MINI-REVIEW

Phosphorylation of K⁺ Channels in the Squid Giant Axon. A Mechanistic Analysis

Eduardo Perozo[1] and Francisco Bezanilla[1]

Abstract

Protein phosphorylation is an important mechanism in the modulation of voltage-dependent ionic channels. In squid giant axons, the potassium delayed rectifier channel is modulated by an ATP-mediated phosphorylation mechanism, producing important changes in amplitude and kinetics of the outward current. The characteristics and biophysical basis for the phosphorylation effects have been extensively studied in this preparation using macroscopic, single-channel and gating current experiments. Phosphorylation produces a shift in the voltage dependence of all voltage-dependent parameters including open probability, slow inactivation, first latency, and gating charge transferred. The locus of the effect seems to be located in a fast 20 pS channel, with characteristics of delayed rectifier, but at least another channel is phosphorylated under our experimental conditions. These results are interpreted quantitatively with a mechanistic model that explains all the data. In this model the shift in voltage dependence is produced by electrostatic interactions between the transferred phosphate and the voltage sensor of the channel.

Key Words: potassium channels; protein phosphorylation; squid giant axon; negative surface charges; electrostatic interactions; kinetic models.

Introduction

Potassium channels are among the most diverse groups of membrane proteins. Although structurally related, many differences can be found in kinetic and conductive properties, due to an abundance of structural genes and to the presence of alternative splicing mechanisms affecting those genes

---

[1] Department of Physiology, Jerry Lewis Neuromuscular Research Center and Ahmanson Laboratory of Neurobiology, University of California at Los Angeles, Los Angeles, California, 90024.

(Schwarz *et al.*, 1988). Given their diversity, K$^+$ channels are frequently the target of modulatory factors including protein phosphorylation (Levitan, 1989), Ca^{+2} ions (Latorre *et al.*, 1989), or GTP-binding proteins (Brown and Birmbaumer, 1990; Brown, this volume). Despite the detailed knowledge about the biochemical mechanisms leading to the modulation of many voltage-dependent channels, the biophysical principles explaining the induced changes in macroscopic or single-channel activities are not well understood.

As the giant axon of the squid is one of the few experimental models in which macroscopic, single-channel, and gating current experiments can be performed, it is the preparation of choice for the study of the modulation of voltage-dependent channels. Ideally, one would like to determine quantitatively the effects of protein phosphorylation on the properties of the delayed rectifier channel in squid, and from those observations try to establish the physical principles underlying the effects. Here, we present an overview of the work done on the effects of phosphorylation on the delayed rectifier current in the squid which has provided some insight into the possible molecular mechanisms induced by phosphorylation on channel gating.

Some Biochemical Properties

We have used the macroscopic ionic currents recorded under voltage clamp conditions as our "biochemical assay" to characterize the basic biochemical properties of this system. In internally dialyzed axons, removal of ATP from the dialyzate depresses K$^+$ currents (I_K) (see Fig. 1). Restitution of ATP in the dialysis solution potentiates the amplitude of I_K. This effect can be repeated through several cycles of washing-out and replenishing of ATP, implying that the dialyzed axon is able to support both the forward and backward reactions responsible for this effect.

A phosphorylation mechanism has been suggested to explain this effect. In dialyzed axons (where the axoplasm is not washed out), addition of nonhydrolyzable analogs of ATP (AMPPCP, AMPPNP) have no effect on I_K (Fig. 2a). In addition, Mg^{+2} is an absolute requirement for the ATP-mediated effect (Bezanilla *et al.*, 1986). The K_m for Mg-ATP is 10 μM, which

---

Fig 1. Effects of ATP on the macroscopic delayed rectifier current. (A) Family of potassium currents recorded from a dialyzed axon in the nominal absence of ATP (upper traces) and in the presence (lower traces) of 2 mM Mg-ATP. The holding potential was − 60 mV. (B) Conductance vs. voltage curve. The conductance was calculated as the sum of the steady-state and tail currents divided by the voltage change. Open symbols are values from an axon after 50 min of dialysis with a 0 ATP solution. The filled circles correspond to the same axon after addition of 2 mM Mg-ATP to the dialysis solution. (From Perozo *et al.*, 1989.)   ⟶
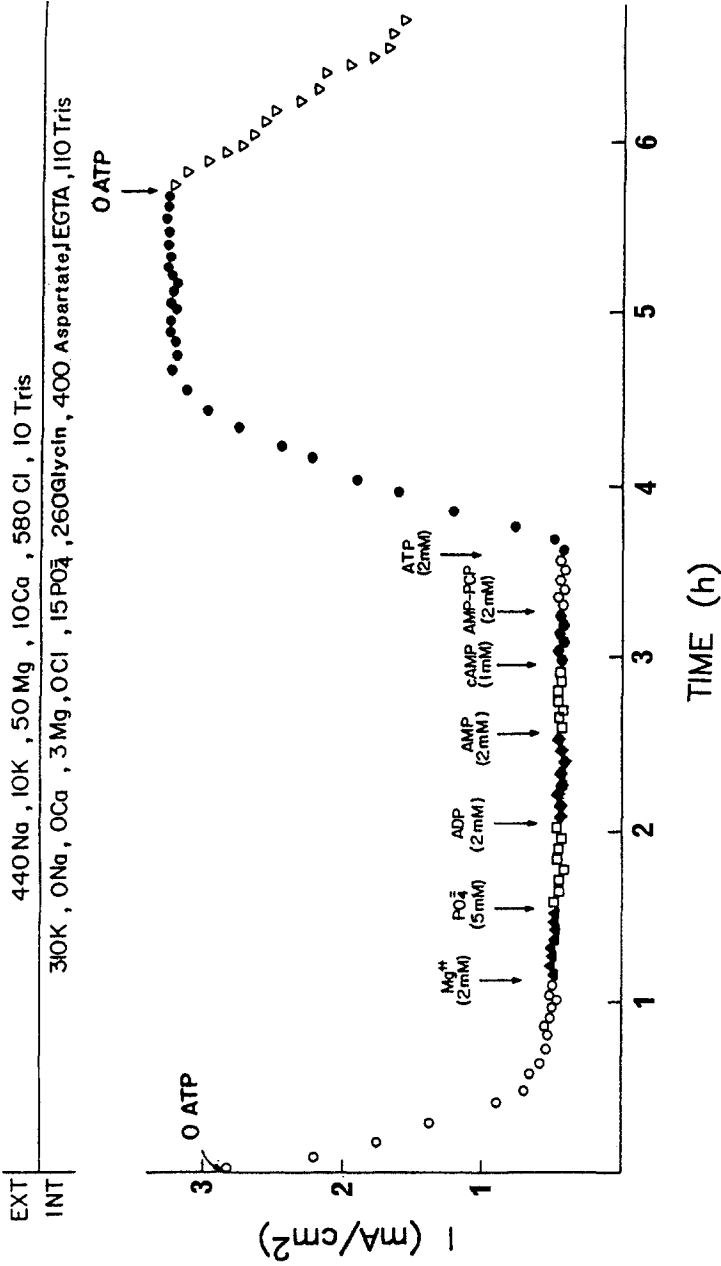
A

I (mA/cm^2)

O ATP

2 mM ATP

TIME (ms)

B

G$_K$ (mS/cm^2)

V (mV)
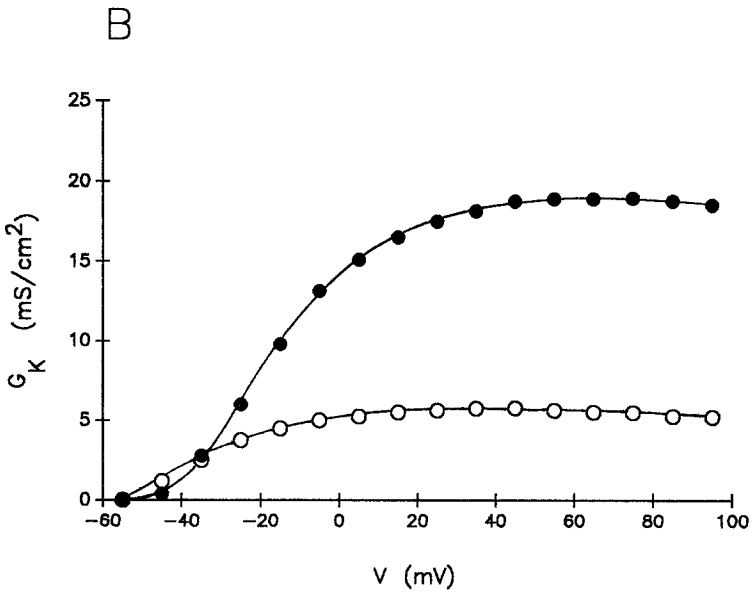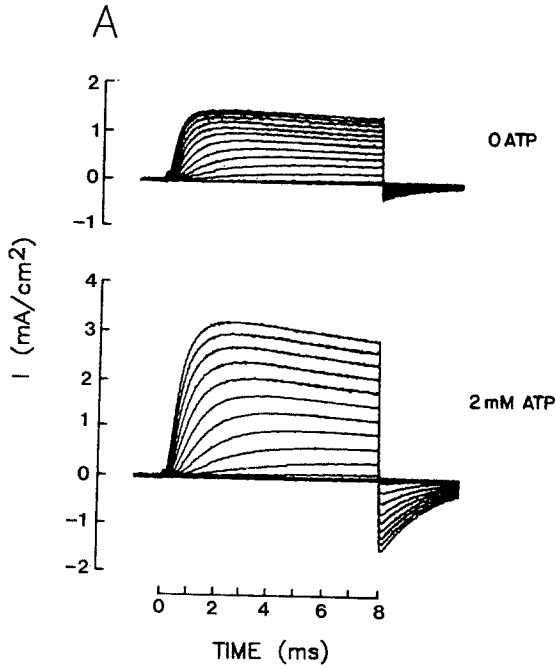
Fig. 2a. Selectivity of the K-current potentiation effect to other nucleotides. (A) Effect of the adenine nucleotide family on I_K amplitude. Each symbol represents a new change of internal solution, as noted by the arrows. (B) Effect of other purinic nucleotides on I_K amplitude. The experimental points correspond to the steady value of I_K elicited by a pulse to 0 mV from a holding potential of -60 mV. (From Perozo et al., 1989.)
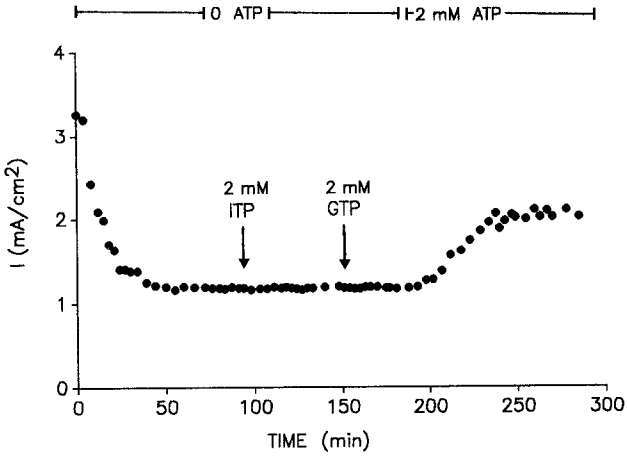
Fig. 2b.

is in the vicinity of the values found for many protein kinases. There is no effect of other adenine nucleotides, or other purinic nucleotides (GTP or ITP) by themselves (Fig. 2b), although GTP-γ-S seems to potentiate the effect of ATP on I_K (Perozo and Bezanilla, 1989).

In experiments using perfused axons (where the axoplasm and all its enzymes are removed), the effects of ATP are not reversible. In the absence of axoplasm, removal of ATP does not return I_K to the control conditions. Reversibility is accomplished, however, by the addition of alkaline phosphatase to the perfusate (Fig. 3), directly suggesting that removal of phosphate
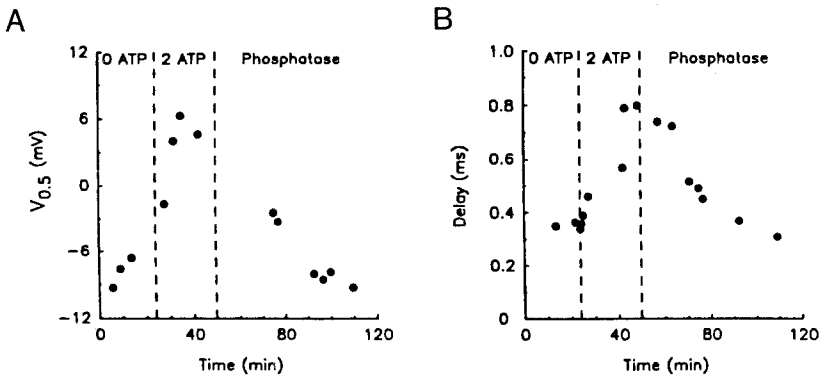

Fig. 3. Reversibility of ATP effects in the perfused axon with alkaline phosphatase. Here, the effects were monitored as changes in the kinetic properties of I_K. (A) Half-maximal activation voltage, obtained from the relative conductance vs. voltage curve. (B) Activation delay for a test pulse of 0 mV from a holding potential of -50 mV. Alkaline phosphatase was added to the perfusate at a final concentration of 100 μg/ml. (From Augustine and Bezanilla, 1990).

groups is a necessary step to reverse the ATP-mediated stimulation of I_K (Augustine and Bezanilla, 1990). These results strongly suggest that the effect of ATP on the K conductance in squid is mediated through phosphorylation, and in the rest of this review we will refer to the effects produced by ATP as phosphorylation effects.

In several preparations I_K can be modulated via extracellular neurotransmitters (Siegelbaum *et al.*, 1982; see Kaczmarek and Levitan, 1987). We have not been able to accomplish this on I_K of the squid axon. In addition, the kinase responsible for the effect has not been identified. These facts limit further speculation on the biochemical characteristics of the phosphorylation effects on the potassium current of the squid axon.

Macroscopic Currents

A combined technique of dialysis and voltage-clamp has been used. Internal dialysis allows the control of the internal milieu without washing out the macromolecular components of the axoplasm, thus presenting an obvious advantage for the study of modulation effects of phosphorylation on ion channels. Ionic currents were measured using the classical axial wire voltage clamp described in detail elsewhere (Bezanilla *et al.*, 1982; Stimers *et al.*, 1987).

When potassium currents are elicited by depolarizing pulses from a holding potential of $-60\,\text{mV}$, the effect of phosphorylation is to increase the magnitude of I_K and to slow down its activation kinetics (Figs. 1 and 4) (Bezanilla *et al.*, 1986; Perozo *et al.*, 1989; Augustine and Bezanilla, 1990). The effect on the magnitude of the current can be explained by a phosphorylation-induced shift of the steady-state inactivation curve toward more positive potentials (Fig. 5) (Perozo *et al.*, 1989). Therefore, at $-60\,\text{mV}$, between 30–50% of the delayed rectifier channel population is inactivated in the absence of ATP, and this number decreases to 5% after addition of ATP to the internal solution.

The effect on the activation process originates from a similar shift in the activation curve. In the presence of ATP, the relative conductance vs. voltage curve is shifted $10–15\,\text{mV}$ toward the more positive potentials. A similar shift is observed in the voltage dependence of the turn-on and turn-off kinetic constants. Consequently, the turn-on kinetics becomes slower upon phosphorylation, while the turn-off kinetics speeds up (Fig. 4). An additional effect of phosphorylation is that the delay induced by hyperpolarizing pulses —the Cole–Moore shift (Cole and Moore, 1960)—increases at all potentials. The interpretation of such an increase is that in a kinetic scheme obeying Markovian processes with multiple closed states ($C_1, C_2, \ldots C_n$), the population
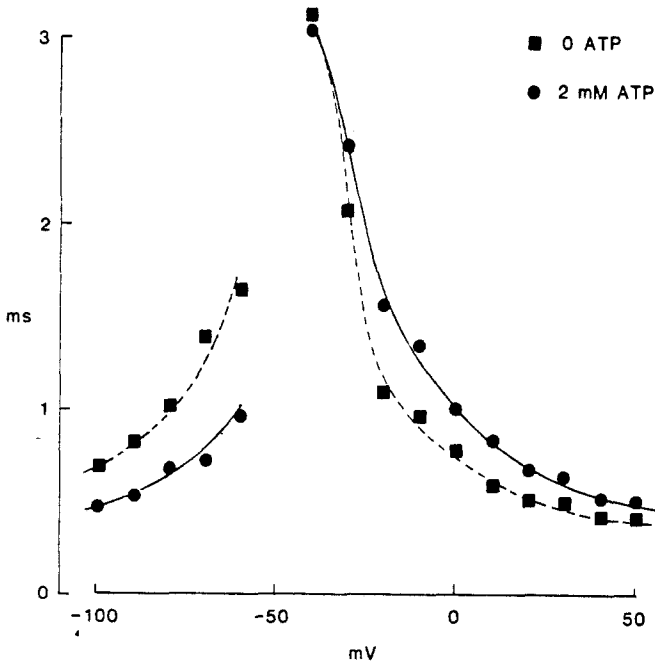
Fig. 4. Shift in the voltage dependence of the turn-on and turn-off time constants by phosphorylation. Data points represent single exponential fits to the rising phase of the currents after the initial lag (turn on) or the tail recovery after a given prepulse. (From Bezanilla et al., 1986.)
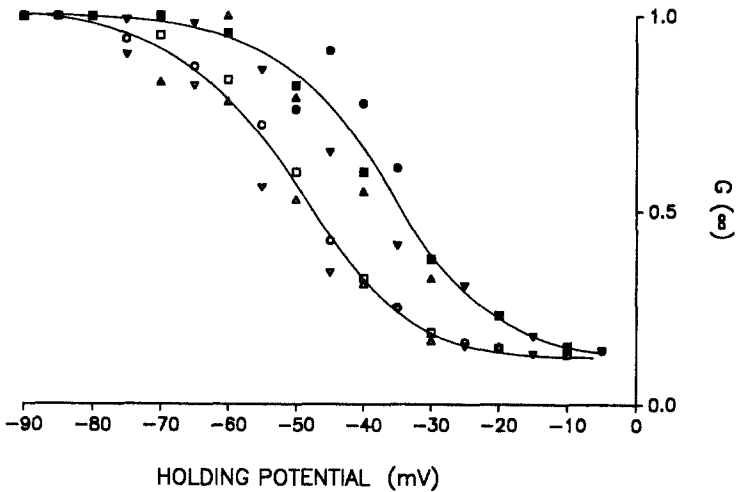

HOLDING POTENTIAL (mV)

Fig. 5. Effect of phosphorylation on the delayed rectifier steady-state inactivation curve. Experimental points were calculated from the ratio of I_K at a given holding potential and I_K at a holding potential of -80 mV (where no inactivation is present). The continuous curve was drawn according to Eq. (3). Open symbols: control in 0 ATP. Filled symbols: axons with 2 mM ATP. Different symbols represent different axons. (From Perozo et al., 1989.)

of channels that is phosphorylated tends to be sharply distributed around the farthest closed state. The nonphosphorylated channel, on the other hand, will show a much broader distribution among all closed states. All these results point to a common interpretation of the origin of the ATP effects, namely that the phosphorylation induces a local hyperpolarization responsible for all the changes in kinetics and macroscopic current amplitude (Perozo et al., 1989).

Single-Channel Experiments

The cut-open axon technique was used in all our experiments to patch the intracellular side of the membrane (Llano et al., 1988; Bezanilla and Vandenberg, 1990); therefore all membrane patches were in the "outside-out" configuration (Hamill et al., 1981). Two types of channels have been identified as the principal contributors to the K^+ conductance in squid. There is a 20 pS channel displaying bursting kinetics, and a 40 pS channel not affected by slow inactivation (Llano et al., 1988). Single-channel phosphorylation experiments were performed by photoreleasing caged ATP (with UV light) in the presence of the catalytic subunit of protein kinase A. It turned out that both the 40 pS and the 20 pS channel can be phosphorylated, which is revealed as a modification in the open probabilities for both channels. Phosphorylation has no effect on the conductive properties of either channel.

The 40 pS channel was studied in steady-state conditions (Perozo et al., 1991a), thus avoiding interferences from the 20 pS channel that readily inactivates within minutes of sustained depolarization. Upon phosphorylation, the 40 pS channel increases its open probability by more than 100 times. This behaviour was obtained by photoreleasing caged ATP in the patch pipette or by predialyzing a whole axon with ATP and then patching a piece of it. The increase in open probability was correlated with a sharp increase in the slow time constant of the closed time distribution without significantly affecting the open time distribution. Under the same experimental conditions, voltage-jump experiments demonstrated that the kinetics of activation of the 40 pS channel was of the order of seconds, too slow to have any contribution to the delayed rectifier current. Therefore, it seems that this form of the 40 pS channel does not have an important participation in the delayed rectifier current, at least under the set of conditions used in these experiments.

The effects of phosphorylation on the 20 pS channel were studied with nonstationary techniques (Perozo et al., 1991b), to remove slow inactivation. Phosphorylation produces shifts in the steady-state activation and inactivation
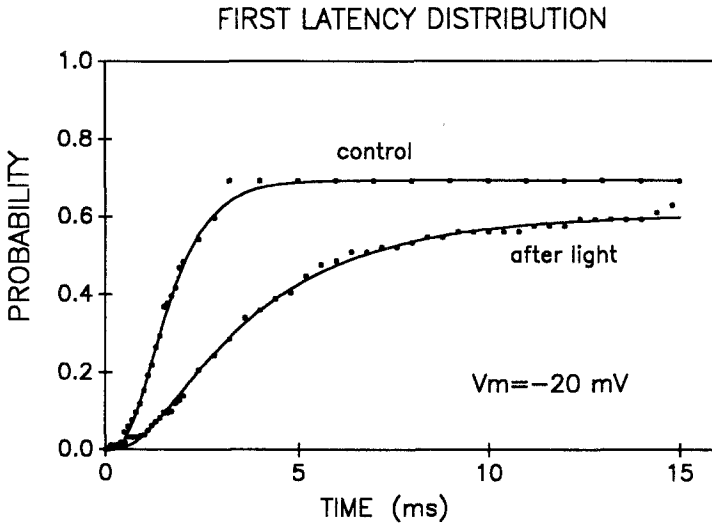
FIRST LATENCY DISTRIBUTION

Fig. 6. Effects of phosphorylation on the accumulated first latency distribution for the 20 pS channel. Phosphorylation of the channel was achieved by photoreleasing caged ATP with UV light. The control data were obtained in the dark. The continuous lines are best fits to the data with a six-state sequential model. Test pulse was -20 mV from a holding potential of -80 mV (Data from Perozo *et al.*, 1991b.)

curves, in close agreement with the results using macroscopic currents. The maximal probability of opening lies around 0.6 for both conditions, due to the fast flickering of the channel. The single-channel inactivation curve is indicative of the probability of having a blank trace during a maximal depolarization at a given holding potential. The 20 pS channel inactivation is incomplete, like the macroscopic inactivation curve, suggesting that the noninactivating component observed in macroscopic current experiments is not due to a different type of channel. Phosphorylation has no effect on the mean open time but it sharply increases the latency to the first opening at all potentials (see Fig. 6 for an example). This suggests that the last closed-to-open transition is voltage independent, and all the voltage dependence is in the transitions between closed states (as suggested by the first latencies). Given its kinetic and steady-state properties (Llano *et al.*, 1988; Perozo *et al.*, 1991b) and its response to phosphorylation, the 20 pS channel appears to be the main contributor to the delayed rectifier current. This is in agreement with tail kinetics analysis of the delayed rectifier current, which indicates the presence of a single exponential time course under a wide variety of conditions, and with slow inactivation studies that show that the inactivating component of the K$^+$ conductance forms up to 89% of the total current (Chabala, 1984; Clay, 1989; Perozo and Bezanilla, 1991).

Gating Current Experiments

Gating currents measure the movement of the "gating particle" of voltage-dependent channels across an electric field. Data from gating currents can give detailed kinetic information about the transitions among closed states, which is of key importance in the discrimination of different types of kinetic models (White and Bezanilla, 1985). Consistent with the results in macroscopic and single channel experiments, phosphorylation shifts the steady-state and kinetic parameters of K^+ gating currents (Augustine and Bezanilla, 1990) toward more positive potentials. Figure 7 illustrates some of the characteristics of the phosphorylation effects on the K^+ gating currents. The upper part of the figure shows an example of K^+ gating currents before and after internal dialysis with 2 mM Mg-ATP. As in the case of the macroscopic currents, there is a clear slowdown of the relaxation kinetics, which can be observed at most potentials. The charge transferred vs. voltage curve is shifted 5 mV toward more positive potentials in perfused axons (Fig. 7b). The same effect is found to be about 10 mV if a dialyzed axon is used, probably because the kinase is more readily available in the dialyzed axon. The voltage dependence of the gating current decay time constant is also shifted in the positive voltage direction (Fig. 7a). Again, the effect is stronger for the dialyzed than for the perfused axon.

The Role of Electrostatic Interactions

Shifts of activation and inactivation parameters along the voltage axis obtained after phosphorylation can be interpreted as the result of electrostatic interactions between an electric point charge near or at the surface of the membrane and the voltage sensor of the channel. To account for the direction of the shifts, this charge must be negative if it is located in the intracellular face of the membrane, and positive if it is located extracellulary. The negative charge could be the phosphate groups transferred from ATP to the channel itself or to another regulatory protein located within electrical distance of the voltage sensor of the channel.

Determination of the density of surface charges in the intracellular face of the membrane showed an increase in surface charges after phosphorylation (Perozo and Bezanilla, 1990). Surface charge density was measured as the voltage shift produced by different concentrations of intracellular magnesium, and calculated according to the Gouy–Chapman–Stern theory of the diffuse double layer (Grahame, 1947). The density of negative surface charges was determined to be $1/320 \, e^-/A^2$ for the nonphosphorylated
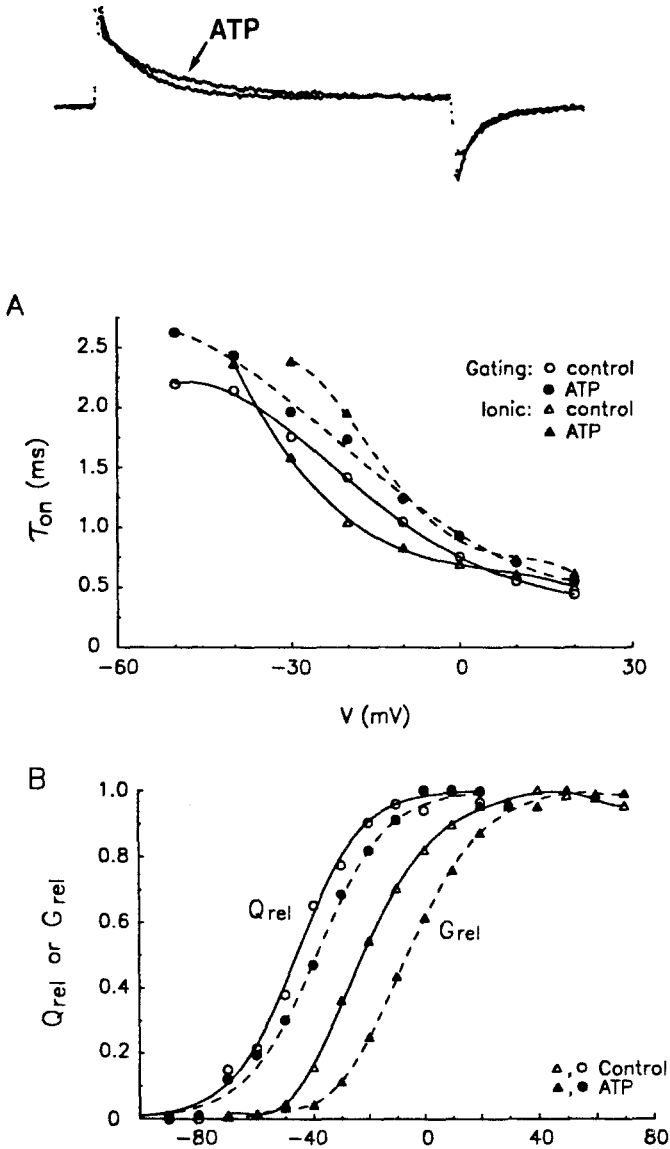
Fig. 7. Phosphorylation effects of potassium gating currents. (A) Ionic (triangles) and gating (circles) current activation kinetics plotted as a function of the test pulse potential before (open symbols) and after (filled symbols) perfusion with ATP and the catalytic subunit of protein kinase A. The holding potential was − 60 and was preceded by a prepulse to − 100 mV. (B) The relative gating charge movement (Q_{ref}, circles) plotted along with the relative conductance (G_{ref}, triangles) as a function of voltage. (From Augustine and Bezanilla, 1990.)
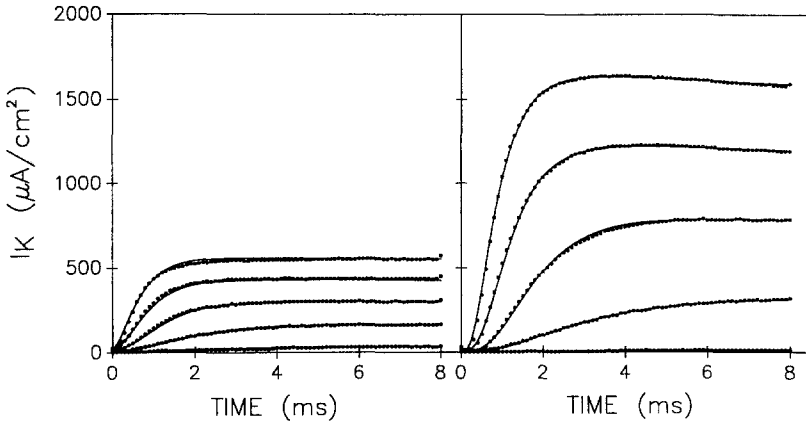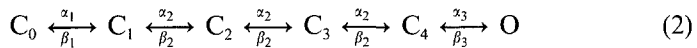
Fig. 8. Predictions of a sequential kinetic model of the delayed rectifier channel with surface charge. The dots correspond to data from experimental traces and the continuous lines are the results of the model. The test voltages are -45, -25, -5, 15, and 35 mV, and the holding potential was -55 mV. Left panel: currents in the absence of ATP. Right panel: family of K currents obtained dialyzing the same axon in the presence of 2 mM Mg-ATP. Best fitted parameters were $\alpha(0)_1 = 1.26 \, \text{ms}^{-1}$, $\beta(0)_1 = 0.05 \, \text{ms}^{-1}$, $z_1 = 2.96$, $x_1 = 0.07$, $\alpha(0)_2 = 4.57 \, \text{ms}^{-1}$, $\beta(0)_2 = 1.48 \, \text{ms}^{-1}$, $z_2 = 0.85$, $x_2 = 0.95$, $\alpha(0)_3 = 1.75 \, \text{ms}^{-1}$, $\beta(0)_3 = 2.04 \, \text{ms}^{-1}$, $z_3 = 0.11$, and $x_3 = 1.0$. (From Perozo and Benzanilla, 1990.)

condition ($\sigma_{0\text{ATP}}$), and increased to $1/155 \, e^-/\text{A}^2$ after addition of Mg-ATP (σ_{ATP}). The difference between σ_{ATP} and $\sigma_{0\text{ATP}}$ gives the density of extra charges ($\Delta\sigma$) resulting from the phosphorylation reaction.

A quantitative model for the phosphorylation of the delayed rectifier channel in squid was proposed (Fig. 8) (Perozo and Bezanilla, 1990) assuming electrostatic shifts in the exponential voltage dependences of the rate constants. The surface potential was considered in the rate constants as follows:

$$\alpha_i = \alpha(0)_i \exp[z_i x_i (V + \phi)/24] \qquad \text{(forward rate)}$$

$$\beta_i = \beta(0)_i \exp[z_i(1 - x_i)(V + \phi)/24] \qquad \text{(backward rate)} \qquad (1)$$

where $\alpha(0)_i$ and $\beta(0)_i$ are the forward and backward rate constants at zero potential, z_i and x_i are, respectively, the valence of the gating particle and its electrical distance to the peak of the energy barrier, and ϕ is the surface potential term. These rate constants govern the transitions among the states of a linear sequential model of the form

$$C_0 \underset{\beta_1}{\overset{\alpha_1}{\rightleftharpoons}} C_1 \underset{\beta_2}{\overset{\alpha_2}{\rightleftharpoons}} C_2 \underset{\beta_2}{\overset{\alpha_2}{\rightleftharpoons}} C_3 \underset{\beta_2}{\overset{\alpha_2}{\rightleftharpoons}} C_4 \underset{\beta_3}{\overset{\alpha_3}{\rightleftharpoons}} O \qquad (2)$$

which was used to compute $I_K(t)$. The slow inactivation component was assumed to be in equilibrium during the time course of the pulse (Clay, 1989)

and is defined by

$$\bar{G}(\infty) \;=\; \frac{1 - a}{1 + \exp\,[(V - V_{1/2})/k]} + a \tag{3}$$

where $\bar{G}(\infty)$ is the steady-state inactivation, a is the noninactivating component of I_K, $V_{1/2}$ is the potential where $\bar{G}_{(\infty)} = (1 + a)/2$ (the midpoint of the voltage-dependent component of $\bar{G}_{(\infty)}$), and k is the steepness factor. The time course of the potassium outward current was calculated from the sequential model, with the inactivation curve equation defining the fraction of channels available at different holding potentials.

The model accurately predicts the amplitude and kinetics of the macroscopic currents with and without ATP, using only one set of rate constants but different surface potentials. The best fit of the data was obtained with a shift of 12 mV in the voltage dependence of the rate constants and 18 mV in the steady-state inactivation curve, respectively. The model also predicts accurately the steady-state behavior and the Cole–Moore shift. These results indicate that direct electrostatic interactions between the voltage sensor of the channel and the negative charges of the phosphate groups is the basic mechanism responsible for the changes produced by ATP in the kinetics and steady-state properties of the delayed rectifier in the squid axon.

Concluding Remarks

We have shown that ATP, most likely through a phosphorylating reaction, induces important changes in the potassium currents of the squid giant axon; as a consequence, ATP also induces clear changes in the time course of the action potential (Perozo *et al.*, 1989). As we do not know what agent or stimulus changes the stage of phosphorylation *in vivo*, we are unable to decide as yet the importance of these effects in the physiology of the nerve.

The effects are mediated through a phosphorylation reaction that changes the probability of opening of at least two types of K$^+$ channels. In the case of the 20 pS channel, the main contributor of the delayed rectifier, the changes are produced by a shift of the voltage dependence to more positive potentials. All the results of gating currents, ionic currents, and single-channel recordings are consistent with the explanation that the phosphate donated by ATP increases the negative potential inside, as seen by the voltage sensor of the channel. Once the primary structure of the squid delayed rectifier is known, it should be instructive to look for phosphorylating sequences in the protein because, as these sites should be located close to

the voltage sensor that gates the channel open, it would help in unravelling the protein structure of the K^+ channel in the membrane.

References

Augustine, C. K., and Bezanilla, F. (1990). "Phosphorylation modulates potassium conductance and gating current of perfused giant axons of squid," *J. Gen. Physiol.* **95**, 245–272.

Bezanilla, F., and Vandeberg, C. (1990. "The cut-open axon technique," in *Squid as Experimental Animals.* (Gilbert, D. L., Adelman, W. J., Jr., and Arnold J. M., eds.), Plenum Press, New York, pp. 153–159.

Bezanilla, F., Vergara, J., and Taylor, R. E. (1982). "Voltage clamping of excitable membranes," in *Methods of Experimental Physics* (Ehrenstein, G., and Lecar, H., eds.), (Vol. 20, Academic Press, New York, pp. 445–511.

Bezanilla, F., Caputo, C., Dipolo, R., and Rojas, H. (1986). "Potassium conductance of the squid giant axon is modulated by ATP," *Proc. Nat. Acad. Sci. USA* **83**, 2743–2745.

Brown, A., and Birmbaumer, L. (1990). "Ionic channels and their regulation by G protein subunits," *Annu. Rev. Physiol.* **52**, 197–213.

Chabala, L. D. (1984). "The kinetics of recovery and development of potassium channel inactivation in perfused squid *(Loligo pealei)* giant axons," *J. Physiol. (London)* **356**, 193–220.

Clay, J. R. (1989). "Slow inactivation and reactivation of the K^+ channel in squid axons. A tail current analysis," *Biophys. J.* **55**, 407–414.

Cole, K. C., and Moore, J. W. (1960). "Potassium ion current in the squid giant axon: dynamic characteristic," *Biophys. J.* **1**, 1–14.

Grahame, D. C. (1947). "The electrical double layer and the theory of electrocapillarity," *Chem. Rev.* **41**, 441–501.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981). "Improved patch-clamp techniques for recording from cell and cell-free membrane patches," *Pflugers Arch.* **391**, 85–100.

Kaczmarek, L. R., and Levitan, I. B. (1987). *The Biochemical Control of Neuronal Excitability,* Oxford University Press.

Levitan, I. B. (1989). "Modulation of ion channels in neurons and other cells," *Annu. Rev. Neurosci.* **11**, 119–136.

Latorre, R., Oberhauser, A., Labarca, P., and Alvarez, O. (1989). "Varieties of calcium. Activated potassium channels," *Ann. Rev. Physiol.* **51**, 385–399.

Llano, I., Webb, C. K., and Bezanilla, F. (1988). "Potassium conductance of the squid giant axon: Single channel studies," *J. Gen. Physiol.* **92**, 179–196.

Perozo, E., and Bezanilla, F. (1989). "ATP-mediated K-channel phosphorylation in squid axons is further potentiated in the presence of GTP-γ-S," *Biophys. J.* **55**, 587a.

Perozo, E., and Bezanilla, F. (1990). "Phosphorylation effects voltage gating of K^+ channel by electrostatic interactions," *Neuron* **5**, 685–690.

Perozo, E., and Bezanilla, F. (1991). "Kinetic and steady-state properties of slow inactivation of the delayed rectifier K^+ channel," *Biophys. J.* **59**, 405a.

Perozo, E., Bezanilla, F., and Dipolo, R. (1989). "Modulation of K channels in dialyzed squid axons. ATP-mediated phosphorylation, *J. Gen. Physiol.* **93**, 1195–1218.

Perozo, E., Vandenberg, C., Jong, D. S., and Bezanilla, F. (1991a). "Single channel studies of the phosphorylation of K^+ channels in the squid giant axon. I. Steady-state conditions," *J. Gen. Physiol.,* in press.

Perozo, E., Jong, D. S., and Bezanilla, F. (1991b). "Single-channel studies of the phosphorylation of K^+ channels in the squid giant axon. II. Nonstationary conditions," *J. Gen. Physiol.,* in press.

Schwarz, T. L., Tempel, B. L., Papazian, D. M., Jan, Y. N., and Yan, L. Y. (1988). "Multiple potassium-channel components are produced by alternative splicing at the Shaker locus in Drosophila," *Nature (London)* **331**, 137–142.

Siegelbaum, S. A., Camardo, J. S., and Kandel, E. R. (1982). "Serotonin and cyclic AMP close single Kp channels in *Aplysia* sensory neurons," *Nature (London)* **299**, 413–417.

Stimers, J. R., Bezanilla, F., and Taylor, R. E. (1987). "Sodium channel gating current. Origin of the rising phase," *J. Gen. Physiol.* **89**, 521–540.

White, M. M., and Bezanilla, F. (1985). "Activation of squid axon K$^+$ channels. Ionic and gating current studies," *J. Gen. Physiol.* **85**, 539–554.