Differential Activation of the Stimulatory and Inhibitory Guanine Nucleotide-binding Proteins by Fluoroaluminate in Cells and in Membranes

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Fluoroaluminate had no effect on cAMP levels in cells but inhibited agonist-stimulated cAMP accumulation. In membranes, fluoroaluminate stimulated adenylate cyclase activity between 1 and 10 mM but not at higher concentrations, and it inhibited agonist-stimulated adenylate cyclase activity at concentrations greater than 1 mM. Fluoroaluminate is known to activate G. and G., the guanine nucleotide-binding (G) proteins that stimulate and inhibit adenylate cyclase. G proteins are heterotrimERIC, with unique α and common βγ subunits, and activation involves dissociation of α from βγ. Pertussis toxin catalyzes ADP-ribosylation of α of heterotrimeric G, but not free α. Fluoroaluminate prevented pertussis toxin-catalyzed ADP-ribosylation of G, in cells and membranes, suggesting that G is activated by fluoroaluminate in both. Cholera toxin catalyzes ADP-ribosylation of the α-subunit of G. In cells, agonist often increased cholera toxin-catalyzed ADP-ribosylation of G, but fluoroaluminate decreased ADP-ribosylation even in the presence of agonist, suggesting that G cannot be activated in the presence of fluoroaluminate. In membranes, both agonist and fluoroaluminate increased cholera toxin-catalyzed ADP-ribosylation, suggesting that G is activated by these agents. We conclude that fluoroaluminate activates G, but not G, in cells and activates both G proteins in membranes. The value of bacterial toxins in assessing the state of G proteins in cells and membranes is demonstrated.

Guanine nucleotide-binding proteins (G proteins) are important in the regulation of cellular responses to environmental stimuli (Gilman, 1987). These G proteins function as signal transducers between receptors on the cell surface and effector proteins within the cell. Among the G proteins that have been well characterized are transducin, which converts the light impinging upon rhodopsin in retinal rod outer segments into phosphodiesterase activation, and the stimulatory (G.) and inhibitory (G.) G proteins, which allow agonist-receptor complexes to stimulate or inhibit, respectively, adenylate cyclase activity. It is also clear that transducin (Kanaho et al., 1985) and G, and G, (Sternweis and Gilman, 1982; Northup et al., 1983; Katada et al., 1984a, 1984b) can be activated by a mixture of fluoride and aluminum ions (fluoroaluminate). As a result, many of the effects of fluoroaluminate have been attributed to the activation of G proteins even though the existence of these G proteins has not always been demonstrated. Examples include fluoroaluminate stimulation of phosphatidylinositol metabolism (Blackmore et al., 1985; Blackmore and Exton, 1986; Paris and Pouyssegur, 1987) and ion flux across membranes (Dunlap et al., 1987).

It also has been observed that in intact cells, fluoroaluminate inhibits the stimulation of adenylate cyclase by agonists whose receptors are coupled to G, but appears to have little if any effect by itself on cAMP levels (Katada et al., 1984b; Blackmore et al., 1985; Blackmore and Exton, 1986; Paris and Pouyssegur, 1987). It has been proposed that this effect of fluoroaluminate is caused by activation of G. This raises an apparent paradox based on the known ability of fluoroaluminate to stimulate adenylate cyclase through G, (see Sternweis and Gilman, 1982). In the course of investigating the effects of fluoroaluminate on phosphoinositide breakdown in MLT-1 cells (Inoue and Rebois, 1989), we made similar observations. We initiated the present study to determine if the inhibitory effect was indeed due to activation of G, or to some other influence of fluoroaluminate on cells. We also used pertussis and cholera toxins as probes for the state of the two G proteins in both cells and membranes in order to determine whether fluoroaluminate has differential effects on their state of activation.

EXPERIMENTAL PROCEDURES

Materials—bCG (13,450 IU/mg, CR 121) was provided by Dr. R. Canfield, Columbia University, through the Center for Population Research for the NICHD. The hormone was iodinated in our laboratory as described previously (Rebois, 1982). [3,8-H]cAMP (33.5 Ci/mmol), [α-32P]ATP (39.9 Ci/mmol), [α-32P]NAD (31 Ci/mmol), and Na21 (17 Ci/mg) were obtained from Du Pont-New England Nuclear. 11-31-Labeled cAMP was from Biomedics Research (Rockville, MD). Bovine serum albumin, dithiothreitol, theophylline, phosphodiesterase, phosphocreatine, creatine phosphokinase, GTP, AMP, ATP, dibutyryl cGMP, L-α-phosphatidic acid (from egg yolk lecithin), digitonin, and deferoxamine mesylate were obtained from Sigma. Ionophore A23187 was obtained from Calbiochem. Pertussis and cholera toxins were from List Biological Laboratories, Inc. (Campbell, CA). Cholera toxin from ICN Biomedicals (Costa Mesa, CA) was iodinated as described by Kassis et al. (1982).

Cell Culture—MLT-1 cells were grown as described by Rebois (1982) except for the omission of antibiotics. Rat glioma C6 cells (Zarembo and Fishman, 1984) and mouse lymphoma L5178Y cells (Kassis et al., 1984) were grown as described previously. Human neuroblastoma SK-N-MC cells were obtained from the American Type Culture Collection. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be sent: Park 5 Bldg., Rm. 408, NIH, Bethesda, MD 20892. Tel. 301-496-2007.
2 The abbreviation used are: G protein, guanine nucleotide-binding protein; G, inhibitory G protein; G, stimulatory G protein; bCG, human chorionic gonadotropin; HEPES, N-2-hydroxyethylpiperezine-N'-2-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylene- nitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate.

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Activation of G. and G. by Fluoroaluminate

Culture Collection and grown as recommended. For experiments to determine cAMP accumulation and agonist binding, cells were cultured in 6 × 35-mm-diameter multicluster dishes. Otherwise, the cells were grown in 75-cm² flasks. In some experiments, C6 cells were made permeable with 0.005% digitonin (Brooker and Pedone, 1986).

The Effects of Aluminum and Fluoride on CAMP Accumulation by Cells and Adenylate Cyclase Activity of Membranes—In experiments to determine the effects of aluminum, cells were incubated in Krebs-Ringer-HEPES buffer containing 132 mM NaCl, 4.8 mM KCl, 2.4 mM MgCl₂, 0.1 mM EGTA, 1 mM CaCl₂, 10 mM glucose, and 20 mM HEPES, pH 7.4. Otherwise, cells were incubated in culture medium without serum. When used, a 50 mM aqueous stock solution of the aluminum chelator deferoxamine was added 10 min before NaF to give a final concentration of 0.5 mM. Stock solutions of fluoride salts and cAMP were stored separately in plastic tubes. The term fluoroaluminate refers to a mixture of fluoride and aluminum salts which was prepared immediately before addition to cells or membranes. Reference to fluoroaluminate concentrations reflect the final fluoride concentrations. Except as noted in figure legends, the final concentration of AlCl₃ was 10 μM. Unless otherwise indicated, cells were exposed to fluoroaluminate for 30 min at 37°C before other additions were made. For CAMP accumulation, MLTC-1 cells were treated with 2 mM hCG; and C6, S49, and SK-N-MC cells were treated with 10 μM isoproterenol. Thirty min after agonist addition, the reaction was stopped and CAMP accumulation determined by radioluminum assay as described previously (Inoue and Rebois, 1984; Zaremba and Fishman, 1984). For permeable cells, CAMP in the medium was determined following acetylation.

To prepare membranes, MLTC-1 cells were homogenized in a Dounce homogenizer with the tight fitting pestle; and C6 cells were homogenized for 10 s with a Polytron homogenizer at 75% of maximum speed, and the homogenates were centrifuged as described previously (Inoue and Rebois, 1989). Adenylate cyclase activity was assayed in MLTC-1 membranes (Inoue and Rebois, 1989) or with C6 membranes (Zaremba and Fishman, 1984) in the presence of different concentrations of fluoroaluminate. Salt concentration was kept constant by the addition of NaCl. In some experiments, C6 membranes were also treated with 10 μM isoproterenol as indicated in figure legends.

ADP-ribosylation of Cells and Membranes with Bacterial Toxins—For studies of toxin-catalyzed ADP-ribosylation, stock solutions containing 1 mg/ml pertussis toxin or 1 mg/ml cholera toxin were prepared as suggested by the supplier and stored at 4°C. The toxins were added directly to cultures so that the final concentration of pertussis or cholera toxin was 0.1 or 1.0 μg/ml, respectively. Generation of the A₁ subunit of cholera toxin, the active ADP-ribosyltransferase peptide, in intact cells was determined as described by Kassis et al. (1982). When fluoroaluminate was used, its addition preceded toxin treatment by 30 min; and when cells were treated with agonist, it was added 1 h after the toxin. The toxins were present for 3 h before cells were washed to remove any free toxin and agonist; in the case of hCG-treated MLTC-1 cells, they were washed with glycine-buffered saline (pH 3.0) to remove bound hCG (Rebois and Fishman, 1983). The cells were scraped from their flasks and homogenized as described above in 50 mM Tris, pH 7.4. The homogenates were centrifuged at 400 × g for 5 min, and the resulting supernatant was centrifuged at 10,000 × g for 15 min. Membrane pellets were suspended to 1 mg of membrane protein/ml in 50 mM Tris, pH 7.4, and used immediately or stored frozen in liquid nitrogen.

Membranes from cells treated as described above or from untreated cells were ADP-ribosylated with pertussis toxin (Bokoch et al., 1983) or cholera toxin (Rebois, 1982) as described previously. For some experiments, the membranes were treated with agonist or with different concentrations of fluoroaluminate for 30 min at 30°C before ADP-ribosylation.

Other Methods—hCG receptor binding was performed as described previously (Rebois, 1982). ATP was determined with a ATP bioluminescent assay kit (Sigma). Protein was determined by the procedure of Lowry et al. (1951). Treatment of cells with phosphatidic acid was performed as described by Murayama and Uri (1987). To determine the effects of calcium and cGMP on agonist-stimulated cAMP accumulation in MLTC-1 cells, we incubated the cells with 10 μM AICl₃ and 0.5 or 2.0 mM CaCl₂ or 2 mM dibutyryl cyclic GMP for 30 min prior to the addition of 2 nM hCG. Thirty min later, cAMP was assayed as described above. Unless otherwise indicated, values represent the mean and standard deviations for triplicate determinations in a single experiment. Each experiment was repeated at least three times with similar results, and representative experiments are shown in the tables and figures.

RESULTS

Characteristics of Fluoroaluminate Inhibition of Agonist-stimulated cAMP Accumulation—Fluoride inhibited cAMP accumulation stimulated by hCG in MLTC-1 cells and by isoproterenol in rat glioma C6 cells in a dose-dependent manner (Fig. 1). Fluoride inhibition was potentiated by the addition of 10 μM AICl₃ and significantly attenuated when the aluminum chelator deferoxamine was added. The effect of aluminum was greatest at low NaF concentrations. Similar results have been observed by other investigators (Blackmore et al., 1985). Since aluminum is required for fluoride to cause optimal attenuation of agonist-stimulated cAMP accumulation, we routinely added 10 μM AICl₃.

We extended our investigation to other tissue culture cells. In all cell types tested, we observed that agonist-stimulated CAMP accumulation was inhibited by fluoroaluminate although the extent of inhibition varied, being greater than 90% for MLTC-1 and C6 cells, 60% for SK-N-MC cells, and 28% for S49 cells. In MLTC-1 cells, agonist response was restored within 2 h after removing fluoroaluminate from the culture medium (Fig. 2), and inhibition of agonist-stimulated CAMP accumulation by fluoroaluminate was rapid (Fig. 3). The level of CAMP accumulation following simultaneous addition of hCG and fluoroaluminate was less than 15% of that

![Fig. 1. The effects of AICl₃ and deferoxamine on the dose-dependent inhibition of agonist-stimulated cAMP accumulation by fluoride in MLTC-1 and C6 cells. Cells were incubated in Krebs-Ringer-HEPES buffer with the indicated concentrations of NaF alone (○) or in the presence of 10 μM AICl₃ (◊) or 0.5 mM deferoxamine (△). C6 (A) and MLTC-1 (B) cells were subsequently stimulated with 10 μM isoproterenol and 2 nM hCG, respectively, and assayed for CAMP accumulation as described under "Experimental Procedures."](http://www.jbc.org/)

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cells when theophylline was present (Table I), indicating that it was an effective inhibitor of phosphodiesterase. Theophylline, however, failed to prevent fluoroaluminate attenuation of hCG-stimulated cAMP accumulation. Similarly, fluoroaluminate inhibited the isoproterenol response in SK-N-MC cells in the presence of isobutylmethylxanthine, another phosphodiesterase inhibitor (data not shown).

The ratio of intracellular to extracellular cAMP in hCG-stimulated MLTC-1 cells was approximately 3:1. Fluoroaluminate did not alter this ratio but only reduced the amount of cAMP produced in the presence of gonadotropin. It has been reported that fluoride inhibits thyroid-stimulated cAMP accumulation in the thyroid (Van Sande et al., 1975), most likely by inhibiting glycocolysis and consequently reducing the availability of ATP, the substrate for adenylate cyclase. However, untreated and fluoroaluminate-treated MLTC-1 cells contained similar amounts of ATP, 10.8 ± 0.3 and 12.7 ± 0.7 µmol of ATP/mg of cell protein, respectively.

Fluoroaluminate can stimulate phosphatidinositol 1,4-bisphosphate metabolism in many kinds of cells, including MLTC-1 cells (Inoue and Rebois, 1989), which in turn may affect the cellular content of phosphatidic acid, calcium, and cGMP (Berridge, 1984; Berridge and Irvine, 1984; Amar et al., 1987). Investigators have reported that adenylate cyclase activity is inhibited by phosphatidic acid and by calcium (Murayama and Ui, 1987; Dorflinger et al., 1984; Pereira et al., 1988), and inhibition of both basal and agonist-stimulated cAMP accumulation in Leydig tumor cells has been shown to occur under conditions in which cGMP increases (Pandey et al., 1985). The latter observation suggests that there may be a causal relationship between increased cGMP and decreased cAMP levels. We therefore tested the effects of all of these substances on hCG-stimulated cAMP accumulation in MLTC-1 cells (Table II). Neither phosphatidic acid nor dibutyryl cGMP, a permeable analogue of cGMP, had any effect on hCG-stimulated cAMP accumulation. Calcium in the presence of the ionophore A23187 reduced the hCG response in MLTC-1 cells, but it was not as effective as fluoroaluminate, and the concentration required for inhibition was 4000-fold higher than the concentrations estimated to be present in cells (500 nM) after phospholipase C activation (Strnad and Wong, 1985; Blackmore and Exton, 1986). These results suggest that fluoroaluminate inhibition is not mediated by any of these substances.

**Fluoroaluminate Effects on Adenylate Cyclase in Permeable Cells**

![Diagram of Fluoroaluminate Effects on Adenylate Cyclase](image)

**FIG. 2. Recovery from fluoroaluminate inhibition of agonist-stimulated cAMP accumulation in MLTC-1 cells.** MLTC-1 cells were incubated with 10 mM fluoroaluminate for 30 min at 37°C, washed with Dulbecco's Ca²⁺/Mg²⁺-free phosphate-buffered saline, and allowed to recover for the indicated time in the culture medium. After the recovery period, the cells were stimulated with 2 nM hCG for another 30 min at 37°C and assayed for accumulated cAMP. hCG caused the accumulation of 470 ± 35 pmol of cAMP/mg of protein in control cells that were not treated with fluoroaluminate.

![Diagram of Fluoroaluminate Effects on Adenylate Cyclase](image)

**FIG. 3. Time dependence of fluoroaluminate inhibition of agonist-stimulated cAMP accumulation in MLTC-1 cells.** MLTC-1 cells were incubated with 10 mM fluoroaluminate and 2 nM hCG. The time scale indicates the time of fluoroaluminate addition relative to agonist addition. hCG was present for 30 min before the cAMP content of the cells was determined. hCG-stimulated cAMP accumulation in control cells that were not treated with fluoroaluminate was 890 ± 100 pmol of cAMP/mg of protein.

in cells receiving hCG alone. The most effective inhibition occurred when fluoroaluminate was added at least 10 min before hCG. Fluoroaluminate by itself did not cause cAMP accumulation in intact cells (data not shown).

**Studies Related to the Mechanism of Fluoroaluminate Action**—Fluoroaluminate might cause inhibition of agonist-stimulated cAMP accumulation by activating G, (Blackmore et al., 1985). However, the apparent inability of fluoroaluminate to activate G, in intact cells gave rise to the bias that G, should similarly be unaffected. Therefore, we investigated other possible mechanisms of fluoroaluminate action.

The phosphodiesterase inhibitor theophylline was used to determine if the effects of fluoroaluminate could be attributed to activation of phosphodiesterase. There was a 4-fold increase in hCG-stimulated cAMP accumulation in MLTC-1
Cells and in Membranes—Fluoroaluminate by itself stimulated cAMP accumulation in C6 (Fig. 4) and MLTC-1 cells (data not shown) after treatment with digitonin to make them permeable. In permeable C6 cells, the stimulation was dose dependent, with a maximum stimulation between 3 and 10 mM fluoroaluminate. Permeable C6 cells maintained good response to isoproterenol whereas the MLTC-1 cells lost their response to hCG when they were made permeable. Isoproterenol-stimulated cAMP accumulation in C6 cells was inhibited by fluoroaluminate in a dose-dependent manner; at 10 mM, the response was the same as with fluoroaluminate by itself, and at 100 mM, there was little response above basal. Activation of adenylate cyclase persisted in membranes prepared from MLTC-1 cells made permeable in the presence of fluoroaluminate. The activity was 202 ± 8 compared with 84 ± 7 pmol of cAMP/mg of protein/10 min for cells treated with digitonin alone. The activity of adenylate cyclase in membranes from cells treated with fluoroaluminate in the absence of digitonin was 85 ± 6 pmol of cAMP/mg of protein/10 min.

Fluoroaluminate also stimulated adenylate cyclase in membranes. Concentrations between 3 and 10 mM were most effective, with higher or lower concentrations causing less stimulation and 100 mM causing no stimulation (Fig. 5). KF could be substituted for NaF with the same result, but the counterion had little to do with the effect since 100 mM NaCl or KCl reduced 10 mM fluoroaluminate-stimulated adenylate cyclase activity by less than 20%. Approximately 30% of the adenylate cyclase activity stimulated by 10 mM fluoroaluminate persisted through three washes of the membranes. In contrast, adenylate cyclase activity was not activated in membranes prepared from fluoroaluminate-treated cells (see above).

Adenylate cyclase activity in membranes prepared from MLTC-1 (not shown) or C6 cells (Fig. 5A) was stimulated by agonist. This response was not affected when cells were treated with fluoroaluminate prior to the preparation of membranes, and adding back the soluble cellular material that had been removed during preparation of the membranes did not inhibit agonist-stimulated adenylate cyclase activity (data not shown). Fluoroaluminate, however, attenuated agonist stimulation of adenylate cyclase in C6 membranes in a dose-dependent manner (Fig. 5A). Concentrations of NaCl to 100 mM had no effect on the agonist-stimulated adenylate cyclase activity in C6 membranes. We could not determine if fluoroaluminate inhibited agonist-stimulated adenylate cyclase activity in MLTC-1 membranes because high salt concentrations prevent hCG stimulation of the enzyme in this preparation (data not shown).

**Pertussis Toxin Effects on Cells and Membranes**—Intoxication of cells with pertussis toxin blocks receptor-mediated stimulation of G, (see Murayama and Ui, 1983). The α2 subunit of the heterorimeric form of G, is a substrate for the ADP ribosyltransferase activity of pertussis toxin (Neer et al., 1984;
Tsai et al., 1984). When membranes from MLTC-1 or C6 cells were incubated with pertussis toxin and \( ^{32}P \text{NAD} \), a 41-kDa protein, probably \( \alpha_2 \), was radiolabeled (Fig. 6, lanes 2 and 8). Treating MLTC-1 or C6 cells with pertussis toxin prior to preparing membranes decreased subsequent toxin-catalyzed ADP-ribosylation of this protein in membranes (lanes 4 and 10). This result indicated that \( \alpha_2 \) was ADP-ribosylated by the toxin in situ. When fluoroaluminate was added to cells before pertussis toxin, there was an increase in pertussis toxin-catalyzed ADP-ribosylation of \( \alpha_2 \) in membranes, which indicated less ADP-ribosylation by the toxin in situ (lanes 6 and 12). Membranes from pertussis toxin-treated cells retained the toxin since some ADP-ribosylation of \( \alpha_2 \) occurred when the membranes were incubated with \( ^{32}P \text{NAD} \) in the absence of additional toxin. The same was true for cells treated with both fluoroaluminate and pertussis toxin, indicating that fluoroaluminate did not prevent the toxin from becoming associated with the cells. Furthermore, pertussis toxin will ADP-ribosylate its own subunits, one of which is 29 kDa (Hildebrandt et al., 1983). We observed that fluoroaluminate did not prevent autolabeling of this subunit, indicating that fluoroaluminate does not inhibit the catalytic activity of pertussis toxin. Based on these observations, we believe it is unlikely that the reduced ADP-ribosylation of \( \alpha_2 \) observed in fluoroaluminate-treated cells is due to inhibition of the initial steps in pertussis toxin action.

We also investigated the effects of fluoroaluminate on ADP-ribosylation of \( \alpha_2 \) in membranes by pertussis toxin. Fluoroaluminate prevented, in a dose-dependent manner, pertussis toxin-catalyzed ADP-ribosylation in both C6 and MLTC-1 membranes (Fig. 7). The effect was most apparent at fluoroaluminate concentrations of 10 mM or more.

**Cholera Toxin-catalyzed ADP-Ribosylation of Cells and Membranes**—Intoxication of cells with cholera toxin causes G, to be irreversibly activated. The \( \alpha \) subunit of G, is the substrate for the ADP-ribosyltransferase activity of cholera toxin (Cassel and Pfeuffer, 1978; Kahn and Gilman, 1984). Incubating membranes from MLTC-1 cells with cholera toxin and \( ^{32}P \text{NAD} \) resulted in toxin-specific ADP-ribosylation of several proteins (Rebois, 1982), among them were what we presume to be the \( \alpha \) subunits of G, (52 and 45 kDa). Treating cells with hCG or fluoroaluminate in the absence of cholera toxin had no significant effect on subsequent ADP-ribosylation of \( \alpha_2 \) in membranes. By scanning densitometry we determined that relative to untreated cells, the percent of ADP-ribosylation in membranes from cells treated with hCG or fluoroaluminate or both was 105 ± 21, 103 ± 25, and 117 ± 33, respectively (n = 3). This observation indicates that ADP-ribosylation of \( \alpha_2 \) in membranes was not affected by treating cells with hCG and/or fluoroaluminate. Treating MLTC-1 cells with cholera toxin prior to preparing membranes (Fig. 8A, lane 1) decreased subsequent toxin-catalyzed ADP-ribosylation of \( \alpha_2 \) in membranes when compared with control cells not treated with toxin, thus indicating that \( \alpha_2 \) is ADP-ribosylated by the toxin in situ. When cells were treated with both cholera toxin and hCG, there was an equivalent amount or sometimes more in situ ADP-ribosylation, resulting in the same or less ADP-ribosylation of \( \alpha_2 \) in membranes (compare Fig. 8A, lanes 1 and 2). However, when cells were treated with fluoroaluminate and cholera toxin, there was increased ADP-ribosylation of \( \alpha_2 \) in membranes. MLTC-1 and C6 membranes were incubated with the indicated concentration of fluoroaluminate (NaF/AlCl₃ in a ratio of 1000:1) followed by \( ^{32}P \text{NAD} \) and pertussis toxin as described under “Experimental Procedures.” Labeling of proteins was determined as described in the legend to Fig. 6, and the autoradiograms show incorporation of label into \( \alpha_2 \).
We investigated the effects of cholera toxin on the membranes of MLTC-1 cells. The enzyme activity in membranes prepared from cholera toxin-treated cells was 4-5-fold higher than that from control cells. However, when cells were treated with floroualuminate in addition to cholera toxin, the adenylate cyclase activity was only 24% (average, n = 3) of that observed when cells were treated with the toxin alone.

We investigated the effects of cholera toxin on the membranes of MLTC-1 cells. The cells were not treated with toxin or effectors prior to the preparation of membranes. In the presence of hCG, which activates Go, cholera toxin-catalyzed ADP-ribosylation of Go was increased (Table III). We also observed that floroualuminate increased the ADP-ribosylation of Go. These results suggest that agonist and floroualuminate have a similar effect on Go in membranes.

**DISCUSSION**

We investigated the effects of fluoride on agonist-stimulated cAMP accumulation in cells. Fluoride alone had no effect on the cAMP content of cells but inhibited agonist-stimulated cAMP accumulation. We found that the effect was dose and time dependent, reversible, and potentiated by aluminum. We were unable to attribute the effect to increased phosphodiesterase activity; depeition of ATP, the substrate for adenylyl cyclase; efflux of cAMP from the cells; decreased receptor number or affinity; or increases in substances such as Ca++, GMP, or phosphatidic acid which are reportedly associated with inhibition of adenylyl cyclase.

There is good evidence that floroualuminate stimulates adenylyl cyclase in the membranes of many cell types by activating Go (see Sternweis and Gilman, 1982). Floroualuminate also inhibits adenylyl cyclase in the membranes of S49 cys cells, a type of cell that lacks Go but contains Gi (Hildebrandt et al., 1983). In addition, floroualuminate inhibits agonist-stimulated adenylyl cyclase in human platelet membranes, presumably through activation of Go (Katada et al., 1984a). More recently, floroualuminate has been reported to inhibit agonist-stimulated cAMP accumulation in isolated rat hepatocytes (Blackmore et al., 1988; Blackmore and Exton, 1986) and cultured hamster fibroblasts (Paris and Pouyssegur, 1987). Activation of Gi also may account for these latter observations, but we know of no experimental evidence to support this proposal. We decided to investigate if floroualuminate caused activation of G proteins, particularly Gi, in cells.

G proteins are heterotrimeric, consisting of unique subunits but having common By subunits (Gilman, 1987). The heterotrimer is generally considered to be inactive whereas the dissociated a and By subunits are thought to be the active form of the protein. Our approach to investigating the effects of floroualuminate on G proteins was to try to determine when these proteins were in an inactive or active form. In order to do this, we used two bacterial toxins, pertussis toxin and cholera toxin, which transfer the ADP-ribose moiety of NAD to the a subunits of Gi and Go, respectively. It is the inactive heterotrimer of G, that is ADP-ribosylated by pertussis toxin rather than the free 41-kDa a subunit produced when G is activated (Neer et al., 1984; Tasi et al., 1984). Treating cells with pertussis toxin caused ADP-ribosylation of Go, in situ, indicating that G was present as the inactive heterotrimer. When C6 or MLTC-1 cells were treated with floroualuminate, there was inhibition of pertussis toxin-catalyzed ADP-ribosylation in situ. We could find no evidence that floroualuminate prevented the toxin from binding to the cells or being activated; therefore, it seems likely that failure of pertussis toxin to ADP-ribosylate a, in the presence of floroualuminate is due to activation and dissociation of Gi subunits. We also found this to be true in membranes in which floroualuminate inhibited the ADP-ribosylation of a by pertussis toxin in a dose-dependent manner.

How is Go affected by floroualuminate in cells and in membranes? As discussed above, there is good evidence that floroualuminate can activate Gi, in membranes, but we found that it was unable to cause cAMP accumulation in cells. We did not observe activation of adenylyl cyclase or inhibition of agonist-stimulated enzyme activity in membranes prepared from floroualuminate-treated cells. This result indicates that the floroualuminate was easily washed away from the affected site during the preparation of membranes. If permeable cells or membranes were treated with 10 mM floroualuminate to activate adenylyl cyclase, the activation persisted, indicating that floroualuminate could not be washed away easily from the affected site in either preparation. To determine if there is a difference in the ability of floroualuminate to interact with Gi, in cells and membranes, we undertook studies with cholera toxin.

When membranes from MLTC-1 cells were treated with cholera toxin in the presence of concentrations of hCG or floroualuminate which stimulate adenylyl cyclase, ADP-ribosylation of Go was increased relative to untreated control membranes. These data suggest that activation of Gi makes it more susceptible to ADP-ribosylation by cholera toxin. When MLTC-1 cells were treated with cholera toxin, Go was ADP-ribosylated in situ. If G was activated by incubating the cells with hCG, in situ ADP-ribosylation of G was often increased, as it was in membranes. In contrast, treating cells with floroualuminate decreased cholera toxin-catalyzed ADP-ribosylation of Go, in situ, and hCG could not prevent the decrease. Floroualuminate did not prevent the activation of cholera toxin to its a subunit, which is the ADP-ribosyltransferase in intact cells (see Kassis et al., 1982). Because floroualuminate had an effect opposite that of hCG on cholera toxin-catalyzed ADP-ribosylation in cells, we believe that floroualuminate does not activate G, in cells but on the contrary blocks activation of Gi and consequently inhibits agonist-stimulated cAMP accumulation by activating Gi.

In membranes and in permeable cells, floroualuminate activated adenylyl cyclase, with the maximum stimulation

**Table III**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ADP-ribosylation</th>
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<tr>
<td>hCG</td>
<td>173 ± 24</td>
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<tr>
<td>Floroualuminate</td>
<td>239 ± 51</td>
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**Effects of hCG and floroualuminate on cholera toxin-catalyzed ADP-ribosylation of G, in MLTC-1 membranes**

MLTC-1 membranes were incubated with no addition, hCG, or floroualuminate and then with activated cholera toxin and [α-32P]NAD in the absence of added GTP. Labeled proteins were determined by SDS-polyacrylamide gel electrophoresis and autoradiography. ADP-ribosylation of proteins corresponding to a, (see Fig. 8) were quantified by scanning densitometry and are reported as a percent of the label incorporated into control samples. Results represent the mean ± S.D. for three independent experiments.

G proteins are heterotrimeric, consisting of unique subunits but having common βγ subunits (Gilman, 1987). The heterotrimer is generally considered to be inactive whereas the dissociated α and βγ subunits are thought to be the active form of the protein. Our approach to investigating the effects of floroualuminate on G proteins was to try to determine when these proteins were in an inactive or active form. In order to do this, we used two bacterial toxins, pertussis toxin and cholera toxin, which transfer the ADP-ribose moiety of NAD to the α subunits of Gi and Gi, respectively. It is the inactive heterotrimer of Gi, that is ADP-ribosylated by pertussis toxin rather than the free 41-kDa α subunit produced when Gi is activated (Neer et al., 1984; Tasi et al., 1984). Treating cells with pertussis toxin caused ADP-ribosylation of Gi, in situ, indicating that Gi was present as the inactive heterotrimer. When C6 or MLTC-1 cells were treated with floroualuminate, there was inhibition of pertussis toxin-catalyzed ADP-ribosylation in situ. We could find no evidence that floroualuminate prevented the toxin from binding to the cells or being activated; therefore, it seems likely that failure of pertussis toxin to ADP-ribosylate α, in the presence of floroualuminate is due to activation and dissociation of Gi subunits. We also found this to be true in membranes in which floroualuminate inhibited the ADP-ribosylation of α by pertussis toxin in a dose-dependent manner.

How is Gi affected by floroualuminate in cells and in membranes? As discussed above, there is good evidence that floroualuminate can activate Gi in membranes, but we found that it was unable to cause cAMP accumulation in cells. We did not observe activation of adenylyl cyclase or inhibition of agonist-stimulated enzyme activity in membranes prepared from floroualuminate-treated cells. This result indicates that the floroualuminate was easily washed away from the affected site during the preparation of membranes. If permeable cells or membranes were treated with 10 mM floroualuminate to activate adenylyl cyclase, the activation persisted, indicating that floroualuminate could not be washed away easily from the affected site in either preparation. To determine if there is a difference in the ability of floroualuminate to interact with Gi, in cells and membranes, we undertook studies with cholera toxin.

When membranes from MLTC-1 cells were treated with cholera toxin in the presence of concentrations of hCG or floroualuminate which stimulate adenylyl cyclase, ADP-ribosylation of α, was increased relative to untreated control membranes. These data suggest that activation of Gi makes it more susceptible to ADP-ribosylation by cholera toxin. When MLTC-1 cells were treated with cholera toxin, α, was ADP-ribosylated in situ. If Gi was activated by incubating the cells with hCG, in situ ADP-ribosylation of Gi was often increased, as it was in membranes. In contrast, treating cells with floroualuminate decreased cholera toxin-catalyzed ADP-ribosylation of Gi, in situ, and hCG could not prevent the decrease. Floroualuminate did not prevent the activation of cholera toxin to its α subunit, which is the ADP-ribosyltransferase in intact cells (see Kassis et al., 1982). Because floroualuminate had an effect opposite that of hCG on cholera toxin-catalyzed ADP-ribosylation in cells, we believe that floroualuminate does not activate Gi, in cells but on the contrary blocks activation of Gi and consequently inhibits agonist-stimulated cAMP accumulation by activating Gi.

In membranes and in permeable cells, floroualuminate activated adenylyl cyclase, with the maximum stimulation

**Table III**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ADP-ribosylation</th>
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<tbody>
<tr>
<td>hCG</td>
<td>173 ± 24</td>
</tr>
<tr>
<td>Floroualuminate</td>
<td>239 ± 51</td>
</tr>
</tbody>
</table>

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occurring at concentrations between 3 and 10 mM. Less activity was observed at lower or higher concentrations, with almost no stimulation above 100 mM fluoroaluminate. We also observed that the agonist-specific stimulation of adenylate cyclase in membranes was inhibited completely when the concentration of fluoroaluminate reached 10 mM. Both the biphasic effect and the inhibitory effect on agonist response have been observed by others (Martin et al., 1980; Katada et al., 1984a). How is it that fluoroaluminate can stimulate adenylate cyclase activity while at the same time inhibiting the agonist-stimulated enzyme in membranes? It seems reasonable to speculate that as the concentration of fluoroaluminate increases, more of the Gs will be activated by fluoroaluminate and less by the hormone receptor complex. Thus, at concentrations of fluoroaluminate which cause maximum adenylate cyclase stimulation, essentially all of the Gs will be fluoroaluminate activated, and there will be no hormone-specific activation. This was the case for both permeable cells and membranes. Why is it then that high concentrations of fluoroaluminate do not activate adenylate cyclase? We have inferred from the ADP-ribosylation data that both Gs and Gi are activated by fluoroaluminate in membranes. Gi usually is present in large excess to Gs (Gilman, 1987). Activation of Gi in membranes increases free αi and βγ. Studies indicate that both free βγ and free αi can cause direct inhibition of adenylate cyclase (Katada et al., 1986). Thus, fluoroaluminate activation of Gi in membranes may produce enough of these peptides to cause inhibition of the enzyme even though Gi is activated by fluoroaluminate.

The proposed model (Gilman, 1987) whereby the common βγ subunits of G proteins play a pivotal role in the regulation of adenylate cyclase activity has been based, to a large extent, on purified preparations of G protein subunits and adenylate cyclase. Our results indicate that this model operates in the intact cell. The present study also shows that pertussis toxin and cholera toxin are useful probes for determining if these G proteins have been activated in cells as well as in membranes. Using these toxins we investigated the effects of fluoroaluminate on G proteins and were able to conclude that fluoroaluminate can activate Gi but not Gs in cells. This explains why fluoroaluminate alone does not stimulate cAMP accumulation in cells but is able to inhibit agonist-stimulated cAMP accumulation. In membranes and in permeable cells, both Gs and Gi appear to be activated by fluoroaluminate. The major unanswered question generated by this study is why both Gs and Gi are sensitive to fluoroaluminate in membrane and permeable cells, but only Gs is sensitive to fluoroaluminate in unperturbed cells.

REFERENCES


Differential activation of the stimulatory and inhibitory guanine nucleotide-binding proteins by fluoroaluminate in cells and in membranes.
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