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Is ATP Required for K^+ Channel Activation in *Vicia* Guard Cells?[1]

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In vivo, K^+ entry into guard cells via inward-rectifying K^+ channels is indirectly driven by ATP via an H^+-ATPase that hyperpolarizes the membrane potential. However, whether activation of the K^+ channels of guard cells requires ATP remains unknown. In the present study, both whole-cell and single-channel patch-clamp techniques were used to address this question. Exogenous ATP, ADP, and adenosine-5′-*O*-(3-thiotriphosphate) applied to the cytoplasm had no effect on whole-cell K^+ currents of *Vicia faba* L. guard cells. Azide, an inhibitor of oxidative phosphorylation, also had no effect. However, an ATP-scavenging system, glucose plus hexokinase, inhibited whole-cell inward K^+ currents by 30 to 40%. Single-channel results acquired from cytoplasm-free inside-out membrane patches showed definite activation of inward K^+ channels by ATP. Other nucleotides, such as ADP, adenosine-5′-*O*-(3-thiotriphosphate), and GTP, did not increase channel activity in the membrane patches. Inward K^+ channel activity in membrane patches preactivated by exogenous ATP was inhibited by glucose plus hexokinase. These results suggest that a low concentration of ATP is required for activation of the inward K^+ channels of the guard-cell plasma membrane. The issue of how ATP as a signal regulates these K^+ channels is discussed.

Stomatal movement is an energy-dependent process (Weyers et al., 1982; Assmann and Zeiger, 1987; Karlsson and Schwartz, 1988). Under conditions that favor stomatal opening, such as illumination, H^+-ATPases in the plasma membrane of guard cells are activated (Assmann et al., 1985; Shimazaki et al., 1986; Serrano et al., 1988; Schwartz et al., 1991; Lohse and Hedrich, 1992), which utilize ATP to pump H^+ out of the cytoplasm and build up an H^+ gradient across the guard-cell plasma membrane. The resultant electrical gradient drives K^+ entry, which is accompanied by Cl^- influx and malate^{2-} synthesis from starch breakdown. As the concentrations of intracellular osmotica increase, guard cells take up water and bend, opening the stomatal pore (Outlaw, 1983). Therefore, ATP-driven H^+ extrusion is a vital process for stomatal regulation in vivo. However, it is not known whether ATP is required for K^+ entry into guard cells other than to fuel the H^+-ATPase. Whether ATP also plays a role as a signal chemical that regulates guard-cell K^+ channels has not been investigated.

In mesophyll protoplasts of *Arabidopsis thaliana*, ATP activation of the K^+ channel PKC1, which can mediate both K^+ influx and K^+ efflux, has been reported (Spalding and Goldsmith, 1993), and an association between K^+ channel activity and photosynthetic activity was inferred. In the same cell type, ATP activation of an outward-rectifying K^+ channel, designated PKC2, was also reported (Spalding and Goldsmith, 1993). Luan et al. (1993) reported evidence for inhibition of the inward-rectifying K^+ channels of guard cells by a Ca^{2+}-dependent phosphatase, which suggests that ATP might be involved in K^+ channel regulation by phosphorylation/dephosphorylation pathways. Hedrich et al. (1990) reported that anion channels of *Vicia faba* guard cells are activated by ATP or other nucleotides, and a nonspecific nucleotide receptor involved in anion channel activation was hypothesized.

Guard-cell K^+ channels have been studied with patch-clamp techniques since 1984 (Schroeder et al., 1984, 1987; Schroeder, 1988, 1989; Schroeder and Hagiwara, 1989; Fairley-Grenot and Assmann, 1991, 1992, 1993; Luan et al., 1993; Wu and Assmann, 1994). ATP was supplied to the cytoplasmic side of the guard cells in most of these investigations, even though it is not known whether addition of ATP was necessary. The ATP concentration supplied varied in different reports, ranging from 1 mM (Schroeder and Hagiwara, 1990) to 2 mM (Schroeder et al., 1987; Schroeder, 1988, 1989; Keller et al., 1989; Hedrich et al., 1990; Cosgrove and Hedrich, 1991; Fairley-Grenot and Assmann, 1991, 1992, 1993; Luan et al., 1993), 4 mM (Schroeder and Hagiwara, 1989; Schroeder and Keller, 1992), and even 9 to 10 mM (Assmann et al., 1985; Marten et al., 1991, 1992; Lohse and Hedrich, 1992). On the other hand, ATP was not supplied, or at least not mentioned, in some other experiments (Schroeder et al., 1984; Raschke and Hedrich, 1989; Blatt, 1992).

Some studies suggest that 1 to 2 mM ATP may be close to the actual physiological ATP concentration in the guard-cell cytoplasm (Blatt, 1987; Michalke and Schnabl, 1987), which is similar to the cytoplasmic ATP level in other plant cells (Roberts, 1984). Schroeder (1988) showed that guard-

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Abbreviations: ATPγS, adenosine-5′-*O*-(3-thiotriphosphate); P_o, open-state probability.

cell pump current was not affected by variation of ATP from 2 to 10 mM and was still 60% of maximal at 1 mM ATP. Therefore, it seems that 1 to 2 mM ATP may be suitable for electrophysiological study of guard cells. In this report, we present results from both whole-cell and single-channel experiments to address the question of whether ATP is required for K^+ channel activation in guard cells. Some data regarding how K^+ channels may be regulated by ATP are also presented and discussed.

MATERIALS AND METHODS

Preparation of Guard-Cell Protoplasts

Plants of *Vicia faba* L. cv Long pod were grown from seeds in potting mix (Metromix 500; W.R. Grace, Cambridge, MA) in growth chambers. Light intensity was 200 μmol m^{-2} s^{-1} for a 10-h daily light period and day and night temperatures were 20 (±2) and 16°C (±2°C), respectively. Guard-cell protoplasts were isolated from the epidermes of young expanded leaves from 3- to 4-week-old plants as described by Kruse et al. (1989). Before use in patch-clamp experiments, isolated guard-cell protoplasts were kept in the dark at 0 to 2°C in a solution containing 5 mM Mes (KOH, pH 5.5), 0.45 M sorbitol, 1 mM $MgCl_2$, and 1 mM $CaCl_2$.

Whole-Cell Clamping Procedure

Experiments were performed using standard whole-cell-recording techniques (Hamill et al., 1981). Guard-cell protoplasts were placed in bath solution containing (in mM) 100 potassium glutamate, 1 $MgCl_2$, 1 $CaCl_2$, 10 Hepes (titrated with KOH to pH 7.20), and mannitol to give a final osmolality of 460 mmol kg^{-1}. The final K^+ concentration of this bath solution was 103 mM. Glass pipettes were pulled from Kimax-51 glass capillaries (VWR, Bridgeport, NJ) and heat polished before use and had resistances of approximately 20 MΩ when filled with solution containing (in mM) 10 Hepes (KOH, pH 7.2), 80 potassium glutamate, 20 KCl, 2 $MgCl_2$, 2 EGTA, and mannitol to give an osmolality of 500 mmol kg^{-1}. The final K^+ concentration for this pipette solution was 107 mM. Whole-cell clamping was performed at room temperature (20 ± 2°C) under green light obtained by placing a Roscolene (Woburn, MA) No. 874 filter in the light path of the microscope. Access resistance was between 2 and 5 GΩ in all experiments. Cell capacitance was measured for each cell using the capacity compensation device of the amplifier (Bookman et al., 1991) and was between 5.0 and 7.2 pF. Data were acquired 10 min after formation of the whole-cell configuration; tests performed with the solutions described above (either with or without 2 mM ATP in the pipette solution) showed that currents remained stable at the level recorded at 10 min for at least 1 to 2 h.

Whole-cell currents were measured using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA), which was connected to a microcomputer (486/33c; Gateway 2000, North Sioux City, SD) via an interface (TL-1 DMA interface, Axon Instruments). pCLAMP (version 5.5.1, Axon Instruments) software was used to acquire and analyze whole-cell currents. After the whole-cell configuration was obtained, membrane potential (V_m) was clamped to −52 mV. Voltage pulse protocols as shown in Figure 1A were generated using pCLAMP software and applied to the clamped cell during data acquisition. Whole-cell current was filtered at 1 kHz by the four-pole Bessel filter of the Axopatch-1D amplifier before acquisition (2 ms/sample) to computer disk.

Whole-cell currents were leak subtracted before generating whole-cell current-voltage relations. Leak currents for each cell were determined from the first one to three data points obtained after V_m was stepped from holding to test voltage. The mean values of whole-cell currents were determined as the average of samples obtained between 3.0 and 3.5 s (250 samples total) after imposition of the test voltage, when current amplitude had plateaued. After subtraction of leak currents, the final time-activated whole-cell currents were expressed per unit capacitance (pA/pF) to account for variation in cell surface area or directly expressed as current/cell (pA/cell).

Single-Channel Recording Procedure

Guard-cell protoplasts were placed in a chamber filled with solution containing (in mM) 80 potassium glutamate, 20 KCl, 10 $CaCl_2$, 2 $MgCl_2$, 10 Hepes (pH 7.2), and mannitol (osmolality adjusted to 480 ± 10 mmol kg^{-1}). When placed in this solution, glass pipettes filled with the bath solution used for whole-cell experiments had resistances between 30 and 40 MΩ. Seals with resistances between 5 and 10 GΩ formed in approximately 20% of attempts. Inside-out membrane patches were obtained after seal formation by retracting the pipette and exposing the pipette tip briefly to the air. After the formation of a stable inside-out patch, the bath solution was changed to the pipette solution used for whole-cell experiments as described above, which had an estimated final Ca^{2+} concentration of 2 nM (Fairley-Grenot and Assmann, 1991). The liquid junction potential caused by the solution change was both measured and calculated as described by Barry and Lynch (1991). All membrane potentials reported in this paper have been corrected for liquid junction potentials.

After the change to the low Ca^{2+} solution was complete, data acquisition was conducted during the subsequent 10 min. For those patches that maintained stable seal resistance and channel activity during this sampling period, the 10-min sample was taken as the control data, and a particular treatment was then applied by solution change. Data were collected again 10 min after imposition of the treatment.

Single-channel currents were measured using the same equipment as for the whole-cell experiments. Data were filtered with a four-pole Bessel filter at 1 kHz, digitized at 250 μS/sample, and stored on computer disk. pCLAMP software (version 5.5.1, Axon Instruments) was used for recording and analysis. P_o's were determined as the ratio of total open time to total recording time. For patches in which greater than one channel was observed, total P_o was

arbitrarily defined as the sum of the P_o's at each of the defined levels of open-channel current. Current amplitude for the open state at a given voltage was obtained from Gaussian fitting of open-state amplitude histograms, typically derived from a 16-s recording period.

Chemical Reagents

Cellulase RS was from Yakult Honsha Co., Ltd. (Tokyo, Japan). Pectolyase Y-23 was from Seishin Pharmaceutical Co., Ltd. (Tokyo, Japan), and Cellulysin was from CalBiochem (La Jolla, CA). ATPγS was from Boehringer. All other reagents were from Sigma.

RESULTS AND DISCUSSION

Whole-Cell Clamping

In vivo, K^+ entry into guard cells is driven by an electrical gradient built up, at least in part, by H^+ pumps that consume ATP. In patch-clamp experiments, the driving force for K^+ flux across the plasma membrane is exogenously provided by voltage clamping the membrane potential and by controlling the K^+ concentrations of external and cytoplasmic solutions. This approach allows one to identify the ionic species responsible for observed currents by analysis of reversal potential. Indeed, the whole-cell currents shown in Figures 1 and 2 were identified as K^+ currents by this method (data not shown; see Fairley-Grenot and Assmann, 1992).

Under the whole-cell configuration, cytoplasmic ATP concentration should be lowered because of the bulk volume of the pipette solution. Consistent with this hypothesis, H^+ pump activity is ATP dependent, and pump currents are inhibited when concentrations of experimentally provided ATP are lowered (Assmann et al., 1985; Schroeder, 1988). However, whole-cell K^+ currents were not affected by the presence or absence of exogenous ATP in the pipette solution (Figs. 1, A and B, and 2). ADP (Figs. 1C and 2) and ATPγS (Figs. 1E and 2) were also tested, and neither one had any effect on whole-cell K^+ currents of guard cells. The addition of GTP in the pipette solutions inhibited whole-cell inward K^+ currents (Fig. 2), consistent with previous reports of current inhibition by G-protein activators (Fairley-Grenot and Assmann, 1991; Wu and Assmann, 1994).

It is possible that because isolated guard-cell protoplasts maintain high mitochondrial activity (Shimazaki et al., 1982; Wu and Assmann, 1993) this activity might maintain cytosolic ATP at the level required for K^+ channel activation, even in the absence of exogenous ATP. If this were the case, K^+ currents might be sensitive to inhibition by azide, an inhibitor of mitochondrial phosphorylation, particularly since these experiments were conducted under low-intensity green light, which has a low quantum efficiency for photophosphorylation. However, whole-cell K^+ currents were not affected by the addition of 1 mM azide in the pipette solution (Figs. 1D and 2). Therefore, these data did not provide evidence that K^+ channel activation requires ATP or ATP-dependent processes. In contrast, the addition

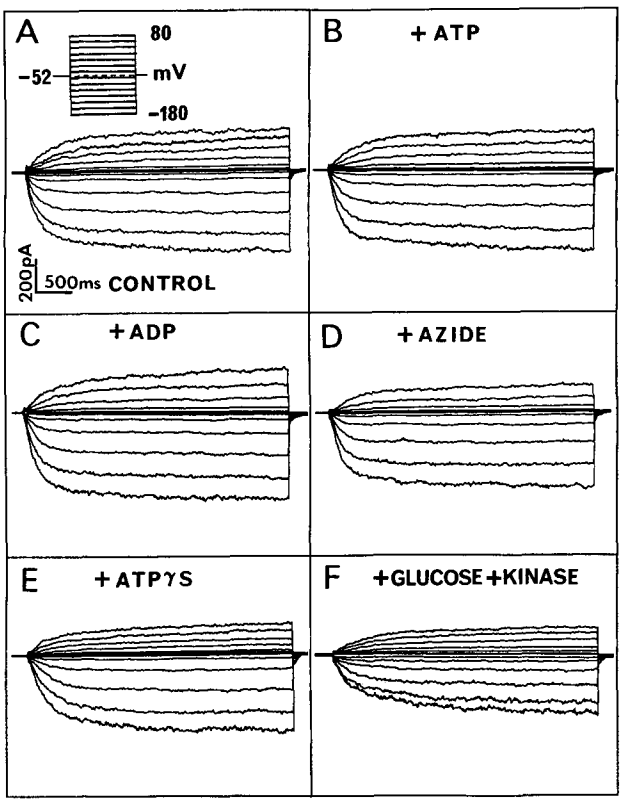

Figure 1. Whole-cell K^+ current recordings of *Vicia* guard cells. No ATP was added in the pipette solutions for A, C, D, E, and F. Two millimolar ATP was supplied in the pipette solution for B, 2 mM ADP for C, 1 mM ATPγS for E, and 1 mM sodium azide for D. For F, 0.5 unit/mL hexokinase and 5 mM Glc were added in the pipette solution. The bars in A give the voltage protocol and current/time scales for all panels. The dotted line indicates the holding potential that was maintained when a voltage step was not being applied.

of Glc and hexokinase together, which is reported to scavenge cytosolic ATP (Hedrich et al., 1990), did inhibit whole-cell K^+ inward currents by 30 to 40% (Figs. 1F and 2).

There are two possible explanations for the Glc/hexokinase results. The first possibility is that, although exogenous ATP is not required for the observation of whole-cell K^+ currents in *Vicia* guard cells, a low level of ATP is still needed for K^+ channel activation to reach its maximum level. ATP levels are lowered to a greater extent by the Glc/hexokinase system than by azide (see "Discussion"); hence, the channels are inhibited. The second possibility is that Glc and/or hexokinase may have an effect on K^+ channel activity that is independent of their effect on ATP concentration. Therefore, to more directly test the involvement of ATP in K^+ channel regulation, single-channel experiments were performed.

Inside-Out Membrane Patch Clamping

Single-channel signals acquired from the inside-out membrane patches shown in Figures 3 to 8 were identified, using several criteria, as the inward K^+ channels respon-

sible for the whole-cell currents. First, open-channel current reverses at the K^+ reversal potential (Fig. 3B). Second, voltage dependence (Fig. 3A and data not shown) of channel P_o correlates with voltage dependence of the whole-cell currents (cf. Fig. 2), with increased P_o at more negative voltages. Third, the channels exhibit the characteristic time dependence of activation that is one of the hallmarks of the whole-cell current (cf. Figs. 1 and 4). Fourth, channels were inhibited by elevated Ca^{2+} levels (data not shown; see Schroeder and Hagiwara, 1989). Taken together, these results indicate that these channels are the molecular basis for the inward currents observed in the whole-cell experiments. Patches selected for subsequent treatment were those that had shown stability during an initial 10-min observation period (see "Materials and Methods"). These patches did not exhibit "run-down" of channel activity;

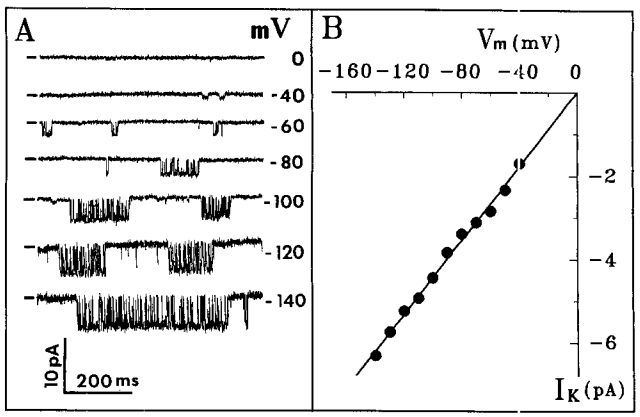

Figure 3. Voltage regulation of inward K^+ channel activity in an inside-out membrane patch derived from a *Vicia* guard-cell protoplast. The cytosolic (bath) solution contained 2 mM ATP. A, Typical recordings, showing increased P_o of the channel as the membrane is hyperpolarized (membrane potential of each recording is indicated on the right; bars on the left indicate the closed level of the channels). B, Open-channel current (I_K) from the patch in A plotted as a function of voltage. Extrapolated reversal potential of the current (0 mV) corresponds with the calculated reversal potential for K^+ (see text).

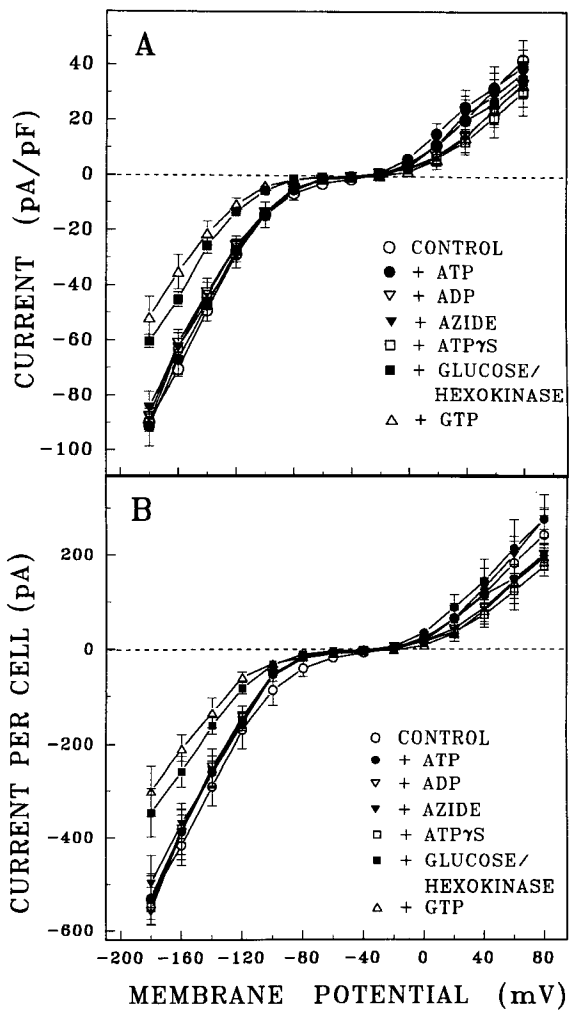

Figure 2. Current/voltage relationships of whole-cell K^+ currents of *Vicia* guard cells. A, The currents are normalized by the capacitances and expressed as current/pF. B, The currents are directly expressed as current/cell. The concentrations of ATP, ADP, GTP, and ATPγS were 2, 2, 1, and 1 mM, respectively. Each data point represents the mean ± SE (n = 6).

activity was stable throughout the standard recording period (see "Control 10 min" in Table II), and even for much longer periods (Fig. 5).

In contrast to the whole-cell data, the addition of exogenous ATP to the cytoplasmic side of cytoplasm-free membrane patches clearly and significantly enhanced inward K^+ channel activity (Fig. 6). This phenomenon was observed in more than 20 membrane patches, of which 8 were analyzed in detail (Table I). As illustrated in Figure 6 and Table I, the inward K^+ channels in isolated membrane patches showed little activity before ATP addition. For example, the total P_o of the inward K^+ channels shown in

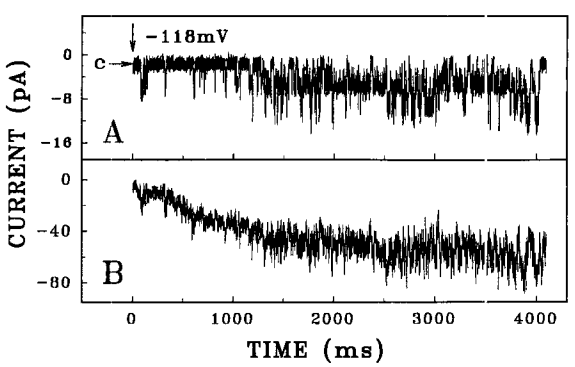

Figure 4. Time-dependent activation of the inward K^+ channel in an inside-out membrane patch. The cytosolic (bath) solution contained 2 mM ATP. A, Channel activity following a step change in the membrane potential from 0 to −118 mV, at the time indicated by the vertical arrow. "c" indicates the closed level of the channels. B, Sum of 23 isolated patch recordings following a step-change in membrane potential from 0 to −118 mV. Total leak-subtracted current plotted as a function of time gradually increased and then plateaued, as is also observed in whole-cell recordings of inward K^+ currents (cf. Fig. 1).

Figure 6A was initially only 0.04, despite the presence of at least two channels in this patch (see also Table I). The addition of 2 mM ATP to the cytoplasmic side of the same membrane patch significantly enhanced channel activity, with total P_o increasing more than 7-fold, to 0.30 (Fig. 6B; see also Table I). If ATP was supplied first to a patch (Fig. 7C), the subsequent addition of Glc and hexokinase together inhibited channel activity (Fig. 7D; Table II), suggesting that Glc/hexokinase may remove ATP from a binding site on the membrane.

The activating effect of ATP on the inward K^+ channels of *Vicia* guard cells is specific and cannot be produced by other nucleotides tested. Thus, addition of GTP (Fig. 7, A and B; Table II), ADP (Fig. 8, A and B; Table II), and ATPγS (Fig. 8, C and D; Table II) did not enhance channel activity. These results clearly demonstrated that ATP is specifically required for the activation of inward K^+ channels in guard cells. The lack of inhibition by GTP of K^+ channel activity in isolated membrane patches, in contrast to the whole-cell data, may be either because the K^+ channels need to be activated by ATP first before inhibition by GTP can be observed or because G-proteins in the isolated membrane patch are primarily in a GDP-binding configuration. In support of the latter explanation, the activating effect on

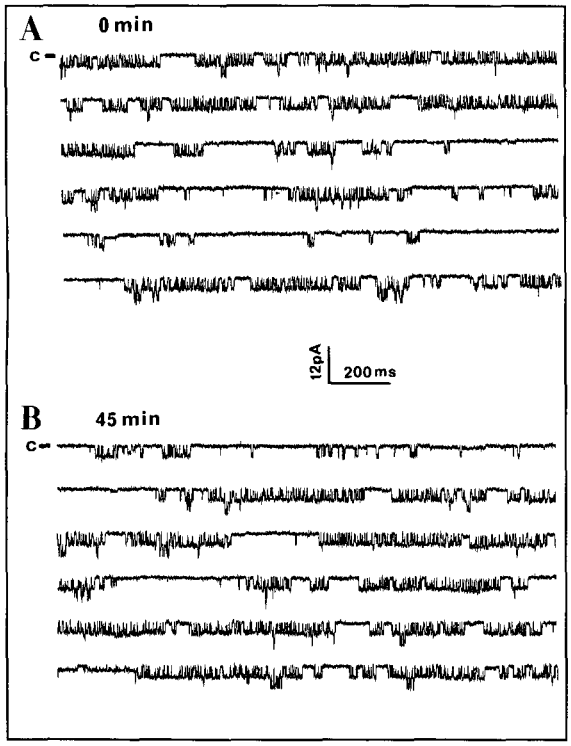

Figure 5. Stability of single-channel recording in an inside-out membrane patch derived from a *Vicia* guard cell. The cytosolic (bath) solution contained 2 mM ATP. A, Initial activity at −118 mV recorded 10 min after change to the low Ca^{2+} cytoplasmic solution (see "Materials and Methods" for details). B, Activity at −118 mV recorded 45 min later from the same membrane patch as in A. Note that the P_o of the channel is similar in A and B, indicating that run-down in channel activity has not occurred.

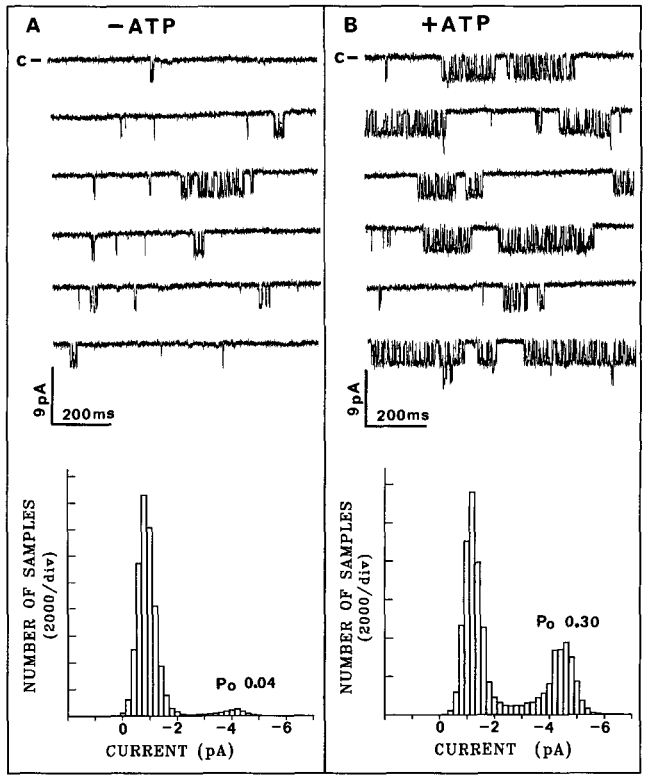

Figure 6. Single inward K^+ channel recordings obtained from an inside-out membrane patch of a *Vicia* guard cell in the absence and presence of ATP. A, No ATP was supplied to the cytoplasmic side of the membrane patch. B, ATP (2 mM) was added to the cytoplasmic side of the same membrane patch in A. Portions of the traces are illustrated in the upper sections of the panels, and all-point histograms derived from the complete traces are presented in the lower sections, with P_o indicated. The membrane potential was clamped at −118 mV for all recordings.

Table I. *Effect of ATP on the P_o's of inward K^+ channels in inside-out membrane patches of Vicia guard cells*

Each pair of P_o's was obtained from the same membrane patch. The data for +ATP were acquired 10 min after 2 mM ATP was added to the cytoplasmic side of each membrane patch. Membrane potentials were held at −118 mV in all recordings. The numbers in parentheses indicate the numbers of channels observed in the tested membrane patch.

Membrane Patch	Total P_o	
	−ATP	+2 mM ATP
1	0.04	0.30 (2)
2	0.07	0.32 (2)
3	0.04	0.21 (1)
4	0.08	0.27 (2)
5	0.03	0.22 (1)
6	0.06	0.25 (1)
7	0.05	0.29 (2)
8	0.04	0.19 (1)
Mean ± SD	0.05 ± 0.017	0.26 ± 0.05

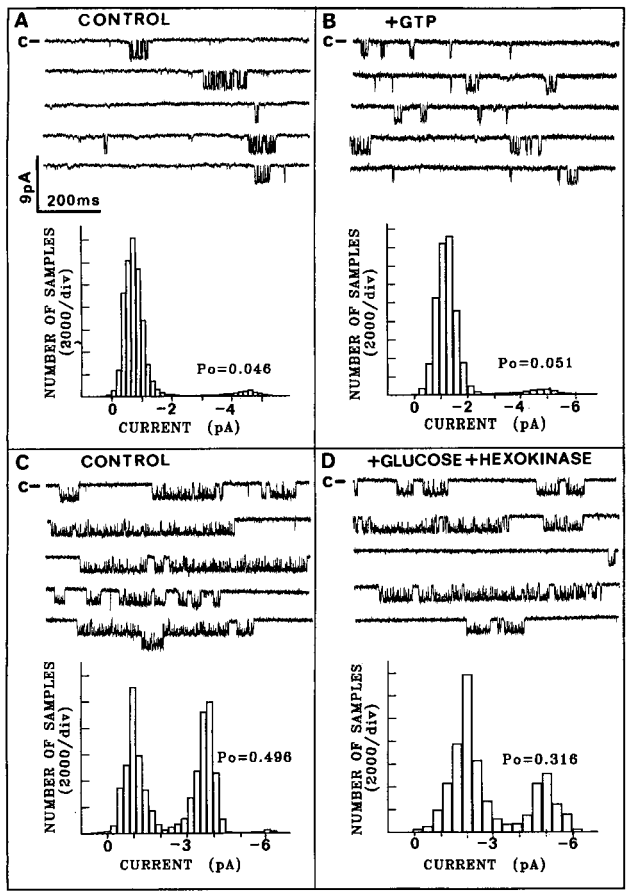

Figure 7. Single inward K^+ channel recordings from inside-out membrane patches of *Vicia* guard cells in the absence and presence of GTP (A and B) and ATP-scavenging agents (C and D). No ATP was added in the bath (cytoplasmic) solutions for A and B. The recordings in A and B (1 mM GTP, Tris salt) were acquired sequentially from the same membrane patch. ATP (2 mM) was provided to the cytoplasmic side of the membrane patch in both C and D. The recordings in C and D (5 mM Glc and 0.5 unit/mL hexokinase) were acquired sequentially from the same membrane patch. Portions of the traces are illustrated in the upper sections of the panels, and all-point histograms derived from the complete traces are presented in the lower sections, with P_o's indicated. All recordings were obtained at −118 mV. The current and time scales refer to the scale bars shown in A.

single K^+ channels of guanosine-5′-O-(2-thiodiphosphate), which locks G-proteins into the GDP-binding configuration, was much less than the inhibiting effect on single K^+ channels of guanosine-5′-O-(3-thiotriphosphate) (Wu and Assmann, 1994), suggesting that the majority of G-proteins were already in their GDP-binding state.

DISCUSSION

The data presented here illustrate a differential requirement for exogenous ATP in the activation of inward K^+ channels in whole-cell versus membrane patch experiments. There are at least three possible explanations for this differential ATP requirement. First, ATP may remain tightly bound to the channel or a channel regulatory protein in the whole-cell configuration; thus, supplemental ATP is not required in the pipette-filling solution. Second, although the cytosol quickly equilibrates with the pipette solution under the whole-cell configuration (Pusch and Neher, 1988), cytoplasmic contents remain inside the cell. The cytoplasmic remains may contain or produce a low level of ATP, which is sufficient to activate inward K^+ channels. Under the conditions of our patch-clamp experiments, the requisite ATP might be provided via substrate level phosphorylation, which is not sensitive to inhibition by azide but would be sensitive to scavenging by Glc/hexokinase (cf. Figs. 1 and 2). The requisite ATP might also be provided because of incomplete exchange of cellular ATP into the large volume of pipette solution. Indeed, it has been estimated (Breitwieser and Szabo, 1988) that initial millimolar concentrations of GTP in mammalian cells are diminished by the whole-cell configuration to 25 to 50 μM, which is still high enough to be considered as a regulator in a signal transduction process. Consistent with either of these first two hypotheses, the addition of Glc and hexokinase together, which is reported to scavenge ATP (Hedrich et al., 1990), did decrease the whole-cell inward K^+ currents (Figs. 1F and 2). A third, more convoluted explanation for the results is that under the whole-cell configuration an unknown deactivating process that is specifically counteracted by ATP is not active; this process becomes active in the cytoplasm-free membrane patch, where it can then be counteracted by addition of ATP. If this hypothesis were to be true, then there would be a cytoplasmic factor(s) involved in regulating the deactivation process.

Table II. *Effects of several nucleotides on the P_o's of inward K^+ channels in inside-out membrane patches of Vicia guard cells*

Each pair of P_o's was acquired from the same membrane patch. The concentrations of ADP, GTP, and ATPγS were 2, 1, and 1 mM, respectively. Data were acquired 10 min after application of each treatment except as otherwise indicated. Glc (5 mM) and hexokinase (0.5 unit/mL) were added together to the cytoplasmic side of membrane patches, which had been preactivated by 2 mM ATP. Membrane potentials were held at −118 mV in all recordings.

Membrane Patch	Total P_o's				Mean ± SD
	1	2	3	4	
Control	0.03	0.02	0.06	0.05	0.04 ± 0.016
Control 10 min[a]	0.04	0.05	0.04	0.07	0.05 ± 0.015
ATP	0.24	0.25	0.18	0.21	0.22 ± 0.027
ATP 10 min[b]	0.25	0.21	0.20	0.23	0.22 ± 0.019
Control	0.08	0.04	0.05	0.06	0.06 ± 0.015
ADP	0.09	0.07	0.02	0.04	0.06 ± 0.027
Control	0.05	0.07	0.07	0.03	0.06 ± 0.017
GTP	0.05	0.09	0.06	0.05	0.06 ± 0.016
Control	0.04	0.07	0.09	0.06	0.07 ± 0.018
ATPγS	0.06	0.05	0.08	0.07	0.07 ± 0.011
ATP	0.19	0.26	0.27	0.22	0.24 ± 0.032
Glc + hexokinase	0.07	0.12	0.10	0.08	0.09 ± 0.019

[a] Data were acquired 10 min after acquisition of control data.

[b] Data were acquired 10 min after acquisition of ATP data.

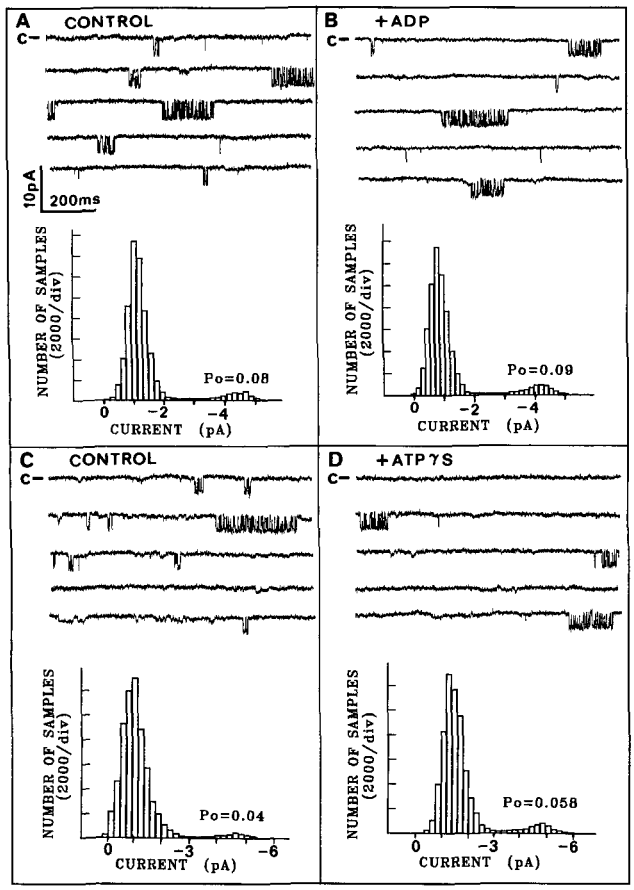

Figure 8. Single inward K^+ channel recordings in the absence and presence of ADP (A and B) and ATPγS (C and D) from inside-out membrane patches of *Vicia* guard cells. In all recordings (A–D), no ATP was added in the bath (cytoplasmic) solutions. The recordings in A (no ADP added) and B (2 mM ADP added, Tris salt) were acquired from the same membrane patch. The recordings in C (no ATPγS) and D (1 mM ATPγS, lithium salt) were acquired from the same membrane patch. Portions of the traces are illustrated in the upper sections of the panels, and all-point histograms derived from the complete traces are presented in the lower sections, with P_o's indicated. All recordings were obtained at −118 mV. The current and time scales refer to the scale bars shown in A.

Whereas the whole-cell data suggested that the inward K^+ channels were activated by ATP, such activation was clearly demonstrated in the isolated patch experiments (Figs. 6 and 7; Tables I and II). Our results indicate that the ATP requirement for inward K^+ channel activation in guard cells is specific, since other nucleotides tested in our experiments, ADP and GTP, did not enhance P_o of inward K^+ channels (Figs. 6–8; Table II). This effect is in contrast to the activation of guard-cell anion channels, which requires nucleotides nonspecifically (Hedrich et al., 1990).

Our data suggest that ATP acts in vivo as a signaling molecule that regulates inward K^+ channels. We have envisioned three theoretical mechanisms by which ATP could regulate channel activity. First, ATP could bind directly to the channel or to a protein that regulates the channel. If this binding were of high specificity, then ATPγS might not be able to substitute for ATP, accounting for the lack of an ATPγS effect. Second, ATP might regulate channel activity via a phosphorylation or dephosphorylation process. Consistent with this hypothesis, we have recently reported evidence that a protein phosphatase is involved in Ca^{2+}-mediated K^+ channel inhibition in guard cells (Luan et al., 1993). However, since ATPγS can substitute for ATP as a substrate for some kinases (Gratecos and Fisher, 1974), yet ATPγS cannot substitute for the ATP effect on isolated K^+ channels (Fig. 8), a kinase may not be involved in our ATP effect. Third, activation of guard-cell inward K^+ channels may require the hydrolysis of ATP. This hypothesis is highly speculative but would account for the observation that the nonhydrolyzable nucleotide, ATPγS, cannot substitute for ATP in activating the channel. Either the channel itself or a channel regulatory protein might hydrolyze ATP during channel activation. Further experimentation will be required to distinguish between the various possibilities listed above; in animal cells, evidence has been presented for both an ATP-binding site and an ATP-regulated phosphorylation site on the "type 1" ATP-sensitive K^+ channels (reviewed by Edwards and Weston, 1993).

A recent study has shown that ATP activates two K^+-selective channels in *Arabidopsis* mesophyll cells, and photosynthetically produced ATP was suggested to be responsible for activation of at least one of the channels (Spalding and Goldsmith, 1993). Guard cells contain chloroplasts and have photosynthetic functions (Shimazaki and Zeiger, 1985; Shimazaki et al., 1989; Poffenroth et al., 1992; Wu and Assmann, 1993). However, the extent to which photosynthetic products, including ATP produced via photophosphorylation, contribute to guard-cell physiological activities requires further study (Reckmann et al., 1990; Wu and Assmann, 1993). Serrano et al. (1988) have shown that addition of the photosynthetic inhibitor DCMU inhibited a light-activated H^+-ATPase on the plasma membranes of guard cells even in the presence of saturating concentrations of exogenous ATP, which suggests that in addition to ATP some other photosynthetically produced "signal" may also regulate H^+ and K^+ fluxes across the plasma membranes of guard cells.

In conclusion, we would like to note that a careful analysis of both whole-cell data and single-channel data from membrane patches may be important for drawing thorough conclusions regarding signal transduction pathways that regulate channel activity.

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