Inhibition of luteinizing-hormone exocytosis by guanosine 5′-[γ-thio]triphosphate reveals involvement of a GTP-binding protein distal to second-messenger generation

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Dual inhibitory and stimulatory actions of guanine nucleotides on luteinizing-hormone (LH) exocytosis were observed in primary sheep gonadotropes permeabilized with staphylococcal α-toxin. At resting cytosolic $[Ca^{2+}]_{free}$ (pCa 7), 5′-[γ-thio]triphosphate (GTP[S]) and guanosine 5′-[βγ-imido]triphosphate (p[NH]ppG) stimulated rapid LH exocytosis, which was maximal between 5 and 10 min. GTP[S] and p[NH]ppG had similar potencies (50 % of maximum effect at 20–50 μM), but the effect of p[NH]ppG was more prolonged. Experiments carried out in the presence of saturating concentrations of phorbol 12-myristate 13-acetate (PMA), or in PMA-desensitized cells, suggested that stimulation by p[NH]ppG is mediated by a mechanism additional to protein kinase C (PKC) activation. Furthermore, p[NH]ppG stimulated LH exocytosis in the presence of saturating cyclic AMP (cAMP) concentrations, although its effect was less than additive. However, when both PMA and cAMP were present, p[NH]ppG did not stimulate a further increase in the rate of LH exocytosis. In contrast, pretreatment of cells with GTP[S] at low $[Ca^{2+}]_{free}$ markedly inhibited subsequent responses to Ca^{2+}, cAMP, PMA, and cAMP plus PMA. This inhibitory effect required lower GTP[S] concentrations than the stimulatory effect (50 % inhibition at 1–10 μM), and was not observed with p[NH]ppG. A similar inhibition was observed with adenosine 5′-[γ-thio]triphosphate, probably by its conversion into GTP[S]. These results suggest that the stimulatory actions of guanine nucleotides can be accounted for by the combined activation of PKC and generation of cAMP, resulting from activation of conventional signal-transducing GTP-binding proteins. The inhibitory effect of GTP[S] can be clearly distinguished and indicates the involvement of a distinct GTP-binding protein in exocytosis at a site distal to second-messenger generation.

INTRODUCTION

GTP-binding proteins play diverse roles in several cellular processes, including protein synthesis, microtubule assembly, intracellular vesicle trafficking, targeting of secretory proteins to the endoplasmic reticulum, and stimulus–secretion coupling (Gilman, 1987; Allende, 1988; Balch, 1989; Rothman, 1989). A family of heterotrimeric GTP-binding proteins (G-proteins) couple certain cell-surface receptors to plasma-membrane ion channels (Houslay, 1987; Rosenthal *et al.*, 1988) and intracellular effectors such as adenylate cyclase, phospoinositidase C and phospholipases A_2 and D (Gilman, 1987; Burgoyne *et al.*, 1987; Fain *et al.*, 1988; Martin & Michaelis, 1989).

Two lines of evidence have accumulated supporting the involvement of GTP-binding proteins in exocytosis. Firstly, studies on a temperature-dependent yeast mutant with a defect in constitutive exocytosis have identified the gene product SEC4 as a 23.5 kDa *ras*-like GTP-binding protein which associates with yeast secretory vesicles (Salminen & Novick, 1987; Goud *et al.*, 1988). Secondly, intracellular application of GTP analogues has been reported to modulate exocytosis in several cell types. GTP analogues stimulate exocytosis in mast cells (Gomperts, 1983; Howell *et al.*, 1987), platelets (Haslam & Davidson, 1984), adrenal chromaffin cells (Knight & Baker, 1985; Bittner *et al.*, 1986), neutrophils (Barrowman *et al.*, 1986), parathyroid cells (Oetting *et al.*, 1986), RINm5F cells (Vallar *et al.*, 1987), gonadotropes (Andrews *et al.*, 1986) and lactotropes (Sikdar *et al.*, 1989). In some of these studies the GDP β-thio analogue GDP[S] inhibited second-messenger-stimulated exocytosis (Barrowman *et al.*, 1986; Bittner *et al.*, 1986; Vallar *et al.*, 1987). In contrast, GTP analogues have also been reported to inhibit exocytosis in bovine adrenal chromaffin cells (Knight & Baker, 1985) and PC12 cells (Ahnert-Hilger *et al.*, 1987).

We have previously characterized the regulation of luteinizing-hormone (LH) exocytosis by Ca^{2+}, protein kinase C (PKC) and cyclic AMP (cAMP) in primary sheep anterior-pituitary cells permeabilized with *Staphylococcus aureus* α-toxin (van der Merwe *et al.*, 1989, 1990; Macrae *et al.*, 1990). In the present study we report that guanine nucleotides exert dual stimulatory and inhibitory effects on LH exocytosis. The stimulatory effect can be accounted for by activation of PKC together with the generation of cAMP. In contrast, the inhibitory effect of guanosine 5′-[γ-thio]triphosphate (GTP[S]) is exerted distal to the generation of second messengers, therefore implicating a GTP-binding protein which acts at a late stage in the exocytotic pathway.

MATERIALS AND METHODS

Materials

Purified *Staphylococcus aureus* α-toxin (Bhakdi & Tranum-Jensen, 1988) was obtained from Dr. Sucharit Bhakdi (Institute of Medical Microbiology, Justus-Liebig University, Giessen, Germany). Sheep LH (NIADDK-oLH-I-3) and antiserum to it (NIADDK-anti-oLH-1) were kindly provided by the National Hormone and Pituitary Program of the NIADDK (Baltimore,

Abbreviations used: LH, luteinizing hormone (lutropin); ATP[S], adenosine 5′-[γ-thio]triphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate; p[NH]ppG, guanosine 5′-[βγ-imido]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; cAMP, cyclic AMP; EC_{50}, concn. giving 50 % of maximum effect; IC_{50}, concn. giving 50 % inhibition.

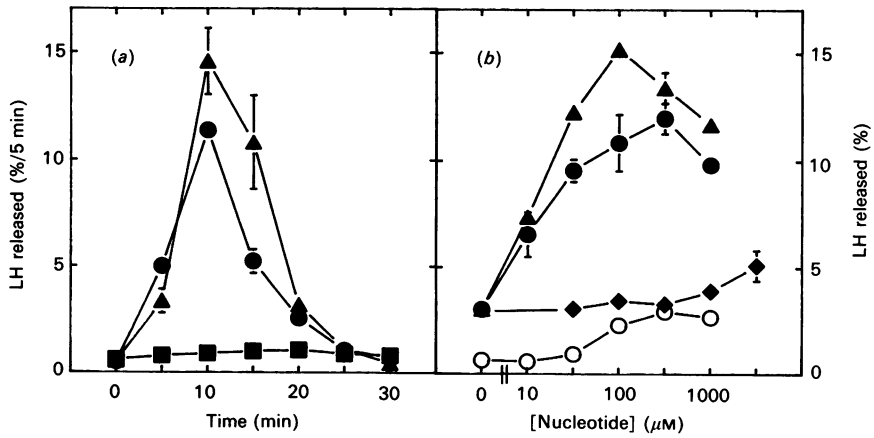

Fig. 1. Stimulatory effects of guanine nucleotides on LH exocytosis

(*a*) Time course. Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 10 mM-CaEGTA (pCa 7) with no guanine nucleotide (■), 300 μM-p[NH]ppG (▲) or 300 μM-GTP[S] (●). LH exocytosis was initiated by replacing with identical stimulation buffer at 37 °C, which was replaced at 5 min intervals. LH release at each time point represents the rate of LH released in the preceding 5 min. The $t = 0$ point represents the rate of LH release per 5 min during the 30 min equilibration period. (*b*) Concentration-dependence. Permeabilized cells were equilibrated at 0 °C for 30 min in buffer IC containing 10 mM-CaEGTA (pCa 7) with 2 mM-$MgCl_2$ (○) or 6.5 mM-$MgCl_2$ plus 6 mM-Na_2ATP (●, ▲, ◆) and the indicated concentration of GTP[S] (○, ●), p[NH]ppG (▲) or GTP (◆). LH exocytosis was initiated by replacing with identical buffer at 37 °C, and LH released after 20 min was determined.

MD, U.S.A.). Adenosine 5′-[γ-thio]triphosphate (ATP[S]), GDP[S], GTP[S] and guanosine 5′-[βγ-imido]triphosphate (p[NH]ppG) were from Boehringer (Mannheim, Germany). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Methods

Cell culture. Primary sheep anterior-pituitary cell cultures were prepared as described previously (van der Merwe *et al.*, 1989). Briefly, fresh pituitaries were dispersed by collagenase digestion, plated at a density of 4×10^5 cells/well in 12-well cell-culture plates (Nunc, Copenhagen, Denmark) and used after 48 h of culture in Minimal Essential Medium containing 10% (v/v) fetal-calf serum (Gibco), penicillin (60 mg/l) and streptomycin (100 mg/l) with CO_2/air (1:19).

Permeabilization and LH-exocytosis stimulation. Anterior-pituitary cells were permeabilized and stimulated as described by van der Merwe *et al.* (1989), with some modifications. Briefly, the cells were washed twice with Buffer I and then once in Ca^{2+}-free Buffer I. Buffer I comprised (mM): NaCl, 140; KCl, 4; $MgCl_2$, 1; $CaCl_2$, 1; glucose, 8.3; Hepes, 20 (pH 7.4); Phenol Red, 6 mg/l; and 0.1% (w/v) BSA. The cells were then permeabilized by incubation for 10 min at 37 °C in intracellular (IC) buffer with 3 μg of α-toxin/ml, 0.5 mM-EGTA, 6.5 mM-$MgCl_2$ and 6 mM-Na_2ATP. IC buffer comprised (mM): sodium propionate, 140; KCl, 4; sodium Pipes, 25 (pH 6.6); Phenol Red, 6 mg/l; and 0.1% BSA. The cell-culture plates were cooled on ice for 10 min and then equilibrated with ice-cold stimulation buffer for 30 min. The stimulation buffer comprised IC buffer with $MgCl_2$ (6.5 mM), Na_2ATP (6 mM) and 10 mM- or 30 mM-CaEGTA buffer with the indicated $[Ca^{2+}]_{free}$. CaEGTA buffers were prepared as described by van der Merwe *et al.* (1989). In all experiments additional nucleotides were added with equimolar $MgCl_2$. LH exocytosis was initiated by warming to 37 °C in a water bath or by replacement with identical medium at 37 °C. After the indicated time (15–30 min) the medium was removed for LH determination.

In experiments examining inhibitory effects of guanine nucleotides, a different protocol was used (Fig. 5). ATP was omitted during the permeabilization and equilibration steps and 2 mM-$MgCl_2$ was present. Cells were equilibrated for 30 min at 0 °C in the presence of guanine nucleotides and then were warmed to 37 °C and preincubated for a further 30 min in the same medium. After preincubation, the medium was removed and the cells were stimulated for 30 min in stimulation buffer (containing 6.5 mM-$MgCl_2$ and 6 mM-Na_2ATP).

Detached cells were removed from all stimulation media by centrifugation (400 *g*, 8 min), and the supernatant was stored at −20 °C until LH determination by radioimmunoassay (van der Merwe *et al.*, 1989). Total cellular LH was measured after solubilizing the cells in Nonidet NP40 (1%, v/v). LH released is expressed as a percentage of the total cellular LH present at the beginning of the stimulation period.

Data presentation. All data shown are representative results from experiments performed two to five times. Data points and error bars represent the mean and range of duplicate determinations. The absence of error bars indicates that the range was smaller than the dimensions of the symbol.

RESULTS

Stimulatory effects of GTP[S] and p[NH]ppG on LH exocytosis

In permeabilized cells buffered at resting cytosolic $[Ca^{2+}]_{free}$ (pCa 7), GTP[S] and p[NH]ppG stimulated rapid LH exocytosis. LH release was maximal between 5 and 10 min and declined to basal levels after 20 min (Fig. 1*a*). Although GTP[S] and p[NH]ppG stimulated LH exocytosis with similar potency (EC_{50} 20–50 μM, $n = 5$), the maximal effect of p[NH]ppG was consistently greater than that of GTP[S] (Fig. 1*b*) and also more sustained (Fig. 1*a*). GTP had little stimulatory effect at concentrations up to 3 mM (Fig. 1*b*). GDP[S] did not stimulate LH exocytosis and inhibited the stimulatory effect of GTP[S] (Table 1). GTP[S]-stimulated LH exocytosis was ATP-dependent (Fig. 1*b*), as has been found for Ca^{2+}-, phorbol ester- and cAMP-stimulated LH exocytosis (van der Merwe *et al.*, 1989; Macrae *et al.*, 1990).

The stimulatory effect of p[NH]ppG consisted of (*a*) an increase in sensitivity to Ca^{2+} (Ca^{2+} EC_{50} was shifted from pCa 5.5 to 6.0)

Table 1. Effect of GDP[S] on GTP[S]-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 30 mM-CaEGTA (pCa 6) and the indicated nucleotides. LH exocytosis was initiated by replacing with identical stimulation buffer at 37 °C, and LH released after 15 min was determined.

	LH released (%)
No addition	6.5±0.1
GTP[S] (300 μM)	28.6±0.9
GDP[S] (2 mM)	4.4±0.8
GTP[S] plus GDP[S]	5.8±0.5

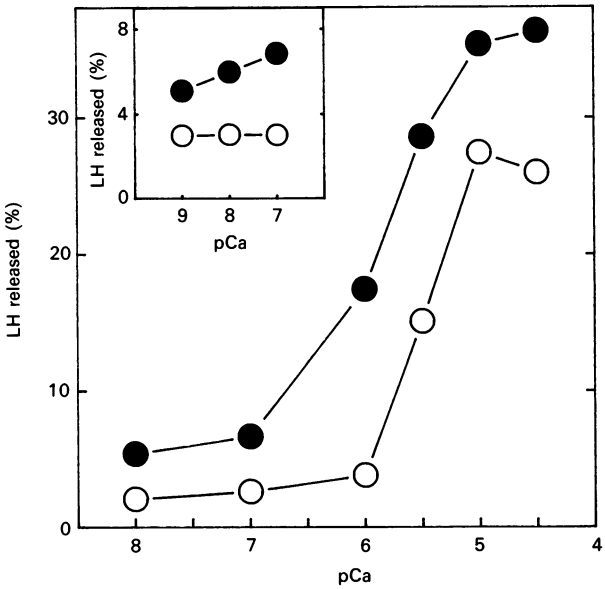

Fig. 2. Effect of $[Ca^{2+}]_{free}$ on p[NH]ppG-stimulated LH exocytosis

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 30 mM-CaEGTA at the indicated $[Ca^{2+}]_{free}$ with (●) or without (○) 300 μM-p[NH]ppG. LH exocytosis was initiated by replacing with identical buffer at 37 °C, and LH released after 20 min was determined. In the inset the experiment was conducted at pH 7.1 to allow buffering of the $[Ca^{2+}]_{free}$ down to pCa 9.

and (*b*) a component which was additive to the effect of Ca^{2+} (Fig. 2). Although diminished, some stimulation by p[NH]ppG was apparent at very low $[Ca^{2+}]_{free}$ (pCa 9; Fig. 2, inset).

A probable mechanism for this stimulatory effect of GTP analogues is through the activation of conventional signal-transducing G-proteins which would result in the generation of diacylglycerol and cAMP. We have previously shown that both the PKC-activating phorbol 12-myristate 13-acetate (PMA) (van der Merwe *et al.*, 1989) and cAMP (Macrae *et al.*, 1990) are able to stimulate LH exocytosis in permeabilized gonadotropes. Furthermore, the stimulatory effects of PMA and cAMP are highly synergistic (Macrae *et al.*, 1990). We therefore examined whether the stimulatory effects of guanine nucleotides result from the generation of these second messengers.

p[NH]ppG was able to stimulate a further increase in LH exocytosis in the presence of maximally effective concentrations of PMA (Fig. 3). In cells desensitized to PMA by prolonged stimulation with high concentrations of PMA, p[NH]ppG was still able to stimulate LH exocytosis (Table 2). Taken together, these findings indicate that p[NH]ppG does not act solely by activation of PKC and suggest that other mechanisms are involved.

GTP[S] was able to stimulate a further increase in LH exocytosis in the presence of maximally effective concentrations of cAMP, although the effects of GTP[S] and cAMP were less than additive (Fig. 3). Therefore, the stimulatory effects of guanine nucleotides are not fully explained either by stimulation of cAMP generation alone or by the isolated activation of PKC. However, it is possible that simultaneous activation of both of these second-messenger pathways could account for the guanine nucleotide effects. In support of this, p[NH]ppG was unable to stimulate further LH exocytosis from cells stimulated with maximal concentrations of cAMP plus PMA (cAMP/PMA) (Table 2).

Inhibitory effects of GTP[S] on LH exocytosis

In contrast with the acute stimulatory effects of guanine nucleotides described above, GTP[S] was found to inhibit LH exocytosis under certain conditions. In cells stimulated with cAMP/PMA, low concentrations of GTP[S] (which were not stimulatory) inhibited LH exocytosis after a lag of 5 min (Fig. 4). In subsequent experiments, cells were preincubated with guanine nucleotides for 30 min at 37 °C in the absence of ATP (see Fig. 5 for an outline of the protocol). The absence of ATP prevented

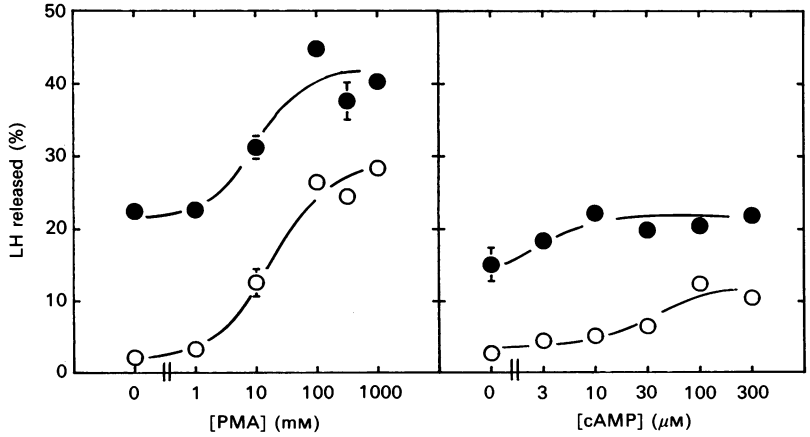

Fig. 3. Effect of p[NH]ppG on LH exocytosis in the presence of PMA or cAMP

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 30 mM-CaEGTA (pCa 7) with (●) or without (○) p[NH]ppG (300 μM) and the indicated concentrations of PMA or cAMP. LH exocytosis was initiated by replacing with identical buffer at 37 °C, and LH released after 20 min was determined.

Table 2. Effects of cAMP, PMA and p[NH]ppG on LH exocytosis in PMA-desensitized cells

Cells were pre-treated with vehicle (dimethyl sulphoxide, 0.13%) or PMA (250 nM) for 18 h. Cells were then washed and permeabilized as usual and then equilibrated at 0 °C for 30 min in stimulation buffer with CaEGTA (30 mM; pCa 7) and the indicated additions. LH exocytosis was initiated by replacing with identical stimulation buffer at 37 °C, and LH released after 20 min was determined. LH release is expressed as a percentage of the cellular LH content immediately before stimulation, which was: dimethyl sulphoxide-pre-treated cells, 920±40 ng/well; PMA-pre-treated cells, 340±10 ng/well. Abbreviation: IBMX, 3-isobutyl-1-methylxanthine.

Treatment	LH released (%)
(*a*) No PMA pre-treatment	
Control	3.7±0.1
PMA (250 nM)	19.0±0.1
p[NH]ppG (500 μM)	11.0±0.3
PMA+p[NH]ppG	23.9±1.2
PMA+cAMP (30 μM) +IBMX (250 μM)	29.3±0.7
PMA+cAMP+IBMX +p[NH]ppG	29.2±2.4
(*b*) PMA-pre-treated	
Control	7.5±0.6
PMA (250 nM)	8.1±0.4
p[NH]ppG (500 μM)	15.2±0.6
PMA+p[NH]ppG	13.3±1.2
PMA+cAMP (30 μM) +IBMX (250 μM)	19.1±1.6
PMA+cAMP+IBMX +p[NH]ppG	17.8±0.9

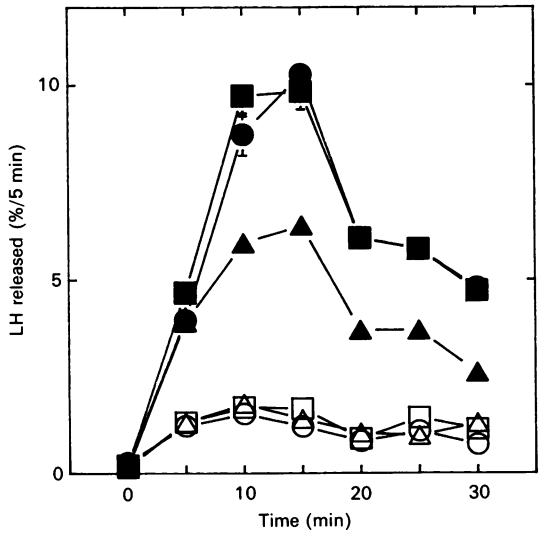

Fig. 4. Time course of inhibition of cAMP/PMA-stimulated LH exocytosis by GTP[S]

Permeabilized cells were equilibrated at 0 °C for 30 min in stimulation buffer containing 10 mM-CaEGTA (pCa 7) with (●, ■, ▲) or without (○, □, △) 3 μM-cAMP plus 10 nM-PMA together with the following guanine nucleotides: none (○, ●), 3 μM-p[NH]ppG (□, ■) or 3 μM-GTP[S] (△, ▲). LH exocytosis was initiated by replacing with identical stimulation buffer at 37 °C, which was replaced at 5 min intervals. LH release at each time point represents the rate of LH released in the preceding 5 min. The $t = 0$ point represents the rate of LH release per 5 min during the 30 min equilibration period.

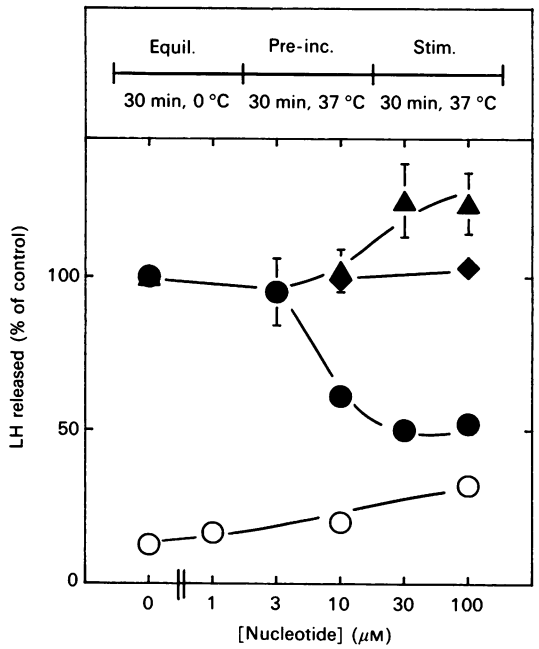

Fig. 5. Inhibitory effects of GTP[S] on Ca^{2+}-stimulated LH exocytosis

An outline of the protocol used in all experiments examining GTP[S] inhibitory effects is shown. Permeabilized cells were equilibrated (Equil.) for 30 min at 0 °C in buffer IC with 2 mM-$MgCl_2$, 10 mM-CaEGTA (pCa 7) and the indicated concentration of GTP (◆), GTP[S] (○, ●) or p[NH]ppG (▲). Cells were then warmed to 37 °C and preincubated (Pre-inc.) for 30 min in the same medium. This medium was removed and replaced with stimulation buffer (Stim.) containing 30 mM-CaEGTA at pCa 5 (◆, ●, ▲) or pCa 7 (○), and LH released after 30 min was determined. To allow the comparison of results from two experiments, LH release is presented as percentage of LH release evoked by pCa 5 in the absence of guanine nucleotides.

exocytosis due to guanine nucleotide stimulation during the preincubation (Fig. 1*b*). After preincubation, cells were stimulated in the presence of ATP but in the absence of guanine nucleotides. Since the inhibitory effect of GTP[S] pretreatment did not require its continued presence during the stimulation period (results not shown), guanine nucleotides were omitted during the stimulation period. This was desirable in order to minimize their stimulatory effects. When this protocol was used, GTP[S] inhibited Ca^{2+}-stimulated LH exocytosis (IC_{50} 10 μM), an effect not observed with p[NH]ppG or GTP at concentrations up to 100 μM (Fig. 5). This inhibition by GTP[S] does not result from depletion of releasable LH during the preincubation, since GTP[S] stimulated minimal LH exocytosis in the absence of ATP (Fig. 1*b*). GTP[S] inhibited cAMP-, PMA- and cAMP/PMA-stimulated LH exocytosis even more potently (IC_{50} 1 μM) (Fig. 6). The inhibitory effect of 30 μM-GTP[S] was prevented by including p[NH]ppG (0.1 mM) and partly prevented by GDP (1 mM) and GTP (1 mM) (Table 3), whereas ATP (6 mM) had no effect (results not shown).

High concentrations of ATP[S] also inhibited both Ca^{2+}-stimulated (IC_{50} 200 μM; Fig. 7*a*) and cAMP/PMA-stimulated (IC_{50} 20 μM; Fig. 7*b*) LH exocytosis. However, this inhibitory effect of ATP[S] was antagonized by UDP (Fig. 7), a competitive inhibitor of nucleoside diphosphate kinase (Seifert *et al.*, 1988), suggesting that inhibition by ATP[S] results from nucleoside diphosphate kinase-mediated synthesis of GTP[S].

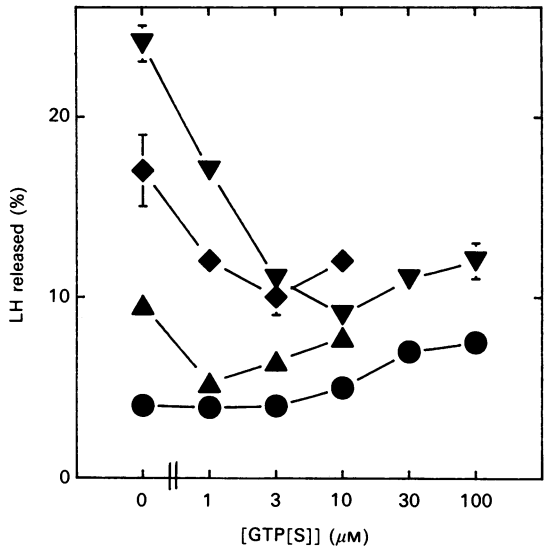

Fig. 6. Inhibitory effects of GTP[S] on cAMP- and PMA-stimulated LH exocytosis

Permeabilized cells were equilibrated for 30 min at 0 °C in buffer IC with 2 mM-$MgCl_2$, 10 mM-CaEGTA (pCa 7) and the indicated concentration of GTP[S]. Cells were then warmed to 37 °C and preincubated for 30 min in the same medium. This medium was removed and replaced with stimulation buffer containing 10 mM-CaEGTA (pCa 7) with no addition (●) or with cAMP (300 μM) (▲), PMA (100 nM) (◆), or cAMP plus PMA (▼), and LH released after 30 min was determined.

Table 3. Antagonism of GTP[S] inhibition by GTP, p[NH]ppG and GDP

Permeabilized cells were equilibrated for 30 min at 0 °C in buffer IC containing 2 mM-$MgCl_2$ and 10 mM-CaEGTA (pCa 7) with the indicated guanine nucleotides. Cells were then warmed to 37 °C and preincubated for 30 min in the same medium. This medium was removed and replaced with stimulation buffer containing 30 mM-CaEGTA at pCa 7 or pCa 5, and LH released after 30 min was determined (n.d., not determined).

Guanine nucleotides present in preincubation	LH released (%)	
	pCa 7	pCa 5
None	5.1±0.1	25.5±1.5
GTP (1 mM)	5.0±0.5	24.0±2.5
GDP (1 mM)	4.5±0.1	22.5±2.5
p[NH]ppG (100 μM)	4.7±0.1	22.4±3.0
GTP[S] (30 μM)	n.d.	11.9±0.2
GTP[S]+GTP (1 mM)	n.d.	16.0±0.1
GTP[S]+GDP (1 mM)	n.d.	19.7±0.4
GTP[S]+ p[NH]ppG (100 μM)	n.d.	21.1±0.1

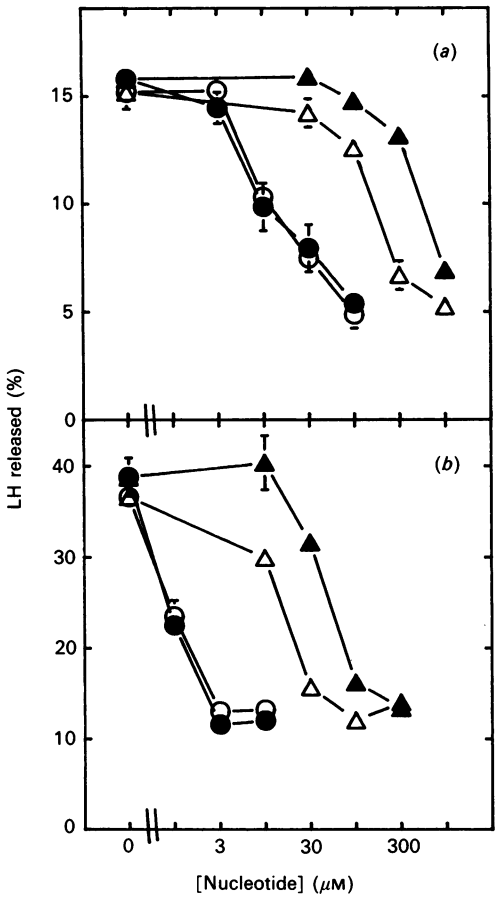

Fig. 7. Inhibitory effects of GTP[S] and ATP[S] on (*a*) Ca^{2+}-stimulated and (*b*) cAMP/PMA-stimulated LH exocytosis

Permeabilized cells were equilibrated for 30 min at 0 °C in buffer IC with 2 mM-$MgCl_2$, 10 mM-CaEGTA (pCa 7) and the indicated concentration of GTP[S] (○, ●) or ATP[S] (△, ▲) with (●, ▲) or without (○, △) 2 mM-MgUDP. Cells were then warmed to 37 °C and preincubated for 30 min in the same medium. This medium was removed and replaced with stimulation buffer containing either (*a*) 30 mM-CaEGTA at pCa 5 or (*b*) 10 mM-CaEGTA (pCa 7) plus cAMP (30 μM) and PMA (100 nM), and the LH released after 30 min was determined.

DISCUSSION

The present study demonstrates that intracellular application of guanine nucleotides has both stimulatory and inhibitory effects on exocytosis in gonadotropes. In several previous studies the stimulatory effects of guanine nucleotides have been interpreted in terms of a stimulatory GTP-binding protein (G_E) directly involved in exocytosis (Cockcroft *et al.*, 1987; Regazzi *et al.*, 1989). In neutrophils (Barrowman *et al.*, 1986), mast cells (Cockcroft *et al.*, 1987) and RINm5F cells (Vallar *et al.*, 1987; Regazzi *et al.*, 1989) there is evidence that the stimulatory effect of guanine nucleotides on exocytosis is not entirely due to the activation of phosphoinositidase C and/or adenylate cyclase. However, it remains possible that in these cell types guanine nucleotides stimulate other G-protein-coupled effector systems such as phospholipases A_2 and D (Burgoyne *et al.*, 1987; Martin & Michaelis, 1989), which themselves stimulate exocytosis.

The present findings are compatible with a conventional scheme in which stimulatory effects result from activation of one or more signal-transducing G-proteins with consequent generation of diacylglycerol and cAMP. Firstly, in time-course experiments, the stimulatory effects of GTP[S] and p[NH]ppG were maximal between 5 and 10 min, whereas those of Ca^{2+}, PKC and cAMP are maximal in the first 5 min (van der Merwe *et al.*, 1989; Macrae *et al.*, 1990). The delay is compatible with guanine nucleotides having less direct mechanisms. Secondly, the combined stimulatory effects of p[NH]ppG and saturating cAMP concentrations were less than additive. Finally, in cells stimulated with saturating concentrations of PMA plus cAMP, p[NH]ppG was unable to stimulate further LH exocytosis. Although this last finding suggests that the stimulatory effects of guanine nucleotides may mediated solely by PKC activation together with cAMP generation, it is possible that, with cAMP/PMA stimulation, LH exocytosis has reached an inherent maximal

rate, thus obscuring any additional effect of p[NH]ppG. Our results do not therefore entirely exclude additional mechanisms for the stimulatory effect of guanine nucleotides.

Several differences between the stimulatory effects and the inhibitory effects of guanine nucleotides indicate that they are exerted at different sites. Unlike the stimulatory effect, inhibition was observed only with GTP[S] (not p[NH]ppG), it had a slower onset, and was produced by a 10-fold lower concentration of GTP[S]. The finding that GTP[S] inhibits LH exocytosis stimulated by a variety of second-messenger pathways (Ca^{2+}, PMA and cAMP) indicates that GTP[S] inhibits exocytosis distal to the generation of second messengers. The delayed inhibitory effect of GTP[S], not found with p[NH]ppG, probably explains the lower maximal stimulation observed with GTP[S] and shorter duration of GTP[S] stimulation as compared with p[NH]ppG.

The finding that the inhibitory effect was observed with ATP[S] as well as GTP[S], but not with p[NH]ppG, raises the question whether the inhibition results from protein thiophosphorylation (Eckstein, 1985; Li *et al.*, 1988). In support of this, GTP (and GTP[S]) is preferred to ATP as a phosphate donor in some protein-phosphorylation reactions (Amir-Zaltsman *et al.*, 1980; Amir-Zaltsman & Salomon, 1989). Since thiophosphorylated proteins are resistant to dephosphorylation (Li *et al.*, 1988), processes requiring dephosphorylation, of which exocytosis may be one (Momayezi *et al.*, 1987), will be inhibited. Our findings (*a*) that much higher concentrations of ATP[S] than of GTP[S] are required for inhibition and (*b*) that the ATP[S] effect is inhibited by a nucleoside diphosphate kinase inhibitor (UDP) suggests instead that ATP[S] inhibition results from its intracellular conversion to GTP[S] (Seifert *et al.*, 1988).

Previously GTP[S] has been reported to inhibit Ca^{2+}-stimulated exocytosis in bovine adrenal chromaffin cells (Knight & Baker, 1985) and PC12 cells (Ahnert-Hilger *et al.*, 1987) over a concentration range similar to that used in the present study. Interestingly, p[NH]ppG was not inhibitory in bovine adrenal chromaffin cells (Knight & Baker, 1985), as we have found in gonadotropes. These similarities suggest that the mechanism of GTP[S] inhibition may be common to many secretory cell types.

It is attractive to hypothesize that GTP[S] inhibition is mediated by a GTP-binding protein directly involved in exocytosis. One possibility is that GTP[S] activates a conventional heterotrimeric G-protein which directly inhibits second-messenger-stimulated exocytosis. In support of this, we have found that pretreatment with pertussis toxin, which catalyses ADP-ribosylation of certain heterotrimeric G-proteins (Bokoch *et al.*, 1983), partially prevents the inhibition by GTP[S] (P. A. van der Merwe & J. S. Davidson, unpublished work). Similar results were obtained in chromaffin cells (Ahnert-Hilger *et al.*, 1987). There is also evidence that the inhibitory effects of somatostatin and α_2-adrenergic-receptor activation on secretion result from coupling to heterotrimeric G-proteins which directly inhibit second-messenger-stimulated exocytosis (Ullrich & Wollheim, 1988; Luini & De Matteis, 1990). However, no receptors have been identified in gonadotropes which mediate inhibition of LH exocytosis.

An alternative possibility is that GTP[S] inhibition is mediated by a non-conventional GTP-binding protein directly involved in exocytosis. Previous studies have identified such a protein (SEC4) which is essential for constitutive exocytosis in yeast (Salminen & Novick, 1987; Goud *et al.*, 1988). SEC4 is a member of a large family of small (20–25 kDa) *ras*-like GTP-binding proteins, some of which have been implicated in intracellular vesicle-trafficking pathways in mammalian cells (Melançon *et al.*, 1987; Bourne, 1988; Balch, 1989; Burgoyne, 1989). Small GTP-binding proteins have been found tightly bound to the cytosolic surface of bovine adrenal chromaffin (Burgoyne & Morgan, 1989) and sheep anterior-pituitary secretory granules (J. S. Davidson & I. K. Wakefield, unpublished work). Bourne (1988) has proposed that hydrolysis of GTP by these GTP-binding proteins establishes unidirectional movement along the secretory pathway. Since this model predicts that non-hydrolysable GTP analogues will inhibit such a pathway, our results are compatible with such a mechanism.

We conclude that GTP-binding proteins are involved in LH exocytosis at two distinct sites. At a proximal site conventional heterotrimeric G-proteins regulate the generation of second messengers which stimulate LH exocytosis. In addition, GTP[S] mediates inhibition of exocytosis at a site distal to second-messenger generation.

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