoproterenol or 8-Br-cAMP from a mode displaying brief repetitive openings.

To examine functional consequences of PKA-mediated phosphorylation, Mundina-Weilenmann et al [25] reconstituted purified dihydropyridine-sensitive Ca^{2+} channels from chick skeletal muscle into liposomes containing the entrapped fluorescent Ca^{2+}-indicator, fluo-3, and measured dihydropyridine- and depolarization-sensitive Ca^{2+} influx. These investigators found that only the α_1 channel subunit was phosphorylated in a time- and concentration-dependent fashion after isoproterenol treatment, and was specific to cAMP elevation because neither raising $[Ca^{2+}]_i$ with A23187 nor activating PKC could induce $[^{32}P]$ incorporation into this protein. Pretreatment of myocytes with isoproterenol prior to channel purification resulted in an increased Ca^{2+} influx that could be mimicked by PKA induced phosphorylation of the channels *in vitro*. In subsequent planar lipid bilayer studies, addition of the catalytic subunit of PKA to the presumptive intracellular face of the dihydropyridine-sensitive Ca^{2+} channel increased channel open probability at all voltages above -50 mV, shifted the voltage-dependence of activation leftward by 7 mV, decreased the rate of inactivation twofold, and increased the frequency with which the channel was observed in the bilayer [24].

PKA-induced phosphorylation also restores the activity of L-type calcium channels after rundown in inside-out patches from rabbit cardiac cells [21]. Addition of MgATP alone caused a small transient increase in channel activity in some patches, but in combination with PKA, the rundown of Ca^{2+} channel activity was reversed. However, single-channel conductance was reduced after exposure of the excised patch to MgATP and PKA. Magnesium itself was responsible and also produced a shift towards more negative potentials in the channel activation kinetics. This voltage shift of activation was probably due to direct screening of inside fixed negative charges in the vicinity of the channel by Mg^{2+}. Pre-exposure of the excised patch to protein kinase inhibitor could prevent the recovery of activity by MgATP and PKA. Okadaic acid, a protein phosphatase inhibitor, greatly diminished the rate of channel rundown.

The study of the gating currents of cardiac Ca^{2+} channels has revealed that isoproterenol or elevation of cAMP speeds the kinetics of this process via a presumed phosphorylation of the channel [20]. Elevation of intracellular cAMP produced a rapid increase in the number of channels available for voltage activation in cardiac muscle and vascular smooth muscle due to the increased probability of slow channel opening as well as an increased mean open time of the channel. Sculptoreanu et al [22, 23], in whole-cell patch clamp experiments, likewise measured an increase in skeletal muscle L-type cardiac Ca^{2+}-channel activity following prepulses of high frequency depolarizations. This increase in channel activity was due to a shift in the voltage dependence of activation to more negative membrane potentials, and directly resulted from cAMP-dependent protein phosphorylation because inclusion of a specific peptide inhibitor of PKA in the patch pipette prevented the effects of conditioning prepulses. Whole-cell current measurements in *Xenopus* oocytes injected with rat cerebellum mRNA, expressing a voltage-dependent P-type Ca^{2+} channel (that is, one sensitive to *Agelenopsis aperta* venom but not to dihydropyridines and ω-conotoxin), revealed increased macroscopic Ca^{2+} channel activity and slowing of channel inactivation after direct intraoocyte injection of cAMP [19]. Direct application of PKA and MgATP to one side of a planar bilayer to which porcine ventricular sarcolemmal membranes containing L-type Ca^{2+} channels had been fused, also increased basal channel open probability and delayed their rundown [56].

Murphy et al [57] studied the role of disulfide, sulfhydryl, and phosphate groups in dihydropyridine binding to the L-type Ca^{2+} channel in rabbit heart sarcolemma and skeletal muscle transverse-tubules. Phosphorylation induced by endogenous protein kinase or cAMP-dependent protein kinase was without effect on $[^3H]$ nitrendipine binding. On the other hand, phosphorylation of cardiac ryanodine receptor-Ca^{2+} channels by PKA increased maximal $[^3H]$ ryanodine binding to cardiac microsomes by 22% [58]. PKA phosphorylation of the skeletal muscle ryanodine receptor has also been demonstrated [59]. However, similr to voltage-dependent Na^+ channels, the functional significance of PKA-mediated ryanodine receptor phosphorylation is controversial [60].

In summary, phosphorylation of voltage-dependent Ca^{2+} channels by cAMP-dependent protein kinase results in an increase in channel mean open probability. In some cases, the voltage dependence of the activation process shifts to more hyperpolarizing potentials, thereby reducing the threshold for channel activity, and the deactivation of the channels is prolonged by phosphorylation. In any case, these phosphorylation-induced changes in channel activity underlie the physiological effects of β-adrenergic receptor agonists' action in both cardiac and skeletal muscle, namely, an increased contractile force and voltage-dependent potentiation. Sculptoreanu et al [23] argue that this voltage-dependent phosphorylation during the cardiac action potential, and dephosphorylation during the repolarization phase, permit a finely tuned beat-to-beat regulatory mechanism.

Protein kinase C

PKC induced phosphorylation of dihydropyridine-sensitive Ca^{2+} channels from skeletal muscle reconstituted into liposomes resulted in an activation of the channels, as assessed by increases in both the rate and extent of Ca^{2+} influx [26]. However, phosphorylation of these channels by either the multi-functional Ca^{2+}/calmodulin dependent protein kinase or by endogenous kinase activity in T-tubule membranes was without functional consequence. All of these kinases preferentially phosphorylated the α_1 subunit, although the 52 kD β subunit was also phosphorylated by PKC, but to a much lesser extent.

Ma et al [27] reported the effects of PKC induced phosphorylation of dihydropyridine-sensitive T tubule membrane Ca^{2+} channel fused with planar bilayers. The non-phosphorylated Ca^{2+} channels exhibited two (a shorter and a longer) open states. When purified PKC was added to the "intracellular" side of the bilayer, the mean open time of the longer open state increased threefold, and the proportion of long versus short openings increased by a factor of five. Unlike PKA-mediated phosphorylation, PKC did not have any significant effect on the voltage dependence of activation. Murphy et al [57] report that promotion of Ca^{2+}/calmodulin-dependent phosphorylation by exogenous calmodulin in cardiac sarcolemmal and skeletal muscle membranes increased maximal [^{3}H] nitrendipine binding up to 20% with no alteration in its apparent affinity. On the other hand, Ca^{2+}/calmodulin-dependent protein kinase induced phosphorylation did not alter the sensitivity of these L-type Ca^{2+} channels toward the dihydropyridines [26].

Reconstitution of the ryanodine-Ca^{2+} release channel of rabbit skeletal muscle sarcoplasmic reticulum into planar bilayers showed that phosphorylation by PKC enhances channel open probability by increasing the sensitivity towards micromolar Ca^{2+} and ATP [61]. Takasago et al [62] determined that [^{3}H] ryanodine binding to cardiac microsomes was increased by PKC by some 15%. Calmodulin alone and calmodulin plus ATP in their experiments decreased [^{3}H] ryanodine binding by 38 and 53%, respectively. Witcher et al [18] showed that Ca^{2+}/calmodulin dependent protein kinase phosphorylated the cardiac ryanodine receptor at only a single site, and that this modification activated the associated Ca^{2+} channel. In fact, phosphorylation by this kinase reversed the depression of P_o produced by calmodulin alone. There was no observed effect on single channel conductance.

Voltage-sensitive K^+ channels

Protein kinase A

Potassium channels are undoubtedly the most thoroughly studied type of ion channel. There are many different kinds of voltage-sensitive K^+ channel, and many of these have already been cloned [63]. These different K^+ channels can be classified primarily according to the way they gate, for example, in addition to voltage, some are activated by intracellular Ca^{2+}, while others are gated by G-proteins or ATP. The alkali metal selectivity of all K^+ channels is fairly uniform, although there are major differences in pharmacological and toxin sensitivities. Like the other classes of ion channel, protein phosphorylation appears to play an important role in modulating K^+ channel activity. There are many examples of K^+ channels in epithelia including the renal nephron [64, 65]. However, relatively few studies exists in which regulation of epithelial K^+ channels by phosphorylation have been examined [66–69].

Phosphorylation of K^+ channels by PKA + ATP invariably results in an enhanced activity, primarily by increasing P_o. Ewald, Williams and Levitan [36] demonstrated an increased activity of individual snail neuron Ca^{2+}-dependent K^+ channels studied in membrane patches and in planar lipid bilayers, resulting directly because of cAMP-dependent protein phosphorylation. PKA has also been reported to activate Ca^{2+}-dependent K^+ channels in cultured aortic smooth muscle cells. Kume et al [38] showed that the open probability of Ca^{2+}-dependent K^+-channels in isolated rabbit tracheal myocytes was reversibly increased by either extracellular application of isoprenaline or intracellular application of PKA, using the patch-clamp technique. Carl et al [37] observed more than a twofold increase of open probability of Ca^{2+}-activated K^+ channels when the catalytic subunit of PKA was applied to inside-out membrane patches excised from smooth muscle cells of the canine proximal colon. The described effect was suggested to be due to a shift in voltage-dependent activation to more negative potentials.

Extracellular application of isoproterenol or forskolin activated ATP-sensitive K^+ channels in cell-attached patches of cultured smooth muscle cells of the porcine coronary artery [39]. Direct application of PKA to the cytoplasmic surface activated these channels in inside-out patch clamp experiments. Interestingly, this activation was not blocked by phorbol 12-myristate 13-acetate, an activator of protein kinase C.

Protein kinase A has been shown to modulate the whole-cell K^+ current through the n type K^+ channel present in human (Jurkat) T lymphocytes. Unlike Ca^{2+}-activated K^+ channels, phosphorylation, by PKA decreased the amplitude of this current [70]. On the other hand, Kim [34] reported an increase of muscarinic-activated K^+ channel activity produced by isoproterenol or cyclic AMP-dependent protein kinase. This activation was associated with a four- to fivefold and twofold increase in the mean open and closed time durations, respectively.

Bezanilla and coworkers, in an elegant series of studies, examined ATP-mediated phosphorylation of delayed rectifier K^+ channels of squid axons in detail, implicating induced electrostatic interactions in channel modulation [29–33, 71]. These investigators found that cAMP-dependent phosphorylation of the K^+ channels of squid axons potentiates the magnitude of potassium conductance and simultaneously slows down its activation kinetics. The onset of K^+ current presents more delay due to local

hyperpolarization of the channel: ATP induced a 10 to 20 mV shift in both activation and inactivation parameters towards more depolarized potentials. Since (i) these effects could be reproduced by Mg-ATP alone, (ii) Mg^{2+} was an obligatory requirement [71], and (iii) other nucleotides like GTP and ITP and nonhydrolyzable ATP analogs were ineffective [29], the authors concluded that the ATP effect most likely was produced via a phosphorylation reaction. This conclusion was justified because the significant increase in macroscopic K^+ current seen in the cut open axon prepration upon photo-release of caged ATP was mimicked at the single channel level by including ATP and PKA within a patch pipette. In this procedure, the inside surface of a cut open axon was patched and, upon phosphorylation, a significant increase in K^+ channel open probability was observed. It also was suggested that the additional negative charge carried by the phosphate group was most probably responsible for the differences in current amplitude with and without ATP due to its coulombic interaction with the voltage sensor of the channel. Using different concentrations of intracellular Mg^{2+}, Perozo and Bezanilla [30] determined the density of surface charges in the vicinity of the K^+ channel voltage sensor before and after phosphorylation. Values for surface charge density near the cytoplasmic side of the channel were between 30 to 40 $e^-/\mu m^2$ for the control (that is, zero ATP) condition, and between 64 to 66 $e^-/\mu m^2$ under phosphorylating conditions.

These authors also developed two methods to shift the population of channels toward a dephosphorylated state. One method consisted of predialyzing a whole axon with solutions containing zero ATP while recording the currents under axial-wire voltage clamp. A piece of axon was then removed, and single channel currents were recorded from the cut open axon. In the second method, the axon was cut open in a solution that did not contain Ca^{2+} or Cl^- in order to allow the access of endogenous phosphatase to the membrane while ATP diffused away, before removing the axoplasm and forming a membrane patch. When a dephosphorylated state was achieved, the steady-state open probability of a 40 pS K^+-channel was very low (< 0.0002), and the channel openings appeared as a series of infrequent, short-duration events. The channel open probability versus voltage curve with a reversed K^+ gradient shifted toward more positive potentials after phosphorylation, and the frequency of channel openings decreased at most voltages. The mean open times under this condition showed no voltage dependence and were not affected by phosphorylation. PKA-mediated phosphorylation was also found to reduce the overall inactivation of the channel at different holding potentials, shifting the inactivation curve toward more positive potentials. These results were interpreted quantitatively with a kinetic model in which the authors simulated the voltage-dependent kinetics with a surface potential term included into the voltage dependence of the steady state activation and inactivation rate constants [33].

Blumenthal and Kaczmarek [72] studied the modulation of a slowly activating voltage-dependent potassium channel (minK) expressed in *Xenopus* oocytes by altering intracellular cAMP levels. They found that the current amplitude increased dramatically by treatments that raise cAMP levels, and decreased by agents that lower cAMP levels. These effects could be prevented by preinjection of the cAMP-dependent protein kinase inhibitor. No changes in the voltage dependence or current kinetics were observed in these experiments. Importantly, the oocyte membrane capacitance increased and decreased in parallel with the observed changes in K^+ current. Mutations that eliminate a potential phosphorylation site on the minK channel protein did not block the effects of activating the kinase. The authors explained their results by a mechanism in which channel proteins can be inserted into and removed from the membrane in response to changes in kinase activity.

Protein kinase C

Protein kinase C can modulate the whole-cell K^+ currents through an n-type K^+ channel present in human (Jurkat) T lymphocytes. Down-regulation of K^+ current was observed following activation of PKC by 12-O-tetradecanoylphorbol-13-acetate [40]. The major 65 kD polypeptide comprising this lymphocyte n-type K^+ channel has been shown in metabolic labeling/immunoprecipitation experiments to be phosphorylated by PKC [73]. Phorbol ester activation of PKC also reduced the open probability and conductance of a small conductance, apically located K^+ channel in cell-attached patches of rat cortical collecting tubule [65]. Direct exposure of the cytoplasmic surface of inside-out patches of the same membrane to PKC also dramatically reduced channel activity. This inhibitory effect of PKC was enhanced by Ca^{2+}. Similarly, potassium currents produced by the Kv 1.3 mRNA (which encodes this n-type K^+ channel) heterologously expressed in oocytes was suppressed by PKC [74], as were smooth muscle ATP-sensitive K^+ channels [75]. In contrast, however, K^+ channels involved in volume regulation of a human parotid adenocarcinoma cell line (HSY) appear to be up-regulated by PKC [76]. Varnum et al [77] reported that PKC stimulates the activity of the slow component of the delayed rectifier K^+ conductance in guinea pig myocytes. The clone that encodes these $minK^+$ channels was expressed in oocytes and its modulation by activators or inhibitors of PKC examined. The stimulatory effects of PKC on this slow K^+ current were only seen in guinea pig myocytes (PKC inhibits this source current in mouse cardiac cells and rat kidney cells [78]. Busch et al [79] mutagenized the serine 103 residue in the rat kidney clone to alanine (S103A) and found that the current decrease produced by PKC phosphorylation was eliminated. The analogous residue in the guinea pig clone is an asparagine. If this asparagine was converted to a serine, PKC depressed rather than stimulated the expressed currents, as predicted [77]. The activity of a Ca^{2+}-activated K^+ channel in guinea pig myocytes was unaffected by manipulations of the activity of PKC [80].

Ca^{2+}-mediated, delayed outward K^+ current in neurons of the land snail was shown to be reduced by the calmodulin inhibitors N-(6-aminohexyl)-1-naphthalene-sulfonamide and N-(6-aminohexyl)-5-chloro-naphthalene sulfonamide [43, 44]. These antagonists caused a slight membrane depolarization and an increase in impulse discharge frequency, whereas the amplitude of the action potentials after hyperpolarization decreased. Intracellular injection of kinase II, a Ca^{2+}/calmodulin-dependent protein kinase, resulted in an increase in both the K^+ current and membrane after hyperpolarization. Weaker effects were produced when a catalytic subunit of cAMP-dependent protein kinase was intracellularly injected.

Thus, PKA phosphorylation of K^+ channels in general results in an increase in single channel open probability. As for the other kinase mediators of channel activity, the influence of PKC phosphorylation on K^+ channel function is either stimulatory or

inhibitory depending upon the type of K^+ channel and the cell type. Interestingly, phosphorylation by Ca^{2+}/calmodulin dependent protein kinase appears to activate K^+ channels.

Amiloride-sensitive renal epithelial Na^+ channels

Protein kinase A, protein kinase C, and G-proteins

The original hypothesis that epithelial Na^+ channels can be regulated by phosphorylation was inspired by the well-known observation that anti-diuretic hormone (ADH; vasopressin) increased net Na^+ reabsorption across epithelia through a cAMP-dependent process [81]. As indicated earlier, the possibilities for the molecular mechanisms underlying regulation of ion channels are numerous, but can be broadly classified into two not necessarily mutually exclusive categories: direct covalent modification of the channel protein leading to changes in the biophysical properties of individual channels, and/or changes in the number of functional channels that are expressed in the plasma membrane. This second mechanism can result by the activation or inhibition of resident channels, or by the incorporation or retrieval of channels into or out of the membrane. Needless to say, evidence for both of these mechanisms exists for cAMP-dependent regulation of epithelial Na^+ channels.

Our laboratory has purified to homogeneity and reconstituted functional bovine renal papillary collecting tubule Na^+ channels [82]. The native channel protein consists of six major polypeptide subunits, ranging in molecular mass from 300 to 40 kD. There also appears to be other proteins associated with the basic complex [83]. Highly specific polyclonal antibodies against this renal Na^+ channel protein have also been generated. These antibodies have permitted experiments designed to test the hypothesis that this renal Na^+ channel complex can act as a substrate for cAMP-dependent protein kinase phosphorylation *in vivo* and *in vitro*. Moreover, the ability to reconstitute this purified protein into planar lipid bilayer membranes allowed us to evaluate the functional consequences of phosphorylation on single channel activity.

PKA specifically phosphorylates only the 300 kD polypeptide of this heteromeric Na^+ channel complex, thereby increasing channel activity [84]. Addition of the catalytic subunit of PKA plus ATP to one side of a planar bilayer membrane containing renal Na^+ channels increased channel open probability but left single channel conductance unaffected. Similar observations were reported for amiloride-sensitive Na^+ channels in renal A6 cells in inside-out and cell-attached membrane patch-clamp experiments [85]. Figure 2 (left side) depicts representative current records for renal Na^+ channels exposed to PKA and ATP at two different holding potentials ($\pm$ 40 mV). The associated amplitude versus dwell time scatter plots display a notable shift in the channel population after phosphorylation (not shown). Non-phosphorylated channels were mostly closed. In this experiment, the unitary single channel current was 2 pA; one channel is apparent, having an average open dwell time of 5 to 10 ms under control conditions. Exposure to PKA and ATP shifts the channels to a more open configuration with a longer open dwell time. Four active channels are seen in the same membrane following PKA-phosphorylation, and the open channel probability increased from 0.01–0.02 to 0.05 (at +40 mV) and 0.69 (at −40 mV). It is apparent that this shift was due to an increased channel open time with no change in unitary conductance. Clearly, the number of channels in the reconstituted bilayer system also increased following PKA plus ATP treatment. We interpret this increase in channel number to result from a shift in resident membrane channels' P_o from low to higher values, and probably not from a fusion of new channel containing vesicles because no proteoliposomes were present in the bath (Fig. 1B). Marunaka and Eaton [86] found that vasopressin pretreatment of A6 cells produced no significant effect on the ohmic nature of current flow through open Na^+ channels, similar to what was observed in the reconstituted system. These observations provide definitive evidence for the direct modulation of channel properties by PKA-mediated phosphorylation, as is the case for numerous voltage dependent channels. In addition, because phosphorylation of the channel protein results in increased Na^+ channel activity, models by which cAMP-induced increases in amiloride-sensitive Na^+ transport across epithelia resulting from insertion of new channels from a subapical membrane pool need not be invoked, but could still be involved in some tissues under certain circumstances.

Kleyman et al [34] provide experimental support for the channel recruitment hypothesis. These authors showed an increase in A6 apical surface iodination of Na^+ channel protein following stimulation of Na^+ transport with vasopressin or forskolin. They also report that pretreatment of A6 cells with brefeldin A, a disruptor of intracellular membrane protein traffic, partially inhibits the forskolin-induced increase in Na^+ transport. These results suggest that recruitment of fresh Na^+ channels from an intracellular pool may be involved in up-regulation of transepithelial Na^+ transport by elevation of intracellular cAMP. However, it should be mentioned that these results are at variance with those published by Oh et al [84]. In these experiments, A6 cells were metabolically labeled with [^{35}S] methionine/cysteine and apical membrane Na^+ channels immunoprecipitated. No intracellular Na^+ channels were isolated because the surface channels were previously biotinylated and separated from the total channel pool using streptavidin. The apical surface of two other groups of A6 cells were effectively stripped clean of any recognizable Na^+ channels by pretreatment with trypsin [58, 87]. Subsequent to trypsin proteolysis, the cells were or were not treated with vasopressin. If new Na^+ channels were recruited to the apical membrane by ADH treatment, their presence in the trypsin-cleaned membrane would be detected by the above protocol. However, no new protein could be detected after ADH treatment. The reasons for the different results between these two groups of investigators are not known, but may relate to differences in culture conditions or in the techniques employed. There are other possibilities to consider as well. A recent study by Prat et al [88] demonstrated that the effects of PKA phosphorylation may be transduced through cytoskeletal proteins. They found that phosphorylation reduced the rate of actin polymerization and that the globular form of actin itself, by interacting directly with some channel component, could also contribute to macroscopic current enhancement. However, by analogy to other channels, the direct covalent modification of Na^+ channel protein with phosphate groups is likely to be the primary mechanism for modulation of function.

This contention is also supported by the change in the biophysical properties of epithelial Na^+ channels following PKA phosphorylation. First, the phosphorylation reaction converts the channel from one whose probability of being open (P_o) is independent of voltage to one whose P_o is dependent on applied potential. Phosphorylation does not alter the single open channel current versus voltage curve. This PKA phosphorylation-induced inward rectification would result in more Na^+ ions entering the

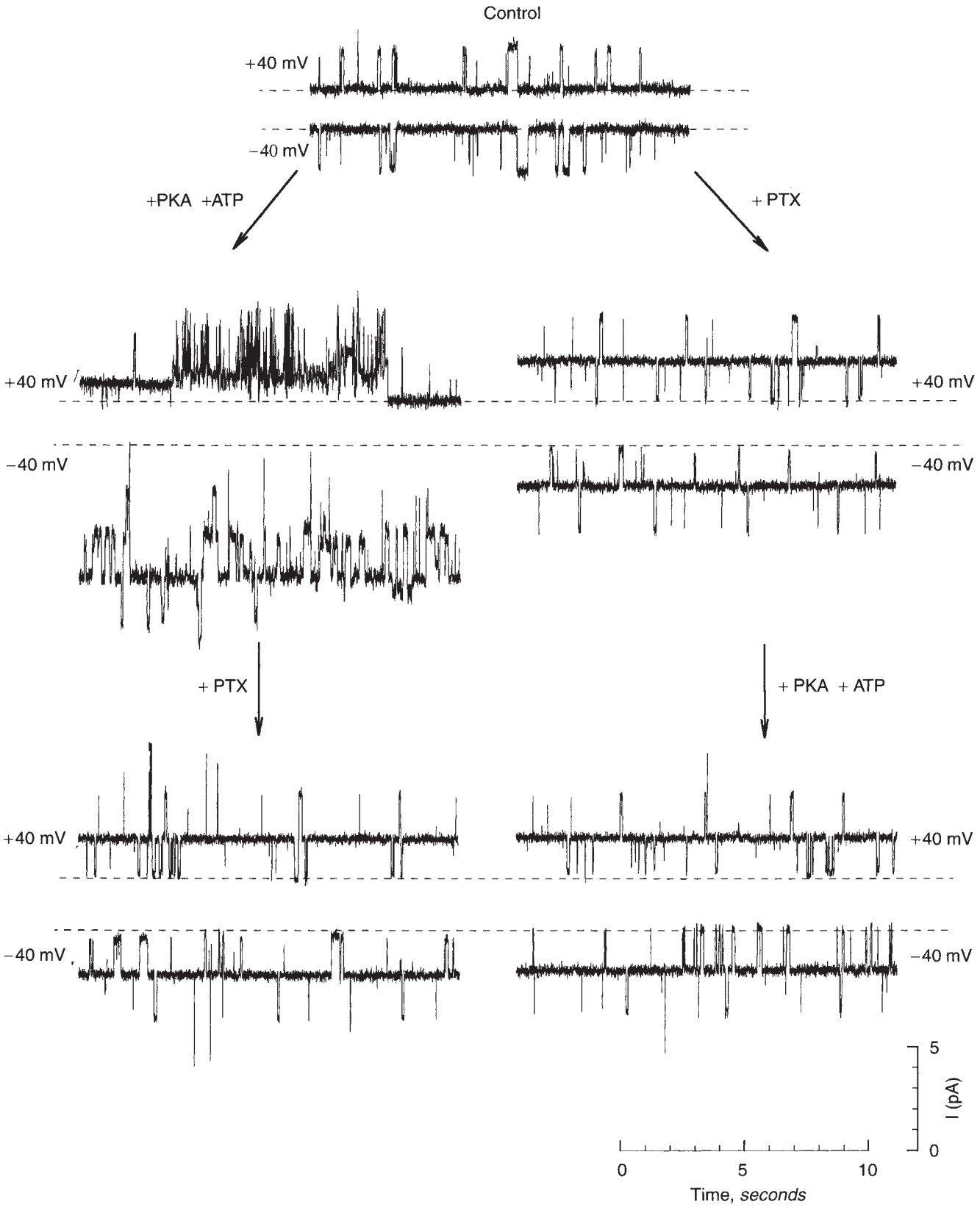

Fig. 2. *Effect of PKA-induced phosphorylation and pertussis toxin treatment on single renal Na^+ channel activity in planar lipid bilayers.* Each group shows current traces at ±40 mV, where the dashed line represents the zero current level. Current records were low-pass filtered at 100 Hz. Current traces before (Control) and after addition of purified catalytic subunit of PKA (1.85 ng/ml) and ATP (100 μM) to the "cytoplasmic" bathing solution, and after ADP ribosylation with pertussis toxin (100 ng/ml) are shown. Bilayers were bathed with symmetrical solutions of 100 mM NaCl, 10 mM MOPS (pH 7.2). In addition, the effects of PTX treatment and PKA-mediated phosphorylation after phosphorylation or ADP-ribosylation, respectively, are also shown.

cell at the negative physiological membrane potential, precisely what is observed in intact epithelia following elevation of intracellular cAMP levels. Second, phosphorylation results in the more frequent appearance of channel subconductance states, at least at negative applied voltages. Although these subconductance states were seen under non-phosphorylating conditions, their existence was much more apparent after PKA-mediated phosphorylation. Third, the fact that PKA-induced phosphorylation produces a change in Na^+ channel kinetic activity without altering single channel conductance indicates that the phosphorylated subunit most probably participates in gating rather than in conduction itself.

It is interesting to note that PKA-mediated phosphorylation induced a voltage-dependence to the large non-selective cation channels produced by the major intrinsic peptide from lens fiber (MIP28) incorporated into planar lipid bilayers [89]. Non-phosphorylated MIP channels were voltage-independent. However, previously phosphorylated MIP channels reconstituted into bilayer and channels that were phosphorylated after reconstitution demonstrated a similar and definite voltage dependence. These authors found that phosphorylation did not alter single channel conductance, but did increase closed channel dwell times as well as the rate of channel closing. Phosphorylated MIP channels did not rectify, but the steady-state currents in multi-channel membranes were reduced by phosphorylation at voltages greater than ± 30 mV.

PKC can also phosphorylate purified renal amiloride-sensitive Na^+ channels [84]. Interestingly, PKC phosphorylates two subunits of this Na^+ channel protein, namely, the 55 and 130 to 150 kD polypeptides, subunits entirely different from the 300 kD substrate of PKA-mediated phosphorylation. PKC treatment abolished single Na^+ channel activity, regardless of whether the channel was phosphorylated with PKA. These results are consistent with those of Yanase and Handler [28], Mohrmann et al [90], and Ling and Eaton [91], who demonstrated that activation of PKC by phorbol esters or diacylglycerol inhibited $^{22}Na^+$ uptake by A6 or LLC-PK_1 monolayers, or greatly diminished single Na^+ channel open probability.

Of relevance to the discussion of epithelial Na^+ channel function by covalent modification is a consideration of GTP-binding protein involvement. Signal transduction mediated by G-proteins is usually thought to occur secondarily via the generation of intracellular second messengers and, through activation of protein kinases, produce a physiological response [92]. However, G-proteins may directly couple to ion channels, or may operate through other membrane derived intermediates such as phospholipids. In some instances, G-proteins are required for ion channel function, such as for the muscarinic atrial K^+ channel, and in other cases, G-protein involvement is strictly modulatory, such as the voltage-sensitive, tetrodotoxin-inhibitable Na^+ channel. It appears that the α subunit of the different G-proteins is most often responsible for coupling to ion channels, but this is not always the case [93].

Ausiello et al [94] demonstrated that a GTP-binding protein, specifically $G\alpha_{i\text{-}3}$, associates with renal amiloride-sensitive Na^+ channels and directly participates in its regulation. In fact, the 40 kD subunit of the channel complex has been immunologically identified as $G\alpha_{i\text{-}3}$. Cantiello et al [95] provide strong evidence that this G-protein regulatory pathway operates through phospholipase-and lipoxygenase-generated phospholipid metabolites. The effects of pertussis toxin (PTX)-induced ADP-ribosylation of epithelial Na^+ channels on channel function have posed a conundrum. One group of investigators have shown that PTX inhibits epithelial Na^+ channels [94–96] while another report an increase in channel mean open time [97]. How can these disparate observations be reconciled? Based upon experiments conducted on purified renal Na^+ channels reconstituted in planar lipid bilayers [98], or on Na^+ currents in human lumphocytes studied using whole-cell patch clamp techniques [99, 100], it has been shown that the effects of ADP ribosylation depend upon the channel protein's phosphorylation history. PTX activated non-phosphorylated channels and inhibited phosphorylated ones. Of course, the disparity in results observed between the different groups in the patch-clamp experiments may be explained because different (at least kinetically) forms of amiloride-sensitive Na^+ channel may have been studied. It is not yet clear whether all types of Na^+ channel are different physiological manifestations of the same gene product or are truly different biochemical entities. But regardless, the most parsimonious explanation for these results is that the differential effect (that is, stimulation or inhibition) of PTX on renal Na^+ channels depends only on whether the channel protein phosphorylated or not. Figure 2 also summarizes single Na^+ channel bilayer experiments examining the role of G-proteins in modulating current flow through epithelial Na^+ channels. Figure 2 shows that PTX-induced ADP-ribosylation increased P_o from 0.01 to 0.02 in control (that is, non-phosphorylated) channels to 0.26. These observations are in agreement with those reported by O'Hara, Matsunaga and Eaton [97] who showed that in cell-attached patches of A6 cells, PTX increased mean open time and P_o of Na^+ channels. However, if the Na^+ channels were first phosphorylated with PKA and ATP, PTX decreased channel activity by decreasing channel mean open time (and thus P_o) and the number of open channels in the bilayer. It is important to note that the effects of all of these covalent modifications were evident only from one side of the bilayer. Addition of these reagents to the opposite (that is, external surface) was without effect. Interestingly, PKA and ATP treatment after PTX resulted in no change in channel activity. PTX treatment after PKA-induced phosphorylation converted the channel back into a non-rectifying state. Figure 3 summarizes mean current-voltage curves obtained under the above described conditions. It is clear that phosphorylation by PKA caused the Na^+ channel to become inwardly rectified, and that ADP-ribosylation does not. Interestingly, PKA-mediated phosphorylation subsequent to PTX treatment does not convert the channels to an inwardly rectified state. The kinetic characteristics of the channel with the blocked inhibitory G-protein (by PTX treatment) are virtually the same, whether the channel was or was not pre- or post-phosphorylated (Fig. 3). It is obvious that G-proteins play a dominant regulatory role, making distinguishable the phosphorylated and nonphosphorylated states of the channel. These results are consistent with the hypothesis that PTX affects epithelial Na^+ channels differently, depending upon their initial state of phosphorylation. In any case, the physiological regulation of these renal Na^+ channels is complex because, in addition to the pathways described here, epithelial Na^+ channel activity is also modulated by methylation [101, 102], arachidonic acid metabolites [95], and by interactions with the cytoskeleton [88, 83].

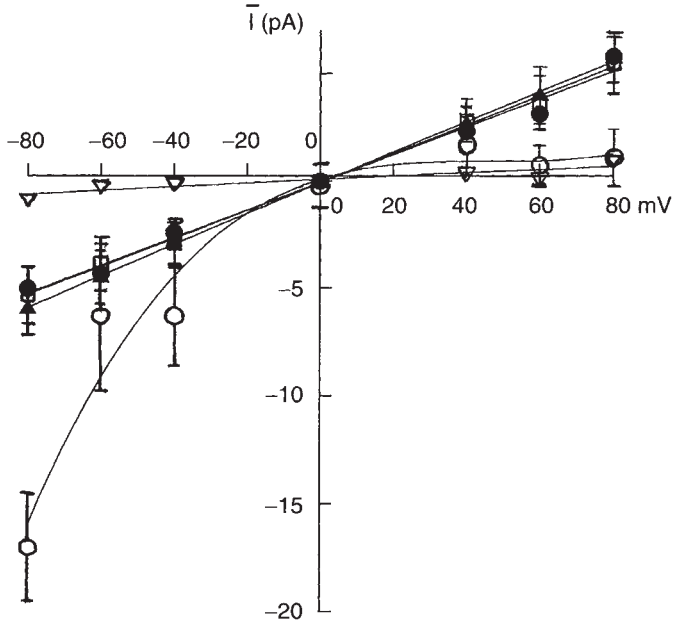

Fig. 3. *Mean current-voltage curves of control (▽), PKA-phosphorylated (○), PTX-treated (in the absence of PKA-phosphorylation) (△), PKA-phosphorylation after PTX treatment (□), and PTX-treated post phosphorylation (●) bovine renal Na^+ channels reconstituted into planar lipid bilayers.* Conditions were the same as in Figure 2, and the results are expressed as mean ± SEM of at least three separate experiments.

Summary

A fundamental property of ion channels is their ability to be modulated by intracellular second messenger systems acting via covalent modifications of the channel protein itself. One such important biochemical reaction is phosphorylation on serine, threonine, and tyrosine residues. Ion channels in the kidney are no exception. Moreover, many ion channels, including many amiloride-sensitive epithelial Na^+ channels, are subject to modulation by a multiplicity of inputs. For example, renal Na^+ channels are not gated by voltage in their unphosphorylated state. However, upon phosphorylation by PKA plus ATP, these channels become voltage-dependent as well as having their open probability increased. Phosphorylation by PKC inhibits channel activity regardless of whether the channel was previously phosphorylated by PKA. Likewise, Na^+ channel ADP-ribosylation by PTX overrides the actions of cAMP-dependent phosphorylation. Consistent with this idea is the fact that the phosphorylation sites for PKA and PKC and the ADP-ribosylation sites occur on different polypeptides comprising the channel complex. Epithelial Na^+ channel activity is also regulated by methylation, arachidonic acid metabolites, and by interactions with cytoskeletal components.

An exciting new age in understanding renal Na^+ channel function has begun. Canessa and collaborators [103, 104] and Lingueglia et al [105] have, for the first time, identified by expression cloning an amiloride-sensitive Na^+ channel from rat distal colon. The messenger RNA encoding the subunits comprising this channel are expressed in the distal tubule and cortical collecting tubule of the kidney (Rossier, unpublished observations). In addition, our laboratory has successfully cloned a mammalian homologue of this same channel from bovine renal papillary collecting ducts [106]. These molecular advances will allow investigations into the specific residues modified by phosphorylation, and what the relationship is between these residues and overall channel structure. The merging of electrophysiological, biochemical, and molecular biological approaches will undoubtedly access new information concerning epithelial Na^+ channel regulation in years to come.

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Reprint requests to D.J. Benos, Ph.D., Department of Physiology and Biophysics, The University of Alabama at Birmingham, BHSB 706 University Station, Birmingham, Alabama 35294-0005, USA. E-mail: Benos @PhyBiol.BHS.UAB.Edu

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