Mechanism of Rapid Desensitization of Muscarinic K⁺ Current in Adult Rat and Guinea Pig Atrial Cells

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Acetylcholine (ACh) activates the muscarinic K⁺ current in atrial cells via the inhibitory GTP binding protein. After activation, the whole-cell K⁺ current decreases rapidly (rapid desensitization) to approximately half of the initial current within ~20 seconds. The mechanism of this rapid desensitization was investigated in adult rat and guinea pig atrial cells. Whole-cell voltage-clamp and patch-clamp techniques were used to study the K⁺ current. In voltage-clamped whole cells, ACh activated a K⁺ current that desensitized rapidly during the initial ~20 seconds followed by a slower decrease over several minutes. The rapid K⁺ current desensitization (a rapid decrease in channel open probability) was also observed at the single-channel level in cell-attached patches and was associated with a progressive shortening of the channel open time and prolongation of the closed time. These changes in channel current and kinetics were abolished by removal of the cytoplasm (by forming inside-out patches) and were partially inhibited by phosphatase inhibitors, suggesting an involvement of cytosolic phosphatase(s) in K⁺ current desensitization. In inside-out patches with ACh in the pipette and GTP in the bath, the open time of muscarinic K⁺ channels and channel open probability were increased by 1 mM Mg²⁺-ATP (but not by the nonhydrolyzable analogue, adenylylimidodiphosphate) and decreased by alkaline phosphatase. These results suggest that the rapid K⁺ current desensitization in adult rat or guinea pig atrial cells is produced by changes in the gating kinetics of the K⁺ channel, possibly mediated via membrane-associated protein kinase and cytosolic phosphatase(s). (Circulation Research 1993;73:89-97)

KEY WORDS • muscarinic K⁺ channel • desensitization • Mg²⁺-ATP • phosphorylation • rats • guinea pigs • atrial cells • patch clamp

It is well known that acetylcholine (ACh) binds to muscarinic receptors in the atrial cell membrane and elicits a sequence of intracellular events that leads to a reduction of the heart rate. One mechanism by which ACh reduces the heart rate may be the reduction of the hyperpolarization-activated (pacemaker) current (Iₑ), which has been found in sinoatrial nodal cells. Moreover, another mechanism for slowing of the heart rate could be the activation of the muscarinic K⁺ current (Iₑ,ACh), which causes hyperpolarization of the atrial cell membrane by increasing K⁺ conductance. A recent study showed that ACh affected Iₑ at concentrations lower than that required to affect Iₑ,ACh; however, the function of each current under physiological conditions in different types of tissues has not been fully established. Since both Iₑ and Iₑ,ACh are present in sinoatrial nodal cells, it seems reasonable to hypothesize that both are involved in the regulation of the heart rate. The effect of ACh on Iₑ has been reported to be stable and show no desensitization, whereas the effect of ACh on Iₑ,ACh has been reported to show rapid desensitization. Thus, the “fade” of the ACh effect on heart rate and K⁺ current is likely to be due to desensitization of Iₑ,ACh.

Previous studies have shown that the K⁺ current activated by ACh is quickly desensitized in a biphasic manner: a fast early phase that lasts ~20 seconds and a slower phase that lasts for many minutes. Although the kinetic properties of the muscarinic K⁺ channel have been studied for almost a decade by many investigators, the mechanism of the rapid phase of desensitization has not been fully elucidated. In neonatal rat atrial cells, it was shown that the rapid desensitization of Iₑ,ACh was associated with alterations in the K⁺ channel open and closed times during the initial 20 seconds. However, it is not known whether the same phenomenon is present in adult atrial cells, which have much higher density of Iₑ,ACh in the cell membrane and also have a higher basal K⁺ channel activity in the absence of ACh than do neonatal cells. In neonatal rat atrial cells, muscarinic K⁺ channel activity was increased by Mg²⁺-ATP, and this was associated with an increased channel open time. Similar effects of Mg²⁺-ATP on K⁺ channel function were not reported in adult rabbit or frog atrial cells. In adult guinea pig or frog atrial cells, Mg²⁺-ATP applied to the cytoplasmic face of the membrane was reported to activate Iₑ,ACh via nucleoside diphosphate kinase; the mean open time of Iₑ,ACh was increased slightly from 1.8 to 2.5 msec by Mg²⁺-ATP. Thus, the modulation of the muscarinic K⁺ channel by second messengers may be different in adult and neonatal atrial cells. In this study, the mechanism of the rapid phase of Iₑ,ACh desensitization was investigated in adult rat and guinea pig atrial cells. The role of membrane-associated

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and cytosolic factors that are involved in $I_{K,AC}$ desensitization was examined.

**Methods and Materials**

**Cell Preparation**

Single atrial cells of adult rat and guinea pig heart (250 to 350 g) were prepared by enzymatic digestion as described previously. Animals were anesthetized with ether, thoracic cavities were open, and hearts were removed. Hearts were retrogradely perfused via the aorta in a Langendorff apparatus with 0.05% collagenase (type II, Worthington Biochemical Corp, Freehold, NJ) and 0.03% hyaluronidase (Sigma Chemical Co, St Louis, Mo) in Ca$^{2+}$-free bicarbonate-buffered physiological solution for 45 minutes. Tissues were then cut into small pieces and mechanically dissociated into single cells. Cells were washed several times with well-oxygenated buffer solution and kept at room temperature (=24°C) in oxygenated (95% O$_2$, 5% CO$_2$) atmosphere. All cells used for experiments were of elongated shape with no visible sign of contracture or blebs. Bicarbonate-buffered solution contained (mM) NaCl, 118; KCl, 4.0; MgSO$_4$, 1.2; KH$_2$PO$_4$, 1.2; NaHCO$_3$, 25; CaCl$_2$, 1; and glucose, 10. All cells were used within 6 hours.

**Electrophysiology**

Gigaseals were formed with Sylgard-coated borosilicate glass pipettes (Kimax) with 1- to 2-MΩ (whole cells) or 4- to 5-MΩ (patches) resistances, and channel currents were recorded using the method described by Hamill et al. In whole-cell studies, the series resistance was compensated (80%) to minimize the voltage drop across it. Channel currents were recorded with an Axopatch 1D patch-clamp amplifier, low-pass-filtered at 5 kHz using an eight-pole Bessel filter (model 902-LPF, Frequency Devices Inc, Haverhill, Mass), and stored on magnetic tape via a digital data recorder (Instrutech Corp, Elmont, NY). Later, digitized data were entered into an Atari computer and analyzed to obtain duration and amplitude histograms and channel open probability (calculated by integrating the current through all channels divided by the total current that would pass through all channels if they were fully open). The multiple channel openings such as that shown below were automatically analyzed as three separate openings with an open time of 1 msec each, not as two-channel openings. Thus, the analysis of overlapping channel events in this way gave open times that represented the minimum possible values. Logarithmic (abscissa) and square-root (ordinate) scales were used to represent dwell-time distributions. Single-channel dwell times were plotted on a logarithmic time scale using binned maximum-likelihood fitting with constant logarithmic bin width (10 bins per decade). The 50% threshold method was used to determine the duration of channel openings. All experiments were performed at 24°C to 26°C. All values are represented as mean±SEM.

**Materials and Solutions**

ACH, potassium fluoride, zinc sulfate, and EGTA were purchased from Sigma. H-7 [1-(5-isoxoquinolino sulfonyl)-2-methylpyrazine dihydrochloride], ryanodine, and calphostin C were purchased from Calbiochem Corp, La Jolla, Calif. Sodium orthovanadate was purchased from Fisher. ATP, GTP, CTP, ITP, UTP, ADP, GDP, γ-S, adenylylimidophosphate (AMP-PPN), and calf liver alkaline phosphatase were purchased from Boehringer Mannheim Corp, Indianapolis, Ind. The standard bath and pipette solutions contained (mM) KCl, 140; MgCl$_2$, 2; and HEPES, 10 (pH 7.2). For whole-cell studies, cells were attached to the bottom of the glass coverslip and perfused at 15 mL/min to produce fast exchange of solutions. The perfusion chamber had a width of 2 mm and a length of 2 cm. Complete exchange of solution was accomplished within 500 msec with this setup. For studies using excised patches, the membrane patch at the tip of the pipette was brought to the mouth of a polypropylene tubing through which solutions flowed by gravity at ~2 mL/min. Exchange of solutions was accomplished by moving the pipette tip to the mouth of another tubing containing the desired solution.

**Results**

**Whole-Cell Currents Activated by ACh**

Atrial cells in the whole-cell configuration were held at ~40 mV to record inward K$^+$ current. GTP (100 μM) and ATP (1 mM) were added to the pipette solution. After ~2 minutes of perfusion with control solution and after a stable baseline current was achieved, cells were perfused with a solution containing 10 μM ACh. ACh elicited a rapid increase in K$^+$ current, and this was followed by a decrease in current in a biphasic manner. An initial rapid decrease lasted only for ~20 seconds and was followed by a more slow monophasic decrease that continued as long as ACh was present. The rapid phase of K$^+$ current desensitization accounted for ~40±8% (mean±SEM, n=7) of the total initial current in both rat and guinea pig atrial cells (Fig 1). Washout of ACh resulted in return of the K$^+$ current to basal level. If these cells were then perfused with the same concentration of ACh immediately after washout, little or no rapid phase of desensitization was observed. Washout of ACh for longer periods of time (>4 minutes) was necessary to cause resensitization. These changes in K$^+$ current produced by ACh were qualitatively similar in atrial cells from adult rat or guinea pig heart.

**Rapid K$^+$ Current Desensitization at the Single-Channel Level**

The presence of rapid K$^+$ current desensitization was examined at the single-channel level in cell-attached patches formed with 10 μM ACh in the pipette. When cell-attached patches were formed quickly, opening of several K$^+$ channels was usually present in a given patch at 80-mV pipette potential. The channel activity quickly decreased during the ~20-second period in every "healthy" atrial cell tested in rats and guinea pigs (Figs 2 and 3). Even with high-resistance pipettes, at least two channel openings were present in cell-attached patches, indicating the high density of $I_{K,AC}$ in adult atrial cells. To characterize the changes in channel kinetics during
this time, single-channel analyses were done on channel openings up to two levels. The open-time analysis program used here took into account the possible overlapping events and provided the minimum single-channel open time (see “Materials and Methods”).

Channel openings at various times after formation of a cell-attached patch are shown in Figs 2 and 3, which show changes in the kinetics of the K⁺ channel behavior. Single-channel analyses showed that the distribution of channel open times during the first 2 seconds could be fit with an exponential function having a time constant of 4.6±0.6 msec in rat (n=5) and 4.8±0.7 msec in guinea pig (n=4) atrial cells. The open time progressively decreased with time such that by 1 minute the distribution of open times could be fit with a single exponential with a time constant of 1.0±0.1 msec in rat and 1.1±0.1 msec in guinea pig atrial cells (Fig 4A). The mean closed time could not be determined accurately with the analysis program used here because of multiple channel openings. However, the total time the channels spent in the closed state increased significantly during the initial 20 seconds by 1.6±0.3-fold (n=5). Thus, the time-dependent decrease in channel activity (the rapid current desensitization) was closely associated with changes in open and closed times. In three patches, channel activity decreased from 0.122 to 0.046 in rat atrial cells, and from 0.082 to 0.028 in guinea pig atrial cells during the 100 seconds in the cell-attached patch (Fig 4B). When inside-out patches were formed from these cell-attached patches, channel activity decreased to basal levels (two or three openings every second) because of washout of GT. Addition of GT to the bath resulted in reactivation of the K⁺ channel. The mean open time of channels activated by GT was 1.1±0.1 msec for both rat and guinea pig atrial cells and remained at this level as long as GT was present. A slow decrease in channel activity was observed in these inside-out patches with time. Thus, the slow phase of desensitization was mainly due to a slow decrease in the frequency of channel opening without a change in the open time (data not shown). Therefore, two separate kinetic mechanisms appear to produce the rapid and slow K⁺ current desensitizations.

In cells that were preincubated with 10 μM ACh for 2 to 3 minutes, formation of cell-attached patches with pipette containing 10 μM ACh only showed channel openings with a mean open time of 1.0±0.1 msec during the initial 2-second period and remained relatively constant throughout (1.0±0.1 msec after 4 minutes, n=4). No rapid decrease in channel activity (rapid desensitization) was present in these cells. When cell-attached patches were formed after washing the cells for >4 minutes in ACh-free solution, the rapid desensitization of the single-channel currents could again be observed. In four cells, the mean open time of the channels activated by ACh during the first 2 seconds was 4.2±0.4 msec. These single-channel desensitization studies are in keeping with those of whole-cell current studies and further show that the changes in the kinetics of channel opening are not artifacts of patch formation.

**Role of Membrane-Associated and Cytoplasmic Factors in K⁺ Current Desensitization**

The gradual decrease in the open time in cell-attached patches during the initial 20 seconds of ACh exposure may be caused by factors in the membrane, cytoplasm, or both. To determine the involvement of membrane-associated or cytoplasmic factors in the changes in the kinetics of channel opening, the following experiments were performed. Cell-attached patches were formed quickly with ACh in the pipette. Within ~2 seconds (ie, before a significant rapid desensitization has occurred), inside-out patches were formed from same cells to wash off the cytoplasmic content. GT was present in the bath or subsequently added to the bath to cause activation of the K⁺ channel. Under these conditions, the muscarinic K⁺ channels showed a mean open time of 5.1±0.6 msec (Fig 5, tracing a; n=5), and this value remained relatively unchanged during the next 10 minutes (5.0±0.9 msec at 5 minutes; 4.8±0.6 msec at 10 minutes). In other cells in which inside-out patches were formed after ~2 minutes in the cell-attached state, addition of GT to the bath activated channels with a mean open time of ~1.0 msec (Fig 5, tracing b; also see Figs 2 through 4). These results support the view that cytoplasmic factors modulate the kinetics of the muscarinic K⁺ channel such that the channel mean open time is decreased during the rapid phase of desensitization.

ACh has been reported to cause release of Ca²⁺ from internal stores in different cell types including heart cells.10-21 Therefore, it is possible that ACh causes desensitization of I_{K\text{ACH}} via an increase in cytosolic [Ca²⁺]. The role of Ca²⁺ was tested by lowering intracellular [Ca²⁺] below 10 nM by dialyzing the cell interior with a pipette containing a solution buffered with 5 mM EGTA in the whole-cell configuration and then forming a cell-attached patch in the same cell with a pipette containing ACh. In these cells, ACh produced changes in K⁺ channel kinetics identical to those without intracellular buffering with EGTA; ie, a gradual shortening of open time from ~5 to ~1 msec was observed. In a separate group of cells, Ca²⁺ release from sarcoplasmic reticulum was prevented by treating the cells with 10 μM ryanodine.22 The rapid phase of K⁺ current desensitization due to the shortening of open time was still present in these cells, indicating that shortening of the channel open time was not caused by a possible increase in cytosolic [Ca²⁺].

**Effect of Mg²⁺-ATP on K⁺ Channel Kinetics**

An earlier study in neonatal rat atrial cells showed that muscarinic K⁺ channel kinetics could be modulated
by adding Mg$^{2+}$-ATP to the cytoplasmic surface membrane in the inside-out configuration. The hypothesis that Mg$^{2+}$-ATP can also affect K$^+$ channel kinetics in adult atrial cells was tested. After ~2 minutes in the cell-attached state with ACh in the pipette, inside-out patches were formed, and GTP (100 μM) was added to the bath to activate the K$^+$ channel (Fig 6). Under these conditions, the mean open time of channels activated by GTP was 1.0±0.1 msec ($n=5$) in rat atrial cells and 1.1±0.1 msec ($n=4$) in guinea pig atrial cells. Changing the concentration of bath GTP from 100 to 1 μM and back to 100 μM caused the channel activity to decrease and increase, respectively. The mean open time remained unchanged at ~1.0 to 1.1 msec throughout the procedure. However, further addition of 1 mM ATP to the bath caused a gradual increase in channel open time such that, at steady state (after ~1 minute), the mean open time was 4.3±0.4 msec ($n=5$) in rat and 5.2±0.7 msec ($n=4$) in guinea pig atrial cells (Fig 6). The increase in open time was associated with an increase in open probability, which increased from 0.032±0.016 to 0.116±0.032 in rat and from 0.016±0.006 to 0.073±0.024 in guinea pig atrial cells. Washout of GTP (with ATP still in the bath) resulted in closing of the channels, indicating that ATP was not activating another population of K$^+$ channels. In these experiments, GTP in the bath solution was washed out slowly, and this is reflected by the slow decrease in channel activity. After washing off ATP completely, readdition of GTP caused activation of channels with a mean open time of 5.5±0.8 msec in rat and 6.5±1.0 msec in guinea pig atrial cells, indicating that the effect of ATP was irreversible. AMP-PNP, a nonhydrolyzable analogue of ATP, failed to mimic the action of ATP (data not shown), suggesting that phosphorylation via a protein kinase may be involved in the modulation of the kinetics of K$^+$ channel opening in both species. Other nucleoside triphosphates or diphosphates such as CTP, ITP, UTP, and ADP failed to substitute for ATP in its action to modify channel open time and activity.

As described above in Fig 5, when inside-out patches were formed 1 to 2 seconds after formation of cell-attached patches in the presence of GTP in the bath, the open time of the K$^+$ channel remained prolonged. Further addition of ATP to the bath had no further effect on the open time (~2.1%) or channel activity (~3.3%), suggesting that the channels were already in the ATP-modified state. After washout of ATP, dia-

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**Fig 2.** Single-channel currents activated by acetylcholine (ACh) in a cell-attached patch from a rat atrial cell. Cell membrane potential was held at ~80 mV. Panel A: Tracings showing muscarinic K$^+$ channels activated by ACh in the pipette during ~2 minutes in the cell-attached patch. Formation of an inside-out patch caused the opening of ATP-sensitive K$^+$ channels, which closed spontaneously except for occasional openings. Bath application of GTP (100 μM) quickly reactivated the K$^+$ channels. Channel openings indicated by tracing a (~2 seconds after formation of seal; mean open time, 4.8 msec), tracing b (1 minute later; mean open time, 1.0 msec), and tracing c (inside-out patch with GTP; mean open time, 1.1 msec) are shown below with an expanded time scale. Panel B: Open-time histograms of channel openings shown in tracings a and c. The mean open times (4.8 msec and 1.1 msec) are indicated by the dotted lines intersecting the abscissa.17
Fig 3. Tracings showing single channels activated by acetylcholine (ACH) in a cell-attached patch from a guinea pig atrial cell. The experimental protocol is the same as that described in the legend to Fig 2. Channel openings observed at 2 seconds (tracing a; mean open time, 4.9 msec), 5 seconds (tracing b; mean open time, 3.8 msec), and 1 minute (tracing c; mean open time, 1.1 msec) after formation of the cell-attached patch and at 1 minute (tracing d; mean open time, 1.2 msec) after addition of GTP to the inside-out patch are shown. Channel openings were analyzed only to the first and second levels.

Lyzed calf intestine alkaline phosphatase (20 U/mL) was applied to the bath together with GTP. This resulted in shortening of the open time from 5.1±0.5 to 2.8±0.4 msec (n=3) in rat and from 5.3±0.6 to 2.2±0.6 msec in guinea pig (n=3) atrial cells. In these cells, the channel activities also decreased 3.3±0.9-fold in rat and 2.9±0.6-fold in guinea pig atrial cells. These results further support the view that the cytoplasmatic factor that shortens open time during the rapid phase of desensitization may be a protein phosphatase.

In all patches used in studies described above, multi-channel openings were present. Although we limited the analysis of open times of current recordings to those that have two-channel openings, the mean open times determined using such recordings will be an underestimation of the true value, as discussed in "Materials and Methods." Unfortunately, in cell-attached patches with ACh in the pipette, patches with a single channel (open only to the first level) could not be obtained even with high-resistance pipettes, presumably because of a high density of \( I_{\text{KAC}} \) in adult atrial cells. However, because the \( K^+ \) channel activity decreased slowly with time, it was possible to record channels that opened only to the first level before and after treatment with Mg\(^{2+}\)-ATP in inside-out patches. In four such patches, the distribution of open times could be fit with a single exponential function with a time constant of 1.2±0.1 or 7.7±0.6 msec in patches treated with GTP alone or with GTP and ATP, respectively (Fig 7). Thus, the mean open time values (=5 msec) analyzed using two-channel patch recordings were underestimated by ≈35%. This suggests that the mean open time of channels activated

Fig 4. Mean open time (panel A) and channel activity (panel B) plotted as a function of time in the cell-attached mode. Channel openings during 0 to 2, 10 to 12, 20 to 22 seconds, and so on, after formation of cell-attached patch with acetylcholine in the pipette were analyzed to obtain mean open times and channel activity. Means from three separate determinations are plotted. Standard error was less than 17% of the mean.

Fig 5. Tracings showing the role of cytoplasmic factors in rapid desensitization of the muscarinic \( K^+ \) current in rat atrial cells. As soon as cell-attached patches were formed with acetylcholine (ACH) in the pipette, inside-out patches were made from the same cell to wash off the cytosolic content. Addition of GTP to the cytoplasmic face of the membrane caused activation of channels that had increased open times (tracing a; mean open time, 5.1±0.6 msec; n=5) compared with those in which inside-out patches were formed after ≈2 minutes in the cell-attached state (tracing b; mean open time, 1.0±0.1 msec; n=6).
FIG 6. Tracings showing modulation of muscarinic K+ currents by ATP in inside-out patches in rat (panel A) and guinea pig (panel B) atrial cells. After \( \approx 2 \) minutes in the cell-attached state with acetylcholine in the pipette, inside-out patches were formed, and GTP was added to the bath to reactivate the channels (tracing a; mean open time, 1.1 msec in rat; mean open time, 1.0 msec in guinea pig). Further addition of ATP (1 mM) to the bath resulted in a gradual increase in channel activity (from 0.032 to 0.116 in rat, from 0.016 to 0.073 in guinea pig) and open time (tracing b; mean open time, 4.3 msec in rat; mean open time, 5.2 msec in guinea pig). Washout of GTP closed the channels. Addition of only GTP to the bath activated channels with increased open time (tracing c; mean open time, 5.5 msec in rat; mean open time, 6.5 msec in guinea pig). Mean open times and channel activity were determined from \( \approx 1000 \) openings to the first and second levels.

by ACh just after the formation of cell-attached patches is also underestimated, and the true values are likely to be \( \approx 6 \) msec.

**Effect of Protein Kinase and Phosphatase Inhibitors on the K+ Current**

The hypothesis that the effect of Mg\(^{2+}\)-ATP on channel kinetics in inside-out patches was via kinase-mediated phosphorylation was tested. The cells were pretreated for \( \approx 10 \) minutes with protein kinase inhibitors H-7 (30 \( \mu \)M, inhibitor of protein kinase A and C) and calphostin C (1 \( \mu \)M, inhibitor of protein kinase C). On formation of cell-attached patches, rapid K+ desensitization was observed in all cells tested (\( n = 5 \) each). After \( \approx 2 \) minutes in the cell-attached state with ACh in the pipette, inside-out patches were formed, and GTP was added to the bath to activate the muscarinic K+ channels. On addition of 1 mM ATP in the presence of the inhibitors, the channel open time and activity increased in a manner similar to those in control patches, which were not treated with the inhibitors. In the presence of inhibitors, the mean open time increased from 1.0 \( \pm \)0.1 to 5.3 \( \pm \)0.4 msec, and channel activity increased 4.6 \( \pm \)0.8-fold (\( n = 3 \)). These results suggest that, if a protein kinase is involved, it is not cAMP or Ca\(^{2+}\) dependent. It is possible that the kinase that is associated with the membrane could be of a different class.

Results described above using Mg\(^{2+}\)-ATP and alkaline phosphatase suggest that \( I_{KAC} \) desensitization may be associated with dephosphorylation. These possibilities were tested by recording either whole-cell currents or single-channel currents in cell-attached and inside-out patches from rat atrial cells. To test the role of dephosphorylation in rapid K+ current desensitization, phosphatase inhibitors (sodium vanadate, 100 \( \mu \)M; potassium fluoride, 1 mM; zinc sulfate, 100 \( \mu \)M) were added to the pipette and bath solution. After breaking the membrane under the pipette, the cell membrane potential was held at \(-40 \) mV. After \( \approx 3 \) minutes, the extracellular perfusion solution was changed to that containing the inhibitors plus 10 \( \mu \)M ACh. In every rat (\( n = 6 \)) or guinea pig (\( n = 4 \)) atrial cell tested, ACh failed to elicit the rapid phase of current decrease (Figs 8B and 8C). In control cells without the inhibitors, ACh-induced rapid desensitization was present as shown previously (Fig 8A). Treatment of cells with the inhibitors not only blocked the rapid desensitization phase but also markedly reduced the ACh-induced initial activation of the current. Thus, these whole-cell data do not clearly distinguish between two possible effects of the inhibitors: block of the rapid desensitization and inhibition of initial activation.

To further test the hypothesis that the rapid K+ current desensitization observed in cell-attached patches is mediated via protein phosphatase(s), cells were pretreated for \( \approx 10 \) minutes with phosphatase inhibitors, sodium vanadate (100 \( \mu \)M), potassium fluoride (1 mM), and zinc sulfate (100 \( \mu \)M). Single-
channel studies identical to those described in Fig 5 were performed, and changes in channel activity and kinetics were determined. As shown in Fig 9, the

phosphatase inhibitors failed to block the opening of channels with longer open times and reduced the rate of decrease in open time and channel activity such that it took considerably longer time to shorten the mean open time from ≈4.5-5.0 to ≈1.0 msec. Thus, the relative absence of the rapid phase of desensitization seen in whole-cell currents (shown in Fig 8) in the presence of the inhibitors is likely to be due to slowing of the desensitization rate. Also, the results showing that the inhibitors did not completely block the change in channel kinetics indicate either that the phosphatase(s) was not fully blocked or that there are other mechanisms that are involved in shortening of the open time.

**Discussion**

Studies reported during the last 5 years indicate that ACh activates $I_{K,AC}$ via dissociation of the receptor-coupled inhibitory GTP binding protein and subsequent interaction of either the $\alpha$ or $\beta\gamma$ subunit ($G_{\alpha}$ and $G_{\beta\gamma}$, respectively) with the channel in a membrane-delimited pathway.25-29 Whether the physiological activator is the $\alpha$ or $\beta\gamma$ subunit of the G protein has not yet been fully resolved.30-33 The present study demonstrates that although the G protein (and GTP) is necessary to activate the $K^+$ channel, the activity and the kinetics of the $K^+$ channel can be modulated by Mg$^{2+}$-ATP and by unidentified cytoplasmic factors. The effects of Mg$^{2+}$-ATP on open time and channel activity in inside-out patches resemble, although opposite in direction, the changes in channel kinetics during the rapid phase of desensitization in cell-attached patches. This suggests that, if the effect of Mg$^{2+}$-ATP is via phosphorylation, then the process underlying the rapid desensitization may be dephosphorylation. The results with Mg$^{2+}$-ATP, alkaline phosphatase, and its inhibitors support the view that channel modulation is probably mediated via kinase and phosphatase. Thus, phosphorylation by mem-

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**FIG 7.** Recordings of the muscarinic $K^+$ current in inside-out patches showing channel openings mostly to the first level with 100 μM GTP (panel A) or 100 μM GTP and 1 mM ATP (panel B) in the bath. τ, Mean open time. Open-time histograms are shown on the right.

**FIG 8.** Whole-cell current recordings activated by acetylcholine (ACh) with and without treatment with phosphatase inhibitors. Experimental protocol is identical to that in Fig 1. Panel A: A control experiment in a rat atrial cell is shown. The peak of the small blip obtained by changing the potential from −40 to 0 mV before the application of ACh is the zero current level. Panel B: Phosphatase inhibitors were added to the pipette and bath solution for ≈3 minutes before ACh was added to the solution perfusing a rat atrial cell. Panel C: The same experiment as in panel B in a guinea pig atrial cell is shown. Note the absence of the initial rapid decrease in $K^+$ current in panels B and C compared with that in panel A.
brane-associated protein kinase(s) to increase the channel activity and dephosphorylation by cytosolic protein phosphatase(s) to reduce the channel activity may be the cause of rapid K⁺ current desensitization in rat and guinea pig atrial cells.

In cell-attached patches without ACh in the pipette, there is a basal level of muscarinic K⁺ channel activity in both rat and guinea pig atrial cells. This basal activity has been shown to be due to slow agonist-independent activation of Gₛ by GTP. ACh binding to the muscarinic receptor accelerates the GTP-GDP exchange of Gₛ to cause agonist-dependent activation of the K⁺ channel. The muscarinic K⁺ channel openings observed under basal conditions have a mean open time of ≤1 msec. As shown in Figs 2 and 3, ACh activated the K⁺ channels with open times of 4 to 5 msec during the initial few seconds in the cell-attached patch. Thus, ACh must have not only increased the frequency of channel opening via activation of Gₛ but also modified the length of time that the channel stays in the open state. Although the exact mechanism for the effect of ACh on open time is not certain, the effects of Mg²⁺-ATP (but not the nonhydrolyzable analogue of ATP) and alkaline phosphatase, ie, an increase and a decrease in the open time in inside-out patches, respectively, suggest that ACh may be causing phosphorylation in addition to activating Gₛ. The phosphorylated entity that could be the channel itself or an associated regulatory protein then undergoes dephosphorylation by cytoplasmic phosphatases, resulting in a gradual decrease in open time. The progressive nature of the changes in open time during rapid desensitization in cell-attached patches or during exposure to ATP in inside-out patches suggests that there are multiple phosphorylation sites on the protein.

In bullfrog atrial myocytes, the rapid phase of desensitization is also present. It was found that eicosatetraynoic acid (an inhibitor of arachidonic acid metabolism) blocked the activation of the peak K⁺ current elicited by ACh, suggesting that lipid metabolites may be responsible for desensitization in bullfrog atrial cells. Replacing ATP in the pipette solution with AMP-PNP failed to affect the desensitization in bullfrog atrial cells, suggesting that phosphorylation may not mediate IₜKₐCS desensitization in this species. Thus, the nature of the muscarinic receptor-G protein-K⁺ channel coupling and its regulation by intracellular factors appears to be species dependent.

It has been shown that GTP in the presence of Mg²⁺ can activate IₜKₐCS even when receptors are not occupied by agonists. The concentration of GTP required to activate IₜKₐCS has been reported to be at least 10⁻fold greater without an agonist than with an agonist occupying the receptor. In the present study, although GTP alone caused activation of the K⁺ channel, the maximal GTP (1 mM)-activated channel activity was only a small fraction (=10%) of activity observed in the presence of ACh. In earlier studies done in guinea pig, frog, and rabbit atrial cells, ATP and Mg²⁺ in the bath also caused agonist-independent activation of the muscarinic K⁺ channel. This was proposed to be via phosphorylation of Gₛ-GDP to Gₛ-GTP by nucleotide diphosphate kinase in the absence of agonist. This is supported by an earlier study in which ATP-γ-S caused activation of IₜKₐCS via nucleotide diphosphate kinase-mediated generation of Gₛ-γ-S in frog atrial cells. In rabbit atrial cells, ATP did not alter the open time of IₜKₐCS. Furthermore, when IₜKₐCS was activated with GTP-γ-S in the presence of ATP and Mg²⁺, no changes in channel kinetics were reported. In rat and guinea pig atrial cells used in the present study, addition of ATP to GTP-γ-S-activated K⁺ channels (IₜKₐCS) caused an increase in open time from 1.1 to 4.3 msec (n = 4). Thus, separate mechanisms of regulation of the muscarinic K⁺ channel may be present in rat (or guinea pig) and rabbit atrial cells.

A study of the nicotinic ACh channel function of Xenopus myocytes reported that ACh-activated single channels obtained with a hard-glass (borosilicate) pipette was different from that obtained with a soft-glass pipette. The time-dependent decrease of current after seal formation was observed with the hard-glass pipette but not the soft-glass pipette. Although the reasons for this disparity are not well known, such a difference indicates that use of an inert-glass pipette may be important. All of the experiments described in this study were done using the hard-glass (borosilicate) pipette. When soft Blu-tip capillary tubes (Labcraft; n = 12) were used, we found a similar rapid phase of desensitization that was due to the shortening of open time in cell-
attached patches and similar increases in channel activity and open time by Mg\textsuperscript{2+}-ATP in inside-out patches.

In summary, ACh caused an increase in the open time of the muscarinic K\textsuperscript{+} channel during the onset of activation. The subsequent decrease in channel activity (rapid desensitization) was associated with a progressive shortening of the open time and prolongation of the closed time that occurred during the initial ~20 seconds of ACh exposure. Because the channel open time could be increased by Mg\textsuperscript{2+}-ATP in an irreversible manner in inside-out patches, it is proposed that a membrane-associated protein kinase is involved in increasing open time and channel activity. Because the rapid desensitization is abolished by washout of cytoplasmic contents, certain factors in the cytosol mediated the shortening of the open time. It is hypothesized that this cystolic factor is a protein phosphatase. Thus, phosphorylation/dephosphorylation processes may be involved in the ACh-induced activation and rapid desensitization of I\textsubscript{K,AC} in atrial cells of adult rat and guinea pigs. Further studies are necessary to identify the protein kinase and phosphatase(s) and other possible cytosolic factors that modulate muscarinic K\textsuperscript{+} channel activity and thus the cardiac rate and rhythm.

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