5-Hydroxytryptamine Stimulates Inositol Phosphate Production in a Cell-free System from Blowfly Salivary Glands

EVIDENCE FOR A ROLE OF GTP IN COUPLING RECEPTOR ACTIVATION TO PHOSPHOINOSITIDE BREAKDOWN*

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Phosphoinositide breakdown has been linked to the receptor mechanism involved in the elevation of cytosolic Ca"++. In a cell-free system prepared from [3H]inositol-labeled blowfly salivary glands, 5-hydroxytryptamine stimulated the rapid production of inositol phosphates. Within 30 s of hormone addition, there was a 100% increase in inositol trisphosphate formation, a 70% increase in inositol bisphosphate formation, and a 90% increase in inositol monophosphate formation as compared to control homogenates incubated for the same length of time. 5-Hydroxytryptamine did not stimulate inositol or glycerol phosphoinositide formation. Half-maximal activation of inositol phosphate production was obtained with 0.33 mM 5-hydroxytryptamine. Ethylene glycol bis(β-aminoethoxy ether)-N',N',N',N'-tetraacetic acid (EGTA) (0.3 mM) inhibited the basal formation of inositol phosphates and decreased the net accumulation of inositol bisphosphate and inositol trisphosphate due to hormone as compared to homogenates incubated in the absence of added Ca"++. EGTA, however, had little effect on the per cent stimulation of inositol phosphate production due to hormone.

In homogenates, ATP, GTP or guany1-5'-yl imidodiphosphate (Gpp(NH)p), was required for a hormone effect. Gpp(NH)p, unlike ATP or GTP, increased the basal formation of inositol phosphates. In membranes, GTP, Gpp(NH)p, or guanosine 5'-[(3-O-thio)trisphosphate (GTPγS) sustained a hormone effect whereas ATP was ineffective. GTP did not affect production while Gpp(NH)p and GTPγS increased inositol phosphate production. Half-maximal effects of Gpp(NH)p and GTPγS on hormone-stimulated inositol phosphate formation occurred at 10 μM and 100 nM, respectively. In the presence of 1 mM GTPγS, 5-methylytryptamine stimulated inositol phosphate formation within 2 s in membranes. These results indicate that in a cell-free system, GTP is involved in mediating the effects of Ca"+-mobilizing hormones on phosphoinositide breakdown.

Hokin and Hokin (1), in 1955, demonstrated that cholinergic stimulation of pigeon pancreases increased 32P incorpo-

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1 The abbreviations used are: PtdIns-4-P, phosphatidylinositol 4-phosphate; PtdIns-4,5-P2, phosphatidylinositol 4,5-bisphosphate; Ins-P, inositol monophosphate; Ins-Pp, inositol bisphosphate; Ins-Ps, inositol trisphosphate; EGTA, ethylene glycol bis(β-aminoethoxy ether)-N',N',N',N'-tetraacetic acid; Gpp(NH)p, guany1-5'-yl imidodiphosphate; GTPγS, guanosine 5'-[(3-O-thio)trisphosphate.
lated phosphoinositide breakdown can be readily demonstrated. Previous studies have shown that the blowfly salivary gland system incorporates [3H]inositol primarily into the pool of phosphoinositides degraded in response to hormone stimulation and linked to the entry of Ca^{2+} (28–30). In salivary gland homogenates and cytosolic-depleted membranes obtained from [3H]inositol-prelabeled salivary glands, 5-hydroxytryptamine stimulated breakdown of [3H]inositol-labeled lipid with the accumulation of inositol and inositol monophosphate (24). The direct activation of phosphoinositide breakdown due to hormone has been difficult to consistently reproduce in our laboratory, suggesting that an uncontrolled variable was essential for demonstration of an effective hormone response. The studies reported in this paper demonstrate that under the appropriate conditions, 5-hydroxytryptamine stimulates a rapid accumulation of inositol phosphates that is GTP-dependent.

MATERIALS AND METHODS

Salivary glands were isolated from adult blowflies (Calliphora erythrocephala) 6–10 days post-emergence from the pupal stage. The blowflies were reared for the isolation and incubation of the salivary glands consisted of 150 mM NaCl, 10 mM KCl, 2 mM CaCl_2, 2 mM MgCl_2, 2.7 mM malate, 2.7 mM glutamate, 10 mM Tris/HCl (pH 7.4), and 5 mM glucose. Approximately 100 salivary glands were incubated in 150 μl of incubation medium containing 30 μCi of [3H]inositol (15.5 Ci/mmol). After 2 h, the salivary glands were removed from the labeling medium and rinsed with 50 μl of ice-cold homogenization buffer containing 320 mM sucrose, 0.5 mM Na_2EDTA, 25 mM LiCl, 100 mM Na_2ATP, and 10 mM Tris/HCl (pH 7.8 at 5 °C). ATP was omitted from the homogenization buffer in experiments designed to determine the effects of ATP. The glands were rapidly homogenized between two glass slides (Corning, frosted glass slides) with 2 × 75 μl of homogenization buffer. This procedure produced a rapid and effective disruption of the glands. The homogenate was transferred to a polypropylene tube. As an additional 150 μl of homogenization buffer was used to rinse the glass slides, and this wash was combined with the initial crude homogenate. The crude homogenate was centrifuged for 5–10 min at 500 × g to obtain a uniform particulate matter. The supernatant (homogenate) was removed and the small pellet discarded. Microscopic examination of the homogenate did not reveal the presence of intact cells. Incubation of the homogenate was started by the addition of 25 μl of homogenate to 125 μl of incubation buffer. The [3H]inositol was from New England Nuclear and was stored with 1% HCl. Chloroform (0.5 ml) and H_2O (0.5 ml) were added to each tube with vigorous vortexing. The lower chloroform phase containing labeled phospholipids was dried in vacuo, resuspended in chloroform, and applied to Uniplate Silica Gel H plates (Analtech). Phosphoinositides were separated by a one-dimensional chromatography system employing the solvent system methanol, chloroform, 4 mM NH_4OH, H_2O (90:9:9:19, v/v), as described by Schacht (32). The silica sections corresponding to labeled phosphoinositides were scraped into vials. Two hundred fifty millimeters of a 1% HCl solution was added to the silica and filtered by 4 ml of ethanol/H_2O (2:1, v/v). After 5 min, the washes were combined and 1.25 ml of acidified methanol/chloroform (2:1, v/v) were added to each sample followed by vigorous vortexing. The lipids were dried in vacuo, resuspended in chloroform, and analyzed by thin-layer chromatography. 5-Hydroxytryptamine (10 μM) stimulated an increase in both the rate and degree of inositol phosphate formation at any time point examined.

RESULTS

The data in Fig. 1 demonstrate that incubation of homogenates, obtained from salivary glands prelabeled with [3H] inositol, resulted in the rapid production of labeled inositol monophosphate, inositol bisphosphate, and inositol trisphosphate. These studies were done in the presence of 29 mM LiCl which inhibits phosphatase-mediated breakdown of inositol monophosphate to inositol (18, 33). The formation of labeled inositol phosphates was observed within 15 s of the start of the incubation at 30 °C. Approximately twice as much labeled inositol trisphosphate as inositol monophosphate was formed within 15 s. Glycerol phosphoinositol did not accumulate appreciably until after 30 s of incubation. There was no detectable change in inositol levels over a 150-s incubation of homogenate at 30 °C (data not shown). An appreciable amount of [3H]inositol was present in the aqueous extract that may have been derived from the labeling medium. 5-Hydroxytryptamine (10 μM) stimulated an increase in both the rate and degree of inositol phosphate formation. A lag in the onset of stimulated inositol monophosphate, inositol bisphosphate, or inositol trisphosphate formation was not observed. 5-Hydroxytryptamine did not affect the rate or degree of glycerol phosphoinositol formation at any time point examined.

The effect of 5-hydroxytryptamine on phosphoinositide breakdown, as determined by analysis of the changes in labeled phosphoinositides, is shown in Fig. 2. In homogenates, approximately 96% of the [3H]inositol label was present in phosphatidylinositol, 2% in PtdIns-4-P and 2% in PtdIns-4,5-P_2. There was an initial 25 ± 6% increase in the level of [3H]PtdIns-4,5-P_2 and a 14 ± 5% increase in [3H]PtdIns-4-P within 10 s of incubation (New England Nuclear). This transient increase in the amount of labeled phosphoinositides indicated that the homogenate contained active phosphatidylinositol kinase and PtdIns-4-P kinase. In the presence of 10 μM 5-hydroxytryptamine, there was a net decrease in the amount
Phosphoinositide Breakdown in a Cell-free System

FIG. 1. Time course for 5-hydroxytryptamine-stimulated water-soluble product formation in salivary gland homogenates. Homogenates, prepared from salivary glands prelabeled with [3H]inositol, were incubated for the specified time in the absence (□—□) or presence (○—○) of 10 μM 5-hydroxytryptamine (5-HT) in buffer containing 100 μM ATP. The amount of label in the phosphoinositides at the start of the incubation was 1085, 670, and 43,000 cpm in PtdIns-4,5-P2, PtdIns-4-P, and phosphatidylinositol, respectively. The amount of water-soluble label present in the homogenate prior to the start of the incubation was as follows: inositol, 11,960 ± 740; glycerol phosphoinositol (GPI), 270 ± 60; inositol monophosphate, 130 ± 20; inositol bisphosphate, 20 ± 4; and inositol trisphosphate, 15 ± 6 cpm, respectively. Results are the mean ± S.E. of three experiments.

FIG. 2. Effect of 5-hydroxytryptamine on phosphoinositide breakdown in salivary gland homogenates. Homogenates, prepared from prelabeled salivary glands, were incubated for the indicated time in the absence (□—□) or presence (○—○) of 10 μM 5-hydroxytryptamine (5-HT) as described in Fig. 1. Data are from the same experiments shown in Fig. 1.

FIG. 3. Effect of ATP concentration on inositol phosphate production in salivary gland homogenates. Homogenates, prepared from [3H]inositol-prelabeled glands, were incubated in the absence or presence of 10 μM 5-hydroxytryptamine for 15, 60, and 300 s in standard incubation buffer containing ATP concentrations as indicated. The ATP was added with a 2-fold excess of MgCl2. Results are the mean ± S.E. of the three combined experiments and are expressed as percent of control for each respective time point.

The results shown in Fig. 1 and 2 were obtained in incubation buffer containing 100 μM ATP. The effect of ATP concentration on hormone-stimulated inositol phosphate formation, determined after a 60-s incubation with 5-hydroxytryptamine, is shown in Fig. 3. In the absence of added ATP, there was little effect of 5-hydroxytryptamine on inositol phosphate formation. Increasing the ATP concentration resulted in a dose-dependent potentiation of the effect of 10 μM 5-hydroxytryptamine. Half-maximal potentiation of the 5-hydroxytryptamine effect was observed with approximately 33 μM ATP for inositol trisphosphate, inositol bisphosphate, and inositol monophosphate formation. There was little effect of ATP concentration greater than 100 μM on the potentiation of the hormone effect. A major effect of ATP was to decrease the basal formation of inositol phosphates. In one experiment, the amount of inositol trisphosphate accumulated after a 60-s incubation was 515 cpm in the absence of added ATP. The addition of 1, 10, 100 or 1000 μM ATP to the incubation buffer decreased by 17, 25, 60, and 60% the amount of inositol.
triphosphate accumulated. Comparable changes due to ATP were observed in inositol bisphosphate and inositol monophosphate levels. These effects of ATP were similar whether inositol phosphate levels were measured at 15, 60, or 300 s (data not shown). The net result of ATP addition was therefore to decrease the basal accumulation of inositol phosphates and possibly augment the effect of hormone.

The effect of 5-hydroxytryptamine concentration on inositol phosphate formation is shown in Fig. 4. 5-Hydroxytryptamine produced a concentration-dependent activation of inositol phosphate formation in salivary gland homogenates. An increase in inositol phosphate formation was obtained with 0.33 μM 5-hydroxytryptamine with maximal stimulation of inositol phosphate formation at approximately 1 μM 5-hydroxytryptamine. The maximal increases due to 5-hydroxytryptamine were slightly greater for inositol bisphosphate and inositol trisphosphate than for inositol monophosphate.

In homogenates, 5-methyltryptamine and 5-fluorotryptamine stimulated inositol phosphate formation to a degree comparable to that of 5-hydroxytryptamine (Table I). Similar results have been reported with intact glands (34). In contrast, 7-methyltryptamine which does not stimulate inositol phosphate production in intact glands (data not shown) was ineffective in stimulating inositol phosphate formation in homogenate. These results demonstrate that the cell-free system exhibits agonist specificity with regard to hormone effects on inositol phosphate formation.

The effect of EGTA and Ca²⁺ on hormone-stimulated inositol formation is shown in Table II. As compared to homogenates incubated in the absence of added Ca²⁺, EGTA produced approximately a 70% inhibition of the basal production of inositol phosphates. There was little effect of EGTA on glycerol phosphoinositol production. The net accumulation of inositol bisphosphate and inositol trisphosphate due to hormone was decreased by the addition of EGTA. EGTA had little effect on the net accumulation of inositol monophosphate due to hormone. The percent increase in inositol bisphosphate and inositol trisphosphate formation due to hormone in the presence of 0.3 mM EGTA was comparable to the increase observed with no added Ca²⁺ or added Ca²⁺. These data show that EGTA does not markedly inhibit the ability of hormone to stimulate inositol phosphate production, although the net accumulation of inositol bisphosphate and inositol trisphosphate (in counts/min) is reduced by EGTA.

GTP-binding proteins are involved in the transduction of receptor activation to increased adenylate cyclase activity. Possibly some effects of ATP on potentiating the hormone response in homogenate were due to the rapid conversion of ATP to GTP via nucleotide transphosphorylation. The effect of ATP, GTP, and Gpp(NH)p on basal and 5-hydroxytryptamine-stimulated inositol phosphate production was there-

Table I

| Agonist                | GPI | Ins-P₁ | Ins-P₂ | Ins-P₃ |
|------------------------|-----|--------|--------|--------|
| 5-Hydroxytryptamine    |     |        |        |        |
| 5-Methyltryptamine     |     |        |        |        |
| 5-Fluorotryptamine     |     |        |        |        |
| 7-Methyltryptamine     |     |        |        |        |

The increase in inositol phosphate production due to hormone was significant with p < 0.050.

The increase in inositol phosphate production due to hormone was significant with p < 0.025.

The increase in inositol phosphate production due to hormone was significant with p < 0.010.

Table II

| Additions | GPI | Ins-P₁ | Ins-P₂ | Ins-P₃ |
|-----------|-----|--------|--------|--------|
| 0.3 mM EGTA | 130 | 36 | 23 | 29 |
| None      | 193 | 106 | 83 | 92 |
| 1 μM Ca²⁺ | 177 | 87 | 74 | 91 |
| 10 μM Ca²⁺ | 170 | 92 | 80 | 99 |

The increase in inositol phosphate formation due to hormone was significantly greater than control with p < 0.005.

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**Fig. 4. Effect of 5-hydroxytryptamine concentration on inositol phosphate production in salivary gland homogenates.** Homogenates, prepared from [3H]inositol-prelabeled salivary glands were incubated in the presence of 100 μM ATP with the indicated additions in the absence or presence of 10 μM 5-hydroxytryptamine for 60 s. Results are the mean ± S.E. of three experiments.
fore examined (Table III). In the absence of ATP, 5-hydroxytryptamine had little effect on inositol phosphate production, consistent with the data shown in Fig. 3. The addition of 100 μM ATP resulted in a depression of basal inositol phosphate production and a 200, 200, and 150% increase in the amount of inositol trisphosphate, inositol bisphosphate, and inositol monophosphate formed due to 5-hydroxytryptamine. GTP, at 100 μM, did not depress the basal formation of inositol phosphates. There was a 2-fold potentiation of the 5-hydroxytryptamine effect in the presence of 100 μM GTP. Gpp(NH)p, a nonhydrolyzable analog of GTP, produced a 2-fold increase in the basal formation of inositol phosphates and a 3-fold increase in the hormone effect.

The effect of guanine nucleotides on basal- and hormone-stimulated inositol phosphate formation in membranes obtained by a 10,000 × g centrifugation of the homogenate is shown in Figs. 5–7. 5-Methyltryptamine was used in these studies since 5-methyltryptamine selectively activates 5-hydroxytryptamine receptors linked to phosphoinositide breakdown, whereas 5-hydroxytryptamine activates 5-hydroxytryptamine receptors as well as 5-hydroxytryptamine receptors which stimulate adenylate cyclase (35, 36). Use of 5-methyltryptamine would avoid complication of the results due to GTP interactions at two receptor sites.

In membranes, there was little effect of ATP on potentiating the 5-methyltryptamine response (Figs. 5–7). GTP had little effect on the basal formation of inositol phosphates, as had been previously observed in homogenates (Fig. 5). GTP, however, markedly increased the effect of hormone on total inositol phosphate production with near-maximal effects observed with 1 μM GTP. Other nucleotides tested were 100 μM GDP and GMP. They were ineffective in supporting a hormone response in membranes (data not shown).

In contrast to GTP, the nonhydrolyzable analog Gpp(NH)p markedly increased the basal levels of inositol phosphates.

### Table III

| Nucleotide | Ins-P₁ | Ins-P₂ | Ins-P₃ |
|------------|--------|--------|--------|
| None       | 98     | 107    | 133    |
| ATP (100 μM) | 88   | 77     | 95     |
| GTP (100 μM) | 106  | 87     | 159    |
| Gpp(NH)p (10 μM) | 185 | 159    | 236    |

The increase in inositol phosphate production due to hormone was significantly greater than control with p ≤ 0.025.

The increase in inositol phosphate production due to hormone was significantly greater than control with p ≤ 0.005.

The increase in inositol phosphate production due to hormone was significantly greater than control with p ≤ 0.010.

The increase in inositol phosphate production due to hormone was significantly greater than control with p ≤ 0.050.

The increase in inositol phosphate production due to hormone was 25 cpm. Results are the mean ± S.E. of three experiments.

(5-MT) or presence (– – –) of 10 μM 5-methyltryptamine (5-MT) with the indicated GTP concentration for 60 s. The amount of label in glycerol phosphinositol after a 60-s incubation in the absence of GTP was 26 cpm while in the presence of 1,000 μM GTP it was 43 cpm. Results are the mean ± S.E. of three experiments.

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The present studies demonstrate that in a cell-free system obtained from ['H]inositol-prelabeled salivary glands, 5-hydroxytryptamine stimulated, in a dose-dependent manner, the rapid formation of inositol monophosphate, inositol bisphosphate, and inositol triphosphate. The hormone sensitivity of this system was comparable to that of intact glands (34, 35). The ability of 5-hydroxytryptamine to stimulate inositol phosphate formation occurred rapidly in membranes.

**DISCUSSION**

The present studies demonstrated that in a cell-free system obtained from ['H]inositol-prelabeled salivary glands, 5-hydroxytryptamine was to stimulate the formation of inositol triphosphate, derived from ['H]PtdIns-4,5-P_2 breakdown, to 200% of the basal inositol triphosphate levels. The formation of inositol monophosphate and inositol bisphosphate was also markedly increased by the addition of 5-hydroxytryptamine (Fig. 1). A linear rate of inositol phosphate formation was not maintained for more than 15 s, and a decline in the enzymatic rate was readily apparent by 30 s. This departure from first-order kinetics suggests that there is a rapid depletion of the hormone-sensitive lipid substrate during incubation of the homogenate to levels where the $V_{max}$ can no longer be sustained.

There was no detectable lag in the production of inositol monophosphate, inositol bisphosphate, or inositol triphosphate when homogenates (Fig. 1) or membranes (Fig. 8) were incubated at 30 °C. Within 2 s of hormone addition, there was an increase in the levels of inositol monophosphate, inositol bisphosphate, and inositol triphosphate (Fig. 8). Both inositol trisphosphate and inositol bisphosphate accumulated to a slightly greater extent than did inositol monophosphate. Since the specific activity of the individual inositol phosphates is not known, it was not possible to establish the enzymatic pathway involved in the conversion of the individual inositol phosphates. However, the simultaneous generation of each inositol phosphate suggests that there may be a rapid phospholipase C-mediated breakdown of the hormone-sensitive pool of phosphoinositides.

Studies with intact blowfly salivary glands have shown that 5-hydroxytryptamine stimulated a rapid and transient increase in labeled inositol bisphosphate and inositol triphosphate. There was a 20-s lag before inositol monophosphate began to accumulate (13). These studies in intact glands were done in the absence of LiCl, and thus, there may have been rapid phosphatase-mediated degradation of inositol monophosphate to inositol. The rapid metabolism of inositol monophosphate to inositol which can be subsequently utilized for the resynthesis of phosphoinositides in intact glands may have precluded an accurate determination of the inositol monophosphate or inositol levels at early time points. Furthermore, small changes in labeled inositol monophosphate or inositol may be difficult to detect in intact glands because of the high basal levels of these compounds.

Analysis of the corresponding lipid changes could give some insight as to the enzymatic events involved in hormone-stimulated phosphoinositide breakdown. However, it was difficult to ascribe significance to the small changes in lipids that occurred at these early time points. In addition, there was appreciable phosphatidylinositol kinase and PtdIns-4-P kinase activity in homogenates that precluded measurements of net loss of labeled lipid. As shown in Fig. 2, incubation of salivary gland homogenates, under standard incubation conditions, resulted in a transient increase in the amount of ['H]PtdIns-4,5-P_2 and ['H]PtdIns-4-P. The level of ['H]polyphosphoinositides then declined presumably as a consequence of increased net hydrolysis by phospholipase C. In the presence of 10 μM 5-hydroxytryptamine, there was a slight net decrease in the amount of labeled polyphosphoinositides and phosphatidylinositol.

The net accumulation of inositol phosphates under basal conditions was markedly inhibited by inclusion of 0.3 mM EGTA in the incubation buffer (Table II). EGTA inhibited, by approximately 70%, the basal formation of inositol phosphates as compared to homogenate incubated in the absence of EGTA. EGTA also inhibited the net accumulation (in counts/min) of inositol bisphosphate and inositol trisphosphate due to hormone. Despite the decreased basal formation...
of inositol phosphates, the per cent increase in inositol phosphate production due to 5-hydroxytryptamine was similar in magnitude to the increase observed in the absence or presence of added Ca++. These results indicate that although EGTA reduces the net accumulation of inositol bisphosphate and inositol triphosphate, it does not markedly inhibit the ability of hormone to stimulate inositol phosphate formation.

Previous studies have shown that 5-hydroxytryptamine stimulated loss of phosphoinositides in the presence of 0.5 mM EGTA within 5 min (24, 34). Similarly, vasopressin (25) and norepinephrine (27) stimulated loss of phosphatidylinositol after a 30-min incubation in buffer containing 0.5 mM EGTA. With these relatively long incubation times, there would have been sufficient phospholipase C activity to allow detection of an effect of hormone on lipid levels.

The results of the present study are consistent with those of Seyfried and Wells (22) who reported that EGTA reduced inositol triphosphate formation in isolated rat liver plasma membranes. In their studies, an effect of vasopressin on [32P]PtdIns-4,5-P2 breakdown was observed if membranes were incubated in medium containing approximately 200 nM Ca++. The reduced accumulation of inositol phosphates due to EGTA may indicate that chelation of Ca++ directly reduces phospholipase C activity. However, it is possible that EGTA exerts its inhibitory effects at sites not linked to the initial breakdown of phosphoinositides by phospholipase C. Hormone-stimulated phosphoinositide breakdown involves receptor activation, possibly a guanine nucleotide binding protein and phospholipase C. The net formation of inositol trisphosphate and inositol bisphosphate may be determined, in part, by the concerted activity of phospholipase C, phosphatidylinositol kinase, and PtdIns-4-P kinase. In addition, EGTA may indirectly affect the activities of enzymes involved in phosphoinositide metabolism. EGTA will chelate Ca++ which is associated with membrane proteins and phospholipids resulting in a change in membrane fluidity. This may have detrimental effects on the activities of the enzymes involved in phosphoinositide breakdown. Further studies are needed to identify the site of action of EGTA.

The phospholipase C mediating phosphoinositide breakdown appears to be membrane-associated since both basal and hormone-stimulated activity was evident in membranes prepared from homogenate. The reduced accumulation in inositol phosphates production due to hormone was comparable to that observed in homogenates. It is not known whether the hormone-stimulated phospholipase C is an integral membrane protein or whether it represents cytosolic phospholipase C which has become associated with the membrane.

GTP was required to obtain a hormone effect on inositol phosphate formation. Initially, it was found that in homogenates, ATP facilitated, in a dose-dependent manner, the effect of 5-hydroxytryptamine (Fig. 3). One obvious effect of ATP was to decrease the basal level of inositol phosphate formation. It is not clear why ATP should depress the formation of inositol phosphates. Possibly ATP may allow the phosphorolysis or dephosphorylation of a protein which regulates the activity of phospholipase C. ATP may also have served as a precursor to GTP via nucleotide transphosphorylation. Early studies on adenylate cyclase had shown that several nucleotides supported a hormone effect in stimulating adenylate cyclase activity. This effect of nucleotides was subsequently shown to occur through the transphosphorylation of ATP and other triphosphates to GTP by nucleotide transferases (37). GTP clearly allowed expression of an effect of 5-hydroxytryptamine on inositol phosphate production in homogenates (Table III). However, in contrast to ATP, GTP did not affect the basal formation of inositol phosphates. The nonhydrolyzable analog of GTP, Gpp(NH)p, produced a marked stimulation of inositol phosphate production with the greatest increase in inositol triphosphate levels. The increase in basal levels of inositol phosphates resulted in an apparent decrease in the effect of 5-hydroxytryptamine in the presence of Gpp(NH)p. The levels of inositol phosphates under basal- and hormone-stimulated conditions were greater in the presence of Gpp(NH)p than in the presence of ATP or GTP (Table III).

In membranes, ATP was no longer an effective nucleotide in supporting a hormone response. This suggests that, in homogenates, ATP may have been converted to GTP via active nucleoside diphosphate kinases present in the cytosol. GTP, Gpp(NH)p, or GTPyS supported a hormone effect. As with the homogenate studies, GTP had little effect on basal inositol phosphate formation while Gpp(NH)p and GTPyS markedly increased the basal levels of inositol phosphates. GTPyS was considerably more potent than Gpp(NH)p in stimulating inositol phosphate production and augmenting the hormone effect. The ability of the nonhydrolyzable analogs to directly activate inositol phosphate production indicates that the effect of nucleotides is exerted at a site involved in the regulation of phospholipase C activity and not simply at a site involved in the binding of hormone to receptor.

These results indicate that GTP, possibly through interaction with a GTP-binding protein, is involved in mediating the effect of 5-hydroxytryptamine on phosphoinositide breakdown. A possible role for GTP in the effects of Ca++-mobilizing hormones on phosphoinositide breakdown has been suggested in the past. The binding of muscarinic cholinergic agonists (38), vasopressin (39), and a,,-adrenergic amines (40) to receptors linked to phosphoinositide breakdown is modulated by guanine nucleotides. Guanine nucleotides stimulate secretion in permeabilized mast cells (41). In permeabilized platelets, guanine nucleotides decreased the Ca++ requirement for induction of secretion due to thrombin (42). Phosphatidylinositol turnover in adipocytes was inhibited by treatment with pertussis toxin which inactivates the guanine nucleotide binding protein linked to the inhibition of adenylate cyclase (43). Pertussis toxin also inhibited the Ca++-dependent histamine release from mast cells (44). These observations suggest that a guanine-nucleotide binding protein may be involved in GTP and hormone effects on phosphoinositide breakdown. The present studies, in a cell-free system, provide the first direct evidence that guanine nucleotides are required for the expression of a hormone effect on phosphoinositide breakdown. It is proposed that, by analogy to the adenylate cyclase system, a GTP-binding protein is involved in mediating the effects of Ca++-mobilizing hormones on phosphoinositide breakdown. GTP modulates the binding of Ca++-mobilizing hormones to their receptors and regulates the activity of the phospholipase C. The GTP-binding protein may be linked to the activation of phospholipase C as is true for adenylate cyclase. Alternatively, a GTP-binding protein may regulate the accessibility of the phosphoinositide substrate by inducing a conformational change in the phosphoinositides, thereby exposing them to hydrolysis by phospholipase C. Further studies will be directed towards characterization of the role of GTP in hormone-stimulated phosphoinositide breakdown.

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