Biochem. J. (1985) 232, 799–804 (Printed in Great Britain)

Guanine nucleotides stimulate production of inositol trisphosphate in rat cortical membranes

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The guanine nucleotides guanosine 5'-[β,γ-imido]triphosphate (Gpp[NH]p), guanosine 5'-[γ-thio]-triphosphate (GTPγS), GMP, GDP and GTP stimulated the hydrolysis of inositol phospholipids by a phosphodiesterase in rat cerebral cortical membranes. Addition of 100 μM-Gpp[NH]p to prelabelled membranes caused a rapid accumulation of [^{3}H]inositol phosphates (< 30 s) for up to 2 min. GTPγS and Gpp[NH]p caused a concentration-dependent stimulation of phosphoinositide phosphodiesterase with a maximal stimulation of 2.5–3-fold over control at concentrations of 100 μM. GMP was as effective as the nonhydrolysable analogues, but much less potent (EC_{50} 380 μM). GTP and GDP caused a 50% stimulation of the phospholipase C at 100 μM and at higher concentrations were inhibitory. The adenine nucleotides App[NH]p and ATP also caused small stimulatory effects (64% and 29%). The guanine nucleotide stimulation of inositide hydrolysis in cortical membranes was selective for inositol phospholipids over choline-containing phospholipids. Gpp[NH]p stimulated the production of inositol trisphosphate and inositol bisphosphate as well as inositol monophosphate, indicating that phosphoinositides are substrates for the phosphodiesterase. EGTA (33 μM) did not prevent the guanine nucleotide stimulation of inositide hydrolysis. Calcium addition by itself caused inositide phosphodiesterase activation from 3 to 100 μM which was additive with the Gpp[NH]p stimulation. These data suggest that guanine nucleotides may play a regulatory role in the modulation of the activity of phosphoinositide phosphodiesterase in rat cortical membranes.

INTRODUCTION

Receptor-stimulated inositol phospholipid hydrolysis is a signal transduction mechanism for a variety of hormones and neurotransmitters in brain and other tissues (Berridge, 1984; Nishizuka, 1984). Agonists bind to specific recognition sites and activate a phospholipase C which hydrolyses membrane inositides, in particular PIP_2 (Kirk *et al.*, 1981; Berridge *et al.*, 1983; Litosch *et al.*, 1983). The phosphodiesteratic cleavage products, IP_3 and DAG, are then released and may act as second messengers to amplify the original receptor signal. IP_3 has been shown to release calcium from intracellular stores and DAG can activate protein kinase C (Streb *et al.*, 1983; Takai *et al.*, 1979). The precise mechanism whereby the receptor–agonist complex is coupled to the phospholipase is not presently known. Radioligand binding techniques have suggested that the binding of agonists to receptors which are known to stimulate inositide hydrolysis (e.g., α_1-adrenergic and muscarinic cholinergic) is modulated by guanine nucleotides (Goodhardt *et al.*, 1982; Evans *et al.*, 1985). Receptors which are coupled to adenylate cyclase characteristically show similar changes in binding of agonists in the presence of guanine nucleotides. It is now established that these changes in binding reflect the presence of a guanine nucleotide binding protein (N-protein) which is involved in the transfer of signal from agonist-occupied receptors to the catalytic unit of adenylate cyclase. Using prelabelled cortical membranes, we now show that guanine nucleotides directly activate a phospholipase C which hydrolyses PIP_2. This suggests that in isolated cortical membranes an N-protein may regulate the activity of phosphoinositide phosphodiesterase.

MATERIALS AND METHODS

Radiochemicals were obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.). Gpp[NH]p and GTPγS were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Phospholipase C (*Clostridium welchii*) and other drugs were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Solvents were obtained from Fisher Scientific Co. (St. Louis, MO, U.S.A.).

Male Sprague–Dawley rats (175–250 g, from the colony at the University of Florida, Gainesville, FL, U.S.A.) were decapitated and cerebral cortical slices were prepared as described (Gonzales & Crews, 1984). The slices were labelled by incubation for 90 min with [^{3}H]inositol in a Krebs–Ringer buffer (KRB) containing no calcium. After the labelling period, the slices were washed with fresh buffer and cooled in an ice bath followed by two additional washes with cold KRB containing 1 mM-EDTA. The labelled slices were homogenized gently using five strokes by hand in a glass/glass homogenizer as described (Chasin *et al.*, 1974). The

Abbreviations used: Gpp[NH]p, guanosine 5'-[β,γ-imido]triphosphate; App[NH]p, adenosine 5'-[β,γ-imido]triphosphate; GTPγS, guanosine 5'-[γ-thio]triphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol trisphosphate; IP_2, inositol bisphosphate; IP, inositol monophosphate; DAG, diacylglycerol.

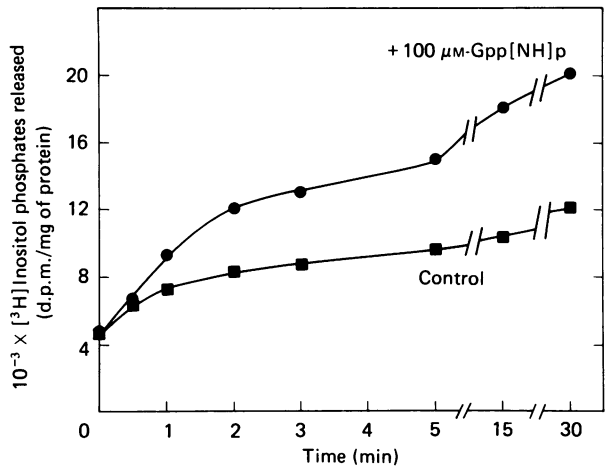

Fig. 1. Time course for Gpp[NH]p stimulation of inositide hydrolysis in prelabelled cortical membranes

Cortical slices were labelled with [^{3}H]inositol and membranes were prepared as described in the Materials and methods section. Membranes were resuspended in 20 mM-Tris/HCl and incubated at 37 °C in the absence and presence of 100 μM-Gpp[NH]p for various times. Aqueous extracts were analysed for [^{3}H]inositol phosphates by Dowex-1 chromatography. Results shown are typical of three separate membrane preparations.

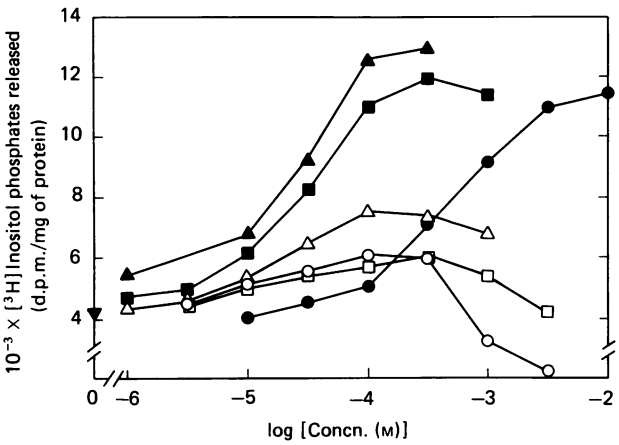

Fig. 2. Concentration–effect curves for guanine and adenine nucleotides for the stimulation of inositide hydrolysis in membranes

Labelled membranes were incubated in the presence of varying concentrations of nucleotides for 15 min. Total [^{3}H]inositol phosphates released were analysed as described in the legend to Fig. 1. Results represent data compiled from triplicate determinations from one or two membrane preparations. ▲, GTPγS; ■, Gpp[NH]p; ●, GMP; △, App[NH]p; □, GDP; ○, GTP.

homogenate was centrifuged at 1000 *g* for 20 min and the pellet was resuspended in ice-cold 20 mM-Tris/HCl (pH 7.4). The membranes were centrifuged a second time (1000 *g* for 20 min) and the resulting pellet was washed once again with Tris. Preparation of membranes which were labelled with [^{3}H]choline was identical except that unlabelled choline was also added to give a final concentration of 62.5μM-[^{3}H]choline.

Release of [^{3}H]inositol phosphates or [^{3}H]phosphocholine from isolated membranes was carried out as described previously except that the buffer used was 20 mM-Tris/HCl instead of Krebs–Ringer containing lithium (Berridge *et al.*, 1983; Gonzales & Crews, 1984). Briefly, 200–300 μg of membrane protein was added to warmed tubes (37 °C) containing 20 mM-Tris/HCl and appropriate drugs or buffer in a final volume of 0.25 ml. After 15 min, 1.0 ml of chloroform/methanol (1:2, v/v) was added and the tubes were shaken. Extraction of lipids and Dowex-1 (formate form) chromatography of the labelled water-soluble organic phosphates was performed exactly as described by Gonzales & Crews (1984).

RESULTS

Time course of Gpp[NH]p stimulation of inositide hydrolysis in membranes

Cortical membrane prelabelled with [^{3}H]inositol was incubated with 100 μM-Gpp[NH]p for various times. Warming of membranes containing [^{3}H]phosphoinositides from 4 to 37 °C resulted in the rapid release of [^{3}H]inositol phosphates for approx. 2 min (1730 d.p.m./min per mg of protein) with a slow release occurring at longer times (Fig. 1). Addition of Gpp[NH]p increased the rate of hydrolysis of [^{3}H]inositides (3750 d.p.m./min per mg of protein) during the initial 2 min rapid release phase. Although the rate of release slowed after 2 min, membranes incubated with Gpp[NH]p continued to release more [^{3}H]inositol phosphates than non-stimulated control membranes for up to 15 min. Further incubation for up to 30 min caused only small increases in both stimulated and non-stimulated samples. Thus, [^{3}H]inositol phosphates are rapidly released for 2 min with maximal release reached at approx. 15 min of incubation.

Nucleotide specificity and concentration-dependence for stimulation of phosphoinositide phosphodiesterase

To investigate the specificity of the nucleotide activation of phosphoinositide phosphodiesterase, several different nucleotides were incubated with [^{3}H]phosphoinositide-labelled membranes. The guanine nucleotides Gpp[NH]p and GTPγS were the most potent and effective compounds tested for release of [^{3}H]inositol phosphates from cortical membranes. Both compounds increased the accumulation of [^{3}H]inositol phosphates approx. 2.5–3-fold at a maximal effective concentration of 100 μM (Fig. 2, Table 1). Other guanine nucleotides which were effective included GTP, GDP, GMP and dibutryl cyclic GMP (Fig. 2, Table 1). These agents in general were less potent than Gpp[NH]p and GTPγS. Curiously, the monophosphorylated GMP was as effective as the nonhydrolysable analogues of GTP although much less potent (EC_{50} 380 μM). Of the adenine nucleotides tested, App[NH]p was the most effective (64% stimulation) and ATP also had a very small effect (29% stimulation). The nonhydrolysable analogue of cyclic AMP, dibutyryl cyclic AMP, had no significant effect on the release of [^{3}H]inositol phosphates from cortical membranes.

Table 1. Guanine and adenine nucleotide effects on inositide hydrolysis in cortical membranes

Nucleotides were added to membranes for 15 min at 100 μM (except where indicated), and liberated [^{3}H]inositol phosphates were analysed by Dowex-1 chromatography. EC_{50} values were determined by probit analysis of full concentration–effect curves from one or two membrane preparations. *Denotes $P < 0.05$, **$P < 0.01$ by paired t-test. N.D., not determined.

Nucleotide	[^{3}H]Inositol phosphates (% of control)	Approx. EC_{50} (μM)	n
Control	100±12	—	7
Gpp[NH]p	266±10**	36	7
GTPγS	278±21**	27	2
GTP	151±5**	9	4
GDP	150±8*	8	3
GMP (1 mM)	243±15*	380	2
Dibutyryl cyclic GMP	122±4*	10	2
App[NH]p	164±9*	24	3
ATP	129±2*	N.D.	2
Dibutyryl cyclic AMP	104±11	N.D.	2

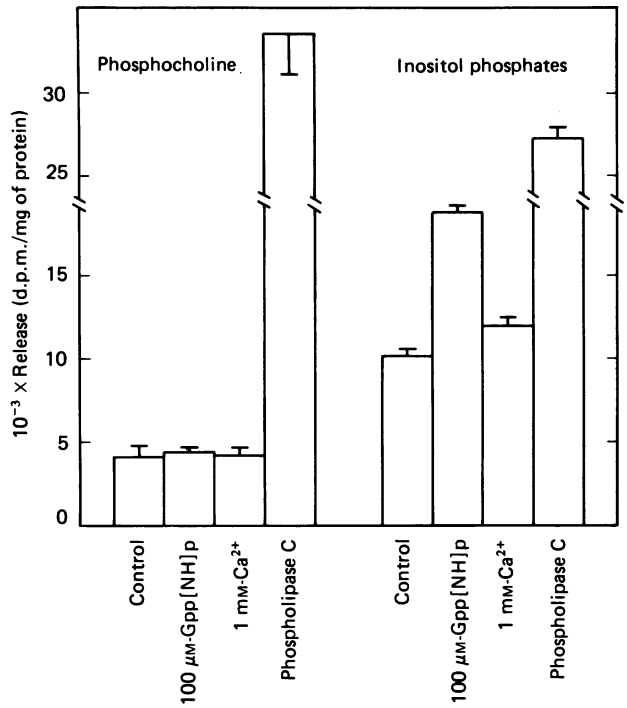

Fig. 3. Effect of Gpp[NH]p, Ca^{2+} and phospholipase C on release of [^{3}H]phosphocholine or [^{3}H]inositol phosphates from prelabelled membranes

Cortical slices were labelled with [^{3}H]choline or [^{3}H]inositol as previously described (Fig. 1). Membranes were resuspended in 20 mM-Tris/HCl and exposed to buffer, 100 μM-Gpp[NH]p, 1 mM-Ca^{2+} or 1 mM-Ca^{2+} and phospholipase C (1.6 mg/ml) for 15 min. Shown are the means ±S.E.M. for triplicate incubations from a single membrane preparation. Similar results were obtained in another experiment.

Substrate selectivity of guanine nucleotide activation of phospholipase C-like activity

To determine if phosphoinositides were specifically hydrolysed by guanine nucleotide stimulation, membranes were prelabelled with [^{3}H]choline or [^{3}H]inositol and the release of [^{3}H]inositol phosphates or [^{3}H]phosphocholine was measured. The presence of 100 μM-Gpp[NH]p caused a significant increase ($P < 0.01$ by ANOVA and Newman–Keuls test) in the release of [^{3}H]inositol phosphates from [^{3}H]inositides (Fig. 3). This stimulation is much larger (314%) if the background, i.e. zero-time, level (6250 d.p.m./mg of protein) is subtracted. In Fig. 3, this was not done to allow comparison between the [^{3}H]choline- and [^{3}H]inositol-labelled membranes. Addition of Ca^{2+} (1 mM) caused only a small increase (17±5%, $P < 0.01$ by ANOVA and Newman–Keuls test) in the release of [^{3}H]inositol phosphates. In contrast, 100 μM-Gpp[NH]p or 1 mM-Ca^{2+} caused no stimulation of the release of [^{3}H]phosphocholine from membranes which had been incubated with [^{3}H]choline to label phosphatidylcholine (Fig. 3). When exogenous phospholipase C was added in the presence of 1 mM-Ca^{2+}, a very large release of both [^{3}H]phosphocholine (812%) and [^{3}H]choline phosphates (266%) was observed. Thus, guanine nucleotides appear to activate a phosphoinositide phospholipase C, but not a phosphatidylcholine phospholipase C.

Guanine nucleotide effects on individual phosphoinositides

To determine if guanine nucleotides released inositol phosphates from polyphosphoinositides, individual [^{3}H]inositol phosphates were separated by stepwise elution from a Dowex-1 column. Gpp[NH]p was found to stimulate the formation of all three inositol phosphates, i.e. IP_3, IP_2 and IP (Fig. 4). IP_3 formation was increased 30-fold, IP_2 by 8-fold and IP by 2-fold. Although IP_3 showed the largest percentage stimulation, 3–4-fold more radioactivity was recovered in the IP fraction, which showed the smallest percentage stimulation. Thus, the percentage stimulation for the total [^{3}H]inositol phosphates is similar to that found previously (Figs. 1 and 2). These results suggest that Gpp[NH]p activates a phospholipase C which can hydrolyse PIP_2. This experiment does not allow conclusions to be made concerning the possible hydrolysis of PIP or PI because the resulting hydrolysis products, IP_2 and IP, could also have been derived from successive dephosphorylation of IP_3.

Interaction of calcium and guanine nucleotides on inositide hydrolysis

In view of the possible relationship between agonist-stimulated PI turnover and calcium gating, we investigated the possible calcium requirements for Gpp[NH]p-stimulated inositide hydrolysis. Since labelled slices are homogenized in EDTA and washed in a calcium-free Tris buffer, there is probably little or no free calcium necessary for guanine nucleotide activation of phosphoinositide hydrolysis. Furthermore, when EGTA (33 μM) was added to the membrane incubation, Gpp[NH]p (100 μM) stimulated release from a control value of 1000±90 to 2140±90 d.p.m./mg of protein. However, we are not sure of the amount of calcium in our membrane

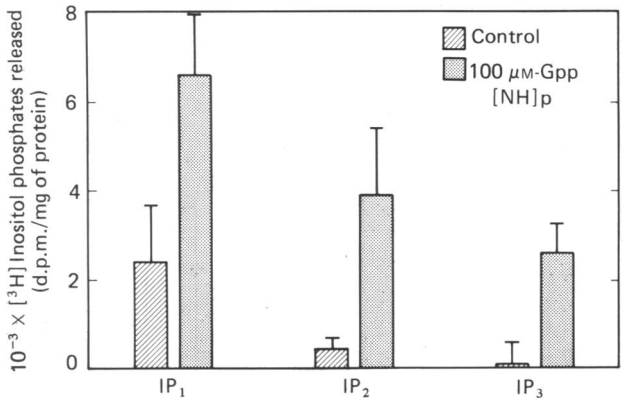

Fig. 4. Effect of Gpp[NH]p on release of individual inositol phosphates from prelabelled cortical membranes

Data were obtained as described in Fig. 1 except that different concentrations of formate buffers were used to elute inositol phosphates sequentially from the Dowex-1 column. Each bar represents the average and range of two experiments each performed in triplicate.

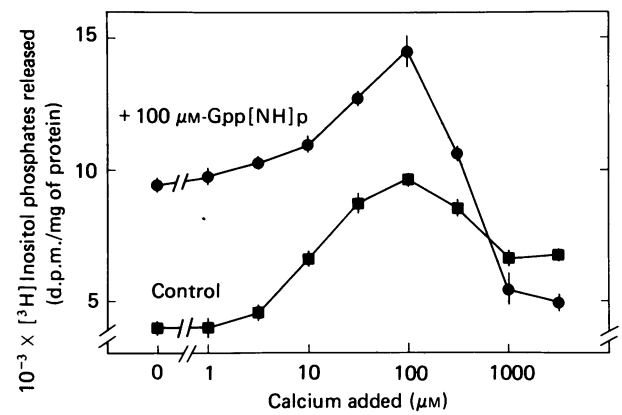

Fig. 5. Effect of calcium on Gpp[NH]p stimulation of [^{3}H]inositol phosphate release from cortical membranes

Data were obtained as in Fig. 1 except that the indicated concentrations of calcium were added to the Tris buffer for a 15 min incubation. Similar results were obtained in two separate experiments.

preparation even with the addition of EGTA. Certain membrane elements have a higher affinity for calcium than does EGTA, e.g. PIP_2. Thus, it is possible that small amounts of calcium are present in our preparation. Addition of calcium by itself caused [^{3}H]inositide hydrolysis by a phospholipase C (Fig. 5). The threshold for Ca^{2+} activation was approx. 3 μM. Maximal stimulation occurred at 100 μM-Ca^{2+}, and phospholipase C activity on [^{3}H]inositides was depressed as the Ca^{2+} levels went beyond 100 μM. A similar calcium curve was obtained in the presence of 100 μM Gpp[NH]p, with no interaction between Gpp[NH]p and Ca^{2+} occurring except at high calcium concentrations (> 100 μM). These results suggest that guanine nucleotide stimulation of inositide hydrolysis requires little or no calcium and that calcium may stimulate inositide hydrolysis through an independent mechanism.

DISCUSSION

A large variety of hormone and neurotransmitter receptors are thought to activate an inositide-specific phospholipase C as an initial event in the receptor-stimulated biochemical cascade (see reviews by Berridge, 1984, and Michell, 1975). Studies on receptors which are linked to adenylate cyclase have shown that a guanine nucleotide binding protein couples agonist-bound receptors to the enzyme adenylate cyclase. Similar studies have recently appeared in the literature which suggest that the enzyme phosphoinositide phosphodiesterase may also be coupled to certain receptors through an N-protein (Cockcroft & Gomperts, 1985; Litosch *et al.*, 1985; Nakamura & Ui, 1985). This concept is supported by the existence of a large variety of N-proteins whose functions are currently not known. We have now shown that guanine nucleotides can stimulate the hydrolysis of phosphoinositides in isolated cerebral cortical membranes, a tissue in which a robust agonist-stimulated PI turnover has been demonstrated previously (Brown *et al.*, 1984; Gonzales & Crews, 1984)

Although we have not been successful in demonstrating direct agonist stimulation of inositide hydrolysis in the membrane preparation we have developed, our data are consistent with the hypothesis that an N-protein mediates the coupling of the receptor to the phospholipase C. The time course of Gpp[NH]p stimulation of inositide hydrolysis is rapid (< 30 s), similar to that previously shown for agonist-stimulated PI turnover (Berridge, 1983). The levelling off of the hydrolysis of inositides after 2 min may be due to depletion of substrate which cannot be replenished in the isolated membranes or to lability of the proposed N-protein. The guanine nucleotide stimulated effect is selective for inositol lipids over choline-containing lipids. Finally, we have shown that PIP_2 is a substrate for the phospholipase C which is activated by Gpp[NH]p. These characteristics of guanine nucleotide stimulated inositide hydrolysis are similar to those observed for agonist stimulated inositol lipid breakdown. Thus, our results suggest that a specific phosphoinositide phosphodiesterase is activated by the presence of guanine nucleotides.

A point of controversy concerning the physiological function of receptor-stimulated inositide hydrolysis has been whether this event represents a calcium 'gate' for the mobilization of calcium and further cell activation (Michell, 1975; Hawthorne, 1982). Evidence that the receptor-stimulated event is independent of calcium has been presented in favour of the calcium gating hypothesis (Kirk *et al.*, 1981). However, there may be tissue- and receptor-specific calcium requirements for the stimulation of inositol lipid hydrolysis (Kendall & Nahorski, 1984; Griffin *et al.*, 1979). Brain tissue has been suggested to support both calcium- and receptor-mediated inositide hydrolysis (Fisher & Agranoff, 1980). It is of interest, therefore, that we have shown both calcium- and guanine nucleotide-stimulated inositide hydrolysis in isolated cerebral cortical membranes (Fig. 4). The pattern of activation of the inositide phosphodiesterase by calcium alone in our membrane is remarkably similar to the pattern obtained with a soluble phosphoinositide phosphodiesterase from brain in a low ionic strength medium (Irvine *et al.*, 1985). A similar calcium-activated enzyme has also been described in mammalian neutrophil membranes (Cockcroft *et al.*, 1984). Gpp[NH]p stimu-

lation is clearly additive with calcium below levels of 100 μM, implying that the guanine nucleotide activation of the phospholipase C is independent of calcium and not controlled by intracellular calcium concentrations. The exact amount of free calcium in our assay system is unknown. However, the slices have been washed exhaustively with calcium-free buffer, homogenized in an EDTA-containing solution, and finally washed in calcium-free buffer. Thus, there are probably only very low levels (less than micromolar) of free calcium available. If the guanine nucleotide stimulated hydrolysis of inositol lipids represents the action of a receptor-coupled phosphodiesterase, this may be evidence that receptor-stimulated PI turnover is also independent of calcium. One must bear in mind that we have not measured rates of enzyme activity but maximum responses, and we cannot exclude the possibility that low micromolar levels of calcium may influence the initial rate of inositide hydrolysis. However, until receptor-coupled inositide hydrolysis is demonstrated in brain membranes, the exact calcium and guanine nucleotide requirements for specific receptors which are coupled to inositide hydrolysis will remain unknown. In keeping with the suggestion of Kendall & Nahorski (1984), it is possible that some receptors which activate inositol lipid breakdown in brain are calcium-mobilizing and others are calcium-requiring.

Guanine nucleotides were found to be the most effective nucleotides which stimulated inositide hydrolysis in isolated cortical membranes. The nonhydrolysable analogues of GTP were most effective; however, GMP was also very effective. The effects of GTP and GDP were interesting in that they possessed a biphasic concentration–effect curve with maximal stimulation at 100 μM and inhibitory effects at higher concentrations (3 mM). These results are remarkably similar to those of Gomperts (1983) who found that high concentrations (> 300 μM) of GMP stimulated histamine release from mast cells and that GDP had inhibitory effects. The presence of phosphatases in the membranes, as well as the inherent instability of these nucleotides, makes the exact concentrations of these compounds difficult to state during the 15 min incubation we used. However, increasing concentrations of GTP and GDP resulted in inhibitory effects rather than stimulatory effects. In addition, inclusion of 1 mM-GTP inhibited the stimulation of inositide hydrolysis by 100 μM-Gpp[NH]p by 60% (results not shown). The differences in potency between the GTP analogues and GMP may reflect a selectivity of one or a number of unidentified N-proteins for specific guanine nucleotides. Although the reason for the biphasic curves for GDP and GTP is not clear, a speculative suggestion may be that these guanine nucleotides might interact with an N-protein which inhibits the inositide phosphodiesterase, analogous to the N_i which mediates the coupling of agonists to the inhibition of adenylate cyclase. Receptor-coupled inhibition of inositide turnover has been demonstrated in pituitary cells (Canonico *et al.*, 1983). Previous attempts to show GTP effects on inositide hydrolysis have been unsuccessful, although GTP has been reported to sustain an agonist-stimulated effect in blowfly salivary gland membranes (Cockcroft & Gomperts, 1985; Litosch *et al.*, 1985).

The significance of the small effects of the adenine nucleotides App[NH]p and ATP on inositide hydrolysis in membranes is also not clear. Litosch *et al.* (1985) similarly reported that ATP was effective in allowing the stimulation of inositol lipid breakdown by serotonin in blowfly salivary gland homogenates. ATP has also recently been reported to stimulate the hydrolysis of inositides in liver cells (Charest *et al.*, 1985). It may be possible that the ATP and App[NH]p stimulation represents a receptor-mediated event. However, ATP is not known to have effects on inositide hydrolysis in cortical slices.

It is well established that a phospholipase C-like enzyme is coupled to receptor activation and is capable of hydrolysing phosphoinositides in brain tissue (Berridge *et al.*, 1983; Jacobson *et al.*, 1985). Although calcium mobilization subsequent to stimulated PI turnover in brain remains to be demonstrated, it seems logical that receptor-mediated inositide hydrolysis in brain represents a signal transduction mechanism for synaptic transmission. In peripheral tissues, evidence is accumulating in support of an N-protein link in the coupling of calcium-mobilizing receptor activation and phosphoinositide hydrolysis (Litosch *et al.*, 1985; Cockcroft & Gomperts, 1985). Irvine *et al.* (1985) have shown that certain proteins can inhibit phosphoinositide phosphodiesterase activity in brain preparations. It is possible that guanine nucleotides interact with certain of these proteins such that they modulate the activity of the enzyme, either increasing or decreasing the activity. A recent report suggests that N_o, a specific N-protein with an as yet unidentified function, can interact with muscarinic receptors in brain (Florio & Sternweis, 1985). It is tempting to speculate that N_o may be a coupling factor for receptor-stimulated inositide hydrolysis. In any case, our finding that guanine nucleotides can stimulate the hydrolysis of phosphoinositides in isolated cortical membranes suggests that phosphoinositide phosphodiesterase(s) can be activated by guanine nucleotides. Thus, inositide hydrolysis in brain appears to have control or regulatory mechanisms similar to what has been found in other cell types. Additional studies will be required to determine if this is a direct activation of the enzyme(s) and/or whether it occurs through some regulatory protein which is related to receptor-activated phosphoinositide hydrolysis.

This work was supported by a grant from NIAAA AA06069. R. A. G. is the recipient of an NRSA from NIAAA (AA05208).

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Received 23 May 1985/13 August 1985; accepted 20 August 1985