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Registry No. GluRS, 9068-76-2; ATP, 56-65-5; D-Glu, 6893-26-1; L-Glu, 56-86-0.

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Effect of Al^{3+} plus F^- on the Catecholamine-Stimulated GTPase Activity of Purified and Reconstituted G_s[†]

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ABSTRACT: The effects of Al^{3+} and F^- on the catecholamine-stimulated GTPase cycle were studied by using reconstituted phospholipid vesicles that contained purified β-adrenergic receptor and the stimulatory GTP-binding protein of the adenylate cyclase system, G_s. Al^{3+}/F^- activated reconstituted G_s to levels previously reported for detergent-solubilized, purified G_s, although both activation and deactivation were faster in the reconstituted preparation. Under these conditions, Al^{3+}/F^- did not inhibit by more than 15% the β-adrenergic agonist-stimulated GTPase activity of the vesicles nor did it significantly inhibit the rates of GTP binding, GTP hydrolysis, or GDP release. When Mg^{2+} (50 mM) was used instead of agonist to promote GTP hydrolysis in the receptor-G_s vesicles, Al^{3+}/F^- was found to inhibit GTPγS binding, GDP release, and steady-state GTPase activity to unstimulated levels. These data can be interpreted as indicating that the receptor catalyzes nucleotide exchange by G_s faster or more efficiently than does Mg^{2+}.

Although stimulation of adenylate cyclase activity by fluoride was noted in the initial description of the enzyme by Rall and Sutherland (1958), little was known about its mechanism of stimulation for over a decade. Ross and Gilman (1977) showed that fluoride acts on a GTP-binding, regulatory protein (G_s)[1] that is distinct from adenylate cyclase itself and acts as an intermediary regulator between receptor and the cyclase [see Ross and Gilman (1980)]. Sternweis and Gilman (1982) later showed that Al^{3+} or Be^{2+} was also required for stimulation of G_s by F^- and suggested that AlF_4^- or FeF_3^- might be the activating species.[2] The mode of action of

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[1] Abbreviations: DTT, dithiothreitol; G_s, stimulatory, GTP-binding protein of the adenylate cyclase system; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

[2] Although it is likely that the AlF_4^- anion is the relevant species, we use Al^{3+}/F^- to refer to the mixture of $AlCl_3$ and NaF, usually 0.1 and 20 mM, respectively, that was used.

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Al^{3+}/F^- is still unclear. It has not been possible to demonstrate directly that a guanine nucleotide is required for activation by Al^{3+}/F^-, activation is generally irreversible in native membranes (Perkins, 1971), and activation of isolated G_s by Al^{3+}/F^- is usually faster than that caused by nonhydrolyzable analogues of GTP [see references in Ross and Gilman (1980)]. On the basis of studies of transducin, a homologous GTP-binding protein from the retinal rod cell, Bigay et al. (1985) suggested that AlF_4^- substitutes for the γ-phosphoryl group of GTP to make bound GDP an activating species. Because G proteins, as isolated, have GDP bound (Ferguson et al., 1986), this mechanism would result in the "activation" of G_s in the absence of added nucleotide. No direct binding data to support this idea are available, however.

In their first report on the catecholamine-stimulated GTPase activity of G_s, Cassel and Selinger (1976) reported that NaF both inhibited the agonist-stimulated activity and also decreased the background level. In a more purified system, Kanaho et al. (1985) showed that Al^{3+}/F^- noncompetitively inhibited the rhodopsin-stimulated nucleotide binding, release, and GTPase activities of transducin, a homologous GTP-binding regulatory protein. Such inhibition is consistent with the tight binding of Al^{3+}/F^- at the nucleotide site, with or without GDP, but is equally consistent with the existence of a separate "fluoride site" on transducin. To date, there have been no similar studies on G_s or other G proteins.

We recently described the regulation of the GTPase catalytic cycle of G_s by agonists using reconstituted phospholipid vesicles containing purified G_s and β-adrenergic receptors (Brandt & Ross, 1986). In the work described here, we have used these vesicles to study the effects of Al^{3+}/F^- on G_s. Although Al^{3+}/F^- rapidly activated reconstituted G_s in the vesicles, we observed minimal effect of Al^{3+}/F^- on hormone-stimulated nucleotide binding, nucleotide dissociation, steady-state GTPase activity, or the steady-state level of G_s–GTP. In contrast, Al^{3+}/F^- inhibited the Mg^{2+}-stimulated rates of nucleotide binding and dissociation by G_s.

Experimental Procedures

Most of the methods used in this study and the sources and purity of most reagents have been described recently (Asano et al., 1984; Brandt & Ross, 1985, 1986).

Materials. $AlCl_3$ and NaF were reagent grade, obtained from Baker Chemical Co. Water used for the preparation of buffers was purified on a Milli-Q apparatus (Millipore Corp.); assay buffers contained approximately 0.2 μM Al^{3+} contamination.

Protein Purification. G_s was purified from rabbit liver according to Sternweis et al. (1981) and stored at −80 °C in 10 mM NaHepes (pH 8)–1 mM EDTA–0.1% Lubrol 12A9. β-Adrenergic receptor was purified from turkey erythrocytes as described by Brandt and Ross (1986) and stored at −80 °C in 20 mM NaHepes (pH 8)–0.5 mM EDTA–0.1 mM EGTA–0.1 M NaCl–0.05% digitonin.

Reconstitution of Receptor and G_s and DTT Treatment of Reconstituted Vesicles. Receptor and G_s were reconstituted into unilamellar phospholipid vesicles composed of phosphatidylethanolamine–phosphatidylserine (3:2) as described previously (Brandt & Ross, 1986). For some experiments, receptor was activated by reduction of disulfides by incubating the vesicles with 5 mM DTT for 1–3 h at 0 °C. Pedersen and Ross (1985) have shown that such treatment enhances the ability of the receptor to promote the binding and release of nucleotides and steady-state GTP hydrolysis by G_s. Subsequent to treatment, vesicles were diluted such that the final concentration of DTT in the assay was always less than 0.5 mM. Because treatment with DTT results in activation of receptor even in the absence of agonist, "basal" activities in vesicles were always determined on untreated vesicles in order to estimate the total stimulation caused by receptor. The incremental effect of receptor on the binding of nucleotides or on GTPase was obtained by subtracting the basal activity for untreated vesicles from the agonist-stimulated activity of either DTT-treated or untreated vesicles.

Assays. The binding of [α-^{32}P]GTP, [γ-^{32}P]GTP, and [^{35}S]GTPγS was measured as described previously (Asano et al., 1984; Brandt & Ross, 1985, 1986). Vesicles were incubated in medium containing 20 mM NaHepes (pH 8), 100 mM NaCl, 0.1 mM ascorbate, and the concentrations of EDTA, $MgCl_2$, guanine nucleotide, $AlCl_3$, NaF, and β-adrenergic agent shown in the text (0.05-mL total volume). For all experiments in which the activities of Al^{3+}/F^--treated and untreated vesicles were compared, the vesicles were first incubated at 0 °C for 1 h with the indicated concentrations of $AlCl_3$ and NaF before assay. This period, longer than necessary, was chosen for convenience. Assays were initiated by the addition of vesicles. Assays were quenched, and nucleotide bound to G_s was separated by filtration on nitrocellulose filters as described. The total amount of G_s in the vesicles was determined by measuring the amount of [^{35}S]GTPγS bound in the presence of 50 mM Mg^{2+}, 0.1% Lubrol 12A9, and 10 μM GTPγS after 30 min at 30 °C (Asano et al., 1984).

GTPase activity was assayed in the same medium used to measure nucleotide binding, except that [γ-^{32}P]GTP was used as a substrate. At the termination of the assay, GTP was adsorbed to activated charcoal suspended in 50 mM NaH_2PO_4 and the [^{32}P]phosphate product remained in tthe supernatant (Brandt & Ross, 1985). This method was modified for a few experiments (not shown) in which high concentrations of Mg^{2+}, Al^{3+}, and F^- caused coprecipitation of [^{32}P]phosphate with the charcoal. Either the charcoal precipitation was carried out in 0.3 M HCl or free [^{32}P]phosphate was extracted into isobutyl alcohol–benzene as the phosphomolybdate complex (Avron, 1960). All three methods gave similar results at low concentrations of Mg^{2+}.

The activation of G_s by Al^{3+}/F^- or by GTPγS was assayed according to the ability of activated G_s to stimulate the catalytic component of adenylate cyclase, as described by Pedersen and Ross (1982). The source of adenylate cyclase was plasma membranes from the cyc$^-$ mutant of S49 lymphoma cells. This procedure measures only activated G_s and is not interfered with by nonactivated G_s (Sternweis et al., 1981; Pedersen & Ross, 1982). For assay, the activation of G_s was quenched by mixing an aliquot of receptor–G_s vesicles with Lubrol 12A9 (0.1% final concentration) to solubilize the G_s, and the mixture was immediately added to an adenylate cyclase assay cocktail that contained cyc$^-$ membranes. The cyclase assay was carried out for 30 min at 30 °C. Activities are expressed in units of G_s activity, defined as the stimulation of 1 nmol/min of adenylate cyclase activity in this assay (Sternweis et al., 1981; Pedersen & Ross, 1982).

Results

When phospholipid vesicles containing β-adrenergic receptors and G_s were incubated with Al^{3+}/F^-, G_s was activated to a state in which it could stimulate the activity of adenylate cyclase. As shown in Figure 1, 100 μM Al^{3+} and 20 mM F^- appeared to be optimal for G_s activation under the conditions used here (3 mM Mg^{2+}, 1 mM EDTA, 100 mM Cl^-). The rate of activation of reconstituted G_s by Al^{3+}/F^- was rapid. Half-maximal activation was achieved in less than 10 s at 30 °C, and activation was complete by 40 s. At 0 °C, activation

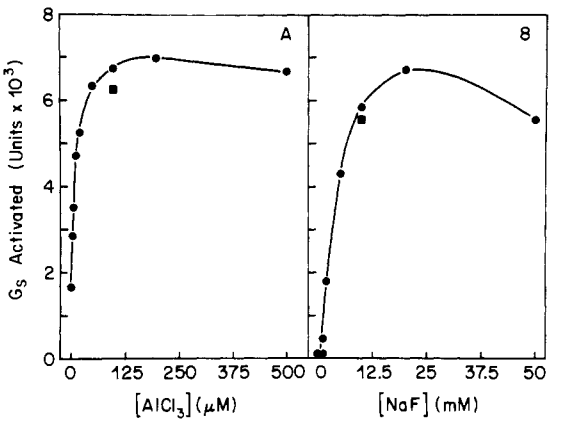

FIGURE 1: Activation of G_s by Al^{3+}/F^- in receptor–G_s vesicles. Panel A: Vesicles were incubated for 1 h at 0 °C in standard assay medium that contained 20 mM NaF, no guanine nucleotide, the indicated concentrations of $AlCl_3$, and either 3 mM Mg^{2+} and 1 mM EDTA (●) or 50 mM $MgCl_2$, 1 mM EDTA, and 0.1% Lubrol 12A9 (■). The vesicles were then solubilized with Lubrol 12A9 (0.1% final) and assayed for activated G_s as described in Experimental Procedures. Each time point represents duplicate assays containing 59 fmol of G_s. The abscissa is not corrected for contamination of buffers with approximately 0.2 μM Al^{3+}. Panel B: Vesicles were incubated and assayed as described in the legend to panel A except that vesicles were activated with 100 μM $AlCl_3$ and the indicated concentrations of NaF.

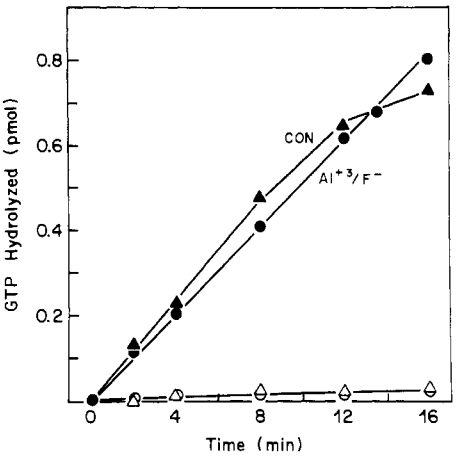

FIGURE 2: Influence of Al^{3+}/F^- on agonist-stimulated GTPase activity in receptor–G_s vesicles. Assays were carried out for the times shown in medium containing 3 mM $MgCl_2$, 1 mM EDTA, 0.2 μM [γ-^{32}P]GTP, and either 10 μM (−)-isoproterenol (▲, ●) or 0.25 μM (−)-propranolol (△, ○). Activity was assayed with (○, ●) or without (△, ▲) 100 μM $AlCl_3$ and 20 mM NaF. All vesicles were preactivated with dithiothreitol. Each point represents the average of triplicate assays of vesicles that contained 4 fmol of receptor and 112 fmol of G_s, 40% of which was sensitive to regulation by receptor as defined previously (Asano et al., 1984; Brandt & Ross, 1986).

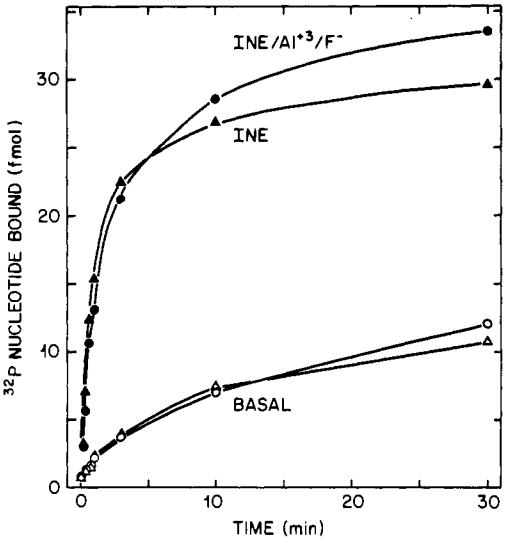

FIGURE 3: Effect of Al^{3+}/F^- on the agonist-stimulated binding of [α-^{32}P]GTP to receptor–G_s vesicles. Binding was assayed in medium containing 2 mM $MgCl_2$ and 1 mM EDTA. This concentration of Mg^{2+} is only slightly suboptimal (Brandt & Ross, 1986). Assays were carried out in the presence or absence of 10 μM isoproterenol (INE). For one set of assays (○, ●), activity was measured in the presence of 10 mM NaF and 40 μM $AlCl_3$. These concentrations are also slightly suboptimal for the activation of G_s (see Figure 1) but still yield substantial effects. The decrease in the concentrations of Al^{3+}, Mg^{2+}, and F^- were necessary to decrease nonspecific and variable binding of [^{32}P] nucleotide, and freshly repurified [α-^{32}P]GTP was used. Bound nucleotide, predominantly [α-^{32}P]GDP and some [α-^{32}P]GTP (Table I), was isolated as described (Brandt & Ross, 1986). Data represent the averages of two separate time courses. Each assay point represents 110 fmol of total G_s and 6.6 fmol of receptor.

was complete in 10 min. This is much faster than the rate observed in detergent solution [see Sternweis et al. (1981)] and is similar to the typically rapid rate of activation of adenylate cyclase by F^- in native membranes. The maximal fluoride-stimulated activity of the G_s in the experiment shown in Figure 1 was 1320 (panel A) or 1260 (panel B) nmol of cAMP produced/min per mg of G_s. These values, which were not altered significantly by detergent, are similar to those observed for soluble G_s by Sternweis et al. (1981). Activation of reconstituted G_s by Al^{3+}/F^- was not changed in rate or extent by the presence of β-adrenergic agonists and/or GTP. Furthermore, treatment of receptor–G_s vesicles with Al^{3+}/F^- did not alter the extent or affinity of agonist or antagonist binding or the stability of reconstituted receptor (data not shown).

While G_s was clearly activated by Al^{3+}/F^-, such treatment did not markedly alter its agonist-stimulated, steady-state GTPase activity (Figure 2). In the experiment shown here, Al^{3+}/F^- decreased the initial rate of GTPase activity from 1.28 to 1.14 mol of GTP hydrolyzed/min per mol of receptor-accessible G_s (11% decrease). This is the largest inhibition that has been observed in five similar experiments. A more consistent effect of Al^{3+}/F^- on GTPase was to increase the linear period of the reaction. The GTPase rate in the presence of Al^{3+}/F^- was constant for at least 20 min in all cases; activity in the absence of Al^{3+}/F^- was linear to only 8 min in the experiment of Figure 2, which was typical. This effect appears to be due to stabilization of the G_s by Al^{3+}/F^-. When the stability of reconstituted G_s was measured under GTPase assay conditions, Al^{3+}/F^- increased the $t_{1/2}$ for denaturation of G_s activity from 6 to 14 min at 41 °C (data not shown). The elevated temperature in this experiment was used to accelerate denaturation.

Although there is only a slight effect of Al^{3+}/F^- on hormone-stimulated GTPase activity, it is the steady-state level of activated G_s–GTP that is central to the regulation of adenylate cyclase. It was therefore of interest to determine if Al^{3+}/F^- altered the steady-state level of this species. The G_s–GTP complex can be measured by using [γ-^{32}P]GTP in order to discriminate bound GTP from the GDP product. The total binding of GTP plus GDP can be measured with either [α-^{32}P]GTP or [^{3}H]GTP (Brandt & Ross, 1985, 1986). As shown in Table I, Al^{3+}/F^- had essentially no effect on the agonist-stimulated steady-state binding of either GTP or GDP.

When the rates of GTP binding and GDP release were measured (Figures 3 and 4A), it was found that Al^{3+}/F^- inhibited the agonist-stimulated initial rate of GTP binding only slightly, about 30%, and did not change the rate of GDP release at all. Basal rates were also minimally changed by

Table I: Agonist-Stimulated Binding of [α-^{32}P]GTP and [γ-^{32}P]GTP in the Presence and Absence of Al^{3+}/F^-[a]

nucleotide	[$AlCl_3$] (μM)	[NaF] (mM)	nucleotide bound (fmol) +DTT	nucleotide bound (fmol) -DTT	[γ-^{32}P]GTP/[α-^{32}P]GTP +DTT	[γ-^{32}P]GTP/[α-^{32}P]GTP -DTT
[α-^{32}P]GTP			80	56	0.16	0.20
[γ-^{32}P]GTP			13	12		
[α-^{32}P]GTP	100	20	66	62	0.19	0.21
[γ-^{32}P]GTP	100	20	12	13		

[a] Vesicles, either untreated or previously treated with DTT, were incubated for 10 min at 30 °C in assay medium with the indicated concentrations of Al^{3+} and F^- and either 0.2 μM [γ-^{32}P]GTP or [α-^{32}P]-GTP. [α-^{32}P]GTP measures both bound GTP and GDP; [γ-^{32}P]GTP measures GTP binding alone (Brandt et al., 1985, 1986). Binding was measured in the presence or absence of 10 μM (-)-isoproterenol by immediate filtration, and the data show the increment in the binding of nucleotide caused by isoproterenol. Each value, the average of triplicate determinations, represents vesicles containing a total of 9.7 fmol of receptor and 270 fmol of G_s.

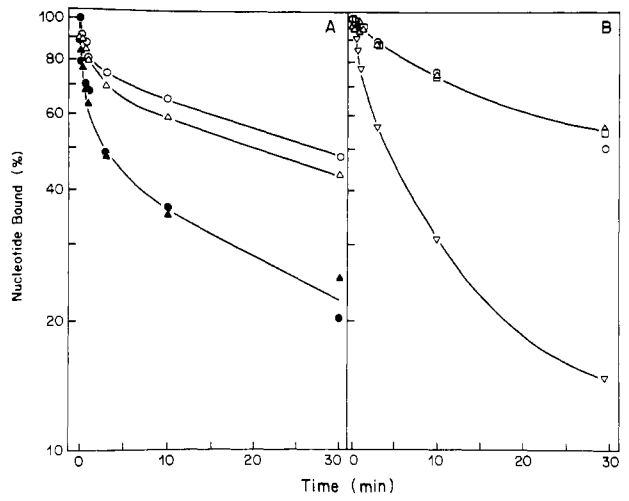

FIGURE 4: Influence of Al^{3+}/F^- on agonist-stimulated and Mg^{2+}-stimulated dissociation of GDP from reconstituted G_s. Panel A: Agonist-stimulated dissociation. Vesicles were incubated at 30 °C in assay medium containing 10 μM (-)-isoproterenol, 3 mM $MgCl_2$, 1 mM EDTA, and 0.2 μM [α-^{32}P]GTP. After 8-min incubation, 0.01 volume of 10 mM (-)-alprenolol (○, △) or an equivalent volume of H_2O (●, ▲) was added. After 2-min additional incubation, 0.01 volume of 20 mM GTP was added to all incubations and vesicles were assayed in duplicate for bound nucleotide at the indicated times. For vesicles that had been preincubated with 100 μM $AlCl_3$ and 20 mM NaF (△, ▲), assay medium was adjusted to these final concentrations of Al^{3+} and F^-. Each time point represents the average of two separate experiments. The amount of nucleotide bound at time zero in the figure was 103 fmol in the absence of Al^{3+}/F^- and 88 fmol in its presence. Vesicles contained 400 fmol of total G_s and 45 fmol of receptor. Panel B: Mg^{2+}-stimulated nucleotide dissociation. Vesicles were incubated at 30 °C as described in the legend to panel A except that 0.25 μM (-)-propranolol was included in the incubation instead of (-)-isoproterenol. After 30-min incubation, 0.035 volume of 5.7 mM GTP (○, □) of 5.7 mM GTP plus 1.35 M $MgCl_2$ (▽, △) was added and vesicles were assayed in duplicate at the indicated times for bound nucleotide. When vesicles were preincubated with 100 μM $AlCl_3$ and 20 mM NaF (△, □), assay medium was adjusted to these final concentrations of Al^{3+} and F^-. The amount of nucleotide bound at time zero in the figure was 64 fmol in the absence of Al^{3+}/F^- and 57 fmol in its presence. Vesicles contained 400 fmol of total G_s and 45 fmol of receptor.

Al^{3+}/F^- in several experiments. There was also no effect of Al^{3+}/F^- on the rate of chemical hydrolysis of bound GTP (not shown), which is not regulated by agonist (Brandt & Ross, 1986). A comparison of the various kinetic parameters for both Mg^{2+}- and agonist-stimulated nucleotide binding, dissociation, and hydrolysis is shown in Table II.

One possible explanation of the lack of significant inhibition of hormone-stimulated binding, dissociation, and overall GTPase activity might be that agonist, or agonist plus nucleotide, reverses the activation of G_s by Al^{3+}/F^-. This was not the case, however. After preincubation with Al^{3+}/F^-, the vesicle-bound G_s maintained a constant level of activation throughout the assay, and this level was unchanged by the presence or absence of agonist plus GTP. To further verify that agonist or nucleotide did not cause dissociation of Al^{3+}/F^-, the rate of deactivation of activated G_s in receptor-G_s vesicles was measured in the presence or absence of agonist plus nucleotide. As shown in Figure 5, isoproterenol, with or without GTP, did not alter the rate of deactivation of Al^{3+}/F^--activated G_s.

Table II: Agonist-Stimulated and Mg^{2+}-Stimulated GTPase Rate Constants (min^{-1}) in Receptor-G_s Vesicles

	agonist-stimulated[a] control	agonist-stimulated[a] $+Al^{3+}/F^-$	Mg^{2+}-stimulated[b] control	Mg^{2+}-stimulated[b] $+Al^{3+}/F^-$
GTPase (steady-state)	1.3	1.1	0.20	0.01
GTP binding	1.0	0.7	ND[c]	ND[c]
GTP hydrolysis[d]	4.6	4.6	ND[c]	ND[c]
GDP dissociation[e]	~7	~7	0.32	0.015
	0.74	0.74		
GTPγS binding[f]	1.2	1.2	0.18	g

[a] Apparent first-order rate constants and turnover numbers were obtained from analysis of the data of Figures 2–4 and 6 and other similar experiments. Determined at 3 mM $MgCl_2$, 1 mM EDTA, 10 μM (-)-isoproterenol. *b* Determined at 50 mM Mg^{2+}, 1 mM EDTA. *c* Not determined. *d* Determined as described by Brandt and Ross (1985). Vesicles were incubated with [γ-^{32}P]GTP in the absence of free Mg^{2+}, and hydrolysis of bound GTP was initiated by the addition of 3 mM Mg^{2+}. *e* Agonist-stimulated nucleotide dissociation is composed of at least two components, a rapid process (~7 min^{-1}) that is not blocked by antagonist and may reflect a performed receptor-G_s complex and a slower (0.74-min^{-1}) receptor-dependent process. See Brandt and Ross (1986). *f* Calculated as described by Asano and Ross (1984). *g* Al^{3+}/F^- reduced the rate of GTPγS binding to basal levels (see Figure 3), i.e. those observed at 3 mM Mg^{2+} and 1 mM EDTA. The rate constant for basal binding was approximately 0.02 min^{-1}.

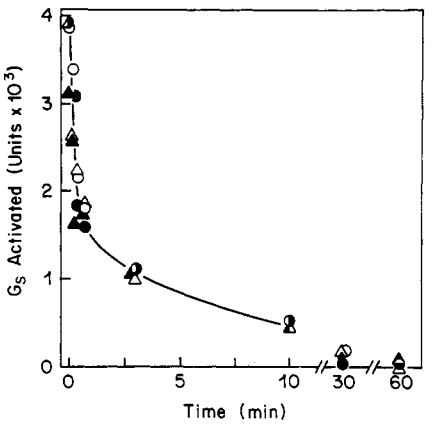

FIGURE 5: Influence of (-)-isoproterenol and GTP on the rate of deactivation of Al^{3+}/F^--activated G_s. Vesicles were incubated on ice for 1 h in assay medium that contained 100 μM $AlCl_3$ and 20 mM NaF. Aliquots were diluted 65-fold with prewarmed medium that contained 3 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, either 10 μM (-)-isoproterenol (●, ▲) or 0.25 μM (-)-propranolol (○, △), and either no added nucleotide (○, ●) or 0.2 μM GTP (△, ▲) and incubated at 30 °C. At the indicated times, aliquots of the reaction mixture were solubilized by the addition of 3.2% Lubrol 12A9 to a 0.1% final concentration and immediately assayed for activated G_s. Each time point represents duplicate assays containing 61 fmol of G_s.

Effects of Al^{3+}/F^- on Mg^{2+}-Stimulated GTPase Activity. High concentrations of Mg^{2+} can also stimulate the binding and release of guanine nucleotides by G_s via a receptor-independent mechanism [Brandt & Ross, 1986, and references therein; see also Sternweis et al. (1981)]. In contrast to the results obtained with the receptor-mediated reaction, Al^{3+}/F^-

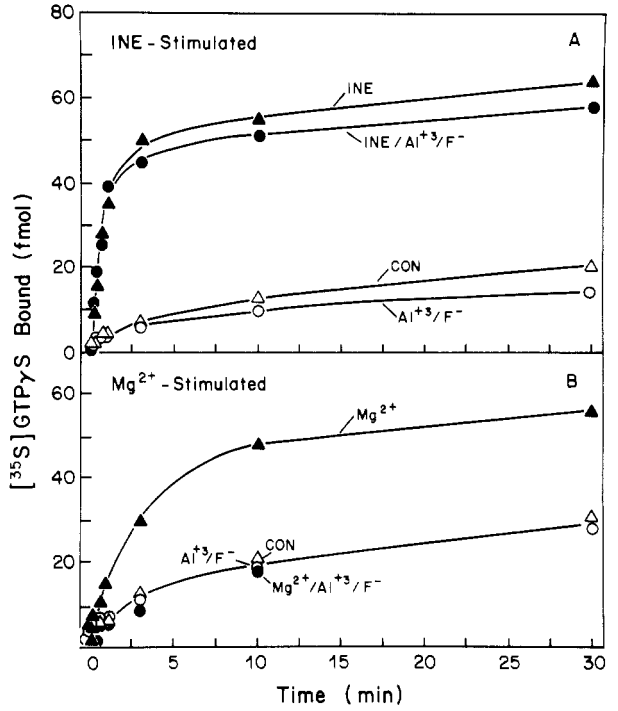

FIGURE 6: Influence of Al^{3+}/F^- on agonist-stimulated and Mg^{2+}-stimulated [^{35}S]GTPγS binding to receptor–G_s vesicles. Panel A: Agonist-stimulated [^{35}S]GTPγS binding. [^{35}S]GTPγS binding assays were performed in duplicate in medium containing 1 mM EDTA, 0.2 μM [^{35}S]GTPγS, and 3 mM $MgCl_2$. Each time point represents vesicles that contained 6.8 fmol of receptor and 179 fmol of G_s. When vesicles were preincubated with 100 μM $AlCl_3$ and 20 mM NaF (○, ●), assay medium was adjusted to these final concentrations of Al^{3+} and F^-. Data were obtained in the presence of 10 μM (−)-isoproterenol (closed symbols) or 0.25 μM (−)-propranolol (open symbols). Panel B: Mg^{2+}-stimulated [^{35}S]GTPγS binding. [^{35}S]GTPγS binding assays were conducted in duplicate at 30 °C for the times shown in medium containing 1 mM EDTA, 0.2 μM [^{35}S]GTPγS, 0.25 M (−)-propranolol, and either 50 mM $MgCl_2$ (▲, ●) or 3 mM $MgCl_2$ (△, ○). Each time point represents vesicles that contained 180 fmol of G_s and 10 fmol of receptor. When vesicles were preincubated with 100 μM $AlCl_3$ and 20 mM NaF (○, ●), assay medium was adjusted to these final concentrations of Al^{3+} and F^-.

markedly inhibited the Mg^{2+}-stimulated functions of G_s (Table II). Al^{3+}/F^- virtually abolised the Mg^{2+}-stimulated release of GDP from reconstituted G_s (Figure 4B). Similarly, when the binding of the GTP analogue [^{35}S]GTPγS was measured, Al^{3+}/F^- strongly inhibited Mg^{2+}-stimulated binding (Figure 6; Table II). In contrast, the agonist-stimulated binding of [^{35}S]GTPγS was not altered by Al^{3+}/F^-, as had been the case with GTP. We also attempted to determine the effect of Al^{3+}/F^- on Mg^{2+}-stimulated GTPase activity and on the rate of binding of GTP itself. Unfortunately, the solubility of Al^{3+}, F^-, and Mg^{2+} at pH 8 and at their optimally effective concentrations is limited, and coprecipitation of nucleotide and inorganic phosphate have caused high and variable blanks in both of these experiments. We have, however, observed substantial inhibitions of Mg^{2+}-stimulated GTPase at suboptimal concentrations of NaF (below 5 mM), and the extent of inhibition appeared consistent with that shown in Figures 4B and 6.

Discussion

The data presented here indicate that Al^{3+}/F^- had a negligible effect on the agonist-stimulated binding, hydrolysis, and release of guanine nucleotide by G_s, as assayed in the reconstituted receptor–G_s vesicles. Conversely, neither GTP nor β-adrenergic agonist altered the rate or extent of activation of G_s by Al^{3+}/F^- or the rate of its deactivation after dilution. This pattern contrasts with the marked inhibition by Al^{3+}/F^- of the rhodopsin-stimulated GTPase and nucleotide binding functions of transducin, as described by Kanaho et al. (1985). Our finding that the Mg^{2+}-stimulated functions of G_s were strongly inhibited by Al^{3+}/F^- is similar to the data on transducin. Al^{3+}/F^- essentially blocked the Mg^{2+}-stimulated binding of GTPγS and the release of GDP (Figures 4B and 6). Mg^{2+}-stimulated GTPase activity and GTP binding were also inhibited, although these data are less complete.

If Al^{3+}/F^- binds at a site apart from guanine nucleotides, the decreased sensitivity of G_s to Al^{3+}/F^- relative to transducin might indicate simply that the Al^{3+}/F^- binding site on G_s is less tightly coupled to the nucleotide binding site. This explanation does not address the difference in sensitivity of G_s when receptor and Mg^{2+} are compared, however. It is plausible that Al^{3+}/F^- binds at the nucleotide-binding site on G proteins, perhaps as a complex with guanine nucleotide as suggested by Bigay et al. (1985). If so, the β-adrenergic receptor may be a sufficiently powerful catalyst of nucleotide binding and release that the blockade of this site on G_s by a nucleotide–Al^{3+}–F^- complex is readily surmountable. Inhibition of G_s would then be observed only under the less efficient stimulation of nucleotide exchange that is provided by Mg^{2+} [see Brandt and Ross (1986)]. The inability of β-adrenergic stimulation to promote deactivation of Al^{3+}/F^--preactivated G_s (Figure 5) sets interesting limits on this interpretation, especially regarding the independence of the association and dissociation of Al^{3+}/F^- and GDP. In general, these results all reiterate the differences in the control of G_s function by receptor or by Mg^{2+} that have been noted previously in this system (Brandt & Ross, 1986). We expect that these hypothetical mechanisms should be testable on vesicles in which the nucleotide that is bound to G_s has been predefined.

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Registry No. GTPase, 9059-32-9; Al, 7429-90-5; F^-, 16984-48-8; Mg, 7439-95-4; adenylate cyclase, 9012-42-4.

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Expression of Growth-Regulated Genes in tsJT60 Cells, a Temperature-Sensitive Mutant of the Cell Cycle†

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ABSTRACT: We have investigated the expression of growth-regulated genes in tsJT60 cells, a temperature-sensitive (ts) mutant of Fischer rat cells, which, on the basis of its kinetic behavior, can be classified as a G_0 mutant. It grows normally at 34 °C and also at 39.5 °C if shifted to the higher temperature during exponential growth. However, if the cell population is first made quiescent by serum deprivation, subsequent stimulation by serum induces the cells to enter S phase at 34 °C but not at 39.5 °C. A panel of growth-regulated genes was used that included three protooncogenes (c-fos, c-myc, and p53), several genes that are induced in G_0 cells stimulated by growth factors (β-actin, 2A9, 2F1, vimentin, JE-3, KC-1, and ornithine decarboxylase), and an S-phase gene (histone H3). The expression of these growth-regulated genes was studied in both tsJT60 cells and its parental cell line, rat 3Y1 cells. All the genes tested, except histone H3, are similarly induced when quiescent tsJT60 cells are stimulated by serum at either permissive or restrictive temperatures. These results raise intriguing questions on the nature of quiescence and the relationship between G_0 and G_1 in cells in culture.

The tsJT60 cells are a temperature-sensitive (ts) mutant of the Fischer rat cell line 3Y1 (Kimura et al., 1975) that displays some interesting characteristics. tsJT60 cells do not manifest a ts phenotype while exponentially growing, but if they enter a resting state (G_0) by serum deprivation of confluent monolayers, they cannot reenter S phase at 39.5 °C after serum stimulation, although they can do so at the permissive temperature of 34 °C (Ide et al., 1984). In this respect, tsJT60 cells behave as a G_0 mutant, i.e., a mutant that is defective in a function required for the transition from a resting to a growing stage but not necessary for cell cycle progression in cycling cells [for a review, see Baserga (1985)].

Recently, a number of genes have been reported whose cognate RNAs are markedly increased when resting (G_0) cells are stimulated to proliferate by growth factors. These include some bona fide protooncogenes like c-fos (Greenberg & Ziff, 1984; Kruijer et al., 1984; Muller et al., 1984), c-myc (Kelly et al., 1983; Campisi et al., 1984; Kaczmarek et al., 1985b), c-myb (Torelli et al., 1985), c-ras (Goyette et al., 1983, 1984; Campisi et al., 1984), and p53 (Reich & Levine, 1984). Others are genes whose products are well-known like core histones (Plumb et al., 1983; Hirschhorn et al., 1984b) and thymidine kinase (Liu et al., 1985). Finally, others have been identified as cDNA clones by differential screening of cDNA libraries (Linzer & Nathans 1983, 1984; Cochran et al., 1983; Hirschhorn et al., 1984b; Matrisian et al., 1985a,b; Edwards et al., 1985; Edwards & Denhardt, 1985; Lau & Nathans, 1985). Interestingly, some of these genes are expressed at roughly constant levels during the cell cycle (G_1 to M), and it is only in G_0 that their RNAs are not detectable or are detectable at very low levels. This is true, for instance, for c-myc (Hann et al., 1985; Thompson et al., 1985) and 2A9, a cDNA clone preferentially expressed in serum-stimulated cells (Hirschhorn et al., 1984b), which is inducible by platelet-derived growth factor and overexpressed in human acute myeloid leukemia (Calabretta et al., 1986).

Table I: List of Genes and Sequences Selected for These Studies

sequence	maximal expression	ref[a]
c-fos	G_0–G_1	Greenberg and Ziff (1984)
c-myc	G_0–G_1	Kelly et al. (1983)
KC-1	G_0–G_1	Cochran et al. (1983)
JE-3	G_0–G_1	Cochran et al. (1983)
4F1 (vimentin)	G_0–G_1	Hirschhorn et al. (1984b)
2F1	G_0–G_1	Hirschhorn et al. (1984b)
β-actin	variable	Campisi et al. (1984)
2A9	mid G_1	Hirschhorn et al. (1984b)
ornithine decarboxylase	mid G_1	Liu et al. (1985)
p53	mid/late G_1	Reich and Levine (1984)
histone H3	S phase	Plumb et al. (1983)

[a] The references given refer to papers showing the inducibility of the genes by growth factors. For simplicity, only one reference is given for each gene, but other references can be found in the text.

Since tsJT60 cells meet the criteria established for a G_0 mutant (Baserga, 1978), we thought it worthwhile to investigate the expression of a panel of growth-regulated genes

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