Na⁺ Activation of the Muscarinic K⁺ Channel by a G-Protein–independent Mechanism

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ABSTRACT Muscarinic potassium channels (KAC₆) are composed of two subunits, GIRK1 and GIRK4 (or CIR), and are directly gated by G proteins. We have identified a novel gating mechanism of KAC₆, independent of G-protein activation. This mechanism involved functional modification of KAC₆ which required hydrolysis of physiological levels of intracellular ATP and was manifested by an increase in the channel mean open time. The ATP-modified channels could in turn be gated by intracellular Na⁺, starting at ~3 mM with an EC₅₀ of ~40 mM. The Na⁺-gating of KAC₆ was operative both in native atrial cells and in a heterologous system expressing recombinant channel subunits. Block of the Na⁺/K⁺ pump (e.g., by cardiac glycosides) caused significant activation of KAC₆ in atrial cells, with a time course similar to that of Na⁺ accumulation and in a manner indistinguishable from that of Na⁺-mediated activation of the channel, suggesting that cardiac glycosides activated KAC₆ by increasing intracellular Na⁺ levels. These results demonstrate for the first time a direct effect of cardiac glycosides on atrial myocytes involving ion channels which are critical in the regulation of cardiac rhythm.

KEY WORDS: KAC₆ • Na⁺-activated K⁺ channel • phosphorylation • cardiac glycosides • arrhythmias

INTRODUCTION

The cardiac muscarinic potassium (K⁺) channel (KAC₆) was the first effector shown to be directly affected by βγ subunits of heterotrimeric GTP-binding (G) proteins (Logothetis et al., 1987). Using immunoprecipitation techniques, KAC₆ was recently shown to be composed of two subunits, GIRK1 (Dascal et al., 1993; Kubo et al., 1993) and GIRK4 (or CIR; Krapivinsky et al., 1995). Moreover, heterologous coexpression of the two subunits in Xenopus oocytes or mammalian cell lines produced large currents with biophysical properties identical to those of KAC₆ (Krapivinsky et al., 1995). A human GIRK4 clone (or KGP with 94% identity to the rat CIR clone), showed similar behavior upon heterologous coexpression with a human GIRK1, (99% identical to the rat GIRK1) (Chan et al., 1996). Homomeric assembly of either subunit alone was not sufficient to produce KAC₆ currents. GIRK4 channels gave rise to currents with properties distinct from those of KAC₆ (Krapivinsky et al., 1995; Chan et al., 1996), while GIRK1 required coassembly with a native oocyte “GIRK4-like” subunit (GIRK5 or XIR) in order to produce functional channels (Hedin et al., 1996).

Heteromeric assembly of the two subunits in oocytes not only reproduced the single-channel characteristics of KAC₆, but also dramatically enhanced both basal (in the absence of agonist) and agonist-induced currents equivalently (Chan et al., 1996). This enhancement of the basal currents was rather surprising, given that agonist is normally required to cause G-protein subunit activation and high levels of channel activity. In addition, channel activity was greatly reduced (run down) immediately upon membrane excision. If KAC₆ were to be gated only by direct G-protein subunit interactions, then could the basal activity, after patch excision, be fully restored by G-protein activation?

In the present study we addressed this question and identified cytosolic components which, when applied to an excised patch, could restore the cell-attached level of basal activity. Such channel activation required two steps. The first step involved channel modification to a distinct functional state, manifested by longer open-time kinetics. This mechanism of channel gating was found to be common to both native KAC₆, and its recombinant constituents expressed heterologously. Thus, in addition to its long-recognized direct G-protein activation, KAC₆ is also an Na⁺-activated K⁺ channel, exhibiting a novel requirement for gating, namely stabilization of a longer-lived open state by ATP hydrolysis, which en-
ables intracellular Na\textsuperscript{+} to further activate the channel, by limiting its residence in the closed state. This mechanism of K\textsubscript{ACb} channel activation was shown to be operative during intracellular Na\textsuperscript{+} accumulation, as with block of the Na\textsuperscript{+}/K\textsuperscript{+} pump, and is likely to be involved in the "direct" electrophysiologic effects of cardiac glycosides, drugs widely used in heart failure or for improvement of the inotropic state of the heart.

**MATERIALS AND METHODS**

**Expression of Recombinant Channels in Xenopus Oocytes**

Recombinant channel subunits (GIRK1 and GIRK4 [or KGP]) were expressed in Xenopus oocytes as previously described (Chan et al., 1996). Briefly, channel subunit coexpression was accomplished by coinjection of equal amounts of each cRNA (4 ng). cRNA concentrations were estimated from two successive dilutions which were electrophoresed in parallel on formaldehyde gels and compared to known concentrations of RNA marker (Gibco BRL, Gaithersburg, MD). Oocytes were isolated and microinjected as previously described (Logothetis et al., 1992). All the oocytes were maintained at 18°C, and electrophysiological recordings were performed 2-10 d after injection.

**Single-channel Recordings on Xenopus Oocytes**

To remove the vitelline membrane, oocytes were placed in a hypertonic solution (Stühmer, 1992) for 5-10 min. Shrunken oocytes were first transferred into a V-shaped recording chamber, then the vitelline membrane was partially removed, exposing just enough plasma membrane for access with a patch pipette. This procedure increased the success rate of forming gigaseals and reduced automatic detaching in long-term cell-attached recordings.

Single-channel activity was recorded in the cell-attached or inside-out patch configurations (Hamill et al., 1981; Methfessel et al., 1986), using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). All microelectrodes used in the experiments were pulled from WPK Borosilicate glass and gave resistances of 3-8 M\textOmega. All experiments were performed at room temperature (20-22°C). Single-channel recordings were performed at a membrane potential of -80 mV and in the absence of agonist in the pipette, unless otherwise indicated. Single-channel currents were filtered at 1-2 kHz with a 6-pole low-pass Bessel filter, sampled at 5-10 kHz and stored directly into the computer's hard disk through the Digidata 1200 interface (Axon Instruments). pClAMP (version 6.01, Axon Instruments) software was used for data acquisition and data analysis, complemented with our own analysis routine. Membrane patches containing multiple channels have been thus far of limited utility in accurately estimating single channel kinetic parameters, such as mean open time. Since our recordings were performed mostly from multichannel patches, we introduced a different method to accurately estimate single-channels parameters. Parameters used for single-channel analysis include activity of all the channels in the patch (or the total open probability, NPo), the opening frequency of all channels in the patch (NFo), and the mean open time (To). pClAMP analysis programs recognize current levels above pre-set threshold values, and count each current level as an event (Heinemann, 1995). For each discrete event, parameters such as the event level, duration, amplitude, etc. are measured and saved in an event-list file. Since it is the current level that defines an event, in records with multiple channels, a given opening may be counted more than once when it overlaps with openings from other channels. For example, two overlapping openings, one of which outlasts the other, would display three current levels and would be counted by pCLAMP as three events. Thus, such event information will not give an accurate account of either the number of openings involved or the duration of each opening. Since each single-channel opening involves one and only one step-up (or step-down) transition, the number of such transitions of adjacent levels within the time period of interest gives the exact number of channel openings and is a measure of opening frequency. In addition, summation of each event segment duration multiplied by the level value will give the total opening duration of all the channels involved within this time period. Therefore, the number of openings and the total time the channels spend in the open state can be accurately obtained, and single-channel activity and kinetic information can be derived from these values. Such analysis is completely independent of the number of channels in the patch and provides valuable and accurate information for channel activity and kinetic changes during the course of the experiment.

**Preparation of Chick Atrial Myocytes and Single-channel Recordings**

The procedure used for isolating cardiac myocytes from chick embryos was modified from that previously described (Clapham and Logothetis, 1988). Briefly, atrial tissue was selected from chick embryos from eggs incubated 14-19 d. Atrial tissue (from 4 to 6 eggs) was incubated for 20-30 min at 37°C in a 15-mL Falcon tube containing 5 ml of Mg\textsuperscript{2+}- and Ca\textsuperscript{2+}-free Hanks' solution supplemented with 1-2% of trypsin-EDTA solution (10X, Gibco BRL). Isolated myocytes were collected by triturating the digested tissue in 5 ml of trypsin-free solutions and stored at 4°C for up to 36 h. The cells were allowed to settle on polylysine-coated coverslips in the recording chamber before recording. Experimental solutions were the same as those used with oocyte recordings with the exceptions of using 140 mM KCl (for mammalian cells) instead of 96 mM (for Xenopus oocytes) and without gadolinium since native atrial stretch-activated channels did not present a problem.

**Single-channel Data Analysis**

Single-channel records were analyzed using pClAMP software, complemented with our own analysis routine. Membrane patches containing multiple channels have been thus far of limited utility in accurately estimating single channel kinetic parameters, such as mean open time. Since our recordings were performed mostly from multichannel patches, we introduced a different method to accurately estimate single-channels parameters. Parameters used for single-channel analysis include activity of all the channels in the patch (or the total open probability, NPo), the opening frequency of all channels in the patch (NFo), and the mean open time (To). pClAMP analysis programs recognize current levels above pre-set threshold values, and count each current level as an event (Heinemann, 1995). For each discrete event, parameters such as the event level, duration, amplitude, etc. are measured and saved in an event-list file. Since it is the current level that defines an event, in records with multiple channels, a given opening may be counted more than once when it overlaps with openings from other channels. For example, two overlapping openings, one of which outlasts the other, would display three current levels and would be counted by pCLAMP as three events. Thus, such event information will not give an accurate account of either the number of openings involved or the duration of each opening. Since each single-channel opening involves one and only one step-up (or step-down) transition, the number of such transitions of adjacent levels within the time period of interest gives the exact number of channel openings and is a measure of opening frequency. In addition, summation of each event segment duration multiplied by the level value will give the total opening duration of all the channels involved within this time period. Therefore, the number of openings and the total time the channels spend in the open state can be accurately obtained, and single-channel activity and kinetic information can be derived from these values. Such analysis is completely independent of the number of channels in the patch and provides valuable and accurate information for channel activity and kinetic changes during the course of the experiment.

Based on these principles, we wrote a Quick Basic (v. 4.5, Microsoft) analysis program which read the event-list file produced by pCLAMP and derived final estimates of NPo, NFo, and To. Among these three parameters, any one can be derived from the other two. In the data presented here, all three parameters were presented in the same figure only when they gave distinct visual information. All these values were plotted in histograms as means of each specified bin duration (normally 2 or 5 s). Implicit assumptions in this analysis were that all the channels in the patch were identical, independent of each other. There were two main limitations to this analysis method. The first one came from the limits of pCLAMP itself, which could only recognize up to five levels of the overlapping channel opening events. Highly active
patches containing more than five levels could not therefore be 
analyzed properly. In such cases, we integrated the total open 
current and calculated NPo values alone by dividing with the uni-
tary current amplitude. The second and more general limitation 
was inherent in the assumption that the probability of an open-
ing transition immediately followed by another opening with no 
time gap was zero. In reality, due to the limited resolution of our 
recording system and the use of filtering, very short separations 
of two such events might have been ignored and treated as a sin-
gle event. Therefore, the results would tend to underestimate the 
opening frequency and overestimate the mean open time. As the 
activity of the channels increases, the error increases as well. Our 
data were normally sampled at 5-10 kHz, therefore, single chan-
nel events shorter than 100 μs might be overlooked, forming a 
source of such errors. However, being fully aware of these limita-
tions, we feel confident that the large and consistent changes in 
our estimates of these three parameters lie outside such analysis 
errors.

**RESULTS**

High Basal Channel Activity Depends on the Presence of 
Cytosolic Components: ATP, Mg²⁺, and Na⁺

Heterologous co-expression of the K⁺ channel subunits 
GIRK1 and GIRK4 (CIR or KGP) in *Xenopus* oocytes has 
been shown to result in channel activity with biophysical 
properties identical to those of atrial KᵦCₐ (Krapivinsky 
et al., 1995; Chan et al., 1996). Basal currents of hetero-
logously expressed channels were much larger than 
those encountered in atrial membranes, presumably 
due to differences in the density of the expressed chan-
nels recorded. Fig. 1 A shows a typical recording (n > 
20) from an oocyte heterologously expressing the hu-
man homologs of GIRK1 and GIRK4 (Chan et al., 1996). 

![Figure 1](image-url)
Inwardly rectifying basal channel activity was high during the cell-attached recording in solutions containing 96 mM K\(^+\) in both the pipette and the bath. Upon excision into an inside-out patch, channel activity decreased to much lower levels (run down). Neither 100 \(\mu\)M GTP nor 10 \(\mu\)M GTP\(\gamma\)S applied to the intracellular surface of the patch restored channel activity. However, when the patch was crammed (Kramer, 1990) back into the oocyte, activity returned to levels similar to those during the cell-attached recording. This rescue of channel activity by patch-cramming was achieved regardless of whether the patch was inserted into the oocyte from which it was obtained \((n > 20)\) or into a non-injected oocyte \((n = 5, \text{data not shown})\). The results of these experiments suggested that soluble components in the oocyte cytosol were critical for the high basal channel activity.

Membrane excision of inside-out patches into a bath solution containing 5 mM MgATP and 20 mM NaCl prevented a decrease in channel activity \((N_{Po})\) such as that seen in Fig. 1 \(A\) (Fig. 1 \(B; n = 5\)). Withdrawal of MgATP and Na\(^+\) from the bath resulted in a decrease of activity with a time course similar to that seen in Fig. 1 \(A\).

Comparison of the single-channel properties in either the cell-attached (Fig. 2 \(A\)) or the inside-out (in the presence of MgATP and Na\(^+\), Fig. 2 \(B\)) modes revealed similar kinetics, current amplitudes, as well as inwardly rectifying properties. Amplitude histograms at \(-80\) mV revealed no change in unitary current between the
cell-attached (−2.47 ± 0.07 pA; n = 13) (Fig. 2 C) and the inside-out patch (−2.40 ± 0.06 pA; n = 13) (Fig. 2 D) configurations. Similar control experiments with noninjected oocytes at the cell-attached or inside-out patch configurations using up to 20 mM internal NaCl confirmed that the channel properties described above were specific to oocyte membranes expressing the recombinant channels (n > 10, data not shown), and did not reveal any native Na⁺-activated conductance. However, in agreement with Egan and colleagues (1992a), at higher internal NaCl concentrations (100 mM) we also observed an endogenous ~95 pS Na⁺-activated K⁺ conductance displaying low activity (NPo = 0.024 ± 0.016, mean ± SEM in 3 out of 11 patches tested) with no dependence on intracellular ATP.

These experiments demonstrated that the presence of intracellular MgATP and Na⁺ were sufficient to prevent run down of the channel activity, suggesting their involvement in maintaining the high basal activity in cell-attached recordings.

Gating of ATP-modified Channels by Intracellular Na⁺

We proceeded to investigate the mechanism by which intracellular MgATP and Na⁺ affected channel activity. Fig. 3 shows an experiment (n > 10) in which only the combined presence of MgATP and Na⁺ was able to restore excised-patch channel activity to levels comparable to those during the cell-attached mode. Channel activity was high during the cell-attached recording showing high frequency of channel opening (NPo) and mean open times (To) ranging between 3–4 ms. Upon excision into MgATP and Na⁺-free solutions, channel opening frequency decreased to low levels and the channel mean open time decreased to ~1 ms. 20 mM NaCl or 1.3 mM MgCl₂ applied individually (Fig. 3) or in combination (data not shown) had little effect on channel activity or opening frequency and no effect on channel mean open time. Application of 5 mM MgATP showed only a small increase in channel activity and opening frequency but restored mean open time to levels comparable to those during the cell-attached recording. This modification to the longer-lived open time required 2–5 min to reach steady state. We will refer to the channels in the increased mean open-time state, induced by MgATP, as being in the ATP-modified state. Once channels had been ATP modified, application of intracellular Na⁺ could cause a rapid increase in channel activity to levels comparable to the cell-attached recording (depending on the Na⁺ concentration, see below). This increase in activity was the result of an increase in channel opening frequency with no further change in the mean open time. Simultaneous removal of MgATP and Na⁺ decreased activity and opening frequency rapidly while mean open time slowly returned to a value comparable to that before ATP modification (not shown). These results indicated that the Na⁺ gating effect required prior ATP modification to a longer-lived open state (To), whereas Na⁺ reduced residence in the closed state of the channel, thus increasing channel opening frequency (NPo).

Functional Modification of the Channel Requires Hydrolysis of Physiological Levels of Intracellular ATP

Fig. 4 A shows a typical experiment (n = 7) in which increasing doses of millimolar ATP concentrations were applied intracellularly in the continuous presence of 1 mM free Mg²⁺ and 10 mM Na⁺. As can be readily appreciated, the ATP-induced functional modification of the channel, manifested by an increase in the mean open time, progressed as a time-dependent process that reached maximal values at physiological intracellular concentrations of ATP. Further increases in ATP

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Functional modification of recombinant KAC₉ channels by hydrolyzable ATP. (A) NPo and To plots of KAC₉ channel records from an oocyte expressing human GIRK1 and GIRK4 (Vm = −80 mV). Concentrations of Na⁺ and free Mg²⁺ were kept constant (10 and 1.0 mM, respectively). Application of increasing millimolar concentrations of ATP are illustrated by the bars. (B) NPo and To plots of channel records from an oocyte expressing GIRK4/GIRK1. The nonhydrolyzable ATP analogue AMP-PNP was perfused at the same concentration (i.e., 2 mM) as ATP (in the continuous presence of Na⁺ (10 mM) and free Mg²⁺ (1.0 mM)).
concentrations did not produce additional changes in the mean open time. However, experiments in which high ATP concentrations were used showed accelerated modification rates to the same maximal mean open time (data not shown).

ATP modification of the channels was completely dependent on the presence of free Mg$^{2+}$ since, in the absence of Mg$^{2+}$, K$_A$ATP ($n = 28$) or Na$_A$ATP ($n = 16$) had neither an effect on mean open time (data not shown) nor could they lead to an increase in activity in the presence of Na$^+$ (20 mM).

We next tested the ability of a nonhydrolyzable analogue of ATP to cause channel modification. Fig. 4B shows a representative experiment ($n = 11$) where 2 mM AMP-PNP failed to cause channel activation or to change mean open time even in the presence of 20 mM Na$^+$ and 1 mM free Mg$^{2+}$. In contrast, hydrolyzable forms of ATP (at 2 mM) could reproducibly modify the channel and enable Na$^+$ gating. These results, together with the requirement of the Mg$^{2+}$ presence for channel modification, indicate that hydrolysis of ATP is required for the functional modification of the channel to the longer-lived open state. Simultaneous withdrawal of ATP and Na$^+$ led to a rapid decline in channel activity and opening frequency but a slower reversal of the mean open time. Reapplication of ATP and Na$^+$ resulted in a similarly slow increase in activity and mean open times, indicating that the ATP-induced channel modification had been mostly reversed, in the mere absence of ATP, and could recur as before. Withdrawal of ATP, in the continuous presence of Na$^+$, showed a much slower reversal in activity and mean open time than the simultaneous withdrawal of ATP and Na$^+$. The slow reversal of the modification process was kinetically similar to that of the modification process itself, suggesting that Na$^+$ could gate open those channels for which the ATP modification was still in effect. Thus, channel modification, rather than the presence of ATP, was the prerequisite for Na$^+$-gating of KACh.

These results, together with those shown in the previous figure, where Na$^+$ failed to cause channel activity when applied prior to modification, further support the assertion that the ATP-dependent channel modification leads the channel into an Na$^+$-sensitive state.

**Gating of ATP-modified Channels Is Initiated at Physiological Concentrations of Na$^+$**

In the experiment of Fig. 5A ($n = 15$), increasing doses of Na$^+$ were applied intracellularly in the maintained presence of 2 mM MgATP. Evidence for steady ATP modification of the channel was manifested by open times in the range of 3–5 ms. Under these conditions Na$^+$ ions could rapidly increase the activity of the modified channels, with an apparent threshold for activation in the 3–10 mM range. The dose-response relationship of the Na$^+$-mediated activity could be fit by a Hill curve (Fig. 5B), showing an EC$_{50}$ of 41 mM and a Hill coefficient of $\sim$2.1, suggesting that at least two Na$^+$ may bind to an ATP-modified heteromeric channel. Na$^+$-mediated activity of non-ATP-modified recombinant channels with 100 mM intracellular Na$^+$ was $<0.1$% of the corresponding stimulation of ATP-modified activity, as determined in the same patches ($n = 3$; data not shown). Given the conductance and probability of opening of endogenous Na$^+$-activated K$^+$ channels

![Figure 5. Dose-dependent activation of the recombinant KACh channel by Na$^+$. (A) NPo, NFo, and To plots of single-channel records in an inside-out patch ($V_m = -80$ mV) from an oocyte injected with human GIRK1 and GIRK4 cRNAs. MgATP concentration was kept constant in the bath at 2 mM. Application of increasing concentrations of Na$^+$ are illustrated by the bars. Activity changes became apparent between 3 and 10 mM Na$^+$. (B) Dose-response plot for activation of the recombinant KACh channels by Na$^+$. Data points were plotted as mean $\pm$ SEM ($n = 8$), and NPo values were normalized for each experiment individually. The dotted line represents the best fit of the data to the equation:

$$
NPo = B + (A - B)/\left[1 + ([Na^+] / EC_{50})^n\right],
$$

with the following values: offset A = 0.09 $\pm$ 0.03; maximum B = 1.18 $\pm$ 0.05; EC$_{50}$ = 41.43 $\pm$ 4.60, Hill coefficient n = 2.07 $\pm$ 0.40; chi$^2$ = 0.0016.
(0.024 × 3/11 = 0.006), their presence in the highly active patches in 100 mM intracellular Na⁺ could not account for >0.1% of the channel activity seen. In addition experiments performed in the same patches using different salts of Na⁺ (e.g., NaCl, n > 50; Na-Acetate, n = 12; Na₃ATP, n > 10) showed no differences in activating the channel.

These experiments indicate that physiologically relevant intracellular levels of ATP (2–5 mM) and Mg²⁺ (1 mM) are sufficient to maintain the channel in an Na⁺-sensitive state, where changes in intracellular Na⁺ levels (5–30 mM) would bring about significant activity changes in K⁺ channel activity.

In experiments where 20 mM Li⁺ was compared to Na⁺ (in the same patches) in its ability to activate the channel, it was found that Li⁺ was 11.2 ± 3.2% as effective as Na⁺ (n = 5, data not shown).

**ATP Modification and Na⁺ Gating of Native Atrial K⁺ Channels**

We proceeded to test the presence of this novel gating mechanism of K⁺ channel in chick embryonic atrial cells. In the experiment of Fig. 6 A (as in 4 out of 4 patches) atrial membranes showed very low basal (i.e., in the absence of agonist) level of K⁺ channel activity as compared to the respective oocyte patches. After patch excision, co-application of 5 mM MgATP and 20 mM NaCl caused a gradual increase in the channel open time and a concomitant increase in the channel activity, in a manner similar to that seen with the recombinant channel subunits in the oocyte membranes. Removal of intracellular Na⁺ in the maintained presence of MgATP revealed that although activity decreased instantly to much lower levels, the mean open times were

![Diagram](http://example.com/diagram.png)
Block of the Na+ /K+ Pump Activates K_ACh

Since high levels of intracellular Na+ increase K_ACh activity, we aimed to raise the intracellular Na+ concentration by blocking the Na+/K+ pump and monitor effects on K_ACh. Direct measurements of intracellular Na+ activity using Na+-sensitive glass microelectrodes has shown that K+-free solutions increased Na+ activity in a reversible manner and with kinetics that depended on the extracellular K+ concentration (0-4 mM/min change in Na+ activity between 0 and 12 mM extracellular K+; Deitmer and Ellis, 1978). Fig. 7 A shows a cell-attached recording (n = 7) in which a chick embryonic atrial cell was bathed in 20 mM K+ and 140 mM Na+. Basal K_ACh activity was low but open-time duration was long (2-4 ms), indicative of K_ACh being in the Na+-sensitive state presumably due to the presence of intracellular Mg2+ and ATP. Replacement of the 20 mM K+ with a K+-free solution, expected to block the Na+/K+ pump, caused a gradual activation of K_ACh, similar in kinetics to that shown for accumulation of internal Na+ (Deitmer and Ellis, 1978). K_ACh activation involved changes in the frequency of channel opening rather than in mean open time. Alternatively, we blocked the Na+/K+ pump using the cardiac glycoside ouabain. Fig. 7 B shows a similar cell-attached recording (n = 5) as that of Fig. 7 A, but with bath application of 0.5 mM ouabain in the maintained external presence of 20 mM K+ and 140 mM Na+. Ouabain block of the Na+/K+ pump (Gadsby and Nakao, 1989) caused significant activation of K_ACh. Again, the effect was due to changes in the frequency of opening rather than mean open time. Block of the Na+/K+ pump caused K_ACh activation indistinguishable from that by Na+. Recovery from block of the Na+/K+ pump was quite variable. Thus, these results are consistent with the interpretation that block of the Na+/K+ pump caused accumulation of intracellular Na+ which in turn activated K_ACh by the novel mechanism we have described. K_ACh activation by Na+/K+ pump block demonstrates for the first time a direct effect of cardiac glycosides on atrial myocytes involving an ion channel critical in the regulation of cardiac rhythm.
We have identified and described a novel gating mechanism of $K_{\text{AC}}$, independent of direct G-protein gating. This mechanism involves two processes: first a modification of the functional state of the channel which depends on ATP hydrolysis, and secondly a subsequent gating of the ATP-modified channel by $Na^+$. Block of the $Na^+ / K^+$ pump activated $K_{\text{AC}}$ in a manner indistinguishable from that of $Na^+$ activation. Activation of $K_{\text{AC}}$ by cardiac glycosides provides the first example of a "direct" effect of these drugs on atrial myocytes.

**Mg$^{2+}$-dependent ATP Modification of $K_{\text{AC}}$**

ATP modification of open-time kinetics of the rat atrial $K_{\text{AC}}$ channel during G-protein activation has been reported previously (Kim, 1991). Kim showed that intracellular ATP and free Mg$^{2+}$, in the continued presence of GTP (and external ACh), caused an increase in rat $K_{\text{AC}}$ channel activity and a progressive increase in the channel mean open times from 1 to 5 ms, while the nonhydrolyzable ATP analogue, AMP-PNP, failed to do the same. Earlier work had reported $K_{\text{AC}}$ activation by ATP (or ATP$\gamma$S which can serve as a substrate for kinases) and free Mg$^{2+}$ (Otero et al., 1988; Heidbüchel et al., 1990; Kaibara et al., 1991) but not by AMP-PNP. Yet, later attempts in guinea pig atrial cells failed to reproduce the effects reported by Kim (reviewed by Kurachi et al., 1992).

Using our modified analysis method (see experimental procedures) we were able to monitor continuously the activity, opening frequency, and mean open time in membrane patches containing multiple channels, throughout the length of the experiment. Our results showed that intracellular ATP in the presence of Mg$^{2+}$ functionally modified recombinant heteromeric channels expressed in oocytes as well as native $K_{\text{AC}}$ channels of chick embryonic atrial cells. The kinetics of the modification process took 2-5 min to reach steady state (depending on the ATP concentration). The dependence on Mg$^{2+}$, the necessity of hydrolyzable forms of ATP for functional modification of $K_{\text{AC}}$, and the slow kinetics of the effect have been suggestive of a phosphorylation process. Involvement of a nucleotide diphosphate kinase (NDPK) has been suggested previously, but direct evidence for involvement of a specific kinase has not been presented. Similarly, withdrawal of ATP results in reversal of the functional modification effect of $K_{\text{AC}}$, with similar slow kinetics, suggestive of a dephosphorylation process. Again, no direct evidence implicating dephosphorylation or a specific phosphatase has been presented.

**Na$^+$ Gating of ATP-modified $K_{\text{AC}}$ Channels**

We have shown $K_{\text{AC}}$ activation by intracellular Na$^+$ in a manner independent of G-protein activation. Na$^+$ gating of $K_{\text{AC}}$ required prior channel modification by a Mg$^{2+}$-dependent process that involved hydrolysis of ATP. We were clued into Na$^+$ gating of $K_{\text{AC}}$, by noticing that in the presence of free Mg$^{2+}$, sodium salts of ATP caused greater channel activation than other ATP salts. Na$^+$ gating of $K_{\text{AC}}$ was independent of the specific sodium salt used and in this respect was not related to the halide dependence of G-protein–mediated activation of $K_{\text{AC}}$ (Nakajima et al., 1992). Na$^+$-activated K$^+$ channels have been described previously in cardiac myocytes and neurons which, unlike $K_{\text{AC}}$, show large single-channel conductance and require high millimolar concentrations of intracellular Na$^+$ for half-maximal activation (Dryer, 1994). The large conductance cardiac Na$^+$-activated K$^+$ channels, like $K_{\text{AC}}$, display considerable inward rectification as compared to their neuronal counterparts (Kameyama et al., 1984; Dryer, 1994). Na$^+$-activated K$^+$ channels of cultured neurons of the rat olfactory bulb, unlike $K_{\text{AC}}$, show a large 170-pS conductance with multiple subconductance levels (in symmetrical 150 mM K$^+$), but like $K_{\text{AC}}$, they require lower Na$^+$ concentrations for half-maximal activation (40–80 mM) and they run down within minutes after excision (Egan et al., 1992b). By comparison, $K_{\text{AC}}$ channels show predominantly a 40-pS conductance; they require prior Mg$^{2+}$-dependent modification by hydrolyzable forms of ATP in order to be gated by intracellular Na$^+$; and they run down after patch excision but activity can be fully restored in the combined presence of MgATP and Na$^+$.

Neuronal recombinant G-protein–activated inwardly rectifying K$^+$ channels, GIRK2, coexpressed with GIRK1 (Lesage et al., 1994) have also been shown to be sensitive to high concentrations of intracellular Na$^+$. Activation of these heteromeric channels by ATP or even AMP-PNP showed a synergistic effect with intracellular Na$^+$. Although clear differences exist between $K_{\text{AC}}$ and other Na$^+$-activated K$^+$ channels, the similarities are rather enticing. Are some of the common functional features between $K_{\text{AC}}$ and other Na$^+$-activated K$^+$ channels due to structural similarities in their subunit composition? Is the functional modification seen with $K_{\text{AC}}$ a determining factor for sensitivity of Na$^+$ gating for other Na$^+$-activated K$^+$ channels as well?

**Significance of the Novel Na$^+$ gating Mechanism in $K_{\text{AC}}$ Function**

Kurachi and colleagues showed, using whole-cell recordings on atrial myocytes, that applications of $\sim$10 $\mu$M ACh caused rapid activation of $K_{\text{AC}}$, which decreased gradually to a steady level (Kurachi et al., 1987). The desensitization kinetics consisted of a rapid (<1 min) and a slow phase (4-5 min). Moreover, GTP$\gamma$S-loaded atrial cells developed currents at the desensi-
tized level of activity, thus making the involvement of receptor-mediated desensitization unlikely. In isolated patches the slow desensitization phase has been correlated with a similarly time-dependent decrease in open time after patch excision (Kim, 1991), but the rapid desensitization phase has not been reproduced at the single-channel level. Given these data Kurachi and colleagues have hypothesized that some unknown soluble factor may be necessary for the initial rapid activation of KAC₄ current which could be lost in the isolated patches (Kurachi et al., 1992). We have shown that cytosolic factors, such as ATP, Mg²⁺, and Na⁺ are involved in ATP-modification and efficient gating of KAC₄, in the absence of direct G-protein activation. ATP modification and intracellular Na⁺ result in a clear increase in mean open-time duration and KAC₄ activity, whereas removal of ATP results in the reversal of this process, which resembles changes in open-time kinetics during desensitization. Is it possible that competitive interactions of this novel KAC₄ gating mechanism with that of direct G-protein gating could be involved in desensitization? Detailed studies examining such questions and possible interactions between the two gating pathways will be necessary to determine their significance when both are operative.

The mechanisms of gating KAC₄ channels to produce high basal activity in heterologous expression systems are not well understood. Our data strongly suggests that physiologic concentrations of Na⁺, Mg²⁺, and ATP are involved in producing the high basal currents of the human recombinant channel subunits, GIRK₁ and GIRK₄. We have shown previously that pertussis toxin (PTX) treatment of oocytes expressing these recombinant subunits caused partial reduction of the basal current while it abolished agonist-induced current (Chan et al., 1996). The relative contribution of each mechanism (i.e., G-protein- versus Na⁺-activation) to the total basal activity remains to be determined.

In atrial cells, we found that when the Na⁺/K⁺ pump is operative (e.g., in the presence of external K⁺), basal activity is low. In contrast, block of the Na⁺/K⁺ pump causes KAC₄ channel activation presumably due to intracellular Na⁺ accumulation. Two lines of evidence support this interpretation. First the rise in intracellular Na⁺ due to block of the pump (by replacement to K⁺-free external solutions) showed similar kinetics (Deitmer and Ellis, 1978) to that of KAC₄ activation. Secondly, KAC₄ activation by block of the Na⁺/K⁺ pump was indistinguishable from that of intracellular Na⁺, involving an increase in channel frequency of opening and no change in the mean open time duration. Cardiac glycosides, such as digitalis, have long been known to affect cardiac rhythmicity, particularly of supraventricular structures including the sinoatrial node, the atrial myocardium, and the atrioventricular node. The cardiac effects of digitalis are concentration dependent and are often referred to as “direct” (acting on cardiac tissues themselves) and “indirect” (acting through increasing activity of the parasympathetic nervous system) (Watanabe, 1985). Although direct effects of digitals have been clearly demonstrated with regard to cardiac inotropy, direct chronotropic effects have been ill defined. Our results demonstrate for the first time that cardiac glycosides exert direct effects on atrial myocytes through activation of KAC₄, an ion channel critical in the control of cardiac rhythm.

Identification of this novel gating mechanism of KAC₄ implies that changes in intracellular Na⁺ concentration in either physiological or pathological conditions could be linked to activation of this channel and thus alteration of atrial membrane excitability.

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vated K+ channels are widely distributed in rat CNS and in *Xenopus* oocytes. Brain Res. 584:319–321.


