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EFFECT OF GUANINE NUCLEOTIDES ON PHOSPHOLIPASE C ACTIVITY IN PERMEABILIZED PITUITARY CELLS: POSSIBLE INVOLVEMENT OF AN INHIBITORY GTP-BINDING PROTEIN

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SUMMARY: Cultured pituitary cells prelabeled with myo-[2-^{3}H] inositol were permeabilized by ATP^{4-}, exposed to guanine nucleotides and resealed by Mg^{2+}. Addition of guanosine 5'-Ø-(3-thio triphosphate)(GTPγS) to permeabilized cells, or gonadotropin releasing hormone (GnRH) to resealed cells, resulted in enhanced phospholipase C activity as determined by [^{3}H] inositol phosphate (Ins-P) production. The effect was not additive, but the combined effect was partially inhibited by guanosine 5'-Ø-(2-thiodiphospate) (GDPβS) or by neomycin. Surprisingly, addition of GDPβS (100-600 uM) on its own resulted in a dose-related increase in [^{3}H]Ins-P accumulation. Several nucleoside triphosphates stimulated phospholipase C activity in permeabilized pituitary cells with the following order: UTP>GTPγS>ATP>CTP. The stimulatory effect of UTP, ATP and CTP, but not GTPγS or GDPβS, could also be demonstrated in normal pituitary cells suggesting a receptor-activated mechanism. GTP and GTPγS decreased the affinity of GnRH binding to pituitary membranes and stimulated LH secretion in permeabilized cells. These results suggest the existence of at least two G-proteins (stimulatory and inhibitory) which are involved in phospholipase C activation and GnRH action in pituitary cells.

Receptor mediated inositol phospholipid (Ins-PL) hydrolysis is involved in the transduction mechanism of various Ca^{2+}-mobilizing hormones (1,2). Recent evidence suggests that Ins-PL breakdown and G-proteins activation may play a role in the mechanism of pituitary hormones secretion (3-8). The nature of the coupling mechanism of Ca^{2+} mobilizing receptors to

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phospholipase C (PLC) remains unknown. G-proteins have been proposed to couple cell surface receptors to PLC, analogous to G-proteins coupling of the adenylate cyclase system (9). A pertussis toxin-insensitive G protein termed Gp was proposed to be involved in Ins-PL hydrolysis in general and in pituitary cells in particular (3,6,10). Permeabilized cells are the model of choice to analyze the effect of guanine nucleotides on PLC activation (11). Our present study was therefore aimed to investigate the effect of guanine nucleotides on PLC activation in permeabilized pituitary cells.

MATERIALS AND METHODS

Myo-[^{3}H] inositol (14 ci/mmol) was obtained from Amersham. UTP, CTP, ITP and, ATP were obtained from Sigma, and GTPγS and GDPβS from Boehringer, Mannheim. Radioimmunoassay reagents for LH were provided through the NIADDK, NIH. [D-Ser (t-Bu)^6]des-Gly^{10}-GnRH N ethylamide (Buserelin) was provided by Dr. J. Sandow (Hoechst A.G., West Germany). IAP, pertussis toxin was kindly donated by Dr. K. Ase of Kaken Pharmaceutic, Japan.

Anterior pituitary glands from Wistar-derived female rats were used for cell preparation as described (6). For [^{3}H]-inositol labeling, cells (5 X 10^6 cells/plate) were cultured for 3 days in medium 199, 5% horse serum, antibiotics and myo-[2-^{3}H] inositol (5 uci/ml). Cells were then washed twice in Krebs Ringer bicarbonate (KRB) pH 7.4 containing glucose 2 mg/ml and BSA (0.1%). The cells were permeabilized by treatment with micromolar concentration of ATP^{4-}(10), in 20 mM Hepes, 137 mM NaCl, 2.7 mM KCl, 5.6 mM glucose and 0.1% BSA, pH 7.7. Initially, ATP (6 um) was added to the cells in the buffer described above in the presence of EGTA (15 uM) and guanine nucleotides or other nucleotides. After 5 min incubation at 37°C, Mg^{2+} (2 mM) was added for 10 min at 37°C. Thereafter Ca^{2+} containing Hepes buffer (1 mM as above) was added and the cells were incubated for 10 min at 37°C. The reaction was stopped by 1 ml of cold methanol, and [^{3}H]Ins-P were resolved by chromatography as previously described (6). GnRH binding to pituitary membranes was performed as previously described (12) using ^{125}I-Buserelin as the ligand. LH release assay from cultured pituitary cells was performed as previously described (6).

RESULTS AND DISCUSSION

When pituitary cells are prelabeled with [^{3}H] inositol, permeabilized with ATP^{4-} and stimulated during permeabilization with GTPγS, or after sealing with GnRH, activation of PLC was observed (Fig. 1). When both stimulants were present together at maximal concentrations, further increases in [^{3}H]Ins-P was observed, but no additivity was found. Surprisingly, GDPβS which maintains G-proteins in the inactive state (9), stimulated PLC activity in pituitary cells (Fig. 1). Although GDPβS was

stimulatory on its own, when added together with GnRH and GTPγS, GDPβS (150 uM) significantly inhibited the production of [^{3}H] Ins-P by the stimulants (Fig. 1, $p<0.02$). When we lowered the concentration of GDPβS to 100 uM, no consistent stimulatory effect was observed but partial inhibition of the separate effects of GTPγS and GnRH could now be detected (not shown). The dose-response for GTPγS-induced [^{3}H]Ins-P formation is shown in Fig. 2. Maximal accumulation of [^{3}H]Ins-P was observed between 50 and 100 um of the nucleotide. As in Fig. 1., GDPβS stimulated PLC activity in permeabilized pituitary cells, maximal effect being observed at about 600 uM. The data suggest the existence of an inhibitory G-protein to PLC (Gpi) analogous to Gi of the adenylate cyclase system (1,9). Inactivation of Gpi by GDPβS will remove inhibition and will therefore raise the basal levels of PLC activity. Since addition of GTPγS to permeabilized pituitary cells might activate both Gp and Gpi, the net effect will be diminished and hence the smaller effect obtained by GTPγS as compared to GnRH which activates probably only Gp (6).

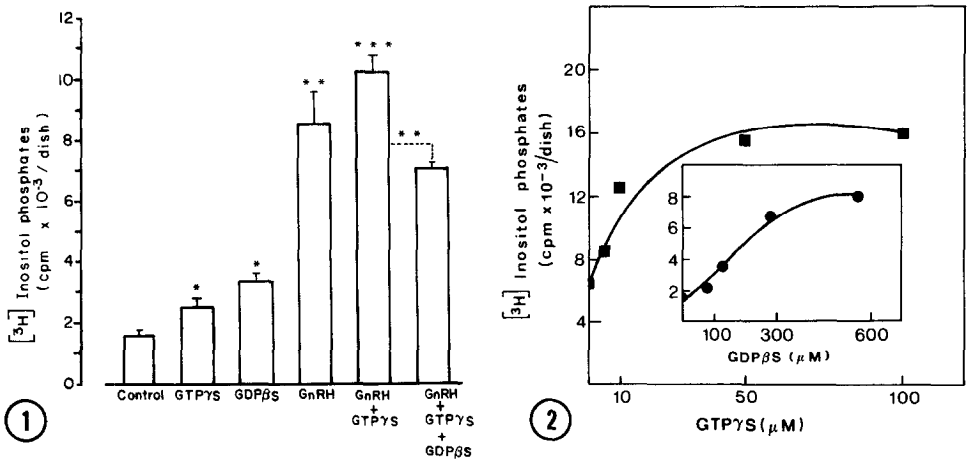

Fig. 1: Effect of GTPγS, GDPβS and GnRH on [^{3}H] inositol-phosphates formation. Pituitary cell cultures were prepared and prelabeled with [^{3}H] inositol as described in Methods. The permeabilization was performed by incubating the cells (5 X 10^6/dish) with ATP^{4-}(6 uM) in the presence of EGTA (15 uM) and the absence of divalent cations for 5 min at 37°C. GTPγS (100 uM) and GDPβS (150 uM) were added during the permeabilization. The permeabilized cells were sealed by incubation with Mg^{2+} (2mM) for 10 min at 37°C. The sealed cells were then treated with or without GnRH (100 nM), in a Hepes buffer containing 1 mM Ca^{2+} and incubation was continued for additional 10 min at 37°C. [^{3}H] inositol phosphate formation was measured after ion exchange chromatography. *$p<0.05$;**$p<0.01$; ***$p<0.001$.

Fig. 2: Dose response study for the effect of GTPγS and GDPβS on phospholipase C activity in permeabilized pituitary cells. For details see legend to Fig. 1.

Neomycin was reported to inhibit the hydrolysis of Ins-PL by PLC (10). When added together with GTPγS and GnRH, neomycin (10 uM) inhibited [^{3}H]Ins-P formation by the stimulants (not shown). Neomycin also inhibited the stimulatory effect of GTPγS but showed no consistent inhibition of the stimulatory effect of GnRH alone.

Further support to involvement of a G-protein in PLC activation in pituitary cells emerges from the observation that NaF stimulated [^{3}H]Ins-P production in cultured pituitary cells (Fig. 3). Since activation of Gi, Gs, and transducin by NaF is potentiated by the presence of Al^{3+}, which by itself has no effect on PLC activity (13,14), we included $AlCl_3$ in the medium. It is thought that the active species interacting with the G-proteins is AlF_4^- which maintains G-proteins in the active state by inhibiting the intrinsic GTPase activity (13,14). We then examined the effect of other nucleoside triphosphates on pituitary PLC activity. At a concentration of 100 uM, UTP was the most potent activator, followed by GTPγS, ATP and CTP (Fig. 4A). The stimulatory effect of UTP, ATP, and CTP might have been exerted also via activation of P_2-purinergic receptors coupled to PLC, as was recently demonstrated in bovine adrenal endothelial cells (15). We therefore investigated the effect of nucleoside triphosphates on PLC activity in intact pituitary cells (Fig. 4B). At a concentration of 100 uM ATP was the most potent activator of PLC ($p<0.001$) followed by UTP ($p<0.02$) and

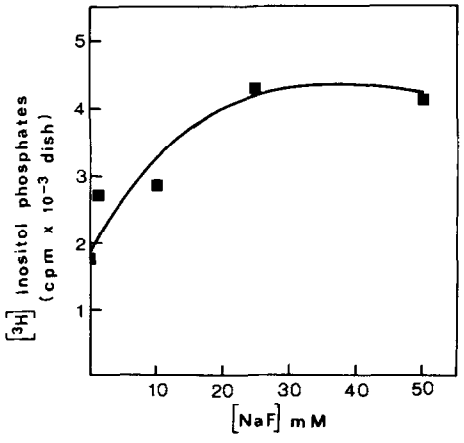

Fig. 3: Effect of NaF on phospholipase C activity in cultured pituitary cells. Prelabeled pituitary cells were exposed to increasing concentrations of NaF in the presence of $AlCl_3$ (100 uM) for 10 min at 37°C. [^{3}H] inositol phosphate formation was measured after extraction and ion exchange chromatography.

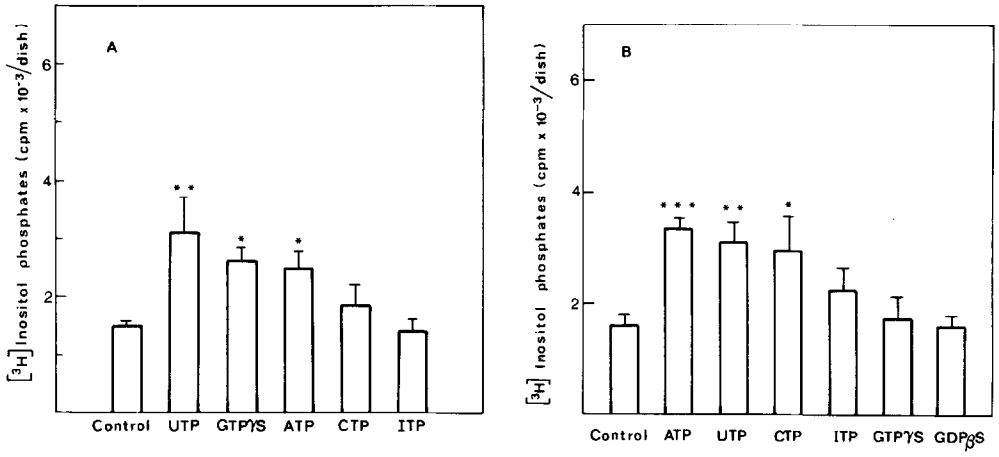

Fig. 4: Effect of various nucleoside triphosphates on phospholipase C activity in permeabilized (A) and normal pituitary cells (B). All nucleotides were added at 100 uM. For further details see legend to Fig. 1.

CTP ($p<0.05$). ITP, GTPγS and GDPβS showed no consistent and significant stimulatory effect. Thus while GTPγS and GDPβS most likely activated directly G-proteins and hence PLC activity in the permeabilized cells, the other nucleoside triphosphates might have exerted their effects via a dual mechanism, a receptor-mediated response (15) and a possible direct activation of a G-protein (16).

To further support the notion that a G-protein is coupled to the GnRH receptor (6, 17) we investigated the effect of guanine nucleotides on GnRH binding (Table 1). The stable GnRH analog Buserelin was used as the ligand (18). Scatchard analysis revealed that GTP and GTPγS induced a ~2.5-fold decrease in the binding affinity of [^{125}I]-Buserelin to pituitary membranes with no significant change in the number of binding sites. The data

Table 1. Effect of GTP and GTPγS on GnRH binding

Treatment	Kd (M)	Bmax (M)
Control	$1.3X10^{-10}$	$5.4X10^{-11}$
GTP (100 uM)	$3.4X10^{-10}$	$6.1X10^{-11}$
GTPγS (100 uM)	$2.7X10^{-10}$	$4.5X10^{-11}$

Pituitary membranes were prepared as described in Methods. [^{125}I] Buserelin (50,000 cpm) was incubated with membranes (20 ug protein) in the presence or absence of GTP or GTPγS (100 uM each) and in the presence of increasing concentrations of Buserelin. The binding assay was performed at 4°C for 90 min. Scatchard analysis was performed using the LIGAND program. Similar results were observed in two experiments.

Table 2. Effect of GTPγS on LH release

Treatment	LH released (ng/ml)	
	EGTA (3 mM)	Ca^{2+} (1 mM)
Control	17.5±3	13.3±3
GTPγS (100 uM)	38.2±4.7**	33 ±4**
GnRH (100 nM)	17 ±1.6	86 ±15**

Permeabilized pituitary cells (as above) were stimulated with or without GTPγS (100 uM) during permeabilization. The cells were then resealed by Mg^{2+} and further incubated with or without GnRH in the presence or absence of added Ca^{2+}. Incubation was then continued for 2 h, at 37°C and the medium was collected and stored at -20°C until assayed for LH by RIA. **p<0.01.

is consistent with modulation by guanine nucleotides of agonist binding to G-proteins coupled receptors (19,20).

We also investigated the effect of GTPγS on LH release in permeabilized pituitary cells. Introduction of GTPγS (100 uM) during permeabilization resulted in enhanced LH release amounting to about 30% of the exocytotic response elicited by GnRH (100 nM) in resealed cells (Table 2). The stimulatory effect of GTPγS, unlike that of GnRH, was Ca^{2+}-independent. Hence, gonadotropin secretion in permeabilized pituitary cells can be elicited by free Ca^{2+} buffers (Ki=0.3 uM, not shown) or alternatively as shown here, by GTPγS in a Ca^{2+}-independent process. It is therefore possible that GTP activation of a G-protein will increase the affinity of the exocytotic response to Ca^{2+}.

The possible existence of Gpi in the pituitary might explain the observation that activation of pituitary D_2 receptors by dopamine results in inhibition of angiotensin II - induced Ins-P formation and prolactin release (21). Activation of Gpi by dopamine will be analogous to activation of pituitary Gi by somatostain leading to inhibition of growth hormone release (22). The existence of a putative Gpi is also inferred from observations that pertussis toxin or GDPβS pretreatment enhanced thrombin stimulated PLC activity in permeabilized platelets (23). Further studies are needed to support the involvement of a G-protein in GnRH action and the proposed existence of Gpi.

In summary our data presented here demonstrate that GTPγS can stimulate PLC activity in permeabilized pituitary cells. Our data suggest a possible involvement of G-protein both in GnRH induced PLC activity and gonadotropin secretion. The early involvement of G-protein in GnRH action is later followed by Ca^{2+} mobilization and protein Kinase C activation (6-8,24-27). We

presented evidence that a putative inhibitory G-protein, Gpi, might complement Gp in regulation of PLC activity in pituitary cells.

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