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 Gᵦ

TOSHIKAMI NAKAJIMA, TSUNEAKI SUGIMOTO, and YOSHIHISA KURACHI

From the Second Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan; and Division of Cardiovascular Diseases, Department of Internal Medicine, and Department of Pharmacology, Mayo Clinic, Mayo Foundation, Rochester, Minnesota 55905

ABSTRACT The effects of various intracellular anions on the G protein (Gᵦ)-mediated activation of the muscarinic K⁺ (Kₐch) 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 Kₐch channel activation in internal solutions containing different anions. The order of potency of anions to induce the Kₐch channel activity at 0.5 µM ACh and 1 µM GTP was Cl⁻ > Br⁻ > I⁻. In the SO₄²⁻ or aspartic acid internal solution, no channel openings were induced by 1 µM GTP with 0.5 µM ACh. In both the Cl⁻ and SO₄²⁻ 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 (Kᵦ) was 0.2 µM in the Cl⁻ and 10 µM in the SO₄²⁻ 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 SO₄²⁻ solutions; i.e., the Hill coefficient was ~3–4 and the Kᵦ 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 SO₄²⁻ solution. These results suggest that intracellular Cl⁻ does not affect the turn-on reaction but slows the turn-off reaction of Gᵦ, resulting in higher sensitivity of the Kₐch 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 Kₐch channel, while neither occurred in the SO₄²⁻ solution. These observations suggest...
that the activation of the \( \beta_{1,2} \) channel by the basal turn-on reaction of \( G_\beta \) or by phosphate transfer to \( G_\beta \) by nucleoside diphosphate-kinase may depend at least partly on the intracellular concentration of \( \text{Cl}^- \).

**INTRODUCTION**

GTP-binding (G) proteins act as transducers between membrane receptors and effectors, including adenyl 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 (\( G_\alpha \cdot \text{GTP} \) and \( G_\beta \cdot \gamma \)), which in turn activate or inhibit the effectors; and (b) GTP bound to \( G_\alpha \) is hydrolyzed to GDP by the intrinsic GTPase activity of \( G_\alpha \). The GDP-bound form of \( G_\alpha \) associates with \( G_\beta \cdot \gamma \) 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 (\( \text{Mg}^{2+} \)) 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 (\( \text{Cl}^- \)) can stimulate the G protein activity by (a) preventing dissociation of \( G_\alpha \) or \( G_\beta \) in the absence of \( \text{Mg}^{2+} \) (Northup, Smiegel, Sternweis, and Gilman, 1983; Higashijima, Ferguson, and Sternweis, 1987b), (b) potentiating dissociation of \( G_\alpha \) into \( G_\alpha \) and \( G_\beta \) subunits (Sternweis, 1986), and (c) disturbing the intrinsic GTPase activity of \( G_\alpha \) (Higashijima et al., 1987b).

In cardiac atrial myocytes, muscarinic acetylcholine (m-ACh) receptors are linked to a specific population of inward-rectifying K\(^+\) channels (\( \beta_{1,2} \)) by PT-sensitive G proteins (\( G_\beta \)) (Breitwieser and Szabo, 1985; Pfaffinger, Martin, Hunter, Nathanson, and Hille, 1985; Kurachi, Nakajima, and Sugimoto, 1986a, b). This \( \beta_{1,2} \) channel activity is also supposed to be regulated by agonist-dependent turn-on and turn-off reactions of \( G_\beta \) in analogy to the regulation of adenyl cyclase (Kurachi, Nakajima, and Sugimoto, 1986c; Logothetis, Kurachi, Galper, Neer, and Clapham, 1987; Nanavati, Clapham, Ito, and Kurachi, 1990). Although the intracellular solution containing various concentrations of \( \text{Cl}^- \) have been used in the electrophysiological experiments on the \( \beta_{1,2} \) channel (Kurachi et al., 1986a, 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 \( \beta_{1,2} \) channel have not yet been examined.

In this study we compared the effects of intracellular chloride and sulfate ions on the \( G_\beta \)-mediated activation of the \( \beta_{1,2} \) channel. Our results clearly show that intracellular \( \text{Cl}^- \) increased the sensitivity of the \( \beta_{1,2} \) channel to intracellular GTP, probably by disturbing the turn-off reaction of \( G_\beta \). The effects of anions on the activation of the \( \beta_{1,2} \) channel by either the basal turn-on reaction of \( G_\beta \) (Ito et al., 1991; Okabe et al., 1991) or the nucleoside diphosphate kinase-mediated phosphate transfer to \( G_\beta \) (Heidbüchel, Callewaert, Vereecke, and Carmeliet, 1990a, 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., 1986b). Briefly, collagenase (0.2% wt/vol) in the Ca²⁺-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): 156.5 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.53 MgCl₂, 5.5 glucose, and 5 HEPES-NaOH buffer, pH 7.4. The Ca²⁺-free bathing solution was the same as the control bathing solution except that CaCl₂ was omitted. The high K⁺/low Cl⁻ solution contained (mM): 70 K glutamate, 25 KCl, 10 KH₂PO₄, 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 K₂SO₄, 2 MgCl₂, 5 EGTA, 3–4 K₃ATP, 0.1 GTP (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 CaCl₂, 1 MgCl₂, 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 K₂SO₄. 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).

**Table I**

<table>
<thead>
<tr>
<th>Compositions of Internal Solutions</th>
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<tr>
<td>130 KCl, 5 EGTA, 2 Mg(NO₃)₂, 5 HEPES</td>
</tr>
<tr>
<td>130 KBr, 5 EGTA, 2 Mg(NO₃)₂, 5 HEPES</td>
</tr>
<tr>
<td>130 KI, 5 EGTA, 2 Mg(NO₃)₂, 5 HEPES</td>
</tr>
<tr>
<td>65 K₂SO₄, 5 EGTA, 2 Mg(NO₃)₂, 5 HEPES</td>
</tr>
<tr>
<td>130 K aspartate, 5 EGTA, 2 Mg(NO₃)₂, 5 HEPES</td>
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pH was adjusted to 7.5 with KOH.
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.

**RESULTS**

**Intracellular Anions as Modulators of the G Protein-gated Muscarinic K⁺ Channel
(KACh) in Single Atrial Myocytes**

Fig. 1 shows the effects of several anions on the KACh 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 KACh channel in the internal solution containing 130 mM Cl⁻. When Cl⁻ was replaced by 65 mM sulfate ion (SO₄²⁻), the channel activity was completely abolished in the continuous presence of GTP (Fig. 1A). When SO₄²⁻ 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 KACh channel. When Br⁻ was replaced by Cl⁻, the channel activity (N ∙ Po; N is the number of channels in the patch, and Po is the open
probability of each channel) slightly increased from 0.1688 to 0.1818 in Fig. 1B. After washout of GTP, the channel activity disappeared (Fig. 1B). 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. 1C, the N ∙ Po of the KACh channel in the I⁻ internal solution was 0.0231 and increased to 0.1553 in the Cl⁻ internal solution. Cl⁻, Br⁻, I⁻, and SO₄²⁻ 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 KACh channel in inside-out patches of the atrial cell membrane.

Fig. 1D compares the KACh 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 ∙ Po of the KACh channel
induced by GTP (1 μM) in the presence of internal anions was expressed with
reference to the N ∙ Po in the 130 mM Cl⁻ internal solution in each patch. The order
of potency of anions to induce the KACh channel activity in the presence of 0.5 μM
ACh and 1 μM GTP was Cl⁻ ≥ Br⁻ > I⁻. In the SO₄²⁻ and L-aspartic acid internal
solutions, no significant openings of the KACh channel were observed.

Fig. 2A shows the KACh 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\textsuperscript{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\textsuperscript{+} 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\textsuperscript{2+}. Their current–voltage relationships were almost superimposable (Fig. 2 B). In addition, the open-time histogram of the GTP-activated \( K_{\text{ACH}} \) channels at ~80 mV could be fit by a single exponential curve with a time constant of ~1 ms in both the 130 mM Cl\textsuperscript{-} and the 65 mM SO\textsuperscript{2-} internal solutions (Fig. 2 C). These observations indicate that the conductance and kinetic properties of the \( K_{\text{ACH}} \) channel were not affected by these anions.

Fig. 3 showed the concentration-dependent effect of Cl\textsuperscript{-} on activation of the \( K_{\text{ACH}} \) channels induced by 1 \( \mu \text{M} \) GTP. The pipette solution contained 0.5 \( \mu \text{M} \) ACh. SO\textsuperscript{2-} in the internal solution was replaced by Cl\textsuperscript{-} as indicated above the trace (Fig. 3 A). GTP (1 \( \mu \text{M} \)) was continuously perfused from the intracellular side of the patch. ACh (0.5 \( \mu \text{M} \)) was present in the pipette. In the 65 mM SO\textsuperscript{2-} internal solution, the channel openings were not observed in the presence of 1 \( \mu \text{M} \) GTP. As the
concentration of internal Cl\textsuperscript{-} ([Cl\textsuperscript{-}]\textsubscript{i}) increased, the K\textsubscript{ACb} channel openings were enhanced in a concentration-dependent manner. Fig. 3 B shows the relationship between [Cl\textsuperscript{-}]\textsubscript{i} and \textit{N'Po} of the K\textsubscript{ACb} channel at 1 \textmu M GTP. The data were obtained from three patches. Relative \textit{N'Po} of the K\textsubscript{ACb} channel at each [Cl\textsuperscript{-}]\textsubscript{i} was calculated with references to the \textit{N'Po} at 130 mM [Cl\textsuperscript{-}]\textsubscript{i}. The relative \textit{N'Po} was 0.05 at 20 mM [Cl\textsuperscript{-}]\textsubscript{i}, and increased in a concentration-dependent manner. The half-maximal activation occurred at \textasciitilde 70 mM [Cl\textsuperscript{-}]\textsubscript{i}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Effect of intracellular anions on the conductance and kinetic properties of the K\textsubscript{ACb} channel. (A) Inside-out patch configuration. The current recordings of the K\textsubscript{ACb} channel induced by GTP (100 \textmu M) at various membrane potentials were shown in the 130 mM Cl\textsuperscript{-} and 65 mM SO\textsubscript{4}\textsuperscript{2-} internal solutions. (B) Current-voltage relationships of the K\textsubscript{ACb} channel in the 130 mM Cl\textsuperscript{-} (•) and 65 mM SO\textsubscript{4}\textsubscript{2-} (○) internal solutions. The data were obtained from three patches in each case. (C) Open-time histograms of the K\textsubscript{ACb} channel induced by GTP (100 \textmu M) in the 130 mM Cl\textsuperscript{-} and 65 mM SO\textsubscript{4}\textsubscript{2-} internal solutions. The membrane potentials were \textasciitilde 80 mV in both cases.}
\end{figure}

**Effects of Anions on the Concentration-dependent Activation of the K\textsubscript{ACb} Channel Activity by GTP and GTP\gamma S**

To clarify the roles of intracellular anions on the activation of the K\textsubscript{ACb} channel, we examined the concentration-dependent effect of intracellular GTP on the channel activity in the 130 mM Cl\textsuperscript{-} and 65 mM SO\textsubscript{4}\textsubscript{2-} internal solutions (Fig. 4). The pipette solution contained 0.5 \textmu M 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]. In the Cl\textsuperscript{-} internal solution, the channel activity reached a maximal value at 0.3–1 \textmu M [GTP] 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].

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 $N'P_o$ of the K$_{ACh}$ channel at each

![Figure 3](attachment:image.png)

**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$^-$]. (B) The relationship between [Cl$^-$], and
the relative $N'P_o$ of the K$_{ACh}$ channel. The relative $N'P_o$ at each [Cl$^-$], was obtained with
reference to that at 130 mM [Cl$^-$]. 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 $N'P_o$ of 10 μM GTP$_\gamma$S-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):

$$y = \frac{V_{max}}{1 + K_d/[GTP]^{1/H}}$$
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 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 GTP$_{yS}$, a nonhydrolyzable GTP analogue. The patch pipette contained 0.5 μM ACh. As $[GTP_yS]$ was raised from 0.01 or 0.03 to 0.1, 1, and 10 μM, the channel activity increased.
μM, channel openings increased in a similar concentration-dependent fashion in both the 130 mM Cl⁻ and 65 mM SO₄²⁻ internal solutions (Fig. 5, A and B). Few or no channel openings were induced at 0.03 μM [GTPγS]ᵢ up to 5–7 min of superfusion. The openings reached a maximal value at 0.1–1.0 μM [GTPγS]ᵢ in both cases. Fig. 5 C shows the relationships between [GTPγS]ᵢ and the quasi-steady-state open probability of the KACh channel in the Cl⁻ and SO₄²⁻ internal solutions. The data for each symbol were obtained from five patches with ACh (0.5 μM) in the pipette. Relative N̄Po of the KACh channel was calculated with reference to the 10 μM

![Diagram](image)

**Figure 5.** Effect of anions on the concentration-dependent effect of GTPγS on the KACh 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 KACh channel openings in the 130 mM Cl⁻ (A) and in the 65 mM SO₄²⁻ internal solutions (B) are shown. (C) The relationships between [GTPγS]ᵢ and the relative N̄Po of the KACh channel in the 130 mM Cl⁻ (●) and 65 mM SO₄²⁻ (○) 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 [GTPγS]ᵢ and the channel activity did not differ significantly between the Cl⁻ and the SO₄²⁻ internal solutions: the Hill coefficient was 3.5 and 3.4, and Kᵦ was 0.06 and 0.08 μM in the Cl⁻ and SO₄²⁻ 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 Gᵦ nor the interaction between the activated Gᵦ and the KACh channel are signifi-
cantly affected by intracellular anions. Therefore, it is likely that the turn-off reaction of GK through hydrolysis of GTP bound to GK may be involved in the effects of anions.

Effects of Anions on the Deactivation of the KAcH Channel

To examine the effects of intracellular anions on the turn-off reaction of GK, the effects of Cl⁻ and SO₄²⁻ 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)

![A](image1)

![B](image2)

FIGURE 6. Effects of intracellular anions on the turn-off of the KAcH 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 SO₄²⁻ (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 SO₄²⁻ 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 SO₄²⁻ internal solution in b. After the application of atropine, the KAcH channel current was abolished but the decay of the current in the Cl⁻ pipette was slower than that in the SO₄²⁻ 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 KAcH channel was 12 ± 4 s (n = 5). On the other hand, in the SO₄²⁻ 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 SO₄²⁻ 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 SO₄²⁻ solution.
Similar results were obtained in whole-cell voltage clamp experiments using pipettes filled with either the 130 mM Cl⁻ or the 65 mM SO₄²⁻ internal solution (Fig. 6 B). Cells were held at ~-40 mV. After the KACH 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 and b). However, the decay of the current in the Cl⁻ internal solution was much slower than that in the SO₄²⁻ 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 SO₄²⁻ pipettes. From these results, it was concluded that intracellular anions affect the turn-off reaction of
GK, resulting in the different sensitivity of the KACh 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 KACh Channel**

Even in the absence of agonists, the KACh 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 (Heidbuechel et al., 1990a, b; Kaibara et al., 1991). The effects of intracellular anions on the GTP-induced basal activation and the ATP-induced activation of the KACh channel were examined in the inside-out patches to reevaluate their physiological roles.

![Figure 8](image)

**FIGURE 8.** Effects of intracellular anions on the activity of the KACh 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 KACh channel, but channel openings were abolished in the SO₄²⁻ 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 P₁-purinergic receptors (Kurachi et al., 1986b). 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], but the maximal channel activity induced by GTP was ~20% of that induced by GTPyS (10 μM) (Fig. 7, A and C; Ito et al., 1991). In the 65 mM SO₄²⁻ 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 KACh 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., 1990a, 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., 1987b; 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, 1987a; 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$_{os}$ (Higashijima et al., 1987b). Although Higashijima et al. (1987b) also showed that Cl$^-$ increased the affinity of G$_{os}$ for GTP$\gamma$S 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 GTP$\gamma$S-induced activation of the $K_{ACh}$ channel in the Cl$^-$
apparently higher concentrations of Cl\(^-\) were necessary to cause significant openings of the \(K_{\text{ACh}}\) channel than those affecting biochemical measurement of GTPase activity of \(G_0\); [Cl\(^-\)] for the half-maximal activation of the \(K_{\text{ACh}}\) channel was \(\sim 70\) mM (Figs. 3 and 4), whereas the [Cl\(^-\)] for the half-maximal inhibition of the GTPase activity of \(G_0\) was 3–20 mM (Higashijima et al., 1987b). This apparent lower sensitivity to intracellular Cl\(^-\) of the GTP-mediated activation of the \(K_{\text{ACh}}\) channel than that of the GTPase activity of \(G_0\) does not necessarily mean that \(G_K\) is different from \(G_0\), since the \(K_{\text{ACh}}\) 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 \(K_{\text{ACh}}\) 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 \(K_{\text{ACh}}\) 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 \(\mu\)M) 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 \(\mu\)M 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, GTP\(_\gamma\)S, 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., 1986b; Kurachi, Nakajima, and Sugimoto, 1987b; Breitwieser and Szabo, 1988; Ito et al., 1991). On the other hand, GTP by itself (up to 2 \(\mu\)M) did not activate the \(K_{\text{ACh}}\) 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 GDP\(_\gamma\)S (1 mM) was added to the Cl\(^-\) pipette solution containing 100 \(\mu\)M GTP to suppress the possible basal activation of the \(K_{\text{ACh}}\) 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 \(K_{\text{ACh}}\) channel in the whole cell, while the GTP-induced basal channel activity in the Cl\(^-\) internal solution was as much as \(\sim 20–40\%\) of the GTP\(_\gamma\)S-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 (\(k_{\text{cat}}\)) 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 $14 \pm 5$ min$^{-1}$ ($n = 5$) in the whole cells using the Cl$^-$ pipette. Since these rates are much higher than that of basal GDP release ($\sim 0.3$ min$^{-1}$) (Higashijima, Ferguson, Smiegel, and Gilman, 1987a; 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 $\sim 4$–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 $\beta$-adrenergic agonist-induced Cl$^-$ current shifted to $\sim 0$ 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 KACh 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., 1987a). 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 KACh 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., 1990a, b; Kikkawa, Takahashi, Takahashi, Shimada, Uj, 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 KACh 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 KACh 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 $K_{ACh}$ 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$^-$]. 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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