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Activation of Inwardly Rectifying Potassium Channels by Muscarinic Receptor-linked G Protein in Isolated Human Ventricular Myocytes

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Abstract. Muscarinic receptor-linked G protein, G_i, can directely activate the specific K^+ channel ($I_{K(ACh)}$) in the atrium and in pacemaker tissues in the heart. Coupling of G_i to the K^+ channel in the ventricle has not been well defined. G protein regulation of K^+ channels in isolated human ventricular myocytes was examined using the patch-clamp technique. Bath application of 1 μM acetylcholine (ACh) reversibly shortened the action potential duration to 74.4 ± 12.1% of control (at 90% repolarization, mean ± SD, n = 8) and increased the whole-cell membrane current conductance without prior β-adrenergic stimulation in human ventricular myocytes. The ACh effect was reversed by atropine (1 μM). In excised inside-out patch configurations, application of GTPγS (100 μM) to the bath solution (internal surface) caused activation of $I_{K(ACh)}$ and/or the background inwardly-rectifying K^+ channel (I_{K1}) in ventricular cell membranes. $I_{K(ACh)}$ exhibited rapid gating behavior with a slope conductance of 44 ± 2 pS (n = 25) and a mean open lifetime of 1.8 ± 0.3 msec (n = 21). Single channel activity of GTPγS-activated I_{K1} demonstrated long-lasting bursts with a slope conductance of 30 ± 2 pS (n = 16) and a mean open lifetime of 36.4 ± 4.1 msec (n = 12). Unlike $I_{K(ACh)}$, G protein-activated I_{K1} did not require GTP to maintain channel activity, suggesting that these two channels may be controlled by G proteins with different underlying mechanisms. The concentration of GTP at half-maximal channel activation was 0.22 μM in $I_{K(ACh)}$ and 1.2 μM in I_{K1}. Myocytes pretreated with pertussis toxin (PTX) prevented GTP from activating these channels, indicating that muscarinic receptor-linked PTX-sensitive G protein, G_i, is essential for activation of both channels. G protein-activated channel characteristics from patients with terminal heart failure did not differ from those without heart failure or guinea pig. These results suggest that ACh can shorten the action potential by activating $I_{K(ACh)}$ and I_{K1} via muscarinic receptor-linked G_i proteins in human ventricular myocytes.

Key words: Patch-clamp technique — Human ventricular myocytes — G proteins — Muscarinic K^+ channel — Inwardly-rectifying K^+ channel

Introduction

G proteins are known to play a major role in intracellular signal transduction from receptors to effectors in the mammalian nervous and circulatory system (Fleming, Wisler & Watanabe, 1992 for review; Wess, 1993 for review). Muscarinic receptor-linked G protein, G_i, is responsible for inhibition of adenylyl cyclase, especially when it is stimulated by catecholamines via β-adrenergic receptor-linked G protein, G_S (Robishaw & Foster, 1989). In cardiac atrial and pacemaking tissues, G_i also couples muscarinic receptors to the specific K^+ channel ($I_{K(ACh)}$) (Breitwieser & Szabo, 1985; Pfaffinger et al., 1985). Acetylcholine (ACh) can directly activate $I_{K(ACh)}$ without prior stimulation of β-adrenergic systems and without formation of intracellular second messengers (Soejima & Noma, 1984; Kurachi, Nakajima & Sugimoto, 1986; Zang et al., 1993). In these tissues, signaling from muscarinic receptors is through dual pathways via G proteins (Kurachi, 1995 for review).

Muscarinic cholinergic modulation in the ventricle

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appears to be different from atrial and pacemaker tissues. Previous studies suggested that ACh could directly increase K^+ conductance in the atrium but not the ventricle (Hino & Ochi, 1980; Josephson & Sperelakis, 1982). Muscarinic stimulation induces minimal direct negative inotropic effects in the ventricle, but it indirectly modifies ventricular myocardial contractility by antagonizing the effects of agonists that increase cAMP and adenyly cyclase levels which enhance the Ca^{2+} current (I_{Ca}). Recently, however, ACh-induced increase of K^+ conductance has been shown to be responsible for the negative inotropic effect of muscarinic stimulation in frog ventricular tissues (Hartzell & Simmons, 1987). The shortening of the action potential duration by ACh was reported in ferret ventricular myocytes which were induced by increased background K^+ conductance (Boyett et al., 1988). A Direct negative inotropic effect of ACh has been shown in rat ventricular myocytes (McMorn et al., 1993). Martin et al. (1987) demonstrated that muscarinic receptor affinity for agonists and regulation of receptor affinity by GTP are the same between atrial and ventricular membranes in chick hearts. These studies suggest that muscarinic cholinergic stimulation can directly affect the effectors in ventricular tissue similar to atrial tissue.

The coupling of G protein to K^+ channels in the human ventricle remains uncertain. The goal of this study was to determine whether or not G proteins can modulate K^+ channels in the ventricle and to clarify the underlying mechanism of its effect in isolated human ventricular myocytes. We focused this study on adult human ventricular K^+ channels because the wide variety of species differences between mammalian hearts make estimation and understanding the behaviors of native human channels less clear.

Materials and Methods

Human Cardiac Specimens

Adult human ventricular specimens were obtained from explanted hearts of transplantation recipients and from patients undergoing cardiac surgery. Institutional and National Institutes of Health guidelines for human experimentation were followed in obtaining surgical specimens. Specimens from a total of 23 patients (14 male and 9 female) were studied. Twelve patients received transplantation with class IV terminal heart failure (HF) with a mean age of 50.7 ± 14.2 years. The Cardiac Index was 2.1 ± 0.27 *l/min/m*^{2} (n = 12), and the ejection fraction was 23.9 ± 2.5% (n = 12) in this group. Eleven patients received corrective cardiac surgery without heart failure (non-HF) with a mean age of 48.9 ± 22.1 years, not statistically different from the HF group. The Cardiac Index was 3.9 ± 0.33 *l/min/m*^{2} (n = 11), and the ejection fraction was 61.0 ± 5.5% (n = 11) in this group. These values were significantly different from HF ($P < 0.001$). The specimen was placed in a chilled transport solution and was transported from the operating room to the laboratory within an hour.

Cell Preparation

Adult human ventricular myocytes were isolated by an enzymatic dissociation method which we have previously described (Koumi et al., 1994, 1995b; Koumi, Backer & Arentzen, 1995*a*). Isolations were carried out using a fine tip needle for tissue perfusion. Specimens from the ventricular free wall of either chamber or from the intraventricular spetum were trimmed to 1 cm^3 using fine scissors, then perfused with Ca^{2+}-free Tyrode's solution via a 25 gauge surgical needle. Twenty ml of perfusion with Ca^{2+}-free Tyrode's solution (1 ml/min) were followed by perfusion with Ca^{2+}-free Tyrode's solution containing 125 U/ml collagenase (Sigma, Type V) and 1 mg/ml of bovine serum albumin (Sigma) for 20–30 min (1 ml/min). The perfusate was bubbled with 100% O_2 and warmed to 37°C. The specimen then was minced with fine scissors in the same enzyme solution. Isolated cells were separated from the minced tissue by gravity filtration through 200 μm nylon mesh and were stored in a modified Kraftbruhe (KB) solution at room temperature. Only Ca^{2+}-tolerant, clearly-striated, rod-shaped cells without any blebs were studied. Isolated cells were morphologically similar between HF and non-HF group. The resting membrane potential in isolated myocytes was −78.0 ± 4.4 mV (n = 34).

Guinea-pig ventricular myocytes were isolated by an enzymatic dissociation method similar to that described previously by Mitra & Morad (1985). To compare the isolated guinea-pig cells with those obtained from humans, we also tested the guinea-pig isolation using the same procedure as that for the human cell isolation in a pilot study. No difference was detected with the different isolation methods (n = 5).

Solutions

The transport solution contained (in mM): NaCl 27, KCl 20, $MgCl_2$, 1.5, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) 5, glucose 274 (pH = 7.0). The control Tyrode's solution contained (in mM): NaCl 140.0 KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, HEPES 5, glucose 5, brought to pH 7.4 with NaOH. Ca^{2+}-free Tyrode's solution was made by omitting $CaCl_2$ from the normal Tyrode's solution. The modified KB solution had the following composition (in mM): KCl 25, KH_2PO_4 10, KOH 116, glutamic acid 80, taurine 10, oxalic acid 14, HEPES 10, glucose 11 at pH 7.0 adjusted by KOH (Isenberg & Klockner, 1982). The pipette solution used whole-cell recording was (in mM): K-aspartate 120, KCl 20, KH_2PO_4 1, $MgCl_2$ 1, Na_2-ATP 5, ethyleneglycol-bis(β-aminoethylether)-N,N′-tetraacetic acid (EGTA) 5, HEPES 5 (pH = 7.2 with KOH). The pipette solution used for both cell-attached and inside-out patch recordings was (in mM): KCl 150, HEPES 5 (pH = 7.4). Normal Tyrode's solution was used as the external solution during whole-cell recordings. A high (150 mM) concentration of K^+ Tyrode's solution was used as the external solution during cell-attached patch recordings. In excised inside-out patch recordings, the cytosolic surface of the membrane was perfused with a bath solution containing (in mM): K aspartate 120, KCl 30, Na_2-ATP 5, $MgCl_2$ 2, HEPES 5 and EGTA 5 (pH = 7.2).

ACh (1 μM) was applied to the bath solution in whole-cell recordings. Guanosine triphosphate (GTP, 100 μM) or guanosine 5′-O-(3 thiotriphosphate) (GTPγS, 100 μM) was added to the bath solution in inside-out patch recordings. In the experiments applying GTP to the bath solution, ACh (1 μM) or adenosine (1 μM) were added to the pipette solution. Pertussis toxin (PTX) was dissolved in KB solution at a final concentration of 5 μg/ml with albumin (3 mg/ml) during myocyte incubations for up to 90 min at 35°C.

The experimental chamber (~0.2 ml) was perfused continuously with solution at a rate of 5–7 ml/min, and complete solution exchange was achieved within 5.0 ± 1.2 sec (n = 16) by measuring the current

change induced by changing the solution from 5.4 mM K^+ solution to 150 mM K^+ solution. To facilitate the rapid exchange of test solutions, they were delivered to the patch from the mouth of a fine bore polyethylene tube positioned within 1 mm from the patch. All agents were purchased from Sigma Chemical (St. Louis, MO).

Electrical Recordings

The action potential, whole-cell and single channel recordings in cell-attached and excised inside-out configurations were made by patch-clamp techniques as described by Hamill et al. (1981). The electrodes were pulled from glass capillary tubes (Kimax-51, Kimble Products), using a horizontal micropipette puller (Model P-87, Sutter Instrument, San Rafael, CA). We used electrodes with resistances of 1.5 to 2.5 MΩ to record whole-cell current and 5.0 to 10.0 MΩ to record single-channel currents. The electrode was connected to the head stage of a patch-clamp amplifier as designed by M. Yoshii (Narahashi, Tsunoo & Yoshii, 1987) The feedback resistance of the headstage was 100 MΩ for recording whole-cell current and 10 GΩ for recording single channel current. For whole-cell recording, the liquid junction potential and the series resistance attributed to the pipette tip and the cell interior were compensated by summing a fraction of the converted current signal to the command potential and feeding it to the positive input of the operational amplifier. Series resistance was compensated to minimize the time course of the capacitative surge; the capacitative transient remaining after series resistance compensation was constant throughout the experiments. The cell capacitance (C_m) was calculated from the equation:

$$C_m = Q/V \quad (1)$$

where Q is total charge movement determined by integrating the area defined by the capacitative transient in response to +10 mV voltage step (holding potential of −40 mV). The mean cell capacitance was 114.8 ± 17.9 pF (n = 20) in human ventricular myocytes. Cell capacitance was not different between HF (117.5 ± 19.4 pF, n = 10) and non-HF (113.0 ± 18.6, n = 10, not significant statistically; NS). The whole-cell membrane currents were filtered at 10 kHz with a two-pole active filter, digitized at a sampling rate of 40 kHz, and stored on the Winchester drive of an LSI 11/73 computer (Digital Equipment, Maynard, MA) for subsequent analysis. Action potentials were measured in the whole-cell current-clamp mode using an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA).

The single-channel currents were monitored with a digital oscilloscope (7101A, Kikusui), collected with an A/D converter and stored continuously on videotape using a PCM converter recording system (Unitrade, Toshiba, Tokyo). The recorded signals were reproduced and filtered offline with a cutoff frequency of 2–5 kHz through an eight-pole low-pass Bessel filter (48 dB/octave, Model 902-LPF, Frequency Devices, Haverhill, MA), digitized with 14-bit resolution at a sample rate of 10–20 kHz and stored on an LSI 11/73 computer (Digital Equipment, Maryland, MA). The data then were analyzed using locally written algorithms based on the half-amplitude threshold analysis only when a single opening level was recorded throughout the experiment (Colquhoun & Sigworth, 1983). Channel transitions were calculated using an averaging technique for channel amplitude. The measurements derived from channel transitions were collected into histograms to allow an analysis of the single-channel kinetics. The mean open lifetimes and the mean closed times were determined from the sum of exponential fits to the distribution of open and closed times recorded from patches with only one channel according to Colquhoun and Hawkes (1983). Experiments were performed at room temperature (20–22°C). For the action potential measurements, the external solution was maintained at 37°C using a Peltier thermo-electrical device.

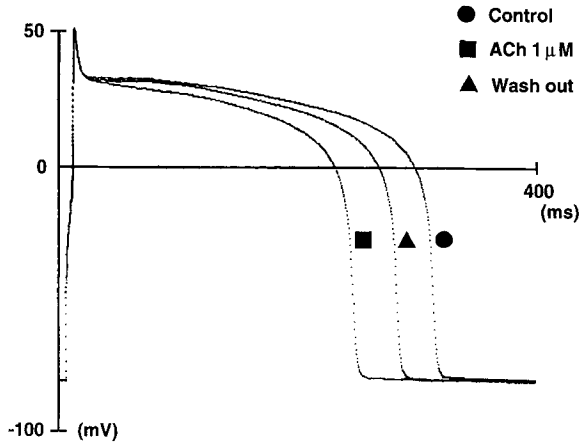

Fig. 1. Effect of ACh on the action potential recorded in human ventricular myocytes. Representative action potential and the response to ACh in an isolated ventricular myocyte from a HF heart recorded under the whole-cell current-clamp configuration at 37°C. Following application of ACh (1 μM) to the bath solution, shortening of the action potential occurred (to 78.2% of control measured at 90% repolarization) with a small hyperpolization of the resting membrane potential (−1 mV, square). The action potential partially recovered following washout of ACh for 10 min (triangle).

Data Analysis and Statistics

The results are expressed as mean ± SD. Statistical analysis was done using Student's t-test or one-way analysis of variance. In the comparison between the data from heart failure specimens and non-heart failure specimens, each data comparison was evaluated by using a two-way analysis of variance (the two-way ANOVA) to consider inter- and intra-patient variability. In addition, an analysis of covariance (ANCOVA) as employed to determine the influence of age and gender in each comparison. A nonparametric procedure in STATISTICAL ANALYSIS SYSTEM (SAS Institute, Cary, NC) on a NeXT computer (NeXT Computer, Redwood City, CA) was used for these analyses. Results were considered to be significant when the $P < 0.05$.

Results

The Action Potential Response to ACh in Isolated Human Ventricular Myocytes

Figure 1 illustrates the typical action potential and the response to ACh in an isolated ventricular myocyte. In the absence of ACh, the averaged action potential duration at 90% of repolarization was 421 ± 30 msec (n = 8). Bath application of ACh (1 μM) caused a shortening of the action potential duration, shifted the plateau level to more negative potentials and increased the resting membrane potentials (~1–2 mV). The action potential shortening by ACh was observed in eight of eight cells from

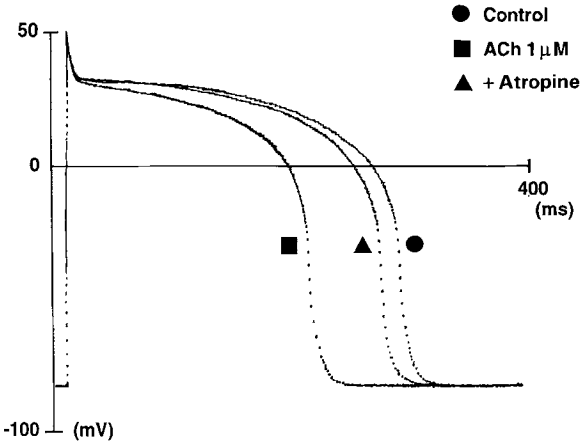

Fig. 2. Effect of atropine on the ACh-applied action potential recorded in human ventricular myocytes. The response to atropine (1 μM) on the ACh-applied action potential in an isolated ventricular myocyte from a HF heart recorded under the whole-cell current-clamp configuration at 37°C. Following application of ACh (1 μM) to the bath solution, shortening of the action potential occurred (square). The action potential duration partially recovered to the control level in the subsequent application of atropine (1 μM) in the presence of ACh (triangle).

both HF and non-HF patients. The averaged shortening of the action potential duration at 90% of repolarization was 74.4 ± 12.1% (n = 8) of control. These responses to ACh in ventricular myocytes were partially recovered after washout of ACh from the bath in eight of eight cells.

Figure 2 shows the reversal effect of atropine on the ACh-exposed action potential in a ventricular myocyte. When atropine (1 μM) was introduced to the bath solution in the presence of ACh (1 μM), the action potential duration was recovered to almost the control level. The atropine-induced recovery of action potential duration was observed in six of six cells studied.

Activation of Whole-Cell Membrane Currents by ACh

Figure 3 shows the effect of 1 μM ACh on whole-cell membrane currents in an isolated human ventricular myocyte. The original whole-cell current families in Figs. 3*A* and *B* were obtained by applying 300 msec duration voltage-clamp steps ranging between −120 and +40 mV from a holding potential of −40 mV. Currents were recorded in the presence of 5 μM tetrodotoxin (TTX) and 2 mM Co^{2+} in the external normal Tyrode's solution to block the Na^+ and Ca^{2+} currents during depolarizations to voltages positive to −30 mV. Under these conditions, bath application of ACh (1 μM) increased net membrane currents (Fig. 3*B*). The average current-voltage (*I-V*) relationship plotted at the end of test pulses (300 msec) after normalization to membrane capacitance demonstrated the increase of both inward and outward current conductances without changing the reversal potential (Fig. 3*C*). ACh-induced current was determined by subtracting the current in the presence of ACh (1 μM) from control. The *I-V* relationship of the difference current was plotted after normalization to membrane capacitance (n = 6, Fig. 3*D*). The averaged ACh-sensitive current exhibited inward-rectification and a reversal potential near the K^+ equilibrium potential (E_K: ~−80 mV). Figure 4 shows the effect of atropine on the ACh-exposed whole-cell currents. Figure 4*A* shows the original current traces at −90 mV. The current magnitude was increased during exposure to ACh (1 μM) which recovered to almost the control level when atropine (1 μM) was introduced to the bath solution. Figure 4*B* illustrates the averaged *I-V* relationships in the control, during exposure to ACh, and additional exposure to atropine. Atropine can reverse the ACh effect in seven different cells. These results suggest that ACh can increase the current by a mechanism directly involving muscarinic receptors. These results suggest that ACh can increase membrance currents in the human ventricle. However, it is unclear whether ACh-induced increase of the current is caused by the activation of the specific type muscarinic K^+ channel ($I_{K(ACh)}$) or the background inwardly-rectifying K^+channel (I_{K1}). To gain further insights into the underlying nature of the membrane current response to ACh, we studied the ACh and G protein effects on the single-channel currents.

Coupling of G Proteins to Ventricular Muscarinic K^+ Channels

Figure 5*A* shows original recordings of single $I_{K(ACh)}$ activity in human ventricular myocytes. In the presence of ACh (1 μM) in the pipette, $I_{K(ACh)}$ activity was observed in a cell-attached patch (initial part of Figure 5*A*). Channel activity exhibited rapid open-close kinetics (flickering) in brief bursts. Upon formation of an excised inside-out patch in the presence of ATP (5 mM) and Mg^{2+} (2 mM) (downward arrow head in Fig. 5*A*), ventricular $I_{K(ACh)}$ activity ceased. Application of GTP (100 μM) to the bath solution (intracellular surface of the membrane) restored the same channel activity as in a cell- attached patch (compare "*b*" with "*a*"). Channel activity disappeared following washout of GTP from the bath. GTP-activated channel activity had characteristics similar to those previously reported in human atrial $I_{K(ACh)}$ (Koumi et al., 1994; Sato et al., 1990). Bath application of GTPγS (100 μM) caused activation of $I_{K(ACh)}$ in the absence of ACh in the pipette solution (Fig. 5*B*). Different from GTP, GTPγS activated the channel

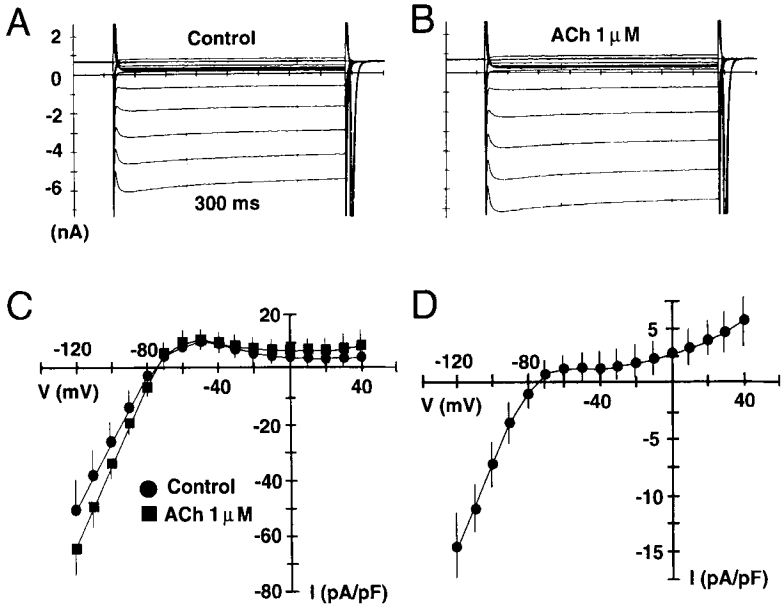

Fig. 3. Effects of ACh on whole-cell membrane currents in isolated human ventricular myocytes. *Panel A*: Control whole-cell membrane currents in the absence of ACh. Test pulses of 300 msec duration to potentials ranging from −120 to +40 mV in 10-mV steps were applied from a holding potential of −40 mV. TTX (5 μM) and Co^{2+} (2 mM) were included in the external normal Tyrode's solution to block Na^+ and Ca^{2+} currents. *Panel B*: Whole-cell currents after exposure to 1 μM ACh (same cell as in Panel *A*). *Panel C*: The averaged steady-state current-voltage (*I-V*) relationship for the membrane currents obtained during the control period (circles) and during exposure to 1 μM ACh (squares) after normalization to cell capacitance. Values were averaged from 6 different cells. *Panel D*: Whole-cell *I-V* relationships of ACh-induced currents obtained from the difference between the control and the membrane currents after exposure to ACh (1 μM) from 6 different cells. Current amplitudes were normalized to cell capacitance.

irreversibly. When ventricular myocytes were preincubated with pertussis toxin (PTX), GTP failed to activate the channel in the inside-out patch configuration (n = 10 out of 10 cells, Fig. 5*C* blank sweep), suggesting that the PTX-sensitive G protein, G_i, may be essential in the activation of ventricular $I_{K(ACh)}$. These results suggest that ACh can activate $I_{K(ACh)}$ by stimulation of muscarinic receptor coupled PTX-sensitive G_i protein.

Figure 6 shows conductance and kinetic characteristics of GTPγS-activated $I_{K(ACh)}$ in inside-out patches. ATP (5 mM) was present in the bath solution to block potential activity of the ATP-sensitive K^+ channel ($I_{K(ATP)}$). Figure 6*A* demonstrates the rapid open-close events of the GTPγS-activated $I_{K(ACh)}$ recorded at holding potential (HP) ranging from −80 to +20 mV. Unitary amplitudes increased with hyperpolarization. Outward currents were not detected at voltages positive to K^+ equilibrium potential (E_K; HP = 0 mV in symmetrical K^+ concentrations). The current-voltage (*I-V*) relationship was linear at HP less than 0 mV. The slope conductance averaged 44 ± 2 pS (n = 25). Fig. 6*C* and *D* shows representative histograms of dwell time distributions of ventricular GTPγS-activated $I_{K(ACh)}$. Open time distributions were best described by a single exponential function with an averaged mean open lifetime of 1.8 ± 0.3 msec (n = 21). The closed times had at least two exponential components. The averaged time constant of the fast component was 1.4 ± 0.4 msec, and that of the slow component was 16.5 ± 2.9 msec (n = 21). Similar $I_{K(ACh)}$ activation by GTP was observed in the presence of adenosine (1 μM) instead of ACh in the pipette solution (n = 5, *not shown*). These results indicate that G_i

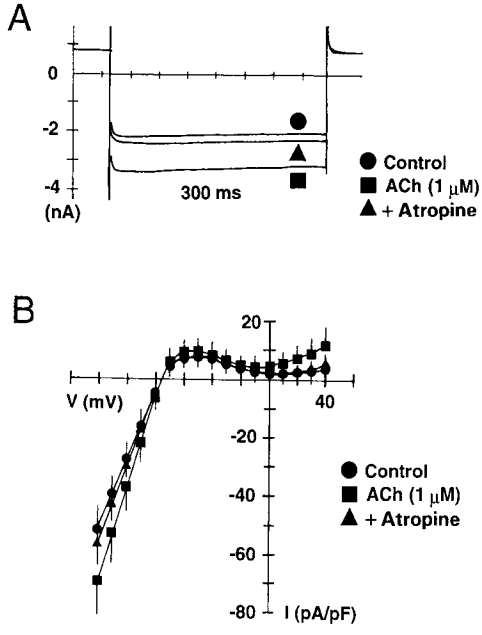

Fig. 4. Effects of atropine on the ACh-applied whole-cell membrane currents in isolated human ventricular myocytes. *Panel A*: Original whole-cell membrane current traces in the control condition (circle), during application of ACh (1 μM, square) and additional application of atropine (triangle). Test pulses of 300 msec duration to potentials of −90 mV were applied from a holding potential of −40 mV. TTX (5 μM) and Co^{2+} (2 mM) were included in the external normal Tyrode's solution to block Na^+ and Ca^{2+} currents. *Panel B*: The averaged steady-state current-voltage (*I-V*) relationship for the membrane currents obtained during the control period (circles), during exposure to 1 μM ACh (squares) and during additional exposure to 1 μM atropine (triangles) obtained from five different cells. Current amplitudes were normalized to cell capacitance.

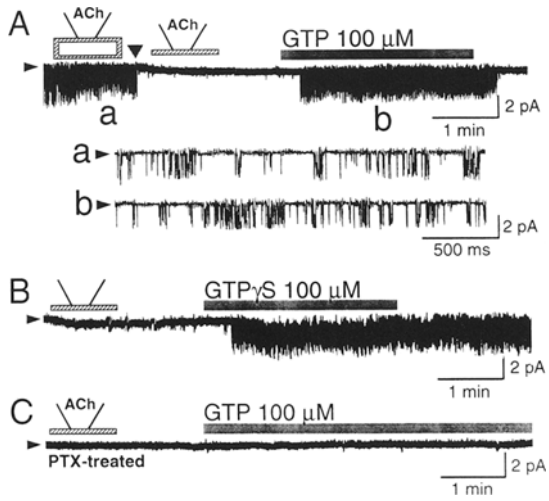

Fig. 5. Activation of the muscarinic K^+ channel by G protein in inside-out patch recordings. *Panel A*: Activation of $I_{K(ACh)}$ by GTP. Initially, $I_{K(ACh)}$ activity was observed in a cell-attached patch configuration in the presence of ACh (1 μM) in the pipette. Upon formation of an inside-out patch (downward arrow), the channel activity ceases. Application of GTP (100 μM) to the bath solution reversibly activated $I_{K(ACh)}$ to a similar degree as that in a cell-attached patch ("*a*" and "*b*" indicate records with a faster sweep speed). Arrowheads indicate the closed state (baseline level). Currents were low-pass filtered at 5 kHz at holding potential (HP) = −60 mV. *Panel B*: Activation of $I_{K(ACh)}$ by GTPγS. No agonist was included in the pipette solution. Application of GTPγS (100 μM) to the bath solution in an inside-out patch caused the activation of $I_{K(ACh)}$. Channel activity continued after washout of GTPγS from the bath. *Panel C*: Failure of GTP-induced activation of $I_{K(ACh)}$ in a myocyte pretreated with pertussis toxin (PTX). Myocytes were preincubated with PTX (5 μg/ml) and albumin (3 mg/ml) for 90 min at 35°C. ACh (1 μM) was included in the pipette solution. GTP (100 μM) was applied to the bath solution in an inside-out patch configuration as a Panel *A*, but could not activate the channel.

can activate $I_{K(ACh)}$ in human ventricular myocytes in a manner similar to atrial myocytes.

Coupling of G proteins to Ventricular Inwardly-Rectifying K^+ Channels

Figure 7 shows the activation of the channel showing slow open-close kinetics following application of GTPγS to the bath solution in inside-out patch recordings in the presence of ATP (5 mM) and Mg^{2+} (2 mM). After formation of an inside-out patch, no channel activity was detected for more than 20 min in this patch. However, channel activity appeared following addition of GTPγS (100 μM) to the bath solution within 30 sec. This channel exhibited slow gating kinetics different from $I_{K(ACh)}$. Pretreating myocytes with pertussis toxin (PTX) prevented GTP from activating the channel in inside-out patch configurations (n = 10), suggesting that the PTX-sensitive G protein, G_i, may be essential in the activation of the channel in human ventricular myocytes.

Figure 8*A* shows the original recording of the GTPγS-activated channel at various holding voltages between −100 and +20 mV in an inside-out patch in the presence of ATP (5 mM). Single-channel activity exhibited slow open-close events in long-lasting bursts (compare Fig. 8*A* with Fig. 6*A*). Slope conductances of the GTPγS-activated channel was 29 pS (Fig. 8*B*). Outward currents were not detected at voltages positive to E_K (HP = 0 mV), indicating inward rectification. The mean slope conductances of the channels activated by GTPγS averaged 30 ± 2 pS (n = 16). These results suggest that the channel is I_{K1}. The open time distribution of this channel was best described by a single exponential function with a time constant of 37.5 msec (Fig. 8*C*). The averaged mean open lifetime was 36.4 ± 4.1 msec (n = 12). The distribution of shut intervals below 100 msec duration of the channel (based on the criteria of critical value of shut time during a burst of ~90 msec by Sakmann and Trube (1984*b*), and ~100 msec by our data) had at least two exponential components (Fig. 8*D*). An averaged time constant for the fast component was 2.77 ± 0.31 msec and 29.8 ± 3.6 msec (n = 12) for the slow component. Distribution of the closed times longer than 100 msec had the time constant of 184.2 msec (n = 12). When the averaged channel open probability (P_o) was calculated, P_o exhibited clear voltage-dependent decreases with membrane hyperpolarization (*not shown*). These conductance and gating characteristics are similar to other mammalian I_{K1} (Sakmann & Trube, 1984*a,b*) and to cell-attached recordings in human ventricular I_{K1} (Koumi et al., 1995*a*).

Figure 9*A* shows an example of simultaneous activation of two distinct K^+ channels by GTP. ACh (1 μM) was included in the pipette solution. Application of GTP (100 μM) to the bath solution caused activation of both $I_{K(ACh)}$ and I_{K1} ("a" in Figure 9*A*). Following washout of GTP from the bath, $I_{K(ACh)}$ activity disappeared within 30 sec. However, I_{K1} remained active in that period ("b" in Fig. 9*A*). Fig. 9 *B* and *C* summarize the effect of GTP on the ability of both channels in several patches. Averaged P_o of $I_{K(ACh)}$ and I_{K1} were evaluated. In the presence of ACh (1 μM) in the pipette solution, bath-applied GTP (100 μM) caused channel activation in inside-out patches at HP = −60 mV. In cell-attached patches, the averaged P_o of $I_{K(ACh)}$ was 0.16 ± 0.08 (n = 8). After formation of inside-out patch configuration, channel activity decreased to nearly zero (P_o < 0.01) in the absence of GTP in the bath. P_o of GTP-activated $I_{K(ACh)}$ averaged 0.15 ± 0.06 (n = 7) which decreased to P_o < 0.01 following washout of GTP from the bath (Fig. 9*B*). In contrast to $I_{K(ACh)}$, P_o of GTP-activated I_{K1} remained constant following washout of GTP (Fig. 9*C*). The averaged P_o of I_{K1} in the cell-attached patch was 0.74 ± 0.10 (n = 7) which decreased to 0.32 ± 0.07 (n = 6) after formation of inside-out patch configuration in the

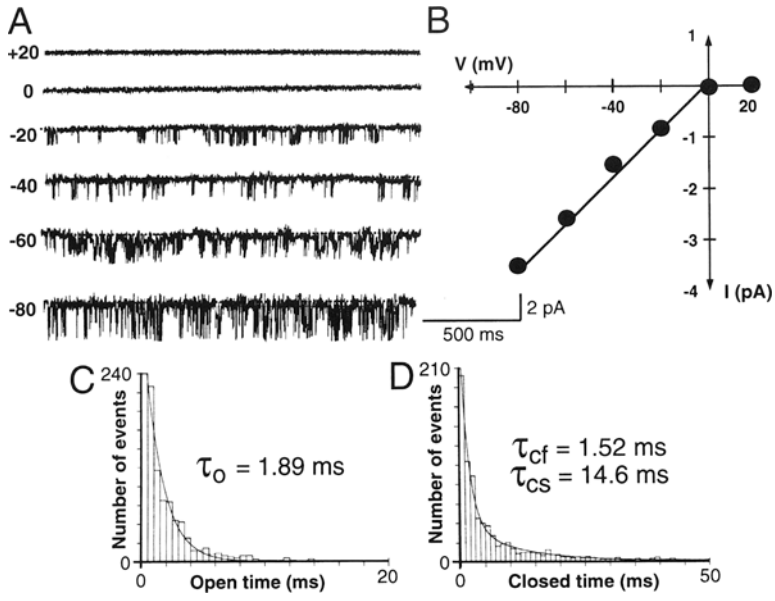

Fig. 6. Characteristics of the GTPγS-activated muscarinic K^+ channel. *Panel A*: Representative unitary currents recorded from a GTPγS-activated muscarinic K^+ channel in an inside-out patch. The holding potential (HP) is indicated to the left of each current trace. Currents were zero at 0 mV (E_K) and outward current conductances were not detected at holding voltages more positive than E_K. *Panel B*: The current-voltage (*I-V*) relationship obtained from the same patch. Inward currents demonstrated a linear slope conductance of 44 pS. *Panel C*: Histogram of open times determined at −60 mV from the same patch. The lifetimes of openings were distributed according to a single exponential function with a time constant of 1.89 msec. *Panel D*: Histogram of closed times determined from the same patch. At least two exponentials were required to describe the closed time distributions with a time constant for the fast component of 1.52 msec and that for the slow component of 14.6 msec. Currents were low pass filtered at 5 kHz.

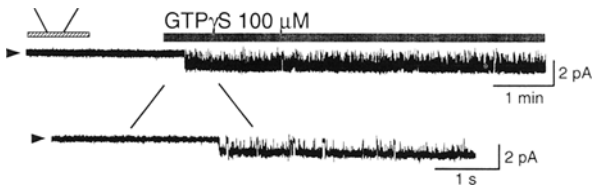

Fig. 7. Activation of the inwardly-rectifying K^+ channel by (I_{K1}) by GTPγS or $G_{i\alpha}$ in inside-out patch recordings. Unitary current traces in inside-out patch configuration at HP = −60 mV in 150 mM K^+ concentration in bath solution. No channel activity was detected in the initial part of the recordings. Application of GTPγS (100 μM) to the bath activated a channel differing from $I_{K(ACh)}$ because of slower gating kinetics and long lasting bursts. Bottom trace is a record with a faster sweep speed. Currents were low pass filtered at 2 kHz.

absence of GTP. Averaged P_O was 0.74 ± 0.12 (n = 6) in the presence of GTP, and 0.77 ± 0.09 (n = 6) after washout of GTP from the bath. These two values were not different significantly (NS). These results suggest that the G protein-activated I_{K1} channel does not require G protein to maintain channel activity, and that the underlying mechanism of I_{K1} channel activation by G protein is different from that of $I_{K(ACh)}$.

Figure 10 illustrates the GTP-concentration-dependence of $I_{K(ACh)}$ and I_{K1}. Relative P_o at each concentration of GTP was estimated. The concentration-dependent activation of the channel was fitted by the least-squares method to the equation:

$$\text{Relative } P_o = 1/\{1 + (IC_{50}/[GTP])^H\} \quad (2)$$

where IC_{50} is the concentration of GTP at the half-maximal channel activation. The relationship between [GTP] and the channel activity fit the equation with a Hill coefficient of 1.8 for both channels. The IC_{50} was 0.22 μM for $I_{K(ACh)}$ and 1.2 μM for I_{K1}.

G Protein Control of K^+ Channels in Ventricular Myocytes from Patients with and without Heart Failure and from the Guinea Pig

To assess the difference of G protein-activated K^+ channel behavior in preexisting patient conditions, individual channel characteristics were compared between patients with HF and without HF (non-HF). Twelve HF patients and 11 non-HF patients were employed in this comparison. The comparison also was made between humans and guinea pigs to determine the difference between species. A total of 40 guinea-pig ventricular cells were studied. The Table summarizes the results. Channel conductances and gating parameters from I_{K1} and $I_{K(ACh)}$ activated by G protein did not show any significant differences between patient states, suggesting that G protein-activated K^+ channel behavior may not be involved in preexisting heart diseases.

In guinea-pig ventricular myocytes, ACh (1 μM) did not produce any effects on the action potential and the whole-cell membrane current (n = 5 for each, not shown). G protein-activated channels in guinea-pig ventricular myocytes exhibited characteristics similar to those from humans with the exception of longer mean open lifetime of I_{K1}.

Discussion

The major findings in this study are as follows: (i) bath application of ACh (1 μM) shortened the action potential

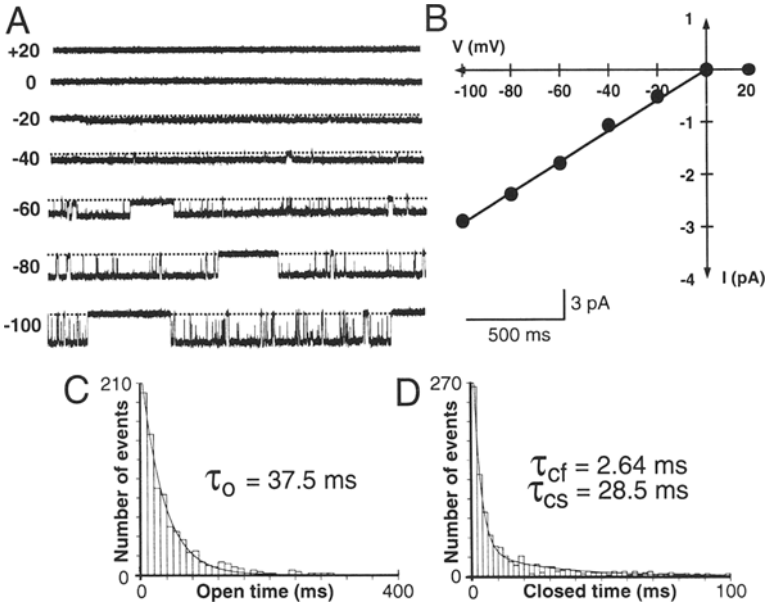

Fig. 8. Characteristics of the GTPγS-activated inwardly-rectifying K^+ channel (I_{K1}). *Panel A*: Original I_{K1} channel current traces in holding voltages between −100 and +20 mV. Unitary amplitudes increased and the channel open probabilities decreased with hyperpolarizing the holding voltages. Outward current conductance was not detected in the positive holding voltages. *Panel B*: The *I-V* plot from the same patch showing the slope conductance of 29 pS. Zero current potential was given at HP = 0 mV in symmetrical K^+ concentrations internal and external. *Panel C*: Histogram of open times in inside-out patch recordings at HP = −60 mV. The measurements were made from a patch containing only one channel. The lifetimes of openings were distributed according to a single exponential function with a time constant (τ_o) of 37.5 msec. *Panel D*: At least two exponentials were required to fit the closed time distributions below 100 msec, with a time constant for the fast component (τ_{cf}) of 2.64 msec while the time constant for the slow component (τ_{cs}) was 28.5 msec. Currents were low pass filtered at 2 kHz.

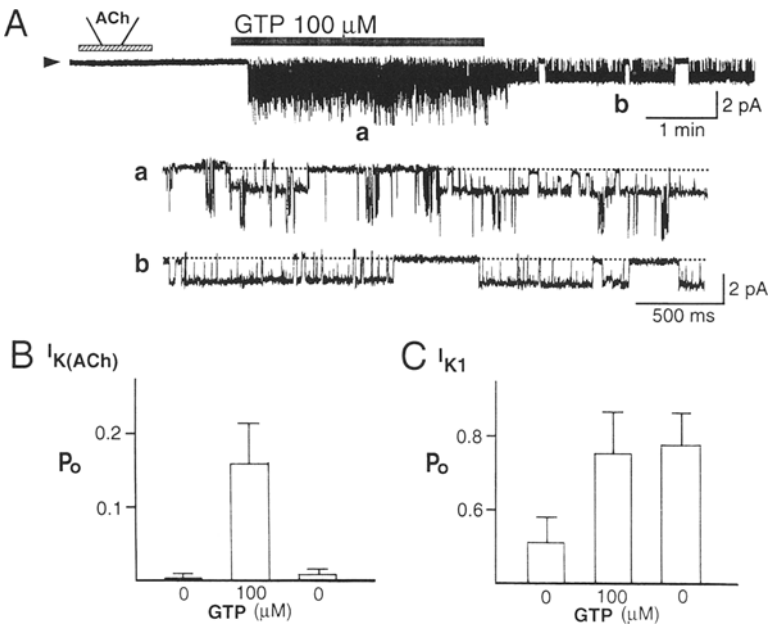

Fig. 9. Effects of GTP on channel activity of muscarinic and inwardly-rectifying K^+ channels in inside-out patches. *Panel A*: A representative record of $I_{K(ACh)}$ and I_{K1} activated by GTP (100 μM) in an inside-out patch configuration at HP = −60 mV. ACh (1 μM) was included in the pipette solution. Application of GTP to the bath solution activated both $I_{K(ACh)}$ and I_{K1}. $I_{K(ACh)}$ activity disappeared following washout of GTP from the bath. In contrast, I_{K1} activity was maintained. Bottom two traces ("*a*" and "*b*") show parts of the top record at the expanded time scale. At "*a*", simultaneous opening of $I_{K(ACh)}$ and I_{K1} was observed. At "*b*", only I_{K1} activity remained. *Panel B*: The averaged channel open probability (P_o) during exposure and after washout of GTP were calculated in inside-out patches at HP = −60 mV. P_o is 0.15 ± 0.06 (n = 7) in the presence of GTP (100 μM), while channel activity disappeared following washout of GTP from the bath, and P_o was nearly zero after washout of GTP (P_o < 0.01). *Panel C*: Effect of GTP (100 μ*M*) on I_{K1} activity. P_o value was measured in the same way as in Panel *B*. P_o was unchanged following washout of GTP from the bath. Averaged P_o values during exposure to and after washout of GTP were calculated. P_o was 0.74 ± 0.12 (n = 6) in the presence of GTP, while it was 0.77 ± 0.09 (n = 6) after washout of GTP from the bath. The two values are not statistically different.

duration and increased the whole-cell membrane current in isolated human ventricular myocytes; (ii) bath application of GTPγS could activate $I_{K(ACh)}$ and I_{K1} in excised inside-out patch configurations; (iii) GTP failed to activate these channels when myocytes were pretreated with PTX; and (iv) G protein-activated channel behavior was substantially similar between myocytes from patients with and without HF and from guinea-pig. These results suggest that activation of PTX-sensitive G protein can shorten that action potential by activating $I_{K(ACh)}$ and

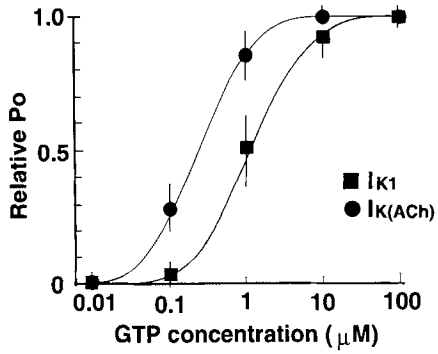

Fig. 10. GTP concentration-dependence of muscarinic and inwardly-rectifying K^+ channels in inside-out patches. GTP concentration-dependent change of channel open probability (P_o) of $I_{K(ACh)}$ and I_{K1}. ACh (1 μM) was included in the pipette solution. The graph shows the relationship between normalized P_o of the channel and the concentration of GTP from 6 different cells in $I_{K(ACh)}$ and 5 different cells in I_{K1}. The continuous line is a curve fit to the equation (2) in the text.

Table. Comparison of the G protein-activated channel characteristics between patients with and without heart failure (HF) and guinea pig

	HF (+)	HF (−)	Guinea pig
$I_{K(ACh)}$			
γ (pS) (*n*)	45 ±2 (15)	44 ±2 (10)	46 ±2 (7)
τ_o (*n*)	1.7 ± 0.4 (13)	1.9 ± 0.4 (8)	1.9 ± 0.3 (7)
I_{K1}			
γ (pS) (*n*)	30 ±2 (9)	30 ±1 (7)	33 ±3 (6)
τ_o (*n*)	35.9 ± 4.8 (7)	37.0 ± 4.1 (5)	44.6 ± 5.2* (6)

HF, heart failure; γ, slope conductance; ρ_o, time constant for open time distributions determined at HP = −60 mV in inside-out patch configurations in the presence of GTP in the bath; *n*, number of cells. Values are presented as mean ±SD. The *n* numbers used are different between γ and T_o, because the latter was calculated from the patch containing only a single channel. *P <0.01 different from other groups. Other statistical values were not statistically different between the groups (NS) estimated by the two-way ANOVA and ANCOVA.

I_{K1} without prior β-adrenergic stimulation in human ventricular myocytes.

Modulation of the Action Potential and the Whole-cell Membrane Current by Muscarinic Cholinergic Stimulation

In human ventricular myocytes, muscarinic stimulation by ACh caused shortening of the action potential duration and increased the whole-cell membrane current. ACh-induced direct increase of K^+ conductance has been reported previously in frog and ferret ventricular tissues (Hartzell & Simmons, 1987; Boyett et al., 1988). In contrast, ACh has little direct effect on ventricular K^+ conductance without prior β-adrenergic stimulation in chick, guinea-pig and other mammalian ventricular tissues (Hino & Ochi, 1980; Josephson & Sperelakis, 1982; Loffelholz & Pappano, 1985). This difference suggests that there are genuine species differences in ventricular response to ACh. In the present study, consistent with previous reports, ACh (1 μM) had no effect on action potentials in the guinea-pig ventricle. In contrast, a previous *in vivo* study in humans have suggested that ACh can directly affect ventricular myocardium (Prystowsky et al., 1981). The action potential shortening by ACh in *in vitro* conditions in the present study is consistent with this report. In addition, our single channel measurements provide new information that both $I_{K(ACh)}$ and I_{K1} may be responsible for the G protein-induced increase of K^+ conductance.

Activation of Ventricular Muscarinic and Inwardly-Rectifying K^+ Channels by G_i

In the present study, GTPγS-activated $I_{K(ACh)}$ in human ventricular myocytes exhibited characteristics nearly identical to those in human atria (Heidbuchel, Vereecke & Carmeliet, 1990; Sato et al., 1990) and to other species (Kurachi et al., 1986). The excised patch experiments revealed that signal transduction from muscarinic receptor-linked G_i to the channel does not require intracellular second messengers. Activation of human ventricular I_{K1} by GTPγS also was found in the study. The basic behavior of the G protein-activated channel was similar to cell-attached patch background I_{K1} in human atria (Koumi et al., 1995*a,b*) and in other mammalian ventricles (Sakmann & Trube, 1984*a,b*; Trube & Hescheler, 1984). The G protein-activated channel was identified as I_{K1} according to the following criteria: (i) Unitary amplitude increased and channel open probability decreased with hyperpolarization of membranes which are consistent with I_{K1} kinetics reported previously in other species (Sakmann & Trube, 1984*b*). (ii) The current was zero when the holding potential was 0 mV (E_K) in symmetrical K^+ concentrations of 150 mM (Fig. 8*A*). (iii) The channel exhibited inward-rectification without outward current conductance detectable at voltages positive to E_K (0 mV) (Fig. 8*A*). (iv) Slope conductance of the *I-V* curve was very close to that for I_{K1} channels recorded in cell-attached patches in human and other mammalian ventricular myocytes (Sakmann & Trube, 1984*b*; Koumi et al., 1995*a*). (v) Single-channel activity exhibited slow gating kinetics during long lasting bursts with mean open lifetime similar to other species (Sakmann & Trube, 1984*a*) (Fig. 8*C*).

The activation pathway of I_{K1} in the ventricle is likely to be similar to that of the $I_{K(ACh)}$. However, different from $I_{K(ACh)}$, G protein-activated I_{K1} remained active following washout of GTP from the bath, suggesting that G protein-activation of I_{K1} may be apparently a simple turn-on reaction (triggering). In Fig. 7, before

application of GTPγS, we kept the excised patch for more than 20 min. During this period, no open events of I_{K1} channels were detected. This long-lasting blank sweep without channel open events is not the interburst interval, because the calculated mean length of an apparent gap between bursts was ~240 msec for human ventricular I_{K1} channels in excised patch configurations (by using the equations in Colquhoun and Hawkes (1981)). The channel in this period is assumed to be nonfunctional. This long-lasting closed period without channel activity has been reported previously (Trube & Hescheler, 1984; Balser, Roden & Bennett, 1991). Based on these observations, the following interpretations can be made about the underlying nature of this G_i-induced I_{K1} channel activation; (i) G protein may simply modify the transition rate before channel opening without affecting gating kinetics because channel bursting behavior itself is similar between background activity of I_{K1} and the G_i-activated channel. (ii) The long closed period may be induced by an alternate gating mode (Balser et al., 1991). G_i may turn-on the channel to escape into the "normal" gating mode resulting in the recovery of burst activity. The second interpretation may be consistent with the result in which the G protein-activated channel remained active following washout of GTP from the bath. This result also suggests that G protein-activated I_{K1} may be different from a ligand-gated channel. In addition to these interpretations, another possibility is that nucleoside diphosphate kinase (NDPK) may contribute to take over channel activation by direct phosphorylation of the G protein bound GDP into GTP (Heidbuchel et al., 1993).

Physiological and Pathophysiological Implications of the G Protein-Control of K^+ Channels

In the present study, ACh shortened the human ventricular action potential duration without prior β-adrenergic stimulation at the physiological level of ATP. The shortening of the action potential duration is caused mainly by activation of the ATP-sensitive K^+ channel ($I_{K(ATP)}$) during myocardial ischemia (Noma & Shibasaki, 1985; Fosset et al., 1988). In contrast, several previous studies have reported that action potential shortening occurs when the intracellular ATP level is maintained in millimolar range in the early stages of ischemia (Allen et al., 1985; Elliott, Smith & Allen, 1989; Nichols & Lederer, 1991), indicating that simple regulation of the channel by ATP alone cannot explain this phenomenon. Our results provide new insights into the action potential shortening before the depletion of intracellular ATP. It is known that serum adenosine level is elevated during ischemia. Enhanced adenosine can activate G_i protein via A_1-purinergic receptor stimulation (Kitakaze, Hori & Kamada, 1993 for review). Prior to the activation of $I_{K(ATP)}$ in early ischemia, activation of $I_{K(ACh)}$ or I_{K1} by G_i may mimic $I_{K(ATP)}$ by contributing to the action potential shortening prior to the depletion of intracellular ATP to the micromolar level in the human heart.

Although these results provide information on the diversity in G protein modulation of K^+ channels in the human ventricle, evaluation of electrophysiological responses under *in vivo* conditions would provide further insights into the channel function related to G protein stimulation.

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