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Fluoroaluminates mimic guanosine 5′-[γ-thio]triphosphate in activating the polyphosphoinositide phosphodiesterase of hepatocyte membranes

Role for the guanine nucleotide regulatory protein G_p in signal transduction

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Fluoride and guanosine 5′-[γ-thio]triphosphate (GTPγS) both activate the hepatocyte membrane polyphosphoinositide phosphodiesterase (PPI-pde) in a concentration-dependent manner. $AlCl_3$ enhances the fluoride effect, supporting the concept that $[AlF_4]^-$ is the active species. Analysis of the products of inositol lipid hydrolysis demonstrate that phosphatidylinositol bisphosphate is the major lipid to be hydrolysed. Guanosine 5′-[β-thio]diphosphate (GDPβS) is an inhibitor of activation of PPI-pde by both fluoride and GTPγS. These observations suggest that the guanine nucleotide regulatory protein (termed G_p) bears a structural resemblance to the well-characterized G-proteins of the adenylate cyclase system and the cyclic GMP phosphodiesterase system in phototransduction.

INTRODUCTION

Activation of polyphosphoinositide phosphodiesterase (PPI-pde) in intact cells occurs due to hormonal stimulation leading to generation of two intracellular second messengers, diacylglycerol (activator of protein kinase C) and inositol trisphosphate (IP_3) (mobilizer of intracellular Ca^{2+}) (Berridge & Irvine, 1984; Nishizuka, 1984). It has been shown recently that the PPI-pde can be directly activated by GTP and its analogues alone or in the presence of a receptor-directed agonist in neutrophils (Cockcroft & Gomperts, 1985; Smith *et al.*, 1985), liver (Wallace & Fain, 1985; Uhing *et al.*, 1985, 1986), cerebral cortex (Gonzales & Crews, 1985), cloned rat pituitary cells (GH_3 cells) (Lucas *et al.*, 1985; Straub & Gershengorn, 1986; Martin *et al.*, 1986), blowfly salivary glands (Litosch & Fain, 1985; Litosch *et al.*, 1985) and smooth muscle (Sasaguri *et al.*, 1985). This supports the concept that the activated receptor is coupled to the PPI-pde via a guanine nucleotide regulatory protein (termed G_p or N_p) (Cockcroft & Gomperts, 1985), analogous to the adenylate cyclase system (Gilman, 1984) and the retinal cyclic GMP phosphodiesterase system (Stryer, 1983). Inhibitory and stimulatory agonists affect cyclic AMP generation by binding to specific receptors which are coupled to adenylate cyclase through two G-proteins termed G_s (stimulatory) and G_i (inhibitory) respectively (Rodbell, 1980; Gilman, 1984). Transducin couples the photoexcitation of rhodopsin to the stimulation of cyclic GMP phosphodiesterase (Stryer, 1983).

G_i, G_s and transducin are structurally and functionally similar; they are heterotrimers consisting of α, β and γ subunits (Hurley *et al.*, 1984; Hildebrandt *et al.*, 1985). The mechanism of adenylate cyclase stimulation is the best understood system and is probably similar to those of other G-proteins. Briefly, GTP (in the presence of an appropriate agonist) or GTPγS cause dissociation of the α subunit from the βγ subunits by binding to the α subunit of the heterotrimer. The released α subunit, with the bound guanine nucleotide, directly stimulates adenylate cyclase. In the case of GTP, the intrinsic GTPase of the α subunit hydrolyses the bound GTP to GDP, thus terminating the stimulation of adenylate cyclase. GTPγS, being non-hydrolysable, binds irreversibly, causing persistent activation (Gilman, 1984).

Fluoride has long been known to inhibit or activate adenylate cyclase and to activate cyclic GMP phosphodiesterase (Sternweis & Gilman, 1982; Bigay *et al.*, 1985; Kanaho *et al.*, 1985). Sternweis & Gilman (1982) showed that Al^{3+} was required for activation of G_s by F^-. $[AlF_4]^-$, like GTPγS, is thought to activate G-proteins by causing the dissociation of the α from the βγ subunits (Kanaho *et al.*, 1985). In the present paper it is demonstrated that fluoride, in the presence of $AlCl_3$, stimulates the PPI-pde of hepatocyte membranes in a manner similar to GTPγS. This suggests that G_p may bear structural resemblance to the well-characterized G-proteins of the adenylate cyclase system, G_s and G_i, and transducin.

MATERIALS AND METHODS

Materials

GTPγS and collagenase were purchased from Boehringer Mannheim. [^{3}H]Inositol was purchased from Amersham International. Medium 199, streptomycin and penicillin were purchased from Flow Laboratories, William's E medium was purchased from Gibco.

Abbreviations used: PPI-pde, polyphosphoinositide phosphodiesterase; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PIP_2, phosphatidylinositol bisphosphate; IP, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; G or N protein, guanine nucleotide regulatory protein; GTPγS, guanosine 5′-[γ-thio]triphosphate; GDPβS, guanosine 5′-[β-thio]diphosphate.

Purification of GTPγS

We have previously shown that GTPγS as purchased from Boehringer is impure; the major impurity is GDP (Cockcroft & Gomperts, 1985). GTPγS was purified by h.p.l.c. on a Hypersil ODS column (purchased from Latek, Heidelberg, Germany) using a gradient of acetonitrile (0–25%, v/v) in triethylamine carbonate (100 mM), pH 7. The gradient was increased linearly over a period of 15 min. The flow rate was 2 ml/min. The GTPγS peak was collected, freeze-dried and redissolved in 0.9% NaCl buffered with phosphate (10 mM), pH 7. Its concentration was determined spectrophotometrically at 252 nm.

Isolation and incubation of hepatocytes

Hepatocytes were made by collagenase perfusion of rat liver by the method of Seglen (1976). Hepatocytes (1.5×10^6 in 2 ml per dish) were plated in Petri dishes coated with Primuria (purchased from Falcon) in William's E medium (supplemented with 5% fetal calf serum, 0.1 μM-insulin, 2 mM-glutamine, 1 μM-dexamethasone and 40 μg of gentamycin/ml). After 2 h the cells had adhered and the medium was then changed to Medium 199 supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), penicillin (50 i.u./ml), streptomycin (50 μg/ml) and [^{3}H]inositol (10 μCi/ml) to label the inositol lipids. Medium 199 is low in inositol and is thus an ideal medium for labelling cells. After 18–20 h, the medium was removed and the cells scraped with a rubber policeman in buffer (pH 7.2) containing 20 mM-Hepes, 100 μM-EGTA and 250 mM-sucrose at 4 °C. The cells were suspended in 12 ml of this buffer and disrupted by sonication for 10 s with an MSE probe sonicator. The homogenate was centrifuged at 600 *g* for 5 min to remove unbroken cells and the resulting supernatant was centrifuged at 4 °C for 20 min at 86500 $g_{av.}$ (35000 rev./min) to pellet the membranes.

The membranes were suspended in buffer (pH 6.8) containing Pipes (20 mM), KCl (150 mM), 2,3-bisphosphoglycerate (2 mM), ATP (5 mM), $MgCl_2$ (10 mM) and free Ca^{2+} (100 nM) buffered with EGTA (3 mM) as described previously (Barrowman *et al.*, 1987). In experiments with NaF and $AlCl_3$ the calcium buffer was omitted. The reaction was initiated by adding 100 μl of membranes to an equal volume of buffer containing the indicated additions at 37 °C. Incubations were terminated either at 10 min for experiments using GTPγS or at 30 min when NaF and $AlCl_3$ were used as an activator. For the time-course, samples were removed from a bulk incubation at the indicated times. The reaction was terminated by addition of 1.5 ml of chloroform/methanol (1:2, v/v). Phase separation of the aqueous from the organic phase was achieved by adding 0.7 ml of water and 0.5 ml of chloroform. After centrifugation, 1.4 ml of the aqueous top phase (containing the water-soluble inositol and its phosphorylated derivatives) was loaded onto Dowex columns (in the formate form) and the inositol phosphates were separated as described previously (Berridge *et al.*, 1983).

Data are presented from individual experiments (±S.D.) performed in triplicate (except for the time-course experiments) and repeated on at least three occasions.

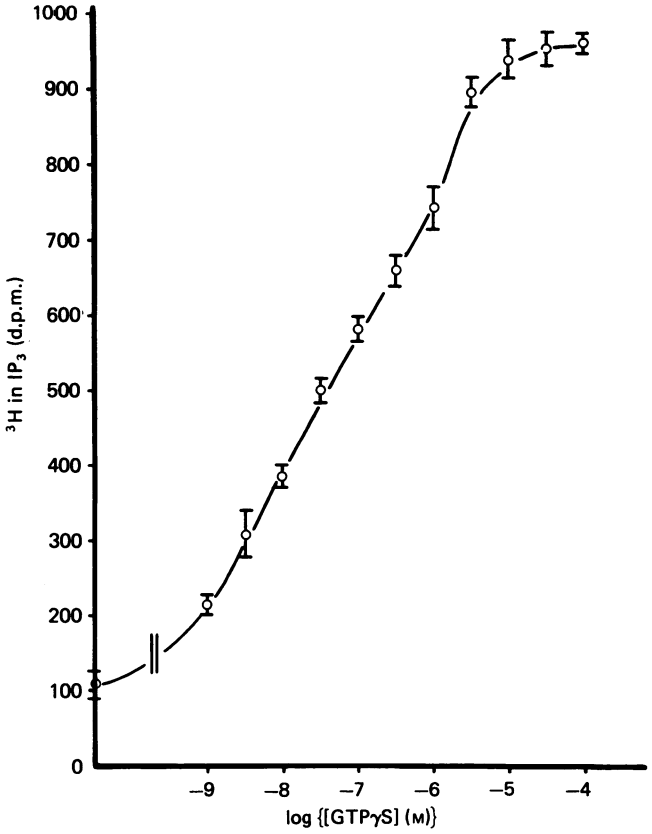

Fig. 1. Concentration-dependence of GTPγS-induced activation of IP_3 production in hepatocyte membranes

[^{3}H]Inositol-labelled rat hepatocyte membranes were incubated with different concentrations of GTPγS for 10 min at 37 °C. After quenching the reaction, the water-soluble IP_3 was analysed by chromatography as described in the Materials and methods section. The results are presented as d.p.m. in IP_3 and the data points are the means of triplicate determinations ±S.D.

RESULTS

Incubation of rat hepatocytes in primary culture for 18–20 h in the presence of [^{3}H]inositol leads to the labelling of all three inositol lipids, PI, PIP and PIP_2. When the cells are disrupted and a membrane fraction isolated, the phosphorylated derivatives of PI become dephosphorylated, but the levels can be regenerated in the presence of Mg-ATP and Mg^{2+} (Cockcroft *et al.*, 1985). The proportions of PI:PIP:PIP_2 in the absence of Mg-ATP is 92.6 (±0.4):6.1 (±0.3):1.3 (±0.1) compared with 86.5 (±1):9.8 (±0.6):3.8 (±0.6) ($n = 8$) in its presence. We have therefore included Mg-ATP (5 mM) and Mg^{2+} (5 mM) to restore the levels of the polyphosphoinositides.

Activation of PPI-pde was monitored by measuring the increase in inositol phosphates derived from the hydrolysis of ^{3}H-inositol-labelled lipids. IP_3 is the unique product of PIP_2 hydrolysis, whereas IP_2 can be derived either from PIP or from IP_3. IP can be derived from PI or from IP_2. IP_3, the product of PIP_2 hydrolysis, is dephosphorylated by a membrane-bound phosphatase to IP_2 (Storey *et al.*, 1984; Joseph & Williams, 1985) and this can be prevented by the addition of 2,3-bisphosphoglycerate (Downes *et al.*, 1982). Thus this was included in all our experiments. In preliminary experiments where 2,3-bisphosphoglycerate was not used,

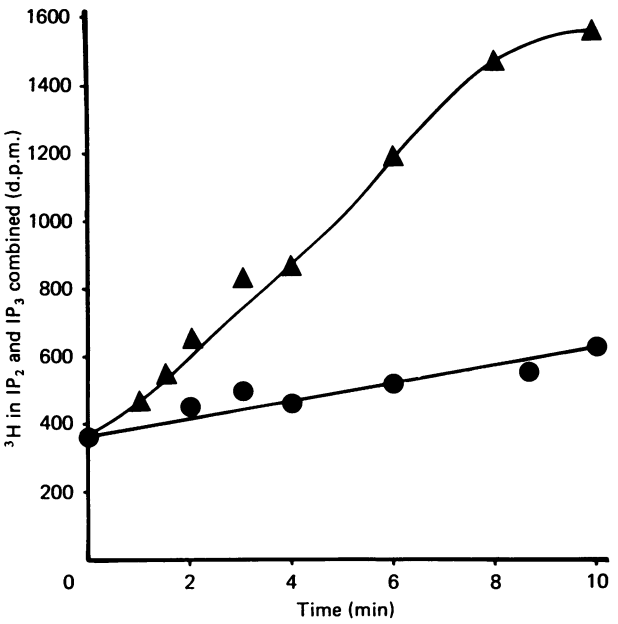

Fig. 2. Time-course of inositol polyphosphate production with GTPγS in hepatocyte membranes

[^{3}H]Inositol-labelled hepatocyte membranes were incubated with GTPγS (10 μM) for 10 min at 37 °C. Samples were withdrawn at the indicated times and the formation of inositol polyphosphates (IP_2 and IP_3 combined) was analysed; ●, control samples; ▲, GTPγS-stimulated samples.

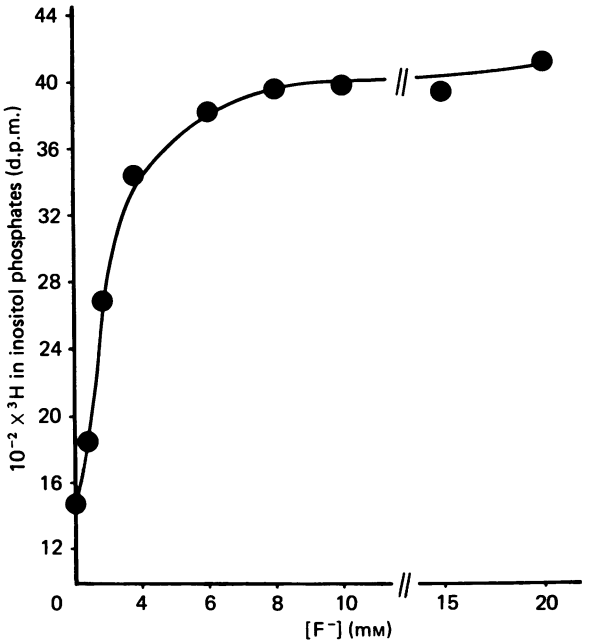

Fig. 3. Concentration-dependence of fluoride activation of PPI-pde

[^{3}H]Inositol-labelled hepatocyte membranes were incubated with different concentrations of NaF for 30 min at 37 °C. Radioactivity (d.p.m.) in the total inositol phosphates (IP, IP_2 and IP_3) was measured. The data points are means from triplicate determinations which were within 3% of each other.

Table 1. Effect of $AlCl_3$ on fluoride activation of PPI-pde

[^{3}H]Inositol-labelled hepatocyte membranes were incubated with NaF at the concentrations indicated in the presence or absence of $AlCl_3$ (20 μM) for 30 min at 37 °C. The radioactivity (d.p.m.) in the total inositol phosphates was determined and the results are means of triplicate observations ± S.D. *P* versus incubation without $AlCl_3$: * < 0.05, ** < 0.01.

	^{3}H (d.p.m.) in total inositol phosphate	
[NaF] (mM)	NaF alone	NaF + $AlCl_3$ (20 μM)
Control (0)	2020 ± 19	2074 ± 6
1	2390 ± 95	2755 ± 108*
2	3248 ± 23	3607 ± 66**

GTPγS stimulated increases in all three inositol phosphates with the greatest increase in IP_2 (results not shown). Similarly, LiCl, which inhibits the inositol 1-phosphatase, was also included to prevent any degradation of the inositol monophosphate back to inositol (Hallcher & Sherman, 1980).

GTPγS stimulates the release of IP_3 in a concentration-dependent manner. Half-maximal activation occurs at 0.3 μM-GTPγS (Fig. 1). The level of free Ca^{2+} was fixed at 100 nM since we have recently shown that PPI-pde requires calcium for maximal activity with GTPγS (Cockcroft, 1986). All three inositol phosphates were analysed and there was a concentration-dependent increase in IP_2 and IP_3. However, over 90% of the increase in radioactivity in the inositol polyphosphates was accounted for by the increase in IP_3, so data for this component only is shown in Fig. 1. The time-course of GTPγS-induced generation of IP_2 and IP_3 (combined) is shown in Fig. 2. The rate of inositol polyphosphate generation is linear for 8 min, after which it reaches a plateau.

In the next set of experiments the activation of fluoride and aluminium was investigated. In the experiments described above the concentration of Ca^{2+} was set at 100 nM, the level that pertains in unstimulated cells. When fluoride was used as an activator of PPI-pde in the presence of 100 nM-Ca^{2+} buffered with 3 mM-EGTA, we found that fluoride did not activate the PPI-pde (results not shown). Since it has been reported that aluminium is essential for fluoride activation (Sternweis & Gilman, 1982) and EGTA chelates aluminium 3-fold more strongly than it does Ca^{2+} (Sillen & Martell, 1968), the calcium buffer was omitted in these experiments. Thus the level of free Ca^{2+} could not be controlled in the experiments using fluoride. The level of contaminating Ca^{2+} from the glassware and the solutions is in the range of 1–5 μM (Cockcroft *et al.*, 1981) which is sufficient to support the activity of GTPγS-dependent PPI-pde (Cockcroft, 1986).

Fluoride, in a concentration-dependent manner, stimulates PPI-pde activity (Fig. 3). In the absence of added Al^{3+} the maximal increase in inositol phosphates is achieved at 10 mM (Fig. 3). Since both ATP and glassware can contribute to contaminating Al^{3+} (Sternweis & Gilman, 1982), it could not be rigorously excluded in our experiments. However, it was possible using low concentrations of fluoride to demonstrate an

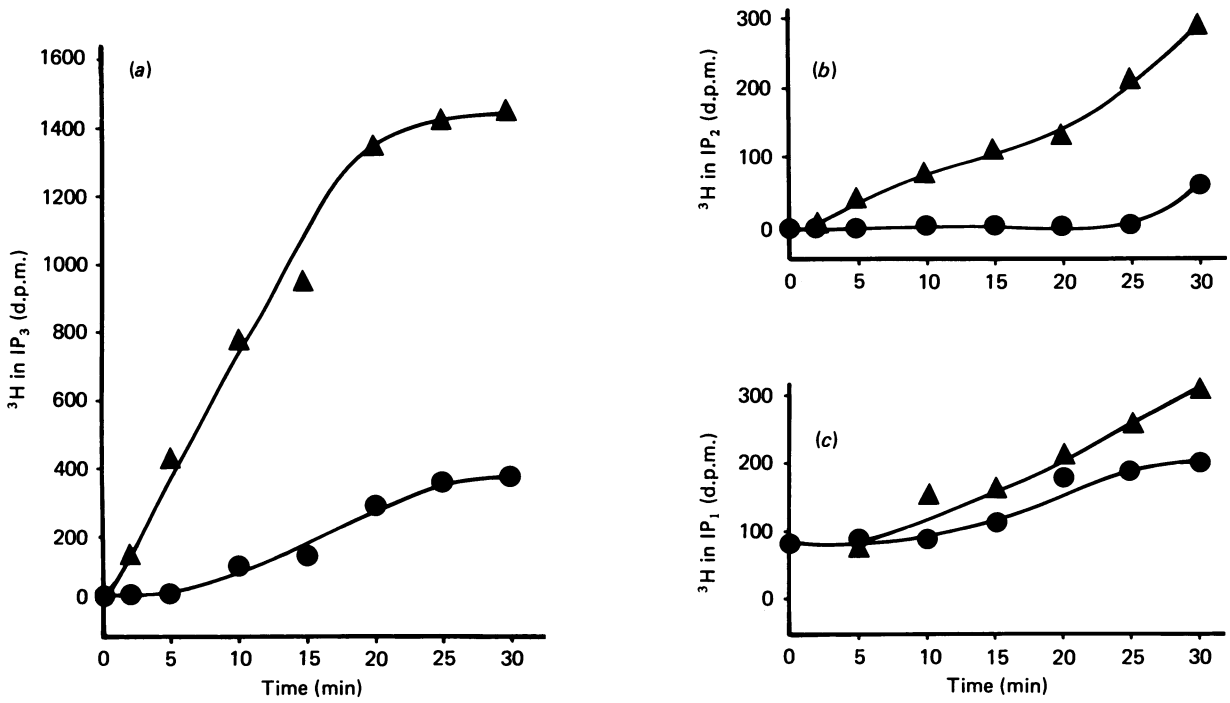

Fig. 4. Time-course of IP, IP_2 and IP_3 production with $[AlF_4]^-$ in hepatocyte membranes

[^{3}H]Inositol-labelled hepatocyte membranes were incubated with NaF (10 mM) and $AlCl_3$ (20 μM) for the indicated times. Radioactivity (d.p.m.) in the individual inositol phosphates [(*a*), IP_3; (*b*), IP_2; (*c*), IP] was measured. ●, Control; ▲, with NaF and $AlCl_3$.

enhancement of inositol phosphate formation with $AlCl_3$ (Table 1).

Figs. 4(*a*)–4(*c*) show the time-course of formation of the individual inositol phosphates stimulated by NaF (10 mM) and $AlCl_3$ (20 μM). IP_3 begins to increase within 2 min and the rate of production is linear for 20 min, after which it reaches a plateau (Fig. 4*a*). Formation of IP_2 is only observed after 5 min and continues to increase at a linear rate for 30 min (Fig. 4*b*). A significant increase in IP is only observed at 10 min, after which the levels increase linearly for 30 min (Fig. 4*c*). IP_3 accounts for the majority of the increase in the total inositol phosphates. From the time-course of the formation of the three inositol phosphates it is clear that the initial product is IP_3, whereas IP_2 is produced from IP_3, and IP subsequently from IP_2.

GDPβS is an inhibitor of GTPγS-induced polyphosphoinositide hydrolysis in neutrophil and liver membranes (Uhing *et al.*, 1986; Cockcroft, 1986). Here we show that GDPβS inhibits activation of PPI-pde by both fluoride and GTPγS (Fig. 5).

DISCUSSION

GTPγS stimulates the hydrolysis of PIP_2 in a concentration-dependent manner with half-maximal activation at 0.3 μM. This concentration of GTPγS is slightly less than that reported by Uhing *et al.* (1985) where half-maximal stimulation with GTPγS was observed at 1 μM. The difference could partly be accounted for by the fact that the GTPγS used in this study was a more highly purified preparation. Another factor that has to be taken into account is that the experiment is done in the presence of ATP, and since hepatocytes do possess receptors for ATP that are likely coupled to the PPI-pde (Creba *et al.*, 1983), it is likely that the GTPγS concentration curve is shifted to lower concentrations because of the presence of the ATP.

Like GTPγS, fluoride is able to stimulate the generation of IP_3 in a concentration-dependent manner. As expected, Al^{3+} potentiates the effect of fluoride, supporting the concept that $[AlF_4]^-$ is the active species. Further support for a metal requirement comes from the observation that fluoride is not active when used in the presence of the chelator, EGTA. It has been recently proposed that $[AlF_4]^-$ mimics the role of the γ-phosphate of GTP and therefore probably activates the G-protein by subunit dissociation in a manner similar to GTPγS (Bigay *et al.*, 1985).

The major lipid that is the substrate for the PPI-pde is PIP_2, as judged by the composition of the recovered inositol phosphates (see Fig. 4). IP_3 accounted for 90% of the released inositol phosphates with fluoride or GTPγS as a stimulus. It is clear that the PPI-pde will hydrolyse both PIP and PIP_2 (Cockcroft & Gomperts, 1985), but when the levels of PIP_2 are maintained by the presence of Mg-ATP, as done in this study, then the enzyme appears to show specificity for PIP_2.

Fluoride has been shown to stimulate a variety of cell functions in different cell types whose mode of action cannot be explained by changes in the levels of cyclic nucleotides. It is more than likely that in these systems, e.g. histamine secretion from mast cells (Patkar *et al.*, 1978; Kuza & Kazimierczak, 1982), respiratory burst in neutrophils (Curnutte *et al.*, 1979; Greenberg *et al.*, 1982), activation of phosphorylase in hepatocytes (Blackmore *et al.*, 1985) and excitation of *Limulus* photoreceptors (Corson & Fein, 1983; Corson *et al.*,

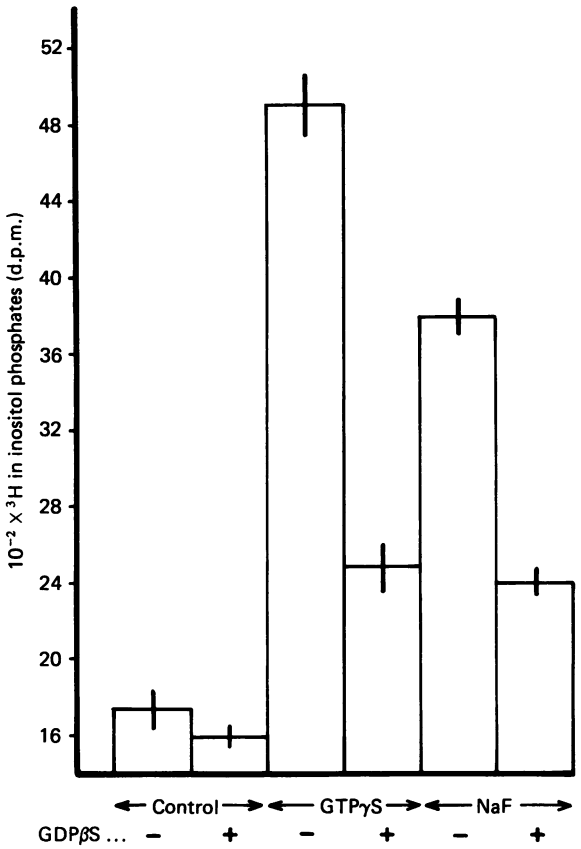

Fig. 5. Inhibition by GDPβS of fluoride- and GTPγS-induced activation of PPI-pde

[^{3}H]Inositol-labelled hepatocyte membranes were incubated with either NaF (10 mM) or GTPγS (10 μM) for 30 min in the presence and absence of GDPβS (1 mM). Results are presented as means of d.p.m. (from triplicate determinations) ± S.D. in the total inositol phosphates (IP, IP_2 and IP_3).

1983) can be explained by the ability of fluoride to stimulate PPI-pde. In intact neutrophils (Strnad & Wong, 1985) and hepatocytes (Blackmore *et al.*, 1985) it has been shown that fluoride can also cause a rise in cytosol Ca^{2+} and in the hepatocyte this is associated with IP_3 formation (Blackmore *et al.*, 1985).

In analogy with the adenylate cyclase and phototransduction systems, the site of fluoride action is likely to be at the level of the G-protein rather than direct activation of the PPI-pde. This is confirmed by the observation that GDPβS can inhibit activation of the PPI-pde by both fluoride and GTPγS in a manner reminiscent of the adenylate cyclase system (Eckstein *et al.*, 1979), suggesting that G_p bears structural homology to the well-defined G-proteins of both the adenylate cyclase and phototransduction systems.

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