Differential effects of fluoride on adenylate cyclase activity and guanine nucleotide regulation of agonist high-affinity receptor binding

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Fluoride ion, presumably an Al^{3+}–F^- complex, has been proposed to activate the guanine nucleotide regulatory protein (G-protein) of the visual system, transducin, by associating with GDP at the nucleotide-binding site and thus mimicking the effects of non-hydrolysable GTP analogues [Bigay, Deterre, Pfister & Chabre (1985) FEBS Lett. **191**, 181–85]. We have examined this proposed model by using the adenylate cyclase complexes of frog erythrocytes, S49 lymphoma cells and human platelets. Preincubation of plasma membranes from frog erythrocytes and S49 cells with 20 mM-fluoride for 20 min at 30 °C strongly stimulated adenylate cyclase activity. In contrast, the preactivated membranes were still able to bind β-adrenergic agonist with high affinity, as determined by radioligand-binding techniques. Moreover, high-affinity agonist binding in fluoride-treated membranes was fully sensitive to guanine nucleotide, which decreased β-adrenergic-receptor affinity for agonist. Very similar results were obtained for [^{3}H]prostaglandin E_1 binding to S49 membranes pretreated with fluoride. Incubation of human platelet membranes with increasing concentrations of fluoride (1–50 mM) resulted in biphasic regulation of adenylate cyclase activity, with inhibition observed at concentrations > 10 mM. Preincubation of platelet membranes with 20 mM-fluoride did not affect agonist high-affinity binding to α_2-adrenergic receptors, nor receptor regulation by guanine nucleotide. These results suggest that the model developed from the study of transducin may not be generally applicable to the G-proteins of the adenylate cyclase system.

INTRODUCTION

The adenylate cyclase complex of mammalian cells is composed of at least three types of membrane proteins: cell surface receptors, the catalytic unit, and guanine nucleotide regulatory proteins (G-proteins) [1, 2]. The G-proteins mediate the transmission of information from receptors to the catalyst and thereby modulate the intracellular concentration of cyclic AMP. The G-proteins of the adenylate cyclase complex appear to be members of a larger family of G-proteins with homologous structures and closely analogous functions in the process of transmembrane signalling [3].

Studies of adenylate cyclase systems have identified two distinct G-proteins that regulate the enzyme's activity [1–4]. These G-proteins, termed G_s and G_i, mediate the stimulation and inhibition of adenylate cyclase activity respectively. Both G-proteins appear to interact directly with distinct receptors. This is manifested in reconstitution experiments using purified protein components. Incorporation of β-adrenergic receptors and G_s into lipid vesicles stimulated the intrinsic GTPase activity of G_s [5, 6]. Moreover, the binding of GTP or its non-hydrolysable analogues to G_s decreased the affinity of the β-adrenergic receptor specifically for agonists and concomitantly stimulated the activity of the catalytic component of adenylate cyclase. Similarly, the reconstitution of purified α_2-adrenergic receptors with pure G_i resulted in agonist-specific increases in GTP[^{35}S] binding and GTPase activity [7].

Fluoride ion has long been recognized as a ubiquitous stimulator of adenylate cyclase activity [8, 9]. The activation of membrane-bound adenylate cyclase by F^- has been generally characterized as robust and essentially irreversible. However, the mechanism by which F^- promotes its effect is not known. G_s was found to be a necessary component for both guanine nucleotide- and fluoride-dependent activation of the adenylate cyclase catalyst [10, 11]. An assay based on the reconstitution of both guanine nucleotide- and fluoride-stimulated adenylate cyclase activity led to the purification of G_s [12]. This protein is a heterotrimer composed of α, β and γ subunits. The α subunit binds guanine nucleotides and interacts with the catalytic component of adenylate cyclase [13]. The α subunit of G_s also contains a site where fluoride interacts with the adenylate cyclase complex [13, 14]. Sternweis & Gilman [15] showed that Al^{3+} is a necessary cofactor for F^--dependent activation of adenylate cyclase, suggesting that an AlF_4^- complex may be the chemical species that triggers activation of the cyclase. Separate sites on G_s for Al^{3+} and F^- were also considered. Aluminium–fluoride complexes have been shown to activate other members of the G-protein family, including G_i [14, 16] and transducin [17]. Ex-

Abbreviations used: G-protein, guanine nucleotide regulatory protein; G_s, the stimulatory G-protein of adenylate cyclase; G_i, the inhibitory G-protein of adenylate cyclase; [^{3}H]DHA, (−)-[^{3}H]dihydroalprenolol; [^{3}H]HBI, (±)-[^{3}H]hydroxybenzylisoprenaline; [^{125}I]ICYP, (−)-[^{125}I]iodocyanopindolol; H, agonist; R_s, receptor coupled to the stimulation of adenylate cyclase; p[NH]ppG, guanyl-5′-yl imidodiphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate; [^{3}H]PGE$_1$, [^{3}H]prostaglandin E_1.

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posure of hepatocytes to Al^{3+}–F^- mimics the effects of hormones that mobilize Ca^{2+} through the hydrolysis of polyphosphoinositol lipids [18, 19]. Al^{3+}–F^- and GTP[S] had similar effects in releasing inositol polyphosphates from isolated hepatocyte membranes [20]. These data are cited as evidence for a G-protein, termed G_p, mediating hormone activation of a phosphotidylinositol-specific phospholipase C.

Recent investigations of the effects of Al^{3+}–F^- on transducin led to a proposed model [21] in which AlF_4^- binds to the terminal phosphate of tightly bound GDP on G-proteins [22] to mimic GTP or its non-hydrolysable analogues, resulting in a slowly reversible activated state. The present study was undertaken to test the applicability of this model to G-proteins of the adenylate cyclase complex.

EXPERIMENTAL

Materials

The radioligands employed were [^{3}H]DHA, 55 Ci/mmol; [^{3}H]HBI, 9 Ci/mmol; [^{3}H]PGE$_1$, 54.2 Ci/mmol; [^{3}H]yohimbine, 87.0 Ci/mmol; and [^{125}I]ICYP, 2200 Ci/mmol; all obtained from New England Nuclear. Cholera toxin was purchased from Schwartz–Mann.

Methods

Cell culture. S49 wt lymphoma cells were grown in suspension culture at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated horse serum in an atmosphere of 10% CO_2 in air [11].

Partially purified frog erythrocyte membranes. Membranes from washed frog erythrocytes were prepared as previously described [23].

Partially purified S49-cell membranes. S49-cell pellets were frozen and thawed before resuspension in lysis buffer [5 mM-Tris/HCl (pH 7.5)/2 mM-$MgCl_2$/1 mM-EDTA] for 10 min at 4 °C. The cell suspension was homogenized with ten strokes in a Dounce homogenizer and then centrifuged for 10 min at 500 ***g*** to remove unbroken cells and nuclei. The supernatant was harvested and centrifuged for 20 min at 40000 ***g***. The resulting pellet was washed twice in buffer A, containing 250 mM-sucrose, 75 mM-Tris/HCl (pH 7.5), 12.5 mM-$MgCl_2$, 1.5 mM-EDTA and 0.5 mM-dithiothreitol, before being resuspended in this buffer for incubation with and without fluoride.

Partially purified human platelet membranes. Membranes from human platelets were prepared as previously described [24], except that the membranes were resuspended in buffer A before assay.

Pretreatment of membranes with NaF. Frog erythrocyte membranes (3 mg/ml), human platelet membranes (5 mg/ml) and S49 lymphoma membranes (3–10 mg/ml) in buffer A were incubated with 20 mM-NaF for 20 min at 30 °C. All incubations were performed in borosilicate glass tubes to provide factors necessary for fluoride activation of adenylate cyclase-coupled G-proteins [15]. Adenylate cyclase activities in frog erythrocyte (3.8 nmol of cyclic AMP/min per mg of protein) and S49-cell (385 pmol of cyclic AMP/min per mg of protein) membranes were nearly linear for 20–30 min and were not stimulated further by increasing fluoride to 40 mM. The addition of 20 μM-$AlCl_3$ with NaF did not alter adenylate cyclase activities or the results of the radioligand-binding experiments.

Cholera-toxin pretreatment of frog erythrocyte membranes. Frog erythrocyte membranes were incubated for 20 min at 25 °C with 0.1 mM-NAD^+ in the presence and absence of 50 μg of cholera toxin/ml (preactivated with 20 mM-dithiothreitol for 10 min at 37 °C). The membranes were washed once in buffer A and resuspended in the same buffer for subsequent binding assays.

Radioligand-binding assays. Erythrocyte or S49-cell membranes (0.1–0.5 mg/ml) were incubated with 2–3 nM-[^{3}H]DHA (frog), 5–10 nM-[^{3}H]HBI (frog), 8–10 nM-[^{3}H]PGE$_1$ (S49) or 50 pM-[^{125}I]ICYP (S49) in a total volume of 0.5 ml as previously described [23, 25]. Human platelet membranes (0.1–0.3 mg/ml) in buffer A were incubated with 3–6 nM-[^{3}H]yohimbine as previously described [24]. Incubations were performed at 37 °C for 10 min, except that a 60 min incubation was employed for [^{125}I]ICYP binding to S49-cell membranes. When present, the final concentration of fluoride was 4 mM. Specific binding in all experiments ranged between 70 and 90%.

Adenylate cyclase assays. These were performed at 30 °C as previously described [23], by using 50 μl incubations containing 0.12 mM-[^{32}P]ATP as substrate. Protein was determined by the method of Bradford [26], with bovine γ-globulin as standard.

RESULTS

The interaction between β-adrenergic receptors and G_s can be monitored directly by radioligand-binding techniques. Agonist (H) binding to the β-adrenergic receptor (R_s) promotes the association of R_s with G_s to form a high-affinity ternary complex H–R_s–G_s [4, 6, 27, 28]. The ternary complex is dissociated by the binding of guanine nucleotide to G_s, leading to decreased affinity of R_s for H. This phenomenon is illustrated in competition-binding experiments as shown in Fig. 1 for frog erythrocyte membranes and in Fig. 2 for S49-cell membranes. The competition binding curves of (−)-isoprenaline for radiolabelled antagonists are distinctly different depending on whether the non-hydrolysable GTP analogue p[NH]ppG is present in the assay (square symbols). In the presence of the guanine nucleotide, the competition binding curve is shifted to the right, indicating a lower receptor affinity for the agonist. Preincubation of the membranes for 20 min at 30 °C in the presence of 20 mM-fluoride did not affect the position of the isoprenaline-competition-binding curves for the frog erythrocyte or S49 membranes, indicating comparable formation of the agonist-induced ternary complex (H–R_s–G_s). Moreover, the ternary complex was fully sensitive to p[NH]ppG. The 20 min pretreatment period was chosen to ensure the activation of G_s by fluoride before any decline in adenylate cyclase activity after prolonged incubation of membranes at 30 °C.

Although fluoride pretreatment of membranes did not affect the ability of isoprenaline to promote a high-affinity state of the β-adrenergic receptor, nor the ability

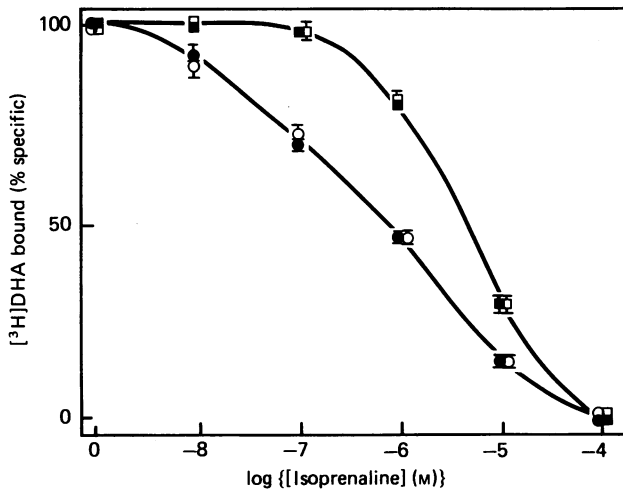

Fig. 1. Isoprenaline competition for [3H]DHA binding to frog erythrocyte membranes pretreated with and without NaF

Frog erythrocyte membranes were incubated without (■, ●) or with (□, ○) 20 mM-NaF for 20 min at 30 °C as described in the Experimental section. Binding assays were performed as described in the Experimental section in the absence (○, ●) and presence (□, ■) of 0.1 mM-p[NH]ppG; 100% specific binding of [3H]DHA was 1.33 ± 0.08 pmol/mg of protein. Each point represents the mean ± S.E.M. of duplicate determinations from three experiments.

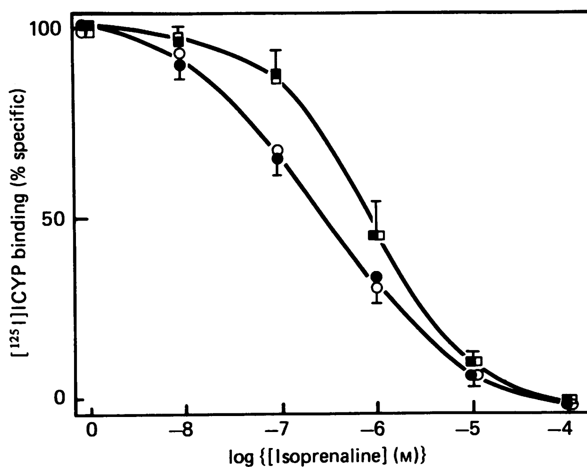

Fig. 2. Isoprenaline competition of [125I]ICYP binding to S49 lymphoma membranes pretreated with and without NaF

S49 lymphoma membranes were incubated without (■, ●) or with (□, ○) 20 mM-NaF for 20 min at 30 °C as described in the Experimental section. Binding assays were performed as described in the Experimental section in the absence (○, ●) and presence (□, ■) of 0.1 mM-p[NH]ppG; 100% specific binding of [125I]ICYP was 72 ± 17 fmol/mg of protein. Each point represents the mean ± S.E.M. of duplicate determinations from three experiments.

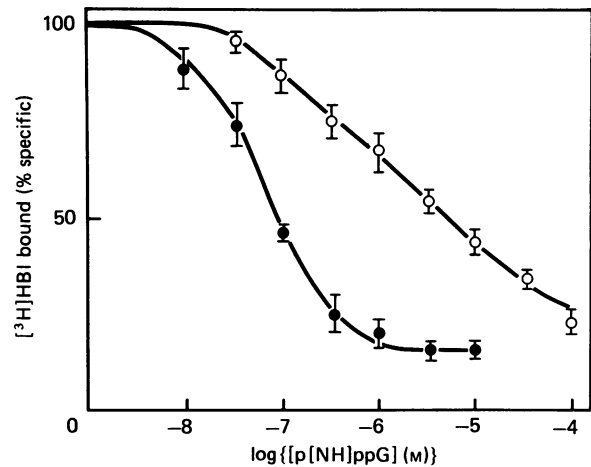

Fig. 3. Inhibition of [3H]HBI binding by p[NH]ppG to frog erythrocyte membranes pretreated with and without cholera toxin

Frog erythrocyte membranes were incubated in the absence (●) and presence (○) of 0.1 mM-NAD+ and 50 μg of cholera toxin/ml as described in the Experimental section. Binding assays were performed as described in the Experimental section; 100% specific binding of [3H]HBI was 533 ± 62 fmol/mg of protein. Each point represents the mean ± S.E.M. of duplicate determinations from three experiments.

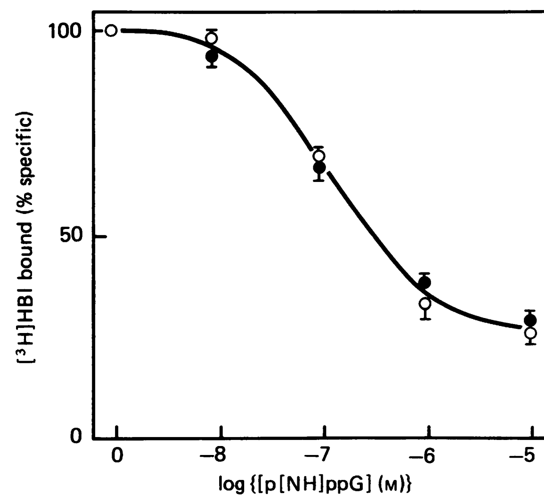

Fig. 4. Inhibition of [3H]HBI binding by p[NH]ppG to frog erythrocyte membranes pretreated with and without NaF

Frog erythrocyte membranes were incubated in the absence (●) or presence (○) of 20 mM-NaF for 20 min at 30 °C as described in the Experimental section. Binding assays were performed as described in the Experimental section; 100% specific binding of [3H]HBI was 814 ± 74 fmol/mg of protein. Each point represents the mean ± S.E.M. of duplicate determinations from three experiments.

of the receptor to be converted into a low-affinity state by maximal concentrations of guanine nucleotide, it was possible that fluoride could perturb the affinity of G_s for nucleotide. Similar effects on G_s have been observed after cholera-toxin-catalysed ADP-ribosylation of G_s in frog erythrocyte membranes [29]. To investigate this possibility, we examined agonist binding to the β-adrenergic receptor directly by using the radiolabelled agonist ligand [3H]HBI [23, 28, 30]. In frog erythrocyte membranes [3H]HBI specifically labels the high-affinity

Table 1. [^{3}H]PGE$_1$ binding to S49 lymphoma membranes pretreated with and without NaF

S49 lymphoma membranes were incubated with and without 20 mM-NaF for 10 min at 30 °C as described in the Experimental section. Binding assays were performed as described in the Experimental section in the absence and presence of 0.1 mM-p[NH]ppG. Binding in the presence of 10 μM unlabelled PGE$_1$ was subtracted to yield specific binding. The data represent the means ± S.D. for duplicate determinations from two experiments.

Treatment	[^{3}H]PGE$_1$ bound (fmol/mg of protein) Total	+ p[NH]ppG
Control	51.9 ± 1.3	13.2 ± 2.5
NaF	48.7 ± 3.7	10.0 ± 0.7

nucleotide-sensitive form of the β-adrenergic receptor [28]. Pretreatment of frog erythrocyte membranes with cholera toxin (50 μg/ml) and NAD$^+$ (0.1 mM) significantly decreased the apparent potency of p[NH]ppG to inhibit [^{3}H]HBI binding to β-adrenergic receptors (Fig. 3). These data, obtained by using steady-state radioligand-binding techniques, are totally consistent with kinetic experiments investigating guanine-nucleotide sensitivity of agonist binding to frog erythrocyte β-adrenergic receptors previously reported [29]. In contrast, Fig. 4 shows that pretreatment of frog erythrocyte membranes with 20 mM-fluoride for 20 min at 30 °C had no effect on the formation of the high-affinity [^{3}H]HBI–β-adrenergic-receptor complex nor its regulation by p[NH]ppG.

These results suggested that fluoride activation of G$_s$ did not perturb its interaction with the β-adrenergic receptor. To extend these observations, we next examined another G$_s$-coupled receptor system, PGE$_1$ [31, 32]. [^{3}H]PGE$_1$ binding to S49 membranes was unaffected by pretreatment with 20 mM-fluoride (Table 1), although the preincubation period was shortened to 10 min, owing to the apparent thermal instability of the PGE$_1$ receptors (results not shown). As with the β-adrenergic receptor, the PGE$_1$ receptor remained fully sensitive to p[NH]ppG after exposure of membranes to fluoride. Thus the absence of an effect of fluoride on nucleotide-sensitive agonist high-affinity binding (H–R$_s$–G$_s$) does not appear to be receptor-specific.

Guanine nucleotides also regulate agonist binding to receptors coupled to the inhibition of adenylate cyclase [1–4]. A well-studied model system for agonist-promoted inhibition of adenylate cyclase is the α_2-adrenergic-receptor complex of the human platelet [33]. As shown in Table 2, increasing concentrations of fluoride initially stimulated adenylate cyclase activity, but at concentrations above 10 mM an inhibition of the enzyme's activity was observed. These data are consistent with the notion that fluoride activates both G$_s$ and G$_i$ in platelet membranes. Fluoride has previously been reported both to activate and to inhibit adipocyte adenylate cyclase [34, 35]. Pretreatment of platelet membranes with 20 mM-NaF for 20 min at 30 °C did not inhibit high-affinity binding of adrenaline to α_2-adrenergic receptors, as determined by competition binding for the radiolabelled antagonist [^{3}H]yohimbine (Fig. 5). Exposure of the membranes to fluoride also did not affect the guanine-nucleotide sensitivity of the agonist–receptor complex, a result that was identical with that observed for receptors coupled to G$_S$. A small but perceptible shift to the right

Table 2. Effects of increasing concentrations of NaF on forskolin-stimulated adenylate cyclase activity in human platelet membranes

Adenylate cyclase assays were performed as described in the Experimental section. Incubations were for 10 min at 37 °C. Forskolin was present throughout at 0.01 mM final concn. in the assay. Similar results were obtained in the absence of forskolin (results not shown). The data represent the means ± S.E.M. of triplicate determinations from three experiments.

NaF (mM)	Forskolin-stimulated adenylate cyclase activity (nmol of cyclic AMP/min per mg of protein)
0	0.71 ± 0.08
1	0.80 ± 0.04
2	0.95 ± 0.08
5	1.14 ± 0.02
10	1.09 ± 0.03
20	0.85 ± 0.04
50	0.64 ± 0.01

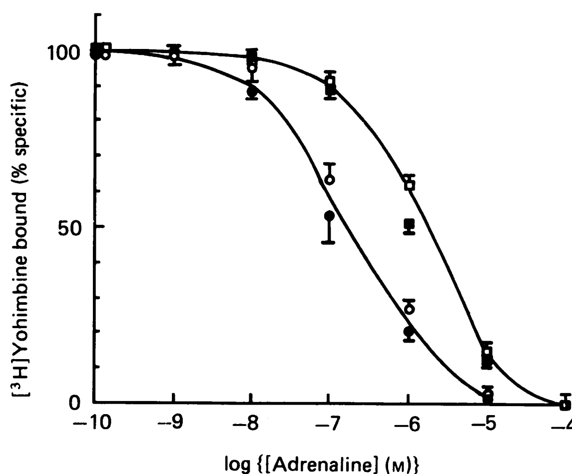

Fig. 5. Adrenaline competition of [^{3}H]yohimbine binding to human platelet membranes pretreated with or without NaF

Human platelet membranes were incubated without (○, □) or with (●, ■) 20 mM-NaF for 20 min at 30 °C as described in the Experimental section. Binding assays were performed as described in the Experimental section in the absence (○, ●) or presence (□, ■) of 0.1 mM-p[NH]ppG. Identical results were obtained if NaF was maintained at 20 mM during the binding assay (results not shown); 100 % specific binding of [^{3}H]yohimbine was 168 ± 33 fmol/mg of protein. Each data point represents the mean ± S.E.M. of duplicate determinations from three experiments.

in the agonist competition-binding curve in both the presence and the absence of p[NH]ppG after NaF treatment was most probably due to the increased Na^+ concentration in the binding assay [36]. Our results demonstrate that a lack of effect by fluoride on agonist-binding properties of receptors coupled to both the stimulation and the inhibition of adenylate cyclase is distinctly different from the regulatory effects of non-hydrolysable GTP analogues.

DISCUSSION

In the present study we have examined receptor–adenylate cyclase complexes from well-characterized model systems to investigate the influence of fluoride on G-proteins. Our studies were prompted by a recently proposed model for the activation of transducin by Al^{3+}–F^- [21]. This model, based on both biochemical and chemical arguments, suggests that the molecular species AlF_4^- binds to the guanine nucleotide site on the α subunit of transducin and complexes with GDP to mimic the activating triphosphate form of guanine nucleotides. The model is supported by a number of observations showing very similar activities of Al^{3+}–F^- and GTP[S] on transducin. Both these agents promote the release of transducin from rod-outer-segment membranes and the subsequent activation of cyclic GMP phosphodiesterase [17, 21]. The peptide-mapping patterns of the α subunit of transducin after binding of GTP[S] or fluoride were indistinguishable, suggesting that these agents induced similar conformational changes in the protein [37]. Rhodopsin stimulation of transducin GTP binding and GTPase were inhibited in a similar manner by GTP[S] and Al^{3+}–F^-, as was pertussis-toxin-catalysed ADP-ribosylation of transducin [37]. These data support the notion that GTP[S] and Al^{3+}–F^- act on transducin in a similar fashion. As the G-proteins of the adenylate cyclase and visual complexes appear to be structurally homologous and subserve similar functions, it seems reasonable to extrapolate from transducin to other G-proteins mediating transmembrane signalling.

Fluoride ion (presumably AlF_4^-) has been extensively studied for its effects on G_s and G_i of the adenylate cyclase system. Guanine nucleotides were shown to modulate the activation of adenylate cyclase by fluoride ion [38], and in a reciprocal fashion fluoride ion inhibited hormone-sensitive GTPase activity [39]. Maximal adenylate cyclase activation by fluoride was supported by either GTP or GDP [38]. Because G-proteins contain tightly bound GDP in their basal state [22], exogenous guanine nucleotide is not needed for fluoride activation of G_s. Moreover, studies of purified G_s showed that fluoride and GTP[S] promoted activation/subunit dissociation by apparently indistinguishable mechanisms [14]. Similarly, purified G_i can be activated through the dissociation of its α and $\beta\gamma$ subunits by Al^{3+}–F^- or GTP[S] by a very analogous mechanism [14, 16]. These data were consistent with the model for G-protein activation proposed for transducin [21], i.e. Al^{3+}–F^- and GTP[S] trigger similar responses of G-proteins, possibly by interacting at the same site. On the other hand, these data do not exclude the possibility of a unique fluoride site on the α subunits of adenylate cyclase-coupled G-proteins.

The results of the present study, however, do not support the extrapolation of the model for fluoride activation of transducin to G_s. Pure G_s, which appears to possess a single guanine-nucleotide-binding site, has been shown to be required for agonist-, fluoride- and guanine-nucleotide-dependent stimulation of adenylate cyclase activity [12]. Reconstitution of pure G_s with pure β-adrenergic receptor into lipid vesicles also re-established high-affinity guanine-nucleotide-sensitive agonist binding to the receptor [6]. These results indicate that a single guanine-nucleotide-binding protein, G_s, modulates both adenylate cyclase activation and receptor affinity for agonists. Our results show that, although fluoride strongly activated adenylate cyclase activity in both frog erythrocyte and S49 lymphoma membranes, there was no apparent effect on receptor–G_s interactions as determined by radioligand-binding techniques. This assertion assumes a near-equal stoichiometry for the receptor and G_s in the plasma membrane [28, 40, 41]. Similar results were obtained in our studies of the G_i-coupled α_2-adrenergic receptor complex in platelet membranes. As GTP and its analogues all modulate the affinity of G_s- and G_i-coupled receptors for agonists, it is not possible to explain these results by the binding of aluminium–fluoride to the guanine-nucleotide-binding site of these G-proteins and thus mimicking the effects of non-hydrolysable analogues of GTP. We propose an independent binding site for fluoride (Al^{3+}–F^-) on the α subunits of G_s and G_i. Additional biochemical experiments will be necessary to define the site(s).

Our results raise additional questions concerning the mechanism(s) of G-protein activation. Studies of pure G_s and G_i in detergent solution have shown that activation by GTP[S] or fluoride promotes the dissociation of the holoproteins into α and $\beta\gamma$ subunits [13, 14]. It is not clear to what extent these studies reflect the functional activation of the G-protein *in situ* in the plasma membrane. If dissociation of the G-proteins takes place in the membrane after exposure to fluoride, the present data would suggest that the α subunit alone can sustain agonist-promoted high-affinity binding to its receptor. The association of the individual α subunits of G_s or G_i with agonist-occupied receptors would point to another distinction between these G-proteins and transducin. For the latter, the trimeric form of transducin is required for fruitful interaction with its receptor, rhodopsin [37]. An alternative explanation might be the existence of another GTP-binding component in plasma membranes that may be involved in the regulation of receptor affinity for agonist.

In summary, the data in the present study argue that fluoride or an Al^{3+}–F^- complex does not bind at the guanine nucleotide site of adenylate cyclase-coupled G-proteins in a manner that mimics a guanine nucleoside triphosphate. Our results are supported by reconstitution experiments using purified β-adrenergic receptor and G_s preparations [42]. Agonist-stimulated GTPase, GTP binding and GDP release were not significantly affected by the addition of Al^{3+}–F^-. However, Al^{3+}–F^- did inhibit these parameters of G_s activity if a high concentration of Mg^{2+} was used as the stimulator. Although there are obvious similarities among G-proteins, both structurally and functionally, the individual characteristics embodied principally in the α subunits may preclude direct extrapolation of certain properties.

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