Chloride Effects on Gs Subunit Dissociation

FLUOROALUMINATE BINDING TO Gs DOES NOT CAUSE SUBUNIT DISSOCIATION IN THE ABSENCE OF CHLORIDE ION

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The stimulatory guanine nucleotide binding protein (Gs) is heterotrimeric (αβγ), and mediates activation of adenylyl cyclase by a ligand-receptor complex. The α subunit of Gs (Gsα) has a guanine nucleotide binding site, and activation occurs when tightly bound GDP is displaced by GTP. Together, GDP and fluoroaluminate (AlF4-) form a transition state analog of GTP that activates Gα. The work of other investigators suggests that AlF4- causes subunit dissociation when it activates Gα. We have observed that in solution AlF4- did not cause Gs subunits to dissociate unless NaCl was also present. The effect of NaCl was concentration dependent (10–200 mM). Omitting F-, Al3+, or Mg2+ prevented the NaCl-induced dissociation of Gs subunits. Na2SO4 could not substitute for NaCl in causing subunit dissociation, but KCl could, suggesting that the anion was responsible for the effect. Gs subunit reassociation occurred when the concentration of Cl1 was reduced even though the concentrations of AlF4- and Mg2+ were maintained. The absence of Cl1 did not prevent AlF4- binding to Gα. We have concluded that AlF4-, a ligand which is capable of activating G proteins, can bind to Gs in solution without causing subunit dissociation.

It has been more than 35 years since F- was first identified as being an activator of adenylyl cyclase (Rall and Sutherland, 1958). In the interim the heterotrimeric (αβγ) stimulatory G protein (Gs)1 was identified as mediating activation of the enzyme by hormone-receptor complexes, by GTP and its analogs, and by F-(Howlett et al., 1979). In order for Gα to activate adenylyl cyclase, GDP which is tightly bound to the guanine nucleotide binding site of the α subunit (Gsα) must be displaced by GTP or a nonhydrolyzable GTP analog such as GTPγS or Gpp(NH)p. This process is facilitated by the hormone-receptor complex which explains its role in the activation of Gα. The intrinsic GTPase activity of Gsα provided a means for terminating activation of the adenyl cyclase (Gilman, 1987; Birnbaumer, 1990; Simon et al., 1991; Clapham and Neer, 1993). The mechanism by which F-activated Gα remained a mystery until it was recognized that Al3+ or Be2+ was also required for the activation (Sternweis and Gilman, 1982). It is believed that together Al3+ and F- form fluoroaluminate (AlF4-), a phosphate analog, that binds to the guanine nucleotide binding site of Gsα and together with bound GDP mimics the effects of GTP (Chabre, 1990). Following the activation of Gα by GTP analogs or AlF4- it is thought that Gsα dissociates from the G protein βγ subunit complex (Gβγ). This dissociation is believed to be a critical part of the activation process. Contributing to the subunit dissociation hypothesis are data suggesting that activation of Gα by AlF4- is accompanied by Gs subunit dissociation (Sternweis et al., 1981; Northup et al., 1983; Kahn and Gilman, 1984). However, we have recently reported that AlF4- does not cause Gs subunits to dissociate (Toyoshige et al., 1994). In an attempt to reconcile our findings with those of other investigators we have discovered that Cl1 is required for AlF4- induced Gs subunit dissociation. Here we report the experimental results of our investigation.

EXPERIMENTAL PROCEDURES

Materials—Anti-Gsα (RM/1) and anti-Gβ (SW/1) antiserum, [35S]iodo-protein A (2–10 μCi/μg), and [125I]iodo-protein A (<800 Ci/mmol) were obtained from DuPont NEN. RM/1 and SW/1 were also obtained as generous gifts from Paul Goldsmith. Protein A-Sepharose CL-4B was purchased from Pharmacia Biotech Inc. Immobilon P came from Millipore Corp. (Bedford, MA). Centrifuge 30 ultrafiltration units were from Amicon, Inc. (Beverly, MA). 1--Tosylamide-2-phenylmethyl ketone-treated trypsin (essentially salt free), bovine serum albumin, soybean trypsin inhibitor, and Lubrol-PX were from Sigma. Purified bovine brain Gβγ at a concentration of 1 mg/ml in 20 mM HEPES (pH 8.0), 0.1 mM EDTA, 1 mM DTT, and 0.25% Lubrol-PX was a generous gift from John Northup. S49 wild type and S49 cyc: cells were grown as described previously (Ross et al., 1977). The cells were collected and homogenized with a Polytron homogenizer (Brinkman Instruments, Westbury, NY), and a plasma membrane fraction was prepared using discontinuous sucrose density gradient centrifugation (Ross et al., 1977). The membranes were stored in liquid nitrogen in a solution containing 20 mM HEPES (pH 7.4); 2 mM MgCl2, 1 mM EDTA, 1 mM DTT, and 10% sucrose at a membrane protein concentration of 1 mg/ml until needed.

Experimental Treatment and Immunoprecipitation of Gα-Gs. Gs was prepared from bovine brain and stored at an estimated concentration of 450 μg of Gs/ml (Roof et al., 1985). For experiments, samples containing approximately 80 ng of bovine brain Gs were incubated in 2 μl of solution A (20 mM HEPES (pH 8.0), 1 mM EDTA, and 1 mM DTT) containing 0.1% Lubrol PX as described previously (Toyoshige et al., 1994) except that the incubation time was reduced from 2 to 1 h, and buffered solutions were prepared with the free acid of HEPES so that the pH could be adjusted with NaOH. The latter change was made in order to avoid the unintentional addition of Cl1 ion. The incubations also included NaCl, Na2SO4, KCl, NaF, AlCl3, MgCl2, and/or MgSO4 at the concentrations designated in the figures. Subsequently, the samples were either immunoprecipitated or used for zonal sedimentation.

For immunoprecipitation the samples were diluted to 100 μl and treated as described previously (Toyoshige et al., 1994). During immunoprecipitation the salt concentrations were maintained, decreased or increased as indicated in the figures. Immunoprecipitates were assayed for Gs subunits by SDS-polyacrylamide gel electrophoresis and immu-

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1 The abbreviations used are: Gs, stimulatory guanine nucleotide binding protein; DTT, dithiothreitol; AlF4-, NaF plus AlCl3; Gα, α subunit of Gs; Gβγ, βγ subunit complex of G proteins; GTPγS, guanosine 5′-O-(3-thiotriphosphate); Gpp(NH)p, guanyl-5′-yl imidodiphosphate.
noblotted (Toyoshige et al., 1994). In addition to the α and β subunits of Gs, the immunobLOTS show a protein with slower electrophoretic mobility than Gα. This is the heavy chain of the RM/1 antibody. When percent dissociation of Gα is reported, it is based on zero percent being defined as the amount of Gα present when Gs was immunoprecipitated following incubation in solution A containing 2 mM MgSO_4 and 0.1% Lubrol PX.

For zonal sedimentation samples of bovine brain Gs incubated as described above were diluted to 100 μl so that, with the exception of Gs, the concentration of components in the solution were unchanged. The samples were then transferred onto the top of sucrose density gradients for zonal sedimentation experiments. Gs from S49 membranes was also used for some zonal sedimentation experiments. Samples containing 250 μg of membrane protein were diluted with one volume of solution A, containing 0.1% Lubrol PX and the combination of salts indicated in the figure legend. The samples were incubated for 30 min at 30°C, and centrifuged at 43,000 × g for 30 min at 4°C to remove insoluble material. The supernatant was then transferred onto the top of a sucrose density gradient for zonal sedimentation experiments as follows.

Zonal Sedimentation of Gs on Sucrose Density Gradients and Adenyl Cyclase Assays—Samples of bovine brain or S49 Gs that had been prepared for zonal sedimentation were layered onto the top of 5 ml of linear density gradients of 5 to 20% sucrose made in solution B (20 mM HEPES (pH 8.0), 1 mM EDTA, 0.1 mM DTT, and 0.025% Lubrol PX) and containing other salts as described in the figure legend. The gradients were centrifuged at 50,000 rpm in an SW 50.1 rotor for about 20 h (υ = 2.0 × 10^−5 rad/s). After centrifugation the gradients were divided into about 32 fractions of equal volume (approximately 160 μl).

Sample volumes of 30 μl from each fraction were then used to reconstitute adenyl cyclase in the membranes of Gs deficient S49 cells. Just prior to use the S49 ccc membranes were thawed and diluted with a large volume of solution A containing 2 mM MgSO_4. The membranes were recovered by centrifugation and resuspended at a membrane protein concentration of 2.5 mg/ml in solution A containing 2 mM MgSO_4. Ten μl of the suspended S49 ccc membranes were added to each sample, and after reconstitution of adenyl cyclase, effector stimulated enzyme activity was assayed as described previously (Toyoshige et al., 1994). Effectors included 10 mM AlF_4⁻ for samples originally treated with AlF_4⁻ and 30 μM GTPγS plus 20 μM isoprenaline for samples of Gα that were not activated before sedimentation. No effect was observed when samples of Gs were activated with GTPγS before zonal sedimentation.

Proteinases of in Vitro Translated Gsα–pBluescript II SK+ containing the DNA for the long form of rat olfactory Gα was prepared and used to produce [35S]methionine-Gsα by in vitro transcription and translation as described previously (Warner et al., 1996). After translation the sample was divided into two 25-μl aliquots. One sample received 0.5 μl (0.5 μg) of brain Gβ and the other received an equivalent volume of solution containing 20 mM HEPES (pH 8.0), 1 mM EDTA, 1 mM DTT, and 0.25% Lubrol-PX. A solution of Lubrol PX was added to make the final detergent concentration 0.1% and to increase the reaction volume to 30 μl. Samples were incubated at 30°C for 1 h, and then any NaCl that might have been present was removed by changing the solution for 30 μl of solution C (20 mM HEPES (pH 8.0), 2 mM MgSO_4, 1 mM EDTA, and 1 mM DTT) with a Centricon 30 ultrafiltration unit. Since Lubrol PX is retained during filtration, the final concentration of detergent remained 0.1%. Three-μl samples were then adjusted to 12.5 μl with solution C containing NaCl and/or AlF_4⁻ so that the final concentrations were 100 and 10 μM, respectively. The samples were incubated for 30 min at 30°C before adding 2.5 μl of solution containing 150 μg of trypsin/ml. The trypsin was made up in solution C containing 0.025% Lubrol PX with the appropriate concentration of NaCl and/or AlF_4⁻. Samples that did not receive trypsin received the same volume of the appropriate solution without the protease. The samples were incubated for 15 min at 30°C before stopping the reactions by adding 1 μl of solution containing 4 mg of soybean trypsin inhibitor/ml. The samples were prepared for electrophoresis by adding a solution containing β-mercaptoethanol and sodium dodecyl sulfate, but the samples were not heat-denatured (Jackson and Hunt, 1983) before applying them to a 10% sodium dodecyl sulfate-polyacrylamide gels.

RESULTS

RM/1 antiserum raised against a synthetic decapeptide corresponding to the carboxyl-terminal of Gα has been used by us (Toyoshige et al., 1994) and others (Simonds et al., 1989; Morris et al., 1990) to immunoprecipitate Gα. The amount of antiserum used for immunoprecipitation is of consequence since either too little or too much will decrease the efficacy of precipitation (Morris et al., 1990). It is reported that RM/1 will immunoprecipitate 30 to >90% of the Gα present in detergent extracts of cell membranes (Simonds et al., 1989; Morris et al., 1990). Using conditions described previously (Toyoshige et al., 1994), we found that RM/1 precipitated 62% of the detected Gα from preparations of bovine brain Gs (Fig. 1). In the absence of RM/1 there was no detectable precipitation of Gα. Sample to sample variation in the amount of Gα precipitated was ± 12% (standard deviation for n = 10 from a representative experiment), and there was no significant difference between the amount of Gα precipitated from samples of dissociated and undissociated Gs (see for example Figs. 2 through 6). Similar results have been reported by other investigators (Morris et al., 1990). By using RM/1 to immunoprecipitate Gsα we found that in the absence of NaCl, AlF_4⁻ was unable to cause Gs subunit dissociation (Fig. 2). NaCl caused a concentration dependent dissociation of Gs subunits in the presence of 2 mM MgSO_4 and AlF_4⁻. Dissociation was easily detectable with 10 mM NaCl, and was nearly complete when it was 200 mM. In the presence of 2 mM MgSO_4 and AlF_4⁻, 150 mM NaCl caused 76 ± 2% dissociation when compared with samples of Gsα incubated in the absence of NaCl. In order to better understand what was required for Gs subunit dissociation we varied the ion composition of the solutions during incubation and immunoprecipitation. Omitting AlCl_3 (Fig. 3A) or NaF (Fig. 3B) during the incubation and subsequent immunoprecipitation prevented Gsα subunit disso-
Cation and anion effects on \( G_\text{s} \) subunit dissociation. Bovine brain \( G_\text{s} \) was incubated with 2 mM MgSO\(_4\) as described under “Experimental Procedures.” Solutions for both the incubation and immunoprecipitation contained 2 mM MgSO\(_4\) as well as 10 mM AlF\(_4\) plus either 150 mM NaCl, KCl, or Na\(_2\)SO\(_4\) as indicated.

Fig. 6. \( G_\text{s} \) subunit reassociation can occur in the presence of AlF\(_4\). Bovine brain \( G_\text{s} \) was incubated with 2 mM MgSO\(_4\) as described under “Experimental Procedures.” The incubations were done in the absence or presence of 10 mM AlF\(_4\) and 150 mM NaCl as indicated in the figure. Subsequently, the samples were diluted for immunoprecipitation, but the addition of 1 \( \mu \)l of RM/1 antiserum was postponed for 1 h while the samples were incubated at 30°C. The dilutions were made in such a way that the MgSO\(_4\) and AlF\(_4\) concentrations were not changed, and the NaCl concentration was either maintained or reduced to 3 mM (150/3) by dilution as indicated. After antiserum was added the immunoprecipitation was completed as described under “Experimental Procedures.”

To investigate AlF\(_4\) binding to \( G_\alpha \), we prepared [\(^{35}\)S]methionine-G\(_{\alpha} \) by in vitro transcription and translation of the cDNA for rat olfactory \( G_\alpha \). This technique produced the \( G_\alpha \) with a molecular mass of 52 kDa as well as two shorter products with molecular masses of 40 and 36 kDa (Fig. 7). The latter two proteins resulted respectively from initiation of translation of this cDNA at the codons for methionines 60 and 110. Only traces of the in vitro translation proteins were able to survive tryptic digestion in the absence of AlF\(_4\). In the presence of AlF\(_4\), a 37-kDa fragment was protected from proteolysis. The ability of AlF\(_4\) to protect this fragment was improved by the presence of NaCl although there was significant protection in the absence of this salt suggesting that AlF\(_4\) binding to \( G_\alpha \) did not require NaCl.

Since sedimentation on sucrose density gradients has been used to show that AlF\(_4\) causes \( G_\alpha \) subunit dissociation, similar experiments were performed for these studies. \( G_\alpha \) was incubated with AlF\(_4\) in the presence or absence of NaCl and subjected to zonal sedimentation. AlF\(_4\) was present throughout the sucrose gradients, and NaCl was included or omitted so as to be consistent with the way \( G_\alpha \) was treated before application to the sucrose gradients. In the absence of NaCl, \( G_\alpha \) from S49 cell membranes (Fig. 8A), and from bovine brain (Fig. 8B) sedimented as a heterotrimer despite the absence of AlF\(_4\). For experiments with \( G_\alpha \) from bovine brain, it was necessary to substitute Na\(_2\)SO\(_4\) for NaCl when the latter was omitted from the gradients. This prevented the purified \( G_\alpha \) from aggregating, and sedimenting to the bottom of the centrifuge tube. However, the addition of Na\(_2\)SO\(_4\) to gradients reduced the sedimentation rate of heterotrimeric \( G_\alpha \) when compared with sedimentation through gradients that did not contain NaCl or Na\(_2\)SO\(_4\) (compare the sedimentation of heterotrimeric \( G_\alpha \) in Panels A and B of Fig. 8). The reduced rate of sedimentation was due, either in
part or entirely, to increased density caused by adding Na2SO4 to the solutions used for making gradients.

In the presence of both AlF4⁻ and NaCl, Gs from S49 cells sedimented at the same rate as the free Gsα-GTPγS subunit (Fig. 8A) when the same conditions employed by other investigators were used (Kahn and Gilman, 1984). These conditions included the addition of 10 mM MgCl2 during incubation and zonal sedimentation. When MgCl2 was replaced with 2 mM MgSO4, Gs from the S49 cell membranes sedimented at a rate intermediate between the free Gsα-GTPγS subunit and heterotrimeric Gs (Fig. 8A). The same phenomenon was observed for Gs from bovine brain even in the presence of 10 mM MgCl2 (Fig. 8B), and the peak of Gs activity was broad, extending to regions of the gradient where both the free Gsα-GTPγS subunit and heterotrimeric Gs sedimented.

**DISCUSSION**

Heterotrimeric G proteins are activated when AlF4⁻ and GDP form an analog similar to the transition state created when Gα hydrolyzes GTP (Chabre, 1990; Sondek et al., 1994; Coleman et al., 1994). The activation is thought to be followed by dissociation of Gγ from Gβγ, and dissociation is considered necessary in order for Gα to interact productively with its effector molecule. The activation of Gs by AlF4⁻ can be accompanied by subunit dissociation (Kahn and Gilman, 1984). However, we have reported that activation of Gs by AlF4⁻ in the presence of 2 mM MgCl2 did not cause Gs subunit dissociation (Toyoshige et al., 1994). We speculated that the difference between our recent results and the earlier results of other investigators was a consequence of experimental design. In an effort to reconcile these differences, and to gain a better understanding of the effects of AlF4⁻ and other ions on Gs subunit interaction we conducted the experiments described in this article.

We found that dissociation of Gs subunit in the presence of AlF4⁻ required the simultaneous presence of 10 mM NaF, 10 µM AlCl3, 2 mM MgSO4, and 10–200 mM NaCl. Higher concentrations of NaF did not cause subunit dissociation in the absence of NaCl (data not shown), and dilution of NaCl to 3 mM allowed the Gs subunit to reassociate even though the concentrations of MgSO4 and AlF4⁻ were maintained. The effects of NaCl are apparently due to Cl⁻. Other investigations (Higashijima et al., 1987) indicate that Cl⁻ causes conformational changes in G proteins when they bind activating ligands, but not when they bind GDP. Recently, we have investigated the ability of ligands other than AlF4⁻ to cause Gs subunit dissociation. In the presence of low concentrations of Mg²⁺ (2 mM or less) neither GTP nor GTPγS caused Gs subunit dissociation (Toyoshige et al., 1994, Basi et al., 1996). However, high concentrations of MgCl2 (Toyoshige et al., 1994) and MgSO4 (data not shown) do cause Gs subunit dissociation both in the absence and presence of guanine nucleotides. While GTP and GDP were equally effective in attenuating the Mg²⁺-induced dissociation of Gs sub-
units (Basi et al., 1996). GTPγS had little influence (Toyoshige et al., 1994) or augmented the effect of Mg2+ (Basi et al., 1996) depending upon how the experiment was done. We have not observed any effect of Cl− on the Mg2+-induced dissociation of Gs subunits in the presence or absence of GTPγS, suggesting that Cl− does not influence subunit dissociation under these circumstances (data not shown).

If AlF4− could not bind to Gs in solution in the absence of NaCl it would not be able to cause subunit dissociation. To investigate this possibility we took advantage of the fact that ligands which activate G proteins can also protect a core fragment ranging in molecular mass from 37 to 41 kDa from proteolytic digestion (Husdon et al., 1981; Fung and Nash, 1983; Hurley et al., 1984). Since the antibodies available to us did not recognize this core fragment we chose to prepare [35S]methionine-Gsα by in vitro transcription and translation. Previously we (Warner et al., 1996) and others (Journot et al., 1994) have found that the properties of in vitro translated Gsα are similar to those of Gsα prepared from animal tissue. AlF4− was able to protect in vitro translated Gsα from trypic proteolysis in the absence as well as the presence of NaCl indicating that the salt was not required for AlF4− binding to Gsα. Mixing in vitro translated Gsα with bovine brain Gβγ allows for the formation of a heterotrimer (Warner et al., 1996), but this had no effect on the ability of AlF4− to protect in vitro translated Gsα from proteolysis by trypsin in the presence or absence of NaCl. Additional evidence that NaCl is not required for the binding of AlF4− to Gs comes from the fact that NaCl is not necessary for the activation of adenyl cyclase in membranes (Sternweis and Gilman, 1982; Northup et al., 1983).3

In previous reports investigators have shown by the technique of zonal sedimentation on sucrose density gradients that AlF4− or NaF causes Gs subunit dissociation (Howlett and Gilman, 1980; Hanski et al., 1981; Sternweis et al., 1981; Northup et al., 1983; Kahn and Gilman, 1984). However, these experiments were done in the presence of 100–300 mM NaCl. Based on our results it seemed likely that Gs subunit dissociation would not have occurred if the sedimentations had been done in the absence of NaCl. In order to demonstrate this we conducted zonal sedimentation experiments in the presence and absence of NaCl. Gsα from S49 cell membranes sedimented as the free Gsα subunit in the presence of NaCl and AlF4− and Mg2+. This result corroborated the findings of other investigators. The experimental design for zonal sedimentation in the presence of NaCl was based on earlier experiments (Kahn and Gilman, 1984), and therefore the sucrose gradients contained 2 M MgSO4 in addition to 100 mM NaCl and AlF4−, and the centrifugations were done at 4 °C. Substituting 2 mM MgSO4 for the MgCl2 caused Gsα from S49 membranes to sediment at a rate intermediate between free Gsα and heterotrimeric Gs. The intermediate rate of sedimentation may have been caused by using 100 mM NaCl, a concentration used in previous investigations (Kahn and Gilman, 1984), but one that is not sufficient to cause complete dissociation of Gs subunits when the Mg2+ concentration was 2 mM (see Fig. 2). Another possibility is suggested by reports that the dissociation of heterotrimeric G protein subunits in the presence of activating ligands is a temperature-dependent process (Codina et al., 1984). G protein subunits dissociated at 32 °C in the presence of MgCl2 and a nonhydrolyzable GTP analog subsequently reassociated when the temperature was decreased to 4 °C. In our experiments, a subunit that dissociated when exposed to NaCl in the presence of AlF4− and Mg2+ probably would not reassociate during immunoprecipitation which was done at 24 °C, but might reassociate during centrifugation at 4 °C, consequently giving rise to a broad peak sedimenting between free Gα and heterotrimeric Gsα.

When NaCl was omitted from the sucrose density gradients, Gsα sedimented as a heterotrimer despite the presence of AlF4− and Mg2+. This result confirmed our prediction that subunit dissociation would not occur in the absence of NaCl during zonal sedimentation. These data also support the results of experiments involving immunoprecipitation which showed that NaCl was required for Gs subunit dissociation in the presence of AlF4− and Mg2+. Based on the data presented here, we have concluded that AlF4−, a ligand that is able to activate heterotrimeric G proteins, can bind to Gsα without causing subunit dissociation.

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