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NaF and guanine nucleotides modulate adenylate cyclase activity in NG108-15 cells by interacting with both G_s and G_i

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1 NaF (10 mM) produced a 2–3 fold increase in adenylate cyclase activity in homogenates of NG108-15 cells incubated in the presence of 1 μM GTP. Higher concentrations of NaF suppressed adenylate cyclase activity.

2 In the presence of the adenosine receptor agonist 5'-(N-ethyl)-carboxamidoadenosine (NECA; 100 μM) or the prostacyclin receptor agonist iloprost (10 nM), NaF produced a much smaller increase in adenylate cyclase activity, whereas in the presence of a saturating concentration of iloprost (1 μM), NaF only inhibited adenylate cyclase activity.

3 Similarly, Gpp(NH)p activated basal adenylate cyclase activity, and inhibited 1 μM iloprost-activated enzyme activity. In the presence of 10 μM forskolin, NaF or Gpp(NH)p increased adenylate cyclase activity synergistically. Analysis of concentration-effect curves indicated that NaF (2 mM) or Gpp(NH)p (100 μM) increased the potency with which forskolin activated adenylate cyclase, whilst reducing the maximum activation of adenylate cyclase by iloprost.

4 Opiate receptors mediate inhibition of adenylate cyclase, and the opiate agonist morphine (100 μM) reduced the capacity of NaF or Gpp(NH)p to inhibit iloprost-activated adenylate cyclase. Unexpectedly, pertussis toxin treatment enhanced the ability of NaF or Gpp(NH)p to inhibit iloprost-activated adenylate cyclase.

5 In the absence of GTP, NaF and Gpp(NH)p remained able both to activate basal adenylate cyclase and to be synergistic with forskolin in activating the enzyme. In contrast the ability of NaF and Gpp(NH)p to inhibit iloprost-activated adenylate cyclase was substantially lost in the absence of added GTP.

6 These results suggest that NaF modulates adenylate cyclase activity in NG108-15 cell membranes by interacting with the α subunits of both G_s and G_i regulatory proteins. The effects of NaF and Gpp(NH)p are critically dependent on the prior mode and extent of activation or inhibition of this transmembrane signalling pathway. This simple system may be of use in assessing alterations in G_s-G_i interaction following manipulations such as hormone receptor desensitization.

Introduction

An understanding of the mechanisms underlying the regulation of adenylate cyclase (ATP: pyrophosphate-lyase (cyclising); EC 4.6.1.1) activity has been greatly increased by the identification of two guanine nucleotide binding regulatory proteins, or G-proteins, which couple hormone receptors to the adenylate cyclase enzyme (Levitzki, 1987; Casey & Gilman, 1988). These proteins are named G_s and G_i and the former mediates stimulation of adenylate cyclase, while the latter mediates suppression of enzyme activity. Both G_s and G_i are heterotrimers with α, β and γ subunits. $G_{s\alpha}$ has a molecular weight of ~45 kDa and $G_{i\alpha}$ ~40 kDa, although α subunits of other molecular weights have been documented (see Gilman, 1987; Spiegel, 1987; Neer & Clapham, 1988). In the presence of a stimulatory agonist, the binding of guanosine 5'-triphosphate (GTP) to $G_{s\alpha}$ leads to its dissociation from $G_{s\beta\gamma}$. The $G_{s\alpha}$·GTP complex then binds to, and activates the adenylate cyclase enzyme. The GTP bound to $G_{s\alpha}$ is then hydrolysed to GDP, and $G_{s\alpha}$ and $G_{s\beta\gamma}$ reassociate to terminate the activation of adenylate cyclase. For adenylate cyclase inhibition, the binding of GTP to $G_{i\alpha}$ again leads to subunit dissociation, but what then follows is less clear. Either $G_{i\alpha}$ inhibits adenylate cyclase directly, or the βγ subunit liberated from G_i binds to $G_{s\alpha}$ and reduces its ability to activate adenylate cyclase. Both processes may occur simultaneously, and at the present time the exact mechanisms are the subject of some controversy (see Birnbaumer, 1987).

Apart from receptor-mediated hormonal activation of adenylate cyclase, a number of non-hormonal agents are able to activate this enzyme. Forskolin can activate directly the catalytic subunit of adenylate cyclase (Seamon & Daly, 1986), while the fluoroaluminate ion AlF_4^- and GTP analogues such as Gpp(NH)p (5'-guanylimidodiphosphate) interact with G-proteins directly, which then leads to effector modulation (Londos *et al.*, 1974; Howlett *et al.*, 1979; Ross & Gilman, 1980; Sternweis *et al.*, 1981). The fluoroaluminate ion (normally formed by combination of fluoride ions from NaF with trace quantities of aluminium ions present in buffers) is thought to activate G-proteins by mimicking the γ-phosphate of GTP when GDP is bound to the α-subunit of the G-protein (Bigay *et al.*, 1985), but this remains to be proven for G-proteins other than transducin (see Chabre, 1989; Stadel & Crooke, 1989). Nevertheless, NaF has been very useful in detecting changes in adenylate cyclase function, such as those that may occur following heterologous desensitization of this effector system (Clark, 1986). In various tissues, heterologous desensitization of adenylate cyclase is commonly seen as a reduction in NaF-activated adenylate cyclase activity following prolonged hormone treatment (for reviews see Harden, 1983 and Clark, 1986). In some cases this may be due to an agonist-induced functional loss of $G_{s\alpha}$ protein from the cell membrane, apparently confirmed by a reduction in the cholera toxin-induced [^{32}P]-ADP-ribosylation of $G_{s\alpha}$ observed under these conditions (Garrity *et al.*, 1983;

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Edwards et al., 1987; Kelly et al., 1990). However, it should be noted that other mechanisms may underlie some forms of heterologous desensitization (Rich et al., 1984). It is known that NaF will activate all G-proteins and not just G_s, implying that changes in NaF-activated adenylate cyclase activity may not simply reflect changes in G_s function (Katada et al., 1984a,b). Furthermore, we have shown in a number of tissues, including the NG108-15 hybrid cell line (Kelly et al., 1990) and human platelets (Edwards et al., 1987), that NaF produces a bell-shaped concentration-effect curve for adenylate cyclase activation, stimulating at lower concentrations, and inhibiting enzyme activity at higher concentrations. In the present study, we have investigated the effects of NaF and guanine nucleotides on adenylate cyclase activity in homogenates of NG108-15 cells under different conditions, in an attempt to determine whether the effects of these agents on adenylate cyclase were due to activation of different G-proteins. Our results indicate that Gpp(NH)p and NaF both activate via G_s and inhibit via G_i. In addition NaF appears to have an inhibitory effect on adenylate cyclase that is unrelated to G-protein activity.

Methods

Cell culture

Cells of the NG108-15 and NCB-20 neuroblastoma somatic hybrid cell lines were both derived by fusions of the 6-thioguanine-resistant clone of a mouse neuroblastoma (N18TG2). Fusions were made with (a) C6BU-1, a 5-bromodeoxyuridine-resistant clone of rat glioma to yield NG108-15, or (b) foetal Chinese hamster brain cells to yield NCB-20 (further details and references to these cells are given in MacDermot et al., 1979). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal calf serum, and supplemented with 1 μM aminopterin, 100 μM hypoxanthine and 16 μM thymidine. Culture flasks (80 cm^2) were maintained at 37°C in a humidified atmosphere of 10% CO_2 and 90% air. Cells were harvested by agitation in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline, and the resultant pellets frozen at −80°C until required.

Adenylate cyclase

Adenylate cyclase activity was measured as described previously (Salomon et al., 1974) with some modifications (Edwards et al., 1987). Cell pellets were thawed and disrupted in a glass Dounce homogeniser using a homogenisation buffer (25 mM Tris-HCl, 0.29 M sucrose, pH 7.4). Reaction mixtures of 100 μl contained 50 mM Tris-HCl pH 7.4, 5 mM magnesium chloride, 20 mM creatine phosphate disodium salt, 10 iu of creatine kinase, 1 mM adenosine 3':5'-cyclic monophosphate (cyclic AMP) sodium salt, 0.25 mM Ro20-1724 as a phosphodiesterase inhibitor, 1 mM [α-^{32}P]-ATP (2 μCi), 1 μM GTP and 200–400 μg of membrane protein. Reactions were incubated at 37°C for 15 min and then terminated by the addition of 800 μl of 6.25% (w/v) trichloroacetic acid. To each tube was added 100 μl of [8-^{3}H]-cyclic AMP (about 20,000 c.p.m.) and the reaction mixtures centrifuged at 4°C for 20 min at 800 g. The [^{32}P]-ATP and [^{32}P]-cyclic AMP were separated by a two step chromatographic procedure (Salomon et al., 1974) and the yield of [^{32}P]-cyclic AMP was corrected for losses on the columns by measurement of the recovery of [^{3}H]-cyclic AMP. Unless otherwise stated, each measurement within an assay was performed in duplicate.

Where applicable, the dose-response data for adenylate cyclase assays were analysed according to a logistic equation describing a single site and giving V_{max}, EC_{50} and n, the Hill coefficient. Statistical significance, where applicable, was determined by two-way analysis of variance or paired t tests. All experiments were performed on at least two separate occasions.

Materials

[8-^{3}H]-adenosine 3':5'-cyclic monophosphate (23.6 Ci $mmol^{-1}$) and [α-^{32}P]-ATP (40–50 Ci $mmol^{-1}$) were obtained from Amersham International. Ro20-1724 (4-(3-butoxy-4-methoxybenzyl)- 2-imidazolidinone) was a kind gift from Roche Products, and iloprost a generous gift from Schering AG Berlin. All other chemicals and drugs were obtained from Sigma Chemical Co. Ltd. or BDH Chemicals Ltd.

Results

Effect of NaF on adenylate cyclase activity

Increasing concentrations of NaF had a biphasic effect on adenylate cyclase activity in homogenates of NG108-15 cells incubated in the presence of 1 μM GTP (Figure 1). Lower concentrations of NaF (1–10 mM) activated adenylate cyclase with a maximum 2–3 fold increase in activity, whilst higher concentrations suppressed adenylate cyclase activity with a near complete abolition of activity at 100 mM NaF. The effect of NaF on hormone receptor-activated adenylate cyclase was then investigated. The prostacyclin receptor agonist iloprost and the adenosine receptor agonist 5'(N-ethyl)-carboxamidoadenosine (NECA) both activate adenylate cyclase activity in NG108-15 cells (Kelly et al., 1990). Saturating concentrations of these agonists (100 μM NECA and 1 μM iloprost) produce around 2–4 fold and 5–10 fold increases in adenylate cyclase activity, respectively (Figure 1a). In the presence of 100 μM NECA, NaF produced a smaller increase in adenylate cyclase activity than observed with NaF alone. Furthermore, in the presence of 1 μM iloprost, NaF only inhibited adenylate cyclase activity, albeit with a 'shoulder' in the inhibition curve (Figure 1a). Figure 1b shows the effect of NaF in the presence of selected concentrations of iloprost. In contrast to the NaF-dependent inhibition of adenylate cyclase in the presence of a saturating concentration of iloprost, in the presence of a low (10 nM) concentration of iloprost, NaF produced only a small increase in enzyme activity. In all cases, very high concentrations of NaF inhibited adenylate cyclase activity below basal levels. The data in Figure 1a were submitted to 2-way analysis of variance, which showed that the three

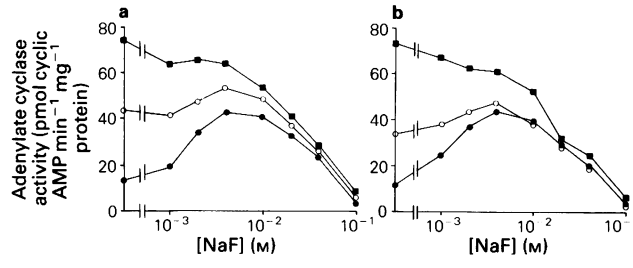

Figure 1 Effect of increasing concentrations of NaF on adenylate cyclase activation in NG108-15 cell homogenates under different conditions of enzyme activation. (a) NaF was added alone (●), in the presence of 100 μM 5'-(N-ethyl)- carboxamidoadenosine (○), or in the presence of 1 μM iloprost (■). (b) NaF was added alone (●), or in the presence of 10 nM (○) or 10 μM (■) iloprost. GTP (1 μM) was present throughout. The full experiment was repeated on one further occasion with the same result. In six separate experiments, enzyme activity measured in the presence of 1 μM iloprost but in the absence of NaF was 75.2 ± 5.4 pmol cyclic AMP $min^{-1} mg^{-1}$ protein (mean ± s.e.mean). This result was significantly different ($P < 0.05$, paired t test) from activities measured in the presence of NaF at the following concentrations: 1 mM NaF (66.8 ± 6.2), 2 mM NaF (63.8 ± 5.6), 4 mM NaF (62.3 ± 5.9), 10 mM NaF (50.8 ± 5.1) and 20 mM NaF (37.0 ± 4.2). It should be noted that activities at 1 and 2 mM NaF were not significantly different from each other, suggesting a complex or biphasic inhibitory curve.

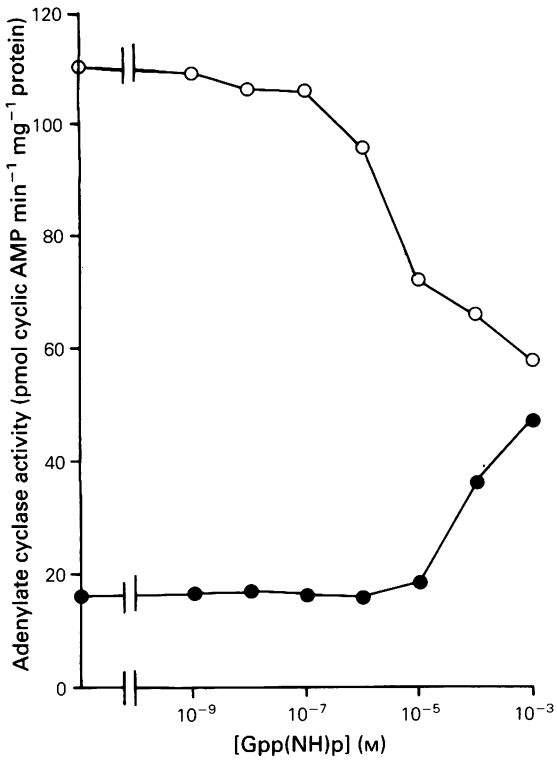

Figure 2 Effect of increasing concentrations of Gpp(NH)p on basal (●) or 1 μM iloprost (○)-activated adenylate cyclase activity in an NG108-15 cell homogenate. GTP (1 μM) was present throughout. This experiment was repeated on one further occasion with similar results.

curves were significantly different from each other ($P < 0.05$). Details of the t tests relating to individual points from six separate experiments are given in the figure legend.

Effect of Gpp(NH)p on adenylate cyclase activity

Gpp(NH)p produced a concentration-dependent increase in basal activity, and inhibited adenylate cyclase activity in the presence of 1 μM iloprost (Figure 2). The results in Figure 2 were analysed according to a logistic equation describing a single site. The EC_{50} for Gpp(NH)p activation of basal adenylate cyclase was 67.4 ± 7.6 μM, and the IC_{50} for Gpp(NH)p inhibition of iloprost-stimulated enzyme activity was 3.1 ± 1.1 μM (means ± s.e. of calculated parameters). A similar estimation for NaF was difficult due to the 'shoulder' observed for inhibition of iloprost-activated adenylate cyclase (Figure 1).

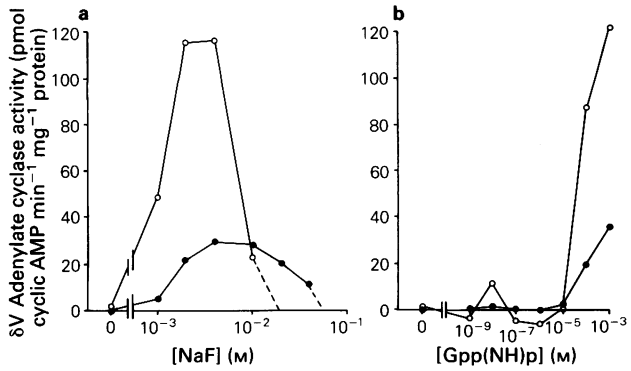

Figure 3 Effect of increasing concentrations of (a) NaF or (b) Gpp(NH)p on basal (●) or 10 μM forskolin (○)-activated adenylate cyclase activity in NG108-15 cell homogenates. Enzyme activation is represented as the increase in adenylate cyclase activity over levels in the absence of NaF or Gpp(NH)p (δV). In experiment (a), adenylate cyclase activity in the absence of NaF or Gpp(NH)p was 13.4 and 243.6 pmol cyclic AMP min^{-1} mg^{-1} protein under basal or 10 μM forskolin-activated conditions respectively. The dashed lines indicate where δV had become negative at high concentrations of NaF. These are not shown in full for clarity. In experiment (b), activity in the absence of NaF or Gpp(NH)p was 16.4 and 310.1 pmol cyclic AMP min^{-1} mg^{-1} protein under basal or 10 μM forskolin-activated conditions. GTP (1 μM) was present throughout. This experiment was repeated on one further occasion with similar results.

Effect of NaF and Gpp(NH)p on forskolin- and iloprost-stimulated adenylate cyclase activity

The diterpene compound forskolin (10 μM) increased adenylate cyclase activity by around 20 fold. In the presence of 10 μM forskolin, the increase in adenylate cyclase activity due to NaF or Gpp(NH)p was much greater than that observed in the absence of forskolin (Figure 3). In other words, forskolin and NaF or Gpp(NH)p activated adenylate cyclase synergistically. However, as observed previously, high concentrations of NaF virtually abolished forskolin-activated adenylate cyclase activity. In further experiments, the effect of single concentrations of NaF and Gpp(NH)p on the concentration-effect curves for adenylate cyclase activation by forskolin and iloprost were examined (Figure 4). The concentrations of NaF (2 mM) and Gpp(NH)p (100 μM) were chosen because these concentrations are able to activate or inhibit adenylate cyclase under different conditions (see Figures 1 and 2). These experiments revealed the contrasting effects of NaF or Gpp(NH)p on adenylate cyclase activation by forskolin and iloprost. Thus 2 mM NaF or 100 μM Gpp(NH)p appeared to enhance the potency with which forskolin activated adenylate cyclase, but markedly inhibited the maximum increase in iloprost-stimulated activity without changing iloprost potency (Figure 4). Also NaF, but not Gpp(NH)p, inhibited the response to high concentrations of forskolin (Figure 4a). The Hill coefficients for forskolin activation of adenylate cyclase activity were less than unity, consistent with the known effects of forskolin in this system, which involves interaction with both G_s and the adenylate cyclase molecule.

In order to assess whether NaF exerted a non-specific ionic action on the adenylate cyclase system, the effects of NaCl on basal, iloprost, and forskolin-activated adenylate cyclase were investigated. In each case, NaCl did not mimic the effects of NaF, although high concentrations of the salt slightly increased enzyme activity under the three different conditions (data not shown).

Inhibition of adenylate cyclase activity by NaF and Gpp(NH)p in the presence of morphine

Activation of opiate receptors inhibits enzyme activity in NG108-15 cells by 30–40% (Kelly *et al.*, 1990; also Figure 5). In the presence of iloprost and morphine, the inhibitory effects of NaF, or Gpp(NH)p were not additive (Figure 5), suggesting that morphine, NaF and Gpp(NH)p utilise at least partially the same mechanism to effect adenylate cyclase inhibition. It should be noted, however, that morphine did not prevent the inhibition of adenylate cyclase by high concentrations of NaF (Figure 5a). To investigate further the interaction of morphine and NaF, we examined the ability of a single concentration of morphine (100 μM) to inhibit adenylate cyclase activity due to increasing concentrations of iloprost or NaF. The results (Figure 6) indicated that morphine-dependent inhibition of iloprost-activated adenylate cyclase is constant irrespective of iloprost concentration, whereas morphine-dependent inhibition of NaF-activated adenylate cyclase decreases with increasing NaF concentration. We also found that morphine-dependent inhibition of Gpp(NH)p-activated adenylate cyclase decreases with increasing Gpp(NH)p concentration (data not shown). These results support the

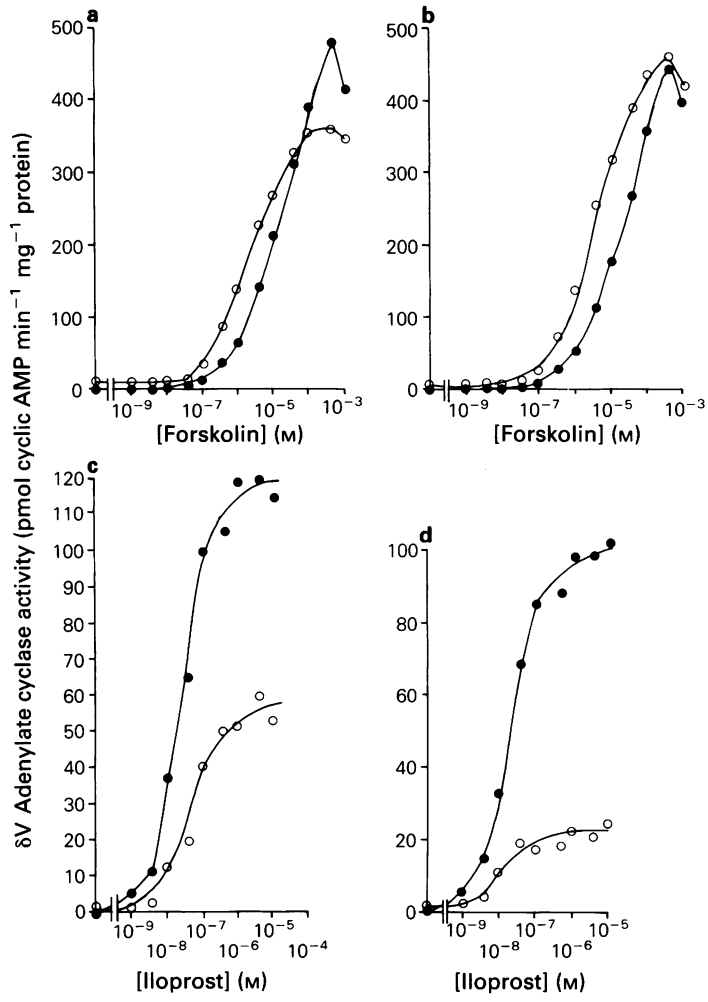

Figure 4 Effect of increasing concentrations of forskolin (a and b) or iloprost (c and d) on adenylate cyclase activity in homogenates of NG108-15 cells measured in the absence (●) or presence (○) of 2 mM NaF (a and c), or in the absence (●) or presence (○) of 100 μM Gpp(NH)p (b and d). GTP (1 μM) was present throughout. Enzyme activation is represented as the increase in adenylate cyclase activity over levels in the presence or absence of NaF or Gpp(NH)p alone (δV). In general 2 mM NaF or 100 μM Gpp(NH)p alone produced a 2–4 fold increase in basal adenylate cyclase activity. Curve parameters for this experiment were estimated as follows: (a) forskolin $EC_{50} = 13 \pm 3$ μM and n (Hill coefficient) $= 0.74 \pm 0.09$, forskolin + NaF $EC_{50} = 2 \pm 1$ μM and $n = 0.75 \pm 0.03$; (b) forskolin $EC_{50} = 18 \pm 4$ μM and $n = 0.73 \pm 0.08$, forskolin + Gpp(NH)p $EC_{50} = 3 \pm 1$ μM and $n = 0.79 \pm 0.06$; (c) iloprost $EC_{50} = 27 \pm 4$ nM and $n = 1.02 \pm 0.13$, iloprost + NaF $EC_{50} = 52 \pm 11$ nM and $n = 1.03 \pm 0.19$; (d) iloprost $EC_{50} = 19 \pm 2$ nM and $n = 1.06 \pm 0.10$, iloprost + Gpp(NH)p $EC_{50} = 11 \pm 4$ nM and $n = 1.00 \pm 0.31$ (all values are means ± s.e. of parameters calculated). The data in (c) and (d) were also analysed without prior subtraction of 'basal' enzyme activity. In these circumstances, the maximum enzyme activity in the presence of saturating iloprost concentrations was still reduced as shown. In (c), maximum enzyme activity was 134 ± 10 and 103 ± 5 pmol cyclic AMP $min^{-1} mg^{-1}$ protein in the absence or presence respectively of 2 mM NaF. In (d), maximum enzyme activity was 115 ± 6 and 65 ± 3 pmol cyclic AMP $min^{-1} mg^{-1}$ protein in the absence or presence of 100 μM Gpp(NH)p. Similar values were obtained in a repeat experiment.

suggestion that NaF and morphine activate a common inhibitory pathway in the regulation of adenylate cyclase activity.

Effects of pertussis toxin

The effects of pertussis toxin treatment of the NaF- and Gpp(NH)p-mediated inhibition of iloprost-activated adenylate cyclase were examined. These results are displayed both as absolute inhibition and % inhibition of iloprost activation (Figure 7). First, pertussis toxin treatment of NG108-15 cells (50 ng ml^{-1} medium; 17 h) markedly enhanced iloprost-activated adenylate cyclase by up to 3 fold (basal enzyme activity in the presence of 1 μM GTP was similarly increased). Secondly, pertussis toxin treatment magnified the inhibitory capacity of NaF and Gpp(NH)p, as seen both with absolute levels of adenylate cyclase activity and with % inhibition of iloprost-stimulated adenylate cyclase activity (Figure 7). The kinetics of this effect were also examined in homogenates of control and pertussis toxin-treated cells. The accumulation of cyclic AMP was linear in the presence of iloprost (1 μM) between 0 and 15 min, in both control and toxin-treated cells (Figure 8). The rate of increase was much greater following toxin treatment. Addition of 100 μM Gpp(NH)p inhibited iloprost-activated adenylate cyclase to a much greater extent in toxin-treated cells. There was also a lag observed before the onset of Gpp(NH)p-mediated inhibition of about 2 min in control cells, which was apparently unchanged following pertussis toxin treatment (Figure 8).

Finally, we examined the effect of NaF and Gpp(NH)p on adenylate cyclase activity in NG108-15 membranes in the absence of GTP (Figure 9). NaF and Gpp(NH)p increased basal and forskolin-activated enzyme activity irrespective of whether GTP was present or not (compare Figure 9 with Figures 1 and 3). Conversely, Gpp(NH)p-mediated inhibition of adenylate cyclase activity in the presence of 1 μM iloprost was virtually abolished in the absence of GTP, as was inhibition of this activity by low concentrations of NaF (up to

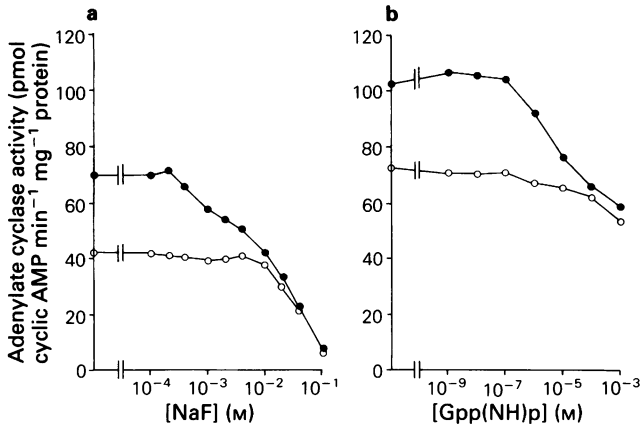

Figure 5 Effect of increasing concentrations of (a) NaF or (b) Gpp(NH)p on either 1 μM iloprost-activated adenylate cyclase activity (●) or 1 μM iloprost plus 100 μM morphine-activated adenylate cyclase activity (○) in homogenates of NG108-15 cells. GTP (1 μM) was present throughout. This experiment was repeated on one further occasion with similar results.

10 mM). However, very high concentrations of NaF were still unable to inhibit adenylate cyclase activity under these conditions.

Discussion

In NG108-15 cell homogenates, NaF produced a bell-shaped concentration-effect curve with relation to adenylate cyclase activity. This characteristic response was modified when stimulatory hormone receptors were co-activated, and in a manner dependent upon the extent of hormonal activation. In the presence of saturating concentrations of NECA or iloprost, which produced about 3 fold and 7 fold increases in adenylate cyclase activity, NaF now produced either a much smaller increase in, or inhibition of enzyme activity respectively. However, these divergent responses observed with different agonists were not due to a qualitative difference in

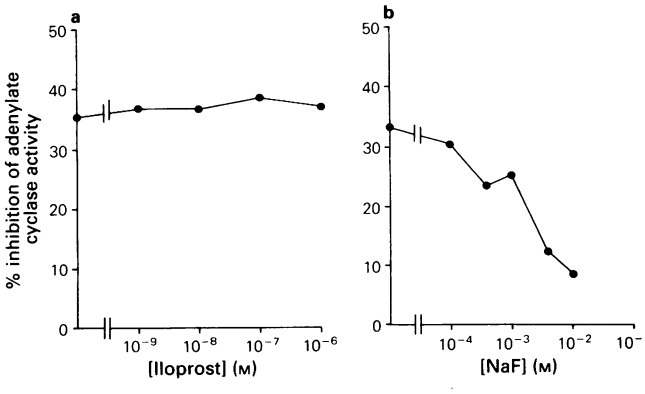

Figure 6 The inhibitory effect of 100 μM morphine on adenylate cyclase activity activated by inreasing concentrations of (a) iloprost or (b) NaF. The effect of morphine is represented as % inhibition of total adenylate cyclase activity in the presence of a particular concentration of iloprost or NaF. GTP (1 μM) was present throughout. In (a), actual enzyme activities due to selected iloprost concentrations in the absence or presence of 100 μM morphine respectively were as follows: no iloprost (basal) 23.9 and 15.3, 1 nM iloprost 27.4 and 17.2, 10 nM iloprost 50.4 and 31.5, 100 nM iloprost 132.9 and 81.8, 1 μM iloprost 163.3 and 102.7 pmol cyclic AMP min^{-1} mg^{-1} protein. Corresponding values for (b) in the presence of increasing NaF concentrations were: no NaF (basal) 12.8 and 8.6, 0.1 mM NaF 12.2 and 8.5, 0.4 mM NaF 12.4 and 9.5, 1 mM NaF 19.1 and 14.4, 4 mM NaF 45.0 and 39.5, and 10 mM NaF 42.9 and 38.1 pmol cyclic AMP min^{-1} mg^{-1} protein. This experiment was repeated with similar results.

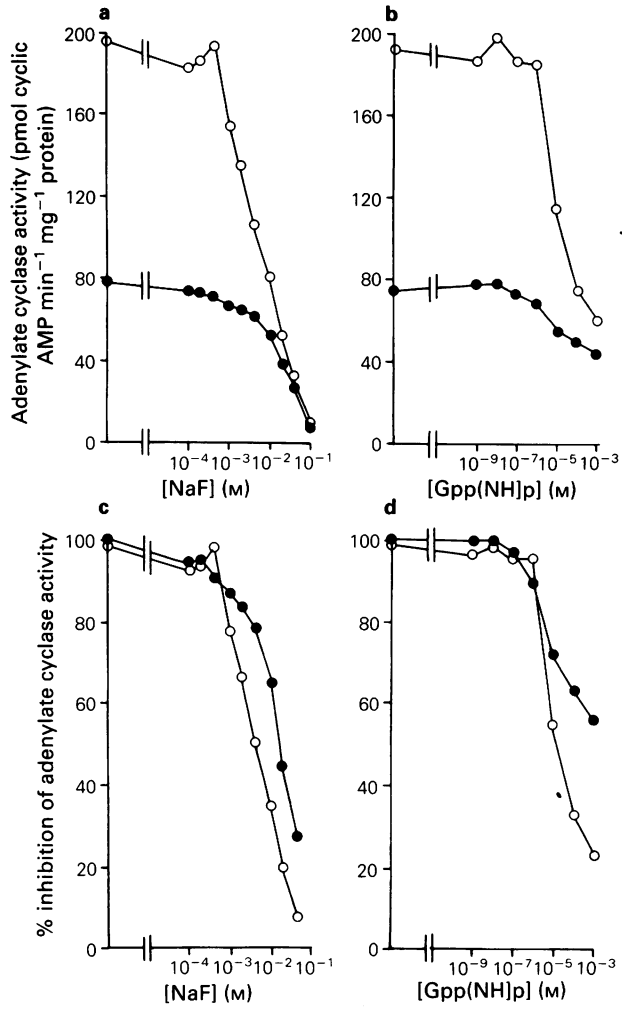

Figure 7 Effect of increasing concentrations of NaF (a and c) or Gpp(NH)p (b and d) on 1 μM iloprost-activated adenylate cyclase activity in homogenates of NG108-15 cells that had been preincubated in the absence (●) or presence (○) of pertussis toxin (50 ng ml^{-1}) for 17 h. GTP 1 μM was present throughout. In (a) and (b) absolute levels of adenylate cyclase activity are shown whereas (b) and (d) show % inhibition of activity in the presence of iloprost, using data transformed from (a) and (b). This experiment was repeated with similar results.

receptor type, but rather to a quantitative difference in the extent of adenylate cyclase activation, since in the presence of a low concentration of iloprost, having an equivalent effect on enzyme activity as the saturating concentration of NECA, an equivalent small increase in adenylate cyclase activity was observed when NaF was included. This indicates that NaF and hormonal activation of adenylate cyclase are not additive, and apparently utilise the same G_s-adenylate cyclase pool in the membrane. When hormonal activation produced high levels of adenylate cyclase activity, as with saturating iloprost concentrations, NaF inhibited enzyme activity. Such an inhibitory effect has been detected in other tissues (Manganiello & Vaughan, 1976; Katada *et al.*, 1984a,b). If NaF is modulating adenylate cyclase by interacting with both G_s and G_i, then under conditions where G_s activity is already high, the more pronounced effect of NaF would be G_i activation with consequent enzyme inhibition. Clearly, this interpretation does not exclude the possibility that the inhibitory effect of NaF might be mediated by protein(s) other than G_i. NaF-dependent inhibition of adenylate cyclase is not observed under basal conditions since NaF would also be interacting with a large and relatively inactive pool of G_s, and consequently stimulation is observed. Interestingly, the inhibition of adenylate cyclase by NaF appeared to be biphasic (Figure 1). This could indicate

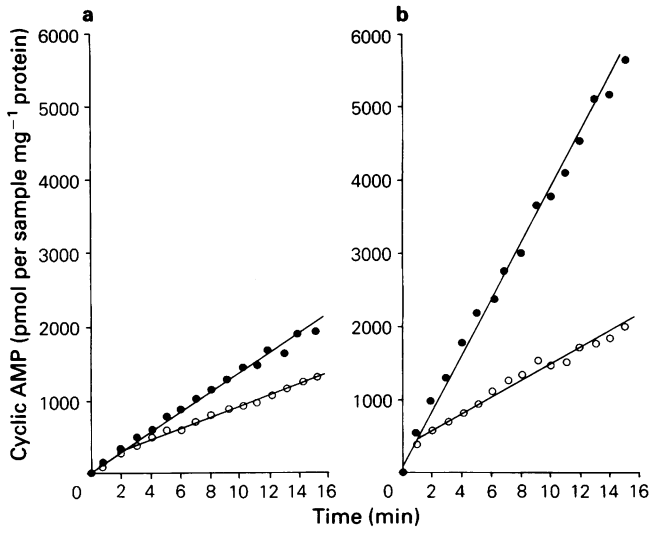

Figure 8 Time course of 1 μM iloprost-activated increase in [^{32}P]-cyclic AMP formation in (a) control and (b) pertussis toxin-treated NG108-15 cells. Incubations were performed in the absence (●) or presence (○) of 100 μM Gpp(NH)p. Each point represents a single determination and 1 μM GTP was present throughout. Two further repeats of this experiment produced similar results.

the involvement of two different mechanisms, or more simply that even in the presence of a saturating concentration of iloprost, NaF remains able to interact with a small fraction of G_s that has remained inactive, thus explaining the apparent 'shoulder' in the inhibition curve.

NaF probably interacts with G-proteins by mimicking the γ-phosphate of GTP when GDP is bound to the α-subunit (Bigay *et al.*, 1985). Poorly hydrolysed guanine nucleotides such as Gpp(NH)p also interact with the α-subunits of G-proteins in an essentially irreversible manner and lead to subunit dissociation (Gilman, 1987). Therefore we postulated

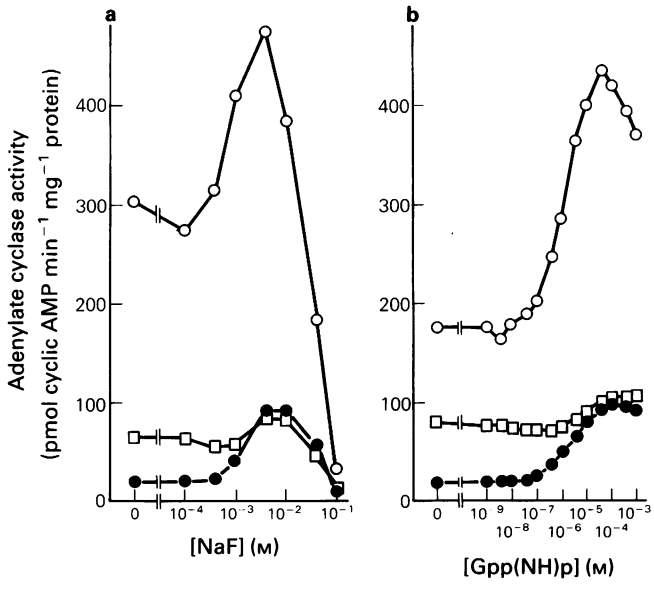

Figure 9 Effect of increasing concentrations of NaF (a) or Gpp(NH)p (b) on basal (●), 1 μM iloprost-activated (□) or 10 μM forskolin-activated (○) adenylate cyclase activity in NG108-15 cell membranes incubated in the absence of GTP. To remove endogenous GTP, membranes were washed three times by spinning at 80,000 *g* for 20 min and resuspended in 50 mM Tris-HCl pH 7.4. This experiment was repeated with the same result. Reference to Figures 1 and 2 indicates the differing effects of NaF and Gpp(NH)p on iloprost-activated enzyme activity in the presence (Figures 1 and 2) or absence (Figure 9) of GTP.

that if both NaF and Gpp(NH)p activated G-proteins by interacting with the α-subunits, then they should have the same effect on adenylate cyclase activity in cell membranes. This was largely confirmed by our finding that Gpp(NH)p activated basal and inhibited iloprost-activated adenylate cyclase, indicating the likelihood that NaF, like Gpp(NH)p, can activate G_s and G_i in this system. However, some differences were apparent in the characteristics of adenylate cyclase modulation by these two agents. Firstly, Gpp(NH)p did not produce a bell-shaped concentration-effect curve for adenylate cyclase activation. This could be because high enough concentrations of the GTP analogue were not employed, or more probably that the inhibition of adenylate cyclase seen at high NaF concentrations is unrelated to G_i function. Secondly, inhibition of adenylate cyclase by Gpp(NH)p occurred at concentrations around 10 fold lower than activation, and no 'shoulder' was observed in the inhibition curve. This latter may again relate to factors other than G-protein activation, whilst the former may reflect the different affinities of G_s and G_i for guanine nucleotide (Jakobs *et al.*, 1985). Interestingly, no obvious difference was observed in the concentrations of NaF that activated or inhibited adenylate cyclase (see Figure 1). However, if the fluoraluminate ion mimics the γ-phosphate of GTP, then its interaction with $G_{s\alpha}$ and $G_{i\alpha}$ would depend upon GDP which is already bound, thus masking any differences in the relative affinities of these proteins for guanine nucleotides.

The interaction of NaF and Gpp(NH)p with forskolin-activated adenylate cyclase indicated a synergistic interaction between these non-hormonal agents. Forskolin is a direct activator of the adenylate cyclase enzyme, but also potentiates hormonal activation of the enzyme by a mechanism involving the G_s protein (Seamon & Daly, 1986). In a number of tissues, Gpp(NH)p inhibits forskolin-activated adenylate cyclase activity (Seamon & Daly, 1982; Jakobs *et al.*, 1983). However, in NG108-15 cell membranes, both NaF and Gpp(NH)p were clearly synergistic with forskolin in activating adenylate cyclase. This was confirmed by constructing full concentration-effect curves for forskolin-activation of adenylate cyclase in the presence or absence of NaF or Gpp(NH)p, revealing that both agents enhanced the potency of forskolin. This effect contrasted markedly with the interaction between NaF or Gpp(NH)p and the iloprost concentration-effect curve. Both NaF and Gpp(NH)p reduced the maximal activation by iloprost, whilst having no apparent effect on the potency of iloprost. These differences may relate to the different sites of action of iloprost and forskolin, that is, hormone receptor and G_s-adenylate cyclase. It is of interest that NaF and Gpp(NH)p increase high affinity [^{3}H]-forskolin binding in rat brain membranes (Seamon & Daly, 1985), thus providing a possible explanation for the functional synergism we have observed. NaF and Gpp(NH)p were synergistic with forskolin, but inhibitory with saturating iloprost concentrations. This is of interest, since both iloprost and forskolin increase adenylate cyclase activity. The most straightforward rationalization of this result would be that iloprost activates the enzyme by promoting the coupling of $G_{s\alpha}$ to adenylate cyclase, whereas forskolin, in the absence of hormone, probably activates adenylate cyclase almost exclusively by a direct interaction with the enzyme, thus leaving open the possibility for activation of G_s by NaF or Gpp(NH)p.

In further attempts to confirm the specificity of action of NaF and Gpp(NH)p on G-proteins, we reasoned that in the presence of an inhibitory receptor agonist such as morphine, the extent of NaF- and Gpp(NH)p-mediated inhibition of adenylate cyclase activity should be attenuated, if all are ultimately utilising the G_i protein. This was found to be the case, suggesting that morphine and the two non-hormonal agents do indeed inhibit adenylate cyclase by a common mechanism involving G_i. Interestingly, morphine did not prevent the inhibition seen at high NaF concentrations (above 10 mM), indicating again the likelihood that this phase of inhibition relates neither to G_i nor to a non-specific ionic effect. The involve-

ment of G_i in NaF-mediated inhibition was further investigated by examining the % inhibition of adenylate cyclase by morphine under conditions of increasing NaF concentration. This revealed a reciprocal relationship between NaF concentration and adenylate cyclase inhibition by morphine, again implicating a common G_i pathway. In contrast to this, the % inhibition of adenylate cyclase by morphine in the presence of increasing iloprost concentrations was constant, which might be expected since iloprost activates the G_s and not the G_i pathway.

Pertussis toxin blocks receptor- and GTP-mediated inhibition of adenylate cyclase activity by ADP-ribosylating the $G_{i\alpha}$ protein (Ui, 1984). If NaF and Gpp(NH)p are inhibiting adenylate cyclase via G_i, then pertussis toxin might be expected to alter their ability to do so by covalently modifying $G_{i\alpha}$. Pertussis toxin treatment markedly increased basal and iloprost-activated adenylate cyclase activity in NG108-15 cell homogenates, consistent with a functional loss of G_i activity (Katada *et al.*, 1982). However, unexpectedly, pertussis toxin treatment greatly enhanced the capacity of NaF and Gpp(NH)p to inhibit iloprost-activated adenylate cyclase activity. It is not easy to assess how covalent modification of $G_{i\alpha}$, which blocks receptor-mediated inhibition of adenylate cyclase by over 70% (data not shown), could lead to an increase in the inhibitory capacity of a non-hydrolysable GTP analogue. A similar effect has been observed previously in C6 glioma cells (Katada *et al.*, 1982). However, the change may relate to the functional loss of GTP activity at the ADP-ribosylated G-protein rather than a real increase in Gpp(NH)p activity. In other systems, pertussis toxin treatment blocks receptor- and GTP-mediated inhibition of adenylate cyclase, but inhibition by non-hydrolysable GTP analogues remains intact (Jakobs *et al.*, 1983). In our control membranes GTP tonically activates G_i, but is unable to do so after toxin treatment, which leads to enhanced basal and iloprost-activated adenylate cyclase activity. Since Gpp(NH)p appears to activate G_i irrespective of whether or not the α-subunit is ADP-ribosylated, the greater inhibition mediated by Gpp(NH)p after pertussis toxin (Figure 7b and d) probably only reflects that the GTP in the assay system is unable to do so. These results suggest that in the whole cell, where $G_{i\alpha}$ has access to GTP, G_i may tonically inhibit adenylate cyclase. In these circumstances, the significance of relatively small receptor-mediated inhibition of adenylate cyclase exerted by some hormones is not easily explained. Although in some other tissues, pertussis toxin pretreatment does not prevent adenylate cyclase inhibition by stable GTP analogues, it does markedly increase the lag phase before the onset of inhibition (Jakobs *et al.*, 1983). However, we were unable to observe any increase in the lag phase before the onset of Gpp(NH)p-mediated inhibition in the presence of pertussis toxin; both before and after toxin, the lag was around 2 min. This indicates the likelihood of differences in the kinetics of G-protein activation between different tissues.

Finally, the effect of GTP itself on the modulation of adenylate cyclase by NaF and Gpp(NH)p was examined. These agents activated adenylate cyclase under basal conditions or in the presence of forskolin irrespective of whether GTP was present or not. Inspection of Figure 9 shows that Gpp(NH)p and low concentrations of NaF have little or no capacity to inhibit adenylate cyclase activity in the absence of GTP. A possible explanation is that the activity of G_i is being masked by the greater effect of G_s. In the presence of iloprost and GTP, NaF or Gpp(NH)p have little further effect on G_s-stimulation of adenylate cyclase activity and, consequently, inhibition is observed.

In conclusion, we have demonstrated that NaF and Gpp(NH)p have multiple effects on adenylate cyclase activity in NG108-15 homogenates. Both agents can activate or inhibit enzyme activity depending upon the assay conditions employed, and these effects appear to relate to interactions with G_s and G_i respectively. A further inhibition of adenylate cyclase activity occurred with concentrations of NaF greater than 10 mM, but this is most probably unrelated to G_i activity. In a situation where NaF or GTP analogues are employed to assess the integrity of a G-protein-effector system, caution should be observed since these agents will in fact reflect the integrity of all G-proteins functionally linked to a particular effector. For instance, a reduction in NaF- or Gpp(NH)p-activated adenylate cyclase activity in a tissue following prolonged agonist treatment may reflect a functional loss of G_s, or a functional increase in G_i (Rich *et al.*, 1984), or both together. Under carefully defined conditions, the use of non-hormonal G-protein activators could be important in assessing G_i function more directly than by using hormone agonists, or to investigate possible 'cross-talk' between G-proteins reciprocally coupled to a common effector. Furthermore, these agents could be used to detect novel G-proteins that inhibit a particular effector, as appears to be the case for phospholipase C (Godfrey & Watson, 1988; Whitworth & Kendall, 1989). Apart from this, NaF and GTP analogues will remain vital tools in any attempt to understand the regulation of G-proteins coupled processes.

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