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G-protein involvement in central-nervous-system muscarinic-receptor-coupled polyphosphoinositide hydrolysis

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Potentiation of muscarinic-agonist-stimulated polyphosphoinositide (PPI) hydrolysis was demonstrated in a rat cerebral-cortical membrane preparation prelabelled with *myo*-[^{3}H]inositol. Accumulation of *myo*-[^{3}H]inositol 1,4,-bisphosphate ([^{3}H]IP_2) was used to assess brain [^{3}H]phosphatidylinositol 4,5-bisphosphate hydrolysis as its immediate metabolite, *myo*-[^{3}H]inositol 1,4,5–trisphosphate, was rapidly hydrolysed to [^{3}H]IP_2. Inclusion of ATP (100 μM) and Mg^{2+} (5 mM) in the assay medium was necessary to demonstrate the effect of GTP analogues on carbachol-stimulated brain [^{3}H]PPI turnover. Carbachol (100 μM) induced only a small increment in [^{3}H]IP_2 accumulation (142% of control) in 1 min. However, its effect was markedly enhanced, to 800% and 300% of control, by 100 μM-guanosine 5′-[γ-thio]triphosphate (GTP[S]) and guanosine 5′-[$\beta\gamma$-imido]triphosphate (p[NH]ppG) respectively. GTP[S] and p[NH]ppG also stimulated [^{3}H]IP_2 accumulation by over 500% and 200% of control, respectively. The GTP-analogue-potentiated carbachol effect was antagonized by 10 μM-atropine, whereas the GTP-analogue stimulation was unaffected. This report confirms the involvement of a G (GTP-binding) protein(s) in brain PPI metabolism and provides new evidence for the role of G protein(s) in the coupling of stimulated muscarinic receptors to PPI hydrolysis in the central nervous system.

INTRODUCTION

Agonist stimulation of receptors coupled to polyphosphoinositide (PPI) hydrolysis leads to phosphodiesteratic cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC) to generate two second messengers, diacylglycerol and *myo*-inositol 1,4,5-trisphosphate (IP_3) (Berridge, 1984). Subsequent results suggest that a GTP-binding protein(s) (G protein) is involved in the coupling of activated receptors to hydrolysis of PPI by PLC (Smith *et al.*, 1986; Merritt *et al.*, 1986; Pfeilschifter & Bauer, 1987). However, there are only sparse data indicating that similar processes are involved in the coupling of receptors to PLC in the central nervous system (CNS).

Detailed elucidation of the mechanism(s) involved in CNS neurotransmitter-receptor-coupled PPI hydrolysis has been hindered by the lack of a suitable membrane preparation for this purpose. Gonzales & Crews (1985) and Jope *et al.* (1987) demonstrated GTP-dependent activation of PPI hydrolysis in prelabelled rat brain membrane preparations, but potentiation of agonist-induced activation of PLC by GTP or its non-hydrolysable analogues was either not attempted or not demonstrated. Hydrolysis of exogenous [^{3}H]PIP_2 was also shown to be markedly enhanced by p[NH]ppG and NaF in rat brain membranes (Litosch, 1987), but again there was no evaluation of whether p[NH]ppG potentiated agonist-induced hydrolysis of [^{3}H]PIP_2. Evidence from receptor-binding studies suggests that muscarinic-agonist-induced receptor activation in CNS involves a GTP-dependent mechanism (Evans *et al.*, 1985). In the present study we report the occurrence of GTP-dependent potentiation of muscarinic-stimulated PPI hydrolysis by PLC in a rat cerebral-cortical membrane preparation, a finding which provides stronger evidence for the involvement of a G protein(s) in the modulation of CNS muscarinic signal transduction.

MATERIALS AND METHODS

Materials

myo-[^{3}H]Inositol (12–17 Ci/mmol), [^{3}H]IP (2–10 Ci/mmol), [^{3}H]IP_2 (2–10 Ci/mmol), [^{3}H]IP_3 (1–5 Ci/mmol) and [^{3}H]IP_4 (1–5 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Carbachol, GDP, GTP, GTP[S], p[NH]ppG, GDP[S], AMP, ADP, ATP and adenosine tetraphosphate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were of analytical grade. Lithium–Krebs-Ringer bicarbonate buffer (Li-KRB, pH 7.4) consisted of the following: NaCl, 108 mM; KCl, 4.7 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 26 mM; glucose, 11 mM; EDTA, 1 mM; LiCl, 10 mM. Tris/HCl buffer (10 mM, pH 7.5) contained the following: $CaCl_2$, 1 μM; $MgCl_2$, 5 mM; EDTA, 0.25 mM; ATP, 0.1 mM; LiCl, 10 mM (Guillon *et al.*, 1986).

Preparation of brain membranes and assay of PPI hydrolysis

Male Wistar rats (300–400 g; Charles River, Montreal, Canada) were killed by decapitation, and the brains were rapidly dissected on ice. Cerebral-cortical mini-prisms

Abbreviations used: IP, *myo*-inositol 1-monophosphate; IP_2, *myo*-inositol 1,4-bisphosphate; IP_3, *myo*-inositol 1,4,5-trisphosphate; IP_4, *myo*-inositol 1,3,4,5-tetrakisphosphate; PPI, polyphosphoinositide; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; p[NH]ppG, guanosine 5′-[$\beta\gamma$-imido]triphosphate; GTP[S], guanosine 5′-[γ-thio]-triphosphate; PLC, phospholipase C; G protein, GTP-binding protein; CNS, central nervous system.

($350\ \mu m \times 350\ \mu m$) were prelabelled in fresh Li-KRB containing *myo*-[^{3}H]inositol (5.7 μCi/ml) with aeration at 37 °C for 90 min as previously described (Li *et al.*, 1988). After incubation, cortical mini-prisms were rinsed with 3×15 ml of cold Tris/HCl buffer, then homogenized manually with a glass homogenizer (five strokes) at 4 °C and then centrifuged at 1000 *g* for 20 min at 4 °C. The supernatant was decanted and the pellet washed with 3×10 ml of Tris/HCl buffer by vortex-mixing and centrifugation. The pellet, comprising cortical membranes, was resuspended in the same buffer to a final protein concentration of 1.0–1.5 mg/ml.

Brain [^{3}H]PPI hydrolysis was initiated by transferring portions (0.26 ml) of membrane preparation into glass tubes (15 mm × 85 mm) containing the respective test agents in a final incubation volume of 0.29 ml. The samples were incubated for 1 min at 37 °C with gentle agitation without aeration in a metabolic shaker, and the reaction was terminated by the addition of 0.3 ml of ice-cold trichloroacetic acid (10%, w/v). The acidified samples were then centrifuged at 12000 *g* for 15 min at 4 °C. The [^{3}H]inositol phosphates in the supernatant were determined by h.p.l.c. as described below. The acid-precipitated pellets were solubilized in 0.5 ml of 0.3 M-NaOH, and the protein content was determined by either the Lowry *et al.* (1951) or Coomassie Blue methods (Sedmak & Grossberg, 1977), with bovine serum albumin as standard.

Determination of [^{3}H]inositol phosphates

Supernatants of the tricholoroacetic acid-treated samples were each washed with 3×3 ml of water-saturated ether to remove the acid. The final aqueous samples were neutralized with 1 M-NH_3, freeze-dried, and the residues were reconstituted in 0.1 ml of water containing 25–50 μg each of the following reference standards: AMP, ADP, ATP and adenosine tetraphosphate. A 50 μl portion of sample was injected into the h.p.l.c. for the analysis of [^{3}H]inositol phosphates.

The [^{3}H]inositol phosphates were separated on a pre-packed Partisil 10-SAX column (25 cm × 0.46 cm i.d.) (Whatman, Clifton, NJ, U.S.A.), by using a stepwise-generated gradient mobile phase of water to 2 M-ammonium formate (buffered to pH 3.45 with H_3PO_4) for 50 min at a flow rate of 0.8 ml/min (35 °C) (Batty *et al.*, 1985). The retention times for the various adenine nucleotide chromatographic reference standards, as determined by their u.v. absorbances at 254 nm, were 15, 20, 31 and 38 min respectively. Serial samples (0.8 ml) of the eluates were collected with a Gilson 201 fraction collector (Villiers LeBel, France) and the radioactivity in each sample was determined by liquid-scintillation spectrometry. The retention times for authentic standards of *myo*-[^{3}H]inositol, [^{3}H]IP, [^{3}H]IP_2, [^{3}H]IP_3 and [^{3}H]IP_4 were 4, 14, 21, 33 and 40 min respectively.

[^{3}H]Inositol phosphate concentrations of samples were expressed as d.p.m./mg of protein. The concentrations of the various [^{3}H]inositol phosphates determined in samples without incubation were defined as background [^{3}H]inositol phosphates. For subsequent data analysis, these background concentrations were subtracted from corresponding [^{3}H]inositol phosphate concentrations obtained from incubated samples. Data were expressed as means ± S.E.M. for the numbers (*n*) of independent experiments performed. Comparison of differences between sample group means was performed by Student's *t* test for paired samples, and $P < 0.05$ was regarded as statistically significant.

RESULTS

Assay conditions

Under the PPI-hydrolysis assay conditions as described by Gonzales & Crews (1985), GTP[S] (100 μM) induced rapid hydrolysis of PPI in pre-labelled rat brain membranes, as indicated by the accumulation of [^{3}H]IP_2 at 1 min (Table 1). Carbachol (100 μM) did not stimulate [^{3}H]IP_2 accumulation, nor was there any potentiation of carbachol by GTP[S] of the latter accumulation. Moreover, there was substantial intra-assay variability in [^{3}H]IP_2 accumulation values within the control (basal) and carbachol-treated samples. Accumulation of [^{3}H]IP (8465 ± 460 d.p.m./mg of protein, $n = 4$) was not affected by carbachol (100 μM) or GTP[S] (100 μM) at the 1 min incubation time used. In comparison, addition of 100 μM-ATP and 5 mM-$MgCl_2$ to the incubation medium from homogenization through to membrane stimulation, as described previously for peripheral tissues (Litosch *et al.*, 1985; Guillon *et al.*, 1986; Pfeilschifter & Bauer, 1987), decreased the magnitudes of basal and stimulated accumulation of [^{3}H]inositol phosphates, but uncovered a clearly distinguishable potentiating effect of carbachol by GTP[S] on [^{3}H]IP_2 accumulation. GTP[S] (100 μM) induced a 2-fold increase in [^{3}H]IP_2 production compared with basal [^{3}H]IP_2 formation (Table 1). Although 100 μM-carbachol did not stimulate [^{3}H]IP_2 accumulation, its effect was markedly potentiated by 100 μM-GTP[S]. Unlike [^{3}H]IP_2, no changes were evident in [^{3}H]IP concentrations compared with controls in the presence of carbachol or GTP[S] at 1 min of incubation. On the basis of the above findings, ATP and Mg^{2+} appeared to have a stabilizing effect on [^{3}H]IP_2 accumulation, and these reagents were subsequently included in the incubation media for studies of muscarinic-stimulated GTP-dependent PPI hydrolysis in prelabelled rat cerebral-cortical membranes.

Identification of [^{3}H]inositol phosphates

In brain membrane samples stimulated with carbachol plus GTP[S] the chromatogram showed three prominent

Table 1. Brain membrane muscarinic-stimulated GTP[S]-dependent [^{3}H]IP_2 formation in the presence and the absence of ATP and Mg^{2+}

For Method A, incubation buffer contained 10 mM-Tris/HCl, pH 7.5, and 10 mM-Li^+; for method B, incubation buffer contained 10 mM-Tris/HCl, pH 7.5, 100 μM-ATP, 5 mM-Mg^{2+}, 0.25 mM-EDTA and 1 μM-Ca^{2+}. Results are expressed as means ± S.E.M. for four determinations on a pooled rat brain membrane preparation. Values in parentheses represent coefficients of variation (as percentages).

Treatment	[^{3}H]IP_2 (d.p.m./mg of protein)	
	Method A	Method B
Control	4650 ± 1570 (78)	1160 ± 81 (14)
Carbachol (100 μM)	3200 ± 1350 (84)	930 ± 33 (7)
GTP[S] (100 μM)	8430 ± 280 (7)	1780 ± 104 (12)
Carbachol (100 μM) + GTP[S] (100 μM)	8240 ± 530 (13)	2600 ± 156 (12)

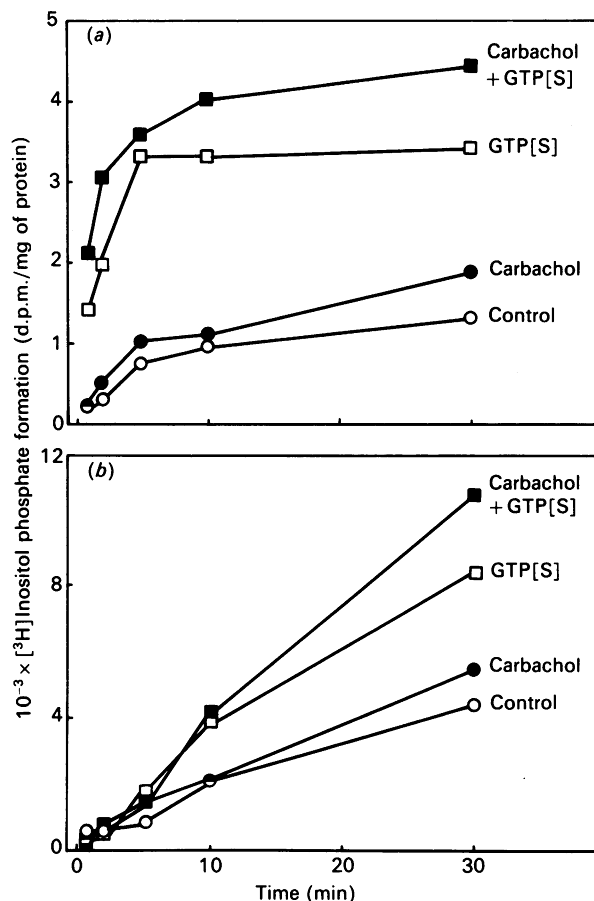

Fig. 1. Time courses for agonist-stimulated GTP-dependent formation of [^{3}H]inositol phosphates in brain membranes

[^{3}H]IP$_2$ (*a*) and [^{3}H]IP (*b*) were determined simultaneously in each sample. Carbachol and GTP[S] concentrations were both 100 μM. Each point represents the average of duplicate determinations in a single experiment. Similar results were obtained from two other separate experiments.

peaks, which were eluted with retention times identical with those of *myo*-[^{3}H]inositol, [^{3}H]IP and [^{3}H]IP$_2$ (see the Materials and methods section) respectively. Accumulation of [^{3}H]IP$_3$ and [^{3}H]IP$_4$ was not observed to occur with any consistency, and the responses for these two compounds were too low [their signals varied from 1 to 4 times background (25 d.p.m.)] to be reliably estimated.

To evaluate whether the observed [^{3}H]IP$_2$ responses induced by carbachol and GTP[S] were indicative of [^{3}H]IP$_3$ turnover, hydrolysis of [^{3}H]IP$_3$ (0.01 μCi) was studied in unlabelled brain membranes. Within 1 min of incubation, most of the added [^{3}H]IP$_3$ was rapidly hydrolysed to [^{3}H]IP$_2$ (74.5 ± 1.4%, $n = 4$), and a small degree of conversion into [^{3}H]IP (3.2 ± 0.2%, $n = 4$) was also evident within this time interval. There was no observable [^{3}H]IP$_4$ formation. Conversion of [^{3}H]IP$_3$ into [^{3}H]IP$_2$ did not occur in trichloroacetic acid-deactivated samples.

Appearance of [^{3}H]inositol phosphates after carbachol and GTP[S] stimulation

Prelabelled rat brain membranes were incubated with carbachol (100 μM) and/or GTP[S] (100 μM) for 0–30 min, and the accumulation of [^{3}H]IP$_2$ and [^{3}H]IP was determined at the times shown in Fig. 1. Basal [^{3}H]IP$_2$ accumulation occurred rapidly over the first 5 min interval, followed by a slower rate of accumulation up to 30 min. Carbachol (100 μM)-induced [^{3}H]IP$_2$ accumulation was not evident until 30 min. GTP[S] induced rapid and marked accumulation of [^{3}H]IP$_2$, and the maximum effect was observed by 5 min (332 ± 44 % of control; $n = 3$ independent experiments performed in duplicate). In the presence of GTP[S], carbachol stimulation of [^{3}H]IP$_2$ accumulation was markedly enhanced at 1 and 2 min (655 ± 157 and 673 ± 147 % of control, respectively) compared with carbachol treatment alone (110 ± 7 and 118 ± 14 % of control, respectively), but the degree of potentiation was decreased thereafter (Fig. 1*a*).

Basal [^{3}H]IP accumulation increased linearly and considerably more slowly than for [^{3}H]IP$_2$ over the 30 min interval studied. Carbachol did not have any stimulatory effect on [^{3}H]IP formation at the various times studied, whereas the stimulatory effect of GTP[S] on [^{3}H]IP production was apparent only after 10 min (179 ± 14 % of control; $n = 3$), and this effect continued up to 30 min (176 ± 19 % of control). Potentiation of carbachol stimulated [^{3}H]IP formation by GTP[S] was only observed at 30 min (248 ± 15 % of control; Fig. 1*b*). Since GTP[S] potentiation of muscarinic-stimulated [^{3}H]PPI hydrolysis occurred very rapidly in brain membranes, as reflected by [^{3}H]IP$_2$ accumulation, the 1 min incubation time was selected for more detailed characterization of the effects of guanine-nucleotide and muscarinic stimulation on [^{3}H]inositol phosphate formation in this preparation.

Guanine-nucleotide potentiation of carbachol-stimulated [^{3}H]IP$_2$ accumulation

Carbachol (100 μM) caused a small and statistically insignificant increase ($P > 0.1$) in [^{3}H]IP$_2$ production (142 % of control) in rat brain membranes (Fig. 2). This small carbachol effect did not appear to be altered by the addition of 10 μM-atropine to the brain membranes, nor did atropine alone have any statistically significant ($P > 0.2$) effect on basal [^{3}H]IP$_2$ accumulation. Treatment of brain membranes with 100 μM-GTP[S] resulted in a marked increase (522 ± 30 %) in [^{3}H]IP$_2$ production over basal values. Furthermore, this GTP analogue significantly potentiated the effect of carbachol on [^{3}H]IP$_2$ accumulation (823 ± 64 % of control). This latter effect was completely abolished in the presence of 10 μM-atropine, whereas the GTP[S]-stimulated [^{3}H]IP$_2$ accumulation was unaffected by this muscarinic antagonist. p[NH]ppG (100 μM) stimulated [^{3}H]IP$_2$ accumulation by over 2-fold (control, 548 ± 33; p[NH]ppG, 1210 ± 147 d.p.m./mg of protein; $n = 3$) in brain membranes, and the effect of carbachol (100 μM) on [^{3}H]IP$_2$ production was also markedly potentiated by p[NH]ppG (carbachol 638 ± 65; carbachol + p[NH]ppG, 1676 ± 201 d.p.m./mg of protein; $n = 3$). GTP (100 μM), and GDP (100 μM) either alone or in combination with carbachol, did not have any significant effect on [^{3}H]IP$_2$ formation. In contrast with the effects of carbachol and/or GTP[S] on [^{3}H]IP$_2$ formation, there were no statistically significant changes in [^{3}H]IP formation at 1 min after any of the above treatments (results not shown).

The rate-limiting step in adenylate cyclase activation by the α-subunit (G_α) of G protein is the exchange of bound GDP for GTP by G protein (Iyengar & Birnbaumer, 1987). We attempted to abolish the stimulation

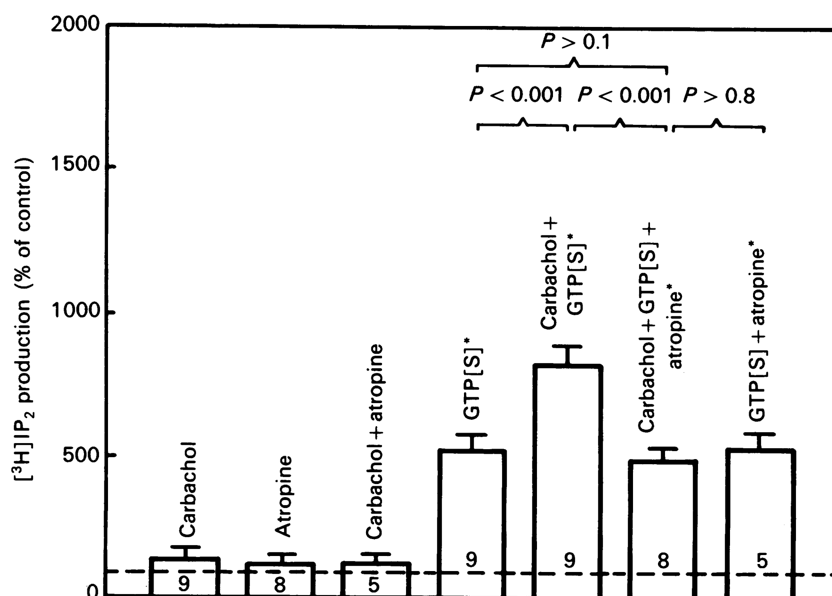

Fig. 2. GTP[S] potentiation of muscarinic-stimulated $[^3H]IP_2$ formation in prelabelled brain membranes

Samples were incubated for 1 min in the absence or the presence of the various drugs. Carbachol and GTP[S] concentrations were 100 μM; that of atropine was 10 μM. Results are expressed as means $\pm$ S.E.M. for the numbers of independent determinations indicated in each column. The broken line indicates the control value (100%); $*P < 0.001$ compared with carbachol, atropine or carbachol + atropine.

of $[^3H]IP_2$ accumulation induced by GTP[S] by using the stable GDP analogue GDP[S]. We observed a small stimulation of $[^3H]IP_2$ accumulation by incubating brain membranes with GDP[S] (100 and 1000 μM), whereas this GDP analogue did not antagonize the effect of GTP[S] (data not shown). This effect of GDP[S] on $[^3H]IP_2$ formation was similar to results reported by Gonzales & Crews (1985), that GDP caused a small increase in [^{3}H]inositol phosphate formation in brain membranes. The observed GDP[S] effect in our experiments may be attributable to the activation of PLC by some non-specific components derived from metabolic degradation of GDP[S], a concern supported by our finding (results not shown) of a number of unidentified peaks on h.p.l.c. analysis of the GDP[S]-treated membrane samples compared with the GDP[S] stock that we used.

DISCUSSION

We employed muscarinic stimulation of [^{3}H]PPI hydrolysis in rat brain cortical membranes as a model to confirm the involvement of a G protein(s) in CNS agonist-induced receptor coupling to PLC activation. In this preparation, non-hydrolysable GTP analogues markedly potentiated carbachol stimulation of [^{3}H]PPI hydrolysis, as reflected in the synergistic increase in $[^3H]IP_2$ accumulation after these combined treatments. These results suggest that carbachol and GTP[S] (and also p[NH]ppG) act at different loci in the chain of muscarinic-receptor-coupled activation of PPI hydrolysis. The finding that atropine blocked the potentiation of carbachol by GTP[S], without affecting GTP[S] stimulation of $[^3H]IP_2$ accumulation, also supports this conclusion. Taken together, these observations provide additional evidence indicating G-protein coupling of muscarinic receptors to PLC activation.

Although it was initially expected that GTP-dependent coupling of muscarinic-activated [^{3}H]PPI hydrolysis would be best reflected functionally in $[^3H]IP_3$ formation (Batty *et al.*, 1985), this was not found to be the case. The explanation for this is likely to lie in factors specific to the brain cortical membrane preparation and the assay conditions employed. Consistent changes in $[^3H]IP_3$ and $[^3H]IP_4$ accumulation could not be identified, as the signal-to-noise ratios for these [^{3}H]inositol phosphates were very low and variable. It is highly unlikely that our inability to quantify $[^3H]IP_3$ (and $[^3H]IP_4$) was related to very low yield of the analytical procedures, as recoveries of $[^3H]IP_3$ processed through the entire procedure exceeded 70%. Furthermore, addition of phytic acid hydrolysate or Tris/maleate as carriers (Wreggett *et al.*, 1987) did not improve the detection of these ^{3}H-labelled compounds. As the prelabelling was performed under conditions which promote labelling of the PPI pool (Gonzales & Crews, 1985; Jope *et al.*, 1987), it is also unlikely that the specific radioactivity of the membrane PPI pools labelled with *myo*-[^{3}H]inositol was insufficient to permit estimation of $[^3H]IP_3$ formation. Since exogenous $[^3H]IP_3$ was found to be rapidly hydrolysed (75%) within 1 min when incubated with the brain membranes, it is most likely that $[^3H]IP_3$ formed *in situ* was very rapidly dephosphorylated by IP_3 phosphomonoesterase to $[^3H]IP_2$ (Storey *et al.*, 1984; Erneux *et al.*, 1986; Batty & Nahorski, 1987; Hansen *et al.*, 1987). Furthermore, $[^3H]IP_4$ was also not detected in the brain membrane preparation, so that phosphorylation of $[^3H]IP_3$ to $[^3H]IP_4$ (Irvine *et al.*, 1985) does not explain the low signal for the former inositol polyphosphate.

Although it is possible that some portion of the $[^3H]IP_2$ measured may derive directly from [^{3}H]PIP hydrolysis, this fraction is not likely to be significant, given the findings that exogenous [^{3}H]PIP hydrolysis in brain membranes is not affected by p[NH]ppG or NaF (Lit-

osch, 1987). In addition, the Ca^{2+} concentrations employed in our incubation medium (1 μM) are far too low to permit ATP-enhanced PI hydrolysis as previously described (Huang & Sun, 1988). Finally, in our preparation the addition of ATP and Mg^{2+} substantially decreased basal [^{3}H]IP_2 accumulation, a finding which also argues against an enhancing effect of ATP on PPI hydrolysis in the present system. However, it is possible that [^{3}H]PI hydrolysis may have contributed to some extent to [^{3}H]IP formation in our preparation. This might account for the fact that [^{3}H]IP accumulation was much less sensitive in discriminating GTP[S] potentiation of the carbachol effect.

In the initial phase of this study, we used the membrane preparation and incubation conditions described by Gonzales & Crews (1985), followed by extraction and determination of [^{3}H]inositol phosphates by a more specific and sensitive h.p.l.c. method (Batty *et al.*, 1985). However, with these conditions we only observed GTP[S]stimulation of [^{3}H]IP_2 accumulation, whereas carbachol failed to produce a response, nor was there any observable potentiating effect by GTP[S]. Inclusion of 100 μM-ATP and 5 mM-Mg^{2+} in the incubation medium decreased but stabilized both basal and stimulated [^{3}H]IP_2 accumulation in the brain membrane preparation. Furthermore, the effect of GTP[S] on carbachol-stimulated [^{3}H]PPI hydrolysis was well discriminated under these modified conditions. These observations concur with previous findings (Litosch *et al.*, 1985), leading these latter authors to propose that ATP might exert its effect through a membrane protein phosphorylation/dephosphorylation process affecting PIP_2 hydrolysis by PLC. The requirement for Mg^{2+} (0.5–5 mM) in GTP-dependent [^{3}H]PIP_2 hydrolysis in rat brain and peripheral tissue membrane preparations has been previously demonstrated (Guillon *et al.*, 1986; Litosch, 1987). The role of Mg^{2+} in GTP-dependent activation of PLC may be analogous to the effect of this metal ion to increase the rate of G_s (stimulatory G protein) activation and GTP binding (Gilman, 1987). Regardless of the exact mechanism of action, inclusion of ATP and Mg^{2+} in the incubation medium during agonist stimulation was essential in our preparation for the demonstration of GTP-dependent potentiation of carbachol-stimulated [^{3}H]IP_2 accumulation.

Accumulation of [^{3}H]IP in prelabelled rat brain membranes did not reflect the characteristic rapid turnover of IP_3 upon stimulation with agonist and/or GTP[S] (Fig. 1*b*). Thus the monitoring of [^{3}H]IP_2 accumulation, and not that of [^{3}H]IP, appears to be more indicative of agonist and/or GTP[S] stimulation of hydrolysis of prelabelled brain membrane PPI.

In summary, we have demonstrated indirectly the involvement of G protein(s) in the regulation of muscarinic-stimulated [^{3}H]PPI hydrolysis in a prelabelled crude rat brain membrane preparation. In this system, accumulation of [^{3}H]IP_2 appears to be a reliable index of [^{3}H]PPI turnover, as the [^{3}H]IP_3 formed is likely to be rapidly dephosphorylated to [^{3}H]IP_2. Inclusion of ATP and Mg^{2+} in the incubation medium was found necessary for the demonstration of potentiation by GTP[S] and p[NH]ppG of the agonist effect on PPI hydrolysis. The modified membrane preparation and assay conditions which we have developed should allow more detailed investigation of the functional role of G proteins in the modulation of neurotransmitter-receptor-activated PPI hydrolysis in CNS.

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REFERENCES

Batty, I. & Nahorski, S. R. (1987) Biochem. J. **247**, 797–800
Batty, I., Nahorski, S. R. & Irvine, R. F. (1985) Biochem. J. **232**, 211–215
Berridge, M. J. (1984) Biochem. J. **220**, 345–360
Erneux, C., Delvaux, A., Moreau, C. & Dumont, J. E. (1986) Biochem. Biophys. Res. Commun. **134**, 351–358
Evans, T., Hepler, J. R., Masters, S. B., Brown, J. H. & Harden, T. K. (1985) Biochem. J. **232**, 751–757
Gilman, A. G. (1987) Annu. Rev. Biochem. **56**, 615–649
Gonzales, R. A. & Crews, F. T. (1985) Biochem. J. **232**, 799–804
Guillon, G., Mouillac, B. & Balestre, M. N. (1986) FEBS Lett. **204**, 183–188
Hansen, C. A., Johanson, R. A., Williamson, M. T. & Williamson, J. R. (1987) J. Biol. Chem. **262**, 17319–17326
Huang, H. M. & Sun, G. Y. (1988) J. Neurochem. **50**, 366–374
Irvine, R. F., Anggard, E. E., Letcher, A. J. & Downes, C. P. (1985) Biochem. J. **229**, 505–511
Iyengar, R. & Birnbaumer, L. (1987) ISI Atlas Sci.: Pharmacol. 213–221
Jope, R. S., Casebolt, T. L. & Johnson, G. V. W. (1987) Neurochem. Res. **12**, 693–700
Li, P. P., Warsh, J. J., Sibony, D. & Chiu, A. (1988) Neurochem. Res. **13**, 1111–1118
Litosch, I. (1987) Biochem. J. **244**, 35–40
Litosch, I. & Fain, J. N. (1986) Life Sci. **39**, 187–194
Litosch, I., Wallis, C. & Fain, J. N. (1985) J. Biol. Chem. **260**, 5464–5471
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Merritt, J. E., Taylor, C. W., Rubin, R. P. & Putney, J. W., Jr. (1986) Biochem. J. **236**, 337–343
Pfeilschifter, J. & Bauer, C. (1987) Biochem. J. **248**, 209–215
Sedmak, J. J. & Grossberg, S. E. (1977) Anal. Biochem. **79**, 544–552
Smith, C. D., Cox, C. & Snyderman, R. (1986) Science **232**, 97–100
Storey, D. J., Shears, S. B., Kirk, C. J. & Michell, R. H. (1984) Nature (London) **312**, 374–376
Wreggett, K. A., Howe, L. R., Moore, J. P. & Irvine, R. F. (1987) Biochem. J. **245**, 933–934

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