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Dual effects of G-protein activation on Ca-dependent exocytosis in bovine lactotrophs

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The whole-cell patch-clamp technique was used to measure cell membrane capacitance (C_m) to monitor exocytosis in single-cultured bovine prolactin-secreting cells (lactotrophs) of the anterior pituitary. The cells were dialyzed with solutions containing different concentrations of ionised Ca and non-hydrolyzable GTP analogues (GTP-γ-S and GMP-PNP) to activate G-proteins. We have identified two distinct effects of G-protein activation on Ca-induced exocytosis: (i) the maximum C_m increase due to intracellular Ca-dependent exocytosis was diminished, suggesting an inhibitory role of G-proteins close to the site of granule fusion, while (ii) the rate of C_m increase ($\Delta C_m/\Delta t$) was facilitated, revealing conversely a stimulatory role of G-proteins in the translocation of secretory granules to the fusion sites.

Ca^{2+}, intracellular; G-protein; GTP analog; Exocytosis; Prolactin; Patch clamp

1. INTRODUCTION

The lactotroph cells of the anterior pituitary gland secrete the hormone prolactin. The neurohormone dopamine (DA) inhibits and thyrotrophin-releasing hormone (TRH) stimulates prolactin release. The receptors to both these factors are coupled to intracellular events via G-proteins [1–6]. Components of the G-protein system have been implicated in regulation of intracellular Ca homeostasis in a variety of cells, but their role in the control of stimulus-secretion coupling is unclear. In some cells, G-protein activation synergizes with Ca-dependent exocytosis [7–9] whereas in others it has an inhibitory effect [10,11]. We have attempted to understand the role of G-proteins in exocytosis of prolactin in single-cultured bovine pituitary lactotrophs. As the fusion of secretory granules with the plasma membrane proceeds during exocytosis, membrane area and hence membrane capacitance (C_m) increase. Using the whole-cell patch-clamp technique [12] we have made real time measurements of C_m to monitor exocytosis [13] during dialysis with solutions containing different concentrations of ionised Ca and non-hydrolyzable GTP analogues to activate G-proteins. We report here the dual effect of G-protein activation on Ca-dependent exocytosis in the lactotrophs.

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2. MATERIALS AND METHODS

2.1. *Materials*

The recording bath solution had the following composition (in mM): 131.8 NaCl, 5 KCl, 1.8 $CaCl_2 \cdot 2H_2O$, 2 $MgCl_2 \cdot 6H_2O$, 0.5 NaH_2PO_4, 5 $NaHCO_3$, 10 Hepes/NaOH, 10 glucose; pH 7.2. The cells were dialyzed with a high Ca (~1 μM free Ca, estimated with a K_d of 0.15 μM [14]) internal solution containing: 140 K gluconate, 10 NaCl, 2 $MgCl_2$, 10 Hepes, 0.5 EGTA, 3.5 Ca-EGTA, pH 7.2, with Trizma base. Ca-free EGTA and Ca-saturated EGTA (Ca-EGTA) were prepared in 100 mM stocks as previously described [8]. The Ca-free solution was: 140 K gluconate, 10 NaCl, 2 $MgCl_2 \cdot 6H_2O$, 10 EGTA, 10 Hepes/Trizma base, pH 7.2. The GTP analogues, GMP-PNP and GTP-γ-S were obtained from Boehringer, all other reagents from Sigma and Aldrich, highest purity obtainable.

2.2. *Methods*

Bovine pituitary lactotrophs were cultured according to the

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methods described previously [15]. The cells were voltage clamped at the holding potential of −70 mV. The reversal potential of the whole-cell current was −50 mV. Membrane capacitance was recorded using a two-phase lock-in amplifier (sinusoidal frequency, 1600 Hz, 1 mV peak-peak) incorporated into a patch-clamp amplifier [16]. The plot of the passive cell parameters: access conductance (G_a), parallel combination of leak and membrane conductance (G_m) and membrane capacitance (C_m) were derived by a computer-aided reconstruction. The d.c. current and the real and imaginary admittance signals were digitised with an A/D converter (CED 1401, Cambridge, England). The software programme (John Dempster, University of Strathclyde) used holding potential and reversal potential in the calculations [17,18].

3. RESULTS AND DISCUSSION

Fig.1 shows a typical response of a lactotroph cell during exocytosis, stimulated to secrete with ~1 μM Ca in the pipette solution, confirming our previous findings [19,20]. The C_m trace consists of an initial flat trace of zero capacitance representing the cell-attached condition. Rupture of the membrane patch spanning the electrode tip was indicated by an upward jump in the capacitance trace due to addition of the resting cell membrane capacitance into the circuit. Changes in C_m are correlated with changes in cell surface area, which increases as the membranes of the secretory granules are incorporated into the plasma membrane during exocytotic fusion [13]. This increase has previously been shown in these cells to be composed of small, abrupt step changes in C_m (2–10 fF), consistent with values expected from single granule fusion [19], which summate to give large overall changes in C_m. With ~1 μM free Ca in the pipette, a slow sigmoidal increase in C_m occurred. For convenience, we have defined the secretory response (in %) as a normalized value for each cell namely, maximal C_m − resting C_m/resting C_m. This was found to be 35 ± 7% (mean ± SE, N = 16) with 1 μM free Ca in the pipette solution, whereas in cells dialyzed with low Ca pipette solutions (see fig.1 legend) only a small slow steady increase of C_m was observed with a minimal secretory response of 4 ± 1% (N = 28).

G-protein activation at low Ca concentrations was not sufficient to invoke a secretory response. Cells were dialyzed with solutions containing 10 mM EGTA with 20–100 μM GTP-γ-S (5′-guanosine γ-thiotriphosphate) added. This GTP analogue binds to G-proteins and can produce irreversible G-protein activation in the absence of receptor activation [21,22]. The secretory responses were indistinguishable from conditions where no GTP analog was included. For example with 20 μM and 40 μM GTP-γ-S, the secretory responses were 3 ± 1% (N = 11) and 5 ± 1% (N = 5), respectively.

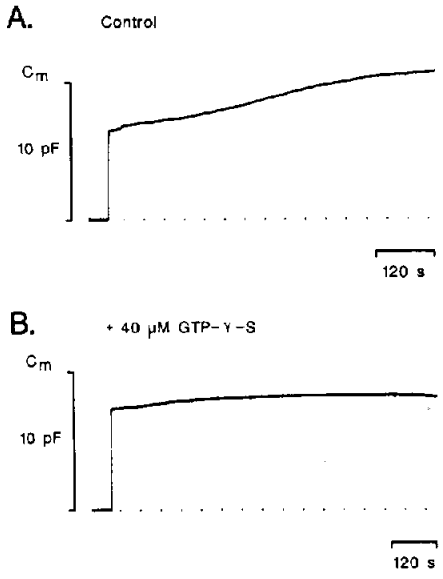

Fig.1. Measurements of C_m in cells dialyzed with ~1 μM Ca only (A), and with the added presence of 40 μM GTP-γ-S in the dialysate (B). Both traces are part of simultaneous plots of access conductance and parallel combination of leak and membrane conductance, but are not displayed as no significant effects on these parameters were observed (see below). The initial resting C_m is indicated by the rapid jump in the C_m trace from the baseline zero capacitance level which indicated the time when the whole-cell recording configuration was attained. Note the slow sigmoidal increase in C_m from the resting C_m value in A, compared to the diminished response in B, indicating attenuation of the Ca-dependent secretory response by the GTP analogue.

However, G-protein activation had very different effects on exocytosis when intracellular solutions with high ionised Ca were used. To ascertain the interaction of G-proteins with Ca-dependent exocytosis, the non-hydrolyzable GTP analogs GMP-PNP (5′-guanolyl imido-diphosphate) and GTP-γ-S were included separately in the pipette solution containing ~1 μM free Ca. Fig.1B shows C_m changes in a lactotroph cell dialyzed with ~1 μM Ca and 40 μM GTP-γ-S, where the secretory response was 12% compared

to fig.1A, where the secretory response was 71%. With 100 μM GMP-PNP, the average secretory response was 27 ± 7% (N = 9), while with 40 μM GTP-γ-S the response was 17 ± 4% (N = 9). Intracellular perfusion of the cells with the GTP analogs therefore attenuated the Ca-dependent secretory response, and the attenuation by GTP-γ-S was statistically significant ($P < 0.02$, fig.2). The different degrees of attenuation produced by the two different GTP analogs can in part be explained by the inherent differences in potency and efficacy with which they activate G-proteins [1,7].

In addition to attenuating the secretory response, the GTP analogues influenced the temporal pattern of the responses in the cells (cf. for instance fig.3A and B). The rate of C_m increase ($\Delta C_m/\Delta t$) was calculated by determining the cell capacitance change (ΔC_m) occurring in the time slot between 10% and 50% of the maximal C_m increase from rest. The rate of C_m increase was highly correlated with the secretory response in the control, GMP-PNP and GTP-γ-S-treated cells with correlation coefficients of 0.78, 0.78 and 0.77, respectively.

Given this correlation, we were interested to determine whether the rate of C_m increase was different in the GTP-analogue treated cells (pooled data of GMP-PNP and GTP-γ-S treatment) compared to control. Linear regression analysis was performed on the rate of C_m increase and secretory response relationship (fig.3), and the predicted estimates from the regression line were found to be significantly different at $P < 0.05$ (t-test) between the control and GTP-analogue-treated group in the region beyond 20% of the secretory response. This suggests that activated G-proteins in lactotrophs influence the rate of exocytosis. A trivial explanation for our data might have been lower access conductances in the treated experiments, allowing a faster diffusional exchange between the pipette and cytosol [17]. This is, however, unlikely as the measured access conductances in the control, GMP-PNP and GTP-γ-S-treated cells were 165 ± 14 (N = 16), 158 ± 15 (N = 9) and 170 ± 18 (N = 9) nS, respectively, and not significantly different.

The granule fusion event itself is virtually instantaneous in mast cells [23] and in bovine pituitary lactotrophs (Zorec, Sikdar and Mason, manuscript in preparation). The slow time course of C_m increase illustrated in fig.1A is thus likely to be deter-

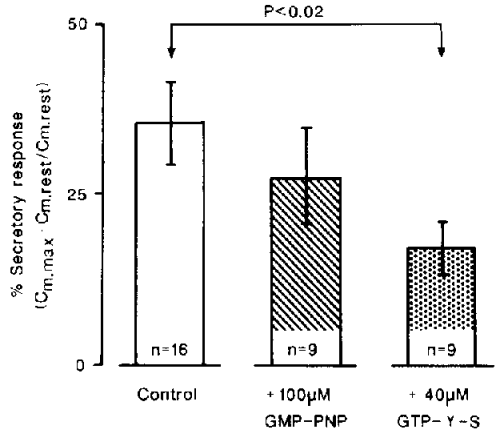

Fig.2. Suppressive effect of GTP analogues on the Ca-dependent secretory response. $C_{m.max}$, maximal C_m recorded; $C_{m.rest}$, initial resting C_m. Note the decrease in the secretory response in the added presence of GMP-PNP and GTP-γ-S in the pipette solution containing ~1 μM free Ca, when compared to the response in the presence of ~1 μM free Ca alone (control). Statistical test: The Welch's test for Behren's Fisher problem [29].

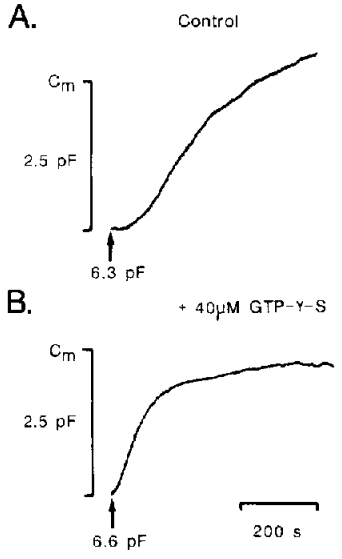

Fig.3. Time course of exocytosis in control (~1 μM intracellular Ca^{2+} only; upper trace) and GTP-γ-S dialyzed cell (lower trace). ΔC_m plots obtained by digital subtraction of the resting initial C_m value from the C_m plot, to show the time course of secretory response. Note the faster time course of secretory response in the GTP analogue treated cell. Both cells were from the same culture batch. The resting C_m, secretory response and the rate of exocytosis ($\Delta C_m/\Delta t$, see text) for the cell shown in the upper trace were 6.3 pF, 48% and 1.1 pF/100 s; while for the cell shown in the lower trace, the corresponding values were 6.6 pF, 36% and 2 pF/100 s.

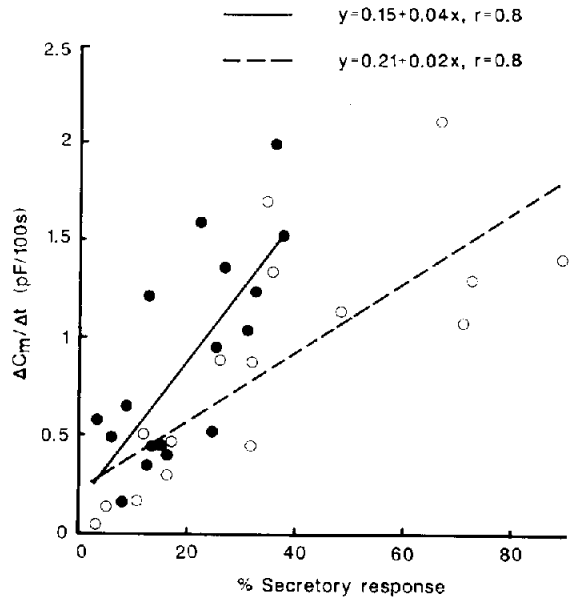

Fig.4. Stimulatory action of GTP analogues on the kinetics of secretory response. $\Delta C_m/\Delta t$ was calculated as described in the text. (○) Control; (●) GTP analogue treated (pooled data of GMP-PNP and GTP-γ-S treatment). The straight lines (dashed line, control; unbroken line, GTP analogue treated) were drawn according to the linear regression equations shown. *r*, regression coefficient.

mined by a slower process than the secretory granule fusion event itself. The likely process governing the slow phenomenon would in part be due to translocation of granules to the plasma membrane [24]. If this is so the effect of GTP analogues in our preparation may be to stimulate the translocation of granules to the plasma membrane. The likely mechanism which facilitates translocation is the reduction or elimination of a possible cytoskeletal barrier which prevents granule approach to the plasma membrane [25–27]. In support of this, a sub-membrane cytoskeletal network can be demonstrated in lactotrophs by TRITC-phalloidin staining (Sikdar and Mason, unpublished). Also, a role for guanine nucleotides in depolymerization of the actin network has recently been suggested in chromaffin cells [9].

To summarize, we have shown that G-protein activation can have both inhibitory and stimulatory effects on Ca-dependent exocytosis, monitored by single-cell capacitance measurements. The inhibitory role is suggested by the suppression of the relative C_m increase (secretory response) by G-protein activation, whereas the stimulatory role is indicated by the faster kinetics of C_m increase indicating more rapid granule translocation. Our results are thus in agreement with mounting evidence that multiple pathways with several functionally different G-proteins are involved in the dynamic control of stimulus-secretion coupling [28]. The two functionally distinct roles of G-proteins demonstrated by the present experiments may thus be important in prolactin secretion controlled by dopamine and TRH (thyrotrophin releasing hormone): evidence suggests that both of these substances are linked to intracellular pathways via G-proteins [1–6]. While the receptor associated G-proteins may additionally control exocytosis indirectly through Ca-homeostasis, our results suggest they may also exert direct actions at (a) site(s) close to the fusion event itself.

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