Modulation of K Channels in Dialyzed Squid Axons

ATP-mediated Phosphorylation

E. PEROZO, F. BEZANILLA, and R. DIPOLO

From the Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Cientificas, Caracas, 1020, Venezuela; and the Department of Physiology, Jerry Lewis Neuromuscular Center and Ahmanson Laboratory of Neurobiology, University of California, Los Angeles, California 90024

ABSTRACT   In squid axons, internally applied ATP potentiates the magnitude of the potassium conductance and slows down its activation kinetics. This effect was characterized using internally dialyzed axons under voltage-clamp conditions. Both amplitude potentiation and kinetic slow-down effects are very selective towards ATP, other nucleotides like GTP and ITP are ineffective in millimolar concentrations. The current potentiation K_m for ATP is near 10 μM with no further effects for concentrations >100 μM. ATP effect is most likely produced via a phosphorylative reaction because Mg ion is an obligatory requirement and nonhydrolyzable ATP analogues are without effect. In the presence of ATP, the K current presents more delay, resembling a Cole-Moore effect due to local hyperpolarization of the channel. ATP effect induces a 10–20 mV shift in both activation and inactivation parameters towards more depolarized potentials. As a consequence of this shift, conductance-voltage curves with and without ATP cross at ~−40 mV. This result is consistent with the hyperpolarization observed with ATP depletion, which is reversed by ATP addition. At potentials around the resting value, addition of ATP removes almost completely K current slow inactivation. It is suggested that a change in the amount of the slow inactivation is responsible for the differences in current amplitude with and without ATP, possibly as a consequence of the additional negative charge carried by the phosphate group. However, a modification of the local potential is not enough to explain completely the differences under the two conditions.

INTRODUCTION

Protein phosphorylation is currently associated with the modulation of membrane properties in different preparations. This process has been related with the action of specific neurotransmitters interacting at the surface of the cell, and its effect is mediated through a second messenger cascade (Nestler and Greengard, 1984). In

this way, ion channels of excitable cells classically considered only dependent on voltage and time, are regulated in their basic properties.

Regulation of channel properties by a phosphorylation process has been reported for Ca channels (Pellmar and Carpenter, 1980; Dunlap and Fishbach, 1981; Doroshenko et al., 1984; De Riemer et al., 1985), different kinds of K channels (Kaczmarek et al., 1980; Castellucci et al., 1982; Benson and Levitan, 1983; Ewald et al., 1985), and intercellular gap-junction channels (Wiener and Lowenstein, 1983). In different preparations, changes in these conductances lead to modifications in action potential duration with increments in synaptic output (Dunlap and Fishbach, 1981; DePeyer et al., 1982), modulation of repetitive firing (Brown and Adams, 1980), or pacemaker activity (Wilson and Wachtel, 1978).

Since the quantitative description by Hodgkin and Huxley (1952a, b), the kinetics and conductive parameters of the potassium conductance (g_K) of the squid has been studied extensively by means of classical macroscopic studies (see Meves, 1984), single-channel events (Conti and Neher, 1980; Llano et al., 1988), or the study of charge movements associated with the opening and closing of K channels (White and Bezanilla, 1985). The amount of information collected makes this conductance the classical delayed rectifier. Recently, this simple view has been modified by the finding that g_K in squid axon can be regulated by internal ATP (Bezanilla et al., 1986). Increments in the intracellular ATP concentration, in voltage-clamped and dialyzed axons, lead to a stimulation in amplitude and a slowing down in the kinetic properties of the K current (I_K) for large depolarizations. Because of the possibility of obtaining highly accurate macroscopic single-channel and gating currents in the same cell, the squid axon is an ideal preparation to study biophysical and biochemical characteristics of ion channel regulation. In fact, regulation studies may be performed to find out what is the effect of the regulatory molecule and the basic mechanisms that are modified in the channel by the presumed phosphorylation.

In this paper, we are concerned with the characterization of the ATP-mediated modificatipn of I_K under internal dialysis conditions. Our results show that ATP at micromolar concentrations is able to selectively stimulate g_K magnitude and slow down its kinetics, and they suggest that the effect is mediated by a phosphorylation that involves phosphate transference to the internal side of the membrane, with concomitant modification in the density of surface charges near the voltage sensor of the channel. The current increase is partially explained by a shift of the slow inactivation parameter toward more depolarized potentials; however, this effect cannot fully explain all the differences found for I_K after ATP treatment. Part of this work has been presented in preliminary form (Perozo et al., 1986).

METHODS

The experiments reported here were performed using live specimens of the tropical squid *Loligo plei* at the Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela; a few experiments were performed at the Marine Biological Laboratory, Woods Hole, MA, using specimens of *Loligo pealei*. After decapitation, the hindmost giant axon was dissected in flowing seawater and carefully cleaned of connective tissue under a dissecting microscope. Mean axon diameter was 410 μm for *L. plei* and 530 μm for *L. pealei*, with resting potentials between −50 and −68 mV.

Experimental Chamber and Voltage Clamp

The experimental chamber resembles the one described by Bezanilla et al., 1982. Briefly, it is divided in three compartments electrically isolated by lucite partitions, and platinum plates collect the current from the central compartment. The voltage clamp, pulse generator, and acquisition system are identical to those described by Stimers et al. (1985). The general arrangement for the dialysis technique under voltage-clamp conditions is similar to the methods reported by Dipolo et al. (1985). A Pt-Pt axial wire was placed inside a dialysis capillary sitting in a four-way Hamilton valve, and inserted from one cut end of the axon. The dialysis capillary is connected to a motor-driven syringe set at a constant flow of 1 μl/min. Ionic currents were recorded using a P/-2 procedure with a subtracting holding potential of -120 mV, filtered at 33 kHz, digitized, and stored in magnetic media for subsequent off-line analysis. Ionic conductance was calculated by dividing the amount of current at the end of a pulse plus the "tail" by the magnitude of the voltage change (Gilly and Armstrong, 1982).

Porosity of the Dialysis Capillary

Dialysis capillaries used throughout all the experiments were made from regenerated cellulose (RCC) (Spectrum Medical Ind., Inc., Los Angeles, CA), with an external diameter of 190 μm and a nominal molecular weight cut-off of 9,000 D. These capillaries are permeable in their whole length and were used as purchased, with no further permeabilization procedures. A protective impermeable coat was applied to the RCC around the air-gap region to avoid mixing with the external solution due to the porosity of the capillary. It was found useful to compare the permeability characteristics of the present capillaries with the ones made of acetate cellulose, which are described extensively elsewhere (Brinley and Mullins, 1967; Dipolo, 1974). Acetate cellulose capillaries (ACC) were obtained from Albany International Research Co., Dedham, MA and permeabilized by soaking in NaOH 50 mM for 24 h. These capillaries possess an external diameter near 150 μm and the porous region extends for ~1 cm in length.

A comparison of the porosity of both capillaries was made by placing them in an experimental dialysis chamber with a system of guards as described by Dipolo et al. (1985), and by measuring the transfer of [22]Na from the chamber to the lumen of the capillary. The solution was then collected in a filter paper. Even though RCC were twice as permeable as the ACC, both suffered changes in porosity that were related to the frequency of use. This phenomenon is unavoidable, but treatment of the capillary with pronase or 4 M urea seems to increase the longevity of the capillary.

Solutions

Internal and external solution composition is shown in Table I. All the solutions were adjusted to an osmolality of 1,000 $\pm$ 4 mosmol/kg H_2O, with a pH of 7.3 for internal solutions and 7.6 for the external solution. ATP and the rest of the nucleotides used were added with an equivalent amount of extra $MgCl_2$, in order to saturate the nucleotide divalent binding site. ATP concentrations in the dialysis solution were verified by a bioluminescence assay using firefly tail powder, and recording the peak of light emission with a photomultiplier connected to a strip chart recorder (Strehler and Totter, 1952; Mullins and Brinley, 1967). ATP was obtained from Boehringer Mannheim, and it was mixed with an equimolar amount of $MgCl_2$ before adding it to the internal solution. The other nucleotides, ADP, AMP, cAMP, ITP (inosine 5' triphosphate), GTP, and AMP-PCP, as well as phosphoarginine were purchased from Sigma Chemical Co., St. Louis, MO. Tetrodotoxin was from Sankyo Co., Tokyo.

TABLE I

Solution Composition

Substance	Artificial seawater	Dialysis solution
Sodium	440	0
Potassium	10	310
Magnesium	50	4
Calcium	10	0
Chloride	580	0
Phosphate	0	30
Tris	10	81
EGTA	0	1
Glutamate	0	310
Glycine	0	288

All the concentrations are in millimoles/liter. Artificial seawater also contains 1 mM NaCN and 300 nM tetrodotoxin. Nucleotide addition to internal solution carries an equivalent amount of Mg.

RESULTS

ATP Increases Outward Current Amplitude

When voltage-clamped axons are dialyzed with ATP-free solutions, they show a gradual decrease in outward current amplitude as revealed by pulsing from a certain holding potential (usually -50 to -60 mV) to 0 mV. This effect can be reversed by adding the nucleotide to the dialysis fluid. Fig. 1 shows an experiment in which an axon was initially dialyzed with a solution containing 310 K^+, 15 mM PO_4^-, 4 mM Mg^{2+}, and 1 mM EGTA, in the nominal absence of Na^+, Ca^{2+}, and ATP (Table I), and in which currents were elicited by pulsing to 0 mV from a holding potential of -60 mV at intervals of 3–5 min. The falling phase of the current amplitude has two clearly distinguishable components: an early one, possibly related to potassium washout (these axons have a mean internal K^+ concentration of ~370 mM, Perozo, 1985) and a second slower phase, probably reflecting ATP washout as would be expected from a process of diffusion with binding (Brinley and Mullins, 1967).
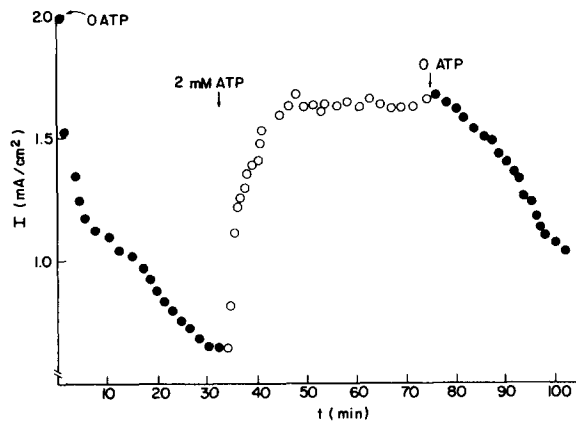

FIGURE 1. Magnitude of the potassium current with changes in the intracellular concentration of ATP. The axon was clamped at -60 mV and pulsed to 0 mV. The ATP values are the concentration of ATP in the fluid entering the dialysis capillary. ●, change to 0 ATP; O, change to 2 mM ATP-Mg. Axon diameter, 460 μm.

Upon addition of 2 mM ATP-Mg, the current amplitude rose steeply until a new steady state value was reached, which in most of the fibers was ~200% the value obtained at 0 ATP; in some cases the increase was up to 300%, depending on the magnitude of the pulse and the holding potential. The effect is reversible as can be noted from the last minutes of the experiment, in which a new removal of ATP induced a slow decrease in I_K, nevertheless, the initial current magnitude before dialysis is never recovered.

Fig. 2 *A* shows a family of potassium currents recorded from an axon at a holding potential of −60 mV by pulsing to potentials up to 60 mV in increments of 10 mV. The currents shown have been corrected for leakage and capacity transients. The upper family was recorded after the axon was dialyzed for 55 min with 0 ATP solution while the lower family corresponds to the same axon after addition of 2 mM Mg-ATP to the dialysis fluid. In a wide range of potentials there is potentiation of current magnitude during the pulse. At the same time tail current amplitude is also potentiated most likely because of the increase in conductance driving force produced by the K transferred to the periaxonal space when I_K is stimulated by ATP.

The conductance-voltage (G-V) curves for both conditions (Fig. 2 *B*) show a complex effect: besides the large ATP-induced potentiation in conductance at highly depolarized potentials, it can be noted that the curves cross at around −40 mV. While the G-V curve for 0 ATP starts to rise at potentials near the holding potential value, the G-V curve in the presence of ATP is quite flat at small depolarizations, and becomes very steep for voltages more positive than −40 mV. The crossing of the G-V curves suggests that ATP shifts the gating parameters toward more depolarized potentials, and this can be verified by normalizing the two curves relative to the maximum conductance in the presence and absence of ATP, in which case a 15–20 mV shift is obtained (Fig. 2 *C*). The fact that the conductances are not equal even at highly depolarized potentials strongly suggests an increase in the maximal conductance when ATP is present.

Changes in Kinetic Parameters

Independent of the amplitude potentiation, ATP also produces a slowing down in outward current kinetics. Normalization of I_K obtained at the same test voltage with and without ATP reveals a considerable change in I_K kinetics. This is shown in Fig. 3 *A*, which compares two current traces from the same axon, elicited with a pulse from a −60-mV holding potential to 0 mV. The current scale corresponds to the trace in the presence of ATP, and I_K in the absence of the nucleotide was multiplied by an arbitrary constant (often near 2) in order to normalize to the maximum current at the steady state. There is a clear increase in the turn-on time constant in the presence of ATP as compared with the control record, but there was a decrease in the turn-off time constant (Fig. 4). A greater lag before the rise of the current is also evident in the presence of ATP (Fig. 3 *A*).

To describe the changes in the rate constants of activation produced by ATP a fitting to a multistate model would be necessary (White and Bezanilla, 1985). However, an estimate could be obtained by analyzing current traces like the ones in Fig. 3 *A* using a phenomenological description based on the Hodgkin and Huxley model
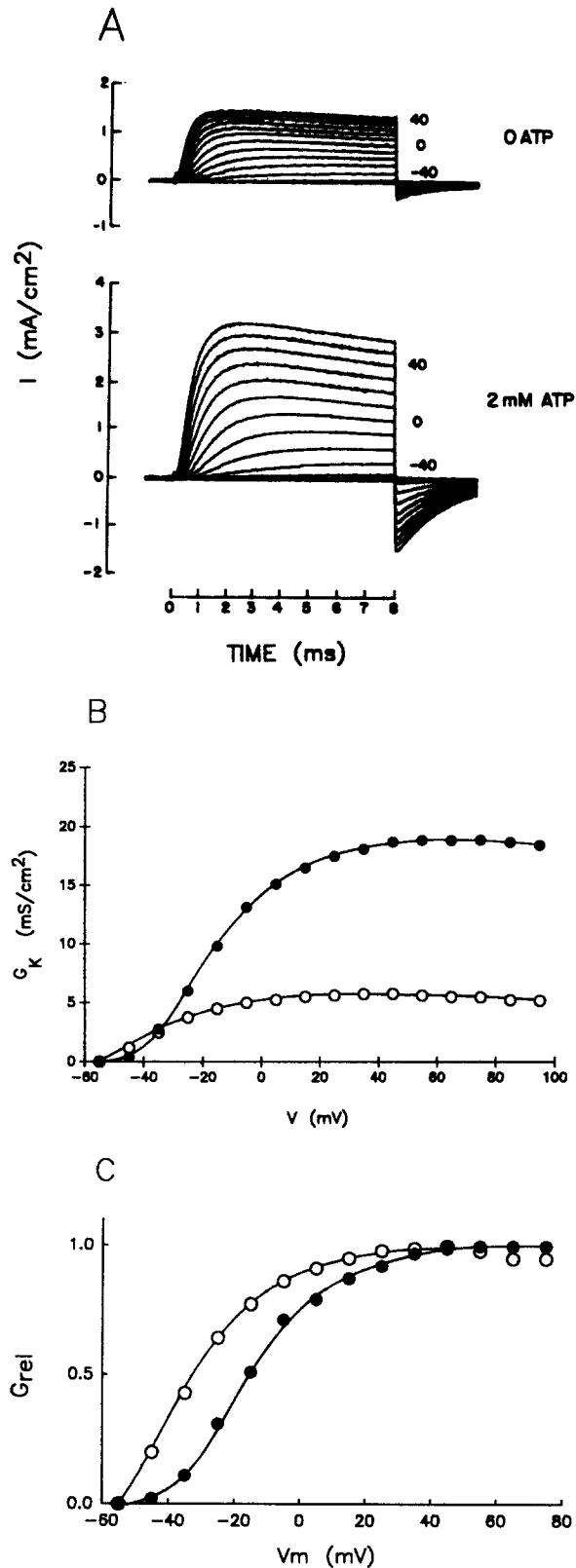
FIGURE 2. ATP potentiation at different test pulses. (*A*) Family of potassium currents recorded in the nominal absence (*upper traces*) and in the presence (*lower traces*) of ATP. Current traces were obtained with 8-ms pulses from −50 to 60 mV and are corrected for leakage and capacitive transients. Internal solution corresponds to solution A in Table I, external solution is ASW with 300 nM tetrodotoxin and 1 mM NaCN. (*B*) Conductance as a function of voltage in an axon before and during internal dialysis with 2 mM ATP. The conductance value is calculated as the sum of the steady state and tail current divided by the voltage change. Open circles are experimental data from an axon after 50 min dialysis in the absence of ATP, filled circles correspond to the same axon after the addition of 2 mM ATP to the dialysis solution. (*C*) Data normalized to the maximum conductance.

(Hodgkin and Huxley, 1952*b*). To this end, each trace was fitted according to the relation:

$$I_K = \bar{g}_K \cdot n(t)^J \cdot (V - E_K) \tag{1}$$

and

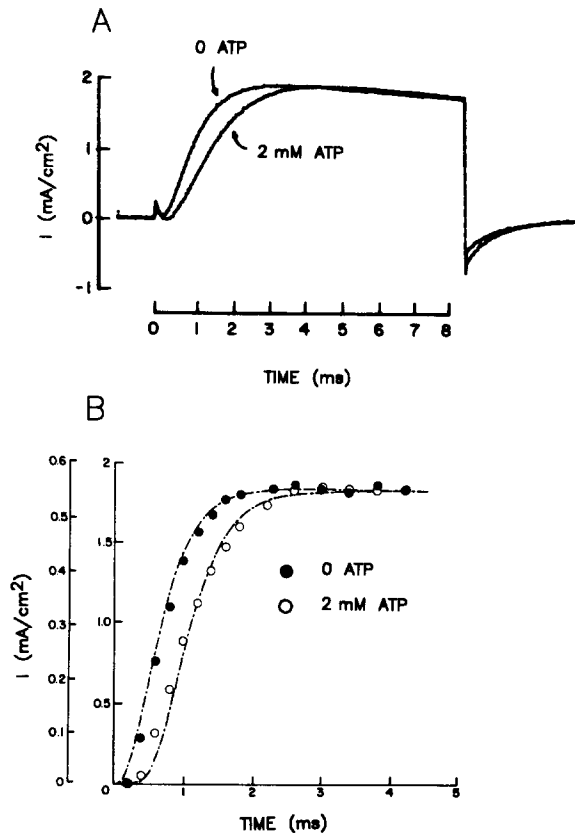$$n(t) = [n_\infty - (n_\infty - n_0)] \exp(t/\tau_n) \tag{2}$$

FIGURE 3. Changes in the kinetic parameters of the potassium current by ATP. (*A*) Current records for a test pulse to 0 mV from a holding potential of −60 mV. Current scale corresponds to the slower trace (with ATP). The current record in the absence of ATP was multiplied by an arbitrary constant (2.23) to normalize the current level at steady state; the arrow points at the tail of the trace without ATP. (*B*) Numerical simulation of the potassium current fitted to experimental values according to the Eq.

$$I_K = \bar{g}_K [n_\infty - (n_\infty - n_o)$$
$$\cdot \exp(-t/\tau_n)]^J \cdot (V - E_K).$$

The first nine experimental points were sampled with a period of 200 μs, and the remainder at 400 μs. Filled circles and the external current scale correspond to an axon dialyzed in the absence of ATP; the curve was fitted with the parameters: $n_\infty = 0.7$, $\tau_n = 35$ μs, $J = 4$, $\bar{g}_K = 25$ mS, and $E_K = -91$ mV. Open circles and the internal current scale correspond to the same axon after the addition of 2 mM ATP; the fitted parameters for the curve were $n_\infty = 0.9$, $\tau_n = 38$ μs, $J = 11$, $\bar{g}_K = 70$ mS, and $E_K = -90$ mV.

where $\bar{g}_K$, n_∞, n_0, τ_n, and E_K have their usual meaning, and J is the power term to which $n(t)$ is raised. The fitting was performed using a minimization protocol based on the simplex program (Nelder and Mead, 1965): six pairs of K current records, with and without ATP, were fitted with Eqs. 1 and 2. The program performed repetitions until the difference between the experimental record and the model was $<10^{-5}$ of the magnitude of traces. The result of the fitting procedure is shown in

Fig. 3 B. ATP induces an increase in n_0, n_∞, and $\bar{g}_K$ without affecting the value of E_K, (which is included in the fitting), implying that apart from the obvious time constant change, there is an increase in both, the maximal conductance and the probability of the channel to be open during a depolarization. One of the most striking changes was obtained in the value of the exponent J which varies from 4 (at 0 ATP conditions) to 11, in the presence of 2 mM ATP. The larger exponent is a representation of the longer delay in the current turn-on produced by ATP and is in close resemblance with the effect of hyperpolarizing prepulses that can make J change from 4 up to 26 (Cole and Moore, 1960). The physical interpretation is that ATP increases the probability that the channel dwells at closed states further away from the open state, perhaps as a consequence of a local hyperpolarization.

Fig. 4 shows the time constants of the K currents as a function of voltage in the presence and absence of ATP. The values for the time constants were obtained from single exponential fittings to the late phase after the current lag, for voltages > −50 mV (turn-on), and to the tail relaxation in instantaneous I-V protocols for voltages

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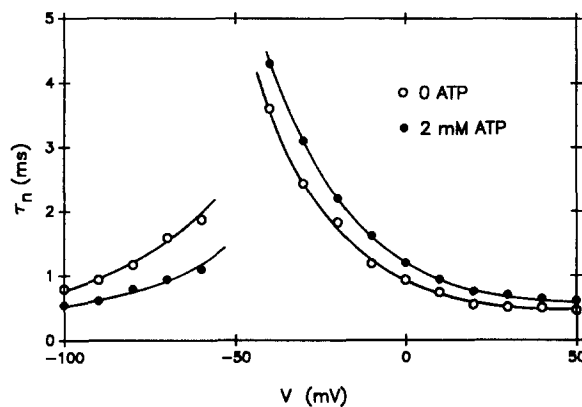o 0 ATP

● 2 mM ATP

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FIGURE 4. Change in the voltage dependence of the turn-on and turn-off time constants in the presence of ATP. Data points were obtained by single exponential fittings to the rising phase of the currents after the initial lag (turn-on, $V > -50$ mV) or the tail recovery after a given prepulse (turn-off, $V < -50$ mV). The continous lines were drawn by eye.

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< −50 mV (turn-off). ATP increases the time constant for the on process but decreases the value of the time constant for the off process. This suggests that ATP is producing a shift of τ_n towards more depolarized potentials, which is in close correlation with the shift in the G-V curve (Fig. 2 B and inset). Changes in kinetics parameters are not as dependent on the holding potential as conductance magnitudes, raising the possibility that they reflect different mechanisms.

Affinity for the Mg-ATP Complex

Fig. 5 shows the change in outward current at the end of an 8-ms pulse to 0 mV as a function of time in an experiment designed to find the apparent $K_{1/2}$ for the potentiation effect of ATP on I_K. The initial washout of ATP was followed for 1.3 h, in the presence of metabolic inhibitors (CN^- and FCCP) even after I_K had already reached a steady value. This was to assure that for our initial conditions, ATP concentration was much lower than the $K_{1/2}$ of the reaction that stimulates I_K. It could be argued that even after prolonged dialysis with 0 ATP solutions there is still a remnant of

ATP and/or ADP in the axoplasm, maintaining a certain level of stimulation; Fig. 5 proves that this is not the case since the addition of 5 mM phosphoarginine, which constitutes (with the help of endogenous kinases) a regenerative system for ATP from axoplasmic ADP (Mullins and Brinley, 1967), is ineffective in producing any current stimulation.

All ATP concentrations were verified before the experiment by the fireflash method (Strehler and Totter, 1952; Mullins and Brinley, 1967) and used in the dialysis solution together with 5 mM phosphoarginine as a way to avoid errors under low ATP concentrations (due to nonspecific ATPase activity). Increasing concentrations of ATP cause a gradual potentiation in the magnitude of outward current. Although 1 μM ATP was ineffective, potentiation was found at quite low ATP concentrations, becoming visible at 5 μM. The maximal effect was found at 100 μM;
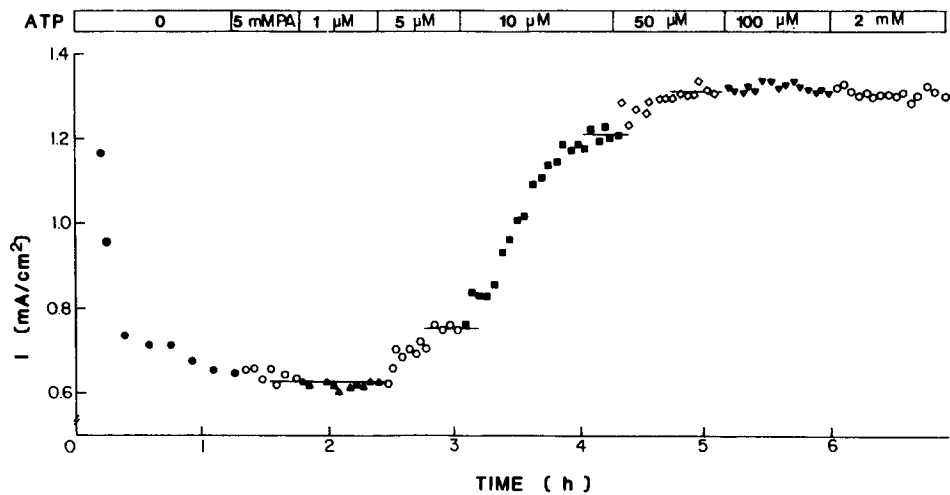

FIGURE 5. Effect of different ATP concentrations on I_K amplitude. The prolonged ATP washout is followed by the addition of 5 mM phosphoarginine (PA) in the absence of ATP. ATP-containing solutions were tested with the fireflash method (see text). Experimental points were elicited by pulsing to 0 mV from a holding potential of -60 mV. Axon KMAY205A, 500 μm diam.

concentrations up to 2 mM showed the absence of a second low affinity binding site for ATP, demonstrating that the system is already saturated. A plot of current values measured at steady state after the effect was established is shown in Fig. 6 as a function of different ATP concentrations. Half of the stimulation is reached near 10 μM ATP.

Discrimination from Different Energy Sources

The previous experiments show that ATP can change the kinetic and steady-state properties of I_K. In an attempt to further characterize the biochemical aspects of the system responsible for the ATP stimulation, a series of experiments were tried using various energy sources other than ATP. In agreement with previous work (Bezanilla
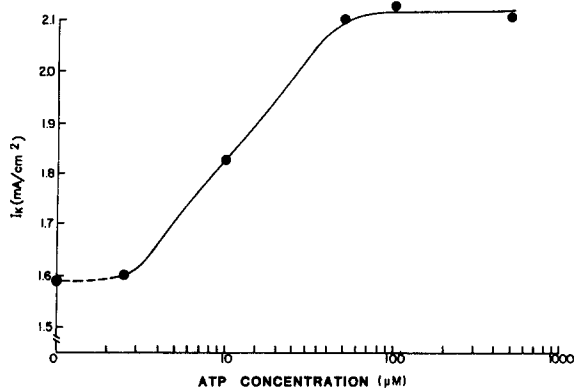
FIGURE 6. Amplitude of the steady value for the delayed outward current as a function of the ATP concentration. The points correspond to the steady-state level of I_K at a given ATP concentration, in an experiment like the one in Fig. 5. Continuous line is drawn as a visual reference. Axon KMAY155A, 430 μm diam.

et al., 1986), Fig. 7 shows that Mg^{2+} alone produces no change in I_K and neither does PO_4^-. Additionally, we tried the effect of the adenine nucleotide series, ADP, AMP, and cAMP and, as is shown in Fig. 7, all of them are ineffective in modifying I_K.

Fig. 8 shows the result of a similar experiment designed to test the effectiveness of other purinic nucleotides like ITP and GTP. At a concentration of 2 mM, neither ITP nor GTP had measurable effects on I_K, under the same conditions in which ATP exerts maximal effect. Nonhydrolyzable analogues like AMP-PCP (Fig. 7) or AMP-PNP (not shown), do not potentiate I_K, suggesting that ATP hydrolysis is necessary in the modulation process. These experiments imply that the enzymatic machinery
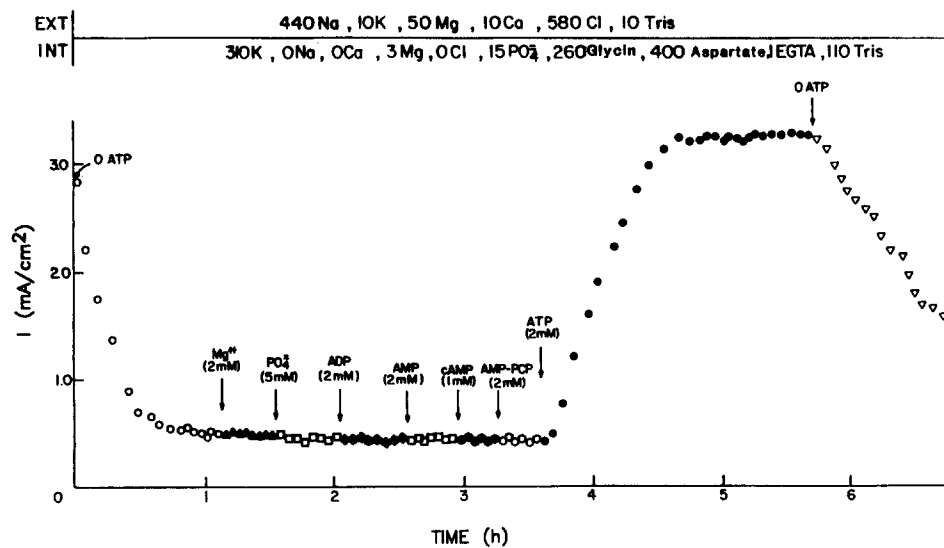

FIGURE 7. Effect of the adenine nucleotide family upon I_K amplitude. Each symbol represents a new change in solution, as noted with arrows. None of the nucleotides tried (with the exception of ATP) affected the current. An additional control with Mg^{++} and PO_4^- was also without effect. The experimental points correspond to the steady value of I_K elicited by a pulse to 0 mV from a holding potential of −60 mV. Axon KMAY035A, 600 μm diam.

responsible for I_K modulation is very selective for ATP since it can recognize ATP among other purinic nucleotides and between adenine nucleotides of lower energy content. A suggestion of the role of endogenous phosphatases comes from experiments with F^- in the internal solution (not shown). In the presence of F^-, the washout process of I_K stimulation is considerably retarded; however, F^- does not prevent a complete recovery of the effect when conditions of 0 ATP are present. F^- is known to inhibit the activity of several phosphatases in vitro (Revel, 1963).

Modification of I_K Slow Inactivation Process

The magnitude of the I_K amplitude stimulation by ATP depends on the holding potential. For holding potentials more negative than -75 mV, ATP has very little effect on I_K amplitude. On the other hand, for potentials slightly more depolarized than -60 mV, ATP exerts an even stronger stimulation. In the absence of ATP, a hyperpolarizing prepulse potentiates the amplitude of the current during the test pulse; nevertheless, if the same protocol is performed in the presence of ATP, only a
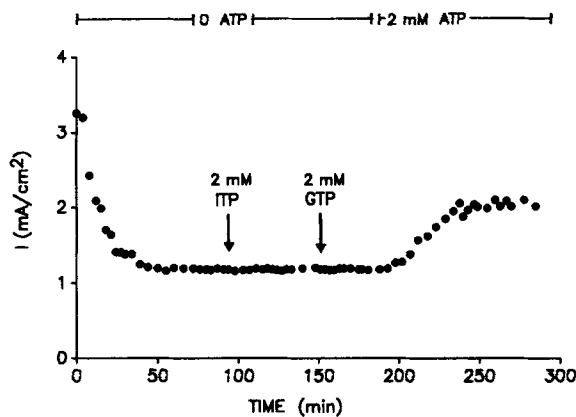

FIGURE 8. Effect of purinic nucleotides on I_K amplitude. Both GTP and ITP are added already complexed with Mg^{++}. Internal solution is 310 K^+, 0 Na^+, 0 Ca^{++}. External solution is artificial seawater, 1 mM NaCN, and 300 nM TTX. Axon KJUN035B, 420 μm diam.

small potentiation is obtained. This observation suggested to us that the most likely process to be modified by ATP is the K channel slow inactivation process (Ehrenstein and Gilbert, 1966).

Due to the slow time constant of the inactivation (Chabala, 1984; Ehrenstein and Gilbert, 1966); it is possible to study this process just by changing the value of the holding potential. Different holding potentials were set and current traces for different ATP concentrations were recorded between 3 and 5 min after the change to let the inactivation fully develop. I_K records were elicited with test pulses to 0 mV from holding potentials of -90 to -10 mV, and the amount of current at steady state was compared. This value was taken to determine the inactivation parameter $\overline{G}(\infty)$, which is defined as:

$$\overline{G}(\infty) = I_{HP}/I_{-80} \tag{3}$$

where I_{-80} is the amount of K current for a pulse to 0 mV from a holding potential of -80 mV, and I_{HP} is the amount of current for the same test pulse from any other holding value. The residual current that originated from the fraction of channels

already open before the test pulse, was substracted from all current traces at each holding potential. Fig. 9 illustrates the result of plotting the inactivation parameter against the holding potential for several axons (each symbol represents a different fiber), in the presence or the absence of 2 mM ATP. The smooth curve was drawn according to the relation:

$$\overline{G}(\infty) = \frac{1 - a}{1 + \exp\left[(V - V_{1/2})/k\right]} + a \tag{4}$$

where V is the holding potential value, $V_{1/2}$ is the potential where half of the inactivation is produced, and k is an empirical value reflecting the voltage dependence of inactivation (Hodgkin and Huxley, 1952b). The curve is asymptotic to the value a,
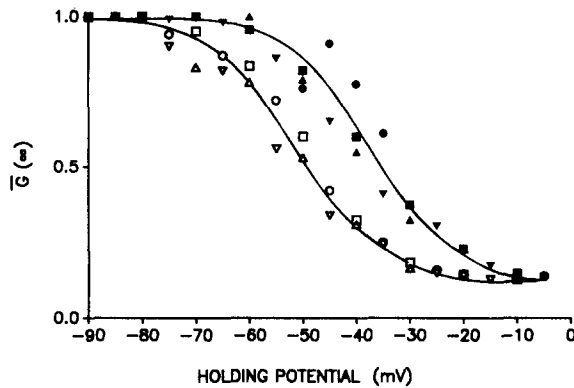

FIGURE 9. Effect of ATP on the inactivation curve of potassium current. Experimental points correspond to the relation $\overline{G}(\infty) = I_{HP}/I_{-80}$. Current traces were obtained from a holding potential varying between -90 and -10 mV and the current magnitude corresponds to the steady value obtained for a depolarization to 0 mV. The continuous curve was drawn according to Eq. 4. V is the holding potential, $V_{1/2}$ is the potential where $\overline{G}(\infty) = (1 + a)/2$ (the middle point of the voltage-dependent component of $\overline{G}(\infty)$, see Eq. 4), and k is an empirical constant representing the voltage dependence of the process. a is the fraction of the potassium conductance independent of inactivation. Different symbols represent independent experiments. Filled symbols are in the presence of 2 mM ATP, open symbols correspond to the control in the absence of the nucleotide.

which represents the amount of potassium conductance independent from the inactivation process.

ATP produced a clear shift of the inactivation curve toward more positive potentials. The shift was ~15 mV, as found by comparison of $V_{1/2}$ values in the two experimental conditions, with a value of $V_{1/2} = -48$ mV in the absence of ATP and $V_{1/2} = -33$ mV in the presence of the nucleotide, although shifts up to 22 mV were found for individual experiments (range, 10–22 mV). All the shifts observed were in the depolarizing direction, and no change in the parameter k could be detected, which suggests that the voltage dependence of the process was not affected. The curve shift induced by ATP offers a clear explanation for the ATP potentiation of outward current amplitude because at a holding potential of -60 mV and in the absence of the nucleotide, 33% of K channels were inactivated before the test pulse; in the presence of ATP however, 95% of the channels were available to open. The shift of the inactivation curve also accounts for the variability in current stimulation,

the fact that no potentiation is present at hyperpolarized holding potentials, and the crossing of the G-V curves around −40 mV.

ATP Effect Under Current-Clamp Conditions

Effect on the resting potential. Fig. 10 shows the time course of the resting potential as the dialysis conditions were changed. After a stable membrane potential was obtained, the axon was dialyzed with solution A, 0 ATP (see Table I). The initial resting potential was −53 mV and, upon ATP washout, a hyperpolarization of 8 mV was produced while the addition of 2 mM ATP induced a 6-mV depolarization, which is in agreement with data reported by Gadsby et al. (1985). This depolarization was not the result of fiber deterioration because when the ATP concentration is again reduced to 0 mM, the membrane tends to repolarize, confirming the reversible nature of the effect.

The magnitude and direction of the voltage change when ATP is added or removed internally is in good agreement with the voltage-clamp data because the
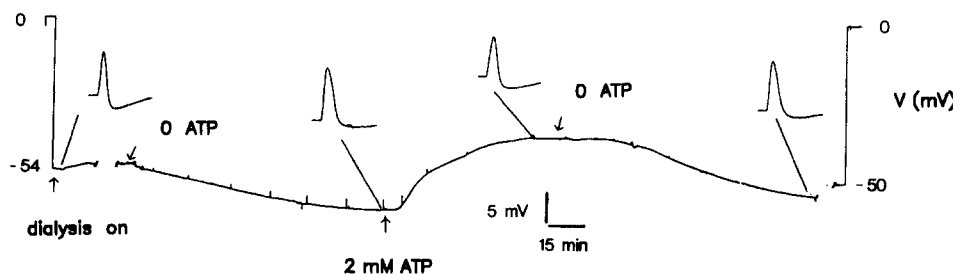

FIGURE 10. ATP effect on the resting potential. Dialysis was performed under the same conditions as in voltage-clamp experiments. Action potentials were recorded under current-clamp conditions, stimulating the axons through the axial wire inside the dialysis capillary. Calibration bars correspond to the continuous voltage trace. At the beginning of the experiment, the resting potential was −54 mV. Solution change and action potential recording times are marked by arrows. Axon PA29586C, 590 μm diam.

G-V curves cross at voltages near resting potential. As can be noted from Fig. 2 *B*, for potentials more negative than −40 mV, I_K is larger in the absence than in the presence of ATP, and the opposite occurs for voltages more positive than −40 mV. The increment in the number of open channels near rest (in the absence of ATP) tends to drive the membrane potential to the K^+ equilibrium potential, producing hyperpolarization of the fiber. This tendency decreases as the presence of ATP reduces the resting potassium conductance.

Changes in the action potential. Considering the kinetic and conductance modifications of I_K by ATP, changes in the properties of the action potential are expected. During the course of an experiment such as the one in Fig. 10, membrane action potentials elicited by a short current pulse were recorded under current-clamp conditions while the ATP washin or washout was in process (Figs. 10 and 11). As can be predicted from the voltage-clamp data, the action potential becomes larger and wider after ATP washout, and this phenomenon is reversed by the addition of inter-

nal ATP. Another major change produced by ATP is an increase in the magnitude and shortening of the duration of the undershoot. This results from both the increased difference between E_K and any voltage during the action potential (due to the fact that the fiber is slightly depolarized), and the shift to more depolarized potentials of I_K activation parameters.

Fig. 11 A shows two superimposed action potentials recorded after each of the effects were fully developed; it also shows the differences in resting potential for both conditions. In Fig. 11 B the baseline of the records has been shifted, superimposing the resting potentials so that the time courses of the action potential could be compared.
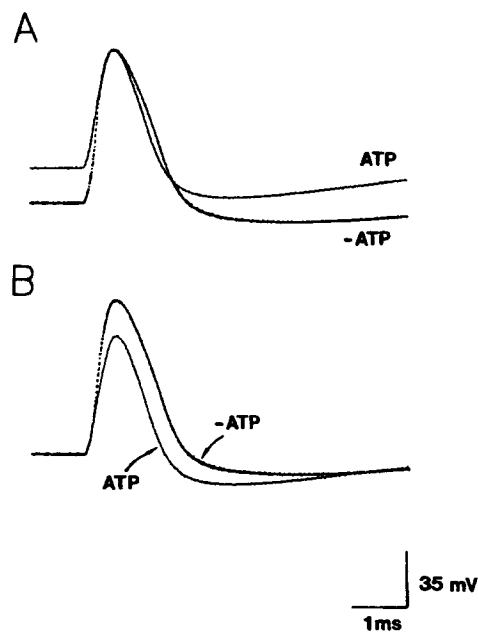
A

B

FIGURE 11. Effect of ATP on the action potential. (A) Direct comparison of records in presence and absence of ATP. (B) Comparison shifting the baselines to contrast the time course of the potential change. (Fine line) Action potential in the presence of 2 mM ATP. (Heavy line) Control in the absence of ATP. The resting potentials are −61 mV in the absence of ATP and −45 mV when ATP is present. Horizontal calibration, 1 ms; vertical calibration, 35 mV.

DISCUSSION

The results of the present study demonstrate that ATP selectively modulates the kinetics and steady-state parameters of the potassium conductance via a phosphorylation mechanism, and that a modification of the K conductance slow inactivation process is involved as the basis of the effect. In the presence of ATP, I_K for large depolarizations is larger and slower than in the absence of the nucleotide, which is in agreement with the previous report of Bezanilla et al. (1986). Modulation of potassium channels has been demonstrated in a variety of preparations (Brown and Adams, 1980; Camardo et al., 1983; Levitan et al., 1983; Cook and Hales, 1984), however, a coherent pattern of regulation can not be drawn from these findings since the type of modulation and the mechanisms underlying the process varies among preparations.

Characteristics of the ATP Effect

ATP action on the intracellular side stimulates the current amplitude up to three-fold. This effect can be very variable, depending on the magnitude of the test pulse and on the value of the holding potential. Under hyperpolarized conditions (holding potential < −60 mV), the effect was normally smaller than for potentials above the normal resting value, this fact accounts for the initial reports about the variability of the ATP stimulation (Bezanilla et al., 1986) and strongly suggests the voltage dependence of the regulation mechanism. On the other hand, the marked change in kinetics induced by ATP is not as holding potential dependent, and kinetic changes can be induced by ATP under a fairly broad range of holding potentials. The Hodgkin-Huxley type of analysis revealed a dramatic change in the order of the reaction sequence that opens the channel, by an increment in the parameter J (Eq. 1) from 4 in the absence of ATP to 11 in the presence of 2 mM ATP. In the classical interpretation of Hodgkin and Huxley's formulation, this implies an increment in the number of independent particles needed to open the channel (Hodgkin and Huxley, 1952b). Instead, by considering the sequence of events that open the channel as a sequential process, the increment in J suggests that the number of intermediate closed states the channel has to transit before it reaches a conducting state, increases (Bezanilla, 1985).

Cole and Moore showed that if a hyperpolarizing prepulse is applied before recording I_K, the turn-on lag increased and that this slowing down in kinetics was proportional to the size of the hyperpolarization (Cole and Moore, 1960). The most straightforward interpretation of this phenomenon is that changes in initial conditions modify the amount of charge that has to mobilize to open the channel. If a linear sequential scheme is considered, the stronger the hyperpolarization of the axon, the more channels will start further away from the open state. Therefore, while hyperpolarizing prepulses set the channel population in a closed state, depolarizing prepulses will speed up the transition to the open state. If amplitude differences are ignored, the ATP-induced delay in I_K activation (Fig. 3 A) can be compared with the slowing down of I_K induced by membrane hyperpolarization (Cole and Moore, 1960), and this is evident from the increase in the fitted J parameter of Eq. 1. It is clear however, that if a simple time shift is taking place due to the ATP effect, complete superposition of the current records could be obtained. Different attempts to superimpose the current traces were unsuccessful, suggesting the presence of a more complex effect by ATP. Nevertheless, a lack of superposition has been reported for the Cole-Moore shift in squid axons (Clay and Shlesinger, 1982), as well as in myelinated nerve (Begenisich, 1979), and crayfish axons (Young and Moore, 1981).

ATP induces a clear shift in the slow inactivation curve of I_K. This effect can account for the voltage dependence of the ATP induced potentiation of current amplitude. The slow inactivation process affecting potassium delayed conductance in squid was first described by Ehrenstein and Gilbert (1966), and has been reported in a variety of preparations such as myelinated nerve (Schwartz and Vogel, 1971), neuroblastoma cells (Moolenar and Spector, 1978), molluscan ganglion cells (Aldrich et al., 1979), and puffer fish supramedullary neurons (Nakajima, 1966). In

a detailed study of inactivation of I_K in squid axons (Chabala, 1984) it was shown that the time course of the inactivation development is described by a two exponential relaxation with time constants of 12.4 and 2.3 s. The recovery from inactivation is fast at hyperpolarized potentials. In our experiments, inactivation was induced by changing the value of the holding potential, and experimental records were taken at least 1 min after the holding potential setting. Under these conditions, the inactivation process is fully developed and considered in steady state. When no ATP is present in the dialysis solution, g_K is half inactivated at ~ -50 mV, and ATP produces (on average) a 15-mV shift towards more positive potentials (Fig. 9). There is an inactivation-independent component in the steady-state curve that accounts for 13% of the total conductance. Our results in the absence of ATP agree fairly well with the inactivation curve presented by Chabala (1984). The a value reported here (0.13) is very similar to the one of Chabala (0.12) and his $V_{1/2}$ parameter, although slightly more negative than ours, is in the range of our experimental variation. It must be considered, however, that Chabala's data represent a single experiment.

Towards a Biophysical Basis for the ATP Effect

ATP affects both activation and inactivation of K channels by displacing its voltage dependence response along the voltage axis. The ATP modification of I_K kinetics can then be explained by a local hyperpolarization due to the addition of a negative charge or elimination of a positive charge on the surface of the channel itself. Since a phosphorylation event is involved, the negative charge of the phosphate group could account for the local hyperpolarization in the intracellular side of the channel. Two possibilities can be suggested for the feasibility of this mechanism: first, the phosphate group could be transferred to the macromolecule in a location in which it can affect electrostatically the voltage sensor of the "gate," or second, it could promote a conformational change of the channel such that other charged or polarized regions of the molecule affect the voltage sensor.

On the basis of macroscopic and gating current experiments, White and Bezanilla (1984) proposed a kinetic model to explain the activation of K channels:

$$C \overset{\text{slowest}}{\underset{}{\rightleftharpoons}} C \cdots C \cdots C \overset{\text{slow}}{\underset{}{\rightleftharpoons}} C \rightarrow C \overset{\text{fast}}{\underset{}{\rightleftharpoons}} O \qquad \text{(Scheme 1)}$$
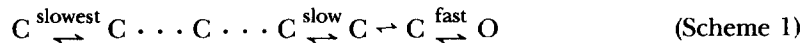
In this scheme, the first transition tends to be the slowest one (or less voltage dependent) and the last one is not rate limiting. One interpretation of our findings is that in the absence of ATP, the probability of being in the most closed state is very low and that probability increases due to the ATP effect, which explains the additional lag in current activation when ATP is present. Another possibility is that ATP changes the values of some of the kinetic constants of the model, increasing the lag and modifying the overall kinetics of the current. A clear prediction of this statement is that potassium gating current should be affected in the presence of ATP. Recently, Webb and Bezanilla (1987) have been able to record K channel-associated asymmetric currents in the presence and absence of ATP, reporting an increase in the turn-on and a decrease in turn-off time constants of the gating current in the presence of the nucleotide. This is consistent with the findings on macroscopic cur-

rents and with the view that ATP shifts the population of closed states towards the very first step of the kinetic scheme.

Shifts in a voltage-dependent property along the voltage axis can be related to local changes of the electric field affecting the voltage sensor for that property. The I_K inactivation curve shifts without changing its intrinsic voltage dependence, and the direction of the shift points to a local hyperpolarization of the channel. As in the case of the kinetic changes, this could be explained on the basis that the phosphate group transferred from ATP affects the voltage sensor of the inactivation process. A similar shift can be observed for the G-V curve, when normalized to the G_{max} value (Fig. 2 C). Here the value of the voltage shift is 11 mV.

The direction of the inactivation parameter shift on the voltage axis may provide a straightforward explanation for the ATP effect on current amplitude, and the local hyperpolarization could account for the kinetics differences. Assuming this hypothesis, one would be able to correct for the differences in currents or conductances with and without the nucleotide by simply scaling the records according to the shift in kinetics and the fraction of inactivated channels. The results presented in Fig. 12 have been analyzed with this idea in mind.

The I-V curve in the presence and in the absence of 2 mM ATP for a typical axon at a holding potential of -50 mV is shown in Fig. 12 A, while Fig. 12 B illustrates the steady-state inactivation curve for the same axon. ATP stimulates I_K nearly 300% for highly depolarized potentials, and the crossing of the I-V curves can be found at ~ -30 mV. The inactivation curves for this axon are shifted by ~ 14 mV, this gives a $\overline{G}(\infty)$ of 0.34 with no ATP and 0.89 in the presence of 2 mM ATP at a holding potential of -50 mV. If the values of inactivation are used to scale the curve without ATP by the fraction of the values of $\overline{G}(\infty)$ with and without ATP, Fig. 12 C is obtained. This correction does not produce superposition of the I-V curves as would be expected if the only mechanism affected by ATP was the slow inactivation process. The two major characteristics are (*a*) current differences after the correction are less at highly depolarized potentials, as can be expected for the differences in the population of inactivated channels, however, currents in the absence of ATP are still smaller. (*b*) At small depolarizations, the currents in 0 ATP are larger than in the presence of ATP for an extended potential range, almost up to 30 mV, in contrast to the unscaled situation where the I-V curves cross at -30 mV (Fig. 12 A). Finally, to account for the shift of the activation parameters, Fig. 12 D shows the result of shifting the ATP I-V curve by 14 mV in the depolarizing direction. In this case, the two I-V characteristics diverge by increasing the depolarization, with the appearance of an unexplained additional current when ATP is present.

A more detailed analysis has been made comparing individual current traces. Again, we have tested the hypothesis that phosphorylation of the channel induces a shift in both activation and inactivation properties. In this view, inactivation differences and initial conditions are corrected by comparing traces at two different holding potentials in such a way that the inactivation parameter is the same. This shift in holding potential should also correct for the presumed change in initial conditions. On the other hand, to account for activation differences (final conditions), traces should be compared at different V_{test} values to correct for the presumed shift that affects the kinetics and steady-state values of the conductance.

Fig. 13 shows the result of such an analysis performed in an axon in which inactivation (Fig. 13 A) and activation (not shown) curves were shifted by nearly 10 mV. Fig. 13 B illustrates the comparison of current traces with and without ATP when initial conditions are taken into account. The traces were taken at a holding potential of −50 mV in the absence of ATP, and of −40 mV when ATP was present. Two test potentials are displayed, −20-mV and 0-mV depolarizations. It is clear that the traces with and without ATP do not superimpose, differing in amplitude and kinet-
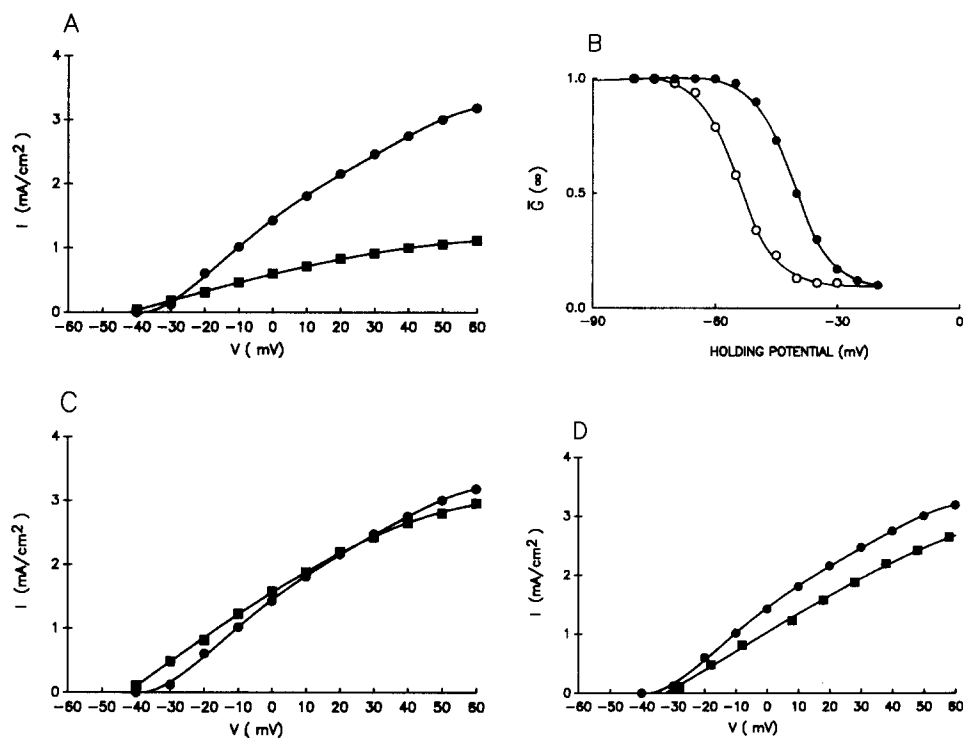

FIGURE 12.   Effect of correcting the I-V relation using the shift in activation and inactivation parameters of the K channel. (A) Normal I-V curves in the presence and absence of ATP. (B) Inactivation curve for the same axon. (C) The I-V curve in the absence of ATP has been corrected for the differences in the inactivation parameter present at a holding potential of −50 mV. (D) Same curve corrected for inactivation and shifted 14 mV to more positive potentials. Filled circles, fiber in the presence of 2 mM ATP; filled squares, fiber in 0 ATP.

ics even after inactivation differences are considered. Normalized traces for each test potential show the extent of the difference in kinetics.

Fig. 13 C demonstrates that the lack of superposition in Fig. 13 B is not due to an ATP-induced shift in the G_{rel} vs. V curve. Here, the compared traces were taken at two different holding potentials (as in Fig. 13 B), but also the shift in the G_{rel} vs. V curve was corrected by comparing currents in ATP that were 10 mV more depolarized than the control ones. The comparison of such records yields even a poorer
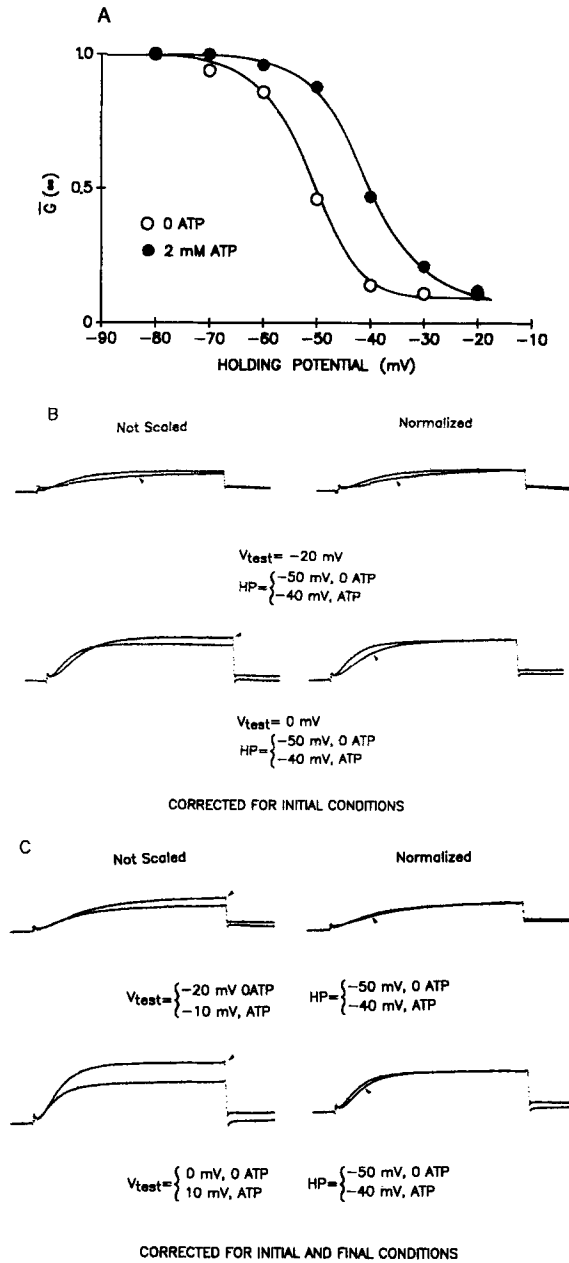
A

O 0 ATP
● 2 mM ATP

HOLDING POTENTIAL (mV)

B

Not Scaled   Normalized

V_{test}= -20 mV
HP= {-50 mV, 0 ATP / -40 mV, ATP}

V_{test}= 0 mV
HP= {-50 mV, 0 ATP / -40 mV, ATP}

CORRECTED FOR INITIAL CONDITIONS

C

Not Scaled   Normalized

V_{test}= {-20 mV 0ATP / -10 mV, ATP}   HP= {-50 mV, 0 ATP / -40 mV, ATP}

V_{test}= {0 mV, 0 ATP / 10 mV, ATP}   HP= {-50 mV, 0 ATP / -40 mV, ATP}

CORRECTED FOR INITIAL AND FINAL CONDITIONS

FIGURE 13. Comparison of corrected current traces with and without ATP. (*A*) Inactivation curve in the presence (*filled circles*) and in the absence (*open circles*) of ATP. The shift induced by ATP is roughly 10 mV. (*B*) Comparison of traces corrected for initial conditions and slow inactivation. Traces with the same inactivation parameter $G(\infty)$ were obtained at different holding potentials (HP). For control records, the holding potential was −40 mV and for records obtained in ATP it was −50 mV. Traces in the left column are unscaled, traces in the right column are normalized to the control record. (*C*) Comparison of traces shifted in the voltage axis for both initial and final conditions. Correction for inactivation is achieved in the same way as in *B*. In an attempt to correct for the shift in activation parameters, traces with the same final conditions (G_{rel}) were obtained at different test pulses (V_{test}), the record in ATP being always 10 mV more depolarized than the control. Traces at the left are unscaled, traces at the right are scaled to the control record. Arrows always point to traces in the presence of ATP.

match in current amplitude, although the kinetic differences are smaller than in Fig. 13 *B*, as can be noted in the normalized records.

The previous analysis strongly suggests that a simple shift in the electrical properties of the delayed outward current is not enough to account for a number of kinetic and conducting differences produced by ATP-mediated phosphorylation.

Various possibilities can be raised to explain this apparent complexity. One possibility is that the potassium conductance is not produced by a homogeneous population of channels. A channel subpopulation can show kinetically different subpopulations with different sensitivity to ATP phosphorylation, or different accessibilities to being phosphorylated. A second possibility is that an additional conductance, previously silent, is activated by ATP phosphorylation, which explains the difficulty in superimposing different current traces or I-V curves. These two possibilities are not mutually exclusive, since a silent channel can always be a K channel subpopulation not active under certain conditions (e.g., the absence of ATP). Evidence that the delayed outward current can be the result of different K current contributions has been demonstrated in molluscan neurons (for review see Adams et al., 1980), and in myelinated nerve (Dubois, 1981). Use of the cut-open axon technique, which records single-channel events in the squid axon preparation, has recently provided evidence for at least two types of K channels (Llano and Bezanilla, 1985; Llano et al., 1988). To assess the relevance of subpopulations of channels, a characterization of the ATP effect at the single-channel level is required.

Regulation by a Protein Phosphorylation Step?

Several pieces of evidence favor the view that ATP modulation of K currents is produced via a phosphorylative process. This mechanism must include hydrolysis of the ATP molecule and transference of the hydrolyzed phosphate group to a target acceptor via an unidentified protein kinase. Neither Na-ATP nor Tris-ATP exert any stimulatory effect on I_K, since a Mg-ATP complex is an obligatory requisite to obtain I_K potentiation. Furthermore, nonhydrolyzable analogues of ATP, like its amino derivative AMP-PNP or the methyl derivative AMP-PCP, cannot elicit any stimulatory effect on I_K (Fig. 7), in contrast to the proposal of a direct stimulation by ATP binding in pancreatic B cells (Cook and Hales, 1984) or cardiac muscle (Kakei et al., 1985). Very recent experiments using ^{32}P-labeled ATP have shown labeling of specific high molecular weight bands directly related to the magnitude of K current stimulation by ATP (Perozo et al., 1988). This suggests that the modulatory effect of ATP is coupled with the transfer of phosphate groups to the membrane.

ATP hydrolysis is carried out very selectively, since addition of other triphosphate nucleotides to the dialysis fluid has no effect on the kinetic and conduction parameters of I_K (Fig. 7, 8). Although limited information is available concerning protein kinases in squid axons, our results agree with the high ATP selectivity reported for other kinases (Flockhart and Corbin, 1982; Sharma, 1982). Additionally, I_K modulation is achieved at low ATP concentrations, given that the K_m for the effect is near 10 μM (Fig. 6). The effect of internal fluoride in slowing down the time course of the reverse effect, supports the view that a phosphatase is involved in the dephosphorylation process observed during ATP washout. This anion is known to be a nonspecific inhibitor of phosphatase enzymes (Revel, 1963), and it has been used successfully by Shuster et al. (1985) to demonstrate the role of phosphatases in the regulation of K channels in *Aplysia* sensory neurons. On the other hand, using perfused axons (which in principle will not show any soluble enzyme activity), Webb and Bezanilla (1986) have demonstrated the irreversibility of the ATP effect, as was

expected because of the lack of a phosphatase. K currents stay stimulated under this condition, only to be reversed by the action of exogenous alkaline phosphatase.

In the present experiments, it is demonstrated, however, that under current-clamp conditions, depleting the axon of ATP has a reversible effect on both the resting potential and the action potential. If we assume a direct relationship between intracellular ATP levels and the phosphorylative state of the channel, after ATP washout all the channels will be in the nonphosphorylated state and hence, by raising ATP concentration most of them will come back to the phosphorylated state. Changes in resting potential can affect directly the excitability level of the axon by influencing inactivation parameters of both Na and K currents.

Given that the axoplasmic ATP concentration has been reported to be ~2 mM (Brinley and Mullins, 1967; Dipolo, 1974), a K_m value of 10 μM for the ATP stimulatory effect makes unlikely the possibility that g_K regulation is carried out by the metabolic state of the fiber. In that case, ATP concentration must drop more than 20-fold (to <100 μM) to reveal any effect. This automatically suggests that g_K regulation in squid axons must be the result of activation-deactivation of the enzymatic components of the proposed biochemical sequence, which include a protein kinase and a phosphatase, in addition to a hypothetical extracellular receptor linked to second messenger production. On the other hand, it is also possible that in this preparation g_K is not subject to a physiologically relevant regulation, and the channel is normally phosphorylated.

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