Rundown of the Hyperpolarization-Activated KAT1 Channel Involves Slowing of the Opening Transitions Regulated by Phosphorylation

Xiang D. Tang and Toshinori Hoshi
Department of Physiology and Biophysics, The University of Iowa, Iowa City, Iowa 52242 USA

ABSTRACT Disappearance of the functional activity or rundown of ion channels upon patch excision in many cells involves a decrease in the number of channels available to open. A variety of cellular and biophysical mechanisms have been shown to be involved in the rundown of different ion channels. We examined the rundown process of the plant hyperpolarization-activated KAT1 K^+ channel expressed in *Xenopus* oocytes. The decrease in the KAT1 channel activity on patch excision was accompanied by progressive slowing of the activation time course, and it was caused by a shift in the voltage dependence of the channel without any change in the single-channel amplitude. The single-channel analysis showed that patch excision alters only the transitions leading up to the burst states of the channel. Patch cramming or concurrent application of protein kinase A (PKA) and ATP restored the channel activity. In contrast, nonspecific alkaline phosphatase (ALP) accelerated the rundown time course. Low internal pH, which inhibits ALP activity, slowed the KAT1 rundown time course. The results show that the opening transitions of the KAT1 channel are enhanced not only by hyperpolarization but also by PKA-mediated phosphorylation.

INTRODUCTION

The KAT1 channel is a K^+-selective ion channel cloned from the higher plant *Arabidopsis thaliana* (Anderson et al., 1992; Schachtman et al., 1992). The amino acid sequence of the KAT1 channel is similar to those of animal depolarization-activated K^+ channels, such as Shaker- and EAG-like channels, and to those of cyclic nucleotide-gated cation channels (Anderson et al., 1992; Schachtman et al., 1992). Despite the sequence similarity, K^+ currents through KAT1 channels are observed only in response to hyperpolarization (Schachtman et al., 1992; Hedrich et al., 1995; Hoshi, 1995). Voltage-dependent gating of the KAT1 channel does not require internal Mg^{2+}, and the inward rectification is considered to be an intrinsic gating property of the channel (Hedrich et al., 1995; Hoshi, 1995; Zei and Aldrich, 1998). The KAT1 gating properties are influenced by the N- and C-terminal deletions and by several other intracellular factors (Hedrich et al., 1995; Hoshi, 1995; Marten and Hoshi, 1997, 1998).

Many ion channels are known to undergo rundown. The channel activity disappears when the membrane patch is excised or the cell is internally dialyzed (Doroshenko et al., 1982; Forscher and Oxford, 1985; Armstrong and Eckert, 1987; Chad et al., 1987; Horn and Korn, 1992; Li et al., 1992; Hoshi, 1995; Becq, 1996). The rundown process is a biologically interesting phenomenon because it illustrates regulation of the channel activity by intracellular factors. However, it is also experimentally inconvenient, as it interferes with data collection in the whole-cell and inside-out configurations. In some channels, rundown is not accompanied by noticeable changes in the gating kinetics, suggesting that the number of channels available to open decreases (Armstrong and Eckert, 1987; Chad et al., 1987; Anderson et al., 1991; Becq, 1996). In addition to the disappearance of the functional activity, establishing the whole-cell configuration, patch excision or internal dialysis is known to cause observable changes in the gating behavior of some ion channels. For example, internal dialysis involving ATP removal decreases the squid axon K^+ current amplitudes at many test voltages (Bezanilla et al., 1986). Voltage dependence of the Na^+ channel is known to shift with the establishment of the whole-cell configuration (e. g., Fernandez et al., 1984). Inactivation of some voltage-dependent K^+ channels is also altered by patch excision (Ruppersberg et al., 1991; Marom et al., 1993; Kupper et al., 1995; Ciorba et al., 1997).

Several different mechanisms have been proposed for ion channel rundown, including dephosphorylation and disruption of cytoskeletal elements (Doroshenko et al., 1982; Horn and Korn, 1992; Rosenmund and Westbrook, 1993; Becq, 1996; Hilgemann and Ball, 1996). Different experimental manipulations are available to at least slow down the rundown time course (Forscher and Oxford, 1985; Kramer, 1990; Horn and Korn, 1992). Dephosphorylation has been shown to promote rundown in many channels, in part because various phosphatases accelerate and some kinases, such as cAMP-dependent protein kinase (PKA), slow the rundown time course (Armstrong and Eckert, 1987; Chad et al., 1987; Becq, 1996). Thus phosphorylation could be considered as a switch that allows the channels to respond to their usual stimuli (Armstrong and Eckert, 1987; Chad et al., 1987).

Using macroscopic current measurements, we previously reported that the plant KAT1 channel heterologously expressed in *Xenopus* oocytes undergoes rundown and that it

Received for publication 2 October 1998 and in final form 4 March 1999.
Address reprint requests to Dr. Toshinori Hoshi, Department of Physiology and Biophysics, Bowen 5660, The University of Iowa, Iowa City, IA 52242. Tel.: 319-335-7845; Fax: 319-335-7330; E-mail: toshinori-hoshi@uiowa.edu.

0006-3495/99/06/3089/10 $2.00

could often be reversed by internal application of ATP (Hoshi, 1995). A KAT1 homolog, the KST1 channel, is also known to run down on patch excision (Müller-Röber et al., 1995). It was thus hypothesized that dephosphorylation may be involved in the KAT1 channel rundown and that it may involve a shift in the voltage-dependent gating of the channel. We directly tested these hypotheses, using both the macroscopic and single-channel measurements. The results obtained show that PKA-mediated phosphorylation regulates the opening transitions of the KAT1 channel and that this regulation is responsible for the shift in the KAT1 voltage-dependent gating observed on patch excision. Thus the KAT1 channel is dually gated by voltage and phosphorylation.

MATERIALS AND METHODS

Channel expression

KAT1 channels were expressed in *Xenopus* oocytes as described previously by RNA injection (Hoshi, 1995). The RNA solution was diluted to give desired current levels. The recordings were typically obtained 2–10 days after RNA injection.

Electrophysiology

Patch recordings were made using an Axopatch 200A amplifier with borosilicate pipettes coated with wax or Sylgard (Dow Corning, Midland, MI). The data acquisition and analysis were performed with Pulse/PulseFit (HEKA, Lambrecht, Germany) and IgorPro (WaveMetrics, Lake Oswego, OR) running on Apple Power Macintosh computers equipped with DA/AD boards (Instrutech, Port Washington, NY). Macroscopic linear leak and capacitative currents were corrected using a modified *P/n* protocol as implemented in Pulse. All of the experiments were performed at room temperature (20–22°C). The external solution contained (in mM) 140 KCl, 2 $MgCl_2$, and 10 HEPES (*N*-methylglutamine) (pH 7.2). The control internal solution contained (in mM) 140 KCl, 11 EGTA, 2 $MgCl_2$, and 10 HEPES (*N*-methylglutamine) (pH 7.2). The low pH internal solution contained (in mM) 140 KCl, 11 EGTA, 2 $MgCl_2$, and 10 2-(*N*-morpholino)-ethanesulfonic acid (*N*-methylglutamine) (pH 5.2 or 6.2).

The single-channel parameters were estimated using a custom routine implemented in Igor that essentially emulates TAC (Bruxton, Seattle, WA). The analysis presented is based on the results obtained from single-channel patches. Values of the four rate constants in the three-state scheme presented later were estimated as described (Hoshi et al., 1990).

Reagents

Adenosine 5′-[β,γ-imido]triphosphate (AMP-PNP) (lithium salt) was purchased from Fluka (Ronkonkoma, NY). The following chemicals were obtained from Sigma (St. Louis, MO): adenosine 5′-monophosphate (AMP) (sodium salt), adenosine 5′-diphosphate (ADP) (potassium salt), adenosine 5′-triphosphate (ATP) (dipotassium salt), adenosine 5′-*o*-(3-thio-triphosphate) (ATPγS) (lithium salt), guanosine 5′-triphosphate (GTP) (sodium salt), guanosine 5′-*o*-(3-thio-triphosphate) (GTPγS) (lithium salt), 3′:5′-cyclic AMP-dependent protein kinase catalytic subunit (PKA), protein kinase C (PKC), alkaline phosphatase (ALP), and protein phosphatase inhibitor 2. All chemicals were diluted to the desired concentrations, and the pH was readjusted.

RESULTS

The rundown time courses of the KAT1 channels are variable in different oocytes

In the cell-attached configuration, macroscopic currents through KAT1 channels elicited by hyperpolarization were quite stable, at least up to ~1 h (Fig. 1, *A* and *B*).

However, on patch excision, the KAT1 currents often progressively decreased in amplitude (Fig. 1, *C* and *D*). The time course and the extent of rundown were quite variable, especially among the patches taken from different oocytes. Fig. 1 *D* shows the KAT1 rundown time courses from five different oocytes to illustrate this variability. After variable intervals following patch excision, the current amplitudes often stabilized to new smaller and relatively stable values, allowing us to record currents in near-steady-state conditions. When the patches were taken from the same oocyte, the time course and the extent of the rundown were more consistent (data not shown).

Rundown does not alter single KAT1 channel current amplitude

The macroscopic ionic current as a function of time t and voltage V, $I(V, t)$, could be described by $N \cdot i(V) \cdot P(V, t)$ where N represents the number of channels available to open, $i(V)$ represents the single-channel amplitude, and $P(V, t)$ represents the probability of the channel being open determined by the channel's gating properties. The decrease in the macroscopic current amplitude observed in rundown could be caused by a decrease in N, $i(V)$, or $P(V, t)$. In the rundown of some other channels, such as voltage-gated Ca^{2+} channels, a decrease in N is typically thought to be responsible for the disappearance of the channel activity (Doroshenko et al., 1982; Armstrong and Eckert, 1987; Chad et al., 1987). Here we will present evidence that a decrease in $P(V, t)$ mediated by slower opening transitions is responsible for the KAT1 channel rundown.

In the KAT1 channel, the progressive decrease in the macroscopic current amplitude on patch excision is accompanied with slowing of the activation time course (Fig. 1, *E* and *F*) (Hoshi, 1995). This kinetic change indicates that a simple change in the number of channels available to open does not account for the KAT1 rundown.

Furthermore, our single-channel recordings show that the unitary current amplitude does not change during the rundown process. Fig. 2 *A* compares representative single-channel openings obtained in the cell-attached configuration and in the excised inside-out configuration after considerable rundown has taken place. The single-channel amplitude histograms in Fig. 2 *B* illustrate that, despite the noticeable change in the gating behavior as shown in Fig. 2 *A*, the single-channel current amplitude remained unaltered by rundown.

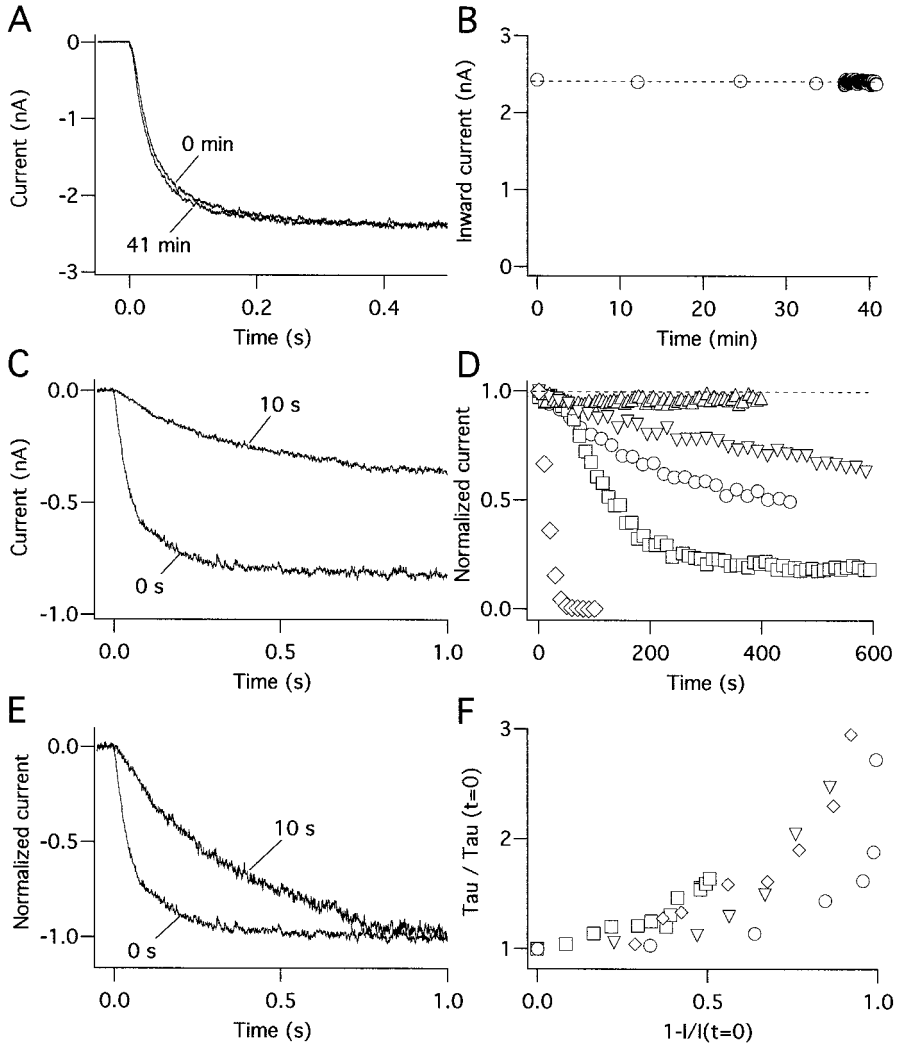


FIGURE 1 Rundown of the macroscopic KAT1 channel currents. (*A*) Comparison of the current traces elicited in response to pulses from 0 mV to −160 mV recorded immediately after the seal formation (0 min) and 41 min later in the cell-attached configuration. (*B*) Time course of the peak amplitudes of the KAT1 currents at −160 mV after the seal formation. (*C*) Comparison of the KAT1 currents recorded in response to pulses from 0 mV to −160 mV immediately after patch excision and 10 s later. (*D*) Time courses of the current rundown in patches taken from five different oocytes. The peak current amplitudes are scaled to the respective cell-attached values and plotted as a function of time after patch excision. Different symbols represent different patches. (*E*) Slowing down in the activation time course after rundown. The two current traces shown in *C* were scaled to compare their activation time courses. (*F*) Time courses of the normalized activation time constants. The current activation time courses were fit with single exponentials, and the time constant values were normalized to the values obtained in the cell-attached configuration. The x axis ($1 - I/I(t = 0)$) represents the fractional decrease in the current amplitude during rundown. $I(t = 0)$ represents the current amplitude in the cell-attached configuration. The normalized time constant values are plotted as a function of the fractional decrease in the current amplitude, showing that the activation time course becomes slower as the fractional current decreases. Different symbols represent results from different patches.

Rundown involves a gradual shift in the voltage dependence of the KAT1 open probability

Based on the macroscopic ramp $I(V)$ data, it was hypothesized that a shift in the voltage dependence of the open channel probability was responsible for the KAT1 channel rundown (Hoshi, 1995). We tested this hypothesis by directly measuring the single-channel open probability values at different voltages before and after rundown. It is clear from the representative recordings shown in Fig. 2 *A* that the overall open probability is lower after patch excision at each voltage. Voltage dependence of the single-channel open probability before and after rundown is compared in Fig. 2 *C*. The voltage dependence in the cell-attached configuration was fit with a fourth power of Boltzmann function with an equivalent charge of 1.4 e_0 and half-activation voltage of −84 mV, in accordance with the previously described macroscopic results (Hoshi, 1995). The voltage dependence of the open probability after excision was also well described by the same Boltzmann function with an offset voltage of −40 mV (Fig. 2 *C*). Similar results were obtained in five other patches analyzed. These observations confirm that the KAT1 channel rundown involves a gradual shift in the voltage dependence of the open probability without any marked change in the voltage sensitivity.

The KAT1 channel rundown and burst behavior

The results presented so far indicate that the rundown process of the KAT1 channel involves changes in the KAT1 gating properties to reduce the open channel probability, excluding the possibility that a change in the number of channels available to open or a change in the single-channel amplitude is involved. To investigate which gating transitions are altered during the rundown process, we examined the single-channel mean dwell times of the KAT1 channel (Fig. 3). Open- and closed-duration histograms recorded from one patch in three different configurations, cell-attached, inside-out, and patch cramming, are shown in Fig. 3 *B*. The open durations were well described by a simple exponential (*upper panel*), whereas the closed durations required a sum of two exponentials (*lower panel*). Patch excision increased the frequency of the long closed-duration

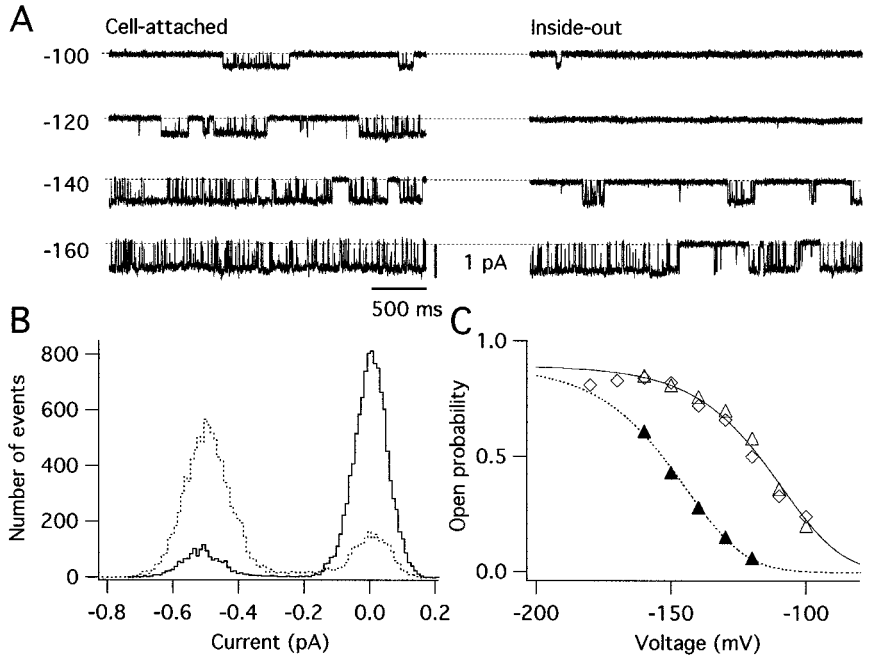
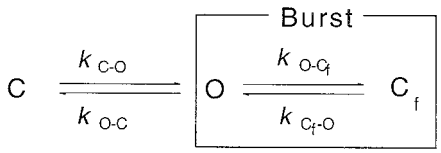


FIGURE 2 Rundown of the single KAT1 channel currents. (*A*) Representative single-channel KAT1 openings recorded at different voltages in the cell-attached configuration (*left*) and in the inside-out configuration after patch excision. Downward deflections indicate the opening transitions. The data were filtered at 1 kHz. (*B*) Comparison of the single-channel amplitude histograms constructed from the single KAT1 currents at −120 mV. ——, Results obtained in the cell-attached configuration; ---, results obtained in the inside-out configuration after patch excision. (*C*) Voltage dependence of the single-channel open probability before (△, ◇) and after (▲) patch excision. The open probability values were calculated from the idealized current records. The voltage dependence in the cell-attached configuration was fit with a fourth power of Boltzmann function with an equivalent charge of 1.4 e_0 and a half-activation voltage of −84 mV (——). The data obtained in the inside-out configuration were fit with the same Boltzmann function with an offset voltage of −40 mV.

events, consistent with the observation that the bursts are often separated by longer intervals in the inside-out configuration (Fig. 3 *A*). Patch excision, however, did not markedly affect the time constant of the fast component in the closed durations, suggesting that the short flicker closures within the bursts are not affected. We found that the steady-state single-channel KAT1 kinetics could be reasonably approximated by the following linear three-state model:



where O represents the open state, C_f represents the short-lived closed state, and C represents the closed state responsible for the interburst intervals (also see Zei and Aldrich, 1998). The values of the four rate constants were estimated using the single-channel records obtained in the cell-attached configuration and those obtained after patch excision. The rate constant values are compared in Fig. 3 *C*. Patch excision did not markedly alter the values of the rate constants within the burst, $k_{O\text{-}C_f}$ and $k_{C_f\text{-}O}$ (*left panel*). However, patch excision noticeably enhanced the burst termination rate constant $k_{O\text{-}C}$ and decreased the burst entry rate constant $k_{C\text{-}O}$ (*right panel*). These changes would produce the longer mean interburst duration and shorter mean burst duration, as illustrated in Fig. 3 *A* (*middle trace*). The intracellular factors lost during rundown thus specifically alter the transitions leading up to the kinetic states of the KAT1 channel involved in the burst behavior, and those states involved in the openings and closings within each burst are not affected by rundown.

Rundown slows down the first latency

We also examined how patch excision alters the first latency of the KAT1 channel. Representative current traces recorded in response to pulses from 0 to −120 mV before and after patch excision are shown in Fig. 4 *A*. Fig. 4 *B* compares the first latency distributions recorded in the cell-attached and the inside-out configurations. The first latency distributions were similar to the time courses of the KAT1 macroscopic currents in the respective configurations (see Fig. 1). Furthermore, the first latency events were similar to the interburst durations observed (see $k_{C\text{-}O}$ in Fig. 3 *C*), suggesting that the interburst events may represent the channel going back to the closed states involved in the activation process. The first latency distribution was markedly slower after patch excision, with the median latency extending from ~150 ms to 2 s. Similar effects of patch excision of slowing the first latency were observed in four other single-channel patches analyzed.

Patch cramming or ATP/PKA restores the KAT1 channel function

We showed previously that patch cramming could enhance the KAT1 macroscopic current after rundown (Hoshi, 1995). The results in Fig. 3 *A* indicate that patch cramming restores the single KAT1 channel activity without altering the unitary channel amplitude. The burst analysis further indicates that patch cramming restores the single KAT1 channel activity by altering the burst entry rate constant and the burst termination rate constant (Fig. 3 *C*). As expected, the effects of patch cramming on the two rate constants were opposite those of patch excision.

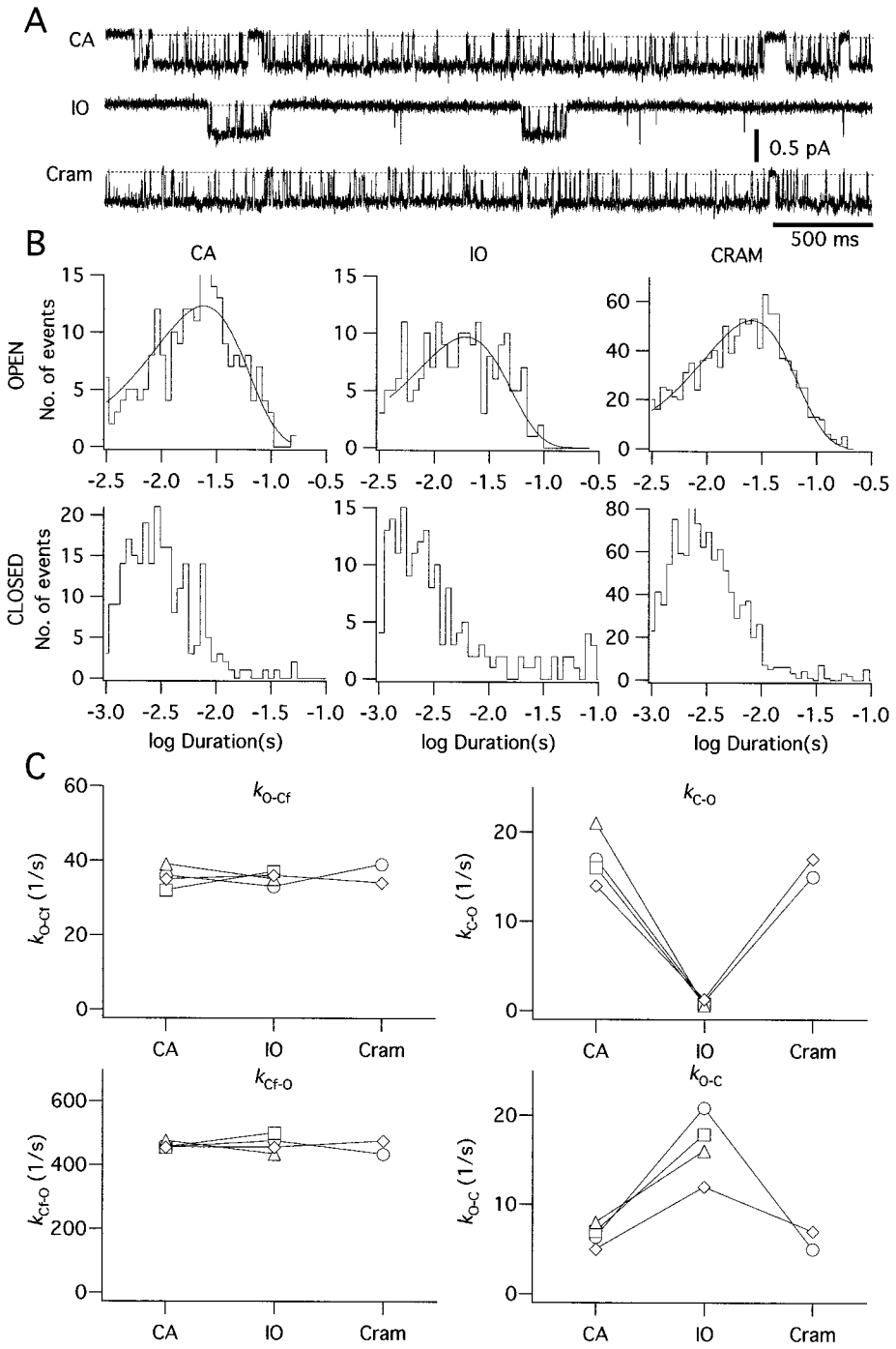


FIGURE 3 Effects of patch excision on the KAT1 single-channel gating parameters. (*A*) Representative single-channel openings at −120 mV recorded in the cell-attached configuration (*top*), inside-out configuration (*middle*), and patch-cramming mode (*bottom*). Downward deflections indicate the opening transitions. The data were filtered at 1 kHz. (*B*) Open duration histograms (*upper panel*) and closed duration histograms (*lower panel*) recorded in the cell-attached, inside-out and cramming configurations from a single-channel patch at −120 mV. CA indicates the results obtained in the cell-attached configuration. IO indicates the results obtained in the excised inside-out configuration. Cram indicates the data obtained after patch cramming. The single-channel events were idealized as described in Materials and Methods. The mean open durations in the cell-attached, inside-out, and cramming configurations were 24, 23, and 25 ms, respectively. (*C*) Comparison of the rate constant values in the linear three-state model as presented in the text in the cell-attached, inside-out, and cramming configurations. Different symbols represent the values estimated from different patches. The burst criterion was 40 ms.

It was shown previously that internal ATP could enhance the macroscopic KAT1 current amplitude, although a considerable amount of variability in the effectiveness of internal ATP was noted (Hoshi, 1995). We found that ATP also restored the KAT1 channel activity without altering the single-channel amplitude (data not shown). Internal ATP without Mg^{2+}, AMP-PNP, a nonhydrolyzable analog of ATP, ATPγS, AMP, ADP, GTP, and GTPγS failed to affect the open channel probability.

Because internal ATP but not its nonhydrolyzable analog AMP-PNP frequently restores the KAT1 channel activity after rundown, we hypothesized that dephosphorylation is responsible for the rundown process and that the equilibrium between the protein kinase activity and the phosphatase activity closely associated with a channel in the membrane patch may determine the voltage dependence of the KAT1 channel after patch excision. The results suggest that PKA-mediated phosphorylation may be involved in regulation of the voltage dependence of the KAT1 channel (Fig. 5). In the results presented in Fig. 5 *A*, after the channel activity had run down, internal ATP restored the current amplitude to the same level as found in the cell-attached

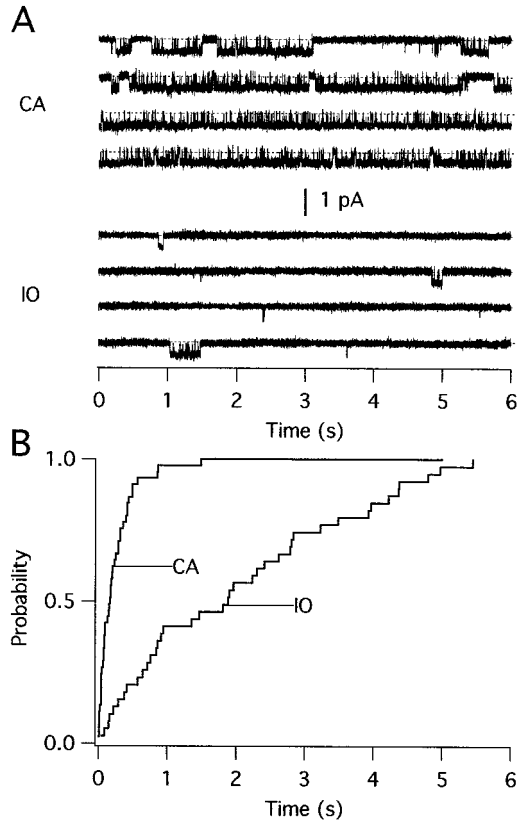


FIGURE 4 Effect of patch excision on the first latency. (*A*) Representative KAT1 openings in the cell-attached configuration (*top*) and the inside-out configuration (*bottom*). The openings were elicited by pulses to −120 mV from the holding voltage of 0 mV every 8 s. The leak and capacitative currents were subtracted using the data sweeps without any opening as the templates. Downward deflections indicate the opening transitions. The data were filtered at 1 kHz. (*B*) Comparison of the first latency distributions obtained using pulses from 0 to −120 mV. The distributions in the cell-attached and inside-out configurations were made from 45 and 40 hyperpolarizing epochs, respectively. The median first latencies were 179 ms and 1.9 s for the events recorded in the cell-attached and inside-out configurations.

configuration. Subsequent withdrawal of ATP decreased the current amplitude. In contrast, the second ATP application was only partially and transiently effective in restoring the KAT1 channel activity, and eventually the current almost totally disappeared, indicating that the factors required to keep the KAT1 channel functioning had been washed away or exhausted. However, addition of the catalytic subunit of PKA (15 U/ml) in the presence of ATP effectively restored the channel activity to the pre-patch excision level. Similar effects of PKA of restoring the channel activity were observed in five other patches. PKA application alone without ATP ($n = 4$) and PKC application (0.2 U/ml) were not effective ($n = 3$, data not shown), suggesting that the effect is mediated by PKA-mediated phosphorylation. The results are consistent with the idea that rundown of the KAT1 channel involves dephosphorylation of the sites that could be phosphorylated by PKA.

ATP-PKA application not only increased the peak current amplitude, but also restored the KAT1 activation time course. The KAT1 currents recorded immediately after patch excision and after ATP-PKA application are shown in Fig. 5 *B* (*right*). Time courses of the currents in the two conditions were indistinguishable. Using ramp $I(V)$ curves recorded immediately after patch excision, after rundown, and after ATP-PKA application, macroscopic $G(V)$ curves were constructed (Fig. 5 *C*). The results show that ATP-PKA application also restored the voltage dependence of the macroscopic KAT1 current.

Nonspecific alkaline phosphatase accelerates the rundown time course

If the rundown process of the KAT1 channel involves dephosphorylation, its time course should be accelerated by the addition of phosphatase. Fig. 6 *A* illustrates the effects of alkaline phosphatase (ALP) application to the KAT1 channels. ALP dephosphorylates proteins in a nonspecific manner (MacComb et al., 1979). In this patch, the rundown was incomplete, and the current amplitude remained stable at ~50% of the pre-patch excision level. ALP (20 U/ml) rapidly decreased the KAT1 current amplitude, and the effect of ALP of reducing the KAT1 channel amplitude was reversible. The addition of ATP (0.5 mM) restored the KAT1 channel current amplitude. Similar results were obtained in five other experiments. Internal Ca^{2+}, fluoride, and protein phosphatase inhibitor 2 (10 μg/ml) did not affect the rundown time course. The results suggest that the KAT1 rundown may be accelerated by ALP-like phosphatase present near the channel proteins in excised patches.

Internal pH regulates the time course of the KAT1 channel rundown

The ALP activity is known to be dependent on pH. The activity is at maximum around pH 9.0, and the enzyme activity is inhibited, especially at lower pH values (MacComb et al., 1979). If the KAT1 channel rundown involves dephosphorylation mediated by ALP-like molecules, the rundown should be dependent on internal pH. The rundown time course should be slower when the cytoplasmic solution pH is lower, which inhibits ALP. We tested this prediction by examining the KAT1 rundown time courses at different pH values. Fig. 6 *B* shows that the KAT1 rundown time course was indeed faster at pH 7.2 than that at pH 6.2 or 5.2, confirming this prediction. Similar effects of internal pH on the rundown time course were observed in seven other patches.

DISCUSSION

Biophysical mechanism underlying the KAT1 channel rundown

Our results show that rundown of the KAT1 channel expressed in *Xenopus* oocytes caused by patch excision in-

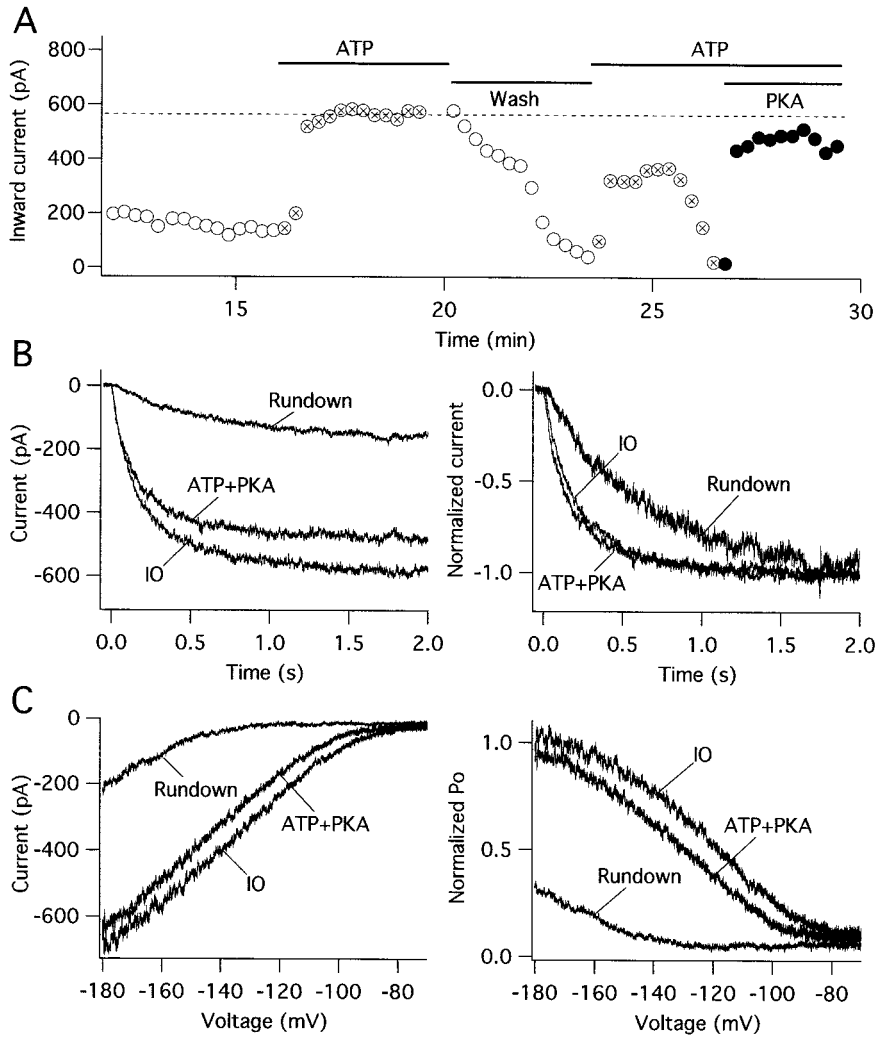


FIGURE 5 Restoration of the KAT1 channel rundown by ATP and ATP-PKA. (*A*) Time course of the peak inward current amplitude recorded at −140 mV after patch excision. The horizontal dotted line represents the current amplitude in the cell-attached configuration. The patch was excised at time $t = 0$, and the current decreased progressively to a near-zero level. ATP (0.5 mM) was applied to the bath in the inside-out configuration, and it restored the current to the preexcision level. The bath was then washed with ATP-free saline and the current amplitude decreased again. The second application of ATP was only partially and transiently effective (24–26 min). PKA (15 U/ml) in the presence of ATP rapidly restored the current amplitude. (*B*) Effect of internal ATP/PKA application on the KAT1 current activation time course. Representative current traces obtained immediately after patch excision (IO), after rundown (Rundown), and after ATP/PKA application (ATP + PKA) (left). The three current traces in the left panel were scaled to facilitate comparison of their activation time courses (*right*). (*C*) Effect of internal ATP/PKA application on the voltage dependence of the KAT1 channel. Ramp $I(V)$ curves obtained immediately after patch excision (IO), after rundown (Rundown), and after ATP + PKA application (ATP + PKA) (*left*). The ramp $I(V)$ curves were elicited by 10-s voltage ramps from +50 mV to −180 mV (Hoshi, 1995). Only the segments between −70 mV and −180 mV are shown. The ramp $I(V)$ curves were then normalized by the driving force to produce normalized macroscopic $G(V)$ curves to indicate the voltage dependence of the normalized open probability (P_O) (*right*).

volves a change in its gating properties. The patch excision does not alter the KAT1 single-channel current amplitude, and the observation that the macroscopic activation time course slows after patch excision suggests that the channel's opening transitions may be slowed on patch excision. Furthermore, our analysis of the single-channel dwell times shows that patch excision specifically affects the gating transitions leading up to the burst states, and it does not affect the transitions within the burst. Thus the KAT1 channel rundown could be biophysically described as a decrease in the open probability caused by slower burst entry and faster burst exit, which result in a shift in the channel's voltage dependence to more negative voltages.

Rundown of the KAT1 channel was most efficiently reversed by patch cramming and by application of PKA and ATP together. The time course and extent of the rundown process were accelerated by application of ALP. These results indicate that dephosphorylation promotes whereas phosphorylation inhibits the KAT1 channel rundown. The results, however, do not directly address whether dephosphorylation of the channel protein itself or of other proteins associated with the channels in excised patches is responsible. Inspection of the KAT1 primary amino acid sequence indicates that there are some threonine and serine residues that could be phosphorylated by PKA. For example, S179 in the S4 segment (RRVS) and T614 (KRVT) in the C-terminus are two easily recognizable PKA-consensus sites (Kemp et al., 1975). The results presented here predict that elimination of the critical phosphorylated residue(s) may mimic the effect of dephosphorylation and shift the channel's voltage dependence to more negative voltages, as found in the inside-out configuration that promotes rundown. T614 can be deleted without marked changes in the electrophysiological properties (Marten and Hoshi, 1997). A mutation around S179 that destroys the PKA consensus sequence (R176S) (Marten and Hoshi, 1997) does not have any obvious effect on the channel's rundown properties (unpublished observation). Those structural elements not involved in the voltage sensing function may be involved because the steepness of the $G(V)$ curve is not noticeably altered during the rundown process. The KAT1 channel in native guard cells is known to be phosphorylated in a Ca^{2+}-dependent manner by a kinase with calmodulin-like domain (CDPK) (Li et al., 1998). The rundown of the KAT1 channel expressed in *Xenopus* oocytes is not appreciably affected by internal Ca^{2+} (unpublished observation), and

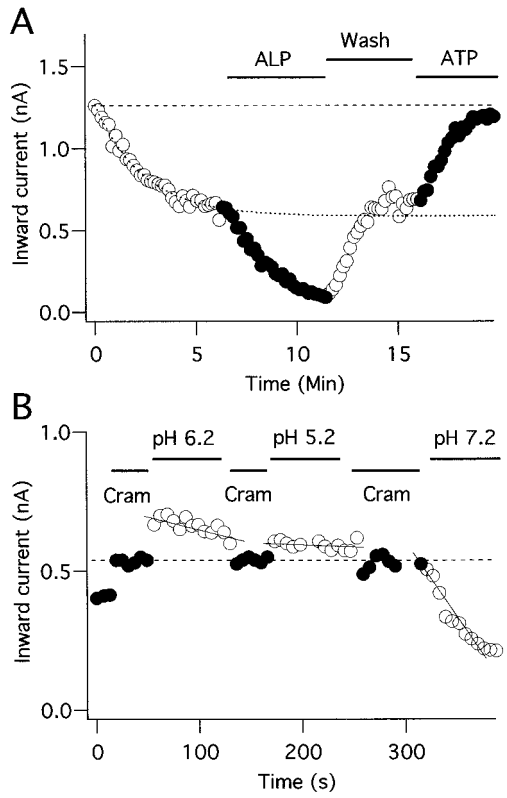


FIGURE 6 Effects of ALP on the KAT1 channel rundown. (*A*) Acceleration of the KAT1 channel rundown by internal ALP application. The peak inward current amplitude recorded at −140 mV is plotted as a function of time after patch excision. The horizontal dotted line represents the current amplitude in the cell-attached configuration. ALP (20 U/ml) rapidly decreased the current amplitude in a reversible manner. Subsequent wash and ATP application restored the channel activity. (*B*) Effects of internal pH on the rundown time course. The peak inward current amplitude recorded at −140 mV is plotted as a function of time after patch excision. The horizontal dotted line represents the current amplitude in the cell-attached configuration. The KAT1 currents were recorded in the excised inside-out configuration at three different pH values. After each pH treatment, the patch was crammed back into the oocyte to restore the channel activity. The rundown rate was approximated by slopes, as indicated by three solid lines superimposed on three dotted-circled segments. Note the difference in steepness of the slopes for the KAT1 rundown at different internal pH. Also note that the KAT1 currents remain at a stable level each time the patch was inserted back into the oocyte.

phosphorylation mediated by the CDPK-kinase is probably not involved.

The opening transition rate constants of the KAT1 channel are enhanced by hyperpolarization (Hoshi, 1995; Zei and Aldrich, 1998). The results presented here show that the equilibrium between phosphorylation of the channel and/or other cellular elements most probably mediated by PKA and dephosphorylation promoted by ALP-like phosphatase also controls the KAT1 opening transitions. Phosphorylation, either directly or indirectly, favors the forward opening transitions, and dephosphorylation interferes with these opening transitions. Patch excision promotes dephosphorylation over phosphorylation, and it decreases the opening rate constant values to decrease the open probability. Much greater hyperpolarization is necessary to observe the same channel activity after rundown. This shift in the KAT1 voltage dependence resembles the shift in the Na^+ channel voltage dependence observed upon establishment of the whole-cell configuration (Fernandez et al., 1984), and similar cellular mechanisms may be involved. The variability in the rundown time course and extent observed among different oocytes (Fig. 1 *D*) may represent different equilibrium phosphorylation states of the KAT1 channels expressed in different oocytes. This may explain some of the variability observed in the electrophysiological properties of KAT1 channels expressed in different systems at different expression levels (Very et al., 1994; Bertl et al., 1995; Marten et al., 1996; Bei and Luan, 1998).

Comparison with other ion channels regulated by phosphorylation/dephosphorylation

Protein phosphorylation is a well-known regulatory mechanism of ion channel function, and many functional effects have been documented (for a review, see Levitan, 1994). Rundown of voltage-gated Ca^{2+} channels is likely to be mediated at least in part by dephosphorylation, and experimental manipulations to keep the channels functional in the whole-cell configuration are often successful (Forscher and Oxford, 1985; Armstrong and Eckert, 1987; Chad et al., 1987). Our results with the KAT1 channel show that patch excision working via dephosphorylation alters the overall voltage dependence of the channel by drastically slowing the forward transition step(s). Other studies have also shown that dephosphorylation and phosphorylation regulate specific transitions involved in the channel gating. The phosphorylation requirement for the function of the cystic fibrosis Cl^- channel is well documented (for a review, see Sheppard and Welsh, 1999). N-type inactivation of transient A-type K^+ channels can be regulated by phosphorylation (Covarrubias et al., 1994; Drain et al., 1994). Perozo and Bezanilla showed that phosphorylation regulates the occupancy probability values in the K^+ channel resting closed states (Perozo and Bezanilla, 1990). Perozo et al. also showed that phosphorylation specifically increased the squid K^+ channel first latency, shifting the voltage dependence of the first latency to a more positive voltage without affecting the open- and closed-duration properties (Perozo et al., 1991). Although phosphorylation affects the first latency properties of both the squid K^+ channel (Perozo et al., 1991) and the KAT1 channel, the overall effects of ATP and phosphorylation are, however, different in these two systems. The positive shift induced in the squid axon would act to inhibit the channel opening, whereas in the KAT1 channel it would promote the channel opening.

Other mechanisms underlying the ion channel rundown

Although rundown of the KAT1 channel as well as other channels appears to be mediated by dephosphorylation (For-

scher and Oxford, 1985; Armstrong and Eckert, 1987), other mechanisms have been found in the rundown of different ion channels. Proteolysis of channel protein by the membrane-associated proteinase calpaine was found to be implicated in the irreversible rundown of Ca^{2+} channels in *Helix* neurons (Chad et al., 1987). Actin filament-depolymerizing agents (cytochalasins and desoxyribonuclease I) were reported to accelerate, whereas an actin filament stabilizer (phalloidin) or F-actin-serving protein inhibitor (phosphatidylinositol biphosphate or PIP2) inhibit the rundown of the cardiac ATP-sensitive K^+ channel (Furukawa et al., 1996; Hilgemann and Ball, 1996) and *N*-methyl-D-aspartate receptor channel in cultured hippocampal neurons (Rosenmund and Westbrook, 1993), indicating that the channel rundown is also regulated by cytoskeleton elements.

Physiological implications of the KAT1 channel regulation by multiple factors

The plant KAT1 channel activity is thus regulated not only by voltage, but also by a variety of other physiological factors. Low internal and external pH can enhance KAT1 macroscopic currents (Hedrich et al., 1995; Hoshi, 1995; Marten and Hoshi, 1997). cGMP decreases the KAT1 channel activity, probably working through the channel's C-terminal domain homologous to that of the cyclic nucleotide gated channel (Hoshi, 1995). This study shows that PKA-mediated phosphorylation also regulates the channel activity. The KST1 channel, a KAT1 homolog, also runs down on patch excision, and its function is restored by internal ATP (Müller-Röber et al., 1995). Thus it is likely that the opening transitions of the KST1 channel are similarly regulated by PKA.

The multitude of regulatory mechanisms available to KAT1 channels may allow plant cells expressing these channels, most likely guard cells (Nakamura et al., 1995), to survive in a variety of growth conditions. Native guard cell channels are regulated by many factors (MacRobbie, 1998). For example, the *Vicia faba* K^+ channels are modulated by cAMP (Li et al., 1994). The effect of the plant hormone ABA on guard cell K^+ channels may be mediated in part by dephosphorylation (Armstrong et al., 1995).

Genes coding for hyperpolarization-activated cation channels in animals have been isolated from different types of animal and human cells (Santoro et al., 1997, 1998; Gauss et al., 1998; Ludwig et al., 1998). These channels are predominantly present in brains and hearts and are considered to be involved in cellular rhythmic activities (Pape, 1996; Santoro et al., 1998). Defects in pacemaking activities can lead to inherited (Spellberg, 1971) and acquired (Bigger and Reiffel, 1979) cardiac arrhythmias and may underlie various neurological diseases (Santoro et al., 1998). Because there is considerable similarity between these animal genes and the plant KAT1 family genes, it will be interesting to see if these animal hyperpolarization-activated channels are regulated in a similar manner.

We thank Dr. Thommandru and Ms. Masropour for technical assistance, Dr. I. Marten for discussion, and Pete Brown for editorial ideas.

This work was supported in part by the National Institutes of Health (GM51474).

REFERENCES

Anderson, J. A., S. S. Huprikar, L. V. Kochian, W. J. Lucas, and R. F. Gaber. 1992. Functional expression of a probable *Arabidopsis thaliana* potassium channel in *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA.* 89:3736–3740.

Anderson, M. P., H. A. Berger, D. P. Rich, R. J. Gregory, A. E. Smith, and M. J. Welsh. 1991. Nucleotide triphosphates are required to open the CFTR chloride channel. *Cell.* 67:775–784.

Armstrong, D. L., and R. Eckert. 1987. Voltage-activated Ca channels that must be phosphorylated to respond to membrane depolarization. *Proc. Natl. Acad. Sci. USA.* 84:2518–2522.

Armstrong, F., J. Leung, A. Grabov, J. Brearley, J. Giraudat, and M. R. Blatt. 1995. Sensitivity to abscisic acid of guard-cell K^+ channels is suppressed by abi1–1, a mutant *Arabidopsis* gene encoding a putative protein phosphatase. *Proc. Natl. Acad. Sci. USA.* 92:9520–9524.

Becq, F. 1996. Ionic channel rundown in excised membrane patches. *Biochim. Biophys. Acta.* 1286:53–63.

Bei, Q., and S. Luan. 1998. Functional expression and characterization of a plant K^+ channel gene in a plant cell model. *Plant J.* 13:857–865.

Bertl, A., J. A. Anderson, C. L. Slayman, and R. F. Gaber. 1995. Use of *Saccharomyces cerevisiae* for patch-clamp analysis of heterologous membrane proteins: characterization of KAT1, an inward-rectifying K^+ channel from *Arabidopsis thaliana*, and comparison with endogeneous yeast channels and carriers. *Proc. Natl. Acad. Sci. USA.* 92:2701–2705.

Bezanilla, F., C. Caputo, R. DiPolo, and H. Rojas. 1986. Potassium conductance of the squid giant axon is modulated by ATP. *Proc. Natl. Acad. Sci. USA.* 83:2743–2745.

Bigger, J. T., Jr., and J. A. Reiffel. 1979. Sick sinus syndrome. *Annu. Rev. Med.* 30:91–118.

Chad, J., D. Kalman, and D. Armstrong. 1987. The role of cyclic AMP-dependent phosphorylation in the maintenance and modulation of voltage-activated calcium channels. *Soc. Gen. Physiol. Ser.* 42:167–186.

Ciorba, M. A., S. H. Heinemann, H. Weissbach, N. Brot, and T. Hoshi. 1997. Modulation of potassium channel function by methionine oxidation and reduction. *Proc. Natl. Acad. Sci. USA.* 94:9932–9937.

Covarrubias, M., A. Wei, L. Salkoff, and T. B. Vyas. 1994. Elimination of rapid potassium channel inactivation by phosphorylation of the inactivation gate. *Neuron.* 13:1403–1412.

Doroshenko, P. A., P. G. Kostyuk, and A. E. Martynyuk. 1982. Intracellular metabolism of adenosine 3′,5′-cyclic monophosphate and calcium inward current in perfused neurons of *Helix pomatia. Neuroscience.* 7:2125–2134.

Drain, P., A. E. Dubin, and R. W. Aldrich. 1994. Regulation of Shaker K^+ channel inactivation gating by the cAMP-dependent protein kinase. *Neuron.* 12:1097–1109.

Fernandez, J. M., A. P. Fox, and S. Krasne. 1984. Membrane patches and whole-cell membranes: a comparison of electrical properties in rat clonal pituitary (GH3) cells. *J. Physiol. (Lond.).* 356:565–585.

Forscher, P., and G. S. Oxford. 1985. Modulation of calcium channels by norepinephrine in internally dialyzed avian sensory neurons. *J. Gen. Physiol.* 85:743–763.

Furukawa, T., T. Yamane, T. Terai, Y. Katayama, and M. Hiraoka. 1996. Functional linkage of the cardiac ATP-sensitive K^+ channel to the actin cytoskeleton. *J. Physiol. (Lond.).* 431:504–512.

Gauss, R., R. Seifert, and U. B. Kaupp. 1998. Molecular identification of a hyperpolarization-activated channel in sea urchin sperm. *Nature.* 393: 583–587.

Hedrich, R., O. Moran, F. Conti, H. Busch, D. Becker, F. Gambale, I. Dreyer, A. Kuech, K. Neuwinger, and K. Palme. 1995. Inward rectifier potassium channels in plants differ from their animal counterparts in response to voltage and channel modulators. *Eur. Biophys. J.* 24: 107–115.

Hilgemann, D. W., and R. Ball. 1996. Regulation of cardiac Na^+, Ca^{2+} exchange and K_{ATP} potassium channels by PIP2. *Science.* 273:956–959.

Horn, R., and S. J. Korn. 1992. Prevention of rundown in electrophysiological recording. *Methods Enzymol.* 207:149–155.

Hoshi, T. 1995. Regulation of voltage dependence of the KAT1 channel by intracellular factors. *J. Gen. Physiol.* 105:309–328.

Hoshi, T., W. N. Zagotta, and R. W. Aldrich. 1990. Biophysical and molecular mechanisms of *Shaker* potassium channel inactivation. *Science.* 250:533–538.

Kemp, B. E., D. V. Bylund, T. S. Huang, and E. G. Krebs. 1975. Substrate specificity of the cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* 81:945–949.

Kramer, R. H. 1990. Patch cramming: monitoring intracellular messengers in intact cells with membrane patches containing detector ion channels. *Neuron.* 4:335–341.

Kupper, J., M. R. Bowlby, S. Marom, and I. B. Levitan. 1995. Intracellular and extracellular amino acids that influence C-type inactivation and its modulation in a voltage-dependent potassium channel. *J. Physiol. (Lond.).* 430:1–11.

Levitan, I. B. 1994. Modulation of ion channels by protein phosphorylation and dephosphorylation. *Annu. Rev. Physiol.* 56:193–212.

Li, J. X., Y. R. J. Lee, and S. M. Assmann. 1998. Guard cells possess a calcium-dependent protein kinase that phosphorylates the KAT1 potassium channel. *Plant Physiol.* 116:785–795.

Li, M., J. W. West, Y. Lai, T. Scheuer, and W. A. Catterall. 1992. Functional modulation of brain sodium channels by cAMP-dependent phosphorylation. *Neuron.* 8:1151–1159.

Li, W., S. Luan, S. L. Schreiber, and S. M. Assmann. 1994. Cyclic AMP stimulates K^+ channel activity in mesophyll cells of *Vicia faba* L. *Plant Physiol.* 106:957–961.

Ludwig, A., X. Zong, M. Jeglitsch, F. Hofmann, and M. Biel. 1998. A family of hyperpolarization-activated mammalian cation channels. *Nature.* 393:587–591.

MacComb, R. B., G. N. Bowers, and S. Posen. 1979. Alkaline Phosphatase. Plenum Press, New York and London.

MacRobbie, E. A. C. 1998. Signal transduction and ion channels in guard cells. *Science.* 353:1475–1488.

Marom, S., S. A. N. Goldstein, J. Kupper, and I. B. Levitan. 1993. Mechanism and modulation of inactivation of the Kv3 potassium channel. *Recept. Channels.* 1:81–88.

Marten, I., F. Gaymard, G. Lemaillet, J. B. Thibaud, H. Sentenac, and R. Hedrich. 1996. Functional expression of the plant K^+ channel KAT1 in insect cells. *FEBS Lett.* 380:229–232.

Marten, I., and T. Hoshi. 1997. Voltage-dependent gating characteristics of the K^+ channel KAT1 depend on the N- and C-termini. *Proc. Natl. Acad. Sci. USA.* 94:3448–3453.

Marten, I., and T. Hoshi. 1998. The N-terminus of the K chanel KAT1 controls its voltage-dependent gating by altering the membrane electric field. *Biophys. J.* 74:2953–2962.

Müller-Röber, B., J. Ellenberg, N. Provart, L. Willmitzer, H. Busch, D. Becker, P. Dietrich, S. Hoth, and R. Hedrich. 1995. Cloning and electrophysiological analysis of KST1, an inward rectifying K^+ channel expressed in potato guard cells. *EMBO J.* 14:2409–2416.

Nakamura, R. L., W. L. McKendree, Jr., R. E. Hirsch, J. C. Sedbrook, R. F. Gaber, and M. R. Sussman. 1995. Expression of an *Arabidopsis* potassium channel gene in guard cells. *Plant Physiol.* 109:371–374.

Pape, H.-C. 1996. Queer current and pacemaker: the hyperpolarization-activated cation current in neurons. *Annu. Rev. Physiol.* 58:299–327.

Perozo, E., and F. Bezanilla. 1990. Phosphorylation affects voltage gating of the delayed rectifier K^+ channel by electrostatic interactions. *Neuron.* 5:685–690.

Perozo, E., D. S. Jong, and F. Bezanilla. 1991. Single channel studies of the phosphorylation of K+ channels in the squid giant axon. II. Nonstationary conditions. *J. Gen. Physiol.* 98:19–34.

Rosenmund, C., and G. L. Westbrook. 1993. Calcium-induced actin depolymerization reduces NMDA channel activity. *Neuron.* 10:805–814.

Ruppersberg, J. P., M. Stocker, O. Pongs, S. H. Heinemann, R. Frank, and M. Koenen. 1991. Regulation of fast inactivation of cloned mammalian IK(A) channels by cysteine oxidation. *Nature.* 352:711–714.

Santoro, B., S. G. Grant, D. Bartsch, and E. R. Kandel. 1997. Interactive cloning with the SH3 domain of N-src identifies a new brain specific ion channel protein, with homology to eag and cyclic nucleotide-gated channels. *Proc. Natl. Acad. Sci. USA.* 94:14815–14820.

Santoro, B., D. T. Liu, H. Yao, D. Bartsch, E. R. Kandel, S. A. Siegelbaum, and G. R. Tibbs. 1998. Identification of a gene encoding a hyperpolarization-activated pacemaker channel of brain. *Cell.* 93:717–729.

Schachtman, D. P., J. I. Schroeder, W. J. Lucas, J. A. Anderson, and R. F. Gaber. 1992. Expression of an inward-rectifiying potassium channel by the *Arabidopsis* KAT1 cDNA. *Science.* 258:1654–1658.

Sheppard, D. N., and M. J. Welsh. 1999. Structure and function of the CFTR chloride channel. *Physiol. Rev.* 79:23–45.

Spellberg, R. D. 1971. Familial sinus node disease. *Chest.* 60:246–251.

Very, A. A., C. Bosseux, F. Gaymard, H. Sentenac, and J. B. Thibaud. 1994. Level of expression in *Xenopus* oocytes affects some characteristics of a plant inward-rectifying voltage-gated K^+ channel. *J. Physiol. (Lond.).* 428:422–424.

Zei, P. C., and R. W. Aldrich. 1998. Voltage-dependent gating of single wild-type and S4 mutant KAT1 inward rectifier potassium channels. *J. Gen. Physiol.* 112:679–713.