Mechanism of Fluoride Activation of G Protein-gated Muscarinic Atrial K⁺ Channels*

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Aluminum fluoride (AlF³⁻) activates the heterotrimeric G protein, Gₐ (stimulatory G protein of adenylcyclase) (Sterneweis, P. C., and Gilman, A. G. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 4888–4891) and Gₜ (transducin), and for Gᵪ, Bigay et al. (Bigay, J., De- 
terre, P., Pfister, C., and Chabre, M. (1985) FEBS Lett. 191, 181–185) have made the intriguing proposal that AlF³⁻ acts by mimicking the γ-phosphate of GTP. The endogenous G protein (probably Gₐ₁α or Gₛ₁α (Yatani, A., Mattera, R., Codina, J., Graf, R., Okabe, K., Padrell, E., Iyengar, R., Brown, A. M., and Birnbauer, L. (1988) Nature 336, 680–682) that stimulates the muscarinic atrial K⁺ (K⁺[ACH]) channel is also thought to be activated by AlF³⁻ (Kurachi, Y., Nakajima, T., and Ito, H. (1987) Circulation 76, 105P). To investigate the AlF³⁻ mechanism, we applied potassium fluoride (KF) to the cytoplasmic face of inside-out membrane patches excised from guinea pig atria. We found that KF activated single K⁺[ACH] channel currents in both a concentration- and a Mg²⁺-dependent manner. Activation persisted following removal of KF, but unlike activation by guanosine 5’-(3-thiotriphosphate) (GTPyS), was fully reversed by removal of Mg²⁺.

Evidence for Al³⁺ involvement was that the Al³⁺ chelator deferoxamine (500 μM) inhibited KF activation and that at low concentrations of KF (<1 mM), micro- molar AlCl₃ concentrations potentiated KF stimulation. The rate of activation produced by KF was far slower than the rate of activation by GTP or GTPyS, and unlike these guanine nucleotides, the rate was unchanged in the presence of agonist. To test the γ-phosphate-mimicking hypothesis, we evaluated the requirement for GDP; and to accomplish this, it was necessary to establish a condition that ensured exchange of guanine nucleotides. This condition was satisfied by using the muscarinic agonist carbachol because both the rate and the extent of activation of the K⁺[ACH] channels produced by GTP were much faster in carbachol, and both were greatly slowed when GDP was added along with GTP. By contrast, the effects of KF were unchanged by carbachol in the presence or absence of GDP. Further evidence that GDP is not essential for activation by AlF³⁻ was provided by the observation that during carbachol activation and following extensive washing with GMP, guanosine 5’-O- (2-thiodiphosphate) at blocking concentrations had no effect on activation produced by KF. We conclude that AlF³⁻ activates the endogenous G protein, but the mechanism appears to be more complicated than implied by the γ-phosphate-mimicking hypothesis.

Ion channels are thought to be G protein effectors, a good example being the muscarinic atrial K⁺ (K⁺[ACH]) channel in guinea pig atrium (1). The responsible G protein is called Gₐ₁α (1–3). Thus, AlF³⁻ (2–4) is probably either of two α subunits, Gₐ₁α, Gₛ₁α (4). Like other heterotrimeric G proteins, Gₐ₁α can be activated reversibly by GTP (5) or irreversibly by GTPyS (3, 6); and in either case, there is an absolute requirement for Mg²⁺. Another agent that activates Giₐ proteins is AlF³⁻ (7), and activation by AlF³⁻ generally introduced as NaF or KF has been used as evidence for G protein effector coupling (8). Adenylylcyclase can be either stimulated or inhibited by NaF- or KF-activated G, or Gᵪ, respectively (7, 9–11). NaF also activates transducin and the G proteins responsible for polyphosphoinositide hydrolysis (12–14); and pertinent to our interest in ion channels as G protein effectors, an abstract has been published indicating that AlF³⁻ may activate Gᵣ (15).

Activation by AlF³⁻ is especially intriguing because it has been proposed that AlF³⁻ activates the α subunit of G proteins by mimicking the γ-phosphate of GTP (12, 13). NMR spectroscopy of Gₐ₁ and Gₛ was interpreted as supporting this view (16). Moreover, the concept that AlF³⁻ may act as a γ-phosphate in the presence of nucleotide diphosphates has been extended to other enzymes that bind phosphate or nucleotide diphosphates (17). We examined the action of KF on K⁺[ACH] channel currents in excised inside-out patches from guinea pig atrial myocytes. KF stimulated the currents in a concentra-
tion-dependent manner, and there was an absolute requirement for Mg²⁺. However, in these experiments, the mechanism appeared to be independent of GDP, leading us to question whether the γ-phosphate-mimicking hypothesis applies to all in vivo circumstances.

MATERIALS AND METHODS

Single atrial cells were dissociated from adult guinea pig heart by collagenase digestion (3). Single channel currents were measured with the gigaseal patch-clamp method (18). Patch pipettes had tip resistances of 5–10 megaohms and were filled with a standard K⁺ solution (140 mM KCl, 2 mM MgCl₂, 5 mM EGTA, 5 mM HEPES (pH 7.3 with Tris base)). The bathing solution (internal solution for inside-out patch recording) had the same composition, unless otherwise noted. For Mg²⁺ experiments, EGTA was replaced by EDTA, and divalent cation concentrations were calculated with an interactive program that used the dissociation constant of Fabiato and Fabiato

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1 The abbreviations used are: G protein, signal-transducing guanine nucleotide-binding protein of subunit structure αβγ; Gᵪ, Gₛ, stimulatory and inhibitory G proteins of adenylcyclase, respectively; Gₐ, Gₜ, transducin; Gₛ₁α, Gₛ₁β, Gₛ₁γ, Gₐ₁α, Gₐ₁β, Gₐ₁γ, Gₜα, Gₜβ, Gₜγ protein that stimulates a class of K⁺ channels; GTPyS, guanosine 5’-(3-thiotriphosphate); GDPyS, guanosine 5’-O-(2-thiodiphosphate); HEPES, 4-(2-hydroxyethyl) piperazineethanesulfonic acid; EGTA, ethylenediaminetetraacetic acid; K⁺[ACH], muscarinic atrial K⁺.
Fluoride Activation of \( \Gamma \)

(19) for EDTA. MgCl\(_2\) and KF were added to the bathing solution; when KF concentrations >10 mM were tested, KCl was replaced by KF. At higher intracellular Mg\(^{2+}\) concentrations (10–20 mM), the current-voltage relationships shifted by ~5 ± 2 mV (n = 4), and this shift was not compensated for. In eight experiments, both EGTA and EDTA were eliminated from the bathing solution. Al\(^{3+}\) was present in two experiments, and KF was added to the bathing solution without any new precisions (12); but in some experiments, Al\(^{3+}\) was removed by adding the Al\(^{3+}\) chelator deferoxamine (14, 20). All test agents were applied by the concentration clamp method (21, 22), which allowed rapid solution changes to occur within 10 ms. All experiments were done at room temperature (20–22 °C).

Single channel currents were displayed on a chart recorder and stored in a video recorder. The data were analyzed using an IBM PC/AT 386 processor. The responses were 38 ms and 5 min. An exponential function was fit to the rising phase after allowing for a delay, and from the fitted value, a half-time (\( t_\text{h} \)) was calculated (see Fig. 3A). Parameter estimates for all currents of interest were determined using a modified likelihood estimator and the Marquardt-Levenberg algorithm for nonlinear least-squares curve fitting. Mean values ± S.E. are given in the text.

KF, AlCl\(_3\), carbachol, atropine, theophylline, and deferoxamine were obtained from Sigma. Nucleotides were from Boehringer Mannheim. Other chemicals were obtained from Fisher. All solutions were made with glass-distilled water. The purity of GTP\(_\gamma\)S was determined by high performance liquid chromatography (Beckman System Gold 126 gradient system with a model 166 detector).

RESULTS

KF Activation of K\(^{+}\)[ACh] Channel Currents—In early experiments, KF effects were tested with a standard K\(^{+}\) solution containing 2 mM Mg\(^{2+}\). Fig. 1A shows the effects of KF on K\(^{+}\)[ACh] channel currents in a cell-free inside-out membrane patch from a guinea pig atrial myocyte. After excision of the membrane patch into GTP-free solution, the channel activity disappeared within 20 s. A concentration jump of GTP (100 \( \mu \text{M} \)) activated the currents within 1 s and, upon jumping back to GTP-free solution, deactivated the currents within 20 s. At this point, application of KF (10 mM) led to the reappearance of currents after a delay of ~30 s. Activity increased slowly and reached steady state with a half-activation time (\( t_\text{h} \)) of 3.1 ± 0.3 min (n = 5). The KF effects were apparent at 1 mM, at which concentration the \( t_\text{h} \) was 5.0 ± 0.8 min (n = 4). In contrast to KF activation, GTP and GTP\(_\gamma\)S produced their effects within seconds (Fig. 1, A and B), and, as presented later, the effects were much faster in the presence of carbachol. Properties of single channel currents activated by KF (10 mM) were compared with those activated by GTP (100 \( \mu \text{M} \)) in the presence of carbachol (10 \( \mu \text{M} \)) in the same patch (Fig. 1C). In both cases, the currents appeared in short bursts separated by longer closed intervals. The unitary currents increased with depolarization (23) and decreased with depolarization, showing the inward rectification (Fig. 1C, panel 3) characteristic of single K\(^{+}\)[ACh] channel currents (23). The current-voltage relationships of KF- and GTP-activated channels were indistinguishable (Fig. 1C, panel 3), and the single channel slope conductances of inward channel current were 38 ± 1 picoamperes (n = 4 for each condition). Analysis of single channel currents in eight patches showed that mean open times in KF- and GTP-activated channels at ~80 mV were 1.3 ± 0.3 and 1.2 ± 0.5 ms, respectively. The burst durations were also identical in both cases. In the experiment shown in Fig. 1C, average burst durations were 4.9 ms for GTP-activated channels (panel 1) and 4.5 ms for KF-activated channels (panel 2).

Influence of Mg\(^{2+}\) on Activation of K\(^{+}\)[ACh] Channels by KF—The rate of activation of K\(^{+}\)[ACh] channel currents by KF was unexpectedly slow compared to that by GTP or GTP\(_\gamma\)S. Upon further examination, we found that Mg\(^{2+}\) was an important cofactor for KF effects on the K\(^{+}\)[ACh] channels. In the presence of higher Mg\(^{2+}\) concentrations, KF (10 mM) activated the channel currents within 10 s (Fig. 2A). Compared to the rates of activation for GTP or GTP\(_\gamma\)S, these rates were still slower, and the concentrations of Mg\(^{2+}\) were much higher.

In the absence of KF, Mg\(^{2+}\) had no effects; and without Mg\(^{2+}\), KF was ineffective (Fig. 2B). In contrast to the channel activation produced by GTP\(_\gamma\)S (24–26), the KF effects were reversed by washing with Mg\(^{2+}\)-free solution (Fig. 2C). However, KF activation was irreversible in the presence of Mg\(^{2+}\) for up to 10 min of washing in KF-free solution (Fig. 2B).

The concentration-dependent effect of Mg\(^{2+}\) on KF activation was examined at a fixed concentration of KF (10 mM) (Fig. 3). The extent of activation was normalized to the maximum response. Mg\(^{2+}\) acted in a concentration-dependent manner on both the extent and the rate of activation (Fig. 3B). The EC\(_{50}\) was 5.9 mM. For comparison, the effects of Mg\(^{2+}\) on carbachol (10 \( \mu \text{M} \)) plus GTP (100 \( \mu \text{M} \)) activation were much smaller, and the EC\(_{50}\) was 3 \( \mu \text{M} \) (27).

Concentration Dependence and Magnitude of KF Activation—Having established the optimum Mg\(^{2+}\) concentration, we turned to the concentration response to KF. Both the magnitude and the rate of activation were concentration-dependent (Fig. 4). The extent of activation measured at steady state was normalized by the maximum response to GTP\(_\gamma\)S (100 \( \mu \text{M} \)) and gave the concentration-response curve shown in Fig. 4B. The EC\(_{50}\) was 3.8 mM. When GTP\(_\gamma\)S (100 \( \mu \text{M} \)) was applied first and allowed to produce its maximum effect, subsequent addition of KF (10 mM) had no further effect (n = 5), indicating that activation by GTP\(_\gamma\)S and KF was not additive. When the opposite sequence was used, GTP\(_\gamma\)S always produced a large increase beyond the maximum increase attained by KF.

KF activated K\(^{+}\)[ACh] channel currents to the same extent in the presence or absence of the muscarinic agonist carbachol (10 \( \mu \text{M} \)) in the patch solution (Fig. 4B). Addition of the muscarinic antagonist atropine (10 \( \mu \text{M} \)) and the purinergic antagonist theophylline (100 \( \mu \text{M} \)) to the pipette solution did not affect the activation (n = 4). The activation produced by KF was always less than the maximum activation obtained by GTP\(_\gamma\)S (Fig. 4B). By contrast, GTP in the presence of agonist produced the same level of activation as GTP\(_\gamma\)S (5). In addition, the rate of activation produced by KF was unaltered by carbachol, whereas the rate of activation produced by GTP was greatly enhanced by carbachol (5).

Requirement of Aluminum Ion for KF Activation—As micromolar concentrations of aluminum ions (Al\(^{3+}\)) were required for F\(^{−}\) activation of \( \Gamma \) (7), we have tested Al\(^{3+}\) effects on KF activation. The effect of KF (1 mM) was potentiated by addition of 100 \( \mu \text{M} \) AlCl\(_3\) (Fig. 5A). In five experiments, AlCl\(_3\) at 100 \( \mu \text{M} \) enhanced the activation by submaximum concentrations of KF at 1 mM by 40 ± 8% (n = 5) in the presence of 2 mM Mg\(^{2+}\). At 20 mM Mg\(^{2+}\), AlCl\(_3\) (100 \( \mu \text{M} \)) had smaller potentiation of 20 ± 6% (n = 3). No significant potentiation by AlCl\(_3\) was obtained at concentrations of KF.
Mg" requires KF to produce K+ channel currents at fixed KF concentration (10 mM). A, time course of KF activation in the presence of 20 mM MgCl₂. B, MgCl₂ requires KF to produce activation, and KF effects persist after removal of KF in the presence of MgCl₂. C, KF-produced channel activity decreased quickly after removal of KF and MgCl₂. The channel activity was restored by GTPγS in the presence of 20 mM MgCl₂. GTPγS activated the K+ channel currents much faster than KF. The concentrations of the test materials and times of washing out of agents (W) are indicated. All test agents were applied using the concentration clamp method (21, 22). The numbers above each segment are times (in minutes) elapsed after onset of the concentration step. Wash-out duration was 2 min. A and B were separate experiments. The holding potential was -80 mV. Both patch pipette and bathing solutions were standard K+ solutions. Current records on slow time resolutions are direct displays from the pen recorder. C, properties of the K+ channel currents produced by GTP (100 μM) plus carbachol (10 μM) in the pipette solution (panel 1) and in KF at 10 mM (panel 2) in the same patch. In both cases, the bathing solution contained 2 mM MgCl₂, and membrane potential was -80 mV. Histograms show frequency distribution of open time constructed from 20-s records. Each of the open time distributions was fit to a single exponential function, and the time constants were identical to those described for Fig. 1 (A and B).

Number of Events

Time (ms)

Panel A

Panel B

Panel C

<10 mM at both MgCl₂ concentrations (n = 4).

The chelator deferoxamine (14) was also tested for its ability to reverse the Al₃⁺ effects. At 500 μM, deferoxamine inhibited the 10 mM KF-stimulated channel currents by ~40% (Fig. 5B), but a complete block could not be obtained. On average, MgCl₂ at 2 mM deferoxamine (500 μM) inhibited the K⁺[ACh] channel currents elicited by KF (10 mM) by 30 ± 8% (n = 4).

In the presence of 20 mM MgCl₂, deferoxamine had no significant blocking effects (n = 3). Deferoxamine at 500 μM alone did not interfere with the K⁺[ACh] channel currents activated...
The data presented in the figure show a concentration-dependent activation of KF (1-100 mM) in the presence of MgCl\textsubscript{2} (20 mM) and different concentrations of AlF\textsubscript{3}. The activation rates produced by AlF\textsubscript{3} were, as noted earlier, markedly slower than those for either of the guanine nucleotides. The rates for GTP\textgamma{S} were slightly slower than those for GTP, possibly as a result of slower association with the α subunit (28).

The lack of any effect of carbachol on the rate of activation produced by AlF\textsubscript{3} suggested that AlF\textsubscript{3} might not be simply mimicking the γ-phosphate of GTP as proposed by Bigay et al. (12, 13), but it was also possible that considerable amounts of G\textsubscript{i} might not have been cleared of GDP in our experiments. To examine this, we tested whether addition of GDP slowed the activation rates produced by either nucleotide or AlF\textsubscript{3}. Fig. 6A shows that this was clearly the case for GTP and GTP\textgamma{S}, but was not the case for AlF\textsubscript{3}. In fact, GDP at concentrations as great as 300 [M] had no effect on activation produced by AlF\textsubscript{3} (n = 8) (Fig. 7A). Furthermore, prolonged exposure of tens of minutes to guanine nucleotide-free bathing solutions in the presence of carbachol did not diminish the subsequent activation produced by AlF\textsubscript{3}.

A strong test of the γ-phosphate-mimicking hypothesis is a block by GDP\textgamma{S}, which prevents AlF\textsubscript{3} from acting as a γ-phosphate. We found that in the presence of agonist, GDP\textgamma{S} at concentrations of 43-86 [M] (n = 4) did not impair activation by KF. However, our high performance liquid chromatography measurements indicated contamination of 0.1-1% with GDP. To eliminate any possible effects of this contamination, in five different experiments, we added GMP at 100 by GTP plus agonist or GTP\textgamma{S} for up to 5 min, but stronger exposure or higher concentrations blocked channel currents (n = 6). The results described here permit us to attribute the effects of KF to AlF\textsubscript{3}. However, we were concerned that the much slower rate of activation produced by KF acting as AlF\textsubscript{3} might have been due to the slow release of Al\textsuperscript{3+} from the C\textsuperscript{a2+} might have been due to the slow release of Al\textsuperscript{3+} from the Ca\textsuperscript{2+}.
Fig. 7. Characteristics of KF activation of atrial K+ channel currents. A, effects of GDP (300 μM) on KF (10 mM) activation; B, effects of GMP (100 μM) and GDP/PS (86 μM) on KF (10 mM) activation. Recording conditions were identical to those described for Fig. 1 (A and B).

μM to the GDP/PS-containing solutions. As shown in Fig. 7B, KF activation was unchanged in the presence of both GMP and GDP/PS, and the average rate of activation in 20 mM Mg2+ for these experiments was 24 ± 5 s.

DISCUSSION

Our results showed that intracellular application of KF activated single-channel K+[ACh] channel currents that had unitary conductances and mean open times identical to the currents produced by muscarinic agonists such as carbachol in the presence of GTP or GDP/yS in the presence of GTP or GDP/PS. The difference, however, was that activation by GTP or GDP/yS was irreversible on the time scale of these experiments even after removal of Mg2+ (24, 26); whereas KF effects were also irreversible after washing out KF, unlike GTP/yS, the KF effects were fully reversed by washing out in Mg2+ -free solution. Activation by KF differed in several other ways from activation by guanine nucleotides. Most notably, the rate of activation was much slower and was independent of agonist. Moreover, the extent of activation was much less than the maximum possible activation.

The concentrations of Mg2+ required for KF activation were ~1000 times higher than those required for physiological activation of the K+[ACh] channels by GTP and carbachol (EC50 = 3 μM) (26, 27). This may account for the fast recovery from KF effects in Mg2+ -free solution. The Mg2+ concentration required for full KF effects found in our experiments exceeded the cytoplasmic free Mg2+ concentration thought to range from 0.4 to 3.5 mM in heart (29, 30).

Activation of G proteins by F- required AlF3, and the active ligand was thought to be AlF3 (7, 11). In our experiments, AlCl3 (10–100 μM) potentiated submaximum effects of KF. However, AlCl3 had no further activation of K+[ACh] channel currents when the KF concentration was >10 mM. The Al3+ chelator deferoxamine (500 μM) reversibly inhibited the KF effects. Similar effects have been reported in KF stimulation of ATP-sensitive K+ channels in an insulinoma cell line (RINm5F) (26). We conclude that the activating agent in our experiments is AlF3.

As to its mechanism of action, we assume that AlF3 acts upon the α subunit of Gα just as it does on the α subunit of Gβ. (31). This is supported by the observation that recombinant α3-3, which is a likely candidate for Gα (4), can stimulate single-channel K+[ACh] channel currents after activation by AlF3 (32). There is no evidence that AlF3 acted at the receptor level since muscarinic and purinergic receptor antagonists had no effects on KF stimulation. Nor is a direct effect on the K+[ACh] channel likely. In four experiments in the presence of 10 μM carbachol, 100 μM GTP, and 2 mM Mg2+, which produced maximum activation of single-channel K+[ACh] channel currents, the addition of 10 mM KF had no effect on mean open time (1.3 ± 0.1 ms) or the single-channel current amplitude at −80 mV (2.1 ± 0.06 pA). In another four experiments in the presence of 100 μM GDP/PS and 2 mM Mg2+, which maximally activated the K+[ACh] channel, addition of 10 mM KF again had no effect on mean open time (1.4 ± 0.05 ms) or amplitude at −80 mV (2.1 ± 0.03 pA).

Our results do not seem to support the attractive γ-phosphate-mimicking hypothesis of Bigay et al. (12, 13). These authors arrived at the conclusion that AlF3 activation of transfducin requires GDP by clear transducin of GDP using excess photoactivated rhodopsin. This result led to the hypothesis that AlF3 interacts with the GDP-bound form of transducin and that AlF3 mimics the role of the γ-phosphate of GTP. Support for this view comes from recent spectroscopic measurements (16). In our experiments, in the presence of agonist, additional GDP had no effect on AlF3 activation, although additional GDP had large effects on activation produced by GTP. Therefore, the Gβ present in the membrane patches that was activating the K+[ACh] channel was capable of releasing its GDP in the presence of GTP and presumably AlF3. If this is so, then AlF3 was producing its effects on Gα proteins that, in the presence of agonist and absence of guanine nucleotides, were cleared of GDP. Another test was our attempt to bind Gα with GDP/PS, which does not support the action of AlF3 as a γ-phosphate. Again, to attempt to clear GDP, we used GMP in great excess. Despite our best efforts, it is possible that some Gα, which did not interact with receptor yet was GDP-bound, was being activated by AlF3. This issue can be resolved when the stoichiometry of the muscarinic receptor, Gα, and the K+[ACh] channel is known. However, another possibility is that the mechanism by which AlF3 activates Gα in these membrane patches differs from the mechanisms reported for membrane-free Gα, Gβ, or Gγ.

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