

Single Channel Studies of the Phosphorylation of K⁺ Channels in the Squid Giant Axon

II. Nonstationary Conditions

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ABSTRACT The effects of phosphorylation on the properties of the 20-pS channel of the squid giant axon were studied using the cut-open axon technique. Phosphorylation of the channel was achieved by photoreleasing caged ATP (inside the patch pipette) in the presence of the catalytic subunit of the protein kinase A. An inverted K⁺ gradient (500 K⁺ external//5 K⁺ internal) was used to study the activation process. Phosphorylation decreased the frequency of openings of the channel at most potentials by shifting the probability vs. voltage curve toward more positive potentials. The mean open times showed no voltage dependence and were not affected by phosphorylation. The distribution of first latencies, on the other hand, displayed a sharp voltage dependence. Phosphorylation increased the latency to the first opening at all potentials, shifting the median first latency vs. voltage curve toward more positive potentials. The slow inactivation process was studied in the presence of a physiological K⁺ gradient (10 K⁺ external//310 K⁺ internal). Pulses to 40 mV from different holding potentials were analyzed. Phosphorylation increases the overall ensemble probability by decreasing the number of blank traces. A single channel inactivation curve was constructed by computing the relative appearance of blank traces at different holding potentials before and after photoreleasing caged ATP. As determined in dialyzed axons, the effect of phosphorylation consisted in a shift of the inactivation curve toward more positive potentials. The 20-pS channel has the same characteristics as the delayed rectifier current in activation kinetics, steady-state inactivation, and phosphorylation effects.

INTRODUCTION

Understanding the modulation of neuronal excitability by protein phosphorylation requires a detailed knowledge of the functional properties of ion channels and their

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regulation. Since it is possible to obtain accurate information from macroscopic, gating, and single channel currents, the squid giant axon constitutes an ideal preparation for studying the molecular basis of ion channel modulation. In the accompanying article (Perozo et al., 1991), it was shown that in the cut-open axon of the squid, phosphorylation of the 40-pS channel increases its probability of opening at steady state. This is a consequence of a decrease in the slow component of the closed time distribution without a considerable effect on the distribution of open times. It was also shown that, for the most part, the contribution of the 40-pS channel to the delayed rectifier current must be small, due to the extremely long latencies to the first opening exhibited during voltage-jump experiments.

In this report we analyze the effects of phosphorylation on the single channel properties of the 20-pS channel of the squid giant axon. This channel has been implicated as the main contributor to the delayed rectifier current (Llano et al., 1988). Because the 20-pS channel is affected by a slow inactivation process, nonstationary techniques were used. Phosphorylation of the 20-pS channel was achieved by changing the concentration of ATP in the patch pipette using caged ATP.

Here it is shown that the voltage dependencies of the single channel inactivation and open channel probability are shifted by phosphorylation toward more depolarized potentials, in agreement with data obtained in dialyzed (Perozo et al., 1989) or perfused (Augustine and Bezanilla, 1990) axons. Kinetic analysis demonstrates that phosphorylation of the 20-pS channel mainly affects the latency to the first opening, without a dramatic effect on the dwell time distribution. Some of the present results have been presented in abstract form (Perozo et al., 1990).

METHODS

Experimental Methods

Details of the methodology used appear in the preceding paper (Perozo et al., 1991). We used the cut-open giant axon of the squid *Loligo pealei* (Llano et al., 1988; Bezanilla and Vandenberg, 1990). Excised patches in the outside-out configuration of Hamill et al. (1981) were used in all the experiments. Patch pipettes were pulled from Corning 7052 glass (Corning Glass Co., Claremont, CA) and filled with a solution containing (in mM): 310 K-glutamate, 140 NMG-PO₄ (*N*-methyl-D-glucamine-PO₄), 1 EGTA, 4 MgCl₂, 5 glutathione, 1×10^{-4} catalytic subunit of protein kinase A, and 2 caged ATP (Calbiochem Corp., La Jolla, CA). The external solution was artificial sea water (ASW) containing 300 nM TTX and 1 mM NaCN.

Photolysis of the caged ATP was achieved by illuminating the tip of the pipette with a 150-W xenon lamp focused on one end of a fused silica fiber optic. Light from the xenon lamp was filtered with an UG-11 filter and with a filter to control the passage of short-wavelength UV. Illumination of the patch proceeded for no more than 10 min.

Channels were dephosphorylated using the "diffusion method" described in Perozo et al. (1991). Briefly, by cutting-open and incubating the axon in a 0 Ca²⁺ -0 Cl⁻ solution, the axoplasm remains on top of the membrane as a gel-like structure. As the diffusion coefficient for ATP is ten times larger than the one for any phosphatase, the membrane-axoplasm interface is quickly depleted of ATP but not of phosphatase. The action of the phosphatase in an ATP-free environment effectively dephosphorylates any available phosphate present.

Data Acquisition and Analysis

Control of the pulse generation, data acquisition, and display was done from a 16-bit IBM type microcomputer with a 12-bit A/D and D/A converter, according to the design of Stimers et al. (1987). Current traces were filtered at 5 kHz with an 8-pole Bessel filter before acquisition at 20 μ s/point, and later filtered digitally at 3 kHz with a Gaussian filter. Acquired data were stored directly in the computer hard disk and later transferred to a high capacity optical drive (IBM 3363; IBM, Tucson, AZ) for subsequent analysis.

Single channel traces were surveyed to locate and accumulate blank traces. Averaged blanks were subtracted from all the traces. Single channel events were idealized following the 50% amplitude criteria (Colquhoun and Sigworth, 1983), and accumulated to generate the ensemble probability traces. Mean open and closed times were calculated from logarithmic dwell time histograms obtained from idealized records, and were fitted using a binned maximum likelihood algorithm (Sigworth and Sine, 1987). The data fitted included potentials with little or no overlap among transitions, in order to avoid biasing of the dwell time histograms toward short openings (Patlak, 1983). Latencies to the first opening were also calculated from idealized traces. In multichannel patches, first latencies were corrected by taking the n th root of the observed first latencies, where n is the number of channels (Aldrich et al., 1983). Nonlinear fits to the accumulated first latencies were performed according to a sequential kinetic scheme (see Scheme A) using SCoP and SCopFit, a simulation control and fitting program from the National Biomedical Simulation Resource at Duke University.

Dialysis Experiments

To compare the effects of phosphorylation on the 20-pS channel studied with single channel recording and the results obtained from macroscopic current experiments, whole axons were dialyzed and simultaneously voltage clamped. The experimental method is described in detail elsewhere (Dipolo et al., 1985; Perozo et al., 1989). Giant axons were dialyzed for 1 h with an internal solution containing (in mM): 310 K-glutamate, 140 NMG-PO₄, 4 MgCl₂, 10 Mops, 1 EGTA, and 0 ATP. The external solution was ASW containing 300 nM TTX and 1 mM NaCN. Potassium currents were measured throughout the dialysis procedure with an axial wire voltage clamp. Currents were monitored by pulsing to 0 mV from a holding potential of -55 mV. Phosphorylation was induced by adding Mg-ATP directly to the dialysis solution.

RESULTS

Phosphorylation Effects on the Activation Process

The activation process of the 20-pS channel was studied in the nominal absence of inactivation. Pulses to various test potentials were applied from a holding potential of -80 mV. At such potential no inactivation was present, as determined in macroscopic current experiments (Ehrenstein and Gilbert, 1966; Chabala, 1984).

Under conditions of a normal potassium gradient (high K⁺ intracellular, low extracellular), it is not possible to determine the open probability at potentials near reversal. This procedure also introduces serious errors in the determination of the middle part of the probability curve. These considerations are particularly relevant for the delayed rectifier channel in squid, with a midpoint of the conductance vs. voltage curve between -30 and -10 mV. We have avoided these complications by reversing the potassium gradient across the membrane patch. Although it has been

reported that high external potassium slows down K channel activation kinetics slightly (Stuhmer, 1980), it does not affect the conclusions of this report.

For these experiments, the external solution contained 500 mM K while the solution inside the patch pipette contained 5 mM K. Fig. 1 shows the steady-state

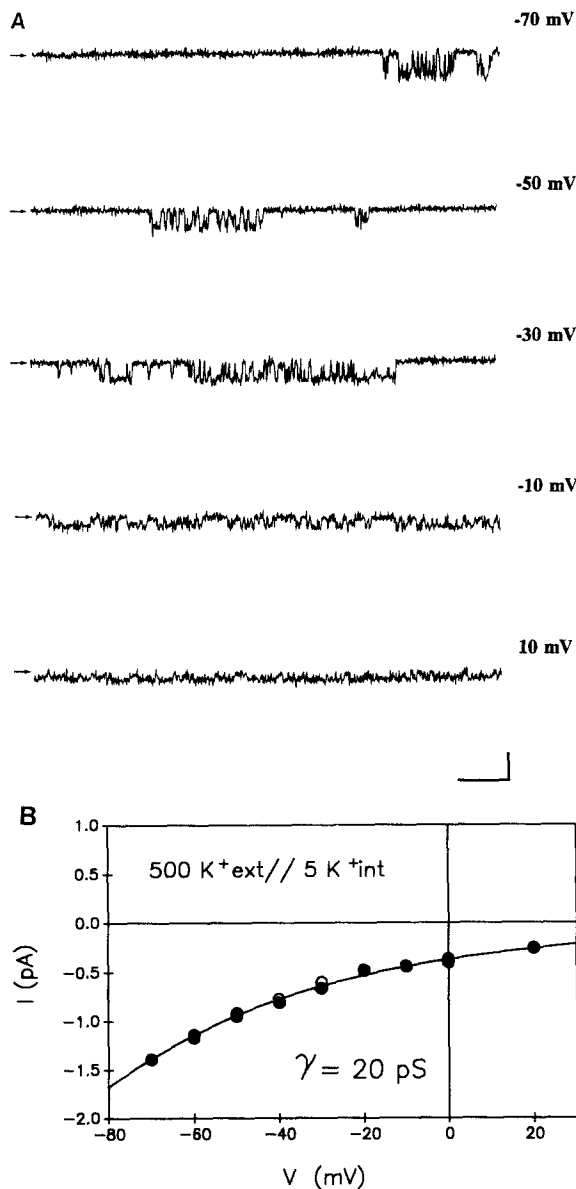


FIGURE 1. Conductive properties of the 20-pS channel in a reverse gradient. (A) Single channel recordings from the cut-open axon. The solution inside the pipette contained 5 mM K⁺ and the external solution contained 500 mM K⁺. Traces shown are from a channel recorded in the dark (presumed not to be phosphorylated). The arrow points to the closed state. Vertical scale, 1.1 pA; horizontal scale, 20 ms. (B) I - V relation for the same channel before and after photorelease of ATP. The continuous line was drawn as visual reference. Single channel conductance was calculated as the slope (at -60 mV) of a polynomial fit to the I - V data points. Open circles, channels recorded in the dark; filled circles, same patch after 4 min illumination. Temperature, 15°C. Traces were filtered at 5 kHz and digitized at 50 μ s/point.

behavior and I - V curve of the 20-pS channel. The I - V curve shows a nonlinear behavior and cannot be fitted with the Goldman-Hodgkin-Katz equation (Hodgkin and Katz, 1949). The single channel chord conductance ($G = i/(V - V_K)$) was calculated as 21.8 pS from a linear fit to the initial part of the I - V curve between -80

and -40 mV. Phosphorylation has no effect on the conductive properties of the channel as the I - V curves are identical before and after caged ATP transformation (Fig. 1 *B*). A direct consequence of the use of a reversed gradient is that it becomes difficult to study the channel for pulses more positive than 20 mV, which sets an upper limit for the present analysis of the activation process of the 20-pS channel.

To determine the effects of phosphorylation on the functional properties of the 20-pS channel, the basic experimental protocol consisted in recording a series of current traces at several test potentials and then repeating the procedure after photoreleasing caged ATP in the presence of the catalytic subunit of the protein kinase A. Traces from such an experiment are shown in Fig. 2 *A*. The left panel displays selected control traces obtained in the dark at -60 , -50 , and -30 mV. The right panel shows recordings from the same membrane patch after 7 min of UV illumination. Clearly there is a decrease in the activity of the channel after phosphorylation. Fig. 2 *B* represents the ensemble probability calculated from the same experiment. Because this experiment requires the recording of an extensive number of transitions at several potentials before and after illumination, the stability of the patches was the main factor that limited its success. Still, we were able to reproduce the phosphorylation effect in five separate preparations. Control experiments were performed by illuminating membrane patches in the absence of caged ATP or in the presence of 1 mM caged Ca (nitro-5). Neither treatment was able to reproduce the phosphorylation effects seen with caged ATP.

The reduction in the burst activity of the 20-pS channel after phosphorylation can be associated with a shift in the steady-state activation curve toward more positive potentials, as revealed in Fig. 3 *A*. This is in agreement with the results obtained in dialyzed axons (Perozo et al., 1989), shown in Fig. 3 *B* for comparison. The same result was obtained in perfused axons (Bezanilla et al., 1986; Augustine and Bezanilla, 1990). Here, the term steady state refers to the maximum value reached by the open probability during the 15-ms pulse, as calculated from the ensemble probability traces. Although the shift of the activation curve produced by phosphorylation is not as large in the relative conductance curve as in the single channel measurements, the values for $V_{1/2}$ of the phosphorylated curves are rather similar (-21.7 and -20 mV). The maximal open probability (P_o) reached in the single channel activation curve tends to stabilize around 0.6, and there is no tendency to increase at higher potentials. This can be explained if the process governing the last transition from closed to the open state is fast and voltage independent, as will be shown below.

The mean open times were calculated by the ratio between the total time the channel is open and the number of open to closed transitions. Fig. 4 shows a plot of the values of mean open time at different voltages. There is no apparent voltage dependence in the mean open duration for a voltage range between -70 and 20 mV. This indicates a voltage-independent transition between the open state and the first adjacent closed state of the channel. Phosphorylation has no effect on the value or voltage dependence of the mean open times. Therefore, it could be suggested that phosphorylation affects the voltage dependence of some of the rate constants of the activation process.

A more detailed analysis was performed by fitting the open time distributions to a sum of exponential functions. Fits to the open time distributions were performed

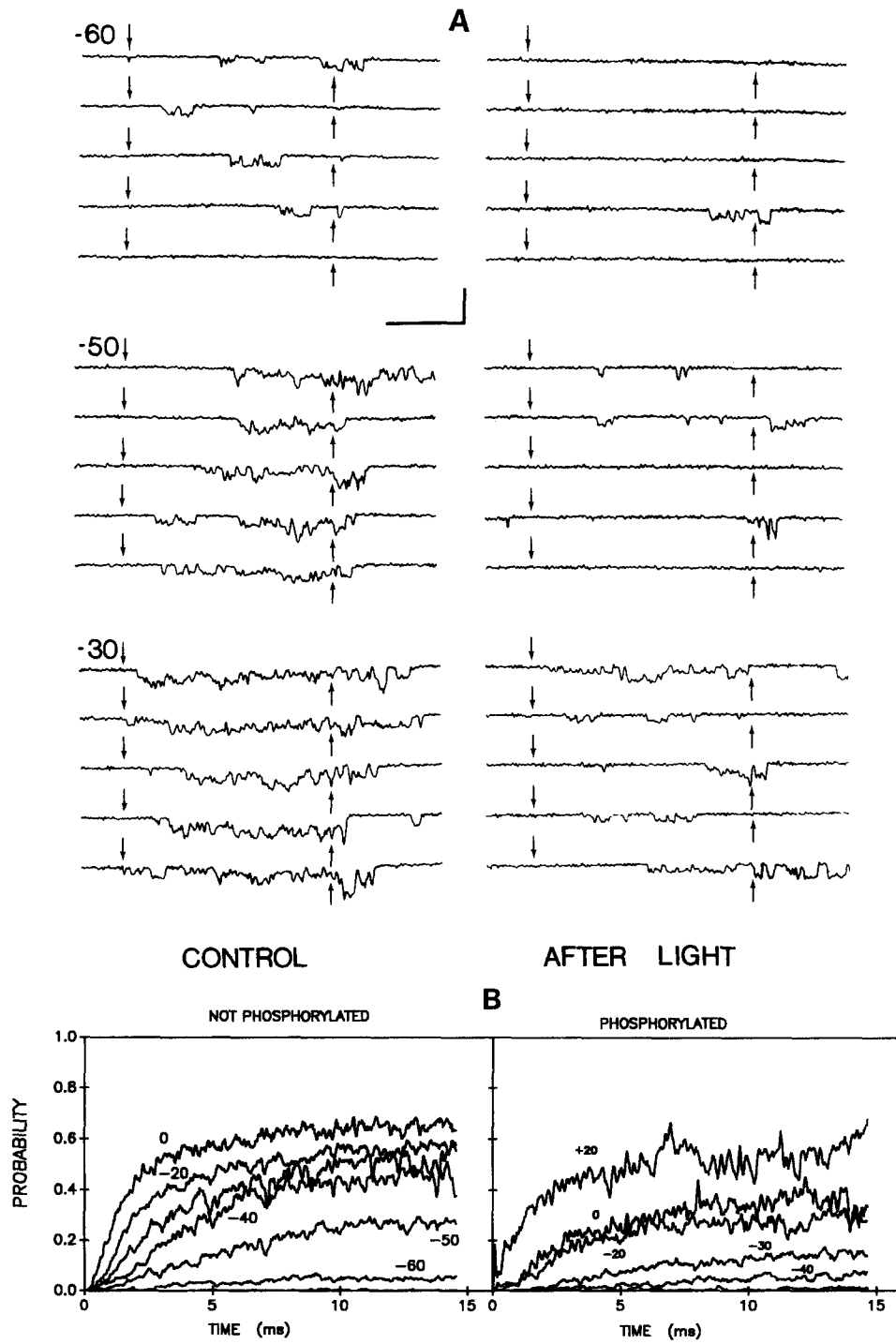


FIGURE 2.

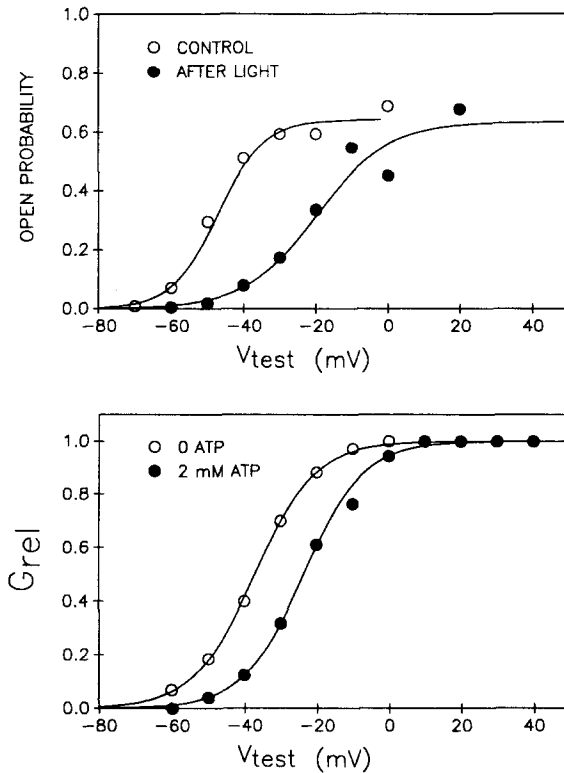


FIGURE 3. Voltage dependence of the activation process. (Top) Probability curve obtained from single channel recordings. (Bottom) Relative conductance vs. voltage curve from an internally dialyzed and voltage clamped axon following the methods of Perozo et al. (1989). In both cases, open circles represent the control, unphosphorylated state, while filled circles denote the phosphorylated condition. The continuous curves represent the best fit to a Boltzmann distribution of the form: $P_o = 1/[1 + \exp\{(V - V_{1/2})zF/RT\}]$. Fitted parameters for the control curves: single channel, $z = 4.2$, $V_{1/2} = -48.6$ mV; macroscopic, $z = 3.1$, $V_{1/2} = -37$ mV. For the curves in the presence of ATP: single channel, $z = 2.8$, $V_{1/2} = -21.7$; macroscopic; $z = 2.3$, $V_{1/2} = -21$ mV. The correction factor α for the top curves is 0.38. Temperature: single channel, 14°C; dialysis experiment, 18°C.

with a two exponential probability distribution function (PDF), using a binned maximum likelihood method according to the equation:

$$PDF = \frac{A_1}{\tau_1} \exp\left(-\frac{t}{\tau_1}\right) + \frac{(1 - A_1)}{\tau_2} \exp\left(-\frac{t}{\tau_2}\right) \quad (1)$$

Fig. 5 shows distributions of open times and their fits at various potentials before and after photoreleasing ATP. As the patch contained four channels, only potentials that showed very little or no overlap between transitions were analyzed. Although it is not

FIGURE 2. (opposite) Effects of phosphorylation on the activation process of the 20-pS channel. (A) Single channel traces before and after the photorelease of ATP. (Left panels) Control traces recorded in the dark; (right panels) same patch after 5 min illumination with UV light. Holding potential was -80 mV. The numbers at the left correspond to the test potential (in millivolts). Arrows signal the beginning and end of the 15-ms pulse. Scale bars are 3 pA and 6 ms. There are four active channels in the control traces and three after phosphorylation. Note that channels remain open after the end of the pulse, generating an inward "tail" current. (B) Ensemble probability. The numbers next to the probability traces are the values of the test potential. Temperature, 14°C. Traces were filtered at 3 kHz and digitized at 20 μ s/point.

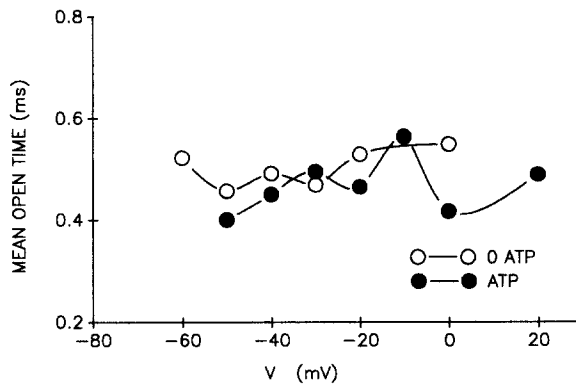


FIGURE 4. Voltage dependence of the mean open time. The mean open time was calculated from the ratio between the total open time of the channel and the number of open to closed transitions. Open circles are from control recordings and filled circles are calculated after the photorelease of ATP.

possible to draw definite conclusions from the present data, the general tendency over a wide voltage range is that the fast exponential component is fairly voltage independent, and seems to determine the value and voltage dependence of the mean open time. On the other hand, the slow time constant shows a marked voltage dependence. Phosphorylation has no apparent effect on the fast time constant, but it seems to shift the voltage dependence of the slow time constant toward more positive potentials.

In clear contrast to the voltage insensitivity of the transition between the open and the last closed state, some or most of the transitions among closed states are highly

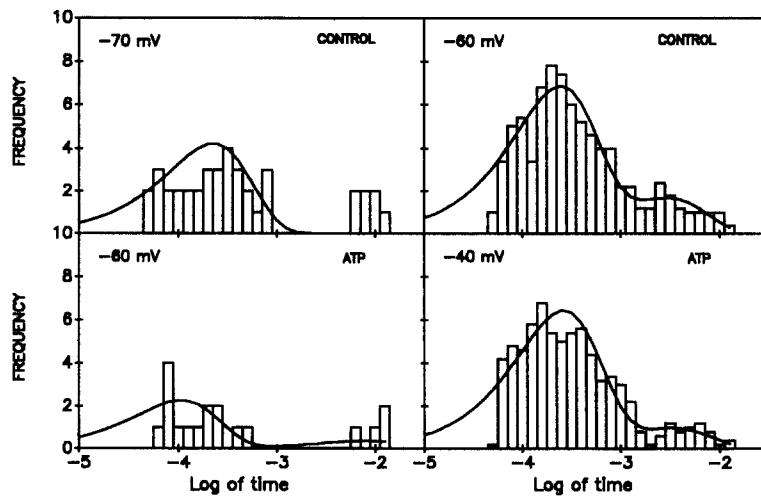


FIGURE 5. Fitted distributions of open times before and after phosphorylation. Data shown correspond to small depolarizations in order to avoid biases due to event superposition. Histograms were fitted using a binned maximum likelihood algorithm according to Eq. 1. The upper panels represent data obtained in the dark, while the lower panels correspond to the same patches after illumination. Test potentials are displayed in the upper left corner of each panel. Data were filtered at 3 kHz and include only binned dwell times longer than the acquisition time. Temperature, 14°C.

voltage dependent. This conclusion derives from the accumulated first latency distributions shown in Fig. 6 *A* at different potentials, which reveal that the delay to the first opening becomes progressively smaller by increasing the amplitude of the voltage pulse. Due to its voltage dependence, the distribution of accumulated first

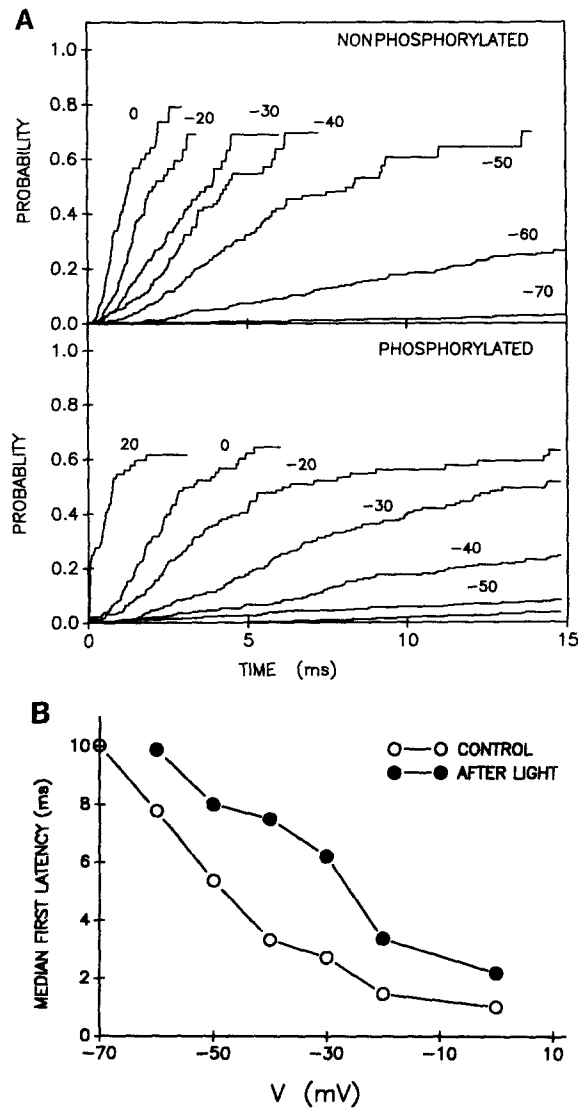
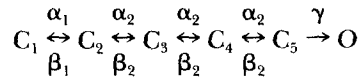


FIGURE 6. Voltage dependence of the accumulated latencies for the first opening. (*A*) The upper panel is the control condition, and the lower panel comes from the same patch after phosphorylation with photoreleased ATP. Numbers next to each trace represent the value of the test potential (in millivolts). (*B*) Voltage dependence of the midpoint of the first latency distribution. Data were filtered at 3 kHz; temperature, 14°C.

latencies is extensively affected by phosphorylation. The lower panel of Fig. 6 *A* contrasts the accumulated first latency at different potentials after photorelease of ATP in the patch pipette. For every test potential there is an increased delay to the first opening when the channel is phosphorylated. This is illustrated as a shift in the midpoint of the first latency distribution (Fig. 6 *B*) toward more positive potentials.

The accumulated first latency distribution was fitted with a sequential scheme with five closed states and one open state, illustrated in Scheme A:



Scheme A

where α_1 and β_1 govern the first transition, α_2 and β_2 the transitions between intermediate closed states, and γ the rate of opening. In the formulation of this

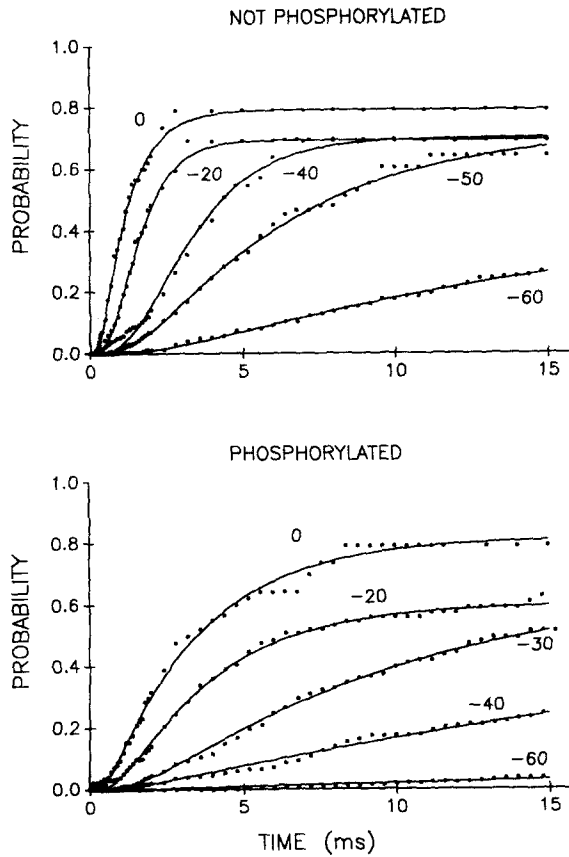


FIGURE 7. Fits of the accumulated first latencies from a sequential model. Numerical solutions to the model illustrated in scheme A were fitted to the data and minimized according to the χ^2 criterion. *Upper panel*, control; *lower panel*, phosphorylated channels. Best fitted parameters for the control curves were $\alpha(0)_1 = 1.3 \text{ ms}^{-1}$, $\beta(0)_1 = 0.045 \text{ ms}^{-1}$, $z_1 = 2.01$, $x_1 = 0$, $\alpha(0)_2 = 6.7 \text{ ms}^{-1}$, $\beta(0)_2 = 0.02 \text{ ms}^{-1}$, $z_1 = 1.91$, $x_1 = 0.46$, $\alpha(0)_3 = 23 \text{ ms}^{-1}$, $\beta(0)_1 = 1.36 \text{ ms}^{-1}$, $z_1 = 0$, $x_1 = 1.0$. For the experimental curves they were $\alpha(0)_1 = 0.36 \text{ ms}^{-1}$, $\beta(0)_1 = 0.005 \text{ ms}^{-1}$, $z_1 = 1.84$, $x_1 = 0.06$, $\alpha(0)_2 = 2.43 \text{ ms}^{-1}$, $\beta(0)_2 = 0.019 \text{ ms}^{-1}$, $z_1 = 1.55$, $x_1 = 0.56$, $\alpha(0)_3 = 23 \text{ ms}^{-1}$, $\beta(0)_1 = 1.36 \text{ ms}^{-1}$, $z_1 = 0$, $x_1 = 1.0$.

model we have considered data from macroscopic, gating, and single channel currents. (a) The first step must be slower than the following steps to produce a rising phase in the K^+ gating current; (b) the last step is not rate limiting and not voltage dependent; (c) the intermediate steps must account for the overall voltage dependence of the first latency distribution (see White and Bezanilla, 1985; Perozo and Bezanilla, 1990). Nonlinear fits to the first latency data were performed numerically, assuming that the rate constants depend exponentially on transmembrane potential

according to the equations:

$$\alpha_i = \alpha(0)_i \exp\left(z_i x_i \frac{FV}{RT}\right) \quad (2)$$

$$\beta_i = \beta(0)_i \exp\left(-z_i(1-x_i) \frac{FV}{RT}\right) \quad (3)$$

where $\alpha(0)_i$ and $\beta(0)_i$ are the values of the rate constants at zero potential, z_i is the number of electronic charges of the gating particle, and x_i is the electric distance to the peak of the barrier (Stimers et al., 1985). V , R , F , and T have their usual meanings. Given those constraints, the only parameters to fit were the values of the rate constants at zero potential and their voltage dependence (z_i and x_i). The results

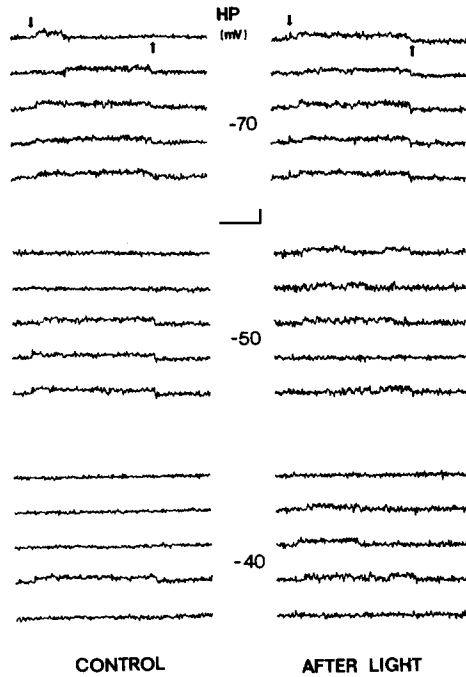


FIGURE 8. Effects of phosphorylation on the inactivation of the 20-pS channel. Single channel recordings obtained in a physiological K gradient: 310 K⁺ internal//10 K⁺ external. Test potential for all records is 40 mV. The numbers between panels represent the holding potential (HP). *Left panels*, control; *right panels*, after photorelease of ATP. Temperature, 14°C. Filter, 5 kHz, digitized at 20 μ s/point. Scale bars are 1 pA and 5 ms.

of the fits are shown in Fig. 7. A direct result from the fit is that phosphorylation shifts the voltage dependence of the rate constants between 9 and 17 mV toward more positive potentials. Therefore, the opening kinetics becomes slower at all potentials.

Phosphorylation Effects on the Slow Inactivation Process

The inactivation of the 20-pS channel was studied under conditions of maximal channel activation. Pulses to 40 mV were applied to activate the channel, and the holding potential (HP) was varied from -80 to -30 mV. At 40 mV, the probability of

opening of the 20-pS channel is near maximal (see Fig. 3 A). Since the test pulse is very positive, all inactivation experiments were performed using a physiological K^+ gradient (10 K^+ external//310 K^+ internal) to improve the resolution in the measurement of the current. The conductive behavior of the 20-pS channel under these ionic conditions is illustrated in the accompanying paper (Perozo et al., 1991, Fig. 3).

Fig. 8 shows some recordings of the 20-pS channel at different holding potentials before and after caged ATP transformation. There is only one channel in the patch.

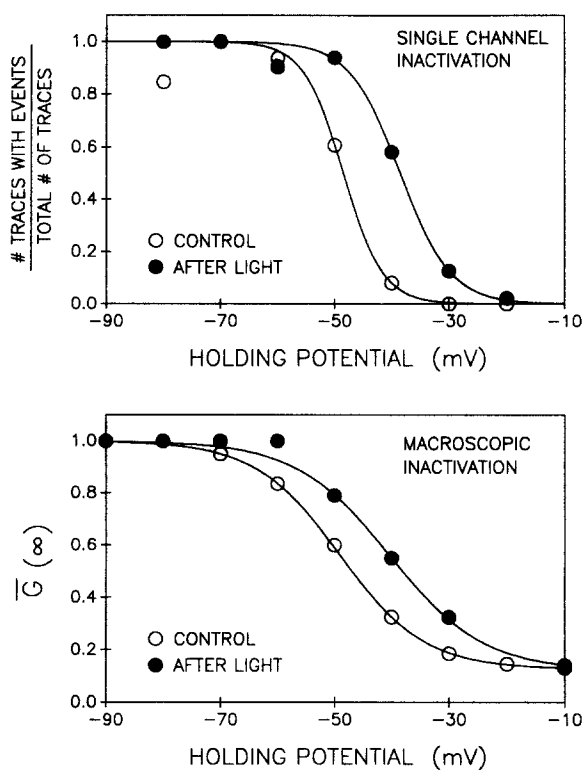


FIGURE 9. Effects of phosphorylation on the voltage dependence of the inactivation process of the 20-pS channel. *Upper panel*, single channel inactivation curve. The inactivation parameter was calculated as the ratio between the number of traces with single channel transitions and the total number of traces. *Bottom panel*, macroscopic inactivation. The inactivation parameter ($\bar{G}_{(\infty)}$) was calculated as the ratio between the current at any given holding potential (HP) and the current at a HP of -80 mV. The curve has been corrected for channels already open at any given HP. *Open circles*, control condition; *filled circles*, phosphorylated channels. Each curve represent the best fit to a Boltzmann distribution of the form $P_o = (1 - a) / [1 + \exp[-(V - V_{1/2})zF/RT]] + a$. Fitted parameters for the control curves: single channel, $z = 7.1$, $V_{1/2} = -48$ mV, $a = 0$; macroscopic, $z = 4.5$, $V_{1/2} = -51$ mV, $a = 0.135$. For the experimental curves: single channel, $z = 5.5$, $V_{1/2} = -38$ mV, $a = 0$; macroscopic, $z = 4.2$, $V_{1/2} = -42$ mV, $a = 0.135$. Temperature for single channel experiments was 14°C , and for macroscopic current experiments 18°C .

control curves: single channel, $z = 7.1$, $V_{1/2} = -48$ mV, $a = 0$; macroscopic, $z = 4.5$, $V_{1/2} = -51$ mV, $a = 0.135$. For the experimental curves: single channel, $z = 5.5$, $V_{1/2} = -38$ mV, $a = 0$; macroscopic, $z = 4.2$, $V_{1/2} = -42$ mV, $a = 0.135$. Temperature for single channel experiments was 14°C , and for macroscopic current experiments 18°C .

At 40 mV, when the 20-pS channel opens, it bursts during the total duration of the pulse. The opening bursts are clustered, separated by blank traces which are interpreted as periods of slow inactivation. At negative HP the channel bursts consecutively with very few blank traces (no blank traces at very negative potentials), but the frequency of blank traces increases as the HP becomes less negative. The result of photoreleasing ATP is to increase the overall activity of the channel by reducing the number of blank traces (Fig. 8, *right*), indicating that another effect of phosphorylation at the single channel level is to reduce the steady-state inactivation.

By computing the relative appearance of blank traces at different holding potentials it is possible to construct a single channel inactivation curve for the 20-pS channel. The inactivation parameter was calculated as the ratio between the number of traces showing at least one transition and the total number of traces. Fig. 9A shows the result of plotting the inactivation parameter vs. HP calculated from the same experiment shown in Fig. 8 before and after photorelease of ATP. The reduction in the steady-state inactivation is the result of a shift of ~ 15 mV in the inactivation curve toward more positive potentials, a conclusion that is in very good agreement with results on dialyzed axons (Perozo et al., 1989).

A comparison between the single channel inactivation curve (Fig. 9A) and the macroscopic inactivation curve (Fig. 9B) reveal some differences. While in this experiment single channel inactivation is almost complete at HP more positive than -30 mV, macroscopic inactivation is never complete, with an inactivation-independent component of $\sim 12\%$ of the outward current (Chabala, 1984; Clay, 1989). This component could be due to the contribution of noninactivating channels like the active 40-pS channel or the 10-pS channel reported in the cut-open axon by Llano et al. (1988). Another difference between the two curves is their apparent voltage dependence. The single channel inactivation curve appears more voltage dependent than the inactivation curve calculated from macroscopic currents. Part of the difference in slope comes from the presence of the inactivation-independent component in the macroscopic curve that effectively compresses the relative shape of the curve. It is also possible that there is some intracellular factor present in the dialysis experiments that modifies the voltage dependence of the inactivation process and is lost during the cut-open axon procedures. Nevertheless, the conclusion that phosphorylation reduces the overall inactivation of the channel by shifting its voltage dependence toward more positive potentials is true in both preparations.

DISCUSSION

There is now consistent evidence supporting the view that the 20-pS channel is the main contributor to the delayed rectifier current in squid, although additional contributions from a variety of K⁺ channels are also present. Not only do reconstructions of the ensemble currents from the 20-pS channel strikingly resemble the time course of the delayed rectifier current, but this channel shows the same general properties regarding slow inactivation and the effects of phosphorylation by ATP measured in the macroscopic delayed rectifier current. In this report we have studied the activation and inactivation processes of the 20-pS channel of squid giant axons and their modification by protein phosphorylation. By photoreleasing caged ATP in the presence of the catalytic subunit of the protein kinase A, it was possible to directly phosphorylate the channel itself (or a very closely associated membrane component) and study its kinetic properties under nonstationary conditions.

There is little data available on the physical basis explaining the modulation of single K channel properties by protein phosphorylation. An important example in the literature is the study of phosphorylation effects on a ~ 20 -pS Ca-activated K⁺ channel from *Helix* by Ewald et al. (1985). They found that phosphorylation of this channel with ATP and the catalytic subunit of the protein kinase A increases its Ca²⁺ sensitivity, as reflected in a shift (toward smaller calcium concentrations) of the

calcium dependence of the open probability curve. Another modulation mechanism could consist, for example, in regulating the number of channels available at any time in a membrane patch. This mechanism is found in the modulation of the S channel in *Aplysia* sensory neurons (Siegelbaum et al., 1982). Nonetheless, there is no detailed knowledge on modulatory mechanisms affecting voltage- and time-dependent K channels. On the other hand, several groups have studied extensively the modulation of single Ca^{2+} channels by phosphorylation. Single channel recordings of cardiac Ca^{2+} channels have shown that phosphorylation increases the open probability by increasing the value of the mean open time and decreasing the value of the mean closed time (Cachelin et al., 1983; Brum et al., 1984). These kinetic changes are not enough to account for the magnitude of the β -adrenergic modulation of I_{Ca} . Using fluctuation analysis, Bean et al. (1984) clearly showed that Ca channel phosphorylation increases the pool of available channels that are able to open with a voltage pulse.

The general effect of the phosphorylation of the 20-pS channel appears to be an induced shift along the voltage axis in all voltage-dependent properties of the channel. This effect occurs without affecting the conductive properties of the open channel. Phosphorylation seems to affect only voltage-dependent parameters, like the single channel activation and inactivation curve, the accumulated first latency distribution, and the slow component of the open time distributions. Voltage-independent parameters such as the mean open time and the fast component of the distribution of open times are not affected by phosphorylation. These results agree well with those obtained from whole axons. For instance, in dialyzed axons phosphorylation shifts the relative conductance vs. voltage curve and the steady-state inactivation curve and slows down the kinetics of activation (Bezanilla et al., 1986; Perozo et al., 1989). In perfused axons, identical results were obtained for the macroscopic currents (Bezanilla et al., 1986), while similar shifts were found in the charge transferred vs. voltage curve derived from gating current measurements (Augustine and Bezanilla, 1990).

Clearly, the consequences of phosphorylation on the activation and inactivation processes of the 20-pS channel act in opposite directions. While the removal of inactivation by phosphorylation increases the number of channels available to open at any given potential, the effects on activation tend to reduce the probability of opening during an opening burst. Due to the relative position of the activation and inactivation curves on the voltage axis, the overall effect of phosphorylation at holding potentials near resting is to increase the amplitude of the macroscopic current (because of the removal of inactivation) and to slow down its turn-on kinetics. However, if the phosphorylation effect is studied in the absence of inactivation (by pulsing from a very negative HP), a decrease in the amplitude of the current is observed from -60 to 50 mV (unpublished observations).

Shifts of voltage-dependent parameters along the voltage axis have been interpreted as the result of electrostatic interactions between the voltage sensor of the channel and electric point charges in the surface of the channel or the membrane interface. Experimentally, surface charge determinations on the internal side of the membrane have demonstrated an increase in the density of negative surface charges after phosphorylation (Perozo and Bezanilla, 1990). In that report it was proposed

that the extra charge is due to the transfer of phosphate from ATP to the channel and that most of the effects of phosphorylation can be explained assuming an electrostatic interaction between the transferred phosphate(s) and the voltage sensor of the channel. Thus, in the presence of ATP, activation and inactivation processes are affected because the voltage sensor of the channel "senses" a more hyperpolarized potential and the rate constants governing the transitions among closed states get shifted toward more positive potentials. On the other hand, the transition between the first adjacent closed and the open state is not affected by phosphorylation (Fig. 4) *because* it is voltage independent. Additional support for this hypothesis comes from preliminary experiments showing phosphorylation-like effects on I_K by changes in the intracellular pH of squid axons (Perozo, 1990).

The fact that phosphorylation does not seem to affect the conductive properties of the channel can be explained by assuming that the extra charge is located far away from the K channel vestibule and its pore. In this way, the increased cation concentration surrounding the extra negative charge will not affect the actual concentration of potassium ions at the mouth of the channel.

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