β-Adrenergic Regulation of the Muscarinic-Gated K⁺ Channel via Cyclic AMP–Dependent Protein Kinase in Atrial Cells

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Cholinergic and β-adrenergic stimulations of ionic currents are major physiological mechanisms in the regulation of heart rate and contractility. Muscarinic receptor stimulation is known to reduce β-adrenergic effects on calcium current via reduction of cyclic AMP. Whether the β-adrenergic stimulation affects the muscarinic response is not known. I report here that the β-adrenergic agonist isoproterenol enhanced the muscarinic-activated K⁺ channel activity in rat atrial cells. Application of cyclic AMP–dependent protein kinase or its catalytic subunit to the cytoplasmic side of the membrane augmented the acetylcholine-activated K⁺ channel activity twofold to threefold. Increases in channel activity produced by isoproterenol or cyclic AMP–dependent protein kinase were associated with fourfold to fivefold and approximately twofold increases in the mean open and closed time durations, respectively. Alkaline phosphatase treatment reversed these effects. These results suggest that cyclic AMP–dependent phosphorylation of the K⁺ channel or associated regulatory proteins modulates the gating kinetics of the channel. This mechanism may be important in the regulation of pacemaker activity and, thus, the heart rate during β-adrenergic stimulation. (Circulation Research 1990;67:1292–1298)

In atrial tissue, the muscarinic agonist acetylcholine (ACh), via the inhibitory GTP binding protein (Gᵢ), activates an inwardly rectifying K⁺ channel,¹–⁵ which contributes to the slowing of the heart. On the other hand, β-adrenergic agonists increase the voltage-dependent Ca²⁺ current and the delayed rectifier K⁺ current and also increase the rate of diastolic depolarization (pacemaker potential) by shifting the activation curve of pacemaker current.⁶,⁷ Most of the effects of β-adrenergic stimulation have been demonstrated to occur via elevation of cyclic AMP (cAMP),⁸–¹² presumably through the activation of the stimulatory G protein (Gₛ). In addition to activation of the K⁺ current in atrial cells, muscarinic agonists reduce the β-adrenergic stimulation of Ca²⁺ current by inhibition of adenyl cyclase activity.⁸ Although the exact mechanism for this effect is not known, there is evidence that the α- or βγ-subunit of Gₛ modulates the activity of the adenyl cyclase.¹⁴,¹⁵

Whether β-adrenergic stimulation affects the muscarinic response is not well known. In two previous studies,¹⁶,¹⁷ addition of cAMP directly to the cells failed to affect ACh-induced hyperpolarization on rabbit sinoatrial nodal cells and carbachol-induced increase in K⁺ conductance in bullfrog atrial cells. These studies were done using multicellular atrial muscle preparations, and muscarinic K⁺ currents were not clearly dissected out from other ionic currents. In initial experiments in the study of the regulation of muscarinic-gated K⁺ channels by β-adrenergic stimulation, it was noted that isoproterenol could significantly increase the ACh-activated K⁺ channel activity. Since this interaction is important in the control of pacemaker potential and heart rate, I investigated the β-adrenergic actions on the muscarinic K⁺ channel activity at the single-channel level.

Materials and Methods

Cell Preparation

Hearts from 1–2-day-old newborn rats were dissociated with collagenase and trypsin. Right and left atrial tissues from whole hearts were cut out and placed in calcium-free Hank’s medium (Sigma

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Chemical Co., St. Louis). The tissues were then cut into small pieces with a sharp blade and placed in Hank’s balanced salt medium containing 0.05% collagenase type II and 0.03% trypsin (Worthington Biochemical Corp., Freehold, N.J.). Tissues were incubated at 37°C for 10 minutes. Suspended cells were then removed and added to a volume of 50% fetal calf serum to inhibit enzyme activity. Remaining tissues were incubated in a new enzyme solution and allowed to dissociate for another 10 minutes. This procedure was done five times. Dissociated cells were collected, centrifuged, and placed in culture medium, which consisted of Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, and 0.1% penicillin–streptomycin. Cells were plated on glass coverslips and incubated at 37°C in 5% CO₂ for 12–24 hours before use.

**Electrophysiology**

Gigaseals were formed with pipettes with 4–6 MΩ resistances by the method of Hamill et al. Membrane patches were brought to the mouth of a polyethylene tubing through which test solutions flowed at approximately 1 ml/min. The pipette and bath solutions contained (mM) K⁺ 140, Cl⁻ 140, EGTA 5, Mg²⁺ 2, and HEPES 10 (pH 7.2). Free calcium concentration was adjusted to 0.1 μM by adding CaCl₂. Single-channel currents were obtained with an Axopatch 1C patch-clamp amplifier, low-pass filtered at 5 KHz, and stored on magnetic tape via digital data recorder (Instrutech, Elmont, N.Y.). Later, digitized data were entered into an ATARI computer and analyzed to obtain duration and amplitude histograms and averaged channel activity. Channel activity was calculated from the first level of openings. The number on top of each tracing in Figures 1–4 represents the channel activity value averaged over a 30-second period of maximal channel activity. All experiments were done at 24–26°C.

**Drugs**

cAMP-dependent protein kinase (PKA), catalytic subunits, dibutyryl cAMP, and isoproterenol were purchased from Sigma. ATP, AMPPNP (a nonhydrolyzable analogue of ATP), GTP, GTP-γ-S, and alkaline phosphatase were purchased from Boehringer Mannheim Corp., Indianapolis. Enzymes were dialyzed against the perfusion solution at 0°C for 8 hours before use. Pertussis toxin was purchased from List Biological, Campbell, Calif.

**Results**

**Stimulation of the Muscarinic K⁺ Channel Activity by Isoproterenol**

In cell-attached patches, ACh in the pipette activated an inwardly rectifying K⁺ channel with kinetic properties similar to those described previously in atrial cells. With 140 mM K⁺ in both pipette and bath solutions, the single-channel conductance was 36±3 pS, and the mean open time was 1.0±0.1 msec (mean±SD, n=12) at −80 mV. Extracellular application of 10 μM isoproterenol produced a striking change in the pattern of channel opening; durations of channel openings and closings were both prolonged. Single-channel analyses revealed that the distribution of open time durations could be fit by a single exponential function with a decay time constant of 3.2±1.1 msec (mean±SD, n=6). Distribution of closed time intervals observed in control patches could be fit by the sum of two exponential functions with time constants of 416±105 μsec (48% of the total number of intervals) and 3.38±2.44 msec (52%). In isoproterenol-treated cells, time constants were 558±197 μsec (70%) and 9.65±3.17 msec (30%). The net result of these effects on open and closed time intervals and on the distribution of the slow and fast components was an increase in the probability of channel openness (Figure 1A). Propranolol (1 μM), a β-adrenergic blocker, abolished the effects of isoproterenol on atrial beating rate and on the K⁺ channel (open time of −1 msec, n=3). When dibutyryl cAMP (50 μM) was applied extracellularly to cell-attached patches, a 1.6±0.4-fold (n=3) increase in channel activity and a 3.2±0.9-fold increase in the mean durations of channel opening were observed (Figure 1B). These results indicate that β-adrenergic stimulation increases the muscarinic-gated K⁺ channel activity by modifying the gating behavior of the K⁺ channel.

**Effect of cAMP-Dependent Protein Kinase on the K⁺ Channel**

Isoproterenol has been shown to modulate Ca²⁺ current, delayed rectifier K⁺ current, and calcium-dependent K⁺ current by stimulation of PKA. To test whether the observed effect of isoproterenol on the muscarinic K⁺ channel is due to phosphorylation by PKA, inside-out patches with ACh in the pipette were formed, and K⁺ channels were activated with GTP in the presence and absence of PKA. GTP-activated channels in nine control patches had a mean open time of 1.0±0.2 msec. In 14 of 15 patches, application of PKA (with cAMP and ATP) to the cytoplasmic side resulted in an immediate increase in open time (4.6±0.4 msec) and closed time (slow component, 4.1 msec; fast component not altered) of the K⁺ channel without a change in the single-channel current amplitude. The net effect of PKA was a 2.6±0.9-fold increase in the channel activity. As shown in Figure 2B, when patches were preincubated with PKA and no GTP for 1–2 minutes, subsequent addition of GTP or GTP-γ-S always activated channels with longer open durations (4.7±0.9 msec, n=6). These effects were blocked by incubation of the patches with 200 μg/ml protein kinase inhibitor (Sigma) in four of four cells (mean open time, 0.96±1.24 msec). PKA alone without GTP did not activate the muscarinic or any other K⁺ channels in six cells. cAMP and ATP at the concentrations used failed to affect the channel ac-
Figure 1. Recordings and histograms showing that isoproterenol augments the activity and open times of the muscarinic K+ channel. ACh, acetylcholine; τo mean open time; τc mean closed time (fast); τs mean closed time (slow); db cAMP, dibutyryl cyclic AMP. Panel A: A cell-attached patch with 10 μM ACh in the pipette shows opening of K+ channels with a mean open time of 0.92 msec before (tracing a) and 4.2 msec after (tracing b) application of 10 μM isoproterenol. Distribution of closed time intervals was fit by the sum of two exponents. Isoproterenol increased the mean closed time of the slow component and increased the channel activity 1.54-fold. On formation of an inside-out patch, GTP activated channels with a mean open time of 5.02 msec. Single-channel openings indicated by tracings a, b, and c are shown at expanded scale. Holding potential was −80 mV. Open and closed time histograms for channel openings shown in tracings a and b are shown in the insert on the right. Note the logarithmic and square root scales used to represent the dwell-time distributions. Mean open and closed times are indicated by dotted lines. In experiments with isoproterenol, isobutylmethylxanthine (50 μM) was used in four cells to block phosphodiesterases. Panel B: Same experiment as in panel A except that db cAMP was used instead of isoproterenol to increase cAMP-dependent protein kinase activity. Single-channel openings are shown below. Both the mean open time and the channel activity were increased by db cAMP. Formation of an inside-out patch resulted in a decrease in channel activity to basal levels. Application of GTP (100 μM) activated the channels with longer open time durations (τ=3.27 msec). The GTP-induced channel activity was not analyzed, since in many cases the channel activities in cell-attached and inside-out patches originating from the same cell are different and, thus, cannot be compared. Patches exposed to control (no drugs) solutions showed channel openings with τ=−1 msec in response to GTP. The change in channel kinetics produced by isoproterenol (seven of 15 cells) or db cAMP (five of 11 cells) was noted in approximately 50% of the cells studied compared with 95−100% in excised patches. This may be due to the presence of active cytosolic protein phosphatases in intact cells.20

Activity in five cells (1.1±0.1-fold change), indicating that PKA may be a cytosolic protein in the heart.

Similar increases (2.4±0.4-fold) in K+ channel activity were obtained in 12 of 12 patches when the catalytic subunit of PKA was applied to the cytosolic surface to produce phosphorylation. The PKA subunit caused an increase in mean open time from 1.1±0.2 to 5.1±0.6 msec (mean±SD) and mean closed time (slow component) from 4.1±1.6 to 8.9±2.8 msec (Figure 3A). Subsequent exposure of the patches to 20 units/ml alkaline phosphatase (Sigma) reversed the effects of PKA subunit on the
duration of channel opening and closing (Figure 3A). Alkaline phosphatase treatment reduced the mean open time to 1.9±0.3 msec in six patches, perhaps indicating that not all sites acted on by the PKA subunit were dephosphorylated by the enzyme. The effect of alkaline phosphatase on the channel was not due to hydrolysis of GTP, because application of both GDP and GTP did not reverse the effect of PKA.

FIGURE 2. Recordings showing that protein kinase A (PKA) increases the open time of the muscarinic K+ channel. ACh, acetylcholine; τ, mean open time. Panel A: Perfusion with GTP of an inside-out patch containing ACh in the pipette activated channels with a conductance of 36 pS and a mean open time of 1.08 msec. Single channels are shown below at expanded scale. Application of PKA (20 units/ml) to a GTP-activated patch resulted in a 2.4-fold increase in channel activity and a 4.6-fold increase in mean open time. Cyclic AMP (0.1 mM) and ATP (1 mM) were also present. PKA alone without cyclic AMP or ATP alone had no effect on the channel activity. Panel B: An inside-out patch was incubated with PKA (cyclic AMP+ATP) for 2 minutes. Subsequent addition of GTP to the patch caused activation of K+ channels with longer open durations (4.78 msec). Amplitude of unitary currents (2.7±0.3 pA, n=5) was not altered by PKA. PKA isolated from bovine heart was dialyzed against the perfusion solution at 0° C for 6 hours before use.

FIGURE 3. Recordings showing that the catalytic subunit of protein kinase A (PKA) and alkaline phosphatase modulate the muscarinic K+ channel kinetics. τ, mean open time. Perfusion of PKA subunit (10 units/ml) to an inside-out patch whose K+ channel has already been activated with GTP resulted in increases in channel activity and the mean open duration. Single channels are shown below at higher time resolution. Washout of PKA subunit and perfusion with dialyzed alkaline phosphatase (20 units/ml) reversed the effects of the subunit (see tracing c compared with b).
Washout of alkaline phosphatase and readdition of PKA resulted in reappearance of channels with longer open times (4.8±0.8 msec, n=3). Washout of GTP at any time quickly reduced the channel activity to basal levels. In initial studies, incubation of cells with 1 μM phorbol 12-myristate 13-acetate (six cells) or 1-oleoyl-2-acetylglycerol (five cells) to stimulate protein kinase C had no significant effect on the K+ channel in all patches either in the presence or absence of ACh.

Effect of PKA on the Unstimulated K+ Channel

To determine whether the K+ channel itself or associated membrane proteins are possible targets of phosphorylation by PKA, we first studied the effects of PKA on the kinetics of the K+ channel that opens spontaneously under basal conditions. We incubated cells with 100 ng/ml pertussis toxin for 8 hours to uncouple muscarinic receptor from G proteins. Under these conditions, ACh in the pipette failed to activate K+ channels, as shown in Figure 4A. The basally active K+ channels, which open once every few seconds, had a mean open time of 0.84±0.12 seconds (mean±SD, n=5). Formation of an inside-out patch did not alter the kinetics of channel opening. Due to long closed times, we did not analyze the closed time duration. On exposure of PKA to the cytosolic side of the membrane (with cAMP and ATP), the time duration of channel openings immediately increased such that the mean open time now was 4.3±1.2 msec (n=4). PKA without ATP or with AMPPNP failed to produce similar results (mean open time of 1.06 msec, n=4), suggesting that the above effects are probably due to phosphorylation. Subsequent application of GTP did not activate the channel, indicating that G was clearly uncoupled from the receptor. Subsequent addition of GTP-γ-S caused activation of the channels with longer mean open times, even after the washout of the kinase. Application of isoproterenol to cell-attached patches also induced similar changes in the kinetics of the basally active K+ channels (open times of 0.86–4.32 msec, n=8). These results suggest that PKA phosphorylates the K+ channel or an associated regulatory protein to cause alterations in the gating mode of the K+ channel. The above results also suggest that the basally active and ACh-activated K+ channel may be the same channel.

Other Activators of the Muscarinic K+ Channel

If PKA directly alters the gating of the K+ channel, then other activators of the K+ channel should, in the presence of the kinase, also cause opening of the channel with similar changes in the gating kinetics. This idea was tested using adenosine, which also activates the same population of K+ channels via a different receptor.25,26 When muscarinic K+ channels were activated with 100 μM adenosine, application of the catalytic subunit of PKA to the inside-out patches also produced increases in channel activity (2.4±0.5-fold) and the mean open time (4.4±1.2-fold) in seven patches (Figure 4B). A1 adenosine receptor antagonist, xanthine amine congener (5 μM), blocked activation of the K+ channel. Although direct evidence is missing, these results suggest that phosphorylation of the K+ channel or associated regulatory protein by PKA modulates the kinetic behavior of the muscarinic K+ channel, as it does the voltage-sensitive Ca2+ channels.

Discussion

Above findings show clearly that isoproterenol can modulate the kinetics of the cardiac muscarinic K+ channel via the cAMP-dependent pathway. This was
due to the effect of PKA on the K⁺ channel kinetics: increases in both open and closed times. Due to a greater effect on the open times than the closed times, the net result was an increase in the K⁺ channel activity. Previous studies reported a lack of effect of cAMP injection on ACh-induced hyperpolarization in rabbit atrioventricular nodal cells17 and on carbachol-induced potassium current in bullfrog atrial cells.16 In these studies, the current measurements may have been complicated by the multiepithelial nature of the preparation and by actions of cAMP on other ionic currents. Furthermore, in one of the studies, the time-dependent changes in pacemaker potential could not be compared, due to a twofold increase in the spontaneous beat rate produced by cAMP. It is possible, however, that the effects of cAMP on the K⁺ channel are tissue and species dependent.

The results of this study indicate that even in the absence of an activator of the K⁺ channel such as ACh or adenosine, isoproterenol or PKA is capable of producing similar changes in the kinetics of channel opening, that is, increase in the channel open time. This is presumably the molecular basis for the β-adrenergic stimulation of the ACh-activated K⁺ channel activity and the enhanced response to ACh in the presence of PKA. Presumably, once the K⁺ channel or an associated regulatory protein is phosphorylated by the kinase, its gating kinetics are altered, and the channel remains in that mode as long as it is phosphorylated. Dephosphorylation by the alkaline phosphatase (see Figure 3) is then able to partially revert the mode of channel behavior. This type of channel behavior is analogous to the effects of isoproterenol on the kinetics of cardiac Ca²⁺ channels, which occur via PKA. It was shown that β-adrenergic stimulation increases open time but shortens closed time durations, resulting in a large net increase in the probability of channel openness.9,27

Although phosphorylation of the muscarinic receptors by PKA cannot be ruled out,28 our results are consistent with the phosphorylation of the K⁺ channel or closely associated regulatory proteins by PKA. Phosphorylation of the receptor itself may potentially alter the probability of channel opening by regulating the amount of G protein subunits (α and βγ) generated to activate the channel but should not alter the gating kinetics of the channel, since the kinetics are not affected by different amounts of the G protein subunits or GTP.29,30 It is possible, however, that the G protein itself is the target of phosphorylation and that the modified α-subunit of the G protein may interact with the K⁺ channel in a way to alter the kinetics of the K⁺ channel opening and closing. Evidences from the experimental results above are also consistent with the existence of one population of ACh-activated K⁺ channels that can be modulated in its kinetics to display a different mode of behavior.

In heart cells, β-adrenergic stimulation has been reported to increase the Ca²⁺ current,10-13 K⁺ current,21,22 pacemaker current (I_p),6,7 and transient outward current33 and activate a Cl⁻ current,32,33 all through the cAMP-dependent pathway. Thus, PKA phosphorylates several membrane proteins, each associated with an ion channel, to modulate cardiac function.8 β-Adrenergic agonists may modulate their positive chronotropic action on atrial cells by phosphorylation of the muscarinic K⁺ channel to increase potassium conductance. In the absence of such a negative feedback mechanism to increase the muscarinic K⁺ channel activity, strong sympathetic stimulation of the heart may result in arrhythmias.

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