

Phosphorylation Modulates Potassium Conductance and Gating Current of Perfused Giant Axons of Squid

CHRISTINA K. AUGUSTINE and FRANCISCO BEZANILLA

From the Department of Physiology, Jerry Lewis Neuromuscular Research Center, and Ahmanson Laboratory of Neurobiology, University of California, Los Angeles, California 90024; and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543

ABSTRACT The presence of internal Mg-ATP produced a number of changes in the K conductance of perfused giant axons of squid. For holding potentials between -40 and -50 mV, steady-state K conductance increased for depolarizations to potentials more positive than ~ -15 mV and decreased for smaller depolarizations. The voltage dependencies of both steady-state activation and inactivation also appeared shifted toward more positive potentials. Gating kinetics were affected by internal ATP, with the activation time constant slowed and the characteristic delay in K conductance markedly enhanced. The rate of deactivation also was hastened during perfusion with ATP. Internal ATP affected potassium channel gating currents in similar ways. The voltage dependence of gating charge movement was shifted toward more positive potentials and the time constants of ON and OFF gating current also were slowed and hastened, respectively, in the presence of ATP. These effects of ATP on the K conductance occurred when no exogenous protein kinases were added to the internal solution and persisted even after removing ATP from the internal perfusate. Perfusion with a solution containing exogenous alkaline phosphatase reversed the effects of ATP. These results provide further evidence that the effects of ATP on the K conductance are a consequence of a phosphorylation reaction mediated by a kinase present and active in perfused axons. Phosphorylation appears to alter the K conductance of squid giant axons via a minimum of two mechanisms. First, the voltage dependence of gating parameters are shifted toward positive potentials. Second, there is an increase in the number of functional closed states and/or a decrease in the rates of transition between these states of the K channels.

INTRODUCTION

Modulation of ion channels by phosphorylation is an important means of regulating cell function (Kaczmarek and Levitan, 1987). Although many channels have been shown to be modulated by ATP-dependent phosphorylation, K channels have proven to be a particularly important locus for modulation via phosphorylation (for

Address reprint requests to Dr. Christina K. Augustine, Max-Planck-Institut für Biophysikalische Chemie, Postfach 2841, 3400 Göttingen, West Germany.

reviews see Levitan, 1985 and Kaczmarek, 1988). For example, the anomalous rectifier K channel in *Aplysia* neuron R15 (Benson and Levitan, 1983) and the Ca-activated K channel in *Helix* neurons (Ewald et al., 1985) are activated by a cAMP-dependent phosphorylation reaction, while the "S" K channel in *Aplysia* sensory neurons (Shuster et al., 1985) is inhibited by a cAMP-dependent phosphorylation reaction. Several components of the delayed K current in *Aplysia* bag cell neurons (Strong and Kaczmarek, 1986) are also modulated by a cAMP-dependent phosphorylation reaction.

The squid axon is an ideal preparation for studying K channel modulation because macroscopic ionic currents, gating currents, and single-channel currents can all be studied to yield a detailed biophysical description of channel modulation. Using either dialyzed or perfused squid axons, Bezanilla et al. (1986) showed that the addition of Mg-ATP to the internal solution resulted in marked changes in the macroscopic K current. These effects were not mimicked by the addition of Mg or ATP alone, other adenosine nucleotides or nonhydrolyzable analogues of ATP, such as AMP-PNP or AMP-PCP (see also Perozo et al., 1989). This suggests that the effects of ATP on the K current are a consequence of a phosphorylation reaction.

This paper provides a detailed examination of the effects of ATP-dependent phosphorylation on the macroscopic K current and K channel gating current of perfused squid axons. The advantage of using perfused squid axons, rather than dialyzed squid axons, is that soluble enzymes are removed as the axoplasm is washed out during perfusion. This provides a relatively defined intracellular environment which can be manipulated to define the role of various regulatory macromolecules in channel modulation. Furthermore, because of the relatively rapid exchange of intraaxonal solution during perfusion, K channel gating currents can be recorded while preserving the macroscopic K current.

We report here that ATP shifts the voltage dependence of channel gating in a manner consistent with its effects in dialyzed squid axons and we offer further evidence that these effects are due to a phosphorylation reaction. K channel gating currents are also altered during internal perfusion with ATP and the changes observed are consistent with those observed in the macroscopic currents. Our results can be explained by proposing a minimum of two mechanisms of action of phosphorylation on K channel gating: a shift in the voltage dependence of gating parameters toward positive potentials and an increase in the number of closed states and/or a decrease in the rates of transition between these states of the K channels. Some of these results have been presented in abstract form (Webb and Bezanilla, 1986, 1987).

METHODS

Giant axons from the squid *Loligo pealei* were isolated, cleaned of connective tissue, and placed in the recording chamber described in Bezanilla et al. (1982a). The axons were internally perfused using methods previously described (Bezanilla and Armstrong, 1972) and voltage-clamped with conventional axial wire recording methods (Armstrong and Bezanilla, 1974). The temperature of the solution in the recording chamber was controlled by a negative feedback circuit connected to a Peltier device mounted in the chamber. Unless otherwise noted, the temperature of the bath solution was maintained at $20 \pm 1^\circ\text{C}$ to speed up the

TABLE I
Solutions for Ionic Current Recording

	K	Glutamate	Internal solutions			Sucrose	Mg ²⁺	
			F	PO ₄	Tris			
200 KFG	200	160	40	—	10	460	2	
200 KPO ₄ G	200	160	—	20	10	460	2	
	Cs	K	Na	External solutions			Ca ²⁺	Mg ²⁺
				Tris	Cl	NO ₃		
xK ASW	—	x	440-x	10	570	—	10	50
yK TrisNO ₃	—	y	—	520-y	—	620	50	—
50 CsNO ₃	50	—	—	490	—	660	10	50
10K NO ₃ SW	—	10	430	10	—	570	10	50
440 CsNO ₃	440	—	—	10	—	570	10	50

Concentrations are in millimolar.

gating of both the Na and K channels and enabling clear measurement of K channel gating currents (White and Bezanilla, 1985).

Pulse generation, data acquisition, and display were performed using a 16-bit microcomputer (Intel 8086-based Lightning-1; Lomas Data Products, Westboro, MA). The system that was used followed the design of Stimers et al. (1987). Ionic and gating currents, recorded through a current-to-voltage converter, were summed with the output of a transient generator to remove large capacity transients (Bezanilla and Armstrong, 1977).

The solutions used are listed in Tables I and II. All external solutions were adjusted to pH 7.6 and 1,000 mosmolal. The osmotic pressure of all internal solutions was adjusted to 980 mosmolal with sucrose, and to pH 7.4 with the appropriate acid. 400 nM tetrodotoxin (TTX; Sigma Chemical Co., St. Louis, MO) was added to all external solutions to block Na channels. Occasionally 1 mM EGTA (Sigma Chemical Co.) was added to the internal solution to buffer Ca²⁺ well below 10⁻⁷ M.

Junction potentials for all solutions used, except those containing 200 mM *N*-methylglucamine (NMG), ranged from 3 to 5 mV; membrane potentials are not corrected for this error. For experiments in which the internal perfusate was 200 NMG the junction potentials were -10 to -15 mV. Membrane potentials were not corrected for this error because we did not attempt to compare gating currents recorded in this solution with currents recorded in other solutions. In the text and figure legends, solutions are referred to as "external solution//internal solution."

TABLE II
Solutions for Gating Current Recording

	Cs	Glutamate	Internal solutions			Sucrose	Mg ²⁺	
			F	NMG	Tris			
200 NMG	—	166	34	200	10	460	2	
350 CsFG	350	300	50	—	10	240	2	
	Cs	K	Na	External solutions			Ca ²⁺	Mg ²⁺
				Tris	Cl	NO ₃		
0K TrisNO ₃	—	—	—	520	—	620	50	—
50 CsNO ₃	50	—	—	490	—	660	10	50

Concentrations are in millimolar.

To examine the effects of ATP on the K conductance, the magnesium salt of ATP (referred to here as simply ATP and obtained from Sigma Chemical Co.) was added to the internal solution at a concentration of 2 (or occasionally 0.2) mM. In some experiments, the catalytic subunit of cAMP-dependent protein kinase (referred to here as catalytic subunit and obtained from Sigma Chemical Co.) also was added to the internal solution at a concentration of 2 $\mu\text{g}/\text{ml}$. Occasionally, 25 or 100 $\mu\text{g}/\text{ml}$ of alkaline phosphatase (Sigma Chemical Co.) was added to the internal solution after perfusion with ATP to reverse the phosphorylation.

Conditions for Measuring Ionic Currents

K ionic currents were filtered at 25 or 50 kHz and digitally sampled at 50 or 100 kHz. The currents were then stored on magnetic media for subsequent off-line analysis. Each ionic current trace shown here represents a single sweep corrected for linear leakage and capacitive currents using the P/-4 or P/-2 pulse procedure described by Bezanilla and Armstrong (1977). The subtracting pulses were made from a holding potential of -100 or -120 mV. Frequently this subtraction method was not used because the voltage steps to -100 or -120 mV tended to remove some of the inactivation present at the usual holding potential of -50 or -60 mV (Ehrenstein and Gilbert, 1966; Chabala, 1984). Instead, 10-mV test pulses were given from a holding potential at which no K currents were elicited (usually -80 mV) and the resulting currents, consisting only of leak current, were scaled as necessary and subtracted from the K currents during off-line analysis. These "leak subtraction" records were taken periodically throughout the experiment.

Accumulation of K in the periaxonal space (Frankenhaeuser and Hodgkin, 1956; Adelman et al., 1973) during a voltage step causes the K current reversal potential (E_K) to change in a manner that depends on the holding potential and the duration and amplitude of the voltage step. This complicates the calculation of steady-state K conductance. To circumvent this problem, steady-state conductance was calculated from the following relation: $G_K = (I_{K1} - I_{K2}) / (V_1 - V_2)$, where I_{K1} is the steady-state current during the test pulse, I_{K2} is the current measured $\sim 70 \mu\text{s}$ after the test pulse, V_1 is the membrane potential during the test pulse, and V_2 is the membrane potential immediately after the test pulse. Generally, V_2 was equal to the holding potential. However, to prevent errors due to the nonlinearity of the instantaneous current-voltage (I - V) curve, V_2 was often set to be 20 mV less than V_1 so that $G_K = (I_{K1} - I_{K2}) / 20$ mV. By minimizing the difference between I_{K1} and I_{K2} in this way it was possible to remain within the linear portion of the instantaneous I - V relationship.

Several terms are used in this paper to describe features of the ionic currents. Conductance refers to the K conductance measured at any time during the test pulse, steady-state conductance is the K conductance measured at the end of the test pulse, and maximal conductance is the highest steady-state conductance value obtained at positive depolarizations.

Ionic currents were fit by the sum of two exponentials plus a delay and the time constants of these exponentials were used to estimate the activation kinetics of the currents. On some occasions, single exponentials were fit to the currents; however, two exponentials were usually needed to fit the current traces. To quantify deactivation kinetics, tail currents similarly were fit with either one or two exponentials. Comparisons of the effect of ATP on current kinetics yielded similar conclusions regardless of whether one or two exponentials were fit to the records.

Shifts in the voltage dependence of gating parameters were measured at the midpoint of the function (i.e., $V_{0.5}$ for relative conductance) or between membrane potentials of -20 and 10 mV (i.e., τ_{on} and delay).

Conditions for Measuring Gating Currents

K channel gating currents (I_g^K) were filtered at 25 or 40 kHz and sampled at 50 or 100 kHz. To insure that all components of I_g^K were recorded, 15-ms test pulses were usually given. The

P/4 or P/-4 pulse procedure was used routinely to compensate for linear leakage and capacitive currents. All test pulses were made from a holding potential of -60 mV and the subtracting pulses were made from a subtracting holding potential of -120 mV. Frequently the test pulse was preceded by a hyperpolarization to -100 mV to induce a delay in the activation of the K channel (Cole and Moore, 1960). This delay provided a clearer breakpoint between I_K and the residual Na channel gating current (Bezanilla et al., 1982b; White and Bezanilla, 1985). Each gating current trace represents the average of 20 sweeps.

The internal solution used most often was 350 CsFG (Spires and Begenisich, 1989). Since Cs is slightly permeant through the K channels, 30 mM tetraethylammonium (TEA; Sigma Chemical Co.; added as TEA-aspartate) or 5–10 μ M 3,4-diaminopyridine (DAP; Sigma Chemical Co.) were often added to the internal 350 CsFG solution to block the K channels. 250 μ M dibucaine (Sigma Chemical Co.) was added to all internal gating solutions to eliminate some Na channel gating current (Gilly and Armstrong, 1980; White and Bezanilla, 1985). To further reduce Na channel gating current, the external solutions contained NO_3^- as the main anion (White and Bezanilla, 1985).

Three methods were used to determine the voltage dependence of the K channel gating charge movement. K channel gating currents in response to a test pulse from -60 mV were fit with one exponential, or two if a single exponential did not provide a good fit. The exponential fit was extrapolated to zero time and integrated to determine the amount of gating charge transferred during the test pulse. Alternatively, the test pulses were preceded by a hyperpolarization to -100 mV. The gating currents were integrated from the breakpoint between I_K and residual Na channel gating current to the end of the test pulse. Finally, gating tail currents were elicited by repolarizing to -60 mV, from a depolarized test pulse. Under the recording conditions of these experiments, Na channel gating current decreases by 50% after 25 μ s at -60 mV (White and Bezanilla, 1985) so K channel gating tail currents were integrated beginning 80–120 μ s after repolarizing to -60 mV.

ON and OFF gating currents were approximated by single exponentials and the time constants of these exponentials were used to estimate activation and deactivation kinetics. Shifts in the voltage dependence of gating parameters were measured as described above for ionic currents.

RESULTS

ATP Alters the K Conductance of Perfused Axons

Addition of 2 mM ATP and 2 μ g/ml catalytic subunit of the cAMP-dependent protein kinase to the internal solution produced marked changes in the magnitude and time course of the K current of perfused axons (Fig. 1). Effects of this treatment on K current kinetics will be considered after describing its effects on steady-state K current.

For the experiment shown in Fig. 1, addition of ATP and catalytic subunit increased the steady-state conductance (i.e., K conductance measured 8 ms after the onset of the test pulse in Fig. 1 A) as much as 90% at membrane potentials more positive than ~ -15 mV but at potentials more negative than this the steady-state conductance decreased by as much as 60% (Fig. 1 B). At a holding potential of -50 mV the mean increase in maximal conductance (i.e., 13 mS/cm² before and 25 mS/cm² during perfusion with ATP in Fig. 1 B) was 40% (SEM = 15%, $n = 8$) for a depolarization to 80 mV. The size of the increase in maximal conductance and the potential at which the G - V curves crossed over (i.e., -15 mV in Fig. 1 B) depended both on the preparation and on the holding potential. When normalized to the max-

imum recorded in each condition, the relative steady-state K conductance (G_{rel}) shifted ~ 15 mV towards more positive potentials when the axon was perfused with ATP and catalytic subunit (Fig. 1 C). The magnitude of this shift in the $G_{rel} - V$ curve varied from 6 to 20 mV in six different preparations held at -50 mV.

As will be discussed below, the qualitative effects of ATP did not depend on the addition of catalytic subunit to the internal solution. Thus, throughout the remain-

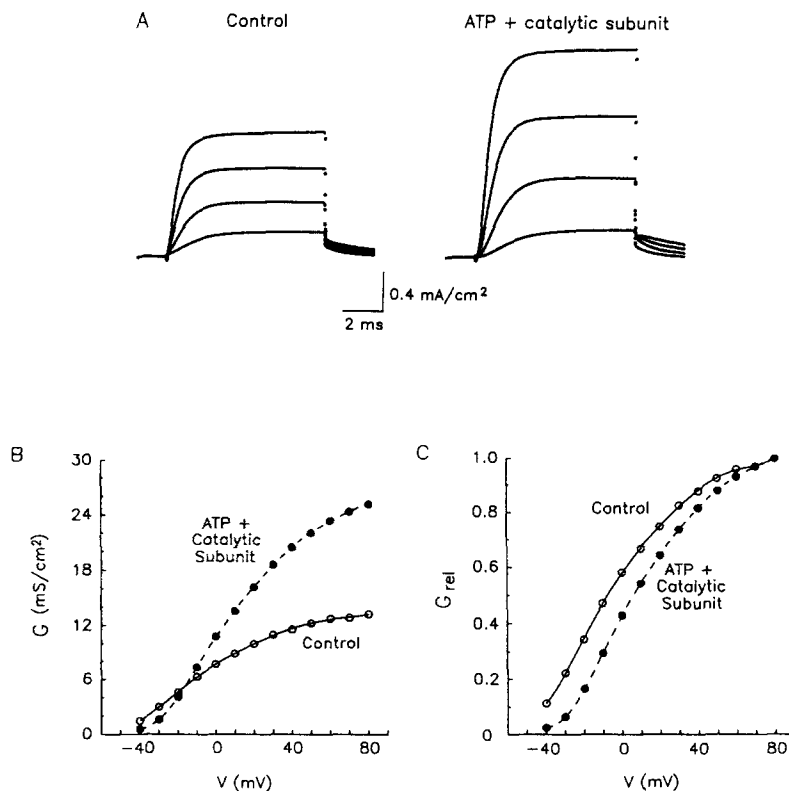


FIGURE 1. Internal ATP alters macroscopic K current. (A) K currents in response to 8-ms test pulses to -20 , 0 , 20 , and 40 mV before (*left*) and after (*right*) 11 min of perfusion with 2 mM ATP and 2 μ g/ml catalytic subunit of the cAMP-dependent protein kinase. Steady-state conductance-voltage curve (B) and relative steady-state conductance-voltage curve (C) for the current records shown in A. Open symbols represent the absence and filled symbols the presence of ATP and catalytic subunit. In C, each point in B was plotted relative to the maximum recorded under each condition. The holding potential was -50 mV and the solutions were 2 K TrisNO₃SW // 200 KFG.

der of this paper, the addition of ATP, either alone or with catalytic subunit, will be referred to as simply the addition of ATP.

ATP Shifts Inactivation of K Channels

The effects of ATP on the steady-state K conductance depended strongly on the holding potential (Fig. 2). At a holding potential of -40 mV the maximal conduc-

tance increased as much as fourfold in the presence of ATP (Fig. 2 A). At a holding potential of -50 mV, the addition of ATP increased the maximal conductance by as much as twofold (e.g., Fig. 1 B). On the other hand, as illustrated in Fig. 2 B, the addition of ATP usually did not increase the maximal conductance at a holding potential of -60 mV. When the membrane was held at a potential of -60 mV, ATP increased the maximal conductance by a factor of 1.02 in only one experiment ($n = 6$).

In the absence of ATP the maximal K conductance depended strongly on the holding potential: it increased as the membrane was held at progressively more hyperpolarized potentials (e.g., control curves in Fig. 2). This is because of a decreased level of steady-state inactivation at more hyperpolarized potentials (Ehrenstein and Gilbert, 1966; Chabala, 1984). In the presence of ATP, however, the maximal conductance changed little with the holding potential (Fig. 2, *filled symbols*), suggesting that ATP altered steady-state inactivation of the K channel.

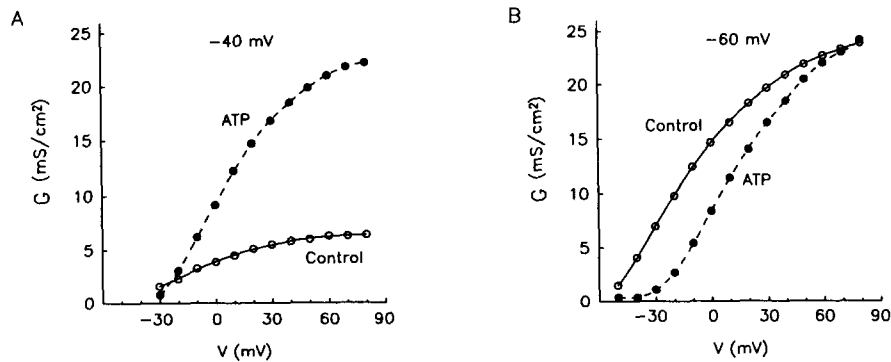


FIGURE 2. ATP effects are dependent on the holding potential. Steady-state conductance-voltage curve before (*open symbols*) and during (*filled symbols*) perfusion with ATP and catalytic subunit. The holding potential was -40 mV (A) or -60 mV (B). The solutions were 2K TrisNO₃SW // 200 KFG.

The magnitude of steady-state inactivation was estimated by repetitively hyperpolarizing the axon using a P/−2 pulse procedure as illustrated in the inset of Fig. 3 A. When this pulse procedure was repeated in quick succession several times (i.e., 10–20 repetitions) inactivation was quickly removed, because of the hyperpolarizing steps to -100 mV, and the K current increased. After several repetitions, a test pulse immediately was delivered, before the K currents were diminished by the slowly developing inactivation. This pulse procedure was performed at a variety of holding potentials in the absence and presence of ATP. For the experiment shown in Fig. 3 A, repetitive hyperpolarizations from a holding potential of -40 mV increased the steady-state conductance elicited by a test pulse to 0 mV nearly threefold in the absence of ATP (*left*). However, in the presence of ATP repetitive hyperpolarizations only increased the steady-state conductance by a factor of 1.1 (*right*).

The increase in the steady-state K conductance (measured at 0 mV) in response to repetitive hyperpolarizations was quantified for each holding potential using the relationship $G(\infty) = G_1/G_2$. In this relationship, G_1 is the steady-state K conductance

before repetitive hyperpolarization, G_2 is the steady-state K conductance obtained after repetitive hyperpolarization, and $G(\infty)$ was taken as a measure of steady-state K conductance inactivation at a given holding potential. When estimated in this way, the voltage dependence of inactivation seemed to be shifted towards more positive potentials in the presence of ATP (Fig. 3 B). At $G(\infty) = 0.9$ this appears to be a shift of ~ 15 mV.

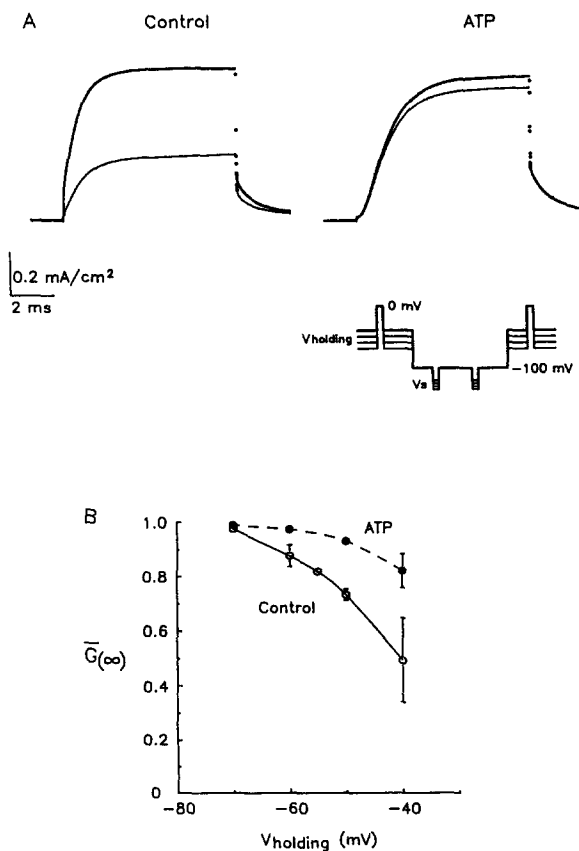


FIGURE 3. ATP reduces steady-state inactivation. (A) Current records in response to an 8-ms test pulse to 0 mV from a holding potential of -40 mV before (*thin line*) and after (*thick line*) repetitive hyperpolarization (see inset for illustration of the pulse protocol used to measure steady state inactivation). The left panel was in the absence and the right panel was in the presence of ATP and catalytic subunit. The solutions were 2K TrisNO₃ SW // 200 KFG. (B) Steady-state inactivation [$G(\infty)$] as a function of the holding potential (V_{holding}) before (*open symbols*) and during (*filled symbols*) perfusion with ATP. Each point represents the mean from two to six experiments except the point at -55 mV which represents one experiment. Error bars represent the standard error of the mean; where these are not visible, the error bar was smaller than the symbol.

ATP Modifies K Current Kinetics

ATP also modified the kinetics of K current activation. To characterize these changes, current records were fit by the sum of two exponentials plus a delay (Fig. 4 A). The fast time constants (τ_{fast} , Fig. 4 B) were very voltage dependent and were slowed by the presence of ATP. This effect of ATP appeared to be a simple shift of the voltage dependence of τ_{fast} toward positive potentials. The magnitude of this shift depended on the preparation and ranged from 10 to 25 mV ($n = 6$).

In contrast to this slowing of activation kinetics, K current deactivation kinetics were faster in the presence of ATP. The voltage dependence of the deactivation time constants (τ_{off}) was determined by repolarizing the membrane to various poten-

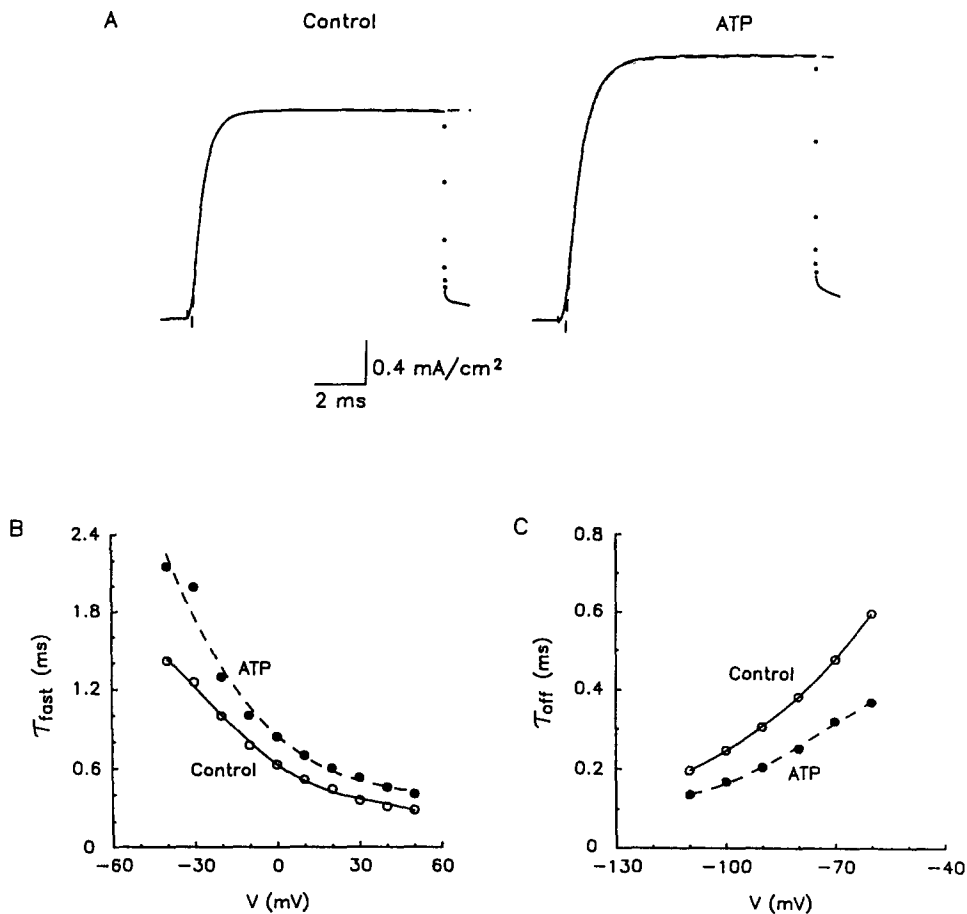


FIGURE 4. ATP modifies K gating kinetics. (A) K currents in response to a 10-ms test pulse to 30 mV from a holding potential of -50 mV before (*left*) and during (*right*) perfusion with ATP. The biexponential fits (*dashed lines*) are superimposed on each current record. The time constants and scaling coefficients for these fits are: 0.413 ms, 3.45 mA/cm² (fast component) and 1.505 ms, 0.08 mA/cm² (slow component) in the absence of ATP (control); 0.562 ms, 4.42 mA/cm² (fast component) and 2.517 ms, 0.12 mA/cm² (slow component) in the presence of ATP. The solutions were 2K TrisNO₃SW // 200 KPO₄G. (B) The fast activation time constant (τ_{fast}) before (*open symbols*) and during (*filled symbols*) perfusion with ATP and catalytic subunit as a function of the test pulse potential. The holding potential was -50 mV. The solutions were 2K TrisNO₃SW // 200 KPO₄G. (C) The deactivation time constant (τ_{off}) as a function of the test pulse potential before (*open symbols*) and during (*filled symbols*) perfusion with 0.2 mM ATP and catalytic subunit. The holding potential was -60 mV. The test pulse was preceded by an 8-ms pulse to 50 mV. The solutions were 0K TrisNO₃SW // 200 KFG.

tials after a depolarization to 50 mV. Under these conditions, ATP sped up tail current decay, effectively causing a 20-mV shift in the voltage dependence of τ_{off} toward more positive potentials (Fig. 4 C). Again, the magnitude of this shift varied from axon to axon.

K currents showed a small delay in activation when the membrane was held at potentials of -50 mV or more negative. In the presence of ATP the activation delay was more pronounced at all holding potentials examined (-40 to -70 mV) and could be most readily seen when current records were scaled to the same steady-state value as in Fig. 5 A. The magnitude of this activation delay was estimated by determining the time at which the biexponential fits to the current records crossed the baseline. This delay decreased as the membrane potential during the test pulse

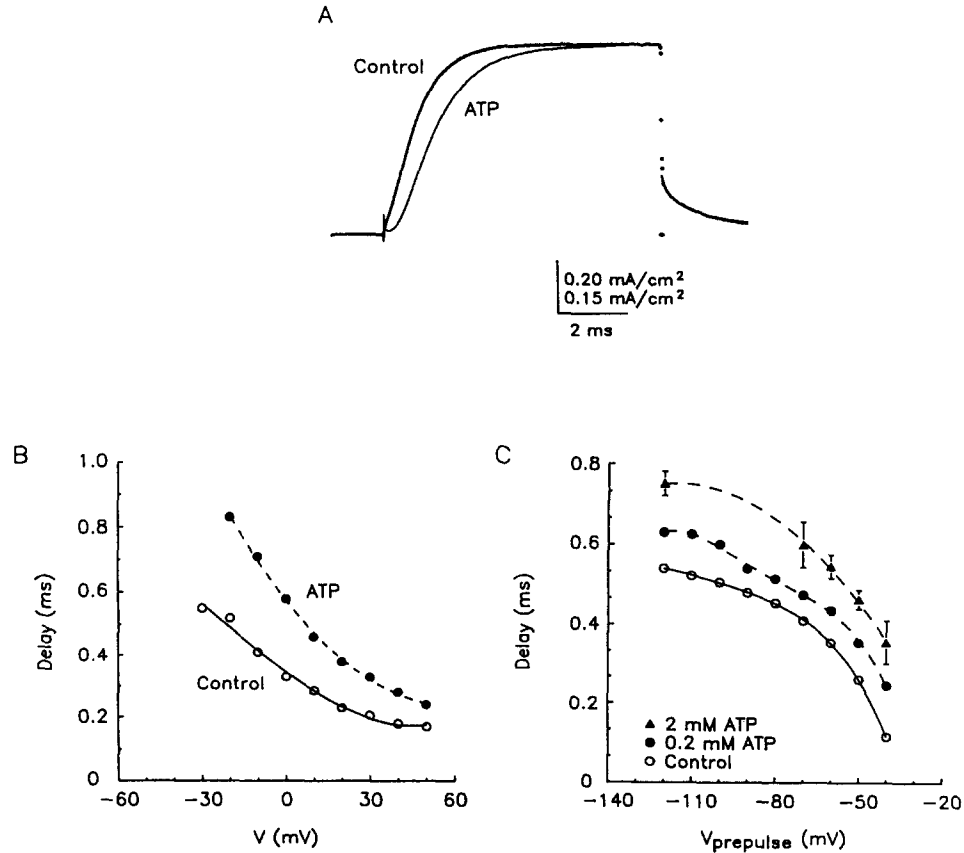


FIGURE 5. ATP increases the activation delay. (A) Current records in response to an 8-ms test pulse to 0 mV from a holding potential of -50 mV before (*thick line*) and during (*thin line*) perfusion with ATP and catalytic subunit. The solutions were 2K TrisNO₃SW // 200 KFG. The records have been scaled so the steady-state currents superimpose. The vertical scale bar represents 0.2 mA/cm^2 for the current recorded in the presence of ATP and 0.15 mA/cm^2 for the control current record. (B) Voltage dependence of the activation delay, measured as described in the text, before (*open symbols*) and during (*filled symbols*) perfusion with ATP. The holding potential was -50 mV. The solutions were 2K TrisNO₃SW // 200 KPO₄G. (C) Activation delay for a test pulse to 10 mV before (*open circles*) and during perfusion with 0.2 mM ATP and catalytic subunit (*filled circles*) or 2 mM ATP (*filled triangles*). Each point in the presence of 2 mM ATP represents the mean from two to seven experiments. Error bars represent the standard error of the mean. The test pulse was preceded by a 10-ms pulse to the potential indicated along the x-axis. The solutions were 0K TrisNO₃SW // 200 KFG.

was made more positive and the voltage dependence was shifted toward more positive potentials in the presence of ATP (Fig. 5 B). The magnitude of this shift ranged from 20 to 35 mV ($n = 6$).

Another means of increasing the activation delay is by applying hyperpolarizing prepulses to -120 mV (Cole and Moore, 1960). The increased delay produced by hyperpolarization was even more pronounced during treatment with ATP and seemed to saturate as the prepulse potential was made more negative, with the maximum delay higher in the presence of ATP (Fig. 5 C). For the experiment illustrated in Fig. 5 C, the maximum delay increased by $\sim 17\%$ in the presence of 0.2 mM ATP. During the addition of 2 mM ATP the maximum delay increased even further (Fig. 5 C). Thus, the presence of ATP seemed to enhance the effect of the hyperpolarizing prepulse on the activation delay.

Role of Phosphorylation in the Effect of ATP

We used the perfused axon because the interior of the axon should be relatively free of soluble kinases and phosphatases. In some experiments (e.g., Fig. 1) the catalytic subunit of cAMP-dependent protein kinase was added to the perfusate, along with the ATP, to substitute for the endogenous kinase we presumed to be removed. This addition of catalytic subunit was not necessary; addition of ATP alone was found to induce changes in K conductance similar to those observed when catalytic subunit was also included. For example, changes in the voltage dependence of the K conductance during the addition of either ATP with catalytic subunit or ATP alone were qualitatively identical (e.g., compare Figs. 1 C and 7 A, below). However, the effects of ATP were slightly larger when catalytic subunit was included in the internal perfusate. In eight experiments in which the catalytic subunit was included in the perfusate, the voltage at which steady-state conductance was half-maximal ($V_{0.5}$) was shifted 14.8 mV (SEM = 2.3 mV) while in seven experiments performed in the absence of catalytic subunit, $V_{0.5}$ was shifted 10.1 mV (SEM = 1.2 mV). The difference between these two means is significant ($P < 0.001$), as determined with a two-tailed *t*-test. Similar results were observed for all of the other effects of ATP on K conductance properties that we have described: ATP alone was sufficient to alter the properties of the K conductance while the catalytic subunit enhanced slightly these effects of ATP. Thus it appears that the kinase responsible for regulating K channel activity is present in the perfused axon.

Further support for the role of phosphorylation in these actions of ATP comes from considering the reversibility of this treatment. The reversibility of ATP actions in perfused axons was examined by quantifying the effects of brief ATP exposure on K conductance activation. The fast activation time constant for a voltage step to 0 mV increased during the addition of ATP to the internal perfusate, but removal of ATP did not reverse this effect even after 20 min of washing (Fig. 6 A). Likewise, the increase in activation delay produced by ATP persisted during washout of the ATP (Fig. 6 B). Additional effects of ATP, such as the shift in the steady-state voltage dependence of activation, reduction in steady-state inactivation, and the increased maximal K conductance, also were not reversed when the ATP was removed (date not shown). This irreversible nature of the ATP effects was independent of the presence of exogenous catalytic subunit in the internal perfusate. Thus,

in contrast to the situation in dialyzed axons (Bezanilla et al., 1986; Perozo et al., 1989), the effects of ATP are not reversible in perfused axons.

If the effects of ATP on K current are due to phosphorylation, one explanation for the irreversibility of ATP actions in perfused axons could be the removal of the necessary phosphatase. This possibility was explored by examining the effects of internal perfusion with alkaline phosphatase. Addition of 25 $\mu\text{g}/\text{ml}$ alkaline phosphatase to the internal solution, with simultaneous removal of ATP, caused the ATP-induced shift in the steady-state voltage dependence of activation to partially reverse (Fig. 7 A). Effects of ATP on K current activation kinetics also were reversed by alkaline phosphatase. The voltage dependence of the activation delay, which was shifted toward more positive potentials in the presence of ATP, was shifted back during removal of ATP and treatment with alkaline phosphatase (Fig. 7 B). In the presence of ATP, the voltage dependence of τ_{fast} was shifted toward more positive potentials relative to control; during washout of the ATP and perfusion with alka-

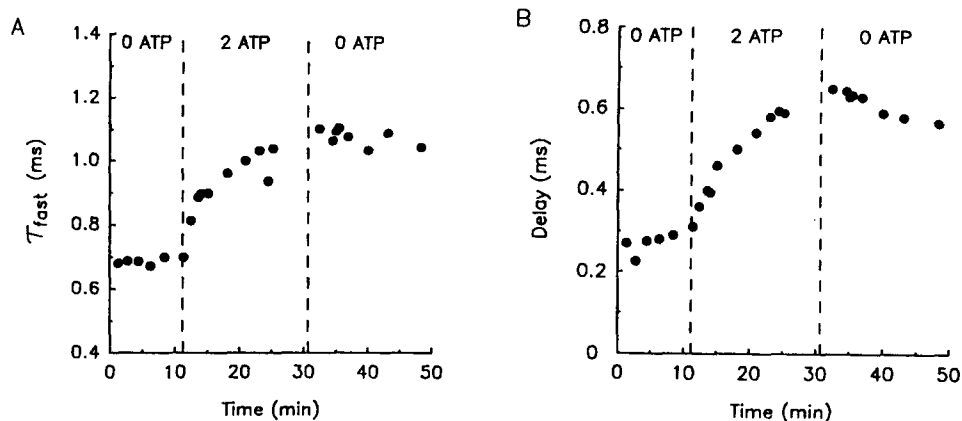


FIGURE 6. The effects of ATP are irreversible. Fast activation time constant (τ_{fast} , A) and activation delay (B) for a depolarization to 0 mV before, during, and after perfusion with ATP and catalytic subunit. The holding potential was -50 mV. The solutions were 2K TrisNO₃SW // 200 KPO₄G.

line phosphatase, the voltage dependence of τ_{fast} shifted back toward more negative potentials (data not shown). The reversibility of these effects of ATP appeared to be a function of the concentration of alkaline phosphatase because 100 $\mu\text{g}/\text{ml}$ alkaline phosphatase had a larger effect than did 25 $\mu\text{g}/\text{ml}$. For example, in Fig. 7 C the increase in $V_{0.5}$ (the potential at which $G_{\text{rel}} = 0.5$) caused by perfusion with ATP was almost completely reversed in the presence of 100 $\mu\text{g}/\text{ml}$ alkaline phosphatase while reversal was less complete with only 25 $\mu\text{g}/\text{ml}$ (e.g., Fig. 7 A). ATP effects on the activation delay also reversed 100% in the presence of 100 $\mu\text{g}/\text{ml}$ phosphatase (Fig. 7 D).

In summary, the K conductance of perfused squid giant axons is modified by ATP-dependent phosphorylation. This phosphorylation appears to be affecting the gating machinery of the channels because the voltage dependence of channel activation, deactivation, and inactivation are all altered in the presence of ATP. To better

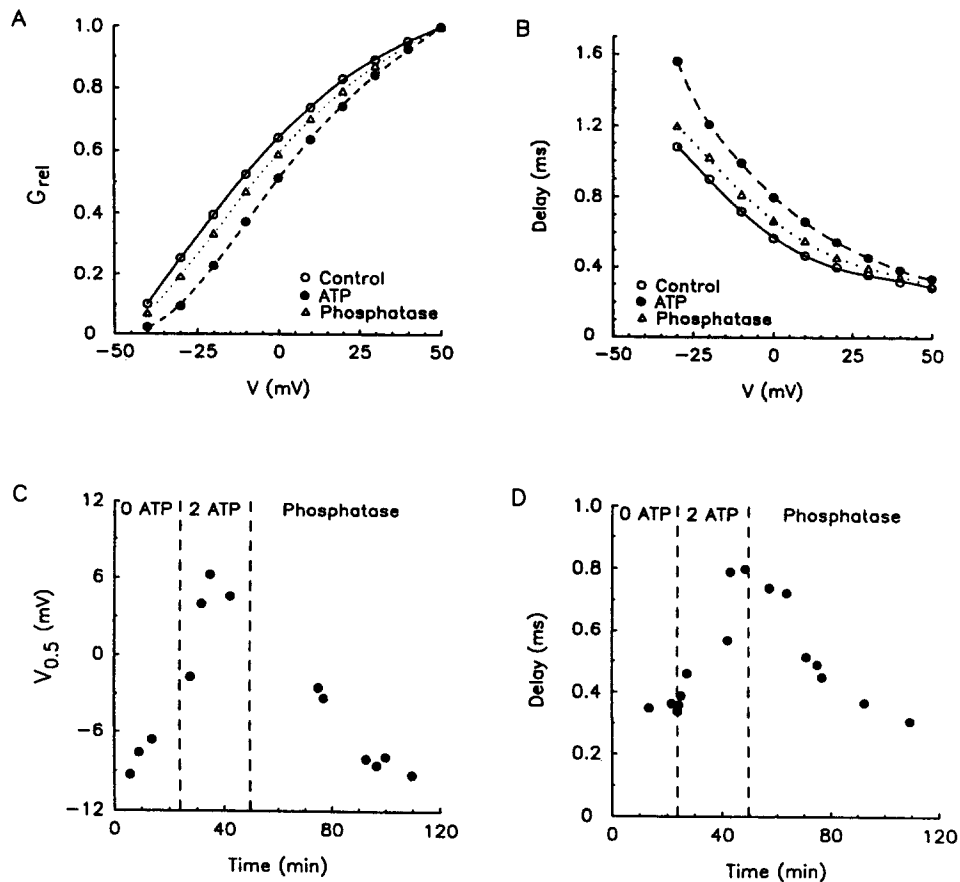


FIGURE 7. Alkaline phosphatase reverses ATP effects on steady-state activation. Relative steady-state conductance-voltage curve (A) and voltage dependence of the activation delay (B) before (open circles) and during (filled circles) perfusion with 2 mM ATP and during treatment (open triangles) with 25 μ g/ml alkaline phosphatase in the absence of ATP. The holding potential was -50 mV; the test pulse was preceded by a 10-ms hyperpolarization to -120 mV. The solutions were 2K TrisNO₃SW // 200 KPO₄G. The half-maximal activation voltage ($V_{0.5}$; C) and the activation delay for a test pulse to 0 mV (D) are shown as a function of time before and during perfusion with 2 mM ATP and during perfusion with 100 μ g/ml alkaline phosphatase in the absence of ATP. $V_{0.5}$ was determined from relative steady-state conductance-voltage curves obtained at holding potentials of -50 , -60 , and -70 mV. The holding potential for D was -50 mV. The solutions were 2K TrisNO₃SW // 200 KPO₄G.

understand these effects of ATP on the gating of K channels, we next examined the effects of ATP on K channel gating currents.

The Effects of ATP on K Gating Currents

Because the properties of K currents and the effects of ATP on these currents vary from axon to axon, we needed to compare the effects of ATP on ionic currents and gating currents recorded from the same axon. This proved challenging for several

reasons. First, K channel gating currents are quite small compared with macroscopic K currents (White and Bezanilla, 1985) and their measurement requires either the complete absence of permeant ions and/or the presence of a blocker that selectively eliminates all ionic currents. We initially adopted the protocol of White and Bezanilla (1985) and perfused the axon with solutions entirely free of permeant ions (0K TrisNO₃ // 200 NMG; see Table II). However, under these recording conditions it was not possible to examine the effects of ATP on both ionic and gating currents in the same axon because the K conductance disappeared because of the absence of permeant ions (Almers and Armstrong, 1980). Further, when axons were bathed in these solutions, addition of ATP to the internal perfusate produced no apparent change in the magnitude, kinetics, or voltage dependence of K channel gating currents ($n = 6$). Thus under these recording conditions, in which K channels become nonconducting, ATP had no effect on K channel gating currents.

We tried to avoid this problem by using internal cesium ions to slow the loss of K conductance (Chandler and Meves, 1970). However, we found that internal Cs alone offered very little protection for the conducting channel. Furthermore, when the squid axons were bathed in 0K TrisNO₃ // 350 CsFG, ATP still had no effect on gating current properties ($n = 5$). While these experiments lend further support to the idea that the phosphorylation reaction requires the presence of conducting K channels, they still did not permit an analysis of the action of ATP on K channel gating currents.

We next tried using Cs in the external medium and found that it offered much more effective protection of the K channel (Almers and Armstrong, 1980). When the axon was bathed in an external solution containing Cs, with no other permeant ions present in either the internal or external solutions, as much as 85% of the ionic K current was recovered upon reperfusion with 200 KFG. Under these conditions, ATP produced changes in the gating currents (Fig. 8 A). Shown are K channel gating currents elicited by a depolarization to -20 mV before (Fig. 8 A, lower panel) and during (Fig. 8 A, upper panel) internal perfusion with ATP. In the presence of ATP, the initial outward K channel gating current at the onset of depolarization was smaller and the decay to the baseline was slower than in the absence of ATP. Upon repolarization, the initial inward K channel gating current was larger and the decay to the baseline more rapid in the presence of ATP. These changes in kinetics can be seen more clearly in Fig. 8 B, which illustrates the integrals of the gating currents shown in Fig. 8 A. Integrated currents in the presence of ATP (*thin line*) are slower during the ON phase and faster during the OFF phase. The amplitude of the integrals did not differ because at -20 mV the gating charge movement was nearly equal under both conditions. For comparison, ionic currents, taken from the experiment illustrated in Fig. 8 A, are shown in Fig. 8 C before and during treatment with ATP. The effects of ATP on the ionic and gating currents were qualitatively similar.

The ON phase of the gating currents could be described by single exponentials. The time constants of these exponentials (τ_{on}) were slower in the presence of ATP, with the voltage dependence of τ_{on} shifted by ~ 7 mV toward more positive potentials (Fig. 9 A, circles). The OFF phase of the gating currents could also be approximated by a single exponential. Although a systematic analysis of the OFF gating kinetics was not performed, the OFF time constant recorded at -60 mV decreased by $\sim 15\%$ during ATP perfusion.

The presence of ATP also altered the voltage dependence of gating charge movement. To measure its voltage dependence, the gating charge was normalized to the maximum recorded under each condition (Q_{rel}). In both the absence and presence of ATP, gating charge was a sigmoidal function of the pulse potential (Fig. 9 B, circles). However, during internal perfusion with ATP and catalytic subunit the $Q_{rel}-V$ curve was shifted by ~ 7 mV toward more positive potentials. These effects of ATP could not be attributed to a difference in junction potential as the addition of Mg-ATP and catalytic subunit to the internal solution did not alter the junction poten-

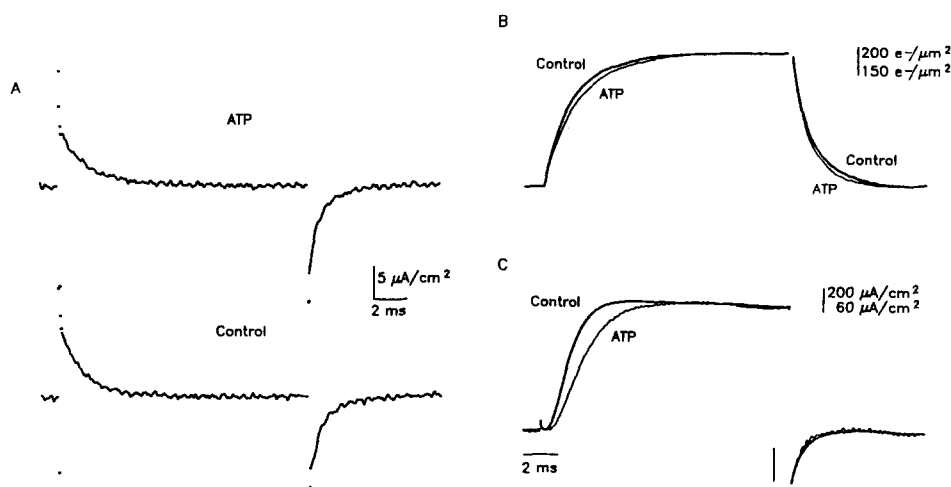


FIGURE 8. K channel gating currents are modified by internal ATP. (A) K channel gating currents elicited by a 15-ms depolarization to -20 mV from a holding potential of -60 mV are shown before (*bottom*) and during (*top*) perfusion with ATP and catalytic subunit. The test pulse was preceded by a hyperpolarization to -100 mV. The solutions were 50 Cs TrisNO₃ // 350 CsFG. (B) Integrals of these gating currents are shown, superimposed, before (*thick line*) and during (*thin line*) perfusion with ATP and catalytic subunit. The 200 $e^-/\mu\text{m}^2$ scale bar corresponds to the ON integral; the 150 $e^-/\mu\text{m}^2$ scale bar corresponds to the OFF integral. (C) Ionic current records before (*thick line*) and during (*thin line*) perfusion with ATP and catalytic subunit. The outward currents are in response to a 15-ms depolarization to -20 mV from a potential of -100 mV; the holding potential was -60 mV. The inward current records are in response to a repolarization to -60 mV from a depolarization to -10 mV. The scale bar for the inward current records is 200 $\mu\text{A}/\text{cm}^2$ before and 60 $\mu\text{A}/\text{cm}^2$ during exposure to ATP. The 2-ms time scale applies to both B and C.

tial of the solution. The solutions used for the recording of K channel gating currents were not otherwise changed during the course of an experiment.

The magnitude of the effects of ATP on the gating currents seemed to be related, in part, to the amount of ionic current recovered upon reperfusion with 200 KFG. For the experiment illustrated in Figs. 8 and 9, up to 85% of the ionic current was recovered upon reperfusion with 200 KFG. The effects of ATP on K channel gating currents were smaller in a second experiment in which only up to 50% of the K ionic current was recovered upon reperfusion with 200 KFG, although the effects of ATP on the K ionic currents in this experiment were comparable to the effects

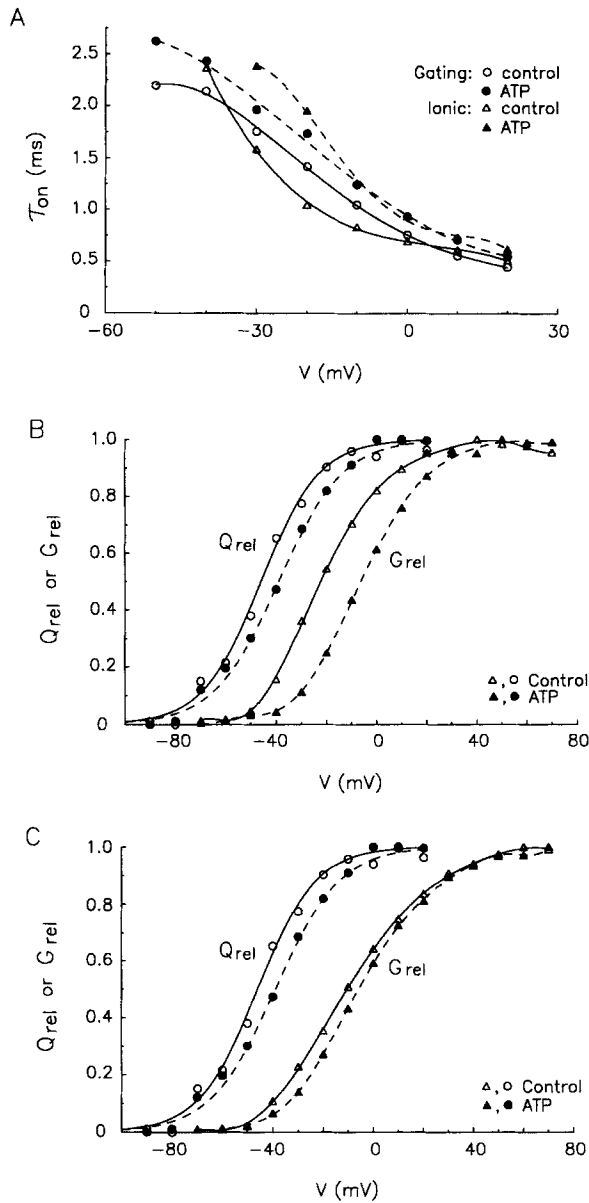


FIGURE 9. Comparison of the effects of ATP on gating and ionic currents. (A) Ionic (triangles) and gating (circles) current activation kinetics (τ_{on}) are plotted as a function of the test pulse potential before (open symbols) and during (filled symbols) perfusion with ATP and catalytic subunit. The holding potential was -60 mV and the test pulse was preceded by a hyperpolarization to -100 mV. The solutions were 50Cs TrisNO₃//200 KFG for ionic current recordings and 50Cs TrisNO₃//350 CsFG for gating current recordings. (B and C) Relative gating charge movement (Q_{rel} , circles) is plotted along with relative conductance (G_{rel} , triangles) as a function of the membrane potential during the depolarizing test pulse. The filled symbols (circles and triangles) represent the presence of ATP and catalytic subunit while the open symbols (circles and triangles) represent the absence of ATP. The holding potential was -60 mV and the test pulse was preceded by a hyperpolarization to -100 mV. The solutions were 50Cs TrisNO₃//350 CsFG for gating current recordings and 0K TrisNO₃//200 KFG (B) or 50Cs TrisNO₃//200 KFG (C) for ionic current recordings.

observed when there was no loss of K conductance. The ON gating current time constants were somewhat slower in the presence of ATP, with τ_{on} shifted ~ 4 mV towards more positive potentials, but this is less pronounced than the effects described above. The voltage dependence of Q_{rel} also was shifted only ~ 5 mV toward more positive potentials in the presence of ATP. Similar results were observed in one other experiment in which K current only partially recovered after Cs treatment.

Comparison of ATP Effects on K Gating and Ionic Currents

Activation and deactivation kinetics. The changes in the activation kinetics of the gating currents produced by treatment with ATP were qualitatively similar to the changes in the activation kinetics of the ionic currents. For example, ionic currents recorded in 2K TrisNO₃ were slower after treatment with ATP, with the voltage dependence of the activation time constants shifted ~15 mV toward more positive potentials (see Fig. 4 B). This was similar to, but larger than, the 7-mV shift in the voltage dependence of the ON gating current time constant (Fig. 9 A, *circles*). This difference in the magnitude of the ATP effect on the activation time constants did not appear to be a consequence of the different external solutions used to record gating and ionic currents. When ionic currents were recorded under conditions more similar to those used for the gating currents (i.e., the external solution was 50Cs TrisNO₃), the shift in ionic current activation time constant still was twofold larger than the shift observed in the ON gating current time constant (Fig. 9 A, *triangles*). This difference in the effects of ATP on the activation time constants of gating and ionic currents may simply be due to the different shapes of the $\tau_{on} - V$ curves for gating and ionic currents (compare open triangles with open circles, and filled triangles with filled circles in Fig. 9 A).

The deactivation kinetics of both ionic and gating currents were faster in the presence of ATP, with the magnitude of these changes comparable for both types of currents. In the presence of ATP, gating current deactivation kinetics at -60 mV were hastened ~16% while ionic current deactivation kinetics at -60 mV were hastened ~23% (data not shown). However, in both the absence and presence of ATP, the ionic tail currents decayed ~20% faster than the gating tail currents, in contrast to the similar gating and ionic current deactivation kinetics reported by White and Bezanilla (1985). The discrepancies may lie in the use of external Cs for the recording of gating currents in these experiments; external Cs has been shown to slow the closing rate of K channels (Matteson and Swenson, 1986).

Voltage dependence of gating charge movement and conductance. The ATP-induced shifts in the steady-state voltage dependence of K conductance and relative charge movement were qualitatively similar (Fig. 9, B and C). Generally, ionic currents were recorded in Tris seawater containing 0 or 2 mM K and the gating currents were recorded in Tris seawater containing 50 mM Cs (i.e., see Fig. 9 B). For the experiment illustrated in Fig. 9 B, ATP shifted the voltage dependence of relative charge movement 7 mV toward more positive potentials, which was ~2.5 times smaller than the 17-mV positive shift observed in the voltage dependence of the relative steady-state conductance in the presence of ATP. This marked dissimilarity in the magnitude of the ATP-induced shifts was observed in all experiments performed under these conditions ($n = 3$). Thus, comparing gating and ionic currents recorded under these conditions, phosphorylation increased the separation between the voltage dependence of gating charge movement and K conductance. For the experiment illustrated in Fig. 9 B, Q_{rel} and G_{rel} were separated by 25 mV in the absence of ATP, compared with 35 mV in the presence of ATP. In another experiment (data not shown), Q_{rel} and G_{rel} were separated by 22 mV before and 40 mV after treatment with ATP.

However, the shifts in voltage dependence produced by ATP were more similar

when macroscopic ionic currents were recorded under conditions more similar to those of the gating currents. This point is illustrated in Fig. 9 C, which compares ionic currents recorded in Tris seawater containing 50 mM Cs with gating currents recorded in the same external solution. These data were from the same experiment illustrated in Fig. 9 B. Under these recording conditions, the shift in the $G_{\text{rel}}-V$ curve after phosphorylation was somewhat smaller than the shift in the $Q_{\text{rel}}-V$ curve so that the voltage separation was ~ 35 mV both in the absence and presence of ATP. In another experiment (data not shown) the shift in both $Q_{\text{rel}}-V$ and $G_{\text{rel}}-V$ curves was ~ 5 mV, and the voltage separation was ~ 45 mV in both the absence and presence of ATP. Thus, when the ionic currents were recorded under conditions similar to those of the gating currents there was no increase in the voltage separation of the $Q_{\text{rel}}-V$ and $G_{\text{rel}}-V$ curves.

These results indicate that a complication is introduced by the use of different ionic conditions for gating and ionic current recordings. The question then becomes which condition provides the most valid comparison between gating and ionic currents. We next performed several experiments to determine how the recording conditions influenced ionic currents and their response to ATP.

Influence of external ions on the ATP response. In the experiment illustrated in Fig. 9 C, the shifts in the $Q_{\text{rel}}-V$ curve and the $G_{\text{rel}}-V$ curve were quite similar. This might support the validity of comparing gating currents with ionic currents recorded in 50Cs TrisNO₃. However, in another experiment done under these conditions (data not shown) there was no shift in either the $Q_{\text{rel}}-V$ curve or the $G_{\text{rel}}-V$ curve. After ATP was removed from the internal medium, this preparation was bathed in 0K TrisNO₃ and $G_{\text{rel}}-V$ curve was shifted ~ 15 mV in comparison to control. This indicates that while the presence of Cs does not prevent the action of ATP it somehow blocks expression of the full magnitude of the action of ATP.

In an attempt to determine how Cs altered the response to ATP we next examined the effect of Cs on K currents. When the external solution was changed from 0K TrisNO₃ to one containing Cs, K current decreased at all test potentials (data not shown). The reduction in K current was voltage dependent, being less prominent at positive potentials (Fig. 10 A, *open symbols*). This voltage-dependent block of K current by external Cs has been reported elsewhere (Adelman and Senft, 1968; Bezanilla and Armstrong, 1972; Adelman and French, 1978). Because of this voltage-dependent reduction, external Cs introduced an apparent positive shift in the conductance-voltage curve (Fig. 10 B). The shift of the midpoint of the curve in the presence of external Cs averaged ~ 20 mV (SEM = 6 mV, $n = 6$). However, the kinetics of activation were not altered significantly when the solution was changed to one containing Cs (data not shown). Thus, like internal ATP, external Cs altered the steady-state voltage dependence of activation of the K conductance.

The above results suggest that Cs and ATP might be producing similar effects on the K conductance. If so, then ATP might be expected to influence the effect of Cs on K conductance. Indeed, the voltage dependent reduction of K current by Cs was markedly reduced, and often eliminated, in axons perfused with ATP (Fig. 10 A, *filled symbols*). ATP also attenuated the Cs-induced shift in the relative $G-V$ curve (Fig. 10 C). In the presence of internal ATP, activation kinetics were, again, not significantly altered by the addition of Cs to the external solution (data not shown).

In summary, the smaller ATP-induced shift in the relative G - V curve in the presence of external Cs may be due to Cs effectively masking the actions of ATP. Because Cs did not alter K channel gating kinetics, it may simply be interfering with ion permeation through the K channels. If so, then Cs should have little effect on the properties of K channel gating current. Thus, the most reliable means of assess-

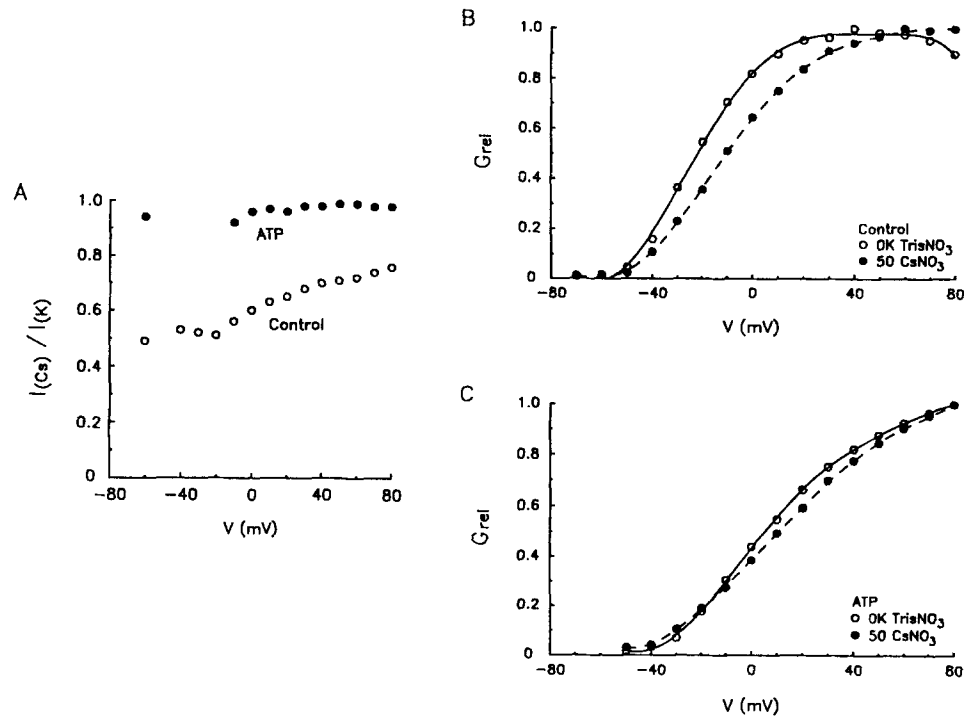


FIGURE 10. The effects of external Cs on the K conductance. (A) Steady-state current recorded in Cs containing seawater was divided by the steady-state current obtained in 0K TrisNO₃. This ratio is plotted as a function of the test pulse potential before (*open symbols*) and during (*filled symbols*) perfusion with 2 mM ATP and catalytic subunit. The holding potential was -60 mV and the test pulse was preceded by a hyperpolarization to -100 mV. The solutions were 0K TrisNO₃ // 200 KFG in the absence or 50Cs TrisNO₃ // 200 KFG in the presence of external Cs. Relative conductance is plotted as a function of the membrane potential during the test pulse in the absence (B) or in the presence (C) of internal ATP before (*open symbols*) and during (*filled symbols*) addition of Cs to the external solution. The holding potential was -60 mV and the test pulse was preceded by a hyperpolarization to -100 mV. The solutions were 0K TrisNO₃ // 200 KFG (*open symbols*) or 50Cs TrisNO₃ // 200 KFG (*filled symbols*).

ing the relative effects of ATP on ionic and gating currents should be to compare gating currents measured in Cs with ionic currents measured in the absence of Cs. This comparison reveals that the relative separation between the voltage dependence of gating charge movement and ionic conductance is increased by ATP (Fig. 9 B).

DISCUSSION

Our results show that the addition of ATP to the internal solution of perfused giant axons of squid produces marked changes in the properties of K ionic and gating currents. The effects of ATP on K channel gating currents were qualitatively similar to its effects on macroscopic K currents. These actions appear to be due to ATP acting as a phosphate donor for an endogenous kinase that is not lost during internal perfusion.

ATP Effects Are Due To a Phosphorylation Reaction

The irreversible effects of ATP in perfused squid axons lend further support to the suggestion that the effects of ATP on the K conductance are due to a phosphorylation reaction (Bezanilla et al., 1986; Perozo et al., 1989). First, they argue against the possibility that the effects of ATP are the result of a direct action of ATP on the channel (e.g., Kakei et al., 1985) by demonstrating that the actions of ATP last far beyond the time of exposure to ATP. Second, the reversal of the effects of ATP by perfusion with alkaline phosphatase indicates that a phosphorylation reaction is involved. Although the concentrations of alkaline phosphatase required were relatively high (180 to 720 nM, assuming a molecular weight of 140 kD [Fosset et al., 1974]), it is possible that the high concentration required was a consequence of the alkaline pH optimum of this phosphatase (Chappelet-Tordo et al., 1974). Alternatively, a higher concentration of alkaline phosphatase might have been required because the phosphorylation site(s) in the axon were not a preferred substrate for this phosphatase.

Although the effects of ATP on the K conductance in the squid axon appear to be a consequence of a phosphorylation reaction, it cannot yet be determined whether the K channel itself or some other component of the axon is the substrate. Because this reaction occurs in perfused axons, in the absence of most cytoplasmic constituents, it is likely that the substrate is intimately associated with the K channel(s). In several cases it has been shown that ion channels are directly phosphorylated (e.g., Costa and Catterall, 1984; Haganir et al., 1984; Ewald et al., 1985). More recently, the primary structures of several K channels have been determined and found to contain consensus phosphorylation sequences (e.g., Baumann et al., 1987, 1988). Thus it is possible that the effects that we have reported are also due to a direct phosphorylation of channel proteins.

Addition of ATP alone to the internal perfusate was sufficient to alter the K conductance in a manner identical to that observed when protein kinase was included. This suggests that the endogenous kinase is so closely associated with the membrane that it can not be removed during perfusion. There are several kinases that are thought to be capable of such close association with membranes. These include protein kinase C (Kikkawa et al., 1982), the type II Ca/calmodulin-dependent protein kinase (Kennedy et al., 1983), and the cAMP-dependent protein kinase (Maeno et al., 1971; Rubin et al., 1979). All of these protein kinases have been implicated in ion channel modulation via ATP-dependent phosphorylation (Levitan, 1985).

Many kinases require second messengers, such as Ca^{2+} , cAMP, or diacylglycerol, to express their enzymatic activity (Nestler and Greengard, 1984). Thus, it is surprising that modulation of the K conductance of squid axons required only the addition

of ATP to the internal perfusate. This suggests that the endogenous kinase was in an active state. How is this kinase activated in perfused axons? One possibility is that the ions or molecules required to activate the kinase either are not washed out by perfusion or can enter the axon from the external solution. It seems likely that small, soluble activators, such as Ca^{2+} and cAMP, would be efficiently removed from the axon by perfusion. However, it is possible that a hydrophobic molecule, such as diacylglycerol, remains associated with the membrane of perfused axons. Another possibility is that the endogenous kinase does not require second messengers to be enzymatically active. One example of such an enzyme is casein kinase (Hathaway and Traugh, 1982). Another, which is distinct from casein kinase, is an independent protein kinase found in squid axoplasm (Pant et al., 1986). Alternatively, the kinase might be artificially activated as a consequence of axon isolation or perfusion. For example, protein kinase C is permanently activated by Ca^{2+} -sensitive proteases (Inoue et al., 1977) of the sort present in squid axoplasm (Pant and Gainer, 1980). Further work will be necessary to define the kinase involved and its mechanism of regulation.

Variability in the Magnitude of the ATP Effects

Although the effects of Mg-ATP on the squid axon K conductance reported here were always observed under the conditions of these experiments ($n > 20$), the magnitude of these effects was quite variable. This variability could be physiological; the basal level of phosphorylation might vary from axon to axon. If the basal level of phosphorylation was high, treatment with ATP would have a smaller effect on the K conductance while with a low basal level ATP treatment would have a larger effect. Alternatively, the variability may arise from subtle differences in the perfusion of individual axons. ATP is smaller than the presumed endogenous phosphatase and will therefore diffuse away faster after the onset of the perfusion. Thus, if the perfusion rate is slow or incomplete the endogenous phosphatase might remain in the axon for a longer period of time and the basal level of phosphorylation would be reduced while with a faster perfusion rate the endogenous phosphatase might linger in the axon for less time thus elevating the basal level of phosphorylation.

Multiple Mechanisms for Phosphorylation Effects on K Conductance

ATP-dependent phosphorylation had a number of complex actions upon the K channels of squid axons. We will briefly list these actions and then try to explain them mechanistically with a qualitative model.

There were two primary actions of ATP on the steady-state properties of the K conductance. First, ATP shifted, toward more positive potentials, the voltage at which the steady-state K conductance was half-maximal. ATP produced similar, but smaller, shifts in the steady-state voltage dependence of K channel gating charge movement. A second action of ATP was to shift the steady-state inactivation of the K conductance toward more positive potentials. Because the ATP effect on the maximal K conductance was more obvious at depolarized holding potentials, it appears that most, or all, of this effect was due to the action of ATP on inactivation.

In addition to these actions upon the steady-state properties of K conductance, ATP also altered the time course of the K conductance. K current activation kinetics

were slower and deactivation kinetics were faster in the presence of ATP. ATP had qualitatively similar actions upon the kinetics of K channel gating currents. It is possible that these kinetic changes are simply due to a shift in the voltage dependence of the gating parameters toward more positive potentials. However, ATP appears to have additional actions on gating because two current records with the same activation time constants, recorded with and without ATP, did not superimpose when scaled to the same steady-state conductance. One such additional action of ATP was an enhancement of the activation delay (see Fig. 5 A).

To explain these actions of ATP-dependent phosphorylation on K channel properties, it is necessary to invoke a minimum of two distinct mechanisms. One mechanism shifts the voltage dependence of gating parameters to more positive potentials. This shift could alter the rates of transition between the various states of the channel and thereby alter the time course of K current activation and deactivation, ON and OFF gating current kinetics, the amount of inactivation present at a given potential, and the steady-state voltage dependence of the K conductance and gating charge movement. It is conceivable that these shifts are a consequence of an addition of negative charge (perhaps from a phosphate group) to the gating moieties of the channels.

However, not all of the actions of ATP can be explained by such a "shift" mechanism. In particular, ATP had smaller effects on the activation kinetics and steady-state voltage dependence of gating currents than on ionic conductance. Such differences are not surprising since macroscopic currents primarily reflect the properties of the transition between the final closed configuration and the open configuration of a channel, while gating current properties reflect the transitions between the closed configurations as well as the final transition to the open state (Armstrong and Matteson, 1984; Bezanilla, 1985). However, the increased separation between the steady-state voltage dependence of gating currents and ionic conductance indicates that there is an increased number of effective closed states of the channel (White and Bezanilla, 1985). This separation results because substantial charge movement can occur between the nonconducting states of the channel(s) before the channel(s) open. Thus, it appears that a second mechanism of phosphorylation is to increase the actual number of closed states of the K channel and/or decrease the rates of transition between some of these states.

The prolongation of K current activation delay by ATP provides additional evidence for this second mechanism. This prolongation is due not only to a shift in the voltage dependence, but also to an increase in the maximum activation delay (see Fig. 5 C). An increase in this activation delay in response to a hyperpolarizing prepulse is thought to be due to a shifting of the channel to a more closed configuration so that the channel must pass through several more states in order to open (Cole and Moore, 1960). By extension, an increase in the maximum value of this delay could indicate an increased number of closed configurations of the channel.

These two mechanisms, however, are not sufficient to explain an additional effect seen only in dialyzed squid axons (Bezanilla et al., 1986; Perozo et al., 1989). Neither a positive shift in voltage dependence nor an increase in the number of closed transitions can explain the large increase in maximal K conductance caused by ATP in dialyzed squid axons. For example, in dialyzed squid axons, the ATP-

induced increase in steady-state K conductance is much larger than that which would be expected if it were simply due to a removal of steady-state inactivation. Most notably, at a holding potential of -60 mV, maximal K conductance increased up to threefold while steady-state inactivation decreased only 1.2-fold in the presence of ATP (Perozo et al., 1989). These observations, then, necessitate a third mechanism whose nature is not clear. Recently it has been shown that a number of different types of K channels with different unitary conductances are present in the squid axon (Llano et al., 1988). It is possible that ATP is changing the relative contributions of these various channels to the macroscopic current to increase its maximal value. Preliminary experiments suggest that this is the case, because ATP increases the activity of the largest conductance (40 pS) channel (Vandenberg et al., 1989). Perhaps the other two proposed mechanisms act on the smaller conductance (20 pS) channel that is thought to be the predominant contributor to the macroscopic K conductance of perfused axons. Furthermore, the absence of this third mechanism in perfused squid axons may indicate that in perfused axons exogenous ATP cannot, for reasons that are not clear, alter the K channel responsible for the increase in the maximal macroscopic K conductance.

Comparison with Previous Results

Our measurements of K channel gating currents coincide with those of White and Bezanilla (1985). Because we have found that the effects of ATP on gating currents were similar to its effects on K ionic currents, our results provide strong additional support for the conclusion that these gating currents are indeed related to K channel gating. We have also found that the time constant of ON gating and ionic currents recorded at positive potentials are strikingly similar, both in the absence and presence of ATP. However, minor disparities between the time course of these two currents arise at negative potentials. Spires and Begenisich (1989) have also reported a disparity between ionic and gating current time constants at negative potentials. While the reported disparities are not identical in the two studies, this is likely because of differences in methods of measurement, such as the use of different pulse durations. At any rate, minor disparities between the time course of ionic and gating currents are predicted by some models of K channel gating (e.g., Spires and Begenisich, 1989) and do not necessarily indicate that the currents are not related to K channel gating.

Our work on perfused axons complements studies on dialyzed axons by providing better defined intracellular conditions and clear measurements of K channel gating currents and their modulation by internal ATP. Our results indicate that ATP alters the gating and voltage dependence of the K conductance of perfused axons in much the same ways as observed in dialyzed axons (Bezanilla et al., 1986; Perozo et al., 1989). A striking difference between these two sets of experiments is that the effects of ATP on K currents recorded from dialyzed, but not perfused, axons are readily reversible. Further, the potentiating effect of ATP is more pronounced and is seen at more hyperpolarized holding potentials in dialyzed squid axons. These differences could be caused by the removal of a phosphatase by internal perfusion. In addition, ATP may be increasing the maximal K conductance in dialyzed axons, as discussed above.

Recently it has been reported that addition of 5 mM Mg-ATP to the internal solution of perfused squid axons resulted in decreases in macroscopic K current and no changes in gating kinetics (Clay and Szuts, 1989). This is in marked contrast to the results reported here and in previous studies. It is difficult to reconcile these results with those reported here. Because the concentration of Mg-ATP used in the present experiments never exceeded 2 mM, and often was as low as 0.2 mM, one possibility is that Mg-ATP has different effects on the K conductance at the very high concentration of 5 mM.

Physiological Significance of K Channel Modulation

As with many other K channels, the potassium channels of the squid giant axon are modified by an ATP-dependent phosphorylation reaction. Although phosphorylation is generally a regulatory mechanism, the physiological function of this particular phosphorylation is not clear. ATP has been shown to influence action potential waveform (Perozo et al., 1989). This, in turn, would affect axonal conduction and also neurotransmitter release (e.g., Augustine et al., 1986). It also is possible that phosphorylation may serve as a molecular tag to identify a protein that is to be removed from (or retained in) the membrane. Whatever the function of this phosphorylation reaction, it is likely to occur in vivo because ATP affects the K conductance at a concentration ($K_m = 10 \mu\text{M}$, Perozo et al., 1989) well below the normal ATP concentration of the axon ($\sim 1 \text{ mM}$; Gainer et al., 1984).

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