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

I. Steady-State Conditions

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ABSTRACT Phosphorylation of the delayed rectifier channel of squid potentiates the macroscopic K^+ current and slows its activation kinetics. We have studied this phenomenon at the single channel level using the cut-open axon technique under steady-state conditions. In 10 mM external K^+//310 mM internal K^+ there are predominantly two types of channels present, a 20-pS and a 40-pS channel. In steady state at depolarized potentials, the 40-pS channel was most active, whereas the 20-pS channel tended to disappear due to a slow inactivation process. Two methods were developed to shift the population of channels toward a dephosphorylated state. One method consisted of predialyzing a whole axon with solutions containing no ATP, while recording the currents under axial-wire voltage clamp. A piece of axon was then removed and cut open, and single channel currents were recorded from the cut-open axon. A second method was based on the difference in diffusion coefficients for ATP and proteins such as the endogenous phosphatase. The axon was cut open in a solution that did not contain Ca^{2+} or Cl^- in order to maintain the axoplasm structurally intact and permit endogenous phosphatase to act on the membrane while ATP diffused away, before removing the axoplasm and forming a membrane patch. When dephosphorylating conditions were used, the steady-state open probability of the 40-pS channel at 42 mV was very low (<0.0002), and the channel openings appeared as a series of infrequent, short-duration events. The channel activity was increased up to 150-fold by photoreleasing caged ATP inside the patch pipette in the presence of the catalytic subunit of protein kinase A. The sharp increase in open probability could be accounted for by a decrease of the slow component of the closed time distribution from 23 s to 170 ms with little change in the distribution of open times (1–2 ms) and no change in the single channel current amplitude. In voltage-jump experiments the contribution

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of the 40-pS channel to the delayed rectifier current was often small due to the large values of the latency to the first opening.

INTRODUCTION

Several lines of evidence have indicated that protein phosphorylation plays a key role in the modulation of ion channels. There is an extensive body of research that links the activities of several well-characterized protein kinases with the electrical properties of excitable tissues (for reviews see Nestler and Greengard, 1984; Kaczmarek and Levitan, 1987). Potassium channels are particularly important in the modulation of neuronal excitability, due to their vast diversity and to the fact that they can be modulated by several distinct mechanisms like phosphorylation (Levitan, 1985), binding of Ca^{2+} ions (Latorre et al., 1989), or interactions with GTP-binding proteins (Yatani et al., 1987). Although the biochemical mechanisms leading to the modulation of many types of K^+ channels are well characterized, the biophysical details that explain changes in macroscopic currents or single channel activities are not well understood.

It has been shown that the potassium conductance in either perfused or dialyzed squid axons is dramatically potentiated by an ATP-mediated phosphorylation (Bezanilla et al., 1986; Perozo et al., 1989; Augustine and Bezanilla, 1990). The potassium conductance was shown to decrease in the absence of ATP and increase in the presence of ATP. Additionally, nonhydrolyzable analogues of ATP were without effect. In perfused axons, the increase in I_K could be reversed by added alkaline phosphatase (Augustine and Bezanilla, 1990). At membrane potentials near resting, phosphorylation increased I_K (elicited by depolarizing pulses) up to threefold, with a simultaneous slow down in turn-on kinetics. Such changes are the consequence of a shift in the steady-state activation and inactivation curves toward more positive potentials (Perozo et al., 1989), with a shift in the distribution of resting potassium channels to closed states further from the open state, as determined from gating current experiments (Augustine and Bezanilla, 1990).

The squid giant axon is an ideal preparation to study the biophysical basis of protein phosphorylation effects in K^+ channels for two reasons: first, the physiology of sodium and potassium channels has been studied extensively, and second, the squid giant axon is so far the only preparation in which potassium macroscopic (Hodgkin et al., 1952), gating (Bezanilla et al., 1982), and single channel currents (Llano et al., 1988) have all been recorded. A quantitative description of the process involved in potassium channel regulation, together with structural information at the atomic level could lead to an understanding of neuroexcitability modulation at molecular and cellular levels.

In this article we have studied the effects of ATP-mediated phosphorylation on the potassium current using single channel recording techniques in steady state. Under these conditions we find two main types of channels, a small 20-pS channel and a larger 40-pS channel. This is in agreement with the work of Llano et al. (1988). Of these two channels, the smaller amplitude channel inactivated almost completely when held at potentials more positive than −30 mV. Therefore, the core of this report deals with the effects of phosphorylation on the 40-pS channel. We found that the activity of the 40-pS channel correlated well with the magnitude of delayed

rectifier current from the whole axon under phosphorylating and dephosphorylating conditions. Also, phosphorylation due to photorelease of caged ATP increased the probability of opening of the 40-pS channel by several orders of magnitude. A detailed study of the effects of phosphorylation on the 20-pS channel is presented in the accompanying paper (Perozo et al., 1991).

A preliminary account of some of this work has appeared previously (Vandenberg et al., 1989).

METHODS

The Cut-open Axon Technique

The technique for recording single channel currents from the cut-open axon has been described in detail previously (Llano et al., 1988; Bezanilla and Vandenberg, 1990). Briefly, the

TABLE I

Solutions

	External					
	K	Na	Cl	Ca	Mg	NMG
B						
0 Ca + + − 0 Cl−	10	—	—	—	60	440
D						
ASW	10	440	570	10	50	—
	Internal					
	K	Glutamate	NMG-PO4	EGTA	Mg	PKA
A						
Patch	310	310	140	1	4	1×10^{-4}
C						
Dialysis	310	300	140	1	4	—

Concentrations are in millimolar. ASW, artificial sea water; NMG, *N*-methylglucamine; EGTA, ethyleneglycol-bis-(B-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; PKA, catalytic subunit of the protein kinase A. Glutamate is the sole counterion in solution B.

hindmost giant axon of the squid *Loligo pealei* was dissected and a small segment (3–6 mm) of axon was tied at the ends with silk thread and carefully cleaned of as much connective tissue as possible. After pinning to a Sylgard-coated chamber, the piece of axon was longitudinally cut open under artificial sea water (ASW; see Table I for composition of solutions). Incubation of the cut-open axon in ASW for 3–4 min at room temperature (20°C) resulted in the liquefaction of the gel-like axoplasm, due in part to the action of Ca^{2+}-activated proteases and to the effects of Cl^- on the axonal cytoskeleton (Pant and Gainer, 1980). Under the dissection microscope, the axoplasm was removed by suction while a stream of ASW flowed over the axon.

A patch pipette was sealed to the cytoplasmic surface of the cut-open axon, resulting in an outside-out membrane patch configuration (Hamill et al., 1981). Patch pipettes were pulled using Corning 7052 or 7040 glass (Garner Glass Co., Claremont, CA) with resistances of 1.5–7 MΩ measured in ASW after fire polishing. Single channel currents were recorded with a List EPC-7 (Medical Systems Corp., Greenvale, NY) or an Axopatch patch clamp with an IHS-1 integrating headstage (Axon Instruments, Foster City, CA). The patch clamp amplifier output

was filtered at 5 kHz (unless otherwise noted) with an 8-pole Bessel filter and later digitized at 20–60 μs per point. Data were recorded continuously on a video cassette recorder through a modified digital audio processor (Bezanilla, 1985).

Methods to Dephosphorylate the Channels

To compare the function of single channels in phosphorylated and dephosphorylated states, we developed two independent methods, using small pieces of axons, to shift the population of K channels toward a dephosphorylated state. Both methods involve removing intracellular ATP to prevent channel phosphorylation while maintaining the axonal membrane in contact with the axoplasm to promote dephosphorylation of the channels by endogenous phosphatases.

The pre-dialysis method. This method is based on the principle of dialysis, in which intraaxonal small molecules are added or removed by diffusional exchange across an internal dialysis capillary inserted into the axon (Mullins and Brinley, 1967). Ions and small solutes are able to cross the dialysis tubing, leaving large molecules such as intracellular enzymes in the axoplasm. The same method allowed us to monitor K channel phosphorylation of the entire population of K channels in the axon under axial-wire voltage clamp conditions. Using this technique, an axon was mounted in a standard dialysis chamber as described previously (Perozo et al., 1989), and then dialyzed for ~ 1 h with an internal solution containing no ATP. The ionic current was monitored using a voltage clamp, pulse generator, and acquisition system as described by Stimers et al. (1987). Dialysis was allowed to proceed until no further changes were observed in the amplitude or kinetics of I_K. Once the current had decreased to its basal level, the dialysis tubing and voltage electrode were removed, and a small segment of axon was tied at both ends while it was still in the dialysis chamber. The piece of axon was transferred to the patch chamber, and then treated as described for the normal cut-open axon technique. An advantage of this technique is that the behavior of the population of channels can be determined with precision at the end of the dialysis by directly measuring I_K. Therefore, the physiological state of the channels is known before the axon is cut open.

The diffusion method. A second technique was developed to promote channel dephosphorylation in the cut-open axon without the need for predialysis. This method is based on the order of magnitude difference estimated for the diffusion coefficients for ATP ($\sim 4 \times 10^{-10}$ m^2s^{-1}; Push and Neher, 1988) compared with that of soluble proteins of the size of a phosphatase ($\sim 5 \times 10^{-11}$ m^2s^{-1} for 70 kD; Push and Neher, 1988; Brautigan, 1989). A small piece of axon was dissected and cut open in 0 Ca^{2+}, 0 Cl^- artificial sea water (solution B). This solution did not activate Ca-dependent intracellular proteases or promote the depolymerization of the cytoskeleton induced by Cl^-. Therefore, the axoplasm could remain on top of the axolemma for several hours. In such a situation, the ATP is expected to diffuse away from the axoplasm–membrane interface in a matter of a few minutes (ATP is estimated to diffuse an average distance of 400 μm, approximately the distance through the axoplasm, in ~3 min; Eisenberg and Crothers, 1979), whereas soluble phosphatases would remain in high concentration close to the membrane for a longer time as expected from the longer diffusion time for a macromolecule. The cut-open axon was incubated in 0 Ca^{2+}, 0 Cl^- solution at room temperature for 20–30 min. The axoplasm was then washed out by perfusing the chamber for 3–5 min in ASW, as in the normal procedure.

Single Channel Analysis

Segments of interest from the data recorded in the VCR tape were digitized and stored directly in the hard disk of the computer using the acquisition system designed by Stimers et al. (1987). The data were then "idealized" with a program for automatic detection of transitions. The transition threshold was set at 50% (Colquhoun and Sigworth, 1983). Dwell time distributions

and open probabilities were calculated from the idealized records. Open and closed time distributions were fitted following the logarithmic dwell time representation of Sigworth and Sine (1987).

Solutions

The compositions of the solutions are listed in Table I. Solution A is the "internal solution" inside the patch pipettes. Solution B is the 0 Ca^{2+}, 0 Cl^- solution used to keep the axoplasm in a gel state with the diffusion method. Solution C is the internal dialysis solution used for the whole axon in the predialysis method. Solution D was the artificial sea water (ASW) used as an external solution in all experiments. Solution D contained, in addition, 300 nM tetrodotoxin (Calbiochem Corp., La Jolla, CA), and for the dialysis experiments contained 1 mM NaCN to inhibit endogenous synthesis of ATP. The osmolality was adjusted to 1,000 mosmol/kg H_2O in all solutions. Internal pH was 7.3 and external pH was 7.6.

For photolysis experiments, the sodium salt of adenosine-5′-triphosphate, P^3-1-(2-nitrophenyl)-ethyl ester (caged ATP; Calbiochem Corp.) was present in the internal solutions at a concentration of 1–2 mM. The internal solution also contained 2–5 mM glutathione, and patch experiments contained 0.1–0.5 μM of protein kinase A catalytic subunit isolated from bovine heart, a generous gift from Dr. Irwin Levitan (Brandeis University, Waltham, MA).

Optical Set-up

Due to the fact that all the seals were formed between the pipette and the intracellular face of the axonal membrane, changes in the composition of the internal solution were not easily achieved by conventional methods. Instead, the concentration of ATP inside the patch pipette was changed using photoreleasable caged ATP. A general diagram of the optical set-up is shown in Fig. 1. Photolysis of caged ATP was performed using a 150-W xenon lamp mounted in a custom housing that permitted extensive heat exchange. Light was filtered through a UG-11 filter and a heat filter to limit the passage of short-wavelength UV and infrared radiation. With a parabolic mirror and a fused silica condenser, the light was focused to a 2-mm^2 spot on one end of a silicone-clad fused silica fiber optic (Quartz Products, Plainfield, NJ). The other end of the fiber optic was positioned very close to the tip of the patch pipette. The area illuminated covered ~1 mm^2 from the very tip of the pipette, which contained ~5 μl of internal solution. Therefore, close to 70% of the total solution volume, including the membrane patch itself, was illuminated, thus avoiding diffusional delays due to the release of ATP in other regions of the pipette. A series of pipettes were illuminated to determine the time necessary to achieve full conversion of the caged ATP. 5-μl aliquots of 10 mM caged ATP solution were placed in patch pipettes and illuminated for different lengths of time from 30 s to 10 min. Illumination of the pipette for 5 min rendered ~80% transformation, and this percentage was not improved substantially by prolonged illumination.

The transformation of caged ATP was verified at the end of every experiment by running a UV spectrum of the solution inside the patch pipette. After illumination, the pipette was broken and its contents were dissolved in 0.5 ml of distilled water. Absorbance spectra were obtained from each sample, and the optical density at 315 nm was computed relative to the difference between a control (not illuminated) and a fully transformed (illuminated with UV light for 30 min) sample (Fig. 1 *B*). The final ATP concentration (if estimated from the calibration experiments), reached from 0.8 to 1.6 mM, depending on the starting concentration of caged ATP. This is a high enough concentration of ATP, given that the K_m for the ATP effects in whole axons is only 10 μM (Perozo et al., 1989).

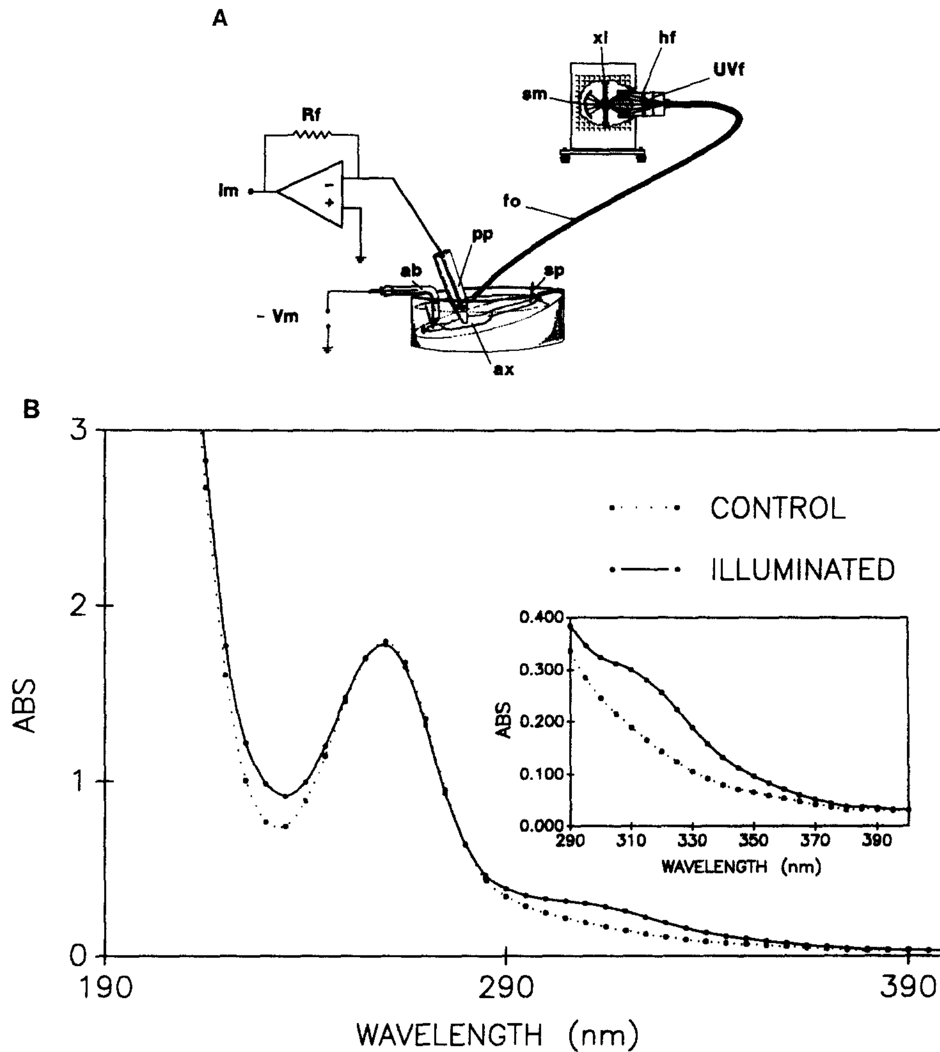

FIGURE 1. (*A*) Scheme for the experimental set-up showing the general arrangement used for all the experiments. The experimental chamber was sitting on the stage of an inverted microscope, in contact with a Peltier-based temperature control system. The fiber optic was positioned with a small micromanipulator after the seal was made. The light source was normally turned on in a different room and later hooked to the fiber optic, thereby avoiding interference on the recording electronics from electromagnetic induction produced by the starter of the power supply. In another configuration, the patch was held at ground and the chamber voltage was controlled. *ab*, agar bridge; *ax*, cut-open axon; *sp*, steel pin that holds the axon to the Sylgard; *fo*, fused silica fiber optic; *hf*, heat filter; *Im*, membrane current output from the patch clamp amplifier; *pp*, patch pipette; *sm*, spherical mirror; *Rf*, 50-GΩ feedback resistor (some experiments were performed using an integrating headstage); *UVf*, ultraviolet filter; *Vm*, transmembrane potential; *xl*, xenon lamp. (*B*) Absorbance spectra for caged ATP before and after photolysis. The control trace (*dotted line*) was obtained from 5 μl of a 10 mM solution of caged ATP in dialysis solution. The experimental trace (*continuous line*) was obtained by illuminating a patch pipette containing the same solution used in the control for 30 min. The inset shows the region of the spectra used to determine the fraction of compound transformed at the end of the experiment.

RESULTS

Caged ATP Studies in Dialyzed Axon

The effect of illumination of caged ATP on K macroscopic currents was examined using whole dialyzed axon under voltage clamp conditions. The optical setup was essentially the same as that described for the single channel patch experiments, except that the light beam was focused directly on the axon using a cylindrical lens without a fiber optic light guide, providing rapid transformation of the caged ATP. Fig. 2 shows the result of 1 min of illumination for an axon that had been dialyzed for 1 h with 2 mM caged ATP in the internal solution. The ionic current was monitored with pulses to 0 mV from a holding potential of −50 mV. After the onset of illumination at 10°C, I_K increased with a lag of 1–2 min. At 10°C the half point of the increase was found to be 12 min. However, when the experiment was performed at

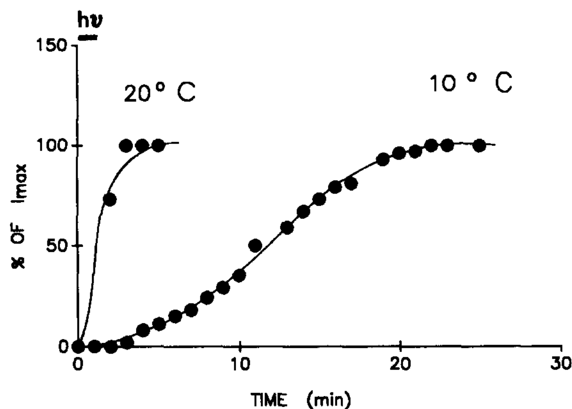

FIGURE 2. Effects of caged ATP on potassium macroscopic current. A squid axon was dialyzed with (in mM): 310 K^+, 140 NMG-PO_4, 10 MOPS, 1 EGTA, 10 glutathione, and 2 caged ATP. The external solution was ASW containing 300 nM TTX and 1 mM NaCN. A xenon lamp was focused directly on the axon with a cylindrical lens. The axon was illuminated for 1 min. The onset of the illumination is marked by a bar (*hν*). Potassium currents were recorded by pulsing to 0 mV from a holding potential of −50 mV. The two curves show recordings from the same axon after similar illumination times but at two different temperatures. There was a 35-min recovery time between the two illuminations to allow a wash-out of the transformed ATP. Notice the sharp temperature dependence of the ATP effect.

20°C this value decreased to 1.8 min. The Q_{10} for the increase in current is ~6.5. When the full effect was established, the kinetic and steady-state characteristics of the current matched those of channels phosphorylated by dialysis with ATP.

Two Types of K⁺ Channels

As was described previously (Llano et al., 1988), under stationary conditions there are two prominent types of K^+ channels in the cut-open axon: a small, ~20-pS channel showing high frequency flicker, and a 40-pS channel with slower kinetics. The small channel was affected by slow inactivation and its opening frequency decreased markedly during prolonged pulses, whereas the 40-pS channel showed activity continuously at a positive potential under steady-state conditions. The behavior of the 40-pS channel varied considerably from patch to patch. At a given potential it

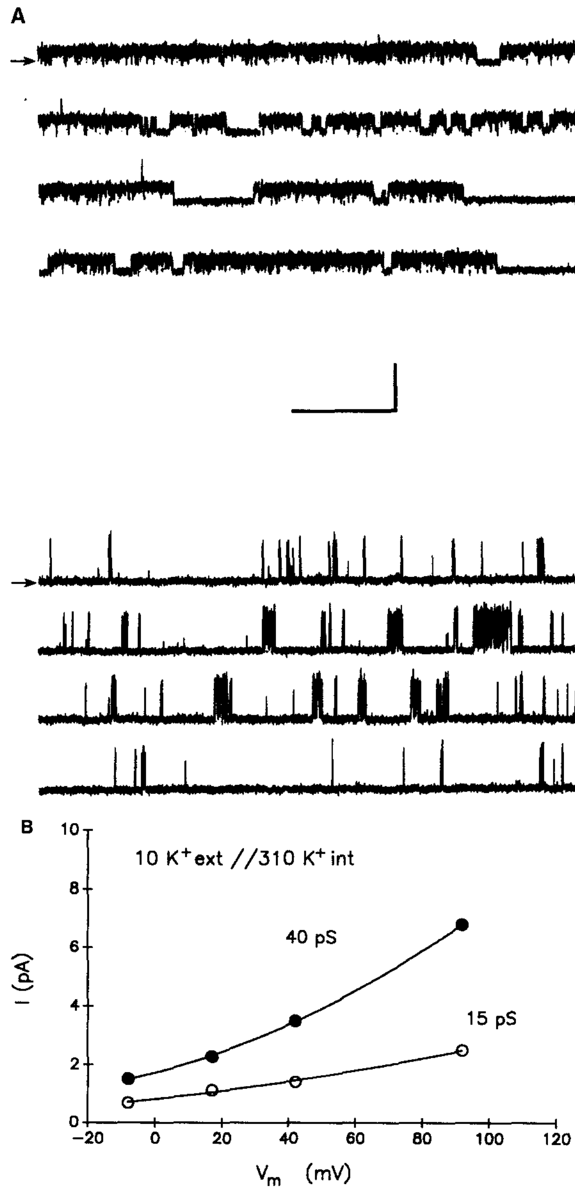

FIGURE 3. Single channels from the cut-open axon in steady state. (*A*) Single channel recordings obtained from an axon cut open in the presence of 2 mM ATP. The holding potential was 42 mV. Traces have been selected to show high activities for both types of channels. (*Top*) Small, inactivating channel with a ~20-pS single channel conductance. (*Bottom*) 40-pS channel. In both cases the arrow points to the closed state. (*B*) *I-V* relation for both channels. The internal solution was in the patch pipette and contained 310 mM K^+. The external solution was ASW containing 10 mM K^+. Temperature was 15°C.

either opened very frequently (high activity state) or showed a very low probability of opening (low activity state).

Fig. 3 *A* shows continuous traces with both channels in their high activity states, and Fig. 3 *B* presents their respective *I-V* curves in 10 mM external K^+//310 mM internal K^+. The slope conductances of the channels were ~20 and 60 pS at 50 mV. The 60-pS channel described here is referred to as a 40-pS channel in Llano et al. (1988), based on its chord conductance rather than on its slope conductance; therefore, we will continue using their nomenclature. Our values are essentially

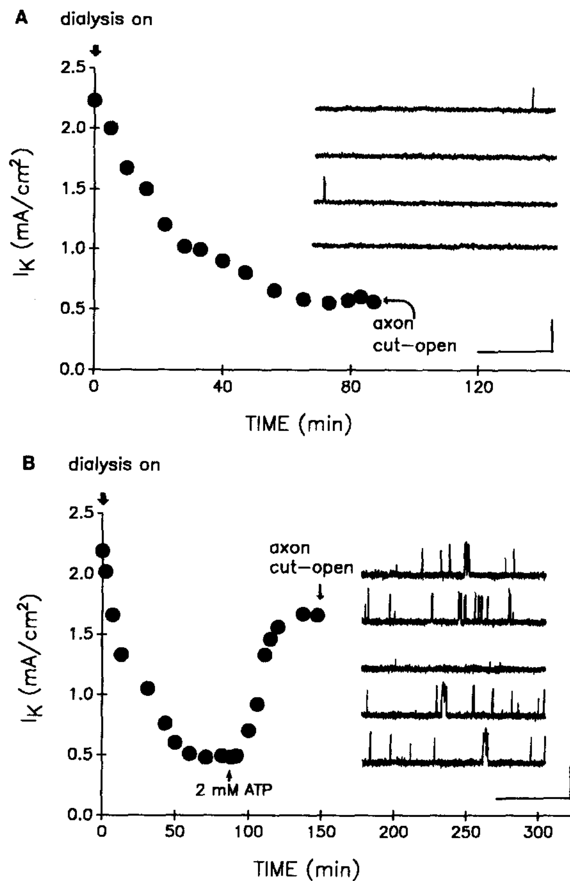

FIGURE 4. Properties of the 40-pS channel studied with the predialysis method. The dots represent outward current measured by pulsing to 0 mV from a holding potential of −55 mV. The single channel recordings shown at the right of each panel correspond to patches obtained from the same axon after termination of dialysis (see Methods). The start and end of dialysis are marked by arrows. (*A*) Predialyzed with internal solution containing no ATP. (*B*) Predialyzed initially with 0 ATP and then with 2 mM ATP. All single channel records were taken at 42 mV. Scale for single channel records: horizontal, 500 ms; vertical, 5 pA. Temperature, 15°C, filtered at 5 kHz.

identical to the slope conductances reported by Llano et al. (1988) for these channels under different ionic conditions (5 mM external K^+//528 mM internal K^+), suggesting that the conductance of the channels is saturated at 310 mM K^+.

Phosphorylation Effects on the 40-pS Channel

The 40-pS channel from a squid axon dialyzed for 85 min with an internal solution containing no ATP shows the pattern of activity illustrated in Fig. 4 *A*. Here, using voltage pulses, the macroscopic delayed rectifier current was followed in time to

monitor the decay of I_K in the absence of ATP. After the amplitude of I_K reached a steady level, the dialysis was stopped and a small piece of axon was retrieved from the dialysis chamber for single channel recording. At a potential of 42 mV the overall probability of opening during a 7-min recording segment was 0.0017. The channel openings appeared as a series of very infrequent short spikes, with an occasional burst of activity lasting 5–10 ms. This corresponds to the "low activity state" of the channel. We have found that the low activity behavior is very reproducible when the axon is predialyzed in 0 ATP (probability of opening = $7.68 \times 10^{-4} \pm 6.1 \times 10^{-4}$ SD, $n = 5$), and the same low probability of opening usually is found when the diffusion method is applied. Therefore, we think that this behavior represents the dephosphorylated state of the channel.

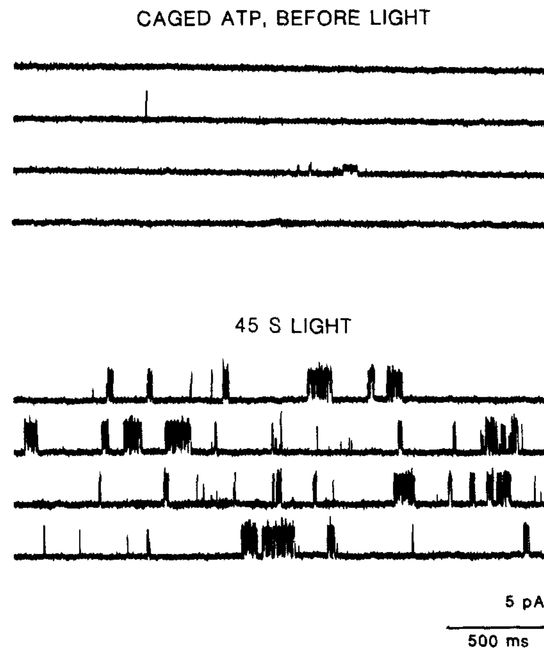

FIGURE 5. Effects of caged ATP on the 40-pS channel. Transformation of the caged ATP was induced by illuminating the tip of the patch pipette with a xenon lamp focused at one end of a fused silica fiber optic (see Methods). Control traces were recorded in the dark to avoid transformation of the caged ATP. The change in the activity of the 40-pS channel was sudden, and occurred ~40 s after the onset of the illumination (see Fig. 6). Illumination was stopped after 1.7 min. Dephosphorylation of the channels was achieved with the diffusion method (see Methods). Holding potential, 42 mV; temperature, 15°C, filtered at 4 kHz.

When dialysis in the absence of ATP was complete and I_K had decayed to a steady level, addition of Mg-ATP to the dialysis solution potentiated the amplitude of I_K as previously reported (Bezanilla et al., 1986; Perozo et al., 1989; Augustine and Bezanilla, 1990). Fig. 4 *B* shows, from a different axon, the macroscopic current amplitude decrease during dialysis in the absence of ATP for 80 min, then its subsequent increase to a new steady state in the presence of 2 mM ATP. The behavior of the 40-pS channel from a segment of the axon retrieved after the increase of macroscopic current with ATP is shown at right. The piece of axon was cut open in ASW containing 2 mM ATP, and the same amount of ATP was also present in the internal solution of the patch pipette. Under these conditions there was a clear increase in the open probability of the channel, which in this experiment was 0.047 for an 11-min recording (mean $P_o = 0.035 \pm 4.2 \times 10^{-3}$, $n = 4$). When the diffusion

method was used with ATP present in all solutions, a similar pattern of activity was found, implying that under conditions that favor phosphorylation reactions, the 40-pS channel functions in the high activity state. Although the activity of the 40-pS channel was always higher when phosphorylating conditions were used, the value of the open probability varied, with values ranging from 0.012 to 0.354.

Direct demonstration of the phosphorylation effects requires the recording of channel activity before and after exposing the channel to ATP. To that end, we have promoted channel dephosphorylation with the diffusion method, and then changed the concentration of ATP inside the patch pipette using caged ATP and UV light in the presence of catalytic subunit of protein kinase A. Fig. 5 illustrates the result of one such experiment. In the upper panel, traces recorded in the dark at 42 mV show very low activity of the 40-pS channel, closely resembling the behavior of the channel after dialysis in 0 ATP solutions. The recording shows a brief appearance of the 20-pS channel (probably returning briefly from inactivation). In sharp contrast, the recording obtained after illuminating the pipette with UV light reveals higher channel

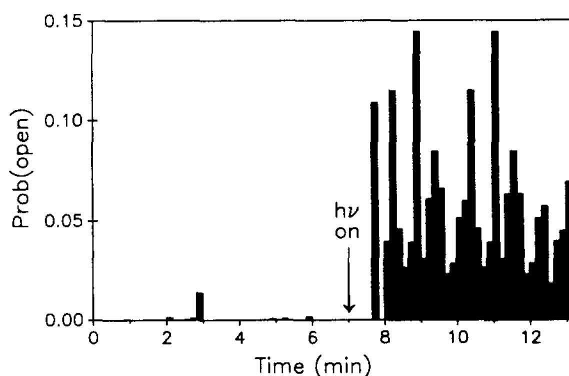

FIGURE 6. Time course of the activation of the 40-pS channel by transformed caged ATP. Bins of 10 s were taken. The probability of opening of the 40-pS channel was calculated as the ratio between the time spent by the channel in the open state and the total recording time in each bin. *hv on* marks the onset of illumination.

activity, as was found when the piece of axon had been predialyzed with ATP. The overall open probability was 0.00038 before illumination and 0.063 after the light was on, representing a 150-fold increase in channel activity (see Table II). Similar results were obtained in other experiments, with a change from 0.00022 ($\pm$0.00015, $n = 7$) to 0.019 ($\pm$0.00096, $n = 3$) in open probability. The time course of the experiment has been summarized in Fig. 6, where the open probability, calculated in bins of 10 s, was plotted against time. The transformation of the channel to a high activity state trailed the onset of the light by approximately half a minute; however, in some experiments the increase in open probability required up to 8 min, and in some cases the channel open probability did not change. After the channel activity increased, it remained at the same high level for the duration of the experiment. Phosphorylation did not change the single channel conductance of the 40-pS channel, as demonstrated by superposition of amplitude histograms at different potentials (not shown). Light by itself had no effect on either macroscopic (Fig. 2) or single channel experiments. Caged Ca^{2+} (nitro-5) was unable to potentiate the 40-pS channel, but induced the opening of a larger 95-pS channel not present before (Vandenberg et al., 1989).

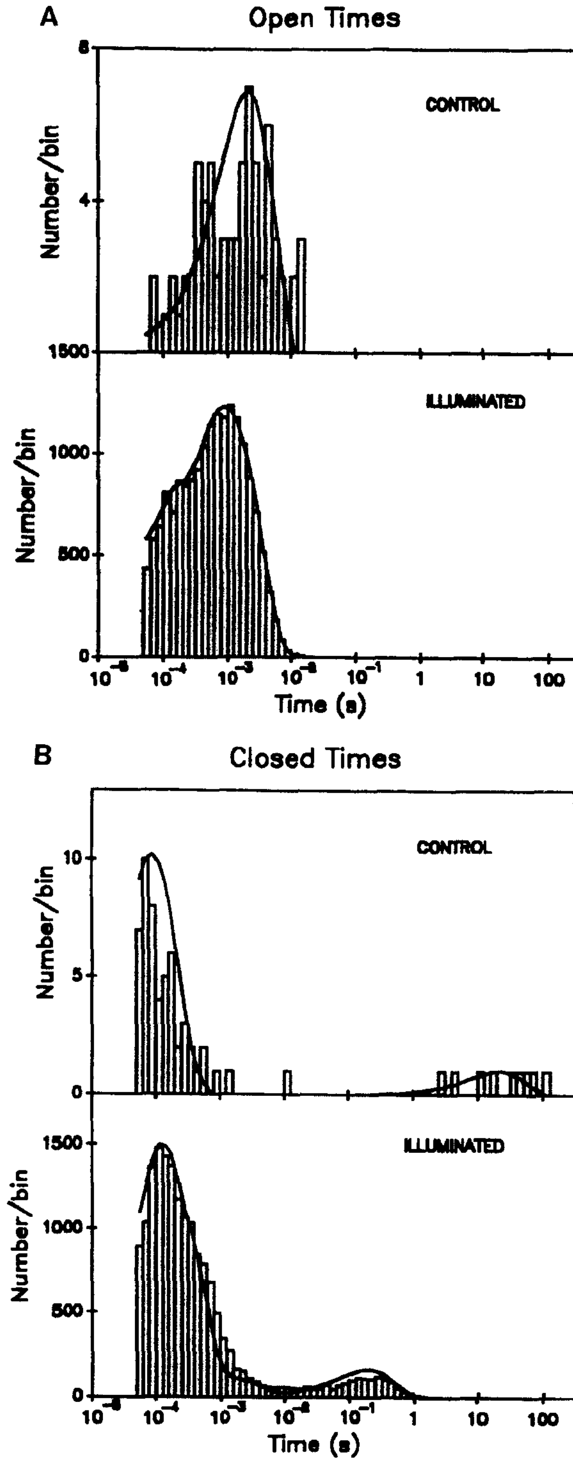

FIGURE 7. Steady-state dwell times for the 40-pS channel before and after transformation of caged ATP. The holding voltage was 42 mV and temperature was 15°C. Data were filtered at 4 kHz. (*A*) Distribution of open times. (*B*) Distribution of closed times. Data not corrected for dead time.

The open time distribution showed little difference between the two activity states, considering the small number of openings present under the dephosphorylating conditions. The distribution of open times is shown in Fig. 7 *A* for the transformation experiment illustrated in Figs. 5 and 6. At 42 mV, the open time distribution of the 40-pS channel after illumination was best described by the sum of two exponentials with tau_1 = 1.2 ms and tau_2 = 0.1 ms, and a relative area of 0.89 for the fast component. The control situation is similar, with a value for tau_1 of 1.7 ms (Table III). Only one open time distribution was fitted for the control open times due to the small number of transitions present. Overall, the mean open time decreased ~50% after illumination (Table II).

The main kinetic difference between the channel before and after illumination was in the closed time distribution (Fig. 7 *B*). Here the histograms were fitted with two or three distinct exponentials: two fast components that did not change with illumination, and a slow component that became faster after the release of ATP. The fast component was tau_2 = 0.29 ms before light, which became tau_2 = 0.53 ms and tau_3 = 0.07 ms after illumination. The slow component for the control condition was tau_1 = 22.7 s, but it decreased greatly to tau_1 = 170 ms after illumination (Table III).

TABLE II

Steady-State Kinetic Parameters

Condition	No. of openings	MOT	MCT	P_o
		ms	*ms*	
Dark	61	1.86	260.56	3.84×10^{-4}
Light	4,357	1.52	23.97	6.3×10^{-2}

MOT, mean open times; MCT, mean closed times; P_o, probability of opening.

Recordings at least 10 min long were analyzed before and after illumination, but due to the low P_o present before illumination we cannot rule out the presence of an intermediate time constant. The increase in the probability of finding the channel in the open state can be largely accounted for by this 130-fold change of the slow component of the closed time distribution. This result implies that the average time the channel spends in the closed state decreases after phosphorylation, with not much change in the mean open time. Since the channel openings appear as bursts of opening that are well separated from each other, these results show that the long time between bursts is decreased by phosphorylation.

The 40-pS Channel Primarily Opens Slowly during Voltage Pulses

We have found that the 40-pS channel shows variable kinetic behavior during voltage-jump experiments. In some experiments the channel opened within a few milliseconds for voltage pulses to +42 mV from a holding potential of −58 mV. This has been previously reported by Llano et al. (1988). Another mode of gating, illustrated by the experiment of Fig. 8, showed very long latencies for opening at several test potentials in a patch containing several 40-pS channels in the presence of

ATP. In this experiment the patch was held at −78 mV, and then the potential was changed to either +52, +72, or +92 mV at the point marked by the arrow. The time to first opening is unusually slow, ranging from 2 to 10 s. Note that the time scale in Fig. 8 is 4 s. This slow gating behavior was found in 97% (n = 68) of the patches studied that showed activity of the 40-pS channel. It therefore appeared to be the predominant gating mode of the 40-pS channel. Still, given the extremely low P_o of the unphosphorylated channel, it is hard to determine with precision the effects of phosphorylation on the kinetics of this channel.

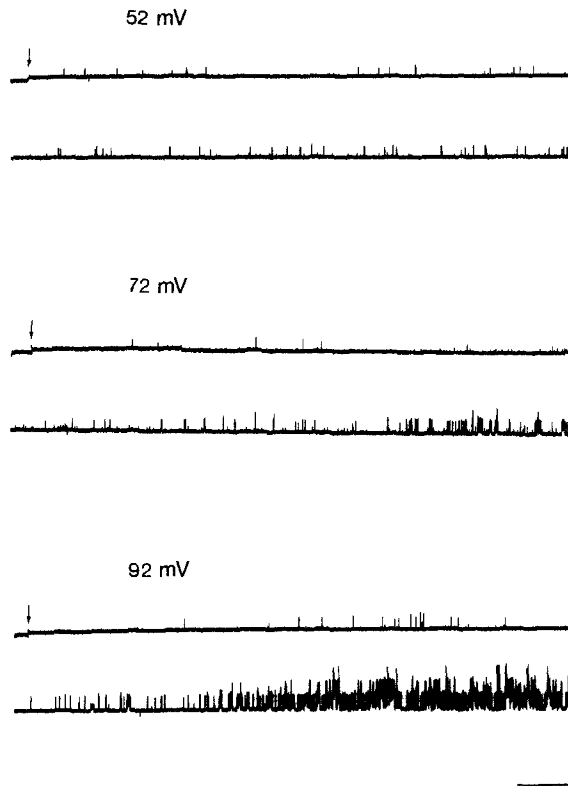

FIGURE 8. Voltage-jump experiments on the 40-pS channel. The axon was cut open in ASW containing 2 mM ATP. The patch was held at −78 mV for 1 min, then suddenly changed to test potential. Each voltage jump is marked by an arrow. Each trace pair is continuous and consecutive. Filter, 5 kHz.

DISCUSSION

In this report it was demonstrated that under stationary conditions the 40-pS potassium channel from squid giant axons can be modulated by ATP in a manner consistent with a phosphorylation process, increasing its probability of being open more than 100-fold.

Using two different approaches, we show that under dephosphorylating conditions the 40-pS channel had a very low open probability (<0.0002), only appearing as a series of very brief, spike-like events. This mode of gating was defined as the "low activity mode" by Llano et al. (1988). It was found here using either the dialysis or the diffusion method to promote dephosphorylation. In sharp contrast, under conditions

that favor phosphorylation reactions, the 40-pS channel showed much higher activity (up to 0.14 probability of being open). This mode of gating closely resembles the "high activity mode" of Llano et al. (1988); however, in our hands the open probabilities were not as high as in their report (up to 0.75 at 50 mV). Conversion from the low activity to the high activity mode of the 40-pS channel was achieved after photoreleasing ATP in the patch pipette, as demonstrated in Figs. 5 and 6. Therefore, we believe that the low probability of opening corresponds to the dephosphorylated state of the 40-pS channel. Likewise, the phosphorylated state is associated with the high activity mode of Llano et al. (1988). Although these experiments do not show that the channel itself is phosphorylated, the experiments with caged ATP and protein kinase A on membrane patches suggest that, if not the channel, at least a membrane-bound entity closely associated with the potassium channel is the site of phosphorylation.

Changes in single channel activity associated with phosphorylation can be explained by differences in the slow time constant of the closed time distribution (Table III), which speeded up considerably after phosphorylation. This shows that the interburst time was reduced sharply by phosphorylation, and the overall open

TABLE III

Parameters from Fitted Dwell Time Distributions

	Open times		Closed times		
	tau_1	tau_2	tau_1	tau_2	tau_3
Control	1.7×10^{-3}	—	22.7	2.9×10^{-4}	—
After light	1.2×10^{-3}	9.5×10^{-5}	1.7×10^{-1}	5.3×10^{-4}	7.2×10^{-5}

Dwell time distributions were fitted with a binned maximum likelihood method. Time constants are in seconds.

probability increased accordingly. The mean open time was also found to be decreased slightly upon phosphorylation (Table II). Although the number of openings in the control situation was low, the change was statistically significant, suggesting an increase in the rates of leaving the open state(s) after phosphorylation. However, we cannot rule out the possibility that phosphorylation could affect the frequency of observing a very long closed state in relation to an intermediate duration closed state. This is a consequence of the reduced amount of data available for the dephosphorylated state.

The time course of the effects of ATP on the single channels varied from 45 s to 8 min between patches when an effect was present. Such variation is not unusual considering the fact that the success of these experiments depends on the effective collisions between a single channel, a protein kinase, and a small number of ATP molecules. Furthermore, the discrete increase in channel activity to a maintained higher activity state is consistent with an enzymatic reaction in which a single event, such as phosphorylation at a particular site, shifts the channel into a more active gating mode. The range of times was also within the expected time for the increase in delayed rectifier current by ATP as was observed for the endogenous kinase in dialyzed whole axons.

In the squid axon, the delayed rectifier current was thought to be carried by a homogeneous population of potassium channels. This has turned out not to be the case, as it has been demonstrated that several types of channels coexist in the axolemma (Fig. 3, and Llano et al., 1988). There are two main types of potassium channels in squid giant axon, a 20-pS and a 40-pS channel, and other types have been observed less frequently. The occurrence of several potassium channels contributing to delayed rectifier current also has been found in other preparations such as neuroblastoma cells (Quandt, 1988), amphibian spinal neurons (Harris et al., 1988), and T lymphocytes (Decoursey et al., 1987).

The relative contribution of each channel to the delayed rectifier current remains to be determined. In voltage-jump experiments, the 40-pS channel displayed different kinetics of activation, often showing very long latencies, but sometimes opening rapidly in response to a depolarizing pulse. Channels that show latencies of several seconds to first opening (Fig. 8) are unlikely to contribute to the delayed rectifier current during an action potential. However, those channels that open during short depolarizing pulses, as studied by Llano et al. (1988) and also observed by us, are likely to be involved in the delayed rectifier current, although their contribution appears to be small.

At the moment, we can only speculate about the physiological role of the 40-pS channel or its modulation by phosphorylation. It is possible that the experimental variations of the behavior of the 40-pS channel represent different channels with similar conductive properties, or that they represent different modes of gating of a single type of channel. There are several reasons that could explain the differences in gating behavior. The amount of phosphorylation obtained during the present experiments may not be enough to fully activate the channel, or the 40-pS channel could be modulated by additional systems such as other protein kinases, G proteins, arachidonic acid, or Ca^{2+}.

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