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Dual regulation of neutrophil adenylate cyclase by fluoride and its relationship to cellular activation

Maan Saad, Colette F. Strnad & [1]Kenneth Wong

Departments of Pharmacology and Medicine, University of Alberta, Edmonton, Alberta, T6G 2G3, Canada

1 Fluoride stimulated (1–10 mM) and inhibited (10–100 mM) adenylate cyclase of neutrophil membranes in a GTP-independent manner. The latter fluoride concentration range corresponded to that shown previously to induce cellular responses.

2 Dual regulation of cyclase activity was also exhibited by a nonhydrolysable GTP analogue, guanosine 5'[γ-thio]triphosphate (GTPγS). Inhibition was observed at 0.1–10 nM GTPγS while stimulation occurred at > 10 nM GTPγS.

3 Relatively high levels (> μM) of formylmethionyl-leucyl-phenylalanine inhibited adenylate cyclase in the presence of GTP (10 μM).

4 *Pertussis* toxin pretreatment abolished adenylate cyclase inhibition mediated by GTPγS and formylmethionyl-leucyl-phenylalanine but did not influence fluoride-induced inhibition.

Introduction

Recent results indicate that receptor-dependent agents such as the chemotactic peptide, *N*-formylmethionyl-leucyl-phenylalanine (FMLP), induce neutrophil responses through the activation of phospholipase C and the subsequent breakdown of polyphosphoinositides to 1,2-diacylglycerol and inositol trisphosphate (Nishizuka, 1984; Berridge & Irvine, 1984; Verghese *et al.*, 1985a). The former messenger in turn activates protein kinase C while the latter mobilizes intracellular calcium stores. Several lines of evidence also point to the coupling of ligand receptor complexes to phospholipase C by a GTP binding protein (G or N proteins). A key observation was that neutrophil responses such as superoxide (O_2^-) generation, chemotaxis, aggregation, degranulation and arachidonic acid release were inhibited by pretreatment of cells with *pertussis* toxin, an AB toxin originally shown to ADP-ribosylate and concurrently inactivate the inhibitory guanine nucleotide regulatory protein (N_i) of adenylate cyclase (Okajima & Ui, 1984; Bokoch & Gilman, 1984; Molski *et al.*, 1984; Verghese *et al.*, 1985a; Lad *et al.*, 1985; Okajima *et al.*, 1985).

Fluoride ions (F^-) stimulate neutrophils and induce responses like O_2^- generation (Curnutte *et al.*, 1979; Okajima *et al.*, 1985). In an earlier study we invoked a GTP binding protein as the site of F^- action (Wong, 1983) on the basis of reports from adenylate cyclase studies that N_s (the stimulatory complex) and N_i are target sites for F^- mediated effects (Insel *et al.*, 1982; Katada *et al.*, 1984a, b). (Strictly speaking, the active complex is AlF_4^-, Al^{3+} being a commonly occurring contaminant (Bigay *et al.*, 1985)). The proposed site of F^- action is also supported by our recent finding that F^- mobilizes intracellular calcium in neutrophils (Strnad & Wong, 1985) and induces the release of inositol trisphosphate to the cytosol (Strnad *et al.*, 1986).

Using fluoride and guanosine 5'[γ-thio] triphosphate as probes, we demonstrated the existence of a functional N_i complex in neutrophil membranes that could conceivably regulate multiple enzyme systems. That is, *pertussis* toxin-sensitive complexes regulating adenylate cyclase and phospholipase C may be closely related proteins.

Methods

Human neutrophils were isolated as described previously (Wong, 1983) and suspended in Hanks balanced salts solution (HBSS), pH 7.4, 37°C, at a density of 1×10^7 cells ml^{-1}. Cells were divided into two groups, one of which was incubated with *pertussis* toxin (1 μg ml^{-1}) for 2 h. For assaying superoxide production, cells were monitored spectrophotometrically by the increase in absorbance at 550 nm occurring upon reduction of ferricytochrome C (Wong, 1983) after addition of drugs, as indicated in

[1] Author for correspondence.

the figures. For adenylate cyclase studies, cells (control and *pertussis* toxin treated) were washed and suspended at a concentration of 5×10^6 cells ml^{-1} in the following ice-cold buffer: Tris-HCl (25 mM), dithiothreitol (5 mM), sucrose (0.25 M) and the protease inhibitor, phenylmethylsulphonyl fluoride (100 μM), pH 7.5 at 25°C. The cells were sonicated in a bath sonicator (Laboratory Supplies Co., N.Y.) for 20 s at 4°C. The sonicate was centrifuged at 1000 *g* for 10 min, the supernate removed and re-centrifuged at 40,000 *g* for 30 min. The pellet from this latter centrifugation was resuspended in ice-cold Tris-HCl (25 mM) buffer containing dithiothreitol (5 mM) pH 7.5. A second centrifugation at 40,000 *g* for 30 min yielded a final pellet which was suspended in the same buffer at a concentration of 1–2 mg protein ml^{-1} and stored under liquid nitrogen until used (within 1 week). Adenylate cyclase activity was determined by the method of Baer (1975) using [α-^{32}P]-ATP at a final concentration of 100 μM as substrate. Duplicate assays were carried out in a volume of 50 μl containing the following: Tris-HCl (25 mM) pH 7.5, $MgCl_2$ (5 mM), creatine phosphate (5 mM), creatine phosphokinase (25 u ml^{-1}), adenosine deaminase (5 u ml^{-1}), cyclic AMP (0.5 mM) and isobutylmethylxanthine (0.5 mM). Protein (15–20 μg per tube) was added to initiate the reaction which was carried out at 37°C for 25–30 min. The results presented are means of duplicate samples with < 10% standard errors and are representative of 3–5 experiments on different neutrophil preparations.

The following materials were purchased from the sources indicated: *pertussis* toxin from List Biological Laboratories (Campbell, CA), *N*-formylmethionyl-leucyl-phenylalanine (FMLP), horse heart ferricytochrome C (type VI) from Sigma Chemicals Co. (St. Louis, MO), guanosine-5′-(3-O-thio) triphosphate (GTPγS), creatine phosphokinase, creatine phosphate, adenosine monophosphate (AMP), adenosine triphosphate (ATP), adenosine deaminase, adenosine 3′: 5′-cyclic monophosphate (cyclic AMP) and guanosine triphosphate (GTP) from Calbiochem-Behring (San Diego, CA). [α-^{32}P]-ATP (25 Ci $mmol^{-1}$) was obtained from ICN Chemicals (Irvine, CA). All other reagents and chemicals were of analytical grade.

Results

Figure 1 shows that, in agreement with recent findings (Lad *et al.*, 1985), FMLP induced O_2^- generation was inhibited by prior treatment of neutrophils with *pertussis* toxin. In contrast, the response to F^- was unaffected.

Fluoride exhibited a biphasic effect on adenylate cyclase activity of neutrophil membranes (Figure 2). At concentrations between 1 and 10 mM, F^- increased enzyme activity to a maximum of 7 fold greater than

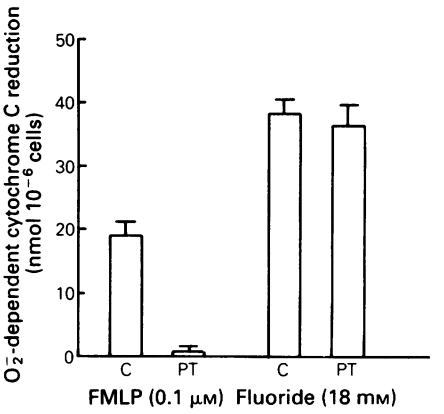

Figure 1 The effect of *pertussis* toxin (1 μg ml^{-1}) pretreatment of neutrophils on superoxide production stimulated by FMLP and fluoride. Control (C) and *pertussis* toxin (PT) pretreated neutrophils were incubated in 0.1 mM ferricytochrome C in the presence of 0.1 μM FMLP for 5 min or 18 nM fluoride for 50 min. The duration corresponded to the cessation of O_2^- generation. Calculations of O_2^- released were carried out as described in Methods. Each column represents the mean and vertical lines s.e.mean of at least 4 experiments.

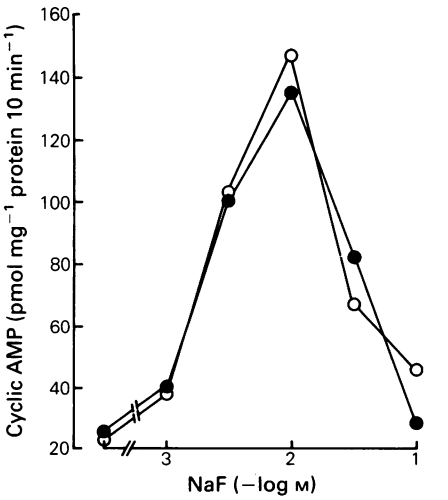

Figure 2 The effect of fluoride ions on adenylate cyclase activity of neutrophil membranes. Control (●) and *pertussis* toxin-pretreated cells (○) were sonicated, the membranes isolated and assayed for adenylate cyclase activity as described in Methods. Each point is the mean of duplicate assays with less than 5% s.e.mean and is representative of at least 4 experiments.

basal levels. In this regard, F^- was more effective than forskolin, a direct activator of the catalytic unit of adenylate cyclase (Seamon *et al.*, 1981) which maximally elevated basal activity 3 fold, or of a β-adrenoceptor agonist, isoprenaline, which in the co-presence of GTP (10 μM), induced a stimulation that was 2–3 fold greater than basal activity (data not shown). At concentrations of F^- over 10 mM, enzyme activity declined and at 100 mM was back to basal levels. Both phases were insensitive to the absence or presence of 10 μM GTP (results not shown). *Pertussis* toxin pretreatment of the cells before cell disruption, membrane preparation and adenylate cyclase assay altered neither the stimulatory nor the inhibitory phase of the effect of fluoride on adenylate cyclase.

In comparison to F^-, FMLP inhibited the adenylate cyclase activity of neutrophil membranes (Figure 3) in assays carried out in the presence of forskolin (10 μM) and GTP. The coupling efficiency of the FMLP-receptor to NADPH oxidase, the O_2^- generating system, appears to be greater than that for adenylate cyclase. Construction of a dose-response curve showed that FMLP maximally inhibited adenylate cyclase at about 10 μM (Figure 3), a result which is in agreement with Lad *et al.* (1985), while maximal O_2^- generation was induced at FMLP concentrations around 0.1–0.2 μM. No inhibition was observed when membrane preparations isolated from cells treated with *pertussis* toxin were used. Furthermore, in the absence of GTP, FMLP did not alter the basal or forskolin stimulated activities. The optimal concentration of GTP was around 10 μM (inset, Figure 3). Previously Lad and coworkers (1985) had also demonstrated inhibition of neutrophil adenylate cyclase activity by FMLP and *pertussis* toxin blockade of this effect. However, Verghese *et al.* (1985b) found that FMLP was neither stimulatory nor inhibitory. Their negative results may be attributable to the absence of forskolin in their cyclase assays; forskolin was used here and in the study of Lad *et al.* (1985) to enhance basal activity and thus permit easier detection of inhibition. A certain variability was found in the present studies in that FMLP failed to inhibit adenylate cyclase in some of the preparations tested. Nevertheless, analysis of all the results showed that 10 μM FMLP significantly inhibited cyclase activity by 25 ± 22% (± s.d., $P < 0.05$, $h = 8$, Student's paired t test).

By comparison to the FMLP effect, the inhibition of adenylate cyclase by GTPγS, a nonhydrolysable analogue of GTP, is marked, reproducible, and can be taken as an index of direct N_i activation (Hudson & Fain, 1983; Katada *et al.*, 1984a, b). We found that GTPγS inhibited the forskolin-stimulated enzyme

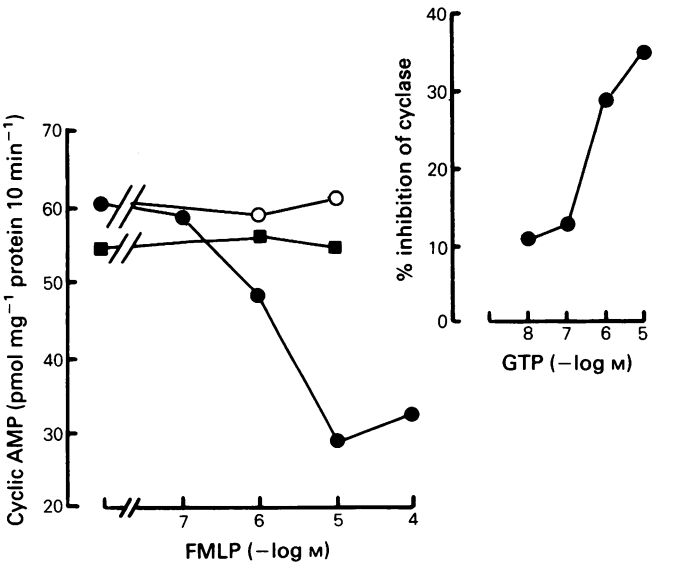

Figure 3 The effect of FMLP on the adenylate cyclase activity of neutrophils. Main figure: (●) forskolin (10 μM) and GTP 10 μM) were present at each point, (○) forskolin (10 μM) but not GTP were added, (■) forskolin and GTP present but membranes from *pertussis* toxin treated cells were used. In the inset, FMLP (10 μM) and forskolin (10 μM) were present together with varying concentrations of GTP as shown. The difference in activity in the absence and presence of GTP was used to calculate % inhibition. All values are the mean of duplicate results with less than 5% s.e.mean and are representative of 5 other experiments.

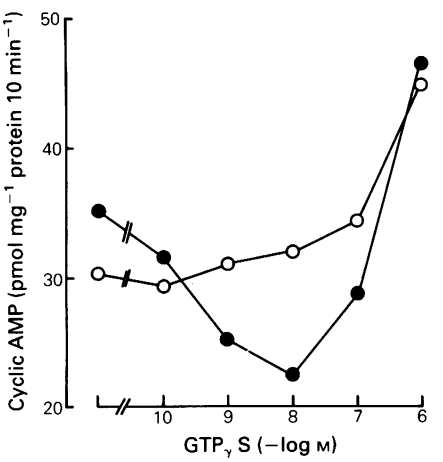

Figure 4 The effect of GTPγS on control and *pertussis* toxin-pretreated neutrophils. Control (●) and pertussis toxin-pretreated (○) cells were sonicated, and the membranes isolated and assayed for adenylate cyclase as described in Methods. Forskolin (10 μM) was included in all incubation mixtures to stimulate the basal activity of the enzyme. The points are the means of duplicates with less than 5% s.e.mean and are representative of at least 3 experiments.

with a maximum effect (35% inhibition) occurring at 10 nM (Figure 4). In the absence of forskolin, the inhibition was less marked. Finally *pertussis* toxin pretreatment abolished the inhibition caused by GTPγS (Figure 4), hence demonstrating attenuation of N_i function. At concentrations above 10 nM, GTPγS stimulated enzyme activity, an effect which was unchanged by *pertussis* toxin.

Discussion

The proposal that FMLP and fluoride induce neutrophil responses by acting at different sites is supported by various observations. For example the kinetics of O_2^- generation differ in cells stimulated with each of the two agents; in addition adenylate cyclase agonists such as isoprenaline and prostaglandin E inhibit FMLP but not fluoride-induced responses (Wong, 1983). The present results showing that pretreatment of cells with *pertussis* toxin blocked FMLP but not fluoride effects on O_2^- generation and adenylate cyclase is consistent with distinct sites of action for these stimulatory agents. A similar result was also obtained in parallel studies in which the intracellular levels of Ca^{2+} were monitored (Strnad & Wong, 1985). Thus it appears that the putative mode of F^- action — dissociation and persistent activation of GTP binding proteins (Katada *et al.*, 1984a, b) — was not affected by ADP-ribosylation of the latter.

Current evidence points to the existence of a family of guanine nucleotide regulatory proteins in human neutrophils. Both β_2- and α_2-adrenoceptors (Galant & Allred, 1980; Panosian & Marinetti, 1983) are found in neutrophil plasma membranes coupled to N_s and N_i, respectively. Specifically a functional N_i was demonstrated by the inhibition of prostaglandin E_1 (or isoprenaline)-stimulated cycle AMP synthesis by a combination of adrenaline and propranolol (Verghese *et al.*, 1985b). The dual regulation of adenylate cyclase by F^- and GTPγS described here confirms the presence of functional N_s and N_i complexes. A similar biphasic effect on adenylate cyclase activity was demonstrated in F^- treated platelet membranes and has been attributed to sequential activation of N_s and N_i (Katada *et al.*, 1984a, b).

On the basis of *pertussis* toxin studies, several groups have advocated a dual role for N_i in the activation of adenylate cyclase and phospholipase C (Okajima & Ui, 1984; Okajima *et al.*, 1985; Lad *et al.*, 1985). Cockcroft & Gomperts (1985) on the other hand postulated the existence of a separate, *pertussis* toxin-sensitive N protein (N_p) coupled to phospholipase C. More recent results indicate that the toxin ADP-ribosylated α subunit of N proteins isolated from neutrophils is different from other known α subunits (Kanaho *et al.*, 1986; Gierschik *et al.*, 1986). The singular nature of neutrophil N_i-α may account for the finding that GTPγS-mediated inhibition of neutrophil adenylate cyclase was abolished by *pertussis* toxin treatment (Figure 4). In contrast GTPγS-dependent inhibition of cyc$^-$ S49 adenylate cyclase is not affected by prior treatment with the toxin (Birbaumer *et al.*, 1985).

Functionally and structurally, N_i and the putative N_p, if not the same complex, are probably closely related. Their susceptibility to modification by *pertussis* toxin is one common attribute. Dose-response studies from adenylate cyclase assays (Figure 2) and O_2^- generation (Curnutte *et al.*, 1979; Wong, 1983) indicate that concentrations of F^- greater than 10 mM preferentially stimulate N_i/N_p. Moreover, the inhibition of adenylate cyclase at relatively high FMLP concentrations (Figure 3) suggests that the FMLP receptor is able to interact with N_i albeit inefficiently. Along the same vein, Cerione *et al.* (1985) reported that rhodopsin, normally regulated by transducin, is capable of coupling to N_i in phospholipid vesicles.

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