Competition Between Internal AlF$_4^-$ and Receptor-Mediated Stimulation of Dorsal Raphe Neuron G-Proteins Coupled to Calcium Current Inhibition

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Chen, Yuan and Nicholas J. Penington. Competition between internal AlF$_4^-$ and receptor-mediated stimulation of dorsal raphe neuron G-proteins coupled to calcium current inhibition. J. Neurophysiol. 83: 1273–1282, 2000. Intracellular aluminum fluoride (AlF$_4^-$), placed in a patch pipette, activated a G-protein, resulting in a “tonic” inhibition of the Ca$^{2+}$ current of isolated serotonergic neurons of the rat dorsal raphe nucleus. Serotonin (5-HT) also inhibits the Ca$^{2+}$ current of these cells. After external bath application and quick removal of 5-HT to an AlF$_4^-$ containing cell, there was a reversal or transient disinhibition (TD) of the inhibitory effect of AlF$_4^-$ on Ca$^{2+}$ current. A short predepolarization of the membrane potential to +70 mV, a condition that is known to reverse G-protein-mediated inhibition, reversed the inhibitory effect of AlF$_4^-$ on Ca$^{2+}$ current and brought the Ca$^{2+}$ current to the same level as that seen at the peak of the TD current. With AlF$_4^-$ in the pipette, the TD phenomenon could be eliminated by lowering pipette MgATP, or by totally chelating pipette Al$^{3+}$. In the presence of AlF$_4^-$, but with either lowered MgATP or extreme efforts to eliminate pipette Al$^{3+}$, the rate of recovery from 5-HT on wash was slowed, a condition opposite to that where a TD occurred. The putative complex of AlF$_4^-$-bound G-protein (GorGDP-AlF$_4^-$) appeared to free G-βγ-subunits, mimicking the effect on Ca$^{2+}$-currents of the G-GTP complex. The on-rate of the inhibition of Ca$^{2+}$ current, after a depolarizing pulse, by βγ-subunits released by AlF$_4^-$ in the pipette was significantly slower than that of the agonist-activated G-protein. The off-rate of the AlF$_4^-$-mediated inhibition in response to a depolarizing pulse, a measure of the affinity of the free G-βγ-subunit for the Ca$^{2+}$ channel, was slightly lower than that of the agonist-stimulated G-protein. In summary, AlF$_4^-$ modified the off-rate kinetics of G-protein activation by agonists, but had little effect on the kinetics of the interaction of the βγ-subunit with Ca$^{2+}$ channels. Agonist application temporally reversed the effects of AlF$_4^-$, making it a complementary tool to GTP-γ-S for the study of G-protein interactions.

INTRODUCTION

Aluminum fluoride (AlF$_4^-$) binds to the α-subunit of heterotrimeric G-proteins (Sternweis and Gilman 1982) by forming a complex with G-GDP (guanosine diphosphate) thus mimicking the terminal γ-phosphate of guanosine triphosphate (GTP) (Bigay et al. 1987; Chabre 1990). The GorGDP-AlF$_4^-$ complex resembles that of the GTP-bound form of the G-protein, and it allows the α- and βγ-subunits to dissociate and interact with effectors downstream, although questions have been raised about the generality of this mechanism (Yatani and Brown 1991). We reasoned that AlF$_4^-$ might be a suitable tool to directly, but reversibly, activate G-proteins in an intact cell and investigate the effect of intracellular AlF$_4^-$ on G-protein coupling to Ca$^{2+}$ currents.

The α-subunits of G-proteins contain a helical domain that is critical for their GTPase activity. The GorGDP-AlF$_4^-$ complex of several Goα-subunits is reportedly a stabilized transition state that results in increased GTPase activity (Scheffzek et al. 1997), but in some systems Al$^{3+}$ or even guanine nucleotide is not required (Vincent et al. 1998). This increase in GTPase activity should be reflected in a faster on and off rate of agonist stimulation. In addition, a new class of GTPase-activating proteins (GAPs) has been discovered to modulate the on- and off-rate of G-protein-mediated effects on K$^+$ channels and other effectors of Goα and Goγ proteins (Koelle 1997; Saitoh et al. 1997). These GAPs also called RGS proteins (regulators of G-protein signaling) bind with a higher affinity to the GorGTP-AlF$_4^-$ complex of Goα-subunits than to the GTP-γ-S bound state (Berman et al. 1996), indicating that they also stabilize the GTPase active conformation of G-proteins. These findings suggest that the kinetics of activation and inactivation of the G-protein(s) involved in Ca$^{2+}$ current modulation, in the presence of AlF$_4^-$, may differ from those observed with agonist stimulation.

Our early observations revealed that AlF$_4^-$ caused a weak stimulation of the G-protein responsible for the modulation of calcium channel gating, but the nature of the stimulation differed markedly from agonist stimulation by activating a 5-HT1A receptor (Penington and Kelly 1990). In addition, serotonin (5-HT) could further inhibit the calcium current, and, unexpectedly, a pronounced transient disinhibition (TD) of the current was observed on removing the agonist. The occurrence of the TD was reminiscent of a prominent rebound facilitation of calcium current, on wash out of an inhibitory agonist from NG108–15 cells grown in conditions that promoted a state of tonic G-protein stimulation (Kasai 1991). In keeping with these observations, muscarinic agonists can also generate a similar rebound inactivation of a G-protein–mediated opening of K$^+$ channels in the heart, when that G-protein is in a state of weak tonic activation by low concentrations of an intracellular GTP analogue, guanylyl imidodiphosphate (Otero et al. 1991). We have tested two hypotheses concerning the actions of AlF$_4^-$ in the inhibitory effect of G-proteins on calcium channel activity.

1) There can be a competition between the receptor and AlF$_4^-$ stimulated G-protein activity, resulting in the short-term removal of tonic G-protein stimulation by AlF$_4^-$.

2) We investi-
gated whether the interaction occurs at the level of the G-protein, or the interaction of the G-βγ-subunit with calcium channels.

METHODS

These results were obtained from acutely isolated serotonergic dorsal raphe (DR) neurons that exhibited stable Ba2+ currents in 5 mM Ba2+.

Cell preparation

Male Sprague Dawley rats (200–250 g) were anesthetized with halothane and then decapitated. Three coronal slices (500 μm) through the brain stem at the level of the dorsal raphe nucleus were prepared using a Vibroslice (Campden Instruments) in a manner that has previously been described (Penington and Kelly 1990; Penington et al. 1991). The slices were placed in cold artificial cerebrospinal fluid (ACSF) containing (in mM) 119 NaCl, 5 KCl, 2 MgCl2, 2 CaCl2, 26 NaHCO3, 1.2 NaH2PO4, and 11 glucose, pH 7.3–7.4 when bubbled with 95% O2–5% CO2. The slices were placed on an agar base, and a tissue were then incubated in a PIPES buffer solution containing 0.07% trypsin (Sigma Type XI) under pure oxygen for 90 min according to the method of Kay and Wong (1986). The pieces of tissue were then triturated in Dulbecco’s modified Eagle’s medium and the isolated cells allowed to settle on a glass coverslip coated with concanavalin A. Within 5 min of plating, the cells were firmly anchored to the coated coverslip.

Recording

The extracellular solution was continually perfused at a rate of ~2 ml/min into a bath containing ~1 ml of recording solution. Neurons with truncated dendrites and a cell soma with one dimension of at least 20 μm were voltage-clamped using an Axopatch 200A (Axon Instruments, Foster City, CA) patch-clamp amplifier in the whole cell configuration. Electrodes were coated with silicone elastomer (Sylgard), and they ranged in resistance from 1.5 to 2.5 MΩ. Leak and capacitance were subtracted from the Ca2+ current records. Leak sweeps consisted of 16 hyperpolarizing steps of 10 mV that were then averaged. The leak sweep currents were scaled to the appropriate size and then subtracted from the individual current records except where noted. Leak sweeps were obtained at regular periods during the experiment. The voltage-clamp data were filtered at 2 kHz and then digitized at 100 μs per point. Voltage protocols were generated and analyzed by an IBM PC Pentium clone using the Axobasic 1 patch-clamp software, and the resultant data were written to disk for analysis off-line. Recordings from neurons acutely dissociated from the adult rat brain were carried out at room temperature (24°C). The measurements of Ca2+ current are expressed as means ± SE, and in some cases the means were tested for equality using a Student’s t-test or paired t-test. Where multiple comparisons of means were attempted a random effects, ANOVA was performed followed by a Student-Newman-Keuls test. Estimates of free Mg2+ and Mg2ATP were obtained using the program WinMaxC version 2.0 (Bers, ccm), which can be found on the internet (Patton 1999).

Solutions

The control pipette solution used in experiments that measured Ca2+ current was 130 mM CsCl, 10 mM HEPES, 10 mM EGTA, 4 mM MgATP, 4 (or 1) mM MgCl2, 300 μM GTP, and 14 mM phosphocreatine (with or without deferoxamine, 500 μM, ± AlCl3, 10 μM; where noted in text), pH 7.3 CsOH. The CsF solution contained 130 mM CsF, 10 mM HEPES, 10 mM EGTA, 2 mM MgATP, 2 mM TrisATP, 1 mM MgCl2, and 300 μM GTP, 14 mM phosphocreatine, ± deferoxamine (500 μM), ± AlCl3 (10 μM), pH 7.3 CsOH. AlCl3 was only used in early experiments because trace Al3+ was found to be sufficient. When deferoxamine was used to chelate Al3+, AlCl3 was not added to the pipette solution. We routinely used the following solution to establish seals for whole cell recording: this contained (in mM) 134 NaCl, 20 HEPES, 10 glucose, 2 CaCl2, 2.5 KCl, and 2 MgCl2. The external recording solution, designed to isolate calcium channel currents (carried by Ba2+), contained 160 TEACl, 5 BaCl2, 10 HEPES, and 20 sucrose, pH 7.3 with TEAOH.

5-HT creatine sulfate, GTP-γ-S tetra lithium salt, and GmpPNP were obtained from Sigma Chemical (St. Louis, MO). 5-HT was added to the bath near the cell using an electric valve-controlled, flow pipe, fast application system.

RESULTS

Aluminum fluoride caused a transient disinhibition of Ca2+ current after washing off 5-HT

AlF3 in the pipette solution was examined for its ability to stimulate the G-protein of dorsal raphe neurons directly. When serotonin was applied, a large TD of Ca2+ current occurred as the serotonin was washed off. Once the response had stabilized, the maximum size of the TD was measured and expressed as a percentage increase, comparing the peak of the TD current to the peak current before the application of 5-HT (Fig. 1A, n = 6, 27.4 ± 1.9%, mean ± SE). With the use of the CsF pipette solution, the current before the application of 5-HT usually activated more slowly [Fig. 1B (a) compared with CsCl solution Fig. 1D (a)]; slow activation is indicative of G-protein stimulation. The rate of activation of Ca2+ current was not quantified in this study because when activated with a square-wave step it is a hybrid of the rate of activation and inactivation (Jones and Elmslie 1997). The TD did not occur when the pipette contained CsCl solution and 10 μM AlCl3 as shown in Fig. 1C, and the baseline Ca2+ current was quite constant when recorded using CsCl. In comparison, the current before and after 5-HT attained the same baseline level. To investigate whether the TD phenomenon was specific for 5-HT, ATP (10 μM) was applied, which inhibited the Ca2+ current and produced a TD similar to that caused by 5-HT (n = 5, not shown).

ATP and free Mg2+ ions are cofactors of the transient disinhibition

The possibility that ATP, or MgATP, was important in the generation of this phenomena was investigated. Two conditions were examined. In the first, control (n = 7) and CsF pipette solutions (n = 6) contained 2 mM MgATP and 4 mM MgCl2; this amount of MgATP did not allow the occurrence of the TD in the CsF condition (Fig. 2A, maximum current after wash of 5-HT was −1.4 ± 5.7% of pre-5-HT level). Using these conditions in recordings from CsF containing cells slowed the recovery from 5-HT because the time to one-half recovery (t1/2) was 35.5 ± 5 s (n = 6, Fig. 2A). In the control group (CsCl) the t1/2 of recovery was significantly faster (18.9 ± 1 s; P < 0.01, n = 7). In another group of cells, 2 mM TrisATP was added back to the solution used in Fig. 2A. A TD measuring 13.4 ± 1.8% occurred (n = 8, Fig. 2B). Taken together, these data suggest that MgATP is required for this phenomenon because our estimate of free MgATP was doubled by adding 2 mM TrisATP when free Mg2+ was halved (Table 1).
The Mg\(^{2+}\) or MgATP dependence of this phenomenon was investigated further by providing the same total concentration of ATP (4 mM) and Mg\(^{2+}\) (4 mM) but in three different forms; all other constituents of the pipette solution were identical.

1. 4 mM MgATP and 0 mM MgCl\(_2\);
2. 1 mM KATP, 3 mM MgATP, and 1 mM MgCl\(_2\);
3. 4 mM TrisATP and 4 mM MgCl\(_2\).

Table 1 shows estimates of free Mg\(^{2+}\) and MgATP obtained using the program WinMaxC. It is notable that all currently available programs would predict identical free Mg\(^{2+}\) and MgATP for these three solutions because they do not take into account the effect of the salts with which they form a complex. In the first case when no MgCl\(_2\) was added to the intracellular solution, the TD was not observed (Fig. 2C), and the peak calcium current was 3.3 ± 0.6% smaller on average after 5-HT was washed off. In the second condition the TD failed to occur in three of four recordings, and one cell showed a small TD (average of 4 cells 1.6 ± 1.8%). In the third condition, 4 mM TrisATP could replace 4 mM MgATP to produce the TD (mean, +13.6% TD; n = 3, not shown); however, calcium current rundown was accelerated. It is possible that the presence of Tris changed the free Mg\(^{2+}\) or MgATP concentrations so that each condition is not equivalent. Further, free Mg\(^{2+}\) is required for the TD, or the response to 5-HT would not occur, and some form of ATP is also implicated (see Fig. 4). From this point on, the standard pipette solution contained 1 mM MgCl\(_2\) unless otherwise stated.

### FIG. 1. Aluminum fluoride (AlF\(_4^−\)) in the pipette solution inhibits barium current and allows a transient disinhibition to occur after application of serotonin (5-HT). A: with CsF in the pipette and (in mM) 4 MgATP and 1 MgCl\(_2\), the recovery current was larger than control after 5-HT (1 μM) was washed off. a) Control current. b) Current during inhibition by 5-HT. c) Current after washing off 5-HT. B: current passing through calcium channels of dorsal raphe (DR) neurons with a CsF pipette solution. The current in B exhibits a transient disinhibition (TD) as 5-HT was washed off. The current at points a, b, and c are equivalent in time to the currents in A, but these traces were not taken from the same cells. C: CsCl pipette solution containing the same concentration of nucleotides and MgCl\(_2\) along with 10 μM AlCl\(_3\) (control) shows a plot of peak Ca\(^{2+}\) current against time, the current returns to control after 5-HT was washed off. D: calcium current with a CsCl pipette solution shows no TD. In all figures time 0 is the time when the earliest recording could be established and is not coincident with going whole cell.

### FIG. 2. ATP is required for transient disinhibition of Ba\(^{2+}\) current. A: halving the concentration of MgATP to 2 mM in the CsF pipette solution did not support a TD, and it dramatically slowed the recovery from 5-HT (1 μM). B: adding back 2 mM TrisATP to the pipette solution in A allowed the TD to occur. C: when no MgCl\(_2\) was added to the intracellular solution containing 4 mM MgATP, the TD was not observed, and the peak calcium current was smaller on average after 5-HT was washed off.

### TABLE 1. Estimated free Mg\(^{2+}\) and Mg2ATP

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<th>Solution, mM</th>
<th>Size of TD, %</th>
<th>Total Added Concentration, M</th>
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<th>Free Mg2ATP Estimate, M</th>
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<td>1.9 (\times 10^{-3})</td>
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<td>2.26 (\times 10^{-3})</td>
<td>1.9 (\times 10^{-3})</td>
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<td></td>
<td></td>
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<td>4 MgCl(_2)</td>
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<td>Mg(^{2+}) (6 \times 10^{-3})</td>
<td>2.26 (\times 10^{-3})</td>
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<td>with ATP</td>
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<td>Mg(^{2+}) (4 \times 10^{-3})</td>
<td>2.03 (\times 10^{-3})</td>
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<td>(4 mM) and</td>
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<td>EGTA (1 \times 10^{-2})</td>
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TD, transient disinhibition.
Role of Al$^{3+}$

To investigate whether F$^-$ or a complex of Al$^{3+}$ and F$^-$ caused the TD, an attempt was made to reduce Al$^{3+}$ in the pipette solution. Deferoxamine (500 μM), a chelator of Al$^{3+}$, was included in the pipette solution (2 mM MgATP, 2 mM TrisATP, and 4 mM MgCl$_2$), and no Al$^{3+}$ was added to the intracellular solution. Unexpectedly, this solution allowed a TD to occur after application of 5-HT ($n = 3$). These cells generated TDs that averaged a 17.4% increase over pre–5-HT baseline. In another group, 2 mM MgATP, 0 mM Tris ATP, and 1 mM MgCl$_2$ plus deferoxamine was used. Under these conditions a large TD developed (19.3 ± 2.2%, $n = 5$, Fig. 3A). We had previously established that when adding only 2 mM MgATP in the pipette solution there was no TD (Fig. 2A). In summary, when the Al$^{3+}$ level was lowered with deferoxamine, but presumably not abolished, the recordings all revealed a TD after 5-HT application.

Subsequently an attempt was made to abolish the Al$^{3+}$ ion from the pipette solution altogether. To do this, the solution, containing deferoxamine (500 μM), was made up in a plastic beaker, and custom patch pipette glass (Warner Instruments No. PG150T-7.5) was used that contains no Al$^{3+}$. Using these conditions the TD was not observed (Fig. 3B) (TD = 1.5 ± 1.8%, $n = 5$), and the recovery from 5-HT was slowed ($t_1/2 = 36 ± 6.7$ s, slower than control at the $P < 0.01$ level). This result suggests that trace Al$^{3+}$ in combination with F$^-$ is sufficient to produce the TD.

5-HT–mediated phosphorylation and dephosphorylation did not appear to mediate the TD

Because ATP appeared necessary for this response, the role of phosphorylation in the effect was investigated. 5′-Adenyl-imido-diphosphate (AmpPNP) is an analogue of ATP that cannot donate a high-energy phosphate in enzyme reactions, and it is not a substrate for kinases. In a group of cells ($n = 3$) 4 mM AmpPNP was used in place of 4 mM MgATP, and the Ca$^{2+}$ current ran down quickly obscuring the interpretation (Fig. 4A). When the peak current was corrected for rundown, there appeared to be a small TD. B: AmpPNP did not prevent the TD. The pipette contained 2 mM MgATP, 2 mM AmpPNP, plus deferoxamine in the CsF pipette solution. C: replacing 2 mM AmpPNP in the pipette solution used in B with 2 mM ATP-γ-S did not prevent or prolong the TD.
ATP-\(\gamma\)-S is another ATP analogue that is less effective than ATP in providing a high-energy phosphate, and a thio-phosphorylated product is resistant to the action of phosphatases. With equal concentrations of ATP-\(\gamma\)-S and MgATP (2 mM) in the pipette solution, a large TD was observed after 5-HT application that measured on average 26.4\% (\(n = 3\)), when 4 mM ATP-\(\gamma\)-S, in place of all forms of ATP, was added to the pipette, 5-HT had no effect on Ca\(^{2+}\) current, although this current could be greatly facilitated by a depolarizing prepulse. The calcium current appeared to be fully modulated as if GTP-\(\gamma\)-S only was present in the pipette.

Role of fluoride

Reducing the CsF concentration from 130 to 10 mM and substituting this with CsCl significantly reduced the size of the TD (Fig. 5A) compared with its size in CsF pipette solution (9.4 ± 2.7\%, \(n = 5\), Fig. 5A vs. 27.4 ± 1.9\%, \(n = 6\), \(P < 0.01\), Fig. 1A).

Disinhibition was G-protein dependent

\(\text{AlF}_4^-\) reportedly activates G-proteins by binding to the GDP of G\(_\alpha\)GDP, thus mimicking G\(_\alpha\)GTP. We attempted to investigate this mechanism by adding GTP-\(\gamma\)-S to the CsF pipette solution at a concentration of 30 \(\mu\)M to activate the G-protein directly. In four cells, the effect of GTP-\(\gamma\)-S completely occluded the TD (not shown).

GmpPNP can also directly activate G-proteins, but it has a lower affinity for the GDP binding site than GTP-\(\gamma\)-S (Otero et al. 1991). Because it was suspected that weak G-protein activation plays a role in the TD, an attempt was made to reproduce the effect of \(\text{AlF}_4^-\) with GmpPNP by adding 10 \(\mu\)M GmpPNP with 300 \(\mu\)M GTP to the control CsCl pipette solution. When 5-HT was applied and washed off in two of four cells, a TD was observed similar to that seen when using the CsF solution. In another three cells the GmpPNP was increased to 20 \(\mu\)M, and in two of three cells a TD was observed (Fig. 5B); in the remaining cell the calcium current was strongly inhibited by the GmpPNP, and this could not be reversed by 5-HT application. All the cells that responded with a TD in the presence of GmpPNP had a similar sized TD that averaged 38.3 ± 7.3\% (\(n = 4\)).

5-HT displaced GDP-\(\text{AlF}_4^-\) from the G-protein GTP-binding site

In the presence of \(\text{AlF}_4^-\) the G-protein was partially activated; this is based on the finding that the Ca\(^{2+}\) current was facilitated to a large degree by a depolarizing prepulse to +70 mV (Fig. 6A) (Bean 1989; Elmslie et al. 1990; Grassi and Lux 1989; Ikeda 1991; Jones and Marks 1989; Scott and Dolphin 1990). After 5-HT was applied and washed off, and the TD current reached its peak, there was always a negligible facilitation of the peak current by a depolarizing prepulse to +70 mV, or in some cases prepulse depolarization inhibited the peak Ca\(^{2+}\) current at the maximal TD (average −5 ± 0.02\%, \(n = 14\)). Later in the recording, in a cell representative of 14 that was previously exposed to 5-HT, a depolarizing prepulse to +70 mV weakly facilitated the peak Ca\(^{2+}\) current in the absence of 5-HT (Fig. 6A, right), suggesting that there was little voltage-dependent G-protein stimulation at this time. Because the degree of facilitation of the Ca\(^{2+}\) current, after a depolarizing prepulse, represents the extent of voltage-dependent interactions of the G-protein \(\beta\gamma\)-subunit with the Ca\(^{2+}\) channel, we compared the decay of facilitation of the Ca\(^{2+}\) current in the absence of 5-HT using a CsF solution in cells that had not been exposed to 5-HT and those that had been repeatedly exposed. Without applying 5-HT (see Fig. 7A for protocol), the decay of the amount of facilitation of peak Ca\(^{2+}\) current was slow and linear (the slope was −1.2 ± 0.3 \times 10^{-3} \frac{\%}{\text{s}}, \(n = 6\)). When 5-HT was applied every 200 s, the facilitation decreased more rapidly with a time course that could be fit with a single exponential decay (\(\tau = 468.2\) s, \(n = 5\), Fig. 6B).

Repeated or prolonged application of 5-HT decreased the degree of G-protein activation by \(\text{AlF}_4^-\) and promoted the development of the TD

The TD required the prior application of 5-HT. However, because the size of the TD appeared to increase with the number of 5-HT applications or time into the experiment, its magnitude could be related to the time of the delay before the first application of 5-HT. To investigate this possibility, the Ca\(^{2+}\) current was elicited every 20 s for 900 s, and a regular test prepulse of 30 ms duration to +70 mV was applied every 60 s, to estimate the degree of G-protein stimulation. When 5-HT was applied for the first time 900 s into the recording, the TD failed to occur in five cells (Fig. 7A). In another experiment 5-HT was applied continuously for a period of 650 s from the beginning of the recording and then washed off. The removal of 5-HT was accompanied by a robust TD in three of four cells measuring 15.6 ± 9.5\% (Fig. 7B). This result indicated that the
TD does not depend on the extent of the delay before 5-HT was applied but rather appears to be related to the total duration of the application, although the duration of 5-HT application was not systematically varied.

Unlike recordings from cells containing CsCl, cells containing AlF$_4^-$ showed smaller Ca$^{2+}$ currents and inhibitory responses to 5-HT. With time after going whole cell, the peak baseline Ca$^{2+}$ current grew larger, as did the absolute response to 5-HT, expressed as a percent inhibition of pre–5-HT baseline current. The relationship between the degree of G-protein activation and the size of the TD was examined and compared with the condition where there was little tonic G-protein activation, e.g., recordings done with CsCl in the patch pipette. Early in the recording, it was found that AlF$_4^-$ appeared to activate the G-protein, as it partially inhibited the calcium current. Figure 7C shows that a graph the size of the baseline Ca$^{2+}$ current using CsF solution (1,977 ± 155 pA, n = 18) was significantly smaller than the size of the peak baseline current using CsCl solution (3,019 ± 393 pA, n = 12). There was little facilitation in control CsCl solution (5.7 ± 0.8%, n = 12) (see also Penington et al. 1991). The average percentage facilitation of the peak Ca$^{2+}$ current with AlF$_4^-$ in the pipette after a conditioning step to +70 mV was 26.3 ± 2.1% (n = 13). When the previous value was compared with the TD, also in AlF$_4^-$ containing solution, expressed as a percentage of the pre–5-HT level (27.4 ± 1.9%, n = 6, Fig. 6B), these values were not statistically different from each other.

![Graph showing relationship between degree of G-protein activation and size of TD](image)

**Fig. 7.** Relationship between the degree of G-protein activation and the size of the TD. A: time course of the action of 5-HT in reversing the effect of AlF$_4^-$. The cell contains CsF. In this experiment a prepulse was applied every 60 s (with no application of 5-HT) for 900 s; then one application of 5-HT (1 μM) produced no disinhibition (n = 5). B: in another group of cells, soon after going whole cell, 5-HT was applied for 650 s. When the 5-HT was washed off, there was a TD that occurred in all 4 cells. C: the graph on the left plots data from cells recorded with CsF or CsCl pipette solutions. The size of the baseline peak Ca$^{2+}$ current and after a conditioning step to +70 mV measured as soon as possible after going whole cell is plotted. The size of the Ca$^{2+}$ current baseline using a CsF solution is significantly smaller than the size of the peak baseline current using a CsCl solution. The size of the prepulse facilitated current using CsF was not significantly different from the size of the current using a CsCl solution. The graph on the right compares the % facilitation of the calcium current using CsF in the pipette at the beginning of the experiment with the % increase in peak calcium current over baseline at the peak of the TD after the TD effect had become maximal. D: with CsF as the pipette solution, both the percentage of the inhibition of baseline calcium current by 5-HT and the size of the TD grew exponentially. Filled squares represent inhibition by 5-HT expressed as a percentage of the baseline current just before the application of 5-HT. Open circles represent the size of the TD. As the inhibition to 5-HT increased, the size of the TD increased.
course to the decay of the facilitation current after multiple 5-HT applications (Fig. 6B).

Decay of the TD may be a close approximation of the on-rate of AlF₄⁻ binding to GaGDP

When 5-HT was removed, the TD reached its peak and the G-protein was presumably in the ground state (GαGDP). After the peak of the TD, the current returned toward baseline, perhaps reflecting the onset of a tonic reactivation of the G-protein. We reasoned that the rate of return of the current to baseline may reflect the rebinding of AlF₄⁻ to GaGDP and measured the rate of this relaxation. Because the rate of inhibition was very slow, we also measured a functional index of the rate of interaction of the Gβγ-subunit freed by AlF₄⁻ with the Ca²⁺ channel (the on-rate of inhibition) to ascertain whether this rate was slowed and could thus account for the slow tonic inhibition. The time course of the decay of the TD was fit well by a single exponential average τ = 34.6 ± 4.2 s (n = 5; Fig. 8A). Despite the fact that the decay was fit by a single exponential, it is probably comprised of at least two steps. 1) AlF₄⁻ slowly binds to GaGDP, mimicking GTP and activating the G-protein (Chabre 1990). 2) By comparison, the activated G-protein βγ-subunit binds to Ca²⁺ channels and inhibits the Ca²⁺ current much faster (Herlitze et al. 1996; Ikeda 1996). The second step of "on-rate of inhibition" was measured by lengthening the interpulse interval between the depolarizing prepulse voltage step of +70 mV that temporarily reverses the G-protein stimulation by Gβγ (Elmslie et al. 1990; Ikeda 1991). The average of the time constants of the on-rate for G-GDP-AlF₄⁻-mediated inhibition (no agonist added) was 129.2 ± 27.8 ms (n = 7, open triangle, Fig. 8B). The on-rate of the G-GDP-AlF₄⁻-mediated inhibition is likely to represent the second step (measured in ms), and the first step: AlF₄⁻ binding to GDP appears to be rate limiting and is measured in seconds. In Fig. 8B the filled square is the on-rate of 5-HT inhibition using a CsF pipette solution; the average of the τs was 66.8 ± 3.1 ms (n = 5). The open square shows the on-rate of 5-HT-mediated inhibition using a CsF pipette solution; the individual τs averaged 69.7 ± 14.3 ms (n = 6). The presence of AlF₄⁻ did not change the on-rate of the 5-HT activated G-protein (βγ) subunit binding to the Ca²⁺ channels.

AlF₄⁻ decreased the off-rate of G-protein binding to the Ca²⁺ channel

AlF₄⁻ changed the off-rate of the effect of 5-HT (Fig. 9A). The off-rate is a measure of the affinity of the G-protein (βγ) subunit binding to the Ca²⁺ channels. In control (CsCl) solution, the average of the off-rates (τs) of the effect of 5-HT was 8.0 ± 0.3 ms (n = 9); this represents the off-rate of the GTP-bound G-protein. The average off-rate of the effect of 5-HT using CsF solution was 10.9 ± 0.5 ms (n = 7). Although the difference between the rates is small, these values are significantly slower (P < 0.05) than with CsCl. In addition, the average off-rate of the G-protein stimulating effect of AlF₄⁻ alone (11.1 ± 1.3 ms, n = 9) is not different from the off-rate of the effect of 5-HT using an AlF₄⁻-containing solution. These data indicate that the product of the AlF₄⁻ bound G-protein (βγ) has a slightly higher affinity for the Ca²⁺ channel than the GTP-bound G-protein (βγ) in its interaction with Ca²⁺ channels.

DISCUSSION

The binding of an agonist to a receptor causes GDP dissociation and GTP binding to the G-protein followed by the
release of the Go from the Gβγ-subunits (Gilman 1987). Simultaneously a conformational change occurs, leading to an increased GTPase activity of the α-subunit. On washing off the agonist, the GTP-bound form converts to the GDP-bound form, which is dependent on the rate of GTPase activity. If the GTPase activity is high, this will be reflected in a faster recovery from the agonist. The main findings of the present study were that intracellular AlF$_4^-$ caused approximately a one-third of maximum tonic stimulation of the G-protein coupled to Ca$^{2+}$ channels of DR neurons (Fig. 7C), consistent with a G-GDP-AlF$_4^-$ complex resulting in a mimicry of the G-GTP complex (G-βγ release).

Based on the literature, a fairly parsimonious explanation of the sequence of events occurring after agonist application to a DR cell containing AlF$_4^-$ is that after several applications of 5-HT, some G-proteins are in the basal state and some are activated (α-GDP-βγ ⇌ α-GDP-AlF$_4^-$ + βγ). This should produce only partial channel inhibition, allowing further inhibition by 5-HT. When 5-HT binds to its receptor, that enhances release of GDP from α-GDP-βγ, and GTP binds, releasing more βγ. Perhaps by mass action (α-GDP-AlF$_4^-$ ⇌ α-GDP → α → α-GTP), or by direct enhancement of the release of GDP-AlF$_4^-$, eventually most G-protein α-subunits are in the GTP bound state. On removal of 5-HT, GTPase activity transiently returns most G-proteins to the basal α-GDP-βγ form, allowing maximal channel activity. Finally, AlF$_4^-$ rebinds. We assume that under these conditions the steady state is a mixture of α-GDP-AlF$_4^-$ + βγ, and α-GDP-βγ. A similar phenomenon has been reported to occur with other weak activators of G-proteins on agonist application (Cassel and Selinger 1977; Otero et al. 1991). With repeated or prolonged application of the agonist, the G-protein should gradually become predominately GDP bound in the presence of agonist, and GDP bound when 5-HT is removed. The observations of the present study can be interpreted within the framework of the above hypotheses. In direct support of this suggestion, we found that low concentrations of GmpPNP, that weakly stimulated the G-protein, produced results similar to AlF$_4^-$.

AlF$_4^-$ bound to Go-subunits of G-proteins produces a stable active transition state of the GTPase activity of Go (Scheffzek et al. 1997); this may contribute to the relatively fast wash off of the effect of 5-HT (TD), because it would speed the removal of GTP. It was puzzling at first to explain why adding deferoxamine (a chelator of Al$^{3+}$) did not prevent the TD while presumably decreasing the amount of the AlF$_4^-$ complex. When steps were taken to remove Al$^{3+}$ completely, this reduced the rate of recovery from the effect of 5-HT and the TD altogether; converting the rate of recovery to one that was much slower than that observed using CsCl in the pipette. In the absence of Al$^{3+}$ (but in the presence of F$^-$) the GTPase activity appears to be greatly inhibited by F$^-$ when the GTPase activating activity of the G-GDP-AlF$_4^-$ complex does not counteract it. A possible explanation for this observation could be that decreasing the amount of G-GDP-AlF$_4^-$ to low levels by lowering (but not abolishing) Al$^{3+}$ may weaken the G-protein stimulation by AlF$_4^-$ and allow it to be more effectively displaced by agonist application, permitting a TD to be observed. Another possibility is that the [Al$^{3+}$] is in excess and as previously proposed it may compete with Mg$^{2+}$ for several binding sites on G-proteins (Chabre 1990), perturbing them, and altering the binding of AlF$_4^-$ to G-GDP. In this fashion, lowering Al$^{3+}$ may increase the effect of the active species. The same argument has been put forward to explain the effect of lowering Mg$^{2+}$ on the effect of AlF$_4^-$-stimulated G-proteins (Chabre 1990).

ATP is required for the efficient coupling of the receptor to the G-protein (Elmslie et al. 1993) and presumably for GTPase activity. With AlF$_4^-$ in the pipette, the rate of recovery from 5-HT was dramatically slowed when added MgATP was lowered from 4 to 2 mM. The fast off-response on washing 5-HT (TD) may be dependent on free Mg$^{2+}$, which might be explained by invoking the dependence of GTPase activity directly (Bourne et al. 1991) or indirectly on free Mg$^{2+}$. It is known that both agonist and AlF$_4^-$-induced stimulation of the GTPase is Mg$^{2+}$ dependent, as is AlF$_4^-$ binding to G-GDP leading to GTPase activation (Chabre 1990; Gilman 1987). Thus in the absence of sufficient ATP or free Mg$^{2+}$, F$^-$ but not
GDP. The peak Ca\(^{2+}\) was occasionally inhibited by a depolarizing prepulse delivered at the peak of the TD. A reduction in N-channel peak current by depolarizing prepulses occurs after complete removal of tonic G-protein inhibition with GDP-\(\beta\)-S (Patil et al. 1998), which supports the suggestion that there is virtually no tonic G-protein activity at this time. The rate of decline of the TD is consistent with the rate of rebinding of the AlF\(_4\) to G-GDP (Bigay et al. 1987); but because the G-GDP bound form is required for AlF\(_4\) binding (Chabre 1990), the rate of rebinding should ultimately depend on the rate of GTPase activity. The slow on-rate of AlF\(_4\) binding confirms that the affinity of this interaction must be quite low (Chabre 1990).

The nonhydrolyzable analogue AmpPNP was able to substitute for MgATP and support the fast off-response of agonist removal in the presence of AlF\(_4\), suggesting that phosphorylation is not involved in this response. However, the finding that AmpPNP or ATP-\(\gamma\)-S can substitute for ATP could be explained by a substitution for MgATP at certain binding sites that do not require ATP hydrolysis, thus effectively freeing up the MgATP complex to take part in reactions that require ATP hydrolysis. When ATP-\(\gamma\)-S completely replaced ATP in the pipette, the calcium current appeared to be fully modulated. A possible explanation for this observation is that nucleoside-di-phosphate kinase produced GTP-\(\gamma\)-S from ATP-\(\gamma\)-S plus GTP (Elmslie et al. 1993).

The size of the TD appeared to be related to the concentration of F\(^-\) in the pipette, but it would be difficult to construct a dose response curve for the concentration of F\(^-\) and the size of the TD. The reason for this is that changing the concentration of free F\(^-\) will alter the concentration of AlF\(_4\) (Chabre 1990). Because it was possible to eliminate the response by eliminating pipette Al\(^{3+}\), our results suggest that the size of the response appears to be related to the concentration of the AlF\(_4\), AlF3(OH)\(^-\) or AlF\(^{-}\) rather than the concentration of F\(^-\).

The on-rate of Ca\(^{2+}\) current inhibition by receptor activation after a depolarizing prepulse appears to be related to the concentration of extracellular agonist, and the amount of activated G-protein \(\beta\gamma\)-subunit (Chen and Penington 1996; Elmslie and Jones 1994; Herlitze et al. 1996; Ikeda 1996). The on-rate of Ca\(^{2+}\) current inhibition by AlF\(_4\) (with no agonist application after a prepulse) was slower than the on-rate of 5-HT inhibition alone, suggesting that there were fewer activated G-GDP-AlF\(_4\) complexes yielding \(\beta\gamma\) interactions with Ca\(^{2+}\) channels compared with the effect of 5-HT receptor stimulation. Under the conditions used in the present study, AlF\(_4\) was not able to maximally activate the G-protein in the manner of a full receptor agonist. After 5-HT was removed, AlF\(_4\) presumably bound again to the GDP-bound G-protein and slowly released G-\(\beta\gamma\) subunits reinhibiting the current. After an equilibrium in the cycle of agonist application became stable, the TD was able to occur and reach a maximum size; this was equal to the average baseline calcium current that would occur if a CsCl pipette solution was used.

Another possible explanation for the TD could be advanced if the affinity of the products of the G-GDP-AlF\(_4\) complex (\(\beta\gamma\)) for the Ca\(^{2+}\) channels was lower than control in the presence of F\(^-\), and they dissociated more quickly. In measuring the off-rate of Ca\(^{2+}\) current inhibition using CsF, it was revealed that the affinity of the Ca\(^{2+}\) channel for G-\(\beta\gamma\) appeared to be a little higher than that of the G-GDP complex. This finding appears to rule out as an explanation an alteration in the kinetics of the interaction between the Ca\(^{2+}\) channel and the \(\beta\gamma\)-subunits produced by G-GTP and G-GDP-AlF\(_4\) complexes. Instead it suggests that the action of AlF\(_4\), which results in the altered properties of G-protein effects on Ca\(^{2+}\) channels, resides with the kinetics of activation and inactivation of the G-protein and the speed with which it releases \(\alpha\)- and \(\beta\gamma\)-subunits.

Previous studies using intracellular F\(^-\), presumably made up in glass containing Al\(^{3+}\), found that high-threshold L-type Ca\(^{2+}\) currents are eliminated by this intracellular solution (Bertolulli et al. 1994). L-type current components show very slow inactivation. Consequently inhibition of a large L-type component of Ca\(^{2+}\) current would increase the relative amplitude of a rapidly inactivating component when using a F\(^-\)-containing recording solution (Kay et al. 1986). In addition, millimolar concentrations of Na or KF have been shown to inhibit various phosphatases, phosphorylases, and ATPases but to activate adenyl cyclase (Murphy and Coll 1992; Murphy and Hoover 1992; Rall and Sutherland 1958). It has also been shown that phosphatase inhibition produces an increase in N-type Ca\(^{2+}\) current inactivation rate (Wertz et al. 1993); thus phosphatase inhibition may partially explain the increased inactivation rate (Kay et al. 1986). Serotonergic dorsal raphe neurons have a small L-type Ca\(^{2+}\) current comprising \(<5\%\) of the total current (Penington et al. 1991), and its abolition by F\(^-\) is not very apparent. We also occasionally observed an increase in the rate of voltage-dependent Ca\(^{2+}\) current inactivation, and a slower activation in comparison to recordings obtained using a CsCl pipette solution. In raphe neurons the latter effect appeared to be reversible and due to G-protein activation.

Usually the TD did not occur at the beginning of the recording, but it developed after applying and washing 5-HT several times (see Figs. 3A, 4C, and 7, A, B, and D). These results indicate that the TD requires prior 5-HT application, although it appeared not to depend on the duration of the recording before agonist application (Fig. 7A) but was related to the total duration of the application. It should be noted that the effects of varying the duration of the 5-HT application on the development of the TD were not systematically tested but only the extremes of repeated short pulses, a continual long application, or finally a short application after 900 s of recording. Nevertheless, the data were consistent with the hypothesis that 5-HT must displace AlF\(_4\) from the G-protein before a TD can result.

There are three main characteristics of the effects of AlF\(_4\) that require explanation. 1) There is usually no TD at the beginning of the recording. 2) The stimulation of the G-protein by AlF\(_4\) appeared to be relatively strong early in the recording, as revealed by large amounts of Ca\(^{2+}\) current facilitation induced by depolarizing prepulses, but becomes weaker with time after several applications of 5-HT. 3) As the recording progressed, the baseline of the calcium current was enhanced.
These observations might be explained by supposing that at the beginning of the recording the majority of the G-protein is bound with AlF₄⁻, leaving a small fraction in a high-affinity state free to interact with the receptor. When the receptors are activated, this would be likely to activate GTPase activity, so that more of the G-GDP-AlF₄⁻ would be replaced by G-GDP on washing the agonist. This could account for the gradual increase in the size of the current and the gradual loss of the ability of AlF₄⁻ to modulate the channels. When more Gα-GDP is available in the cell, G-βγ will bind to Gα-subunits more quickly, and recovery from 5-HT will be more rapid (Wickman and Clapham 1995). Presumably it takes ~1 min for all of the AlF₄⁻ to rebind to Gα-GDP (τ = 33.6 s) (Chabre 1990; this study). The percentage of G-proteins bound to AlF₄⁻, after an equilibrium state with respect to agonist application frequency has been achieved, would be less than at the start of the recording; this may explain the reduction in tonic G-protein stimulation later in the experiment. These findings clarify a number of questions about the modulation of G-proteins and Ca²⁺ currents by AlF₄⁻. Further study will explore the potential usefulness of AlF₄⁻ in studies of G-protein modulation by neurotransmitters.

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