

# Stimulation of $\text{Ca}^{2+}$ -independent exocytosis in rat pituitary gonadotrophs by G-protein

Frederick W. Tse and Amy Tse

*Department of Pharmacology and Division of Neuroscience, University of Alberta,  
Edmonton, Alberta, Canada T6G 2H7*

(Received 24 September 1999; accepted after revision 29 March 2000)

1. We employed the whole-cell recording technique in conjunction with fluorometry to measure cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) and exocytosis (capacitance measurement) in single, identified rat gonadotrophs.
2. Direct activation of G-protein (via intracellular dialysis of non-hydrolysable analogues of GTP, but not of GDP) triggered a slow rise in capacitance even in the presence of a fast intracellular  $\text{Ca}^{2+}$  chelator.
3. The broad-spectrum kinase inhibitors H7 and staurosporine did not prevent this  $\text{Ca}^{2+}$ -independent exocytosis, ruling out the involvement of the cAMP and PKC pathways.
4.  $\text{AlF}_4^-$ , a potent stimulator of heterotrimeric G-proteins, failed to stimulate any exocytosis when the intracellular  $\text{Ca}^{2+}$  store was depleted, implicating the involvement of  $\text{AlF}_4^-$ -insensitive G-protein(s).
5. Maximal stimulation of  $\text{Ca}^{2+}$ -independent exocytosis by GTP analogues did not reduce the number of readily releasable granules that were available subsequently for  $\text{Ca}^{2+}$ -dependent release.
6. The last finding raises the possibility that the G-protein-stimulated  $\text{Ca}^{2+}$ -independent exocytosis may regulate a pool of granules that is distinct from the  $\text{Ca}^{2+}$ -dependent pool.

In rat pituitary gonadotrophs, stimulation of gonadotropin-releasing hormone (GnRH) receptors activates the  $G_q$ -coupled phosphoinositide pathway that in turn triggers rhythmic release of  $\text{Ca}^{2+}$  from the  $\text{IP}_3$ -sensitive store (Tse & Hille, 1992; Kukuljan *et al.* 1992). The GnRH-triggered  $[\text{Ca}^{2+}]_i$  oscillation is closely accompanied by exocytosis and the inclusion of intracellular  $\text{Ca}^{2+}$  buffer strongly suppresses exocytosis, indicating that the GnRH-stimulated exocytosis is  $\text{Ca}^{2+}$  dependent (Tse *et al.* 1993). However, in permeabilized sheep gonadotrophs in which  $[\text{Ca}^{2+}]_i$  was strongly buffered, activation of G-protein by non-hydrolysable analogues of GTP has been reported to stimulate luteinizing hormone (LH) release at resting or low  $[\text{Ca}^{2+}]_i$  and this action has been attributed to the combined activation of the cAMP and protein kinase C (PKC) pathways (Macrae *et al.* 1990; van der Merwe *et al.* 1991). In rat gonadotrophs, on the other hand, cAMP was reported to have negligible effect on stimulation of LH secretion (Turgeon & Waring, 1986; Billiard *et al.* 1997) but the activation of the PKC pathway can trigger exocytosis even when  $[\text{Ca}^{2+}]_i$  remains near the resting level (Billiard *et al.* 1997). Here, we investigated whether G-protein activation in rat gonadotrophs could directly affect exocytosis independently of  $[\text{Ca}^{2+}]_i$  elevation. Our results indicate that

G-protein activation can directly stimulate a component of exocytosis that is independent of  $[\text{Ca}^{2+}]_i$  elevation, as well as the activation of the cAMP or PKC pathway. This G-protein-mediated and  $\text{Ca}^{2+}$ -independent exocytosis appears to be acting on a pool of vesicles or granules that is distinct from the  $\text{Ca}^{2+}$ -dependent pool. Preliminary data of this work have been published in an abstract (Tse & Tse, 1997).

## METHODS

### Cell preparation

The anterior lobe of the pituitary gland was removed from male Sprague-Dawley rats (age 5–6 weeks) that had been killed with halothane in accordance with the standards of the Canadian Council on Animal Care. Anterior pituitary glands were dissociated enzymatically using collagenase and trypsin as previously described (Tse & Hille, 1994). Single gonadotrophs were identified from the heterogeneous population by reverse haemolytic plaque assay (Smith *et al.* 1984), using polyclonal antibodies to LH (kindly provided by Dr J. D. Neill, University of Alabama, Birmingham). The procedures were similar to those described previously (Tse & Hille, 1994). The cells were maintained under standard culture conditions in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) horse serum,  $50 \mu\text{g ml}^{-1}$  penicillin G and  $50 \mu\text{g ml}^{-1}$  streptomycin (all from Gibco, Grand Island, NY, USA). Recordings were performed on cells cultured for 2–4 days.

### Chemicals and solutions

Stock of GnRH (Peninsula Laboratories, Belmont, CA, USA) was dissolved in 0.1 M acetic acid, lyophilized, and kept at  $-20^{\circ}\text{C}$ . Indo-1 (Calbiochem, La Jolla, CA, USA) and indo-1 FF (Texas Fluorescence Labs, Austin, TX, USA) were kept as stock solutions in distilled water at  $-20^{\circ}\text{C}$ . Guanosine 5'-triphosphate (GTP- $\gamma$ -S), guanylyl-5'-imidodiphosphate (GppNHP), guanosine 5'-O-(2-thio-diphosphate) (GDP- $\beta$ -S), 2,5-di-(*t*-butyl)-1,4-hydroquinone (BHQ), staurosporine and pertussis toxin were obtained from Calbiochem. EGTA, apamin, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine HCl (H7) and BAPTA were obtained from Sigma. DM-nitrophen and caged GTP- $\gamma$ -S were obtained from Molecular Probes (Eugene, OR, USA).

The standard bath solution contained (mM): 150 NaCl, 10 Hepes, 8 glucose, 2.5 KCl, 2 CaCl<sub>2</sub> and 1 MgCl<sub>2</sub> (pH 7.4). In experiments where extracellular Ca<sup>2+</sup> was removed, Ca<sup>2+</sup> was omitted from the standard bath solution but 3 mM MgCl<sub>2</sub> and 1 mM Na-EGTA were added. In experiments involving flash photolysis of caged Ca<sup>2+</sup>-DM-nitrophen, apamin (0.4  $\mu\text{M}$ ) was included in the standard bath solution to inhibit the SK type Ca<sup>2+</sup>-activated K<sup>+</sup> current (Tse & Hille, 1992). The standard pipette solution contained (mM): 120 potassium aspartate, 20 KCl, 20 K-Hepes, 1 MgCl<sub>2</sub>, 2 Na<sub>2</sub>ATP, 0.1 Na<sub>4</sub>GTP and 0.1 indo-1 (pH 7.4). In experiments where [Ca<sup>2+</sup>]<sub>i</sub> was elevated via flash photolysis of caged Ca<sup>2+</sup>, the pipette solution contained (mM): 70 caesium aspartate, 40 Cs-Hepes, 20 tetraethylammonium-Cl, 0.1 Na<sub>4</sub>GTP, 0.1 indo-1 FF and 6.5–8 DM-nitrophen (~90% saturated with Ca<sup>2+</sup>) (pH 7.4). As the purity of DM-nitrophen varied slightly between batches, the loading of each patch of DM-nitrophen with Ca<sup>2+</sup> was determined empirically. In experiments involving pertussis toxin treatment, cells were incubated with pertussis toxin (100 ng ml<sup>-1</sup>) in DMEM at 37 °C for 20–24 h before recording.

### Measurement of [Ca<sup>2+</sup>]<sub>i</sub>

[Ca<sup>2+</sup>]<sub>i</sub> was measured fluorometrically using the ratiometric Ca<sup>2+</sup> indicator indo-1 or indo-1 FF. The Ca<sup>2+</sup> indicator was dialysed into the cell via the whole-cell patch pipette. Details of the instrumentation and procedures of the [Ca<sup>2+</sup>]<sub>i</sub> measurement were as described previously (Tse & Hille, 1994; Tse & Tse, 1998). Briefly, indo-1 or indo-1 FF in single gonadotrophs was excited by 365 nm (band-pass filtered) light delivered from a HBO 100 W mercury lamp via a  $\times 40$ , 1.3 NA UV fluor oil objective (Nikon). Photon counts were collected at 405 and 500 nm by two photomultiplier tubes (Hamamatsu H3460-04) and then translated into logic signals counted simultaneously by a CYCTM-10 counter card (Cyber Research Inc., Branford, CT, USA) on an IBM-compatible computer. [Ca<sup>2+</sup>]<sub>i</sub> was calculated from the ratio (*R*) of fluorescence at 405 and 500 nm, using the equation of Grynkiewicz *et al.* (1985):

$$[\text{Ca}^{2+}]_i = K^*(R - R_{\min})/(R_{\max} - R), \quad (1)$$

where  $R_{\min}$  is the fluorescence ratio of Ca<sup>2+</sup>-free indicator and  $R_{\max}$  is the ratio of Ca<sup>2+</sup>-bound indicator.  $K^*$  is a constant that was determined empirically. Calibrations were determined from groups of single gonadotrophs ( $n = 5-8$ ) dialysed with one of the three pipette solutions as described previously (Tse & Tse, 1998). For indo-1 measurement,  $R_{\min}$  was measured in cells loaded with (mM): 52 potassium aspartate, 10 KCl, 50 K-EGTA, 0.1 indo-1 and 50 K-Hepes (pH 7.4); and  $R_{\max}$  was measured in cells loaded with (mM): 136 potassium aspartate, 15 CaCl<sub>2</sub>, 0.1 indo-1 and 50 K-Hepes (pH 7.4).  $K^*$  was calculated from eqn (1) using *R* values obtained from cells loaded with (mM): 60 potassium aspartate, 50 K-Hepes, 20 K-EGTA, 15 CaCl<sub>2</sub> and 0.1 indo-1 (pH 7.4), which had a calculated free Ca<sup>2+</sup> concentration of 212 nM at 24 °C (Blinks *et al.* 1982). For all indo-1 measurements reported here, the values

of  $R_{\min}$ ,  $R_{\max}$  and  $K^*$  were 0.403, 5.05 and 2.62  $\mu\text{M}$ , respectively. In experiments involving flash photolysis of caged Ca<sup>2+</sup>-DM-nitrophen, [Ca<sup>2+</sup>]<sub>i</sub> was measured with the low affinity Ca<sup>2+</sup> indicator indo-1 FF. For these measurements,  $R_{\max}$  was measured in cells loaded with (mM): 109 potassium aspartate, 25 CaCl<sub>2</sub>, 10 DM-nitrophen, 0.1 indo-1 FF and 40 K-Hepes (pH 7.4).  $K^*$  was calculated from eqn (1) using *R* values obtained from cells loaded with (mM): 70 diglycolic acid, 15 potassium aspartate, 50 K-Hepes, 10 CaCl<sub>2</sub> and 0.1 indo-1 FF (pH 7.4), which had a free Ca<sup>2+</sup> concentration of 24  $\mu\text{M}$  at 24 °C (measured with a Ca<sup>2+</sup> electrode). For all indo-1 FF measurements reported here, the values of  $R_{\max}$  and  $K^*$  were 2.56 and 79.2  $\mu\text{M}$ , respectively.  $R_{\min}$  was obtained individually for each cell as the average of 5–10 measurements taken immediately before the flash and the values ranged from 0.21 to 0.25.

### Electrophysiological recording

Single gonadotrophs were voltage clamped with the whole-cell, gigaseal method (Hamill *et al.* 1981) using an EPC-9 patch clamp amplifier. Changes in cell capacitance ( $C_m$ ) during intracellular dialysis of GTP analogues were measured with the capacitance-tracking feature of the EPC-9 amplifier (Tse *et al.* 1997). During flash photolysis of caged Ca<sup>2+</sup>, the rapid increase in  $C_m$  was measured at high temporal resolution with a dual-phase lock-in amplifier (Tse *et al.* 1997). Values of [Ca<sup>2+</sup>]<sub>i</sub> and  $C_m$  were first recorded on VCR tapes with a NeuroData PCM recorder and digitized later. The pipettes were made from haematocrit glass (VWR Scientific Canada Ltd, London, Ontario, Canada) and the resistance was 2–4 M $\Omega$  after filling and 5–10 M $\Omega$  during whole-cell recording. Recordings were done at room temperature (22–25 °C). In all the experiments reported here, the cell membrane potential was held at  $-70$  mV, a potential at which most voltage-gated channels in the gonadotrophs were closed (Tse *et al.* 1993). Values given in the text are means  $\pm$  s.e.m. To photolyse caged molecules, a UV flash from a modified XF-10 xenon flash lamp (Hi-Tech Ltd, Salisbury, UK) was delivered to the cell via a fused silica focusing lens which replaced the microscope's condenser (Tse *et al.* 1997).

## RESULTS

### Activation of G-protein(s) triggers Ca<sup>2+</sup>-independent exocytosis

Figure 1 shows a simultaneous measurement of [Ca<sup>2+</sup>]<sub>i</sub> and cell membrane capacitance ( $C_m$ ) in a gonadotroph during G-protein activation. In order to bypass the GnRH receptors and directly activate G-protein, we have employed GTP- $\gamma$ -S, a non-hydrolysable analogue of GTP. In this experiment, the cell was recorded with 100  $\mu\text{M}$  caged GTP- $\gamma$ -S in the pipette solution. About 3 min after whole-cell recording had begun, a UV flash was delivered (at time zero) to photolyse the caged molecule and release GTP- $\gamma$ -S. Shortly following the flash (~10 s),  $C_m$  started to rise, reflecting exocytosis.  $C_m$  continued to rise and at ~100 s after the UV flash, the cumulative increase in  $C_m$  reached ~0.09 pF. This increase in  $C_m$  was subsequently followed by a decrease, reflecting endocytosis. Although GTP- $\gamma$ -S has been postulated to inhibit some types of endocytosis (Carter *et al.* 1993; Takei *et al.* 1995), endocytosis could still occur in gonadotrophs even in the continued presence of GTP- $\gamma$ -S (see also Fig. 6B). Note that the exocytosis in Fig. 1 is not accompanied by any change in [Ca<sup>2+</sup>]<sub>i</sub>. [Ca<sup>2+</sup>]<sub>i</sub> remained near its resting level

(~50 nM) throughout the recording period. A similar result was observed in three other cells. In all the cells examined, exocytosis started with a delay of 10–20 s after the delivery of the UV flash. This delay cannot be due to a slow release of GTP- $\gamma$ -S because the kinetics of release of GTP- $\gamma$ -S from the cage during flash photolysis is rapid (milliseconds). Instead, the delay suggests that the GTP- $\gamma$ -S effect in the gonadotrophs is intrinsically slow.

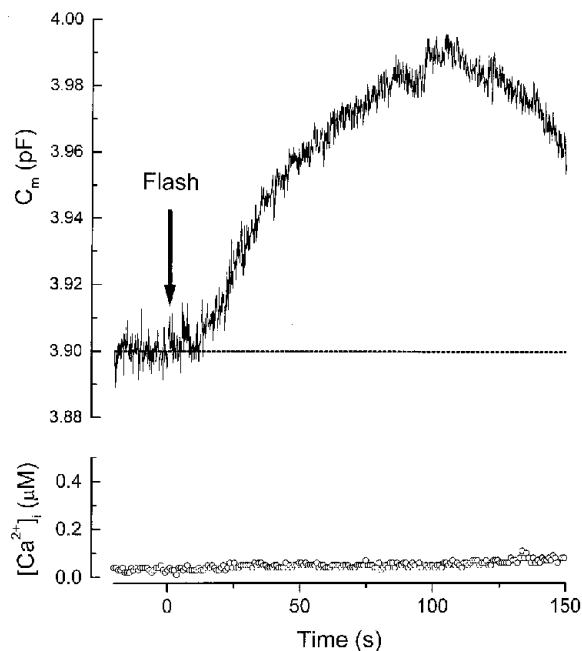
Although flash photolysis of caged GTP- $\gamma$ -S has the advantage of inducing rapid elevation in the concentration of GTP- $\gamma$ -S inside the cell, the fraction of caged GTP- $\gamma$ -S photolysed by each flash cannot be controlled precisely. The uncertainty about the concentration of GTP- $\gamma$ -S creates difficulties in making comparisons among cells. Therefore, for the rest of the experiments in this study, a known concentration of non-hydrolysable GTP analogue was dialysed into the cell via the whole-cell pipette.

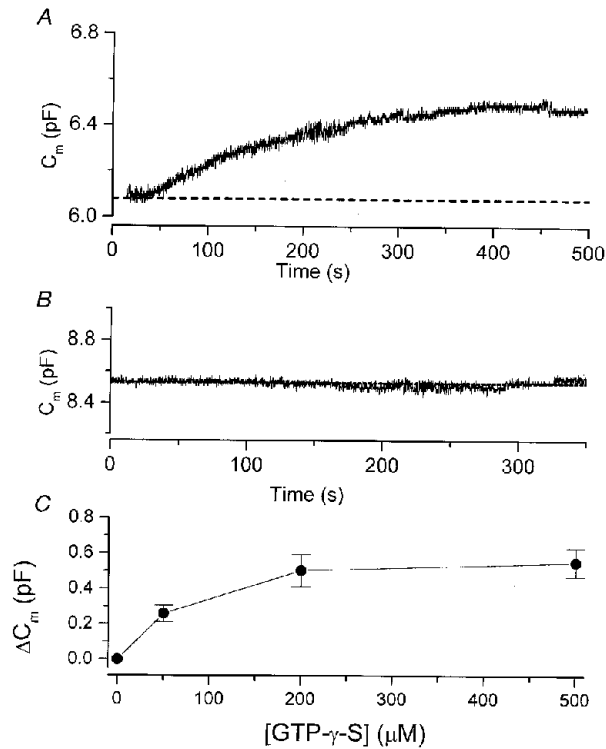
To examine whether the GTP- $\gamma$ -S-triggered exocytosis was indeed independent of any rise in [Ca<sup>2+</sup>]<sub>i</sub>, we included 10 mM BAPTA in the whole-cell pipette solution to buffer [Ca<sup>2+</sup>]<sub>i</sub> to a low concentration. In all cells recorded with BAPTA, [Ca<sup>2+</sup>]<sub>i</sub> (data not shown) remained near zero (~R<sub>min</sub> of indo-1) throughout the experiment. Figure 2A shows that during the first 25 s of the whole-cell recording, C<sub>m</sub> remained near the resting level. Subsequently, C<sub>m</sub> started to rise. This cell was recorded with 200  $\mu$ M GTP- $\gamma$ -S in the pipette solution. The delay observed here is in part due to the time taken for sufficient GTP- $\gamma$ -S to diffuse into the cell. In a previous study (Tse & Hille, 1994), we have shown that the diffusion of indo-1 (MW = 750), a molecule which is comparable in size to GTP- $\gamma$ -S (MW = 563), into a

gonadotroph (typically ~5 pF) has a time constant of ~65 s (pipette series resistance of 6 M $\Omega$ ). Thus, under our current experimental conditions, the diffusion of GTP- $\gamma$ -S into the cell should reach an equilibrium within 200–300 s. Figure 2A shows that C<sub>m</sub> continued to rise slowly and appeared to reach a plateau after ~5–6 min. In this and all other cells recorded with GTP analogues (e.g. Fig. 3A), C<sub>m</sub> remained near a plateau level during the time course of recording (~8–10 min). This probably reflects a saturation of the effect of GTP- $\gamma$ -S. Alternatively, it may reflect a balance between exocytosis and endocytosis in the cell. Nevertheless, Fig. 2A shows that the cumulative C<sub>m</sub> increase triggered by 200  $\mu$ M GTP- $\gamma$ -S reached ~0.4 pF. Note that this stimulatory action of GTP- $\gamma$ -S could be observed even in the absence of extracellular Ca<sup>2+</sup> (see Fig. 5C). Thus the GTP- $\gamma$ -S-triggered exocytosis is independent of a rise in [Ca<sup>2+</sup>]<sub>i</sub> or the presence of extracellular Ca<sup>2+</sup>. On the other hand, in cells recorded with a pipette solution containing 10 mM BAPTA but no GTP- $\gamma$ -S, C<sub>m</sub> remained near its resting level for > 5 min (e.g. Fig. 2B; n = 5). Figure 2C shows that the amplitude of the GTP- $\gamma$ -S-triggered C<sub>m</sub> rise was dependent on the concentration of GTP- $\gamma$ -S. In cells stimulated with 50, 200 or 500  $\mu$ M GTP- $\gamma$ -S in the pipette solution, the cumulative increase in C<sub>m</sub> was 0.259  $\pm$  0.047 pF (n = 13), 0.502  $\pm$  0.09 pF (n = 4) and 0.549  $\pm$  0.08 pF (n = 9), respectively. Note that increasing the concentration of GTP- $\gamma$ -S from 200 to 500  $\mu$ M caused little additional increase in C<sub>m</sub> and the maximal increase in C<sub>m</sub> was ~0.5 pF. In rat gonadotrophs, the total number of granules present in a single cell is estimated to be ~10 000 (Tse *et al.* 1993) and there are few if any microvesicles (Tse *et al.* 1997). Using flash photolysis of caged Ca<sup>2+</sup>, we have previously

**Figure 1. Flash photolysis of caged GTP- $\gamma$ -S triggers slow exocytosis in gonadotrophs without [Ca<sup>2+</sup>]<sub>i</sub> elevation**

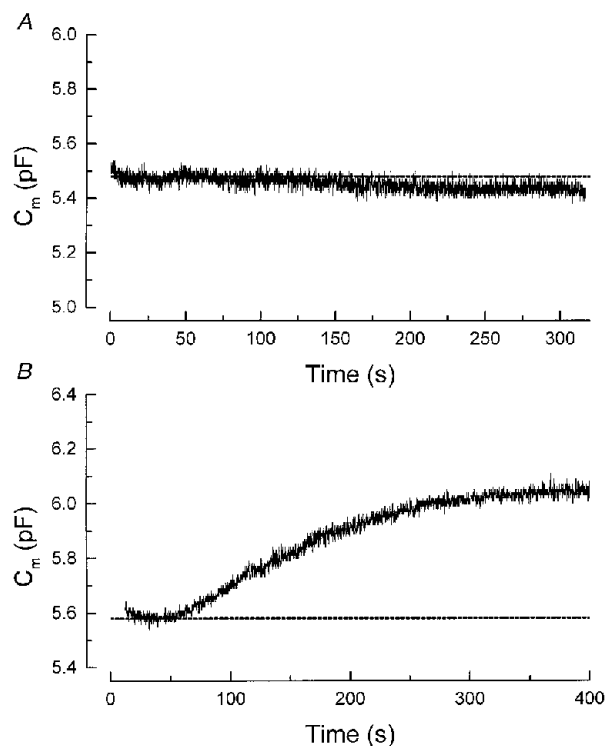
The pipette solution contained 100  $\mu$ M caged GTP- $\gamma$ -S and 100  $\mu$ M indo-1. A UV flash was delivered at time zero (indicated by the arrow). Note that there was a delay of ~10 s before the membrane capacitance (C<sub>m</sub>) started to rise.





**Figure 2. The GTP- $\gamma$ -S-triggered exocytosis is  $Ca^{2+}$  independent**

*A*, intracellular dialysis of GTP- $\gamma$ -S triggered exocytosis even in the presence of intracellular  $Ca^{2+}$  chelator. The pipette solution contained 200  $\mu M$  GTP- $\gamma$ -S and 10 mM BAPTA. At time zero, the whole-cell recording began. *B*, no exocytosis could be observed when the pipette solution contained BAPTA but no GTP- $\gamma$ -S. *C*, the amount of exocytosis is dependent on the concentration of GTP- $\gamma$ -S. The pipette solution contained 0, 50, 200 or 500  $\mu M$  GTP- $\gamma$ -S. Each data point is generated from 4–13 cells. Note that at  $[GTP-\gamma-S] > 200 \mu M$ , the effect of GTP- $\gamma$ -S appeared to saturate at  $\sim 0.5$  pF.



**Figure 3. The GTP- $\gamma$ -S response is due to G-protein activation**

*A*, intracellular dialysis of GDP- $\beta$ -S, a non-hydrolysable analogue of GDP, failed to mimic the GTP- $\gamma$ -S response. The pipette solution contained 1 mM GDP- $\beta$ -S and 10 mM BAPTA. *B*, intracellular dialysis of another non-hydrolysable GTP analogue, GppNHP, also triggered slow  $Ca^{2+}$ -independent exocytosis. The pipette solution contained 500  $\mu M$  GppNHP and 10 mM BAPTA.

shown that only ~140 granules are readily releasable (Tse *et al.* 1997). The average diameter of the granules in gonadotrophs is ~250 nm (Tse *et al.* 1997), and each granule is expected to have a capacitance of 1.9 fF. Therefore, a  $C_m$  increase of 500 fF by GTP- $\gamma$ -S reflects the exocytosis of ~260 granules, almost 2-fold the size of the pool of readily releasable granules.

The effect of GTP- $\gamma$ -S may be mediated by at least two distinct mechanisms: the binding of GTP- $\gamma$ -S to a G-protein and thus directly activating the G-protein or the inhibition of a GTPase. To distinguish between these two mechanisms, we examined the action of a non-hydrolysable analogue of GDP (GDP- $\beta$ -S). Similar to GTP- $\gamma$ -S, GDP- $\beta$ -S can inhibit GTPase activities. Thus a mimicking of the GTP- $\gamma$ -S effect by GDP- $\beta$ -S will imply an inhibition of GTPase. On the other hand, GDP- $\beta$ -S cannot activate G-protein and a failure of GDP- $\beta$ -S to mimic the action of GTP- $\gamma$ -S will indicate an involvement of G-protein activation. Figure 3A shows that in cells recorded with 1 mM GDP- $\beta$ -S for >5 min, no significant increase in  $C_m$  could be detected ( $n=4$ ). This result suggests that the GTP- $\gamma$ -S-triggered exocytosis is mediated by the stimulation of a G-protein rather than via GTPase inhibition. Consistent with this, in cells recorded with 500  $\mu$ M GppNHP (another non-hydrolysable analogue of GTP), an increase in  $C_m$  could be observed following the diffusion of GppNHP into the cell (Fig. 3B). In six cells examined with GppNHP (500  $\mu$ M), the mean cumulative increase in membrane capacitance (at 240–300 s after whole-cell recording) was  $0.478 \pm 0.109$  pF,

closely resembling that triggered by 500  $\mu$ M GTP- $\gamma$ -S ( $0.549 \pm 0.08$  pF;  $n=9$ ).

#### The G-protein action is not mediated via the PKC or cAMP pathway

The experiments above strongly suggest that the effect of non-hydrolysable analogues of GTP (GTP- $\gamma$ -S or GppNHP) on exocytosis is mediated by the activation of G-proteins. The non-hydrolysable analogue of GTP employed here will stimulate all heterotrimeric G-proteins in the gonadotrophs. These will include  $G_s$  which is coupled to the cAMP pathway and  $G_q$  which is coupled to the phospholipase C and PKC pathway. To examine whether the G-protein-triggered Ca<sup>2+</sup>-independent exocytosis observed here is caused by the activation of protein kinases, we employed two broad-spectrum protein kinase inhibitors, H7 and staurosporine. H7 inhibits cAMP-dependent kinase and PKC with  $K_i$  values of 3.0 and 6.0  $\mu$ M, respectively (Hidaka *et al.* 1984). Staurosporine, a more potent inhibitor, inhibits these two kinases with  $K_i$  values of 8.2 and 2.7 nM (Takahashi *et al.* 1990). In rat pituitary cells, both of these inhibitors have been reported to inhibit phorbol ester-mediated LH release with an IC<sub>50</sub> of 1.7  $\mu$ M for H7 and 44 nM for staurosporine (Thomson *et al.* 1993). Figure 4A shows a gonadotroph that had been treated with H7 (50  $\mu$ M) for 15–25 min before whole-cell recording began. Intracellular dialysis of GppNHP still triggered robust Ca<sup>2+</sup>-independent exocytosis. In six cells treated with H7, the mean increase in capacitance triggered by GppNHP (500  $\mu$ M) was  $0.425 \pm 0.070$  pF, similar to that of the controls ( $0.478 \pm 0.109$  pF;  $n=6$ ).

#### Figure 4. Second messenger pathway

A, the broad spectrum kinase inhibitor H7 failed to inhibit the G-protein-triggered exocytosis. The cell had been exposed to the kinase inhibitor H7 (50  $\mu$ M) in the bath for 20 min before whole-cell recording began. Intracellular dialysis of GppNHP still triggered robust exocytosis. B, the PKC inhibitor staurosporine (200 nM) did not prevent the G-protein stimulation of exocytosis. The cell was exposed to staurosporine in the bath for 15 min before the establishment of the whole-cell configuration. In both A and B, the pipette solution contained 500  $\mu$ M GppNHP and 10 mM BAPTA.

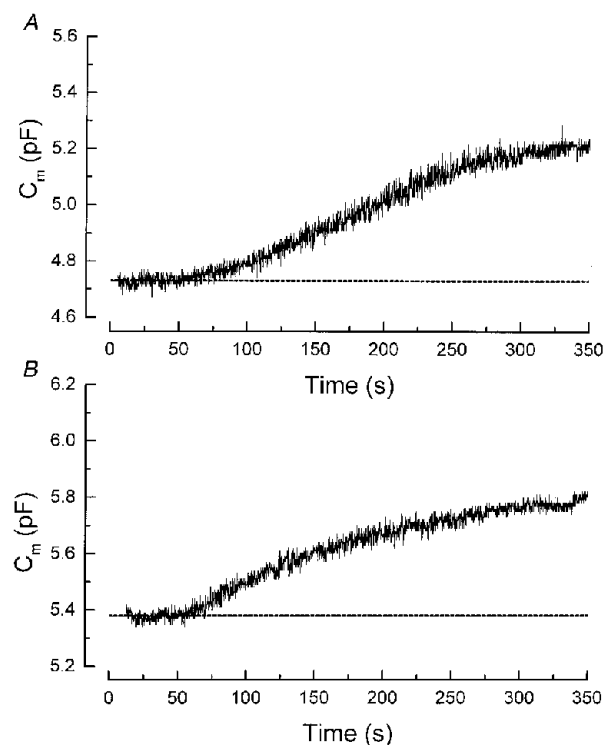
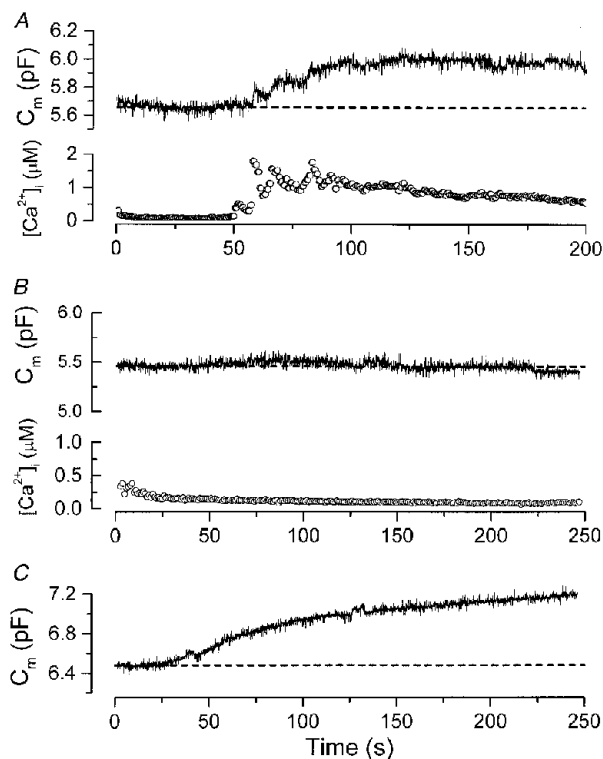


Figure 4B shows that pretreatment with staurosporine (200 nM) for 15 min also failed to inhibit the GppNHP response. In seven cells treated with staurosporine, the mean increase in membrane capacitance triggered by GppNHP (500  $\mu\text{M}$ ) was  $0.471 \pm 0.101$  pF. At the concentrations employed here, H7 and staurosporine would inhibit the cAMP-dependent kinase as well as PKC (Hidaka *et al.* 1984; Takahashi *et al.* 1990). Thus it is unlikely that these protein kinases are involved in the G-protein-triggered  $\text{Ca}^{2+}$ -independent exocytosis.

To examine further the involvement of a second messenger pathway underlying the G-protein response, we asked whether  $\text{AlF}_4^-$ , a potent activator of all heterotrimeric G-proteins (including  $G_s$ ,  $G_q$ ,  $G_i/G_o$ ) can stimulate  $\text{Ca}^{2+}$ -independent exocytosis. Since most  $\text{Ca}^{2+}$  chelators, including BAPTA, bind strongly to  $\text{Al}^{3+}$  and can reduce the effect of  $\text{AlF}_4^-$ , we omitted BAPTA from the pipette solution in this series of experiments. As expected from the stimulatory action of  $\text{AlF}_4^-$  on  $G_q$  which in turn leads to the generation of  $\text{IP}_3$  and activation of PKC, Fig. 5A shows that the diffusion of  $\text{AlF}_4^-$  (100  $\mu\text{M}$ ; generated as  $\text{AlF}_x^-$  where  $x = 3-5$  as described in Okano *et al.* 1993) into the gonadotroph stimulated  $[\text{Ca}^{2+}]_i$  oscillations that were accompanied by  $\text{Ca}^{2+}$ -dependent exocytosis ( $n = 3$ ). While this result shows that  $\text{AlF}_4^-$  indeed stimulates heterotrimeric G-proteins in gonadotrophs, it is difficult to separate the  $\text{Ca}^{2+}$ -independent component from the  $\text{Ca}^{2+}$ -dependent component of exocytosis. In order to study the  $\text{Ca}^{2+}$ -independent component of exocytosis in isolation, we

reduced intracellular  $\text{Ca}^{2+}$  release in gonadotrophs by depleting the intracellular  $\text{Ca}^{2+}$  stores. In a previous study (Tse *et al.* 1994), we have shown that during GnRH stimulation, the sarco-endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) has an essential role in the reuptake of  $\text{Ca}^{2+}$  into the intracellular stores. Here, we exploited this property to deplete the intracellular stores by exposing the cell to the SERCA inhibitor BHQ (1  $\mu\text{M}$ ), in the absence of extracellular  $\text{Ca}^{2+}$ . The cell was then challenged with GnRH (100 nM) for 3 min to stimulate intracellular  $\text{Ca}^{2+}$  release. Following GnRH removal, the cell was exposed continuously to BHQ in the absence of extracellular  $\text{Ca}^{2+}$  for another 5 min before whole-cell recording began. Under such experimental condition, intracellular  $\text{AlF}_4^-$  failed to elicit any  $[\text{Ca}^{2+}]_i$  rise or exocytosis (Fig. 5B;  $n = 5$ ). To examine whether the experimental manipulation to deplete intracellular  $\text{Ca}^{2+}$  stores inhibited  $\text{Ca}^{2+}$ -independent exocytosis, the same experiments were repeated in cells recorded with GTP- $\gamma$ -S and BAPTA in the whole-cell pipette. Figure 5C shows that even following the depletion of intracellular  $\text{Ca}^{2+}$  stores, GTP- $\gamma$ -S can still trigger robust  $\text{Ca}^{2+}$ -independent exocytosis ( $n = 6$ ). Since  $\text{AlF}_4^-$  activates both  $G_s$  and  $G_q$ , the failure of  $\text{AlF}_4^-$  to trigger  $\text{Ca}^{2+}$ -independent exocytosis in gonadotrophs further confirms that neither the cAMP nor the PKC pathway is involved. In addition, this result suggests that the pertussis toxin-sensitive heterotrimeric G-proteins  $G_i$  and  $G_o$  are not involved in the GTP- $\gamma$ -S-triggered  $\text{Ca}^{2+}$ -independent exocytosis. Consistent with this, GTP- $\gamma$ -S could still trigger  $\text{Ca}^{2+}$ -independent exocytosis in



**Figure 5. The G-protein action does not involve activation of heterotrimeric G-proteins**

A,  $\text{AlF}_4^-$ , a potent activator of heterotrimeric G-proteins, stimulated  $[\text{Ca}^{2+}]_i$  oscillation which was accompanied by  $\text{Ca}^{2+}$ -dependent exocytosis. The pipette solution contained  $\text{AlF}_4^-$  (100  $\mu\text{M}$ ) and 100  $\mu\text{M}$  indo-1 but no BAPTA. B, when the intracellular  $\text{Ca}^{2+}$  store was depleted,  $\text{AlF}_4^-$  failed to trigger any exocytosis. To deplete the intracellular  $\text{Ca}^{2+}$  store, the cell was bathed continuously in a solution containing 1 mM EGTA (no added  $\text{Ca}^{2+}$ ) plus the SERCA inhibitor BHQ (1  $\mu\text{M}$ ). It was then challenged with GnRH (100 nM) for 3 min. About 5 min following the removal of GnRH, the whole-cell recording began. Note that  $[\text{Ca}^{2+}]_i$  remained near 100–200 nM throughout the time course of the recording, suggesting that most of the intracellular  $\text{Ca}^{2+}$  store was depleted by this experimental manipulation. Same pipette solution as in A. C, the depletion of the intracellular  $\text{Ca}^{2+}$  store does not affect the GTP- $\gamma$ -S triggered  $\text{Ca}^{2+}$ -independent exocytosis. The cell was subjected to the same experimental procedure as described in B. Intracellular dialysis of GTP- $\gamma$ -S still triggered robust exocytosis. The pipette solution contained 500  $\mu\text{M}$  GTP- $\gamma$ -S and 10 mM BAPTA. Note that in both B and C, the cells were continuously bathed in a  $\text{Ca}^{2+}$ -free solution containing BHQ (1  $\mu\text{M}$ ).

gonadotrophs treated with pertussis toxin ( $n = 5$ ; data not shown). Note that for the experiment shown in Fig. 5C, the cell was bathed in a Ca<sup>2+</sup>-free bath solution. Thus GTP- $\gamma$ -S can stimulate exocytosis even when the intracellular Ca<sup>2+</sup> stores were depleted and in the absence of extracellular Ca<sup>2+</sup> ( $n = 6$ ).

### G-protein may regulate a pool of granules that is distinct from the Ca<sup>2+</sup>-dependent pool

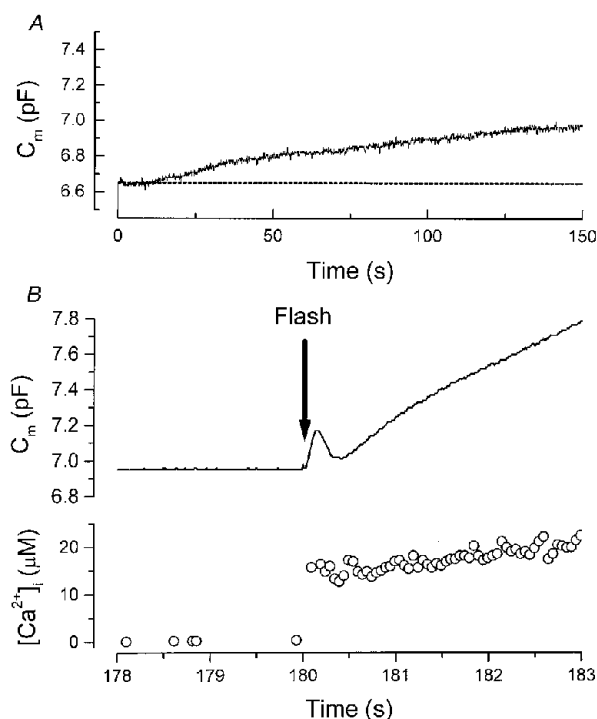
The saturation of the GTP- $\gamma$ -S-triggered exocytosis at  $\sim 0.5$  pF (Fig. 2C) raised the possibility that the persistent stimulation of G-protein might eventually exhaust the pool of readily releasable granules. In a previous study (Tse *et al.* 1997), we have shown that in gonadotrophs, rapid elevation of [Ca<sup>2+</sup>]<sub>i</sub> beyond 15  $\mu$ M triggered kinetically distinct phases of exocytosis. The fastest kinetic phase reflects the exhaustion of a pool of readily releasable granules. We examined whether the same pool of readily releasable granules was exhausted by the G-protein-triggered Ca<sup>2+</sup>-independent exocytosis. Any reduction in the amplitude of the fastest Ca<sup>2+</sup>-dependent exocytic burst will suggest that the G-protein-stimulated Ca<sup>2+</sup>-independent exocytosis has partially exhausted the pool of readily releasable granules. In this series of experiments, the pipette solution contained a caged Ca<sup>2+</sup> compound (DM-nitrophen) and GTP- $\gamma$ -S (50 or 500  $\mu$ M). After the establishment of whole-cell recording, the G-protein-stimulated exocytosis was monitored for 3–4 min. A UV flash was then delivered to elevate [Ca<sup>2+</sup>]<sub>i</sub> rapidly to trigger Ca<sup>2+</sup>-dependent exocytosis. An example of such an experiment is shown in Fig. 6. In this cell, at 150 s after whole-cell recording began, the cumulative increase in

membrane capacitance stimulated by GTP- $\gamma$ -S (50  $\mu$ M) was  $\sim 0.2$  pF. At 180 s after whole-cell recording, a UV flash was delivered to photolyse the caged Ca<sup>2+</sup>. [Ca<sup>2+</sup>]<sub>i</sub> rose rapidly to  $\sim 15$   $\mu$ M and triggered two distinct kinetic phases of exocytosis: an initial fast exocytic burst followed by a rapid endocytosis, and a slower phase of exocytosis. The rapid endocytosis was seen in about half of the cells recorded with 50  $\mu$ M (8/13 cells) or 500  $\mu$ M GTP- $\gamma$ -S (5/12 cells) as well as in control cells (8/15 cells). This suggests that the rapid endocytosis in gonadotrophs is unaffected by GTP- $\gamma$ -S. As the fastest exocytic burst reflected the exhaustion of the most readily releasable pool of granules, the size of the pool of readily releasable granules can be measured from the amplitude of this burst (Tse *et al.* 1997).

The results from these experiments are summarized in Fig. 7. Since gonadotrophs vary in size, we normalized the cumulative exocytosis and the amplitude of the fast exocytic burst to the initial membrane capacitance of individual cells. The normalized data were plotted as the percentage increase in cell surface area. In cells recorded with 50  $\mu$ M GTP- $\gamma$ -S, the G-protein-stimulated cumulative exocytosis (before the UV flash) was  $4.36 \pm 0.91\%$  ( $n = 13$ ). The mean size of the pool of readily releasable granules from these cells was  $3.61 \pm 0.18\%$ , closely resembling the value of the control cells ( $3.56 \pm 0.22\%$ ;  $n = 15$ ). In cells recorded with 500  $\mu$ M GTP- $\gamma$ -S, the G-protein-stimulated cumulative exocytosis was  $8.09 \pm 1.67\%$  ( $n = 12$ ), almost 2-fold larger than that triggered by 50  $\mu$ M GTP- $\gamma$ -S. However, even in these cells, the mean size of the pool of readily releasable granules ( $3.76 \pm 0.36\%$ ) was similar to that of the control cells.

### Figure 6. Multiple components of exocytosis in a gonadotroph

*A*, slow Ca<sup>2+</sup>-independent exocytosis triggered by intracellular GTP- $\gamma$ -S. The pipette solution contained GTP- $\gamma$ -S (50  $\mu$ M), 100  $\mu$ M indo-1 FF and the caged Ca<sup>2+</sup> compound (DM-nitrophen). Before the delivery of the UV flash, DM-nitrophen was a strong Ca<sup>2+</sup> chelator, thus the resting [Ca<sup>2+</sup>]<sub>i</sub> was buffered to a low level. *B*, fast Ca<sup>2+</sup>-dependent exocytosis triggered by the rapid [Ca<sup>2+</sup>]<sub>i</sub> elevation in the same cell as shown in *A*. At 180 s after whole-cell recording began, a UV flash (denoted by the arrow) was delivered to photolyse caged Ca<sup>2+</sup>. [Ca<sup>2+</sup>]<sub>i</sub> rose rapidly to  $\sim 15$   $\mu$ M and triggered at least two kinetically distinct phases of exocytosis. The fastest burst reflected the exhaustion of a pool of most readily releasable granules.



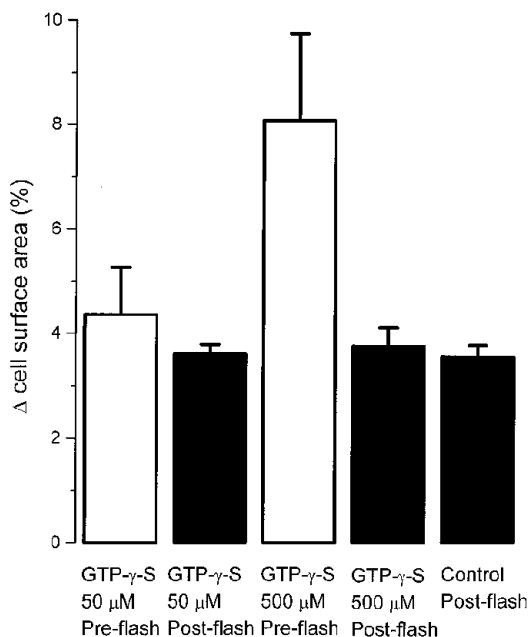
## DISCUSSION

### G-protein directly stimulates exocytosis

The general phenomenon that non-hydrolysable GTP analogues stimulate  $\text{Ca}^{2+}$ -independent exocytosis has been reported in a variety of cell types including melanotrophs (Okano *et al.* 1993), chromaffin cells (Burgoyne & Handel, 1994) and mast cells (Fernandez *et al.* 1984). However, the mechanism(s) involved in different cell types may not be identical. For example, in gonadotrophs and melanotrophs, both GTP- $\gamma$ -S and GppNHP trigger robust  $\text{Ca}^{2+}$ -independent exocytosis. In contrast, GppNHP was reported to be far more effective than GTP- $\gamma$ -S in triggering capacitance increase (Burgoyne & Handel, 1994) as well as in stimulating noradrenaline release in chromaffin cells (Ahnert-Hilger *et al.* 1992). This discrepancy probably reflects fundamental differences in the regulation of the exocytic machinery of different cell types.

Several lines of evidence suggest that the action of GTP analogues on gonadotrophs in this study cannot be due to the activation of cAMP or PKC pathways via heterotrimeric G-proteins. First, a previous study has shown that the PKC-mediated LH secretion from rat gonadotrophs could be completely abolished by intracellular BAPTA (Billiard *et al.* 1997). In contrast, the GTP analogue-stimulated exocytosis in this study persisted even in the absence of extracellular  $\text{Ca}^{2+}$  (Fig. 5C) and when  $[\text{Ca}^{2+}]_i$  was buffered to a very low concentration by intracellular BAPTA (Fig. 3A). Second, treatment with the broad-spectrum protein kinase inhibitors H7 (50  $\mu\text{M}$ ) or staurosporine (200 nM) failed to prevent the action of the GTP analogues (Fig. 4). At similar concentrations, these protein kinase inhibitors have been shown to be effective in inhibiting PKC-mediated LH release (Thomson *et al.* 1993) or PKC-mediated enhancement of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current in rat gonadotrophs (Tse *et al.* 1995). Third, in the absence of a  $[\text{Ca}^{2+}]_i$  rise,  $\text{AlF}_4^-$ , a potent stimulator of heterotrimeric G-proteins, including  $\text{G}_q$

and  $\text{G}_s$ , failed to mimic the action of GTP analogues (Fig. 5). The non-involvement of  $\text{G}_s$  was further supported by a previous study showing that cholera toxin, a potent stimulator of  $\text{G}_s$ , was an ineffective LH secretagogue in rat gonadotrophs (Hawes *et al.* 1993). Overall, our results suggest that the exocytosis examined here is equally well stimulated by GTP- $\gamma$ -S or GppNHP, and is independent of any rise in  $[\text{Ca}^{2+}]_i$ , phosphorylation or  $\text{AlF}_4^-$ -sensitive G-proteins. To our knowledge, the triggering of exocytosis under all of the above conditions has been reported only in rat melanotrophs (Okano *et al.* 1993). Our results contrast with those in mast cells where the GTP- $\gamma$ -S-stimulated exocytosis is mediated via the activation of an  $\text{AlF}_4^-$ -sensitive heterotrimeric G-protein,  $\text{G}_{13}$  (Aridor *et al.* 1993). Although monomeric G-proteins were previously thought to be insensitive to  $\text{AlF}_4^-$ , recent reports have suggested that  $\text{AlF}_4^-$  can affect the interactions between some monomeric G-proteins and their GTPase-activating proteins (of Ras and Rho) or effectors (of Rho) (Mittal *et al.* 1996; Hoffman *et al.* 1998). Thus our observation also rules out the involvement of any monomeric G-proteins that can be activated by  $\text{AlF}_4^-$ .  $\text{Ca}^{2+}$ -independent exocytosis triggered by intracellular dialysis of short peptides derived from the putative effector domain of the monomeric G-protein Rab3a have been reported in mast cells (Oberhauser *et al.* 1992), pancreatic cells (Padfield *et al.* 1992) and chromaffin cells (Senyshyn *et al.* 1992). However, interpretation of these data has been complicated by the observations that some of these peptides (even when the sequences are scrambled; MacLean *et al.* 1993) may stimulate heterotrimeric G-proteins directly, perhaps because they have a structure similar to mastoparan (Law *et al.* 1993). In addition, a study (Johannes *et al.* 1994) has suggested that the GTP-bound form of Rab3a has a negative regulatory role in  $\text{Ca}^{2+}$ -dependent exocytosis. In the anterior pituitary gland, Rab3b has been shown to be the dominant Rab protein, and the inhibition of Rab3b expression in lactotrophs (via antisense oligonucleotides)



**Figure 7. The size of the pool of most readily releasable granules during  $\text{Ca}^{2+}$ -dependent exocytosis is not affected by the G-protein-triggered  $\text{Ca}^{2+}$ -independent exocytosis**

Plot of the cumulative exocytosis triggered (in 3–4 min) by GTP- $\gamma$ -S (50 or 500  $\mu\text{M}$ ) before the delivery of the UV flash (Pre-flash) and the size of the readily releasable pool (Post-flash; measured as the amplitude of the fastest exocytic burst following  $[\text{Ca}^{2+}]_i$  elevation). Exocytosis was normalized to the initial capacitance of individual cells and plotted as the percentage increase in cell surface area. A two-tailed Student's *t* test showed that the post-flash increases in cell surface area in cells stimulated with 50 and 500  $\mu\text{M}$  GTP- $\gamma$ -S were not statistically different from control cells ( $P = 0.87$  and  $0.62$ ). Thus GTP- $\gamma$ -S has no significant effect on the size of the readily releasable pool. The experimental procedure is as described in Fig. 6. In control cells, the pipette solution contained no added GTP- $\gamma$ -S. Since no increase in membrane capacitance was detected in control cells before the UV flash, the value of the pre-flash for control cells was not included here. The mean value for each group of cells was generated from 12–15 cells.



reduces Ca<sup>2+</sup>-dependent exocytosis (Lledo *et al.* 1993). Further experiments are needed to determine whether Rab proteins or other monomeric G-proteins mediate the G-protein-stimulated Ca<sup>2+</sup>-independent exocytosis in gonadotrophs.

### Ca<sup>2+</sup>-independent exocytosis may affect a distinct pool of granules

Our study also shows that the G-protein stimulated-Ca<sup>2+</sup>-independent exocytosis is different from the Ca<sup>2+</sup>-dependent exocytosis in several aspects. First, the G-protein-stimulated exocytosis has a slow onset. Even in experiments involving flash photolysis of caged GTP- $\gamma$ -S, where the concentration of GTP- $\gamma$ -S in the cell rose within milliseconds, exocytosis was triggered only at tens of seconds following the delivery of the UV flash (Fig. 1). This slow onset may reflect the time of G-protein turnover in the cell. In contrast, Ca<sup>2+</sup>-dependent exocytosis was triggered within tens of milliseconds following the photolysis of caged Ca<sup>2+</sup> (Tse *et al.* 1997; see also Fig. 6B). Second, the rate of G-protein-stimulated exocytosis is slow. Even in cells stimulated with 500  $\mu$ M GTP- $\gamma$ -S or GppNHP, the maximum rate of exocytosis was only  $\sim 6$  fF s<sup>-1</sup>. A similar slow rate of exocytosis was also observed in chromaffin cells (Burgoyne & Handel, 1994) and melanotrophs (Okano *et al.* 1993) stimulated with GTP analogues. In contrast, the rate of Ca<sup>2+</sup>-dependent exocytosis in gonadotrophs could range from several femtofarads per second (at [Ca<sup>2+</sup>]<sub>i</sub> of 1–2  $\mu$ M) to several thousand femtofarads per second (at [Ca<sup>2+</sup>]<sub>i</sub> of > 20  $\mu$ M; Tse *et al.* 1997). Third, during maximal stimulation by GTP analogues, the total exocytosis triggered via the Ca<sup>2+</sup>-independent process is almost twice the pool size of the readily releasable granules that is available for Ca<sup>2+</sup>-dependent release (Fig. 7). However, subsequent stimulation of Ca<sup>2+</sup>-dependent exocytosis still elicited an exocytic burst that was similar to that of the control cells (Fig. 7). This raises the possibility that G-protein-stimulated Ca<sup>2+</sup>-independent exocytosis may affect a pool of granules that is distinct from the Ca<sup>2+</sup>-dependent pool.

Since the capacitance measurement monitors only changes in cell surface area during exocytosis, it is not certain whether the G-protein-stimulated Ca<sup>2+</sup>-independent exocytosis observed here indeed reflects exocytosis of granules containing LH and/or follicle stimulating hormone (FSH) in rat gonadotrophs. Discrepancies between the time course of the rapid phase of capacitance increase (triggered by flash photolysis of caged Ca<sup>2+</sup>) and amperometry signals (reflecting fusion of granules containing oxidizable products) have been reported in mast cells and chromaffin cells (Oberhauser *et al.* 1996), and this has led to the suggestion that capacitance increase may not always reflect fusion of granules containing oxidizable products. Yet, another study on chromaffin cells has shown that the rapid increase in capacitance triggered by flash photolysis of caged Ca<sup>2+</sup> (when [Ca<sup>2+</sup>]<sub>i</sub> < 100  $\mu$ M) is always accompanied by amperometry signals (Xu *et al.* 1998). In mast cells, the GTP- $\gamma$ -S-triggered increase in capacitance was accompanied by amperometry signals,

indicating GTP- $\gamma$ -S indeed caused exocytosis of granules containing oxidizable products (Oberhauser *et al.* 1996). Since LH and FSH cannot be detected by amperometry, similar experiments cannot be performed here. Considering that microvesicles are rarely seen in gonadotrophs (Tse *et al.* 1997), the Ca<sup>2+</sup>-independent exocytosis probably came from fusion of granules. However, it is not clear whether every granule present in gonadotrophs contained LH and/or FSH. Nevertheless, in permeabilized sheep gonadotrophs, GTP- $\gamma$ -S has been reported to stimulate LH release even when [Ca<sup>2+</sup>]<sub>i</sub> was buffered below 100 nM (van der Merwe *et al.* 1991). Therefore, it is likely that in rat gonadotrophs, LH/FSH release indeed occurred during GTP- $\gamma$ -S stimulation. Since GnRH induced negligible exocytosis when [Ca<sup>2+</sup>]<sub>i</sub> was buffered (Tse *et al.* 1993), the G-protein-stimulated Ca<sup>2+</sup>-independent pathway probably contributes little to secretion during GnRH stimulation. However, gonadotrophs exhibit basal release of LH/FSH. The basal release was slow and manyfold smaller than that triggered by GnRH stimulation but persisted in the presence of voltage-gated Ca<sup>2+</sup> channel blockers, intracellular Ca<sup>2+</sup> buffers or removal of extracellular Ca<sup>2+</sup> (Chang *et al.* 1988; Billard *et al.* 1997). Since the G-protein-stimulated Ca<sup>2+</sup>-independent pathway triggers slow exocytosis, it is possible that this pathway may have a role in the basal release of LH/FSH.

- AHNERT-HILGER, G., WEGENHORST, U., STECHER, B., SPICHER, K., ROSENTHAL, W. & GRATZ, M. (1992). Exocytosis from permeabilized bovine adrenal chromaffin cells is differently modulated by guanosine 5'-[ $\gamma$ -thio]triphosphate and guanosine 5'-[ $\beta$ -imido]triphosphate. *Biochemical Journal* **284**, 321–326.
- ARIDOR, M., RAJMILEVICH, G., BEAVEN, M. A. & SAGI-EISENBERG, R. (1993). Activation of exocytosis by the heterotrimeric G protein G<sub>13</sub>. *Science* **262**, 1569–1572.
- BILLIARD, J., KOH, D., BABCOCK, D. F. & HILLE, B. (1997). Protein kinase C as a signal for exocytosis. *Proceedings of the National Academy of Sciences of the USA* **94**, 12192–12197.
- BLINKS, J. R., WIER, W. G., HESS, P. & PRENDERGAST, F. G. (1982). Measurement of Ca<sup>2+</sup> concentrations in living cells. *Progress in Biophysics and Molecular Biology* **40**, 1–114.
- BURGOYNE, R. D. & HANDEL, S. E. (1994). Activation of exocytosis by GTP analogues in adrenal chromaffin cells revealed by patch-clamp capacitance measurement. *FEBS Letters* **344**, 139–142.
- CARTER, L. L., REDELMEIER, T. E., WOOLENWEBER, L. A. & SCHMID, S. L. (1993). Multiple GTP-binding proteins participate in clathrin-coated vesicle-mediated endocytosis. *Journal of Cell Biology* **120**, 37–45.
- CHANG, J. P., STOJILKOVIC, S. S., GRAETER, J. S. & CATT, K. (1988). Gonadotropin-releasing hormone stimulates luteinizing hormone secretion by extracellular calcium-dependent and -independent mechanisms. *Endocrinology* **123**, 87–97.
- FERNANDEZ, J. M., NEHER, E. & GOMPERTS, B. D. (1984). Capacitance measurements reveal stepwise fusion events in degranulating mast cells. *Nature* **312**, 453–455.
- GRYNKIEWICZ, G., POENIE, M. & TSIEN, R. Y. (1985). A new generation of Ca<sup>2+</sup> indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440–3450.

- HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.
- HAWES, B. E., BARNES, S. & CONN, P. (1993). Cholera toxin and pertussis toxin provoke differential effects on luteinizing hormone release, inositol phosphate production, and gonadotropin-releasing hormone (GnRH) receptor binding in the gonadotrope: evidence for multiple guanyl nucleotide binding proteins in GnRH action. *Endocrinology* **132**, 2124–2130.
- HIDAKA, H., INAGAKI, M., KAWAMOTO, S. & SASAKI, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23**, 5036–5041.
- HOFFMAN, G. R., NASSER, N., OSWALD, R. E. & CERIONE, R. A. (1998). Fluoride activation of the Rho family GTP-binding protein Cdc42Hs. *Journal of Biological Chemistry* **273**, 4392–4399.
- JOHANNES, L., LLEDO, P.-M., ROA, M., VINCENT, J.-D., HENRY, J.-P. & DARCHEN, F. (1994). The GTPase Rab3a negatively controls calcium-dependent exocytosis in neuroendocrine cells. *EMBO Journal* **13**, 2029–2037.
- KUKULJAN, M., STOJIKOVIC, S. S., ROJAS, E. & CATT, K. J. (1992). Apamin-sensitive potassium channels mediate agonist-induced oscillations of membrane potential in pituitary gonadotrophs. *FEBS Letters* **301**, 19–22.
- LAW, G. J., NORTHROP, A. J. & MASON, W. T. (1993). Rab3-peptide stimulates exocytosis from mast cells via a pertussis toxin-sensitive mechanism. *FEBS Letters* **333**, 56–60.
- LLEDO, P.-M., VERNIER, P., VINCENT, J.-D., MASON, W. T. & ZOREC, R. (1993). Inhibition of rab3B expression attenuates Ca<sup>2+</sup>-dependent exocytosis in rat anterior pituitary cells. *Nature* **364**, 540–544.
- MACLEAN, C. M., LAW, G. J. & EDWARDSON, J. M. (1993). Stimulation of exocytotic membrane fusion by modified peptides of the rab3 effector domain: re-evaluation of the role of rab3 in regulated exocytosis. *Biochemical Journal* **294**, 325–328.
- MACRAE, M. B., DAVIDSON, J. S., MILLAR, R. P. & VAN DER MERWE, P. A. (1990). Cyclic AMP stimulates luteinizing-hormone (lutropin) exocytosis in permeabilized sheep anterior-pituitary cells. *Biochemical Journal* **271**, 635–639.
- MITTAL, R., AHMADIAN, M. R., GOODY, R. S. & WITTINGHOFFER, A. (1996). A formation of transition-state analog of the Ras GTPase reaction by Ras-GDP, tetrafluoroaluminate, and GTPase-activating proteins. *Science* **273**, 115–117.
- OSBERHAUSER, A. F., ROBINSON, I. M. & FERNANDEZ, J. M. (1996). Simultaneous capacitance and amperometric measurements of exocytosis: a comparison. *Biophysical Journal* **71**, 1131–1139.
- OSBERHAUSER, A. F., MONCK, J. R., BALCH, W. E. & FERNANDEZ, J. M. (1992). Exocytotic fusion is activated by rab3a peptides. *Nature* **360**, 270–273.
- OKANO, K., MONCK, J. R. & FERNANDEZ, J. M. (1993). GTP $\gamma$ S stimulates exocytosis in patch-clamped rat melanotrophs. *Neuron* **11**, 165–172.
- PADFIELD, P. J., BALCH, W. E. & JAMIESON, J. D. (1992). A synthetic peptide of the rab3a effector domain stimulates amylase release from permeabilized pancreatic acini. *Proceedings of the National Academy of Sciences of the USA* **89**, 1656–1660.
- SENYSHYN, J., BALCH, W. E. & HOLZ, R. W. (1992). Synthetic peptides of the effector-binding domain of rab enhance secretion from digitonin-permeabilized chromaffin cells. *FEBS Letters* **309**, 41–46.
- SMITH, P. E., FRAWLEY, L. S. & NEILL, D. (1984). Detection of LH release from individual pituitary cells by the reverse hemolytic plaque assay. *Endocrinology* **115**, 2484–2486.
- TAKAHASHI, I., KOBAYASHI, E., NAKANO, H., MURAKATA, C., SAITOH, H., SUZUKI, K. & TAMAOKI, T. (1990). Potent selective inhibition of 7-o-methyl UCN-01 against protein kinase C. *Journal of Pharmacology and Experimental Therapeutics* **255**, 1218–1221.
- TAKEKI, K., MCPHERSON, P. S., SCHMID, S. L. & DECAMILI, P. (1995). Tubular membrane invaginations coated by dynamin rings are induced by GTP- $\gamma$ S in nerve terminals. *Nature* **374**, 186–190.
- THOMSON, F. J., JOHNSON, M. S., MITCHELL, R., WOLBERS, W. B., ISON, A. J. & MACEWAN, D. J. (1993). The differential effects of protein kinase C activators and inhibitors on rat anterior pituitary hormone release. *Molecular and Cellular Endocrinology* **94**, 223–234.
- TSE, A. & HILLE, B. (1992). GnRH-induced Ca oscillations and rhythmic hyperpolarizations of pituitary gonadotropes. *Science* **255**, 462–464.
- TSE, A. & HILLE, B. (1994). Patch clamping studies on identified pituitary gonadotropes *in vitro*. In *Pulsatility in Neuroendocrine Systems*, vol. 20, *Methods in Neurosciences*, ed. LEVINE, J. E., pp. 85–99. Academic Press, Orlando, FL, USA.
- TSE, A. & TSE, F. W. (1997). G-protein activation stimulates Ca<sup>2+</sup>-independent exocytosis in pituitary gonadotrophs. *Society for Neuroscience Abstracts* **23**, 95.
- TSE, A. & TSE, F. W. (1998).  $\alpha$ -Adrenergic stimulation of cytosolic Ca<sup>2+</sup> oscillations and exocytosis in identified rat corticotrophs. *Journal of Physiology* **512**, 385–393.
- TSE, A., TSE, F. W., ALMERS, W. & HILLE, B. (1993). Rhythmic exocytosis stimulated by GnRH-induced calcium oscillations in rat gonadotropes. *Science* **260**, 82–84.
- TSE, A., TSE, F. W. & HILLE, B. (1994). Calcium homeostasis in identified rat gonadotrophs. *Journal of Physiology* **477**, 511–525.
- TSE, A., TSE, F. W. & HILLE, B. (1995). Modulation of Ca<sup>2+</sup> oscillation and apamin-sensitive, Ca<sup>2+</sup>-activated K<sup>+</sup> current in rat gonadotropes. *Pflügers Archiv* **430**, 645–651.
- TSE, F. W., TSE, A., HILLE, B., HORSTMANN, H. & ALMERS, W. (1997). Local Ca<sup>2+</sup> release from internal stores controls exocytosis in pituitary gonadotrophs. *Neuron* **18**, 121–132.
- TURGEON, J. L. & WARING, D. W. (1986). cAMP augmentation of secretagogue-induced luteinizing hormone secretion. *American Journal of Physiology* **250**, E62–68.
- VAN DER MERWE, P. A., MILLAR, R. P., WAKEFIELD, I. K. & DAVIDSON, J. S. (1991). Inhibition of luteinizing-hormone exocytosis by guanosine 5'-[ $\gamma$ -thio]triphosphate reveals involvement of a GTP-binding protein distal to second messenger generation. *Biochemical Journal* **275**, 399–405.
- XU, T., BINZ, T., NIEMANN, H. & NEHER, E. (1998). Multiple kinetic components of exocytosis distinguished by neurotoxin sensitivity. *Nature Neuroscience* **1**, 192–200.

### Acknowledgements

We thank Drs J. D. Neill for the gift of antibodies and W. F. Dryden for comments, and Mrs C. Shiu for technical assistance. This work was supported by the Canadian Medical Research Council and the Alberta Heritage Foundation for Medical Research (AHFMR).

F.W.T. and A.T. contributed equally to this work.

### Correspondence

F. W. Tse and A. Tse: Department of Pharmacology, 9-70 Medical Science Building, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.

Email: fred.tse@ualberta.ca

