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Topical Review

Phosphorylation of Ion Channels

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Introduction

Ion Channels

All living cells maintain ionic gradients across their plasma membrane, and the movement of ions down these concentration gradients, through ion channels in the membrane, is of fundamental importance for many cellular functions. The central role of ion channels has, of course, been recognized for many years in the case of excitable cells such as neurons and muscle cells, but only recently has the development of powerful new techniques allowed an appreciation of the significance of ion channels in the functioning of many other cell types. The continued refinement of macroscopic physiological approaches, the emergence of single-channel recording techniques, the increasing sophistication of biochemical purification methods, the feasibility of studying purified channel functions in artificial membranes, and the dramatic advances in molecular genetic manipulation, have all contributed to our ability to study ion channels as molecular entities rather than simply as membrane processes. In short, these integral membrane proteins, which for many years were little more than an esoteric concept of biophysicists, have emerged from relative obscurity to become valued and highly visible members of the cell and molecular biology community.

It is also becoming evident that ion channels in biological membranes are dynamic entities. They of course act as pores through which ions can move down their electrochemical gradients, but more than that many of them are *regulatable* pores. Channels can exist in more than one functional state, and rapid transitions between these states can be regulated by the voltage across the membrane, or by the binding of a small molecule to a specific receptor which is intimately associated with the channel. Furthermore, it is now becoming clear that these rapid transitions are themselves subject to longer term modulation, which may last for seconds, minutes or even hours. This review will address a possible molecular mechanism for such long-term modulation of ion channel activity.

Modulation of Ion Channels

What molecular mechanisms might give rise to long lasting modulation of ion channels? Cyclic AMP (cAMP) and calcium ions are well established as intracellular second messengers in the regulation of carbohydrate metabolism, where they modulate the activities of certain enzymes by causing them to be phosphorylated (for a recent review *see* Cohen, 1982). It has long been suspected that they might play a similar role in the regulation of ion channels, and evidence from several laboratories has confirmed that this is indeed the case (Siegelbaum & Tsien, 1983). A large number of investigators have applied cAMP to cells while monitoring their membrane properties (Tsien, 1973; Reuter, 1974; Treistman & Levitan, 1976; Kaczmarek, Jennings & Strumwasser, 1978; Klein & Kandel, 1978; Deterre et al., 1981; Pellmar, 1981; Green & Gillette, 1983; Connor & Hockberger, 1984). In some cases membrane-permeable derivatives of cAMP have been applied in the extracellular medium, while in others cAMP itself or derivatives which are resistant to hydrolysis by phosphodiesterases have been injected intracellularly via microelectrodes. In other experiments intracellular cAMP levels have been elevated by application of phosphodiesterase inhibitors, which inhibit cAMP breakdown, or activators of adenylate cyclase, which increase cAMP synthesis. Results from all of these approaches indicate that cAMP can indeed modulate membrane properties, and in some of these studies voltage-clamp analysis has allowed the identification of particular

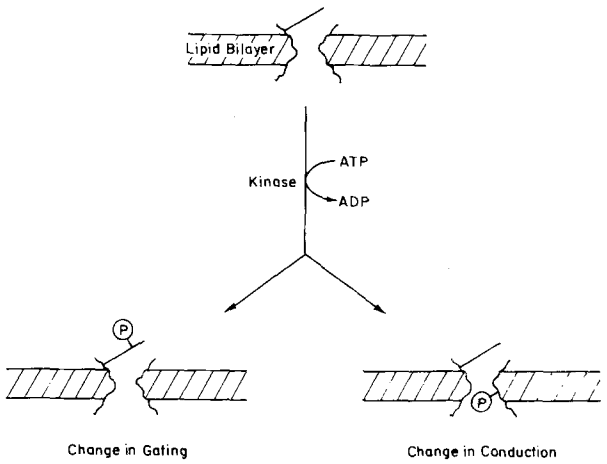

Fig. 1. Possible ways in which the activity of an ion channel might be modulated by protein phosphorylation. At the top an ion channel is depicted schematically as a conducting pathway or pore, with a gate whose opening and closing might be governed by such factors as the trans-membrane voltage, or occupation of a closely-associated receptor by a ligand. Phosphorylation Ⓟ of the gate (lower left) might change the open *versus* closed probability (p), whereas phosphorylation of the conducting pathway (lower right) might alter the single-channel current (i). It is also possible that phosphorylation might alter N, the number of functional channels in the membrane, without affecting p or i values for those channels which are functional. Finally, it is conceivable that phosphorylation of some regulatory component, which is not an integral part of the channel but may bind to it when phosphorylated, could modulate N, p or i

ionic currents which can be affected by cAMP. There appears to be no single ion current that is a universal target for cAMP action, but rather the current that is modulated is different from cell to cell, and in fact in at least some cells several different currents can be regulated.

A demonstration of a *pharmacological* effect of cAMP on membrane properties is, of course, not in itself evidence that cAMP plays a *physiological* role. However, in several of the studies referred to above it has been shown that cAMP mimics the action of a particular physiological agonist and that the physiological agonist can cause the stimulation of adenylate cyclase and the accumulation of cAMP in the target cell. These and other pharmacological, biochemical and electrophysiological experiments have indeed provided strong circumstantial evidence for a physiological role for cAMP in some cells. Among the physiological responses that appear to be mediated by cAMP are the activation of the slow inward calcium current by β-adrenergic stimulation of cardiac cells (Tsien, 1983; Reuter, 1983) and the inactivation (Deterre et al., 1981; Klein, Camardo & Kandel, 1982) or activation (Drummond, Benson & Levitan, 1980; Lemos & Levitan, 1984) of several distinct potassium currents by serotonin in several different molluscan neurons.

Particularly compelling evidence for second messenger involvement in physiological responses has been provided by use of single-channel patch recording techniques. Since the gigohm seal between a patch-recording electrode and a membrane forms a lateral diffusion barrier, it follows that neurotransmitter applied outside the electrode cannot activate receptors in the patch of membrane inside the electrode. Thus, if channels inside a patch are affected by transmitter applied outside, this must be due to occupancy of receptors located outside, and these receptors can only communicate with the channels inside the patch by means of a diffusible intracellular messenger. This general test has been used in at least two laboratories to identify second messenger-mediated responses. Siegelbaum, Camardo and Kandel (1982), working with sensory neurons from the abdominal ganglion of *Aplysia,* have demonstrated that serotonin applied in the extracellular medium can cause the closure of individual potassium channels inside a patch electrode. Other experiments have shown that the second messenger that mediates this response is cAMP, but this knowledge was not necessary for the demonstration that *some* second messenger must be involved. Maruyama and Petersen (1982) have used a similar approach to show that cholecystokinin and acetylcholine activation of single calcium-dependent cation channels in pancreatic acinar cells is mediated by intracellular calcium ions. These elegant experiments have introduced a new level of sophistication to the way we think about and study second messenger-mediated physiological responses in excitable cells.

Protein Phosphorylation as a Modulatory Mechanism

It is thought that all actions of cAMP and many actions of calcium in eukaryotic cells result from activation of protein kinases and subsequent phosphorylation of specific substrate proteins (Kuo & Greengard, 1969; Glass & Krebs, 1980; Cohen, 1982). In analogy to alterations in enzyme activities following phosphorylation, it seems possible that direct phosphorylation of ion channel proteins may alter their gating or conduction properties (Fig. 1) and that this is the molecular mechanism by which cAMP and calcium exert the variety of effects on ionic currents described above. The purpose of this review is to describe the kinds of experimental approaches that have been taken to investigate this

possibility, and to assess the evidence that implicates phosphorylation of ion channels as a fundamental regulatory mechanism in the control of cellular functions.

Experimental Approaches

The most productive approach in assessing the role of protein phosphorylation in ion channel function has been to introduce purified kinases or specific inhibitors directly into cells and to ask what this does to membrane properties. In several cases this approach has been extended to an examination of the effects of kinases on single ion channel activity in isolated membrane patches or in reconstituted systems. In addition, this review will discuss recent evidence that certain purified ion channels are excellent substrates for protein kinases *in vitro*, although the functional significance of these latter findings is not yet clear.

The availability of purified protein kinases, and highly specific inhibitors of protein kinases, has been essential for all of these approaches, and several of the molecular probes that have been most useful for these studies are described below. Although some of these reagents are available commercially, the commercial products are far from homogeneous. The importance of using *purified* enzymes for such experiments must be emphasized, since the introduction of protein and nonprotein impurities into cells may confound the interpretation of the results.

Molecular Probes for the Study of Protein Phosphorylation

cAMP-Dependent Protein Kinase

The most thoroughly understood protein kinase system is the cAMP-dependent protein kinase, which exists in cells as an inactive holoenzyme consisting of two regulatory (R) and two catalytic (C) subunits. At least two different types of enzyme, with identical C subunits but different R subunits, are present in most cells (Glass & Krebs, 1980). cAMP activates the enzyme by binding to the regulatory subunits and freeing the catalytic subunits, which in their unbound form are active and can catalyze the transfer of the terminal phosphate from ATP to threonine or (most often) serine residues of a protein to produce a phosphoprotein. There is no evidence for phosphorylation of tyrosine residues by cAMP-dependent protein kinase. Many (perhaps all) eukaryotic cells also contain a specific inhibitor of the cAMP-dependent protein kinase (Walsh et al., 1971). This 10,000-dalton protein kinase inhibitor (PKI) binds with high affinity to the free C subunit and inhibits its catalytic activity (*see* Fig. 1 in Levitan, Lemos & Novak-Hofer, 1983). Although the cellular function of PKI is not understood, its synthesis appears to be under hormonal control in at least some cell types (Means et al., 1980), and thus it is conceivable that it plays some important regulatory role.

The C and R subunits of cAMP-dependent protein kinase, as well as the protein kinase inhibitor, have been purified to homogeneity from a variety of tissues (*see*, for example, Peters, Demaille & Fischer, 1977; Demaille, Peters & Fischer, 1977). It appears that the active sites of these proteins have been remarkably well conserved during evolution, since the C subunit from mammalian cardiac or skeletal muscle can phosphorylate proteins in molluscan nervous system (Kaczmarek et al., 1980; Novak-Hofer & Levitan, 1983), and PKI from rabbit muscle is an excellent inhibitor of molluscan C subunit (Adams & Levitan, 1982). This high degree of conservation is interesting in its own right and also is particularly convenient, since these purified proteins from mammalian sources can be used as probes of membrane physiology in molluscan neurons.

Calcium/Calmodulin-Dependent Protein Kinases

Although calcium/calmodulin-dependent protein kinases were first described in the nervous system less than ten years ago (Schulman & Greengard, 1978), they have been studied intensively in a number of laboratories, and many of their properties are becoming well understood. Brain contains two well-characterized calcium/calmodulin-dependent kinases also found in nonneuronal tissues, myosin light-chain kinase and phosphorylase b kinase (Kennedy & Greengard, 1981); both of these enzymes have limited substrate specificities, and it is not clear what role they play in brain function. In addition, there are at least two other brain calcium/calmodulin-dependent protein kinases (Kennedy & Greengard, 1981), one of which has a broader substrate specificity. Purification of such a calcium/calmodulin-dependent kinase activity capable of phosphorylating a number of different protein substrates has been reported by several laboratories (Bennett, Erondu & Kennedy, 1983; Goldenring et al., 1983; Yamauchi & Fujisawa, 1983; Kuret & Schulman, 1984; McGuinness, Lai & Greengard, 1985), and it appears that these various enzymes are very similar and probably identical.

Table. Ion channels whose activity has been modulated by protein phosphorylation

Cell type	Channel modulated	Type of phosphorylation	Relevant references
Aplysia bag cell neurons	Transient K^+ channel	cAMP-dependent	Kaczmarek et al., 1980
	Delayed K^+ channel (slow)	cAMP-dependent	Strong, 1984
	Delayed K^+ channel (fast)	cAMP-dependent	Kaczmarek & Strumwasser, 1984 Strong & Kaczmarek, 1984
	Ca^{++} channel	Protein kinase C	DeRiemer et al., 1985
Aplysia sensory neurons	Serotonin-sensitive K^+ channel[a]	cAMP-dependent	Castellucci et al., 1980 Castellucci et al., 1982 Shuster et al., 1985
Aplysia neuron R15	Anomalously rectifying K^+ channel	cAMP-dependent	Adams & Levitan, 1982 Benson & Levitan, 1983
Hermissenda photoreceptors	Transient K^+ channel	cAMP-dependent	Alkon et al., 1983
	Delayed K^+ channel	Phosphorylase kinase	Acosta-Urquidi et al., 1984*a*
Helix neurons	Ca^{++} channel	cAMP-dependent	Doroshenko et al., 1984 Eckert & Chad, 1984 Chad & Eckert, 1985
	Ca^{++}-dependent K^+ channel[a]	cAMP-dependent	DePeyer et al., 1982 Ewald et al., 1985
Squid giant axon	Delayed rectifying K^+ channel	cAMP-dependent	Bezanilla et al., 1985
Cardiac cells	Ca^{++} channel[a]	cAMP-dependent	Osterrieder et al., 1982 Brum et al., 1983
Cultured cells	Gap junctional channel	cAMP-dependent	Wiener & Loewenstein, 1983
		Tyrosine kinase	Azarnia & Loewenstein, 1984

[a] Single channel measurements suggest the phosphorylation target is closely associated with the channel.

Protein Kinase C

The most recent addition to the protein kinase family is protein kinase C, which was first described by Nishizuka and his collaborators (Takai et al., 1977) as a proteolytically-activated enzyme. It was shown subsequently to be a calcium- and phospholipid-dependent, but calmodulin-*independent*, enzyme present in high concentration in a variety of tissues including brain (Kuo et al., 1980), and evidence is accumulating that it plays an important role in signal transduction across the plasma membrane (for a review *see* Nishizuka, 1984). Protein kinase C has also been purified from mammalian brain (Kikkawa et al., 1982) and is available for use as an intracellular probe.

Protein Phosphorylation Can Modulate Membrane Properties

Most of the studies of the regulation of ion channel activity by protein phosphorylation have involved cAMP-dependent phosphorylation (Table). This is in part because a wide variety of cAMP-induced electrophysiological responses have been reported, as described above. In addition, the molecular probes of the cAMP-dependent protein kinase system are the most thoroughly studied and have been most readily available. It will also be noted that molluscan neurons have been the cellular system of choice for virtually all these studies, with only a few exceptions such as cardiac myocytes (which may be considered honorary molluscan neurons for our purposes), and this is no accident. The large size of many of these neurons has permitted combined biochemical, pharmacological and electrophysiological experiments to be carried out on individual, identified nerve cells, and such an interdisciplinary approach is essential for a detailed understanding of the molecular mechanisms involved in the long-term modulation of ion channel activity.

Aplysia Bag Cell Neurons

The abdominal ganglion of *Aplysia* contains two apparently homogeneous clusters of about 300 neurosecretory neurons called bag cell neurons, which

synthesize and release several neuroactive peptides involved in triggering egg laying behavior. Secretion of these peptides is associated with a long-term period of repetitive firing known as an afterdischarge, and Kaczmarek and coworkers (1978) have provided evidence that cAMP is causally involved in the generation of the afterdischarge in bag cell neurons. Voltage-clamp analysis of this response indicates that cAMP enhances bag cell excitability by decreasing one transient and two delayed outward potassium currents evoked by depolarizing voltage steps (Kaczmarek & Strumwasser, 1984; Strong, 1984; Strong & Kaczmarek, 1984). Decreases in the two kinetically distinct components of delayed outward current are observed in cells internally dialyzed with EGTA-containing solutions (Strong & Kaczmarek, 1984), indicating that neither is calcium dependent.

One consequence of this inhibition of at least three different voltage-dependent potassium currents is that the bag cell action potentials are enhanced in both amplitude and duration, and Kaczmarek et al. (1980) have demonstrated that the intracellular injection of *C* subunit from bovine heart can produce a similar enhancement of action potentials. Although these experiments were not carried out under voltage clamp, *C* subunit does cause an increase in the input resistance of the bag cell neurons (Kaczmarek et al., 1980), consistent with a decrease in potassium conductance. In more recent experiments it has been shown that intracellular injection of PKI blocks the enhancement of action potentials, produced either by electrically triggering an after-discharge or by elevating cAMP levels by treatment with the adenylate cyclase activator forskolin (Kaczmarek, Nairn & Greengard, 1984). Furthermore PKI injection blocks the forskolin-induced decreases in potassium currents measured under voltage clamp (L.K. Kaczmarek, A.C. Nairn and P. Greengard, *in preparation;* L. Kaczmarek, *personal communication*). This series of experiments provides strong evidence that cAMP-dependent protein phosphorylation can modulate the activity of several distinct potassium currents in bag cell neurons and that this modulation plays an important role in the normal physiological functioning of these cells.

Kaczmarek and his colleagues (DeRiemer et al., 1985) have also examined the effects of protein kinase C on the physiological properties of bag cell neurons. Intracellular injection of protein kinase C, or treatment of bag cells with a phorbol ester (which activates protein kinase C—*see* Nishizuka, 1984), leads to an increase in the amplitude of the action potential. However, this appears to result not from a decrease in potassium currents, but rather an increase in calcium current following phorbol ester treatment (DeRiemer et al., 1985). It is of interest that protein kinase C and cAMP-dependent protein kinase both enhance action potentials in bag cell neurons, but they do so by modulating different ionic currents. The physiological significance of this intriguing finding remains to be investigated.

Aplysia Sensory Neurons

Another group of *Aplysia* abdominal ganglion neurons whose activity can be modulated are the sensory neurons involved in the animal's well-characterized gill withdrawal reflex (Kandel & Schwartz, 1982). Serotonin can evoke a cAMP-dependent increase in the duration of action potentials in these cells (Klein & Kandel, 1978), and voltage clamp studies have shown that this, too, involves a decrease in a potassium current (Klein, Camardo & Kandel, 1982). This current, the gating of which is not dependent on calcium and is only slightly dependent on voltage (Klein et al., 1982; Siegelbaum et al., 1982), has been given the name "*S* current." Intracellular injection of *C* subunit can produce action potential broadening identical to that produced by serotonin or cAMP (Castellucci et al., 1980), and injection of PKI can prevent the serotonin-evoked spike broadening (Castellucci et al., 1982). Again these experiments were not done under voltage clamp, but subsequent single-channel studies (to be discussed below) make it likely that these effects of *C* subunit and PKI do indeed reflect modulation of *S* current. It is interesting that PKI injection can rapidly reverse the fully developed spike broadening produced by prior application of serotonin (Castellucci et al., 1982). This indicates that the phosphorylation of the relevant substrate protein(s) is rather labile and suggests that a continuously active kinase is necessary to maintain the substrate(s) in the phosphorylated state.

Aplysia Neuron R15

Aplysia neuron R15 is an endogenously "bursting" neuron. Its normal pattern of electrical activity consists of bursts of action potentials separated by interburst hyperpolarizations (Frazier et al., 1967; Strumwasser, 1967). Although this pattern is generated endogenously by several voltage- and time-dependent ionic currents in R15's membrane (Adams, 1985; Adams & Levitan, 1985; Kramer & Zucker, 1985*a*,*b*) and does not require synaptic input for its generation (Alving, 1968), the endogenous pattern can be modulated for very long periods of time by synaptic stimulation or application of neurotransmitters (Levitan, Harmar & Adams, 1979). This combination, a fascinating endogenous activity to-

gether with the capacity for long-term modulation, has made R15 probably the most thoroughly studied of all nerve cells (Adams & Benson, 1985).

Serotonin causes neuron R15 to hyperpolarize and stop firing (Drummond, Benson & Levitan, 1980). This effect of serotonin is mimicked by intracellular injection or extracellular application of cAMP analogs (Drummond et al., 1980) and is blocked by the intracellular injection of the adenylate cyclase inhibitor GDPβS (Lemos & Levitan, 1984). Furthermore, serotonin stimulates adenylate cyclase activity in membranes from R15 (Levitan, 1978). These and other biochemical, pharmacological and electrophysiological experiments (summarized in Levitan & Benson, 1981) have established that this serotonin response in R15 is mediated by cAMP. The hyperpolarization produced by serotonin/cAMP is due to activation of an anomalously rectifying potassium current (Benson & Levitan, 1983), although cAMP appears to affect some other ionic currents in the cell as well (Ewald & Eckert, 1983; D. Lotshaw and I.B. Levitan, *in preparation*). Intracellular injection of PKI into R15, under voltage-clamp conditions, completely blocks the serotonin-induced activation of the anomalously rectifying potassium current, but has no effect on the dopamine-induced, cAMP-*independent* change in another ionic current in the same cell (Adams & Levitan, 1982). This selective block by PKI of the serotonin effect on the anomalous rectifier indicates that this response to serotonin is indeed mediated by cAMP-dependent protein phosphorylation.

Hermissenda Photoreceptors

One type of associative learning in *Hermissenda*, another marine mollusc, involves changes in the membrane properties of the type B photoreceptors located in the caudal-dorsal part of each eye (Alkon, 1984). Voltage-clamp experiments have suggested that two potassium currents, a rapidly inactivating transient potassium current and a delayed calcium-dependent potassium current, are markedly reduced in type B photoreceptors from associatively conditioned animals (Alkon, Lederhendler & Shoukimas, 1982; Alkon, 1984). Furthermore, the decrease in these two currents itself is dependent on intracellular calcium (Alkon, 1984). To investigate whether these changes involve protein phosphorylation, Alkon and his colleagues have iontophoretically injected several different protein kinases into type B photoreceptors and have examined their effects on membrane currents under voltage clamp. *C* subunit injection causes a decrease in both the early and delayed potassium currents, although the predominant effect is on the latter (Alkon et al., 1983). These changes induced by *C* subunit do not appear to require calcium, in contrast to the decreases in these currents observed in conditioned animals (Alkon, 1984).

Acosta-Urquidi, Alkon and Neary (1984*a*) have also injected phosphorylase *b* kinase, a calcium/calmodulin-dependent protein kinase, into type B photoreceptors. Again such injections reduce both the transient and delayed potassium currents, but in this case the transient current is more strongly affected (Acosta-Urquidi et al., 1984*a*). Furthermore, the effects of the kinase are calcium dependent, leading Alkon and his colleagues to suggest (Alkon, 1984) that the calcium-dependent changes in ionic currents in type B photoreceptors during associative conditioning might be mediated by calcium/calmodulin-dependent protein phosphorylation. There are several curious features about these experiments. It is somewhat surprising that these large proteins can be injected iontophoretically, and indeed other workers in this field have chosen either pressure injection or intracellular perfusion to introduce kinases into cells. However, the authors do report that they can iontophoretically eject sufficient kinase from their electrodes to be detected in a test tube assay (Alkon et al., 1983; Acosta-Urquidi et al., 1984*a*). Another curious aspect has to do with the strict substrate specificity of phosphorylase *b* kinase for phosphorylase *b* and glycogen synthase (*see*, for example, Kennedy & Greengard, 1981; Cohen, 1982), and in view of this it is surprising that the injection of this enzyme can produce changes in membrane ionic currents.

More recently Acosta-Urquidi et al. (1984*b*) have injected into other *Hermissenda* neurons one of the calcium/calmodulin-dependent kinases isolated from brain (Goldenring et al., 1983) and have reported that such iontophoretic injections alter calcium current, voltage-dependent potassium current, and calcium-dependent potassium current. However, the various currents increase in some cells, decrease in other cells, and sometimes do not change at all (Acosta-Urquidi et al., 1984*b*), and in view of the complexity and inconsistency of the effects of this kinase, it must be concluded that a role for calcium-dependent protein phosphorylation in this system remains to be demonstrated.

Snail Neurons

Internal perfusion, a technique first introduced by Kostyuk and his colleagues, allows the experimental control of a neuron's cytoplasmic contents. Among the advantages of this technique is the ability to introduce a *particular concentration* of a

given molecular probe into a cell, in contrast to intracellular pressure injection where it is difficult to control accurately the amount injected, and iontophoretic injection where there is virtually no control whatsoever. This is particularly important in the case of the *C* subunit, since at sufficiently high concentrations this kinase will phosphorylate proteins which may not be physiological substrates. The total intracellular concentration of *C* subunit (both free and bound to *R* subunit) has been estimated to be 0.3-1 μM (Hofmann, Bechtel & Krebs, 1977), and DePeyer et al. (1982) have introduced *C* subunit in this concentration range into internally perfused *Helix* neurons while measuring membrane currents under voltage clamp. Perfusion with *C* subunit causes a large increase in the steady-state outward current evoked by depolarizing voltage pulses (DePeyer et al., 1982); this current consists of at least two components, a delayed rectifying potassium current and a calcium-dependent potassium current (Meech & Standen, 1975). When the calcium-dependent component is eliminated by internal perfusion with EGTA, *C* subunit has no effect on the remaining delayed rectifier, suggesting that phosphorylation selectively enhances the calcium-dependent potassium current in these neurons (DePeyer et al., 1982). The tentative conclusion from these experiments, that *C* subunit is directly phosphorylating the potassium channel and increasing its affinity for calcium (Fig. 2), has received strong support from single-channel studies (discussed below).

Although many membrane currents are stable in internally perfused neurons, selective loss of calcium current has been seen in molluscan neurons (Byerly & Hagiwara, 1982; Doroshenko, Kostyuk & Martynyuk, 1982) and vertebrate cells (Kostyuk, Veselovsky & Fedulova, 1981; Fenwick, Marty & Neher, 1982). Kostyuk and his colleagues have found that the loss of calcium current in perfused *Helix* neurons can be prevented or reversed by inclusion of either cAMP (Doroshenko et al., 1982) or *C* subunit (Doroshenko et al., 1984) in the intracellular medium, and have concluded that loss of calcium current is due to washout of cAMP during perfusion. A similar conclusion has been drawn by Eckert and Chad (1984), who carried out the same kind of experiments on *Helix* neurons and found that calcium current loss is slower in the presence of *C* subunit.

These investigators have also examined the possibility that protein phosphorylation/dephosphorylation might be involved in the well-established phenomenon of calcium-dependent inactivation of calcium current (for a review *see* Eckert & Chad, 1984). They found that calcium-dependent inactivation is enhanced when calcineurin, a calcium/calmodulin-dependent phosphoprotein phosphatase (Stewart et al., 1982), is included in the internal perfusate (Chad & Eckert, 1985). These results have led the authors to suggest that calcium-dependent inactivation requires calcium-dependent dephosphorylation, and that cAMP-dependent phosphorylation is then required to return the channel to an activatable state (Chad & Eckert, 1985). If this scheme is correct, then loss of calcium current with perfusion might be due to calcium-dependent inactivation which cannot be reversed because the kinase has been washed out of the cell.

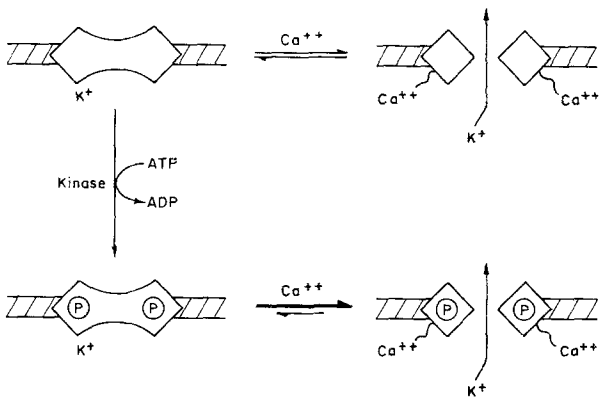

Fig. 2. Hypothesis for the modulation of calcium-dependent potassium channel activity by protein phosphorylation. The opening of the channel is dependent on trans-membrane voltage, and also requires the binding of calcium to some regulatory site on the channel (top). DePeyer et al. (1982) have suggested that phosphorylation Ⓟ of the regulatory site or some other site closely associated with the channel increases the affinity of the regulatory site for calcium, thus shifting the closed/open equilibrium in favor of the open state at a given voltage and calcium concentration (bottom). Support for this hypothesis has been obtained from single-channel experiments (*see* text and Ewald et al., 1985)

It is worthy of note that in *Lymnaea* neurons, calcium current loss during perfusion (Byerly & Hagiwara, 1982) can be prevented by addition of magnesium, ATP, and a strong calcium buffer to the internal perfusate *without* adding a kinase (Byerly & Yazejian, 1985), and these authors suggest that the effects of cAMP and *C* subunit seen by other workers might result from enhancement of the cell's calcium buffering capability rather than from some direct effect on the calcium channel. It is, of course, conceivable that *Lymnaea* and *Helix* use different mechanisms to regulate their membrane ionic currents, but in any event this finding is not necessarily at odds with the conclusions of Chad and Eckert (1985), since Byerly and Moody (1984) have found that both ATP and EGTA are required to buffer calcium adequately near the plasma membrane of these neurons. It is possible that under these good

buffering conditions (Byerly & Yazejian, 1985) calcium-dependent inactivation does not have a chance to occur, and thus it is not necessary to have a kinase present to reactivate channels and prevent loss of calcium current. It should be emphasized that these are preliminary and ongoing experiments, but they promise to provide significant insights into the physiological regulation of calcium currents.

Squid Giant Axon

In spite of its enormous value as an experimental system for elucidating the ionic changes underlying the action potential, the squid giant axon has been thought of as rather boring by investigators interested in modulation of ion channels. The axon goes rapidly from being a potassium battery at rest to a sodium battery during the action potential, and then back again, and there has been no evidence for long-term modulation of these all-or-none transitions. However, recent experiments using internally perfused or internally dialyzed squid axon suggest that cAMP-dependent protein phosphorylation may regulate the axonal potassium conductance. In *dialyzed* axons, in which small molecules (such as ATP) are washed away but proteins (including kinases) presumably remain behind, addition of ATP causes a large increase in the outward potassium current evoked by depolarization (Bezanilla et al., 1985). This appears to result from a shift in the voltage dependence of both the activation and inactivation of the potassium current (F. Bezanilla, *personal communication*). In *perfused* axons, which presumably lose proteins as well as small molecules, ATP itself has some effect but the changes in potassium current are enhanced when *C* subunit is added together with the ATP (Bezanilla et al., 1985; F. Bezanilla, *personal communication*). These experiments are still preliminary, but in view of the wealth of information available about squid axon potassium currents, this system may be particularly favorable for investigating the details of changes in channel gating and conduction mechanisms induced by protein phosphorylation.

Squid Giant Synapse

An intriguing recent study has implicated calcium/calmodulin-dependent protein phosphorylation in the regulation of neurotransmitter release at the squid giant synapse (Llinas et al., 1985). These investigators made use of synapsin I, a phosphoprotein associated with synaptic vesicles (Nestler & Greengard, 1983), and a purified calcium/calmodulin-dependent kinase from brain which phosphorylates synapsin I at a specific site (Nestler & Greengard, 1983). Injection of dephospho-synapsin I into the presynaptic terminal of the squid giant synapse produces a decrease in the amplitude and rate of rise of the postsynaptic potential, which is taken as a measure of neurotransmitter release (Llinas et al., 1985). Conversely, intraterminal injection of the calcium/calmodulin-dependent kinase increases the amplitude and rate of rise, and decreases the latency, of the postsynaptic potential. These changes in neurotransmitter release do not appear to be accompanied by changes in the presynaptic calcium current, and the authors suggest that the availability of synaptic vesicles for release may be regulated by the phosphorylation state of synapsin I (Llinas et al., 1985). The authors point out that the results are preliminary and many controls remain to be done during the next squid season, and, in addition, there is no evidence at the moment that this phenomenon involves phosphorylation of ion channels. Nevertheless, I present it here as a particularly good example of the usefulness of, and indeed the necessity for, purified and highly specific reagents for probing the physiological role of protein phosphorylation. The availability of other molecular probes of this system, including antibodies against synapsin I, should allow the molecular regulation of transmitter release to be investigated in considerable detail.

Cardiac Myocytes

Cardiac cells were among the first excitable cells whose membrane properties were shown to be regulated by cAMP (Tsien, 1973; Reuter, 1974). β-adrenergic stimulation of the heart results in a cAMP-mediated increase in the slow inward calcium current responsible for the plateau phase of the cardiac action potential (for reviews *see* Tsien, 1983; Reuter, 1983). The possible role of cAMP-dependent protein phosphorylation in this phenomenon has been investigated by the injection of *C* subunit into isolated ventricular cells (Osterrieder et al., 1982; Brum et al., 1983). *C* subunit injection dramatically prolongs the plateau phase of the action potential and increases the amplitude of the slow inward calcium current measured under voltage clamp. Furthermore, once the action potential duration has been maximally increased by the *C* subunit, no further prolongation can be produced by treatment of the cell with a β-adrenergic agonist (Brum et al., 1983); this occlusion of the β-adrenergic response provides evidence that there is a common target for the actions of receptor agonists and the kinase. An interesting feature of this study was the

demonstration (Osterrieder et al., 1982) that intracellular injection of *R* subunit can *decrease* the duration of the action potential, apparently by binding to and inactivating free *C* subunit. Furthermore, adrenaline can reverse this action of *R* subunit, apparently by stimulating production of intracellular cAMP, which binds to the injected *R* subunit and prevents it from "mopping up" free *C* subunit. This elegant series of experiments has provided particularly convincing evidence for a physiological role for protein phosphorylation in the regulation of cardiac ionic currents.

Cultured Avian and Mammalian Cells

Loewenstein and his collaborators have been investigating the regulation of permeability through cell-to-cell "gap junctional" channels in a variety of cell types. These channels can be modulated by a number of physiological regulators (Loewenstein, 1981), one of which appears to be cAMP-dependent protein kinase. Mutant Chinese hamster ovary cells, which are deficient in one of the isoenzymes of cAMP-dependent protein kinase, are also deficient in cell-to-cell junctional permeability (Wiener & Loewenstein, 1983). Furthermore, in revertant cells junctional permeability reappears in concert with kinase activity. When mutant cells are permeabilized (reversibly) and are loaded with purified *C* subunit, the defect in junctional permeability can be "cured" over a period of hours, suggesting that cAMP-dependent protein phosphorylation regulates some aspect of the formation or function of the cell-to-cell channel (Wiener & Loewenstein, 1983).

There is also evidence implicating tyrosine phosphorylation in the modulation of junctional permeability. Cultured quail and chick embryo cells, and mouse fibroblasts, were infected with a temperature-sensitive mutant of the Rous sarcoma virus; tyrosine phosphorylation controlled by the viral *src* gene product $pp60^{src}$ occurs only at the permissive temperature in such cells (Radke & Martin, 1979). When the temperature is shifted from the nonpermissive to the permissive temperature and back again, junctional permeability falls and rises in concert with the appearance and disappearance, respectively, of tyrosine phosphorylation (Azarnia & Loewenstein, 1984). This apparent down-regulation by tyrosine phosphorylation overrides the up-regulation associated with the serine/threonine phosphorylation catalyzed by the cAMP-dependent protein kinase. In view of the rapidity of the changes in junctional permeability induced by the temperature shifts, the authors speculate that the cell-to-cell channel itself might be the phosphorylation target, but point out that this is by no means proven (Azarnia & Loewenstein, 1984).

Purified Ion Channels as Substrates for Protein Kinases

Two membrane ion channels, the voltage-dependent sodium channel (Agnew et al., 1980; Weigele & Barchi, 1982; Hartshorne & Catterall, 1984) and the acetylcholine receptor/channel (reviewed by Karlin, 1980; Changeux, 1981), have been solubilized and purified to homogeneity. Both of these purified channels have been shown to be phosphorylated by protein kinases *in vitro*.

Sodium Channel

The rat brain sodium channel consists of a high molecular weight α subunit and two smaller β subunits (Catterall, 1984). The α subunit can be phosphorylated *in vitro* by *C* subunit, with up to 3 moles of phosphate incorporated per mole of α subunit (Costa, Casnellie & Catterall, 1982). The concentration of C subunit used in these experiments and the rate and extent of the phosphorylation are consistent with the possibility that the sodium channel is a physiological substrate for cAMP-dependent protein kinase. Furthermore, the sodium channel can be phosphorylated in lysed synaptosomes by exogenous *C* subunit and in intact synaptosomes in the presence of 8-bromo cAMP (Costa & Catterall, 1984), which presumably acts by activating an endogenous cAMP-dependent kinase. Some effects of 8-bromo cAMP-stimulated phosphorylation on the slow influx of radioactive sodium into synaptosomes have been observed (Costa & Catterall, 1984), but the significance of these findings to the physiological regulation of normal sodium channel gating remains to be determined.

Acetylcholine Receptor/Channel

The other membrane ion channel that has been purified and thoroughly characterized is the nicotinic acetylcholine receptor/channel. The receptor/channel complex is a 255,000-dalton protein consisting of two α subunits and one each β, γ, and δ subunits. Postsynaptic membranes isolated from the ray *Torpedo californica,* which have an extremely high density of acetylcholine receptors, contain endogenous protein kinases that phosphorylate the receptor (Gordon et al., 1977; Teichberg, Sobel & Changeux, 1977; Saitoh & Changeux, 1981). An endogenous cAMP-dependent protein kinase

phosphorylates the γ and δ subunits, and this can be blocked by PKI (Huganir & Greengard, 1983). Furthermore, the purified receptor can be phosphorylated on these same subunits by exogenous *C* subunit, and again the rate and extent of the phosphorylation are consistent with the possibility that this is a physiologically significant modification (Huganir & Greengard, 1983). There are also at least two endogenous calcium-dependent protein kinases present in *Torpedo* postsynaptic membranes, one of which is calcium/calmodulin-dependent (Smilowitz et al., 1981; Huganir & Greengard, 1983) and the other of which is protein kinase C (R. Huganir, K. Albert & P. Greengard, *in preparation*; R. Huganir, *personal communication*). Although the calcium/calmodulin kinase does not phosphorylate the acetylcholine receptor (Huganir & Greengard, 1983), the endogenous protein kinase C specifically phosphorylates the δ subunit in a calcium and phosphatidylserine-dependent manner, and exogenous protein kinase C can specifically phosphorylate the purified receptor on the δ subunit (R. Huganir, K. Albert & P. Greengard, *in preparation*). Finally Huganir, Miles and Greengard (1984) have shown that, when endogenous cAMP-dependent and calcium-dependent kinases are blocked by addition of PKI and EGTA, it is possible to demonstrate phosphorylation of the β, γ, and δ subunits of the receptor on tyrosine residues by an endogenous tyrosine-specific protein kinase.

As in the case of the sodium channel, the kinetics and stoichiometry of these phosphorylations of the acetylcholine receptor/channel, together with the existence of multiple phosphorylation sites on the various subunits, suggest that phosphorylation might modulate the functioning of this channel. It is now possible to reconstitute the purified acetylcholine receptor under conditions that allow measurement of its single-channel properties with good time resolution (Suarez-Isla et al., 1983; Tank et al., 1983), but to date no effects of phosphorylation on the gating or conduction of this reconstituted receptor/channel complex have been reported.

Phosphorylation Modulates the Activity of Single Ion Channels

From the experiments discussed in the previous two sections it seems clear that ion channels can serve as substrates for protein kinases *in vitro*, and that the introduction of kinases into cells leads to changes in membrane properties. A number of investigators have suggested, on the basis of the latter kind of experiment, that the changes in membrane properties result from the direct phosphorylation of ion channel proteins (*see*, for example, Fig. 2). However, it is important to emphasize that this experimental approach does not allow the identification of the phosphorylation target; although phosphorylation of the channel seems a reasonable possibility, it is also conceivable that phosphorylation of some nonchannel membrane or even cytoplasmic protein might initiate a cascade of events which leads ultimately to the modulation of the cell's properties, without any need for direct phosphorylation of ion channels. The question of the identity of the phosphorylation target has been addressed directly in several recent studies using single-channel recording techniques, both in isolated membrane patches and in a reconstituted artificial bilayer system.

Cardiac Calcium Channels in Isolated Patches

Although it has been possible to measure the activity of individual calcium channels in cell-attached membrane patches (reviewed in Reuter, 1983; Tsien, 1983), many investigators have noted that calcium channel activity disappears when patches are detached from the cell in the so-called "inside-out" configuration, in which the cytoplasmic face of the membrane is exposed to the bathing medium (Hamill et al., 1981). It seems possible that this phenomenon is related to the loss of macroscopic calcium current in internally perfused neurons (*see* above), and thus might involve protein phosphorylation or dephosphorylation. In recent experiments Tsien and his colleagues have found that single calcium channel activity, which normally disappears within several minutes in inside-out patches from cardiac cells, can be maintained for as long as 20–30 min in patches excised into a solution containing *C* subunit, ATP and magnesium (P. Hess, E. McCleskey & R. Tsien, *in preparation*; R. Tsien, *personal communication*). These preliminary findings suggest that, at least in cardiac cells, cAMP-dependent phosphorylation of some membrane protein which comes away with the excised patch may be necessary for calcium channel activity.

S Channels in Isolated Patches

The broadening of action potentials by serotonin in *Aplysia* sensory neurons is, as discussed above, mimicked by injection of *C* subunit and blocked by injection of PKI (Castellucci et al., 1980, 1982). This effect of serotonin is due to inhibition of *S* current, a novel potassium current present in these neurons (Klein et al., 1982), and it has been shown (Siegel-

baum et al., 1982) that serotonin acts on cell-attached membrane patches to cause prolonged closures of the individual *S* channels which underlie the *S* current. Shuster et al. (1985) have examined the modulation of *S* channel activity in isolated inside-out membrane patches from sensory neurons. They have found that application of *C* subunit together with ATP and magnesium to the cytoplasmic side of the membrane produces all-or-none closures of individual *S* channels in the isolated patch, simulating most of the effects of serotonin on cell-attached patches (Shuster et al., 1985). It is of interest that on occasion prolonged *S* channel closures occur in the absence of exogenous *C* subunit, suggesting that endogenous kinase may sometimes come away with the patch of membrane when it is pulled off the cell. Similarly exogenous *C* subunit sometimes does not cause channel closures, or the closures it does produce often reverse "spontaneously," and the authors suggest (Shuster et al., 1985) that some cell-free patches may lack a regulatory component necessary for complete channel modulation or may contain an endogenous phosphoprotein phosphatase. In support of this latter idea is the finding that addition of fluoride ion, a nonspecific phosphatase inhibitor, together with *C* subunit, increases the percentage of successful experiments (Shuster et al., 1985). On the basis of these experiments it seems likely that the closure of *S* channels by serotonin is mediated by cAMP-dependent phosphorylation of some membrane protein. The relevant substrate might be the *S* channel protein itself, or some other element, for example a cytoskeleton component, which comes away with the patch when it is excised.

Calcium-Dependent Potassium Channels in Isolated Patches and Reconstituted in Artificial Phospholipid Bilayers

As described above DePeyer et al. (1982) found that *C* subunit perfusion can increase calcium-dependent potassium current in *Helix* neurons, and they suggested that *C* subunit might phosphorylate the potassium channel and increase its affinity for calcium (Fig. 2). This possibility has been tested using single-channel measurements. When *C* subunit together with ATP and magnesium is added to the cytoplasmic surface of an isolated inside-out patch containing calcium-dependent potassium channels, the activity of the channels increases (Ewald, Williams & Levitan, 1985), indicating that, as in the case of *S* channels, the phosphorylation target comes away with the isolated patch. The spontaneous reversals and occasional failures seen with the *S* channel phosphorylations are not observed in this case, suggesting that for unknown reasons the *Helix* neuron patches may come away more cleanly, or with a more complete complement of regulatory components, than those from the *Aplysia* sensory neurons. Because the patches always contain more than one calcium-dependent potassium channel, it has been difficult in these experiments to determine whether phosphorylation is increasing the number of active channels in the patch, or the probability that an individual channel is open (Ewald et al., 1985).

A complementary approach is to examine individual calcium-dependent potassium channels reconstituted in artificial phospholipid bilayers. For these experiments a crude membrane vesicle preparation from *Helix* neurons is reconstituted with exogenous phospholipid, and a bilayer is formed on the tip of a patch-recording electrode (Wilmsen et al., 1983). This marriage between the patch and bilayer technologies provides particularly favorable conditions for single-channel recording (Coronado & Latorre, 1983; Suarez-Isla et al., 1983; Wilmsen et al., 1983), and Ewald and coworkers (1985) have observed single calcium-dependent potassium channels from *Helix* neurons in such bilayers. When *C* subunit is added to the side of the membrane on which the calcium-sensitive site is present, there is a dramatic increase in the activity of the channel, corresponding to a large increase in the single-channel open probability (Ewald et al., 1985). The voltage dependences of channel open probability, before and after addition of *C* subunit, are consistent with the hypothesis that phosphorylation is increasing the affinity of the channel for calcium (D. Ewald, A. Williams and I.B. Levitan, *unpublished*). Furthermore, an important conclusion that can be drawn from this experiment is that the phosphorylation target is either the potassium channel itself or some regulatory component so intimately associated with the channel that it travels with it in the bilayer.

Summary and Conclusions

The introduction of highly specific reagents such as enzymes and inhibitors directly into living cells has proven to be a powerful tool in studying the modulation of cellular activity by protein phosphorylation. The use of *exogenous* kinases can be thought of as a pharmacological approach: this demonstrates that phosphorylation *can* produce modulation, but does not address the question of whether the cell actually uses this mechanism under normal physiological conditions. The complementary approach, the introduction of highly specific inhibitors such as *R* subunit or PKI, does ask whether *endog-*

enous kinase activity is necessary for a given physiological response. Together these two approaches have provided rather compelling evidence that cAMP-dependent and calcium/phospholipid-dependent protein phosphorylations can regulate membrane excitability. In several cases single-channel analysis has allowed the demonstration that an ion channel itself or something very close to the channel is the phosphorylation target, and it seems reasonable to assume that this will also be the case for many if not all of the other systems described above.

Have any general principles emerged from the results to date? Certainly it seems clear that protein phosphorylation regulates not one but many classes of ion channels. As summarized in the Table, different channels can be modulated in different cells, some channels are activated while others are inhibited, and in some cells more than one channel is subject to modulation by phosphorylation. The list in the Table is probably not yet complete, and indeed it is not inconceivable that all ion channels can under appropriate conditions be regulated by phosphorylation.

What aspect of channel function is altered by phosphorylation? The total membrane current, I, carried by a particular species of ion channel is given by Npi, where N is the number of active channels in the membrane, p is the probability that an individual channel will be open, and i is the single-channel current. In principle a change in I, the quantity measured in whole cell experiments, could be caused by a change in any one (or more) of the parameters, N, p or i (*see* Fig. 1). In the two cases in which single-channel measurements have allowed this question to be investigated, changes in N (Shuster et al., 1985) and p (Ewald et al., 1985) have been observed. Here again it seems unlikely that any one mechanism operates in all cases, and it would not be surprising to find that phosphorylation of some other channel results in a change in i.

Finally, what does the future hold, what experimental approaches are likely to provide fundamental information about the regulation of ion channels by protein phosphorylation? Certainly it will be of interest to extend the single-channel approach to more of the systems described above, to characterize fully the parameters which are modulated, but the most significant advances are likely to come if one or more of these regulatable channels can be purified and characterized in isolation. Although substantial progress has been made recently towards the purification of calcium channels, it would be misleading to pretend that purification of regulatable potassium channels is likely to be achieved soon. The effort has begun to *identify* phosphorylated proteins which might be potassium channel components or regulatory elements (Lemos, Novak-Hofer & Levitan, 1985), but *purification* is likely to be successful only with a suitable routine assay, and no ligands which bind specifically and with high affinity to these regulatable channels are yet available. Nevertheless, this remains an important long term goal. The detailed picture we have of the structure and function of the nicotinic acetylcholine receptor/channel is an excellent example of the power of the purification approach in understanding channel function, and a fantasy of many channelologists is to achieve the same degree of understanding of other channels whose activity is regulated by protein phosphorylation.

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References

Acosta-Urquidi, J., Alkon, D.L., Neary, J.T. 1984*a*. *Science* **224:**1254–1257

Acosta-Urquidi, J., Neary, J.T., Goldenring, J.R., Alkon, D.L., DeLorenzo, R.J. 1984*b*. *Soc. Neurosci. Abstr.* **10:**1129

Adams, W.B. 1985. *J. Physiol. (London)* **360:**51–68

Adams, W.B., Benson, J.A. 1985. *Prog. Biophys. Molec. Biol.* **46:**1–49

Adams, W.B., Levitan, I.B. 1982. *Proc. Natl. Acad. Sci. USA* **79:**3877–3880

Adams, W.B., Levitan, I.B. 1985. *J. Physiol. (London)* **360:**69–93

Agnew, W.S., Moore, A.C., Levinson, S.R., Raftery, M.A. 1980. *Biochem. Biophys. Res. Commun.* **92:**860–866

Alkon, D.L. 1984. *Science* **226:**1037–1045

Alkon, D.L., Acosta-Urquidi, J., Olds, J., Kuzma, G., Neary, J.T. 1983. *Science* **219:**303–306

Alkon, D.L., Lederhendler, I., Shoukimas, J.J. 1982. *Science* **215:**693–695

Alving, B.O. 1968. *J. Gen. Physiol.* **51:**29–45

Azarnia, R., Loewenstein, W.R. 1984. *J. Membrane Biol.* **82:**191–205

Bennett, M.K., Erondu, N.E., Kennedy, M.B. 1983. *J. Biol. Chem.* **258:**12735–12744

Benson, J.A., Levitan, I.B. 1983. *Proc. Natl. Acad. Sci. USA* **80:**3522–3525

Bezanilla, F., DiPolo, R., Caputo, C., Rojas, H., Torres, M.E. 1985. *Biophys. J.* **47:**222*a*

Brum, G., Flockerzi, V., Hofmann, F., Osterrieder, W., Trautwein, W. 1983. *Pfluegers Arch.* **398:**147–154

Byerly, L., Hagiwara, S. 1982. *J. Physiol. (London)* **322:**503–528

Byerly, L., Moody, W.J. 1984. *J. Physiol. (London)* **352:**637–652

Byerly, L., Yazejian, B. 1985. *J. Physiol. (in press)*

Castellucci, V.F., Kandel, E.R., Schwartz, J.H., Wilson, F.D., Nairn, A.C., Greengard, P. 1980. *Proc. Natl. Acad. Sci. USA* **77:**7492–7496

Castellucci, V.F., Nairn, A., Greengard, P., Schwartz, J.H., Kandel, E.R. 1982. *J. Neurosci.* **2**:1673–1681
Catterall, W.A. 1984. *Science* **223**:653–661
Chad, J., Eckert, R. 1985. *Biophys. J.* **47**:266*a*
Changeux, J.-P. 1981. *Harvey Lect.* **75**:85–254
Cohen, P. 1982. *Nature (London)* **296**:613–620
Connor, J.A., Hockberger, P. 1984. *J. Physiol. (London)* **354**:139–162
Coronado, R., Latorre, R. 1983. *Biophys. J.* **43**:231–236
Costa, M.R.C., Casnellie, J.E., Catterall, W.A. 1982. *J. Biol. Chem.* **257**:7918–7921
Costa, M.R.C., Catterall, W.A. 1984. *J. Biol. Chem.* **259**:8210–8218
Demaille, J., Peters, K., Fischer, E. 1977. *Biochemistry* **16**:3080–3086
DePeyer, J.E., Cachelin, A.B., Levitan, I.B., Reuter, H. 1982. *Proc. Natl. Acad. Sci. USA* **79**:4207–4211
DeRiemer, S.A., Strong, J.A., Albert, K.A., Greengard, P., Kaczmarek, L.K. 1985. *Nature (London)* **313**:313–316
Deterre, P., Paupardin-Tritsch, D., Bockaert, J., Gerschenfeld, H.M. 1981. *Nature (London)* **290**:783–785
Doroshenko, P.A., Kostyuk, P.G., Martynyuk, A.E. 1982. *Neuroscience* **7**:2125–2134
Doroshenko, P.A., Kostyuk, P.G., Martynyuk, A.E., Kursky, M.D., Vorobetz, Z.D. 1984. *Neuroscience* **11**:263–267
Drummond, A., Benson, J., Levitan, I.B. 1980. *Proc. Natl. Acad. Sci. USA* **77**:5013–5017
Eckert, R., Chad, J.E. 1984. *Prog. Biophys. Molec. Biol.* **44**:215–267
Ewald, D., Eckert, R. 1983. *Cell. Molec. Neurobiol.* **3**:345–353
Ewald, D., Williams, A., Levitan, I.B. 1985. *Nature (London)* **315**:503–506
Fenwick, E.M., Marty, A., Neher, E. 1982. *J. Physiol. (London)* **331**:599–635
Frazier, W.T., Kandel, E.R., Kupfermann, I., Waziri, R., Coggeshall, R.E. 1967. *J. Neurophysiol.* **30**:1288–1351
Glass, D., Krebs, E. 1980. *Annu. Rev. Pharmacol. Toxicol.* **20**:363–388
Goldenring, J.R., Gonzalez, B., McGuire, J.S., Jr., DeLorenzo, R.J. 1983. *J. Biol. Chem.* **258**:12632–12640
Gordon, A.S., Davis, C.G., Milfay, D., Diamond, I. 1977. *Nature (London)* **267**:539–540
Green, D.J., Gillette, R. 1983. *Nature (London)* **306**:784–785
Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. *Pfluegers Arch.* **391**:85–100
Hartshorne, R.P., Catterall, W.A. 1984. *J. Biol. Chem.* **259**:1667–1675
Hofmann, F., Bechtel, P., Krebs, E.G. 1977. *J. Biol. Chem.* **252**:1441–1447
Huganir, R.L., Greengard, P. 1983. *Proc. Natl. Acad. Sci. USA* **80**:1130–1134
Huganir, R.L., Miles, K., Greengard, P. 1984. *Proc. Natl. Acad. Sci. USA* **81**:6968–6972
Kaczmarek, L., Jennings, K., Strumwasser, F. 1978. *Proc. Natl. Acad. Sci. USA* **75**:5200–5204
Kaczmarek, L.K., Jennings, K.R., Strumwasser, F., Nairn, A.C., Walter, U., Wilson, F.D., Greengard, P. 1980. *Proc. Natl. Acad. Sci. USA* **77**:7487–7491
Kaczmarek, L.K., Nairn, A.C., Greengard, P. 1984. *Soc. Neurosci. Abstr.* **10**:895
Kaczmarek, L.K., Strumwasser, F. 1984. *J. Neurophysiol.* **52**:340–349
Kandel, E.R., Schwartz, J.H. 1982. *Science* **218**:433–443
Karlin, A. 1980. *In:* The Cell Surface and Neuronal Function. G. Poste, G. Nicolson, and C. Cotman, editors. pp. 191–260. Elsevier/North-Holland Biomedical, New York
Kennedy, M.B., Greengard, P. 1981. *Proc. Natl. Acad. Sci. USA* **78**:1293–1297
Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S., Nishizuka, Y. 1982. *J. Biol. Chem.* **257**:13341–13348
Klein, M., Camardo, J., Kandel, E.R. 1982. *Proc. Natl. Acad. Sci. USA* **79**:5713–5717
Klein, M., Kandel, E. 1978. *Proc. Natl. Acad. Sci. USA* **75**:3512–3516
Kostyuk, P.G., Veselovsky, N.S., Fedulova, S.A. 1981. *Neuroscience* **6**:2431–2437
Kramer, R.H., Zucker, R.S. 1985*a*. *J. Physiol. (London)* **362**:107–130
Kramer, R.H., Zucker, R.S. 1985*b*. *J. Physiol. (London)* **362**:131–160
Kuo, J.F., Andersson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, I., Brackett, N.L., Katoh, N., Shoji, M., Wrenn, R.W. 1980. *Proc. Natl. Acad. Sci. USA* **77**:7039–7043
Kuo, J., Greengard, P. 1969. *Proc. Natl. Acad. Sci. USA* **64**:1349–1355
Kuret, J., Schulman, H. 1984. *Biochemistry* **23**:5495–5504
Lemos, J.R., Levitan, I.B. 1984. *J. Gen. Physiol.* **83**:269–285
Lemos, J.R., Novak-Hofer, I., Levitan, I.B. 1985. *J. Biol. Chem.* **260**:3207–3214
Levitan, I.B. 1978. *Brain Res.* **154**:404–408
Levitan, I.B., Benson, J.A. 1981. *Trends Neurosci.* **4**:38–41
Levitan, I.B., Harmar, A.J., Adams, W.B. 1979. *J. Exp. Biol.* **81**:131–151
Levitan, I.B., Lemos, J.R., Novak-Hofer, I. 1983. *Trends. Neurosci.* **6**:496–499
Llinas, R., McGuinness, T.L., Leonard, C.S., Sugimori, M., Greengard, P. 1985. *Proc. Natl. Acad. Sci. USA* **82**:3035–3039
Loewenstein, W.R. 1981. *Physiol. Rev.* **61**:829–913
Maruyama, Y., Peterson, O.H. 1982. *Nature (London)* **300**:61–63
McGuinness, T.L., Lai, Y., Greengard, P. 1985. *J. Biol. Chem.* **260**:1696–1704
Means, A.R., Welsh, M.J., Dedman, J.R., Tash, J.S. 1980. *Adv. Cycl. Nucleotide Res.* **12**:373–385
Meech, R.W., Standen, N.B. 1975. *J. Physiol. (London)* **249**:211–239
Nestler, E.J., Greengard, P. 1983. *Nature (London)* **305**:583–588
Nishizuka, Y. 1984. *Nature (London)* **308**:693–698
Novak-Hofer, I., Levitan, I.B. 1983. *J. Neurosci.* **3**:473–481
Osterrieder, W., Brum, G., Hescheler, J., Trautwein, W., Flockerzi, V., Hofmann, F., 1982. *Nature (London)* **298**:576–578
Pellmar, T.C. 1981. *Cell. Molec. Neurobiol.* **1**:87–97
Peters, K., Demaille, J., Fischer, E. 1977. *Biochemistry* **16**:5691–5697
Radke, K., Martin, G.S. 1979. *Cold Spring Harbor Symp. Quant. Biol.* **44**:975–982
Reuter, H. 1974. *J. Physiol. (London)* **242**:429–451
Reuter, H. 1983. *Nature (London)* **301**:569–574
Saitoh, T., Changeux, J.-P. 1981. *Proc. Natl. Acad. Sci. USA* **78**:4430–4434
Schulman, H., Greengard, P. 1978. *Nature (London)* **271**:478–479
Shuster, M., Camardo, J., Siegelbaum, S., Kandel, E.R. 1985. *Nature (London)* **313**:392–395
Siegelbaum, S.A., Camardo, J.S., Kandel, E.R., 1982. *Nature (London)* **299**:413–417

Siegelbaum, S.A., Tsien, R.W. 1983. *Trends Neurosci.* **6**:307–313

Smilowitz, H., Hadjian, R.A., Dwyer, J.; Feinstein, M.B. 1981. *Proc. Natl. Acad. Sci. USA* **78**:4708–4712

Stewart, A.A., Ingebritsen, T.S., Manalan, A., Klee, C.B., Cohen, P. 1982. *FEBS Lett.* **137**:80–84

Strong, J. 1984. *J. Neurosci.* **4**:2772–2783

Strong, J., Kaczmarek, L.K. 1984. *Soc. Neurosci. Abstr.* **10**:1101

Strumwasser, F. 1967. *In:* Invertebrate Nervous Systems. C. Wiersma, editor. pp. 291–319. University of Chicago, Chicago

Suarez-Isla, B.A., Wan, K., Lindstrom, J., Montral, M. 1983. *Biochemistry* **22**:2319–2323

Takai, Y., Kishimoto, A., Inoue, M., Nishizuka, Y. 1977. *J. Biol. Chem.* **252**:7603–7609

Tank, D.W., Huganir, R.L., Greengard, P., Webb, W.W. 1983. *Proc. Natl. Acad. Sci. USA* **80**:5129–5133

Teichberg, V.I., Sobel, A., Changeux, J.-P. 1977. *Nature (London)* **267**:540–542

Treistman, S.N., Levitan, I.B. 1976. *Nature (London)* **261**:62–64

Tsien, R.W. 1973. *Nature New Biol.* **245**:120–122

Tsien, R.W. 1983. *Annu. Rev. Physiol.* **45**:341–358

Walsh, D.A., Ashby, C.D., Gonzalez, C., Calkins, D., Fischer, E.H., Krebs, E.G. 1971. *J. Biol. Chem.* **246**:1977–1985

Weigele, J.B., Barchi, R.L. 1982. *Proc. Natl. Acad. Sci. USA* **79**:3651–3655

Wiener, E.C., Loewenstein, W.R. 1983. *Nature (London)* **305**:433–435

Wilmsen, U., Methfessel, C., Hanke, W., Boheim, G. 1983. *In:* Physical Chemistry of Transmembrane Ion Motions. pp. 479–485. Elsevier, Amsterdam

Yamauchi, T., Fujisawa, H. 1983. *Eur. J. Biochem.* **132**:15–21

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