Effects of Anions on the G Protein–mediated Activation of the Muscarinic K^+ Channel in the Cardiac Atrial Cell Membrane

Intracellular Chloride Inhibition of the GTPase Activity of G_K

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ABSTRACT The effects of various intracellular anions on the G protein (G_K)–mediated activation of the muscarinic K^+ (K_{ACh}) channel were examined in single atrial myocytes isolated from guinea pig hearts. The patch clamp technique was used in the inside-out patch configuration. With acetylcholine (ACh, 0.5 μM) in the pipette, 1 μM GTP caused different magnitudes of K_{ACh} channel activation in internal solutions containing different anions. The order of potency of anions to induce the K_{ACh} channel activity at 0.5 μM ACh and 1 μM GTP was $Cl^- \geq Br^- > I^-$. In the SO_4^{2-} or aspartic acid internal solution, no channel openings were induced by 1 μM GTP with 0.5 μM ACh. In both the Cl^- and SO_4^{2-} internal solutions (with 0.5 μM ACh) the relationship between the concentration of GTP and the channel activity was fit by the Hill equation with a Hill coefficient of ~3–4. However, the concentration of GTP at the half-maximal activation (K_d) was 0.2 μM in the Cl^- and 10 μM in the SO_4^{2-} solution. On the other hand, the quasi–steady-state relationship between the concentration of guanosine-5′-*o*-(3-thiotriphosphate) and the channel activity did not differ significantly between the Cl^- and SO_4^{2-} solutions; i.e., the Hill coefficient was ~3–4 and the K_d was ~0.06–0.08 μM in both solutions. The decay of channel activity after washout of GTP in the Cl^- solution was much slower than that in the SO_4^{2-} solution. These results suggest that intracellular Cl^- does not affect the turn-on reaction but slows the turn-off reaction of G_K, resulting in higher sensitivity of the K_{ACh} channel for GTP. In the Cl^- solution, even in the absence of agonists, GTP (>1 μM) or ATP (>1 mM) alone caused activation of the K_{ACh} channel, while neither occurred in the SO_4^{2-} solution. These observations suggest

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that the activation of the K_{ACh} channel by the basal turn-on reaction of G_K or by phosphate transfer to G_K by nucleoside diphosphate-kinase may depend at least partly on the intracellular concentration of Cl^-.

INTRODUCTION

GTP-binding (G) proteins act as transducers between membrane receptors and effectors, including adenylyl cyclase, phospholipase C, and ion channels (Gilman, 1987; Neer and Clapham, 1988; Brown and Birnbaumer, 1990). The activity of G proteins is supposed to be regulated in a cyclic manner by the turn-on and turn-off reactions: (*a*) agonist binding to the membrane receptors facilitates the release of GDP and subsequent binding of GTP to the G protein (turn-on reaction), resulting in the functional dissociation of the G protein into its subunits (G_α-GTP and $G_{\beta\gamma}$), which in turn activate or inhibit the effectors; and (*b*) GTP bound to G_α is hydrolyzed to GDP by the intrinsic GTPase activity of G_α. The GDP-bound form of G_α associates with $G_{\beta\gamma}$ to return back to the trimeric inactive G protein (turn-off reaction). Various toxins, substances, and ions such as cholera toxin, pertussis toxin (PT), heparin, *ras* p21-GTPase activating protein, and magnesium (Mg^{2+}) affect various steps in these reactions and can modulate the activation of the G protein (Gilman, 1987; Ito, Takikawa, Iguchi, Hamada, Sugimoto, and Kurachi, 1990; Yatani, Okabe, Polakis, Halenbeck, McCormick, and Brown, 1990). It is also reported that the intracellular chloride ion (Cl^-) can stimulate the G protein activity by (*a*) preventing dissociation of GTPγS from G_s or G_o in the absence of Mg^{2+} (Northup, Smiegel, Sternweis, and Gilman, 1983; Higashijima, Ferguson, and Sternweis, 1987*b*), (*b*) potentiating dissociation of G_i into $G_{i\alpha}$ and $G_{\beta\gamma}$ subunits (Sternweis, 1986), and (*c*) disturbing the intrinsic GTPase activity of G_o (Higashijima et al., 1987*b*).

In cardiac atrial myocytes, muscarinic acetylcholine (m-ACh) receptors are linked to a specific population of inward-rectifying K^+ channels (K_{ACh}) by PT-sensitive G proteins (G_K) (Breitwieser and Szabo, 1985; Pfaffinger, Martin, Hunter, Nathanson, and Hille, 1985; Kurachi, Nakajima, and Sugimoto, 1986*a*, *b*). This K_{ACh} channel activity is also supposed to be regulated by agonist-dependent turn-on and turn-off reactions of G_K in analogy to the regulation of adenylyl cyclase (Kurachi, Nakajima, and Sugimoto, 1986*c*; Logothetis, Kurachi, Galper, Neer, and Clapham, 1987; Nanavati, Clapham, Ito, and Kurachi, 1990). Although the intracellular solution containing various concentrations of Cl^- have been used in the electrophysiological experiments on the K_{ACh} channel (Kurachi et al., 1986*a*, *b*; Breitwieser and Szabo, 1988; Ito, Sugimoto, Kobayashi, Takahashi, Katada, Ui, and Kurachi, 1991; Okabe, Yatani, and Brown, 1991), the effects of anions on the G protein–mediated activation of the K_{ACh} channel have not yet been examined.

In this study we compared the effects of intracellular chloride and sulfate ions on the G_K-mediated activation of the K_{ACh} channel. Our results clearly show that intracellular Cl^- increased the sensitivity of the K_{ACh} channel to intracellular GTP, probably by disturbing the turn-off reaction of G_K. The effects of anions on the activation of the K_{ACh} channel by either the basal turn-on reaction of G_K (Ito et al., 1991; Okabe et al., 1991) or the nucleoside diphosphate kinase-mediated phosphate transfer to G_K (Heidbüchel, Callewaert, Vereecke, and Carmeliet, 1990*a*, *b*; Kaibara, Nakajima, Irisawa, and Giles, 1991) were also examined in the present study.

MATERIALS AND METHODS

Cell Preparation

Single atrial cells were isolated by an enzymatic dissociation from guinea pig hearts using methods described previously (Kurachi et al., 1986*b*). Briefly, collagenase (0.2% wt/vol) in the Ca^{2+}-free bathing solution was perfused for 15–20 min through the coronary arteries using a Langendorff apparatus (37°C). Thereafter, the heart was stored in the high K^+/low Cl^- solution at 4°C for later experiments. Single atrial cells were dispersed by trituration in a recording chamber filled with the control bathing solution. This procedure yielded an acceptable number of quiescent and relaxed cells.

Solutions and Drugs

The control bathing solution contained (mM): 136.5 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.53 $MgCl_2$, 5.5 glucose, and 5 HEPES-NaOH buffer, pH 7.4. The Ca^{2+}-free bathing solution was the same as the control bathing solution except that $CaCl_2$ was omitted. The high K^+/low Cl^- solution contained (mM): 70 K glutamate, 25 KCl, 10 KH_2PO_4, 10 taurine, 0.5 EGTA, 11 glucose, and 10 HEPES-KOH buffer, pH 7.4. In the whole-cell voltage clamp experiments the pipette solution contained (mM): 130 KCl or 65 K_2SO_4, 2 $MgCl_2$, 5 EGTA, 3–4 K_2ATP, 0.1 GTP

TABLE I

Compositions of Internal Solutions

130 KCl, 5 EGTA, 2 $Mg(NO_3)_2$, 5 HEPES
130 KBr, 5 EGTA, 2 $Mg(NO_3)_2$, 5 HEPES
130 KI, 5 EGTA, 2 $Mg(NO_3)_2$, 5 HEPES
65 K_2SO_4, 5 EGTA, 2 $Mg(NO_3)_2$, 5 HEPES
130 K aspartate, 5 EGTA, 2 $Mg(NO_3)_2$, 5 HEPES

pH was adjusted to 7.3 with KOH.

(sodium salt), and 10 HEPES-KOH buffer, pH 7.4. In the inside-out patch experiments, the pipettes were filled with a solution containing (mM): 130 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES-KOH, pH 7.4. 0.5 μM ACh was added to the pipette solution when indicated in the text. The composition of the internal solutions for inside-out patches is listed in Table I. To change the concentration of Cl^-, KCl was substituted with equimolar K_2SO_4. ACh, guanosine-5′-triphosphate (GTP, sodium salt), adenosine 5′-triphosphate (ATP, potassium salt) were purchased from Sigma Chemical Co. (St. Louis, MO). Guanosine-5′-*o*-(3-thiotriphosphate) (GTPγS) was from Boehringer-Mannheim (Mannheim, Germany). In each experiment, the stock solutions of the drugs, stored at −80°C, were diluted in the internal solution to desired concentrations just before use. All experiments were performed at 35–37°C.

Electrophysiological Methods and Data Analysis

The currents were measured by using the patch clamp technique in the inside-out patch and whole-cell configurations (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) through a patch clamp amplifier (EPC-7; List, Darmstadt, Germany) and monitored with a storage oscilloscope (VC-6025; Hitachi, Tokyo, Japan). The resistance of the patch electrodes was 3–5 MΩ, and the tip of each electrode was coated with Sylgard and fire-polished. The data were stored in a video cassette recorder (BR6400; Victor, Tokyo, Japan) using a PCM converter system (RP-880; NF Electronic Circuit Design, Tokyo, Japan, or VR-10; Instrutech, Elmont,

NY, with a 10 kHz bandwidth), reproduced and low-pass filtered at 1.5–2 kHz (at −3 dB) by a Bessel filter (FV-625A, NF Electronic Circuit Design; 48 dB/octave slope attenuation), sampled at 5 kHz, and analyzed off-line on a computer (PC-9800 VM2; NEC, Tokyo, Japan). For single channel analysis, the threshold used to judge the open state was set at half of the single channel amplitude (Colquhoun and Sigworth, 1983). Data were expressed as mean ± SD.

Perfusion Method

The chamber was continuously perfused by the bathing and internal solutions. A very narrow recording chamber (2 mm in width and 15 mm in length) was used. The depth of the perfusion solution was ~0.7–1 mm. The solution was perfused at a rate of ~5–7 ml/min with gravity. The rate of exchanging the internal solution in the inside-out patches in Fig. 6 was estimated by measuring the current alteration induced by changing the solution from the KCl internal solution containing 0.1 mM GTP to the internal solution containing 70 mM KCl, 60 mM NaCl, 2 mM $MgCl_2$, 0.1 mM GTP, 5 mM EGTA, and 5 mM HEPES-KOH (pH 7.3). 90% exchange occurred at 0.77 ± 0.16 s (mean ± SD, n = 20).

RESULTS

Intracellular Anions as Modulators of the G Protein–gated Muscarinic K^+ Channel (K_{ACh}) in Single Atrial Myocytes

Fig. 1 shows the effects of several anions on the K_{ACh} channel in an inside-out patch of the guinea pig atrial cell membrane. With 0.5 μM ACh in the pipette solution, the application of GTP (1 μM) caused full activation of the K_{ACh} channel in the internal solution containing 130 mM Cl^-. When Cl^- was replaced by 65 mM sulfate ion (SO_4^{2-}), the channel activity was completely abolished in the continuous presence of GTP (Fig. 1 *A*). When SO_4^{2-} was changed to Cl^-, the channel activity resumed. In the 130 mM bromide ion (Br^-) or iodide ion (I^-) containing internal solutions, GTP (1 μM) also induced openings of the K_{ACh} channel. When Br^- was replaced by Cl^-, the channel activity ($N \cdot P_o$; N is the number of channels in the patch, and P_o is the open probability of each channel) slightly increased from 0.1688 to 0.1818 in Fig. 1 *B*. After washout of GTP, the channel activity disappeared (Fig. 1 *B*). The channel activity induced by 1 μM GTP in the I^- internal solution was much less than that in the Cl^- internal solution: In Fig. 1 *C*, the $N \cdot P_o$ of the K_{ACh} channel in the I^- internal solution was 0.0231 and increased to 0.1553 in the Cl^- internal solution. Cl^-, Br^-, I^-, and SO_4^{2-} alone did not activate the channel in the absence of GTP. These results provide direct evidence that intracellular anions modulate the GTP-dependent activation of the K_{ACh} channel in inside-out patches of the atrial cell membrane.

Fig. 1 *D* compares the K_{ACh} channel activity induced by 1 μM GTP in the internal solutions containing various anions. The data for each anion were obtained from four patches in the presence of 0.5 μM ACh. The relative $N \cdot P_o$ of the K_{ACh} channel induced by GTP (1 μM) in the presence of internal anions was expressed with reference to the $N \cdot P_o$ in the 130 mM Cl^- internal solution in each patch. The order of potency of anions to induce the K_{ACh} channel activity in the presence of 0.5 μM ACh and 1 μM GTP was $Cl^- \geq Br^- > I^-$. In the SO_4^{2-} and L-aspartic acid internal solutions, no significant openings of the K_{ACh} channel were observed.

Fig. 2 *A* shows the K_{ACh} channel openings induced by 100 μM GTP with 0.5 μM ACh at various membrane potentials (see also Fig. 4). In both the 130 mM Cl^- and

65 mM SO_4^{2-} internal solutions, similar pulse-like channel openings were observed. With hyperpolarization, the unit amplitude of the channel increased. With depolarization, the amplitude decreased and became zero at around the K^+ equilibrium potential (~ 0 mV in the present experimental condition). At more positive potentials, small outward currents were observed. In both cases, the unitary conductance of the GTP-activated channels was 40–45 pS and showed a strong inward rectification in the presence of 2 mM intracellular Mg^{2+}. Their current–voltage relationships were almost superimposable (Fig. 2 *B*). In addition, the open-time histogram of the GTP-activated K_{ACh} channels at −80 mV could be fit by a single exponential curve

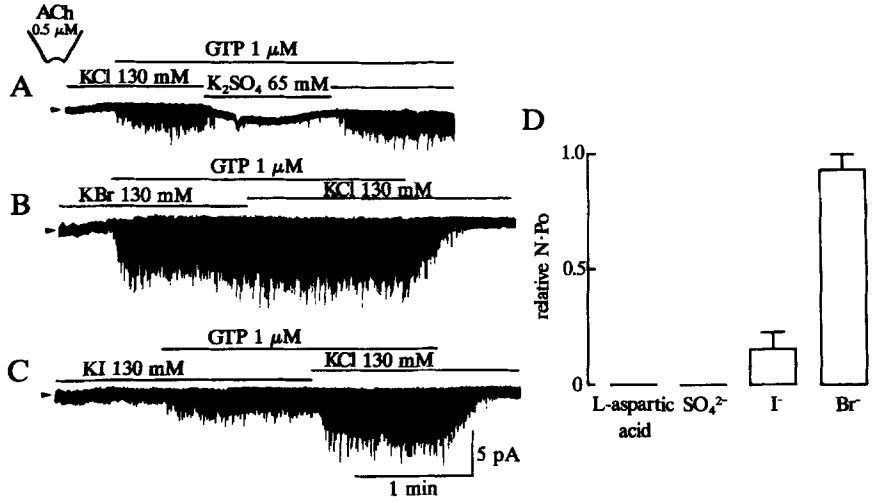

FIGURE 1. Effects of intracellular anions on the muscarinic K^+ channel in inside-out patches. (*A*, *B*, *C*) Inside-out patch experiments. The holding potential was −80 mV. The pipette solution contained 0.5 μM ACh. The protocol for perfusing various internal solutions and GTP are indicated by the bars above each current trace. Arrowheads indicate the zero current level. Effects of the Cl^- and SO_4^{2-} internal solutions on the 1 μM GTP-induced activation of the K_{ACh} channel were compared in *A*, Br^- and Cl^- in *B*, and I^- and Cl^- in *C*. Note that each anion by itself did not activate the channel. (*D*) Comparison of the effects of anions on the open probability of the K_{ACh} channels activated by GTP (1 μM). The relative $N \cdot P_o$ of the K_{ACh} channel activated by GTP (1 μM) in various internal anions (SO_4^{2-}, Br^-, I^-) and L-aspartic acid was calculated with reference to the $N \cdot P_o$ in the 130 mM Cl^- internal solution. The data for each symbol were obtained from four patches in each case.

with a time constant of ~ 1 ms in both the 130 mM Cl^- and the 65 mM SO_4^{2-} internal solutions (Fig. 2 *C*). These observations indicate that the conductance and kinetic properties of the K_{ACh} channel were not affected by these anions.

Fig. 3 showed the concentration-dependent effect of Cl^- on activation of the K_{ACh} channels induced by 1 μM GTP. The pipette solution contained 0.5 μM ACh. SO_4^{2-} in the internal solution was replaced by Cl^- as indicated above the trace (Fig. 3 *A*). GTP (1 μM) was continuously perfused from the intracellular side of the patch. ACh (0.5 μM) was present in the pipette. In the 65 mM SO_4^{2-} internal solution, the channel openings were not observed in the presence of 1 μM GTP. As the

concentration of internal Cl^- ($[Cl^-]_i$) increased, the K_{ACh} channel openings were enhanced in a concentration-dependent manner. Fig. 3 *B* shows the relationship between $[Cl^-]_i$ and $N \cdot P_o$ of the K_{ACh} channel at 1 μM GTP. The data were obtained from three patches. Relative $N \cdot P_o$ of the K_{ACh} channel at each $[Cl^-]_i$ was calculated with references to the $N \cdot P_o$ at 130 mM $[Cl^-]_i$. The relative $N \cdot P_o$ was 0.05 at 20 mM $[Cl^-]_i$, and increased in a concentration-dependent manner. The half-maximal activation occurred at ~70 mM $[Cl^-]_i$.

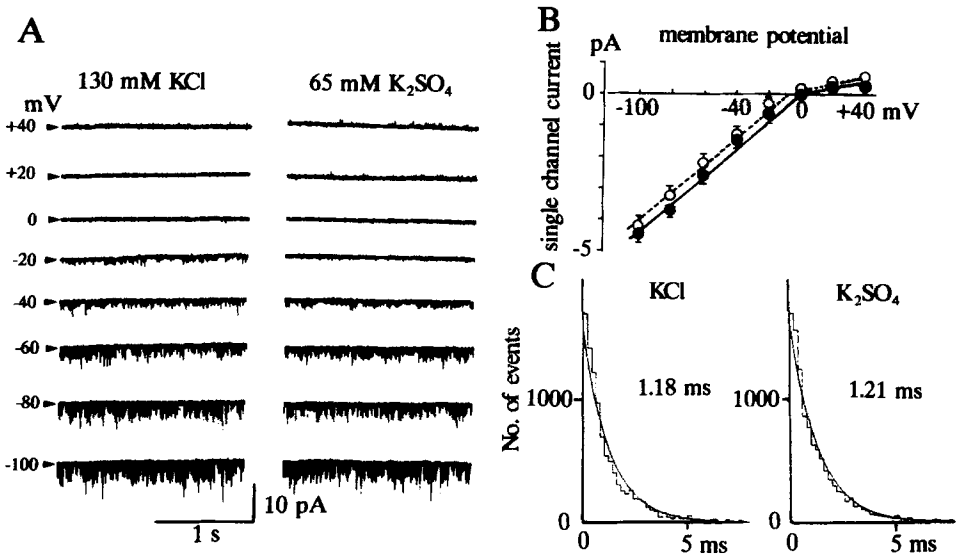

FIGURE 2. Effect of intracellular anions on the conductance and kinetic properties of the K_{ACh} channel. (*A*) Inside-out patch configuration. The current recordings of the K_{ACh} channel induced by GTP (100 μM) at various membrane potentials were shown in the 130 mM Cl^- and 65 mM SO_4^{2-} internal solutions. (*B*) Current–voltage relationships of the K_{ACh} channel in the 130 mM Cl^- (●) and 65 mM SO_4^{2-} (○) internal solutions. The data were obtained from three patches in each case. (*C*) Open-time histograms of the K_{ACh} channel induced by GTP (100 μM) in the 130 mM Cl^- and 65 mM SO_4^{2-} internal solutions. The membrane potentials were −80 mV in both cases.

Effects of Anions on the Concentration-dependent Activation of the K_{ACh} Channel Activity by GTP and GTPγS

To clarify the roles of intracellular anions on the activation of the K_{ACh} channel, we examined the concentration-dependent effect of intracellular GTP on the channel activity in the 130 mM Cl^- and 65 mM SO_4^{2-} internal solutions (Fig. 4). The pipette solution contained 0.5 μM ACh. Various concentrations of GTP ([GTP]) were applied to the internal side of the patch membrane. In both internal solutions, channel openings increased dramatically with increasing $[GTP]_i$. In the Cl^- internal solution, the channel activity reached a maximal value at 0.3–1 μM $[GTP]_i$ as reported

previously (Kurachi, Ito, and Sugimoto, 1990; Ito et al., 1991). On the other hand, in the SO_4^{2-} internal solution it reached a maximal value at ~100 μM $[GTP]_i$.

Fig. 4 *C* compares the $[GTP]_i$ dependence of the open probability of the K_{ACh} channel in the Cl^- and SO_4^{2-} internal solutions. The data for each symbol were obtained from five different patches. The relative $N \cdot P_o$ of the K_{ACh} channel at each

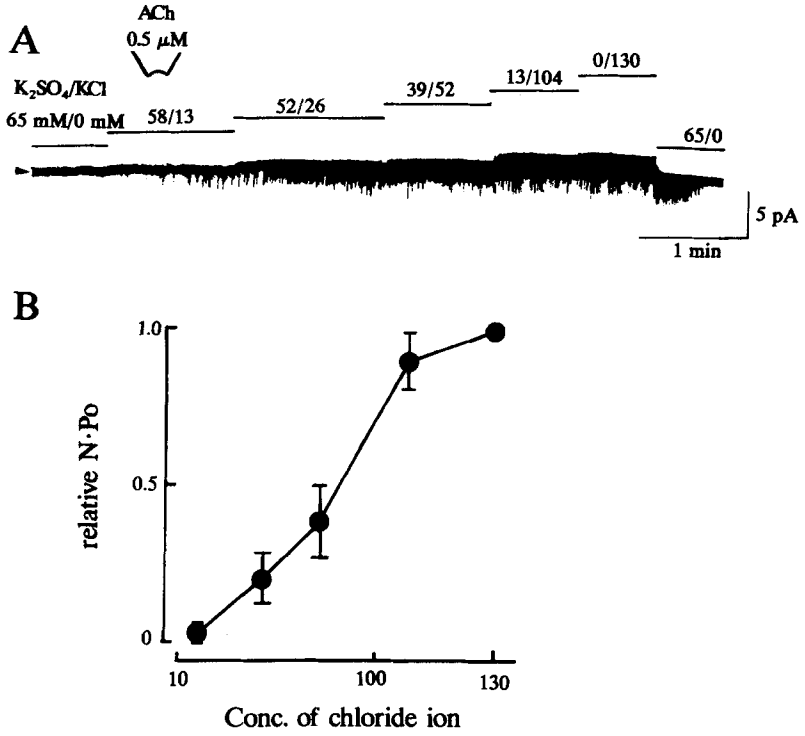

FIGURE 3. Concentration-dependent effect of intracellular Cl^- on the activity of the K_{ACh} channel induced by GTP. (*A*) A typical experiment of inside-out patches. The pipette solution contained 0.5 μM ACh. GTP (1 μM) was perfused throughout the experiment. The membrane potential of the patch was −80 mV. The protocol for perfusing the internal solution containing various concentrations of Cl^- was indicated above the trace. Cl^- was replaced by SO_4^{2-}. Note that channel openings increased with increasing $[Cl^-]_i$. (*B*) The relationship between $[Cl^-]_i$ and the relative $N \cdot P_o$ of the K_{ACh} channel. The relative $N \cdot P_o$ at each $[Cl^-]_i$ was obtained with reference to that at 130 mM $[Cl^-]_i$. The patch contained 0.5 μM ACh, and the channel activity was induced by 1 μM GTP. Symbols and bars are mean ± SD (n = 3).

$[GTP]_i$ was calculated with reference to the $N \cdot P_o$ of 10 μM GTPγS-induced channel openings in each patch. The concentration–response relations in the Cl^- and SO_4^{2-} internal solutions were fit by the Hill equation using the least-squares method (Yamaoka, Tanigawara, Nakagawa, and Uno, 1981):

$$y = V_{max}/\{1 + K_d/[GTP])^H\}$$

where y is the relative $N \cdot P_o$, V_{max} is the maximal relative $N \cdot P_o$, K_d is $[GTP]_i$ at the half-maximal activation of the channel, and H is the Hill coefficient.

The relationship between $[GTP]_i$ and the channel activity fit the Hill equation with the Hill coefficient of 3.7 in the 130 mM Cl^- internal solution and 3.6 in the 65 mM SO_4^{2-} solution. However, the relationship in the SO_4^{2-} internal solution was shifted to the right compared with that in the Cl^- internal solution: The K_d value was 0.18 μM

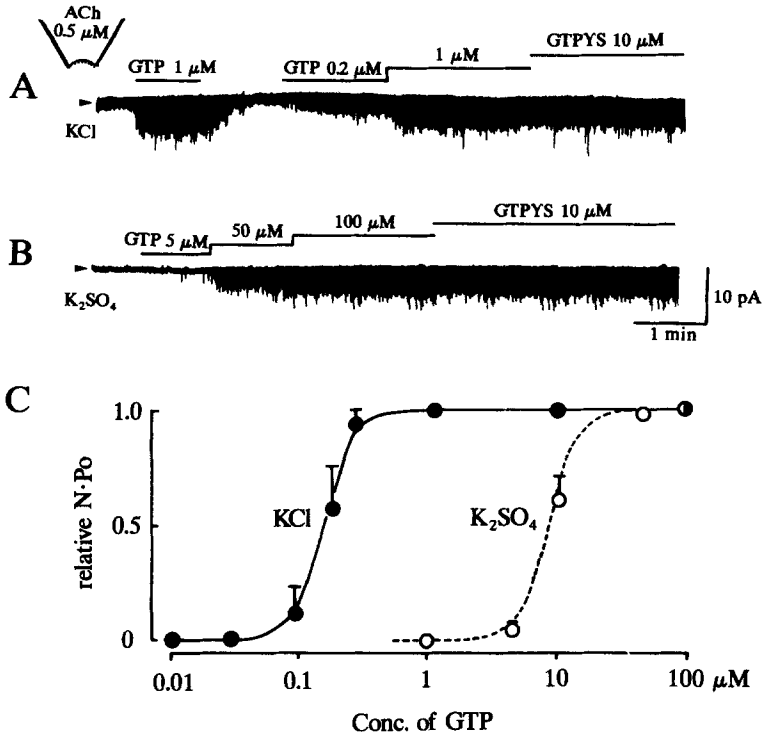

FIGURE 4. Effects of intracellular chloride and sulfate ions on concentration-dependent activation of the K_{ACh} channel by GTP in the presence of ACh. Inside-out patch experiments. The holding potential was −80 mV. The pipette solution contained 0.5 μM ACh. Various concentrations of GTP and 10 μM GTPγS were added to the 130 mM Cl^- internal solution in *A* and to the 65 mM SO_4^{2-} internal solution in *B*. (*C*) The relationships between the concentration of GTP and the relative $N \cdot P_o$ of the K_{ACh} channel with reference to that induced by GTPγS (10 μM) in the 130 mM Cl^- (●) and 65 mM SO_4^{2-} (○) internal solutions. Symbols and bars are mean ± SD (n = 5). The continuous curves are fit by the Hill equation with the least-squares method.

in the Cl^- internal solution and 11 μM in the SO_4^{2-} internal solution. These results indicate that intracellular anions affect the sensitivity of the K_{ACh} channel to intracellular GTP.

In Fig. 5, we compared the effects of Cl^- and SO_4^{2-} on the activation of the K_{ACh} channel induced by GTPγS, a nonhydrolyzable GTP analogue. The patch pipette contained 0.5 μM ACh. As $[GTP\gamma S]_i$ was raised from 0.01 or 0.03 to 0.1, 1, and 10

μM, channel openings increased in a similar concentration-dependent fashion in both the 130 mM Cl^- and 65 mM SO_4^{2-} internal solutions (Fig. 5, *A* and *B*). Few or no channel openings were induced at 0.03 μM $[GTP\gamma S]_i$ up to 5–7 min of superfusion. The openings reached a maximal value at 0.1–1.0 μM $[GTP\gamma S]_i$ in both cases. Fig. 5 *C* shows the relationships between $[GTP\gamma S]_i$ and the quasi-steady-state open probability of the K_{ACh} channel in the Cl^- and SO_4^{2-} internal solutions. The data for each symbol were obtained from five patches with ACh (0.5 μM) in the pipette. Relative $N \cdot P_o$ of the K_{ACh} channel was calculated with reference to the 10 μM

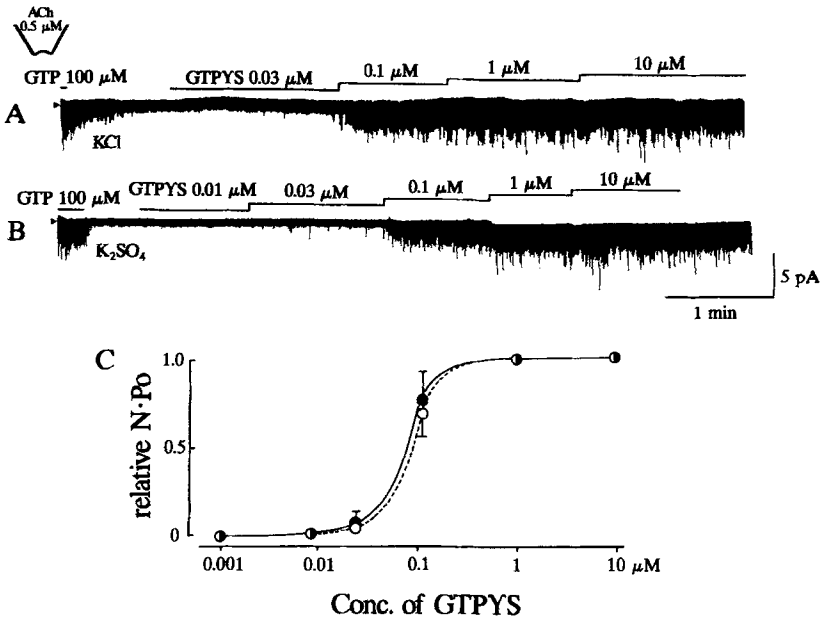

FIGURE 5. Effect of anions on the concentration-dependent effect of GTPγS on the K_{ACh} channel. Inside-out patch experiments. The holding potential was −80 mV. The patch pipette contained 0.5 μM ACh. Concentration-dependent effects of GTPγS on the K_{ACh} channel openings in the 130 mM Cl^- (*A*) and in the 65 mM SO_4^{2-} internal solutions (*B*) are shown. (*C*) The relationships between $[GTP\gamma S]_i$ and the relative $N \cdot P_o$ of the K_{ACh} channel in the 130 mM Cl^- (●) and 65 mM SO_4^{2-} (○) internal solutions. Symbols and bars are mean ± SD (n = 5). The continuous curves are fit by the Hill equation with the least-squares method. Note that these curves are almost superimposable.

GTPγS-induced channel openings in each patch. Each concentration–response curve was fit by the Hill equation. In contrast to the case of GTP illustrated in Fig. 4, the relationships between $[GTP\gamma S]_i$ and the channel activity did not differ significantly between the Cl^- and the SO_4^{2-} internal solutions: the Hill coefficient was 3.5 and 3.4, and K_d was 0.06 and 0.08 μM in the Cl^- and SO_4^{2-} internal solutions, respectively. Similar findings were observed in the absence of ACh in the patch pipette (not shown). These results indicate that neither the turn-on reaction of GTP analogues on G_K nor the interaction between the activated G_K and the K_{ACh} channel are signifi-

cantly affected by intracellular anions. Therefore, it is likely that the turn-off reaction of G_K through hydrolysis of GTP bound to G_K may be involved in the effects of anions.

Effects of Anions on the Deactivation of the K_{ACh} Channel

To examine the effects of intracellular anions on the turn-off reaction of G_K, the effects of Cl^- and SO_4^{2-} internal solutions on the decay rate of the channel activity upon washout of GTP were compared (Fig. 6 *A*). The pipette solution contained 0.5 μM ACh. In the Cl^- internal solution (Fig. 6 *A*, *a*), after washout of GTP (100 μM)

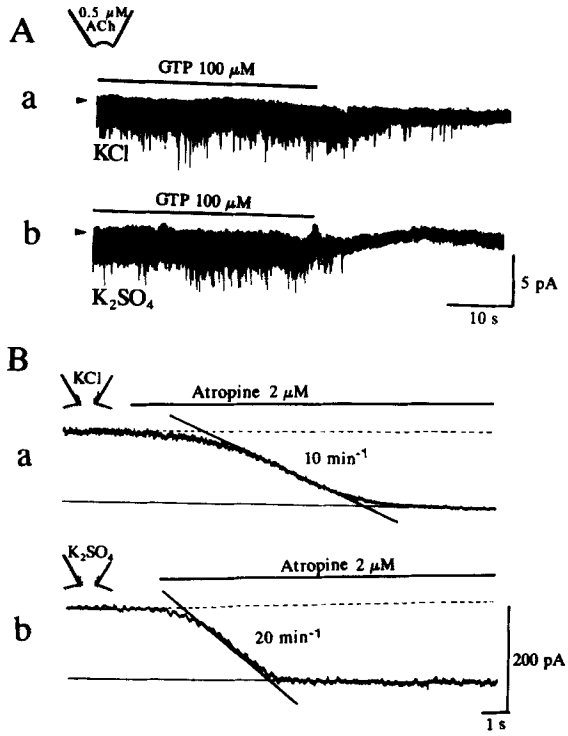

FIGURE 6. Effects of intracellular anions on the turn-off of the K_{ACh} channel activity. (*A*) In the inside-out patch configuration, GTP (100 μM) was quickly washed out in the 130 mM Cl^- (*a*) and the 65 mM SO_4^{2-} (*b*) internal solutions. The pipette solution contained 0.5 μM ACh. The holding potential was −80 mV. The protocols are indicated above the current recordings. Note that the decay of the channel activity in the 130 mM Cl^- solution was much slower than that in the 65 mM SO_4^{2-} solution. (*B*) The atrial cells were held at −40 mV under the whole-cell clamp condition. Atropine (2 μM) was added to the bathing solution containing 0.5 μM ACh as indicated by the bars above the current traces. The pipette solution contained the 130 mM Cl^- internal solution in *a* and the 65 mM SO_4^{2-} internal solution in *b*. After the application of atropine, the K_{ACh} channel current was abolished but the decay of the current in the Cl^- pipette was slower than that in the SO_4^{2-} pipette.

the channel activity gradually declined and disappeared within 15–30 s (see also Fig. 1, *B* and *C*). The half-time for complete decay of the K_{ACh} channel was 12 ± 4 s (n = 5). On the other hand, in the SO_4^{2-} internal solution, the channel activity was abolished within 2–5 s after washout of GTP (Fig. 6 *A*, *b*). The half decay time was 3 ± 1 s (n = 5). Note that the half decay time in the SO_4^{2-} internal solution might have been overestimated due to the present perfusion technique (see Materials and Methods). Thus, the decay of the channel activity on washout of GTP in the Cl^- solution was much slower than that in the SO_4^{2-} solution.

Similar results were obtained in whole-cell voltage clamp experiments using pipettes filled with either the 130 mM Cl^- or the 65 mM SO_4^{2-} internal solution (Fig. 6 *B*). Cells were held at −40 mV. After the K_{ACh} channel current was activated by ACh (0.5 μM), the muscarinic antagonist, atropine (2 μM), was added to the bath solution. In both cases, atropine abolished the ACh-induced outward K^+ current (Fig. 6 *B*, *a*

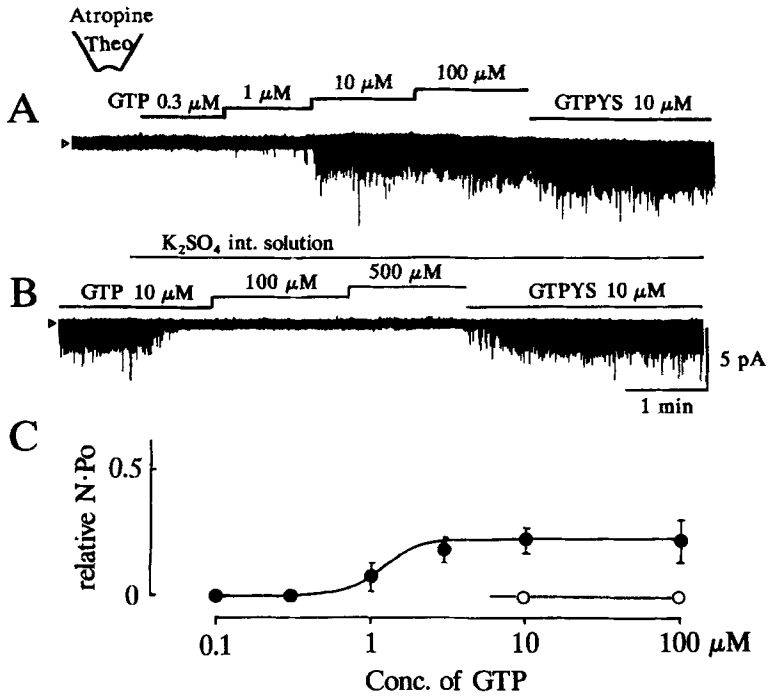

FIGURE 7. Effect of intracellular anions on the activity of K_{ACh} channels by GTP in the absence of ACh. Inside-out patch experiments. The holding potential was −80 mV. The pipette contained atropine (1 μM) and theophylline (200 μM). (*A*) In the 130 mM Cl^- internal solution, various concentrations of GTP and 10 μM GTPγS were applied. GTP alone activated the K_{ACh} channel in a concentration-dependent manner, but the maximal channel activity was smaller than that by GTPγS (10 μM). (*B*) GTP (10 μM) caused activation of the K_{ACh} channel in the Cl^- internal solution, which was abolished by changing the internal solution from Cl^- to SO_4^{2-}. In the 65 mM SO_4^{2-} solution, GTP (10–500 μM) did not activate the K_{ACh} channel, but GTPγS (10 μM) could activate the channel fully. (*C*) The relationships between $[GTP]_i$ and the relative $N \cdot P_o$ of the K_{ACh} channel with reference to the $N \cdot P_o$ induced by GTPγS (10 μM) in the 130 mM Cl^- (●) and 65 mM SO_4^{2-} (○) internal solutions. The symbols and bars are mean ± SD. The continuous curves are fit by the Hill equation. Note that in the SO_4^{2-} internal solution, GTP (up to 500 μM) did not activate the channel at all.

and *b*). However, the decay of the current in the Cl^- internal solution was much slower than that in the SO_4^{2-} internal solution. The half decay time was 8 ± 2 s (n = 5) in the Cl^- pipettes, while it was 4 ± 1.5 s (n = 5) in the SO_4^{2-} pipettes. From these results, it was concluded that intracellular anions affect the turn-off reaction of

G_K, resulting in the different sensitivity of the K_{ACh} channel to intracellular GTP in the internal solutions containing various anions.

Effects of Anions on the Basal Activity and the ATP-induced Activation of the K_{ACh} Channel

Even in the absence of agonists, the K_{ACh} channel in the inside-out patch condition is activated by intracellular GTP alone (basal activation; Ito et al., 1991; Okabe et al., 1991) or by ATP alone via phosphate transfer by nucleoside diphosphate (NDP) kinase (Heidbüchel et al., 1990*a*, *b*; Kaibara et al., 1991). The effects of intracellular anions on the GTP-induced basal activation and the ATP-induced activation of the K_{ACh} channel were examined in the inside-out patches to reevaluate their physiological roles.

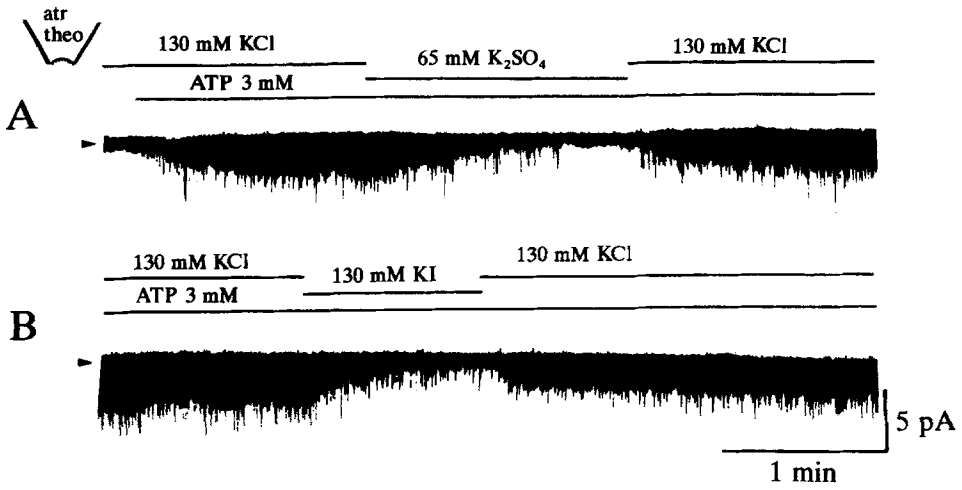

FIGURE 8. Effects of intracellular anions on the activity of the K_{ACh} channel induced by ATP. Inside-out patch experiments. The holding potential was −80 mV. The patch contained atropine (1 μM) and theophylline (200 μM). In the 130 mM Cl^- internal solution, ATP (3–4 mM) could activate the K_{ACh} channel, but channel openings were abolished in the SO_4^{2-} internal solution (*A*) and dramatically decreased in the I^- internal solution (*B*).

In Fig. 7, the patch pipette contained 1 μM atropine and 200 μM theophylline to block the muscarinic and P_1-purinergic receptors (Kurachi et al., 1986*b*). In the 130 mM Cl^- internal solution, various concentrations of GTP from 0.01 to 100 μM were applied to the internal side of the patches. Even in the absence of agonist, the channel openings were enhanced by increasing $[GTP]_i$, but the maximal channel activity induced by GTP was ~20% of that induced by GTPγS (10 μM) (Fig. 7, *A* and *C*; Ito et al., 1991). In the 65 mM SO_4^{2-} internal solution, on the other hand, GTP (10–500 μM) did not activate the channel at all (Fig. 7, *B* and *C*).

Intracellular ATP can activate the K_{ACh} channel in the absence of agonist in inside-out patches of the atrial cell membrane, possibly through phosphate transfer

from ATP to G_K by the membrane-bound NDP-kinase (Heidbüchel et al., 1990*a*, *b*; Kaibara et al., 1991). Fig. 8 shows the effects of anions on the activity of the K_{ACh} channel induced by ATP. ATP (3–4 mM) with Mg^{2+} activated the K_{ACh} channel in the 130 mM Cl^- internal solution as illustrated in Fig. 8, *A* and *B*. However, when Cl^- was completely replaced by 65 mM SO_4^{2-} or 130 mM I^-, the channel activity induced by ATP was either abolished or markedly suppressed.

These findings indicate that intracellular Cl^- is essential for the significant openings of the K_{ACh} channel induced by physiological concentrations of GTP through the basal turn-on reaction of G_K and by millimolar concentrations of ATP through possible phosphate transfer to G_K by NDP-kinase.

DISCUSSION

The major findings of this study are as follows: (*a*) intracellular anions, such as Cl^- and SO_4^{2-}, affect the agonist-dependent, GTP-induced activation of the K_{ACh} channel in single atrial cells; (*b*) Cl^- appears not to affect the turn-on reaction but may slow the turn-off reaction of G_K in the presence of Mg^{2+}, resulting in higher sensitivity of the K_{ACh} channel to intracellular GTP; and (*c*) activation of the K_{ACh} channel either by the basal turn-on reaction of G_K or by phosphate transfer to G_K by NDP-kinase is affected to a large extent by intracellular Cl^-.

Effect of Intracellular Anions on the K_{ACh} Channel

It has been reported that anions have several effects on GTP-binding proteins, i.e., G_s, G_o, and G_i (Downs, Spiegel, Singer, Reen, and Aurbach, 1980; Higashijima et al., 1987*b*; see also Gilman, 1987). It is well known that F^- with Al^{3+} can activate various G proteins in the absence of GTP or agonist. It was also reported that AlF_4^- could activate the K_{ACh} channel, which was probably due to the direct activation of G_K (Kurachi, Nakajima, and Ito, 1987*a*; Yatani and Brown, 1991). In this study we examined the effects of several intracellular anions other than F^- on the K_{ACh} channel activity. Intracellular anions examined in the present study, i.e., Cl^-, Br^-, I^-, SO_4^{2-}, and aspartic acid, did not activate the channel by themselves with or without Al^{3+} (not shown), but required intracellular GTP for activation. Therefore, the effects of these anions on the K_{ACh} channel are not due to the direct activation of G_K.

Our results show that intracellular anions, especially Cl^-, affect the sensitivity of the K_{ACh} channel to intracellular GTP mainly by regulating the turn-off reaction of G_K: the decay of the channel activity on washout of GTP in the inside-out patches or upon application of atropine under the whole-cell configuration in the Cl^- internal solution was much slower than that in the SO_4^{2-} internal solution (Fig. 6). This suggests that intracellular Cl^- slows the turn-off reaction of G_K, probably by inhibiting the intrinsic GTPase activity of G_K, resulting in the increased sensitivity of the K_{ACh} channel to intracellular GTP (Fig. 4). This notion is consistent with the biochemical observation that Cl^- inhibits the catalytic rate of GTP by $G_{o\alpha}$ (Higashijima et al., 1987*b*). Although Higashijima et al. (1987*b*) also showed that Cl^- increased the affinity of $G_{o\alpha}$ for GTPγS and GTP in the absence of Mg^{2+} primarily by decreasing the rate of dissociation of the nucleotides, we could not observe a significant difference of the GTPγS-induced activation of the K_{ACh} channel in the Cl^-

and SO_4^{2-} internal solutions. This is probably because the internal solutions used in the present study contained 2 mM Mg^{2+}, which should have increased the affinity of $G_{K\alpha}$ for GTPγS and GTP maximally.

Apparently higher concentrations of Cl^- were necessary to cause significant openings of the K_{ACh} channel than those affecting biochemical measurement of GTPase activity of G_o: [Cl^-] for the half-maximal activation of the K_{ACh} channel was ~70 mM (Figs. 3 and 4), whereas the [Cl^-] for the half-maximal inhibition of the GTPase activity of G_o was 3–20 mM (Higashijima et al., 1987*b*). This apparent lower sensitivity to intracellular Cl^- of the GTP-mediated activation of the K_{ACh} channel than that of the GTPase activity of G_o does not necessarily mean that G_K is different from G_o, since the K_{ACh} channel is activated by the GTP-bound form of G_K, probably in a positive cooperative manner (Kurachi et al., 1990; Ito et al., 1991), and is not the direct indicator of the GTPase activity of G_K.

Effects of Intracellular Anions on the Activation of the K_{ACh} Channel by the Basal Turn-on Reaction of G_K or Phosphate Transfer to G_K by NDP-Kinase

Under the inside-out patch conditions, GTP alone activated the K_{ACh} channel even in the absence of agonist in the Cl^- internal solution (Fig. 7 *A*), which may be due to the basal turn-on reaction of G_K (Ito et al., 1991; Okabe et al., 1991). However, when Cl^- was replaced by SO_4^{2-}, the channel activity induced by GTP (up to 500 μM) was completely abolished (Fig. 7). This may be due to the faster turn-off reaction of G_K in the SO_4^{2-} internal solution than that in the Cl^- internal solution (Fig. 6): in the SO_4^{2-} internal solution, the turn-off reaction of G_K should exceed the agonist-independent, GTP-induced basal turn-on reaction of G_K (at least up to 500 μM GTP) and vice versa in the Cl^- internal solution. Thus, the Cl^--induced inhibition of turn-off reaction of G_K may be essential for the basal channel activation induced by physiological concentrations of GTP in the inside-out patches.

In the absence of agonists, GTPγS, a nonhydrolyzable GTP analogue, caused a gradual irreversible activation of the channel current in the whole cell as well as in the inside-out patch due to the basal turn-on reaction of G_K (Kurachi et al., 1986*b*; Kurachi, Nakajima, and Sugimoto, 1987*b*; Breitwieser and Szabo, 1988; Ito et al., 1991). On the other hand, GTP by itself (up to 2 mM) did not activate the K_{ACh} channel current under the whole-cell condition even when the pipette was filled with the Cl^- internal solution (Nakajima, T., and Y. Kurachi, unpublished observations). Furthermore, when GDPβS (1 mM) was added to the Cl^- pipette solution containing 100 μM GTP to suppress the possible basal activation of the K_{ACh} channel by G_K, the steady-state membrane currents of atrial cells were not affected at all (Nakajima, T., and Y. Kurachi, unpublished observations). These observations suggest that the basal turn-on reaction of G_K does not cause significant activation of the K_{ACh} channel in the whole cell, while the GTP-induced basal channel activity in the Cl^- internal solution was as much as ~20–40% of the GTPγS-induced maximal channel activity in the inside-out patch (Fig. 7; Okabe et al., 1991; Ito et al., 1991). Thus, the turn-off reaction of G_K may be faster than the basal turn-on reaction in the whole-cell condition even when the pipettes are filled with the Cl^- internal solution.

Electrophysiologically, Breitwieser and Szabo (1988) estimated the rate of GTPase activity (k_{cat}) of G_K as >200 min^{-1} in the frog atrial whole cell with the pipette filled

with the solution containing 60 mM K aspartate and 50 mM KCl. Similarly, we could estimate the k_{cat} of G_K from the experiments in Fig. 6: in the whole cell using the 65 mM SO_4^{2-} pipette, k_{cat} was $\sim 29 \pm 9$ min^{-1} ($n = 5$) in guinea pig atrial myocytes. The value of k_{cat} was reduced to 14 ± 5 min^{-1} ($n = 5$) in the whole cells using the Cl^- pipette. Since these rates are much higher than that of basal GDP release (~ 0.3 min^{-1}) (Higashijima, Ferguson, Smiegel, and Gilman, 1987*a*; see also Breitwieser and Szabo, 1988), which may reflect the basal turn-on reaction of G proteins, the GTPase activity measured in the whole-cell condition even with the 130 mM Cl^- pipette would be high enough to account for the lack of receptor-independent channel activation by GTP.

Since the intracellular [Cl^-] is supposed to be ~ 4–10 mM and the remaining intracellular anions, such as various proteins, amino acids, PO_4^{3-}, and HCO_3^-, are much larger molecules than Cl^- (Ganong, 1991), the mobility of these anions may be significantly lower than that of Cl^-. Thus, the concentration of intracellular Cl^- might be much lower than that in the pipette solution under the whole-cell clamp condition. This might be one reason for the relatively high turn-off reaction of G_K in the whole cell with the Cl^- pipette. Bahinski, Nairn, Greengard, and Gadsby (1989), however, showed that the reversal potential of the β-adrenergic agonist-induced Cl^- current shifted to ~ 0 mV in the whole ventricular cell with the 135 mM Cl^- pipette, suggesting that the intracellular concentration of Cl^- under the whole-cell configuration with the Cl^- pipettes can be equilibrated to high enough levels to inhibit the turn-off reaction of G_K as in the inside-out patch. If this is the case, a high concentration of intracellular Cl^- may not be the only factor that causes the GTP-induced basal activation of the K_{ACh} channel in the inside-out patch. Actually, the k_{cat} value measured biochemically either in isolated membranes or solubilized preparations of G proteins is on the order of 2 min^{-1} in the buffer solution containing 50 mM Na-HEPES and 1 mM Na-EGTA (pH 8.0) (Higashijima et al., 1987*a*). This value is much smaller than those estimated from the electrophysiological measurements, suggesting that some unknown factors cause acceleration of the k_{cat} value in the whole cell. One of the candidates for such a factor is the GTPase activating protein (GAP) that interacts with small GTP-binding proteins such as *ras* p21 (Trahey and McCormick, 1987). However, Yatani et al. (1990) showed that GAP together with *ras* p21 uncouples the muscarinic ACh receptors and G_K. Further studies are needed to elucidate the possibility of such an intracellular regulatory factor for the GTPase activity of G_K.

In addition to the basal turn-on reaction of G_K, it has been indicated that the K_{ACh} channel can be activated by phosphate transfer from ATP to G_K by NDP-kinase (Kimura and Shimada, 1988; Otero, Breitwieser, and Szabo, 1988; Heidbüchel et al., 1990*a*, *b*; Kikkawa, Takahashi, Takahashi, Shimada, Ui, Kimura, and Katada, 1990; Kaibara et al., 1991). Actually, our results also indicated that in the Cl^- internal solution the application of ATP (3–4 mM) could activate the K_{ACh} channel even in the absence of GTP or agonist. However, when Cl^- in the internal solution was replaced by I^- or SO_4^{2-}, the K_{ACh} channel activity was greatly reduced or abolished (Fig. 8). This observation suggests that inhibition of the turn-off reaction of G_K by Cl^- is also essential for the activation of G_K by NDP-kinase–mediated phosphate transfer in the inside-out patch. Otero et al. (1988) also showed in the whole-cell voltage clamp

experiments of frog atrial cells that ATPγS, but not ATP, could induce activation of the K_{ACh} channel in the absence of agonist, suggesting that the rate of activity of GTPase in the whole cell is high enough to preclude the development of a significant pool of GTP-bound G_K by phosphate transfer by NDP-kinase.

Possible Physiological Role of Intracellular Anions in the K_{ACh} Channel Regulation

The present results showed that intracellular Cl^- increased the openings of the K_{ACh} channel induced by GTP in the presence of agonist in a concentration-dependent manner. Thus, it is likely that under agonist stimulation, the increase or decrease of intracellular [Cl^-] may affect the K_{ACh} channel activity. Since in the heart Cl^- channels are activated by various neurotransmitters, such as isoproterenol and histamine (Bahinski et al., 1989; Harvey and Hume, 1989), these agonists may affect the activity of the K_{ACh} channel induced by ACh through an indirect effect on $[Cl^-]_i$. In addition, it is likely that a number of channels regulated by G proteins other than the K_{ACh} channel can also be modulated by intracellular anions. Thus, intracellular anions may act as a regulator of G protein–gated channels in various kinds of cells. However, further studies are needed to elucidate the physiological and pathophysiological roles of anions in the regulation of the K_{ACh} channel.

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