Dual effect of fluoride on phosphoinositide metabolism in rat brain cortex

Stimulation of phospholipase C and inhibition of polyphosphoinositide synthesis

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We have studied the effects of fluoride, guanosine 5'-[γ -thio]triphosphate (GTP[S]) and carbachol on phospholipase C and polyphosphoinositide synthesis. The experimental system consisted of membranes from rat brain cortex, with exogenous [3 H]phosphatidylinositol ([3 H]PtdIns) as substrate. In such systems, we have not found evidence to support carbachol and/or GTP[S] stimulation of PtdIns phosphorylation. Fluoride inhibited synthesis of PtdIns4P and PtdIns(4,5) P_2 from PtdIns. Consequently, under conditions where breakdown of polyphosphoinositides by phospholipase C was dependent on PtdIns kinase activity, fluoride inhibited activation by GTP[S] plus carbachol of phospholipase C. When conditions allowed direct breakdown of PtdIns and precluded PtdIns kinase activity, the stimulatory effects of fluoride and GTP[S] plus carbachol on phospholipase C activity were additive.

INTRODUCTION

Although it has not yet been directly demonstrated, it is thought that G-proteins are involved in the regulation of hormone and neurotransmitter stimulation of phosphoinositide breakdown [1–3]. The most consistent evidence supporting this involvement is the ability of hydrolysis-resistant GTP analogues to stimulate phospholipase C in permeabilized cells or cell-free preparations. Moreover, guanine nucleotides are required to demonstrate coupling of receptors to phospholipase C [4–6]. Fluoride in combination with aluminium is another known activator of G-proteins, and its ability to stimulate phospholipase C lends further support to the existence of putative Gp protein(s) [7–9].

As recently reviewed by Linden & Delahunty [10], activation of a number of receptors can lead to the inhibition of phosphoinositide metabolism. In some cases, this effect can be indirect, but in other instances it could be a direct result of receptor-mediated inhibition of phospholipase C. To illustrate this, we have recently found that, in a cell-free preparation from rat brain cortex, dopamine inhibits the carbachol-stimulated breakdown of exogenous PtdIns, and this effect appears to be mediated by D-1 receptors [11].

Reports on fluoride inhibition of carbachol-stimulated phosphoinositide breakdown in brain and ileum have been interpreted to indicate the existence of an inhibitory Gp protein [12]. In this paper we show that fluoride inhibition of phosphoinositide breakdown, rather than being due to inhibition of phospholipase C, is probably secondary to inhibition of PtdIns kinase.

EXPERIMENTAL

Brain cortical membranes were prepared as described in ref. [6]. Briefly, cross-chopped tissue slices were incubated for 30 min at 37 °C in Krebs–Henseleit buffer without added Ca^{2+} , at pH 7.4 equilibrated with O_2/CO_2 (19:1). All subsequent operations were done at 4 °C. The slices were washed with 20 mM-Tris/HCl

buffer, pH 7.0, containing 1 mM-EGTA, then homogenized in the same buffer with a Teflon/glass Dounce homogenizer operated by hand. After a first centrifugation at 3300 g for 15 min, pellets were resuspended in the same buffer, homogenized again and incubated for 15 min. After a third homogenization, the membranes were centrifuged twice at 3300 g for 15 min. The final pellets were kept at –80 °C for not more than 3 weeks.

Phospholipase C assays using exogenous [3 H]PtdIns were done in a final volume of 100 μ l of 8 mM-Tris/maleate adjusted to pH 6.8 with KOH, containing 100 μ g of membrane protein, 1 mM-sodium deoxycholate, 6 mM-MgCl $_2$, 8 mM-LiCl, 3 mM-EGTA and CaCl $_2$ to yield the indicated free Ca^{2+} concentrations [13]; 100 μ M-[3 H]PtdIns was used as substrate in the absence or presence of 2 mM-ATP as noted. Incubations were usually run for 10 min at 37 °C, and stopped with 1.2 ml of chloroform/methanol (1:2, v/v). Then 0.5 ml each of 0.25 M-HCl and chloroform were added and the two phases separated by centrifugation at 1500 g for 10 min. Total [3 H]inositol phosphates present in the aqueous (upper) phase were determined by counting radioactivity of 1 ml samples. Separation of [3 H]inositol phosphates by h.p.l.c. was done with a Whatman Partisil 10 SAX column and an ammonium formate gradient. [3 H]Inositol-containing phospholipids present in the organic phase were dried under vacuum, then resuspended in 30 μ l of chloroform/methanol (9:1, v/v) containing unlabelled PtdIns, PtdInsP and PtdInsP $_2$ as carriers, and separated by t.l.c. on silica-gel HL plates (Analtech), with chloroform/methanol/ NH_4OH /water (90:90:10:19, by vol.) as mobile phase. After staining with iodine vapour, spots were scraped and counted for radioactivity. Before assays, commercial [3 H]PtdIns was re-purified by t.l.c. in the same system described above, and extracted from the silica by sonication in chloroform/methanol (1:1, v/v).

Results referring to production of [3 H]inositol phosphates, [3 H]PtdInsP and [3 H]PtdInsP $_2$ are expressed as pmol and nmol, and were derived from the specific radioactivity of the added [3 H]PtdIns. No correction was made for dilution by endogenous phospholipid.

Hydrolysis of PtdInsP and PtdInsP $_2$ owing to phospho-

monoesterase activity was studied in the absence of ATP, to preclude PtdIns and PtdInsP kinase activities, and with no added Ca^{2+} (in the presence of 3 mM-EGTA), to minimize breakdown of substrates by phospholipase C.

Phosphatidyl[2- ^3H]inositol (^3H]PtdIns, 5 Ci/mmol), phosphatidyl[2- ^3H]inositol 4-phosphate (^3H]PtdInsP, 4.5 Ci/mmol) and phosphatidyl[2- ^3H]inositol 4,5-bisphosphate (^3H]PtdInsP₂, 6.7 Ci/mmol) were obtained from New England Nuclear. Unlabelled phosphoinositides, carbachol, deoxycholate, EGTA, ATP and NaF were from Sigma, and guanosine 5'-[γ -thio]triphosphate (GTP[S]) was from Boehringer Mannheim.

RESULTS

In rat brain membranes PtdInsP₂ and PtdInsP phosphatases, PtdIns and PtdInsP kinases, as well as phospholipase C are present [6]. It has been suggested that GTP[S] stimulates a PtdInsP kinase activity in membranes from rat brain [14]. We decided to estimate PtdIns phosphorylation by a different approach from that used by Smith & Chang [14]. Using exogenous ^3H]PtdIns as substrate in the presence of ATP, and at a low free Ca^{2+} concentration to inhibit direct PtdIns breakdown by phospholipase C [6], the amount of ^3H]inositol phosphates released is primarily dependent on PtdIns phosphorylation to PtdInsP and PtdInsP₂. The results in Table 1 illustrate an experiment where accumulation of total inositol phosphates and newly synthesized PtdInsP and PtdInsP₂ was examined under different conditions. In the presence of 0.1 or 30 nM free Ca^{2+} , GTP[S] alone or in the presence of carbachol stimulated the production of inositol phosphates. As expected, the stimulation elicited by the ligands was higher at 30 nM free Ca^{2+} .

Although Ca^{2+} facilitates phospholipase C activity [15], it inhibits PtdIns kinase [16] and PtdInsP kinase [17] in the sub-micromolar range. Thus there was a somewhat higher accumulation of PtdInsP and PtdInsP₂ at 0.1 nM free Ca^{2+} , but the data on inositol phosphate production indicate an overall greater PtdIns flux through PtdInsP and PtdInsP₂ at 30 nM free Ca^{2+} . These data suggest that, although ligands may stimulate PtdIns phosphorylation, the main mechanism seems to be stimulation of phospholipase C activity without any change in net accumulation of labelled PtdInsP and PtdInsP₂.

On the other hand, whereas 10 mM-NaF gave a very small stimulation of inositol phosphate production at 30 nM free Ca^{2+} ,

Fig. 1. Effect of NaF on GTP[S]-plus-carbachol-stimulated polyphosphoinositide breakdown studied by using exogenous PtdIns in the presence of ATP

Brain cortical membranes (100 μg of protein) were incubated for 10 min at 37 °C with 100 μM - ^3H]PtdIns (2.2 Ci/mol) in the presence of 2 mM-ATP, 6 mM-MgCl₂, 8 mM-LiCl, 1 mM-deoxycholate and 30 nM free Ca^{2+} buffered with 3 mM-EGTA, under control conditions (○) or with 30 μM -AlCl₃ (△), with 1 μM -GTP[S] plus 1 mM-carbachol (●) or with 1 μM -GTP[S] plus 1 mM-carbachol plus 30 μM -AlCl₃ (▲), and with the indicated concentrations of NaF. Results are expressed as nmol of inositol phosphates released.

at both Ca^{2+} concentrations the formation of PtdInsP and PtdInsP₂ was inhibited by about 80–85%. Clearly, at 0.1 nM free Ca^{2+} the inhibitory effects of NaF on PtdInsP and PtdInsP₂ formation predominate, and there is no stimulation of direct PtdIns breakdown.

Data in Fig. 1 illustrate the dual effect of NaF. When the phospholipase C assay was done with exogenous ^3H]PtdIns in the presence of 2 mM-ATP and 30 nM free Ca^{2+} as in Table 1,

Table 1. Effect of GTP[S], carbachol, NaF and Ca^{2+} on synthesis of PtdInsP and PtdInsP₂ and inositol phosphates production from exogenous PtdIns in the presence of ATP

Brain membranes were incubated for 10 min with no addition, with 1 μM -GTP[S] alone or in combination with 1 mM-carbachol, and with 10 mM-NaF, in the presence of 100 μM - ^3H]PtdIns, 2 mM-ATP, 8 mM-LiCl, 6 mM-MgCl₂, 1 mM-deoxycholate, 3 mM-EGTA and no CaCl₂ added (~ 0.1 nM free Ca^{2+}) or the appropriate concentration of CaCl₂ to yield a free Ca^{2+} concentration of 30 nM. After the incubation, total ^3H]inositol phosphates were counted for radioactivity and ^3H]PtdInsP and ^3H]PtdInsP₂ were separated by t.l.c. Results are expressed in pmol, as means $\pm$ S.E.M. of six determinations from two experiments. To quantify direct ^3H]PtdIns breakdown, controls were run in the absence of ATP. At 0.1 nM free Ca^{2+} , the production of inositol phosphate was 35 ± 4 and 41 ± 4 pmol under control conditions and with GTP[S] plus carbachol, respectively. At 30 nM free Ca^{2+} , these values were 43 ± 1 and 96 ± 6 pmol. Neither labelled PtdInsP or PtdInsP₂ was found at the end of the 10 min incubation in the absence of ATP.

	No Ca^{2+} added			30 nM free Ca^{2+}		
	Inositol phosphates	PtdInsP	PtdInsP ₂	Inositol phosphates	PtdInsP	PtdInsP ₂
Control	69 ± 4	283 ± 8	116 ± 8	93 ± 5	243 ± 14	83 ± 1
1 μM -GTP[S]	91 ± 3	277 ± 3	121 ± 2	183 ± 3	257 ± 13	87 ± 2
1 μM -GTP[S] + 1 mM-carbachol	132 ± 8	266 ± 2	100 ± 3	393 ± 16	236 ± 18	89 ± 13
10 mM-NaF	73 ± 7	51 ± 11	24 ± 1	146 ± 8	38 ± 15	16 ± 5

Fig. 2. Concentration-effect relationship for NaF inhibition of formation of PtdInsP and PtdInsP₂ from PtdIns

Membrane protein (100 μ g) was incubated for 10 min at 37 °C with 100 μ M-[³H]PtdIns (2.2 Ci/mol) in the presence of 2 mM-ATP, 6 mM-MgCl₂, 8 mM-LiCl, 1 mM-deoxycholate and 30 nM free Ca²⁺ buffered with 3 mM-EGTA. Reactions were stopped and organic phases were dried under vacuum. The lipids were separated by t.l.c. Areas corresponding to PtdInsP and PtdInsP₂ were scraped from the plates and counted for radioactivity. Results, which represent incubations done under control conditions (○) or with 1 μ M-GTP[S] plus 1 mM-carbachol (●), are expressed in nmol of PtdInsP (a) or PtdInsP₂ (b).

fluoride alone had little effect, but inhibited the stimulation by 1 μ M-GTP[S] plus 1 mM-carbachol. Inclusion of 30 μ M-AlCl₃ enabled a stimulatory effect of low concentrations of fluoride to be demonstrated that was maximal at 2 mM-NaF. On the other hand, the fluoride inhibition of the stimulation by GTP[S] plus carbachol was unaffected by the presence of Al³⁺ (Fig. 1).

Fluoride effects on the PtdInsP and PtdInsP₂ accumulation are shown in Fig. 2. Fluoride concentrations of 10 mM and higher totally inhibited the conversion of labelled PtdIns into PtdInsP and PtdInsP₂. Despite the higher production of inositol

phosphates in the presence of GTP[S] and carbachol (Fig. 1), there were no differences in labelled PtdInsP and PtdInsP₂ contents between control and GTP[S]-plus-carbachol-stimulated samples (Fig. 2).

Fig. 3 shows the time course for inositol phosphate production and PtdInsP accumulation by using exogenous [³H]PtdIns and ATP. NaF blocked the GTP[S]-plus-carbachol-stimulated production of inositol phosphates (Fig. 3a). This effect was due to the inhibition of PtdIns conversion into PtdInsP (Fig. 3b), the preferred phospholipase C-sensitive substrate at 30 nM free Ca²⁺.

Fig. 3. Time course for formation of inositol phosphates and PtdInsP from exogenous PtdIns in the presence of ATP

Membranes (100 μ g) were incubated at 37 °C for the indicated times with 100 μ M exogenous [³H]PtdIns (2.2 Ci/mol), 2 mM-ATP, 6 mM-MgCl₂, 8 mM-LiCl, 1 mM-deoxycholate and 30 nM free Ca²⁺ buffered with 3 mM-EGTA, under control conditions (○), with 1 μ M-GTP[S] plus 1 mM-carbachol (●) or with 1 μ M-GTP[S] plus 1 mM-carbachol plus 15 mM-NaF (▲). Results represent nmol of inositol phosphates (a) and nmol of PtdInsP (b) produced at each time.

Fig. 4. Effect of NaF on phosphomonoesteratic cleavage of PtdInsP₂ and PtdInsP

Cortical membranes were incubated for the indicated time periods with no added Ca²⁺, 6 mM-MgCl₂, 8 mM-LiCl, 1 mM-deoxycholate, 3 mM-EGTA and in the absence of ATP, with 10 μ M-[³H]PtdInsP₂ (15.7 Ci/mol) (a) or 10 μ M-[³H]PtdInsP (b), under control conditions (○) or with 10 mM-NaF (△). At the end of the incubations, lipids were extracted and separated by t.l.c. Results in (a) represent PtdInsP₂ disappearance (continuous lines) and PtdInsP appearance (broken lines), and in (b) PtdInsP disappearance (continuous lines) and PtdIns appearance (broken lines).

In agreement with data in Table 1, there were no major differences in net accumulation of labelled PtdInsP between control incubations and those in the presence of GTP[S] plus carbachol.

Fluoride inhibits PtdIns kinase, but may have other actions on components such as phosphomonoesterases. As shown in Fig. 4(a), fluoride at optimally active concentrations did not stimulate PtdInsP₂ phosphomonoesterase activity. In contrast, this was not the case with PtdInsP phosphomonoesterase activity, which was activated by fluoride. However, this activation does not entirely account for the almost absolute blockade of PtdInsP formation shown in Figs. 2 and 3.

Although fluoride impairs the net accumulation of PtdInsP by both inhibiting kinase and stimulating PtdInsP phosphomonoesterase, assay conditions can be designed in such a way that these effects are not reflected in an inhibition of inositol phosphate formation. As shown in Fig. 5, at relatively high free Ca²⁺ concentrations (300 nM), 15 mM-fluoride did not inhibit the appearance of inositol phosphates stimulated by GTP[S] and carbachol. The obvious interpretation of the results is that these Ca²⁺ concentrations allow a guanine-nucleotide-dependent carbachol stimulation of direct PtdIns phosphodiesteratic breakdown as demonstrated previously [6]. Table 2 further illustrates this, since it shows that, at a free Ca²⁺ concentration of 180 nM, fluoride blocked the formation of Ins(1,4)P₂ elicited by GTP[S] and carbachol. In contrast, fluoride stimulated that of Ins1P, which is derived only from the cleavage of PtdIns in our rat brain membrane preparations [6].

When the assay conditions allowed direct PtdIns breakdown (300 nM free Ca²⁺) and precluded kinase activities (no ATP) as shown in Fig. 6, NaF stimulated PtdIns breakdown. When fluoride was added together with GTP[S] and carbachol, their effects were additive. In the presence of 30 μ M-AlCl₃ two different effects were observed. First, there was an enhancement of PtdIns breakdown in the absence of NaF, both under control conditions

Fig. 5. Concentration-effect relationship for the influence of free Ca²⁺ on the NaF inhibition of stimulation by GTP[S] and carbachol of inositol phosphates production from exogenous PtdIns in the presence of ATP

Membranes were incubated for 10 min with 100 μ M exogenous [³H]PtdIns (2.2 Ci/mol), 2 mM-ATP, 6 mM-MgCl₂, 8 mM-LiCl and 1 mM-deoxycholate, under control conditions (○), with 1 μ M-GTP[S] plus 1 mM-carbachol (●), with 15 mM-NaF (△), or with 1 μ M-GTP[S] plus 1 mM-carbachol plus 15 mM-NaF (▲). Free Ca²⁺ concentrations were buffered with 3 mM-EGTA to the indicated values.

and in the presence of GTP[S] and carbachol. Second, there was an increase in the potency of fluoride. Whereas this second effect is consistent with the role of a fluoroaluminate complex as the functional form in the activation of G-proteins [18], the first effect does not seem to involve G-proteins, since it is observed only in the absence of fluoride. It has been reported that Al³⁺ stimulates PtdIns breakdown by a partially purified phospholipase C preparation from bovine heart, and this effect disappeared when the cation was complexed by fluoride, citrate

Table 2. Effect of NaF on GTP[S]- and carbachol-stimulated production of Ins1P and Ins(1,4)P₂, by using exogenous [³H]PtdIns in the presence of ATP

Membranes were incubated with 100 μ M-[³H]PtdIns (sp. radioactivity 5 Ci/mol) in the presence of 2 mM-ATP, 6 mM-MgCl₂, 8 mM-LiCl, 1 mM-deoxycholate and 180 nM free Ca²⁺ buffered with 3 mM-EGTA, and with the indicated additions. Inositol phosphates were separated by h.p.l.c. and the peaks eluted were identified with the corresponding standards. There was no appearance of Ins(4)P or inositol trisphosphates. Values represent d.p.m. and are means of two determinations. Numbers given in parentheses are percentages of control values.

	Ins1P (d.p.m.)	Ins(1,4)P ₂ (d.p.m.)
Control	718 (100%)	330 (100%)
1 μ M-GTP[S] + 1 mM-carbachol	1534 (214%)	1974 (598%)
1 μ M-GTP[S] + 1 mM-carbachol + 15 mM-NaF	1617 (225%)	234 (71%)
15 mM-NaF	1359 (189%)	286 (87%)

Fig. 6. Concentration-effect relationship for the NaF effects on GTP[S]-plus-carbachol-stimulated direct breakdown of exogenous PtdIns

Membranes were incubated for 10 min in the absence of ATP, with 100 μ M exogenous [3 H]PtdIns (2.2 Ci/mol), 6 mM-MgCl₂, 8 mM-LiCl, 1 mM-deoxycholate and 300 nM free Ca²⁺ buffered with 3 mM-EGTA, under control conditions (○), with 30 μ M-AlCl₃ (△), with 1 μ M-GTP[S] plus 1 mM-carbachol (●), or with 1 μ M-GTP[S] plus 1 mM-carbachol plus 30 μ M-AlCl₃ (▲), and with the indicated concentrations of NaF. Results are expressed as nmol of inositol phosphates released.

or phosphate [19]. These authors suggested that the Al³⁺ effect is probably due to a direct interaction with the substrate, although other interactions with the enzyme cannot be ruled out. Alternatively, Al³⁺ is known to interact with EGTA more strongly than Ca²⁺ does, so this competition could lead to a rise in the free Ca²⁺ concentration and the subsequent stimulation of phospholipase C activity. In support of this, 30 μ M-Al³⁺ did not stimulate PtdIns breakdown in the absence of added Ca²⁺ or EGTA, although it still potentiated the effects of fluoride (results not shown).

DISCUSSION

The PtdIns-kinase-inhibitory characteristics of fluoride have been studied in rat brain [20], yeast [21], mast-cell granules [22] and rat liver [23]. On the other hand, phospholipase C stimulation by fluoride, although more recently described, is much more extensively documented. Whereas it is well accepted that this second action of fluoride is mediated by a guanine-nucleotide

regulatory protein, controversy exists regarding the first one. Biffen & Martin [23] suggested that fluoride inhibition of PtdIns kinase could be mediated by a G-protein, based on the similar effects of fluoride as compared with those of cholera toxin. However, fluoride inhibited a partially purified preparation of PtdIns kinase [21], although it could have retained a regulatory G-protein. Our results show that fluoride stimulation of phospholipase C is potentiated by Al³⁺. However, the inhibition of GTP[S]-plus-carbachol-stimulated production of inositol phosphates by fluoride is independent of the presence of Al³⁺, suggesting that the inhibition of PtdIns phosphorylation is not related to G-protein activation.

Fluoride has been reported to inhibit the carbachol stimulation of phosphoinositide breakdown in rat cerebral-cortical slices [12]. This effect was attributed to activation of an inhibitory G-protein. However, our results suggest that the inhibitory action of fluoride could be due to inhibition of polyphosphoinositide synthesis.

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