Guanine nucleotide and NaF stimulation of phospholipase C activity in rat cerebral-cortical membranes

Studies on substrate specificity

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Guanylyl-5'-yl imidodiphosphate (p[NH]ppG) stimulated a rapid phospholipase C-mediated breakdown of exogenously added phosphatidylinositol 4,5-bisphosphate (PIP₂) in rat cerebral-cortical membranes, with half-maximal activation at approx. 33 μ M. NaF stimulated phospholipase C activity, with half-maximal activation at 0.5 mM. Stimulation of phospholipase C activity by NaF exhibited pH optima at approx. 5.5 and 7.0, with the stimulatory activity at pH 7.0 greater than that at pH 5.5. With p[NH]ppG, only stimulation at pH 7.0 was observed. Neither p[NH]ppG nor NaF stimulated hydrolysis of added phosphatidylinositol (PI) or phosphatidylinositol 4-phosphate (PIP). Mg²⁺ (0.5 mM) potentiated p[NH]ppG-stimulated breakdown of PIP₂. Ca²⁺ increased basal and p[NH]ppG-stimulated breakdown of PIP₂. PI breakdown was stimulated only by high Ca²⁺ concentrations and was unaffected by p[NH]ppG at any Ca²⁺ concentration examined. These results indicate that, in cerebral-cortical membranes, activation of phospholipase C by guanine nucleotides or fluoride directly increases a phospholipase C activity which specifically hydrolyses PIP₂.

INTRODUCTION

Evidence from several laboratories has implicated a role for a regulatory guanine-nucleotide-binding protein in transducing the effect of hormones on phospholipase C-mediated breakdown of phosphoinositides (for reviews, see Berridge & Irvine, 1984; Litosch & Fain, 1986). Direct activation of phospholipase C by hydrolysis-resistant analogues of GTP was shown in neutrophil membranes (Cockcroft & Gomperts, 1985). Guanine nucleotides and hormone produced a synergistic activation of phospholipase C activity in permeabilized platelets (Haslam & Davidson, 1984) and in membranes prepared from blowfly salivary glands (Litosch *et al.*, 1985), neutrophils (Smith *et al.*, 1985), GH₃ cells (Lucas *et al.*, 1985; Shaub & Gershengorn, 1986), hepatocytes (Uhing *et al.*, 1986) and WRK1 cells (Guillon *et al.*, 1986), thereby providing biochemical evidence that receptor activation was coupled to phospholipase C activation.

Litosch & Fain (1985) and Sasaguri *et al.* (1985) showed that guanine-nucleotide-stimulated phospholipase C activity could be monitored by using exogenously added phosphoinositide substrate. The present studies utilize membranes prepared from rat cerebral cortex and added phosphatidylinositol 4,5-bisphosphate (PIP₂), phosphatidylinositol 4-phosphate (PIP) or phosphatidylinositol (PI) substrate to examine further the characteristics of phospholipase C activation and the substrate specificity of the enzyme. In rat cerebral cortex, a variety of hormones, including α_1 -adrenergic amines and carbachol, stimulate phosphoinositide breakdown (Brown *et al.*, 1984; Gonzalez & Crews, 1985), an event which may be involved in neurotransmission (Hawthorne & Pickard, 1979). The present report demonstrates that,

in cerebral-cortical membranes, non-hydrolysable guanine nucleotides and fluoride activate a phospholipase C activity which hydrolyses PIP₂, but not PIP or PI.

MATERIALS AND METHODS

Male rats (100–150 g; Sprague–Dawley) were decapitated and the brain was rapidly removed from the skull. The cerebral cortex was dissected from the brain, chopped into small pieces with a razor blade and then incubated for 30 min in modified Krebs–Henseleit buffer of the following composition: 118 mM-NaCl, 4.7 mM-KCl, 3.0 mM-CaCl₂, 1.2 mM-MgSO₄, 0.5 mM-EDTA, 25 mM-NaHCO₃, 10 mM-glucose and 20 mM-Tris/HCl buffer (pH 7.2) equilibrated with O₂/CO₂ (19:1) at 37 °C. After 30 min, the buffer was aspirated and the tissue homogenized in 14 ml of homogenization buffer [280 mM-sucrose, 0.5 mM-EDTA, 60 μ g of soya-bean trypsin inhibitor (Type II; Sigma)/ml and 10 mM-Tris/HCl buffer (pH 7.5 at 5 °C)] with a Brinkmann Polytron (setting 4 for 10 s). The homogenate was centrifuged at 500 *g* for 10 min, after which the supernatant was removed and centrifuged at 32000 *g* for 10 min. The resulting pellet was resuspended in homogenization buffer and centrifuged at 32000 *g* for 10 min. This washing procedure was repeated twice. The final pellet was resuspended in homogenization buffer to a protein concentration of 1 mg/ml. These membranes were used without further purification. All procedures were carried out at 5 °C with freshly prepared membranes.

Incubation was started by the addition of 10 μ l of membrane protein to 40 μ l of reaction mixture, consisting of 0.5 mM-MgCl₂, 12 mM-LiCl, 25 mM-Hepes buffer

Abbreviations used: p[NH]ppG, guanylyl-5'-yl imidodiphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate.

(pH 7.0), [^3H]PIP₂ (950 c.p.m./pmol), [^{32}P]PIP (500 c.p.m./pmol) or [^3H]PI substrate (700 c.p.m./pmol) plus effectors at 35 °C. Before use, the lipid substrates were dried *in vacuo*, resuspended in 200 μl of incubation buffer containing 0.01% sodium deoxycholate and sonicated for 40 s. Deoxycholate was used to facilitate the sonication process. The sonicated substrate was diluted with sufficient reaction mixture such that the final detergent concentration in the assay tube did not exceed 0.0025%. Free Ca²⁺ concentrations were set by using a Ca²⁺-EGTA buffer system (Portzehl *et al.*, 1964). The incubation was terminated by the addition of 100 μl of ice-cold 50 mM-cyclohexane-1,2-diaminetetra-acetic acid (neutralized to pH 7.0), followed by 1.25 ml of methanol/chloroform (2:1, v/v) containing 0.1% HCl. Chloroform (0.5 ml) and water (0.5 ml) were added, followed by vortex-mixing. After 60 min on ice, the tubes were centrifuged for 10 min at 3000 rev./min (Beckman TJ-6 rotor). The upper phase was collected and neutralized. The aqueous phase was incubated for 15 min with 0.5 ml of resin slurry (AG1-X8; formate form). The slurry was poured into small columns, and water-soluble products of breakdown were analysed as described previously (Litosch *et al.*, 1985).

In some experiments, the lipid phase was analysed by t.l.c. on Uniplat Silica-Gel HL plates (Analtech). The solvent system used to separate the phosphoinositides was methanol/chloroform/4 M-NH₃/water (90:90:9:19, by vol.), as described by Schacht (1976). The labelled lipids were detected by fluorography by using Enhance

(New England Nuclear). Membrane protein was determined by the Bradford dye-binding method (Bio-Rad). [^3H]PIP₂ (2 Ci/mmol) and [^3H]PI (8.4 Ci/mmol) were from New England Nuclear. [^{32}P]PIP was isolated from brain membranes which had been incubated in standard incubation buffer containing 5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of 10 mM-MgCl₂ for 30 min. The lipids were extracted and [^{32}P]PIP was isolated by the protocol described by Irvine *et al.* (1984). Purity of the isolated lipid was determined by co-migration with PIP standard. Lipid standards were from Sigma. p[NH]ppG and GTP were from Boehringer Mannheim. AG1-X8 (formate form) was from Bio-Rad.

RESULTS

Effects of guanine nucleotides and fluoride

Cerebral-cortical membranes slowly hydrolysed added PIP₂ substrate in the absence of added guanine nucleotide (Fig. 1). p[NH]ppG rapidly stimulated the rate and extent of PIP₂ hydrolysis. The stimulated rate of PIP₂ hydrolysis, however, was not maintained, and after 30 s there was a decrease in the rate of hydrolysis.

It has been reported that cholate is essential to obtain p[NH]ppG stimulation of phospholipase C activity in membranes (Jackowski *et al.*, 1986). The effect of cholate on p[NH]ppG stimulation of phospholipase C is shown in Fig. 2. Although cholate at low concentrations (1–10 mM) increased both basal and p[NH]ppG-stimulated activity, the degree of stimulation by p[NH]ppG was not augmented by cholate. Thus, for cortical membranes, cholate was not essential for demonstration of a p[NH]ppG effect. At 20 mM-cholate, both basal and p[NH]ppG-stimulated activity was inhibited.

The dose-response curve for p[NH]ppG stimulation of PIP₂ breakdown is shown in Fig. 3. p[NH]ppG at less than 1 μM did not stimulate phospholipase C activity. Half-maximal activation of phospholipase C occurred with approx. 33 μM -p[NH]ppG, with maximal activation occurring at 100 μM .

NaF produces both a stimulation (Rall & Sutherland, 1958) and an inhibition (Harwood & Rodbell, 1973; Manganiello & Vaughn, 1976) of adenylate cyclase

Fig. 1. Time course for p[NH]ppG-stimulated breakdown of PIP₂ by phospholipase C in membranes

Membranes prepared from rat cerebral cortex were incubated in buffer containing 0.5 mM-MgCl₂, 12 mM-LiCl, 25 mM-Hepes buffer (pH 7.0) and 20 pmol of PIP₂ for the indicated time in the absence or presence of 100 μM -p[NH]ppG. The cumulative production of inositol phosphates from PIP₂ breakdown is shown.

Fig. 2. Effect of cholate on basal and p[NH]ppG-stimulated phospholipase C activity

Membranes were incubated for 60 s in incubation buffer, containing 0.5 mM-MgCl₂, 12 mM-LiCl, 25 mM-Hepes buffer (pH 7.0), 20 pmol of PIP₂ and with or without 100 μM -p[NH]ppG at the indicated cholate concentration.

Fig. 3. Effect of p[NH]ppG concentration on breakdown of PIP₂ by phospholipase C

Membranes were incubated for 60 s in incubation buffer containing 0.5 mM-MgCl₂, 12 mM-LiCl, 25 mM-Hepes buffer (pH 7.0) and 20 pmol of PIP₂, in the absence or presence of the indicated p[NH]ppG concentration. Results are means $\pm$ S.E.M. for three experiments.

activity. This effect is mediated through a GTP-binding protein (Sternweis *et al.*, 1981; Katada *et al.*, 1984a,b). Since a putative regulatory GTP-binding protein is thought to be involved in hormone activation of phospholipase C activity, the effect of NaF on phospholipase C activity was examined. As shown in Fig. 4, NaF produced a marked activation of phospholipase C activity that was discernible at 60 s and sustained throughout the 15 min incubation period. Activation of phospholipase C was dependent on the NaF concentration (Fig. 5), with maximal activation at 1 mM-NaF.

The pH optimum for NaF and p[NH]ppG activation of phospholipase C is shown in Figs. 6 and 7. In the absence of NaF or p[NH]ppG, there was little phospholipase C activity detected in the pH range 4.0–7.0. Inclusion of 5 mM-NaF elicited two major peaks of

Fig. 4. Effect of NaF on breakdown of PIP₂ by phospholipase C

Membranes were incubated in standard incubation buffer in the absence or presence of 10 mM-NaF for the indicated time.

Fig. 5. Effect of NaF concentration on breakdown of PIP₂ by phospholipase C

Membranes were incubated for 2.5 min in incubation buffer containing the indicated NaF concentration. Results are means of two experiments.

Fig. 6. Effect of pH on NaF activation of phospholipase C in membranes

Membranes were incubated for 60 s in the absence or presence of 5 mM-NaF at the indicated pH. The pH of the incubation buffer was varied with 25 mM-Hepes (pH 6.0–8.0) or 25 mM-acetate (pH 4.0–6.0) buffers. Results are means of four experiments.

Fig. 7. Effect of pH on p[NH]ppG-stimulated phospholipase C activity in membranes

Membranes were incubated for 60 s at the indicated pH in the absence or presence of 100 μ M-p[NH]ppG with 20 pmol of PIP₂. Results are the means of two experiments.

phospholipase C activity. A broad peak in the pH range 4.0–6.0, with an optimum at pH 5.5, was evident. In addition, a sharp peak at pH 7.0 was evident that was greater than the peak at pH 5.5 (Fig. 6). When membranes were stimulated with 100 μ M-p[NH]ppG, there was a similar major peak of activation in the pH range 6.5–7.0. A smaller broad peak of activation was also observed at approx. pH 5.5 (Fig. 7). These data demonstrate that activation of phospholipase C by these effectors occurred within a similar pH range, suggesting that the same enzyme was being activated.

A comparison of the effect of NaF, p[NH]ppG and GTP on breakdown of exogenously added PIP₂, PIP and PI substrate is shown in Table 1. Both NaF and

Table 1. Effect of guanine nucleotides and NaF on phosphoinositide hydrolysis

Membranes were incubated for 90 s in standard incubation buffer containing 10 pmol of PIP₂, PIP or PI substrate with the indicated additions. The basal inositol phosphate production for PIP₂, PIP and PI was 0.06, 0.10 and 0.03 pmol respectively. Results are means $\pm$ S.E.M. for three experiments. Significance of the increase in activity compared with controls: * P < 0.050; ** P < 0.025 (by Student's t test for paired analysis).

Condition	Substrate...	Hydrolysis (% of control)		
		PIP ₂	PIP	PI
No addition		100	100	100
5 mM-NaF		314 $\pm$ 27**	96 $\pm$ 5	94 $\pm$ 5
50 μ M-p[NH]ppG		313 $\pm$ 46**	100 $\pm$ 3	102 $\pm$ 5
5 mM-NaF + 50 μ M-p[NH]ppG		377 $\pm$ 81	—	94 $\pm$ 6
50 μ M-GTP		76 $\pm$ 10	—	107 $\pm$ 6

p[NH]ppG stimulated phospholipase C activity on PIP₂, in agreement with the data shown in other Figures. The stimulation of phospholipase C was slightly greater in the presence of p[NH]ppG and NaF than with either alone. GTP did not stimulate phospholipase C activity, and actually caused a depression in basal activity.

PIP was hydrolysed by membranes, but the degree of hydrolysis was unaffected by either p[NH]ppG or NaF. Similarly, neither p[NH]ppG nor NaF stimulated hydrolysis of PI. Thus, under the present experimental conditions, breakdown of exogenously added PIP or PI was not stimulated by activators of PIP₂ hydrolysis.

Modulation of phospholipase C by Mg²⁺ and Ca²⁺

It has been reported that Mg²⁺ both inhibits (Sasaguri *et al.*, 1985; Jackowski *et al.*, 1986) and stimulates phospholipase C activity (Uhing *et al.*, 1986). The effects of Mg²⁺ on basal and p[NH]ppG-stimulated phospholipase C activity is shown in Fig. 8. In the absence of added Mg²⁺, there was little basal phospholipase C activity. p[NH]ppG produced a slight stimulation of phospholipase C activity under these conditions. Addition of 1.0 mM-Mg²⁺ had little effect on basal phospholipase C activity, but greatly augmented the effect of p[NH]ppG. Mg²⁺, at concentrations greater than 1 mM, slightly increased both basal and p[NH]ppG-stimulated phospholipase C activity. Mg²⁺ had little effect to stimulate PI hydrolysis. The production of inositol monophosphate was 0.01 pmol in the absence of added Mg²⁺ and 0.03 pmol in the presence of 5 mM-Mg²⁺. Stimulation of PI breakdown by p[NH]ppG was not observed under any of these conditions (results not shown).

The possibility that the inability to observe p[NH]ppG stimulation of PI breakdown by phospholipase C was

Fig. 8. Effect of Mg²⁺ on basal and p[NH]ppG-stimulated breakdown of PIP₂ by phospholipase C

Membranes were incubated for 90 s in incubation buffer containing 12 mM-LiCl, 25 mM-Hepes buffer (pH 7.0), 20 pmol of PIP₂ and various Mg²⁺ concentrations, in the absence or presence of 100 μ M-p[NH]ppG.

Fig. 9. Effect of Ca^{2+} concentration on basal and p[NH]ppG-stimulated breakdown of PIP_2 and PI

Membranes were incubated for 90 s in buffer containing the indicated Ca^{2+} concentrations in the absence (●) or presence (■) of 100 μM -p[NH]ppG plus the indicated phosphoinositide substrate. Results are means $\pm$ S.E.M. for three experiments.

due to insufficient Ca^{2+} in the incubation buffer was examined. The effect of increasing Ca^{2+} concentrations on phospholipase C-mediated breakdown of PIP_2 is shown in Fig. 9. Ca^{2+} in the concentration range 0.1–100 μM stimulated phospholipase C activity. p[NH]ppG produced a marked stimulation of phospholipase C activity over the entire Ca^{2+} -concentration range.

The production of inositol monophosphate from PI breakdown was little affected by increasing the Ca^{2+} concentration from 0.1 μM to 100 μM (Fig. 9). However, at 1000 μM - Ca^{2+} , there was a marked stimulation of inositol monophosphate production. p[NH]ppG did not stimulate breakdown of PI at any of the Ca^{2+} concentrations examined.

DISCUSSION

In the present study, exogenously added substrate was used to characterize further the activation and substrate-specificity of the p[NH]ppG-regulated phospholipase C using membranes from rat cerebral cortex. p[NH]ppG produced a rapid stimulation of phospholipase C activity (Fig. 1), which declined after 30 s despite the utilization of less than 10% of the available substrate (Fig. 1). Sequential addition of an equivalent amount of substrate produced a slight increase in activity, which was also not maintained (results not shown). The basis for this loss of stimulatory activity is not clear. In studies where the time course for p[NH]ppG activation has been documented, a similar decline in p[NH]ppG-stimulated activity occurred with either endogenously labelled phosphoinositide substrate (Litosch *et al.*, 1985; Wallace & Fain, 1985; Shaub & Gershengorn, 1986; Gonzales & Crews, 1986) or exogenous added substrate (Litosch & Fain, 1986). This decline in stimulated activity may be

characteristic of the presently used systems, and the basis for it remains to be determined.

In addition to guanine nucleotides, fluoride also stimulated phospholipase C activity (Figs. 4 and 5). The degree of stimulation by the combination of fluoride and p[NH]ppG was only slightly greater than with either alone (Table 1), suggesting that both effectors activate phospholipase C through a similar mechanism, presumably through the putative GTP-binding protein. Fluoride activation of phospholipase C has also been reported in GH_3 -cell membranes (Martin *et al.*, 1986) and intact hepatocytes (Blackmore *et al.*, 1985). Activation by NaF provides additional supportive data that the phospholipase C system is regulated by a GTP-binding protein in a manner analogous to the adenylate cyclase and transducin systems.

There were two pH optima for phospholipase C activity when the enzyme was assayed in the presence of NaF (Fig. 6). This may indicate that there are two enzymes whose activity is stimulated by NaF, or alternatively one enzyme may exhibit two pH optima. It is of interest that studies with crude soluble PI-specific phospholipase C have shown that two pH optima are exhibited by that enzyme (Kemp *et al.*, 1961; Allan & Michell, 1974). PI kinase also exhibits two pH optima (Phillips, 1973). It is possible that the dual pH profile for phosphoinositide-metabolizing enzymes reflects an effect on the physical state of the substrate. In contrast with NaF, with p[NH]ppG there was a major peak of activity at neutral pH, and only a slight stimulation observed in the acidic range (Fig. 7). The prominent guanine-nucleotide-stimulated activity at physiological pH has been reported in blowfly salivary-gland membranes (Litosch & Fain, 1986) and epithelial membranes (Jackowski *et al.*, 1986).

Activation of PIP_2 breakdown by p[NH]ppG was clearly facilitated by Mg^{2+} (Fig. 8). The inhibitory effect of Mg^{2+} reported in some studies (Sasaguri *et al.*, 1985; Jackowski *et al.*, 1986), but not others (Uhing *et al.*, 1986), may reflect Mg^{2+} activation of a lipid phosphatase activity which converts PIP_2 into PIP or PI , thereby causing a depletion of PIP_2 . In the present studies, most incubations were done within 2 min. Analysis of lipids, by t.l.c., indicated that during this time there was no detectable conversion of PIP_2 into PIP or PI (results not shown). The mechanism for Mg^{2+} activation of phospholipase C remains to be determined. In the adenylate cyclase system, Mg^{2+} facilitates the rate of p[NH]ppG activation of the GTP-binding protein (Maguire *et al.*, 1976; Iyengar & Birnbaumer, 1981), and a similar process could occur for the phospholipase C system. However, other possible sites of Mg^{2+} action could be at the substrate level or phospholipase C.

Low Ca^{2+} concentrations stimulated phospholipase C activity on PIP_2 . Unlike the situation with PIP_2 , whose breakdown was apparent at low Ca^{2+} concentrations, at least 1 mM- Ca^{2+} was needed to activate PI breakdown. These data suggest that hydrolyses of PI and PIP_2 were mediated through separate regulatory mechanisms. Since high Ca^{2+} concentrations, which would not be normally attained in cells, were needed to stimulate appreciable PI breakdown, it is possible that other, as yet unidentified, factors regulate the activity of a PI -directed phospholipase C. A similar situation may be true for PIP , since its hydrolysis was unaffected by p[NH]ppG or NaF (Table 1). A similar lack of effect by p[NH]ppG on PI and PIP hydrolysis has been reported for membranes prepared from a mink lung epithelial-cell line (Jackowski *et al.*, 1986). Clearly, further clarification of the route of metabolism of the individual phosphoinositides will depend on the purification and reconstitution of the individual components of the phosphoinositide-receptor system.

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