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The Heterotrimeric GTP-Binding Protein, G_S, Modulates The Cl^- Conductance of Rat Parotid Acinar Secretory Granules

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$G_{s\alpha}$ has been reported to be present in rat parotid acinar secretory granule membrane (SGM) fractions. In the present study, we evaluated epitope orientation of $G_{s\alpha}$ on the secretory granule (SG) and the ability of G_s to modulate the Cl^- conductance of isolated granules by measuring granule lysis. $G_{s\alpha}$ was found to be associated with the cytoplasmic face of the SGM. Aluminum fluroide (AlF_4^-, 20μM Al^{3+} and 10 mM F^-) significantly increased granule lysis and this effect was blocked by GDPβS. Cholera toxin (5μg/ml) mimicked the effects of AlF_4^- on granule lysis, whereas pertussis toxin (0.5μg/ml) was without effect. GTPγS, however, reduced granule lysis in a concentration-dependent manner. The orientation of $G_{s\alpha}$ on the SGM as well as the effects of AlF_4^- and cholera toxin on granule lysis lends support for a role of G_s in the exocytotic process.

Recent data show that heterotrimeric GTP-binding proteins (G-proteins) consisting of α,β and γ subunits are not restricted to the plasma membrane (PM) where they link receptors to effectors of the signal transduction cascade (2,3). G_α subunits have been shown to be associated with intracellular membranes/structures such as the Golgi complex (4), cytoskeletal components, actin and microtubules (5,6), endoplasmic reticulum (ER) and SGs (7,8,9,10), and to be involved in vesicular transport (11) and coat formation (12). The identification of G_α subunits on intracellular organelles, such as SGs, suggests that they may play a role in exocytosis. In the rat parotid gland, several G-proteins have been identified in the SGM fraction with $G_{s\alpha}$ being the most prominent (1). $G_{s\alpha}$ has also been found on the SG of anterior pituitary cells and is thought to play a role in targeting and/or exocytosis (8). However, its precise role is not clear.

GTP and GTP analogues have been demonstrated to modulate Cl^- conductance found to be present in zymogen granules (ZGs) isolated from rat pancreatic acini (13,14,15) and in SGs isolated from parotid acini (14,15,16). Further, it was concluded that Cl^- influx into the granule, followed by water movement, is required for granular swelling and the flushing out of granular contents into the lumen (14,17). Thus, the aim of the present study was to investigate the functional significance of $G_{s\alpha}$ in rat parotid SGs by examining the effects of bacterial toxins, cholera and pertussis and $GTP_\gamma S$ on granule lysis (Cl^- conductance).

MATERIALS AND METHODS

Materials were obtained as follows: aluminum fluoride (AlF_4^-) from EM Science (Cherry Hill, N.J.); pertussis and cholera toxins from List Biological Laboratories Inc. (Campbell, CA); renografin-60 from E.R. Squibb (New Brunswick, N.J.); Nonidet P-40 (NP-40), rabbit anti-human α-amylase fractionated serum, trypsin, trypsin inhibitor, guanosine 5′-[γ-thio]triphosphate (GTPγS), GTP, ATP, NAD^+ from Sigma (St. Louis, MO); sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE), dithiothreitol (DTT), TRIS, glycine and sucrose from Bio-Rad (Encinitas, CA); Mark 12, molecular weight protein standards, NuPAGE 10% bis[2-Hydroxyethyl]-iminotris[hydroxymethyl]methane (BIS-TRIS) gels (1 mm, 10 well), 3-[N-morpholino] propane sulfonic acid (MOPS) SDS running, sample preparation and transfer buffers from Novex (San Diego, CA); Ficoll 400 from Pharmacia (Uppsala, Sweden); Enhanced Chemiluminescent (ECL) reagents and Hyperfilm MP autoradiography film from Amersham Life Sciences, Inc. (Arlington Heights, IL); horseradish peroxidase (HRP) conjugated donkey anti-rabbit IgG from Jackson ImmunoResearch (West Grove, PA). Affinity-purified antiserum (RM/122), raised against a peptide specific for high and low mass $G_{s\alpha}$ proteins was generously supplied by Dr. Allen Spiegel, NIDDK/NIH (Bethesda, MD). Purified recombinant $G_{s\alpha}$ was generously provided by Dr. Maurine Linder and Dr. Alfred Gilman, University of Texas (Dallas, TX). All other chemicals were of reagent grade.

Preparation of rat parotid secretory granule membranes. Secretory granules were isolated using protocols of Iversen et al., (18); their purity was previously characterized (1). Used as isolated for lysis; granules were purified additionally by sedimentation through 1.45 M sucrose, 5% wt/vol Ficoll 400, 2 mM sodium MOPS, 1 mM EDTA, pH 7.0 onto a cushion of 2 M buffered sucrose as described by von Zastrow and Castle (19) for characterization of $G_{s\alpha}$ association with the SG.

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Characterization of SG associated with $G_{s\alpha}$. Proteolytic digestion experiments were performed using purified intact SGs suspended in 0.3 M sucrose, 2mM sodium MOPS and 1mM EDTA. Granules were incubated with 50 μg/ml trypsin in the absence or presence of 0.1% NP-40 for 25 min, and at room temperature to expose the cytoplasmic surface of granules to proteolysis. The digestion was stopped in the presence of 100 μg/ml trypsin inhibitor. Samples were centrifuged for 5 min at 16,000 g for granule particulate collection. Amylase was monitored by immunoblot analysis to assess efficacy of trypsin and detergent permeabilization conditions.

Gel electrophoresis and immunoblot analysis. Secretory granule proteins were resolved concomitantly with proteins of standard molecular weight by SDS/BIS-TRIS 10% PAGE with MOPS SDS running buffer (20) using NuPAGE mini-gels, the Xcell II Mini-Cell electrophoresis system, and protocols of Novex (San Diego, CA). Resolved proteins were transferred to Immobilon-P^{SQ} polyvinyldene filters (PVDF) from Millipore Corp., (Bedford, MA), using the Mini Trans-Blot system of Bio-Rad Laboratories (Hercules, CA), placed overnight in 2X NuPAGE transfer buffer (50 mM, N,N-bis[2-hydroxyethyl]-glycine (bicine), 1 mM EDTA, 0.5 mM chlorobutanol, pH 7.3) and antioxidant (Novex) at 4°C at a constant 30 V. The presence of $G_{s\alpha}$ on SGs was assessed by immunoblot analysis using HRP conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc. (Burlingame, CA), ECL reagents and protocol of Amersham Life Sciences, Inc. (Arlington Heights, IL).

Measurement of Cl^- conductance in isolated secretory granules. The Cl^- conductance of isolated rat parotid SGs was determined as described by Gasser et al. (14). Aliquots of granules were placed in buffer consisting of 150 mM KCl, 1.0 mM EGTA, 0.1 mM $MgSO_4$ and 20 mM N-2-hydroxyethylpiperazine-N′-2 ethanesulfonic acid (HEPES), pH 7.0 and placed into 1 ml quartz cuvettes in a Beckman spectrophotometer at 37° C for 30-35 min. The Cl^- conductance was determined by measuring the decrease in absorbance, i.e., Δ absorbance (ΔA) with time at 540 nm. Lysis rates (ΔA_{540} h^{-1}) were calculated from the slopes of the curves at fixed times (7 and 12 min) following the introduction of valinomycin (1 μM) to initiate lysis. In the presence of valinomycin, the K^+ permeability is no longer rate limiting and, therefore, the rate of decrease in the absorbance of the SG suspension is a measure of the Cl^- conductance. Data are normalized to control A values.

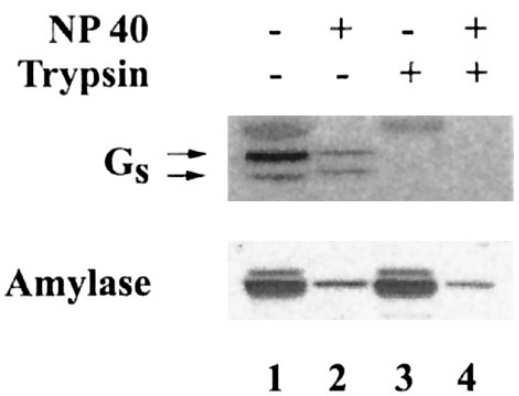

FIG. 1. Membrane orientation of $G_{s\alpha}$ associated with SGs of the rat parotid gland. Intact granules (160 μg of protein) were incubated in buffered (pH 7.0) media containing 0.3M sucrose, 2mM Na MOPS, and 1mM EDTA in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 0.1% Nonidet P-40 (NP-40) and exposed (lanes 3 and 4) or not exposed (lanes 1 and 2) to protease digestion as described in Methods. Granule proteins (22 μg) were resolved in SDS/BIS-TRIS 10% PAGE and Western blotted to PVDF, and the presence of $G_{s\alpha}$ and amylase was assessed by immunoblot analysis using rabbit anti-$G_{s\alpha}$ affinity purified polyclonal antibody (RM/122; 2 μg/ml) or rabbit anti-human α-amylase (salivary) fractionated antisera (0.25 μg/ml) for 1 h and HRP conjugated donkey anti-rabbit IgG (1:20000 dilution) for 1 h prior to ECL detection.

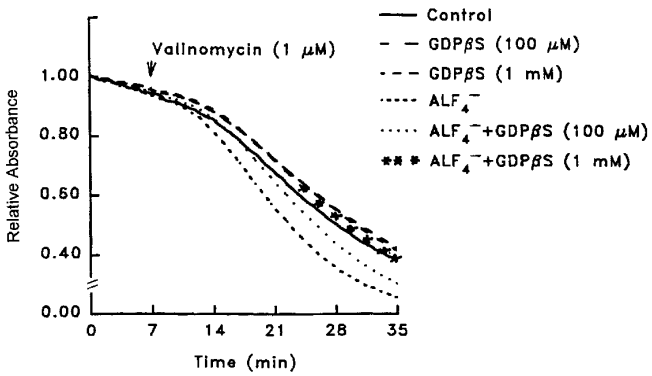

FIG. 2. Effects of AlF_4^- (20 μM Al^{3+} and 10 mMF^-) on rat parotid acinar SG lysis in the absence and presence of GDPβS (100 μM and 1 mM). Intact granules were suspended in a buffered KCl solution at 37° C in a 1 ml cuvette. AlF_4^- or GDPβS was added 5 min prior to the addition of 1μM valinomycin (at arrow), and lysis was followed for 30 min. Results are representative of four experiments.

Statistical analysis. For statistical analysis of the data, a Student's t-test for paired samples was used. Data are presented as the mean ± S.E. Results with levels of $P < 0.01$ were considered significant.

RESULTS

Since exocytosis is known to involve an interaction of the cytosolic face of the SG with the cytosolic face of the PM, initial experiments were conducted to determine epitope orientation of $G_{s\alpha}$ on the SG. Purifed intact SGs were exposed to proteolysis with trypsin, in the presence or absence of nonionic detergent (NP-40) used to permeabilize granules. As shown in Fig. 1, $G_{s\alpha}$ was not detected in trypsin-treated granules either in the absence or presence of detergent. Further, the internal surface of the granule became accessible to proteolysis only after granule permeabilization with NP-40, as evidenced by α amylase levels being unaffected by trypsin digestion, and by the marked reduction in detectable α amylase in granules exposed only to detergent. These data indicate that $G_{s\alpha}$ is associated with the granule membrane on its cytosolic face. Interestingly NP-40, at 0.1% concentration, not only permeabilized the granule membrane, but also solubilized much of the $G_{s\alpha}$ protein as evidenced in Fig. 1 (lanes 1 vs 2).

In functional studies, aluminum flouride (AlF_4^-), a specific activator of all G-proteins (21), which was used successfully to ascertain the involvement of G_α subunits in vesicular transport in a cell-free system (11), significantly increased the rate of granule lysis at 20μM Al^{3+} and 10mM F^- from 1.19 ± 0.08 h^{-1} to 1.53

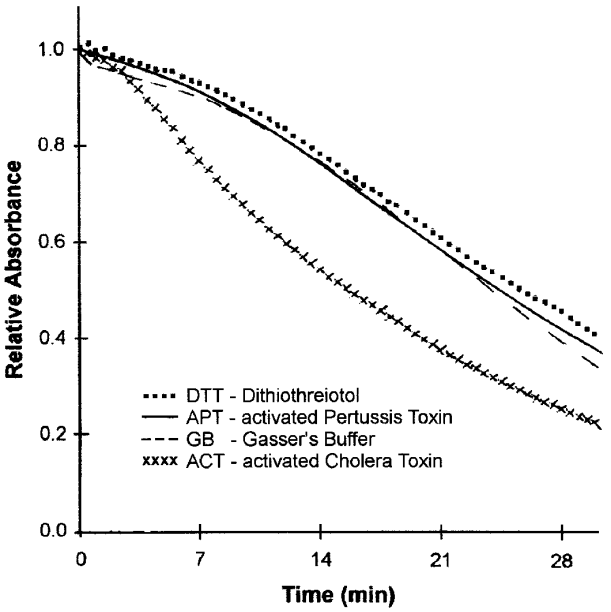

FIG. 3. Effects of activated cholera toxin (ACT, 5μg/ml) and pertussis toxin (APT, 0.5μg/ml) on isolated rat parotid SG lysis. Toxins were activated with 40 mM DTT just prior to use. Granules were suspended in a buffered KCl solution at 37°C in a 1 ml cuvette; agents were added together with 1μM valinomycin at time zero, and lysis was followed for 30 min. Results are representative of three experiments.

$\pm$ 0.11h^{-1} ($P < 0.01$) in the absence and presence of AlF_4^-, respectively, in four different preparations (Fig. 2). These results are consistent with those of Thevnod et al., (15) who also reported that AlF_4^- increased lysis of rat pancreatic zymogen granules (ZGs). We also found that GDPβS (100μM and 1mM) inhibited the effects of AlF_4^- by approximately 50% and 92%, respectively; GDPβS alone had little effect. Similar results were obtained using granules prepared from isolated acinar cells (data not shown).

To provide further evidence that the effects of AlF_4^- on the Cl^- conductance were related to activation of $G_{s\alpha}$, we utilized cholera toxin which is a specific activator of G_s. As shown in Fig. 3, cholera toxin (5μg/ml), activated in the presence of 40 mM (DTT) as described previously (1), significantly increased the rate of granule lysis from 0.99 $\pm$ 0.21 h^{-1} to 1.58$\pm$ 0.27 h^{-1} ($P < 0.01$) in the absence and presence of cholera toxin, respectively. Because $G_{i\alpha}$ proteins are also detected in rat parotid acinar SGM fractions, albeit to a lesser extent than $G_{s\alpha}$ (1), the effect of pertussis toxin on granule lysis was also determined. As shown in Fig. 3, there was no significant change in the lysis rate with pertussis toxin (0.5μg/ml). Concentrations of cholera and pertussis toxins used were based on previous ADP-ribosylation studies (1).

In contrast to the results obtained with AlF_4^- and cholera toxin, GTPγS reduced the rate of SG lysis in a concentration-dependent manner (Fig. 4). Lysis rates were 1.39 $\pm$ 0.22 h^{-1} and 1.19 $\pm$ 0.19 h^{-1} ($P < 0.01$) in the absence and presence of 100 μM GTPγS, respectively. These results are in agreement with those of Thevenod et al., (15), but opposite to results obtained by Piiper et al. (22) in pancreatic zymogen granule membranes (ZGMs). The differences noted may reflect incubation conditions, i.e. a much higher concentration, 25μM, of valinomycin was used by Piiper et al., (22).

In contrast to our results and those of Gasser et al., (14) and Thevenod et al., (15), Takuma et al., (23), did not find detectable Cl^- channels responsible for osmotic swelling of parotid SGs. Although it is not clear why these results differ, at least from our results, it appears that the experiments may not have been carried out long enough, following the addition of valinomycin, to observe significant changes in absorbance at 540nm.

DISCUSSION

Our previous finding that $G_{s\alpha}$ is the major G-protein of the SGM (1), together with new data showing that AlF_4^- and cholera toxin increase granule lysis, is interpreted as indicating a functional role for $G_{s\alpha}$ in stimulating the Cl^- influx in SGMs, which may be important in the exocytotic process. Although AlF_4^- was shown previously to increase a Cl^- conductance in pancreatic ZGs (15), the studies presented here represent the first

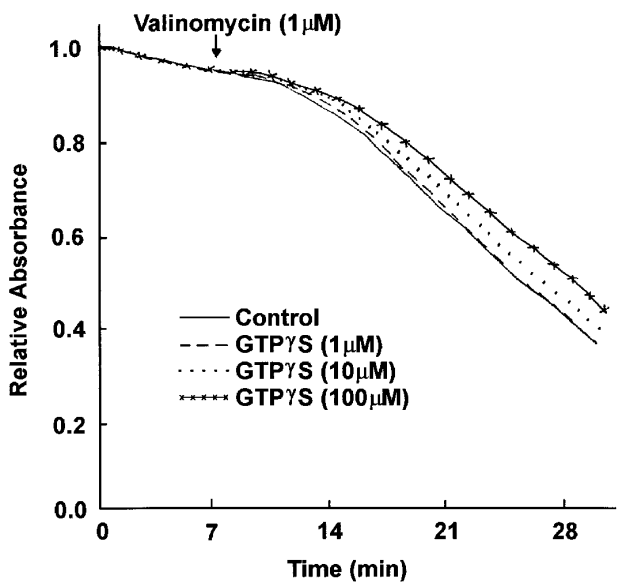

FIG. 4. Concentration effects of GTPγS on Cl^- conductance of isolated rat parotid SG lysis. Granules were suspended in a buffered KCl solution at 37°C in a 1 ml cuvette. GTPγS was added 5 min prior to the addition of 1μM valinomycin (at arrow), and lysis was followed for 30 min. The results are representative of four experiments.

attempt to identify the G-protein involved. The inhibition of ALF_4^--induced lysis by GDPβS further supports a role for a G-protein in the regulation of the Cl^- channel. Further, the presence of $\beta\gamma$ subunits in the SGM fraction (Watson et al., unpublished data) suggests that $G_{s\alpha}$ is activated since the $G_{\beta\gamma}$ complex is required for the activation of G_α subunits. The lack of effect of pertussis toxin on granule lysis, on the other hand, is consistent with previous immunoblot data which showed low immunoreactivity of the SGM fraction to antisera generated against $G_{i\alpha}$ and G_o.

G_s on the cytoplasmic face of the parotid SG may be the unidentified GTP-binding protein, G_E, or one of several other GTP-binding proteins, located downstream from the plasma membrane and suggested to be involved in exocytosis in mast cells (24). Interestingly, a putative G-protein on chromaffin granules has been suggested to inhibit exocytosis (25). In the present study, GTPγS caused a significant decrease in SG lysis. One explanation is that the effects of GTPγS represent the sum of effects on both stimulatory and inhibitory GTP-binding proteins. Given that AlF_4^-, activates heterotrimeric G-proteins, both stimulatory and inhibitory (21), but not monomeric proteins, and that GTP analogues modulate both monomeric and heterotrimeric GTP-binding proteins, suggests that a monomeric GTP-binding protein(s) may be contributing to the inhibitory effect on Cl^- conductance. Monomeric GTP-binding proteins have been detected in isolated SG fractions (26), and they may have both stimulatory and inhibitory roles.

If G_s regulates exocytosis, the mechanism(s) is not clear. One possibility is via changes in ion conductances known to be present in the rat parotid and pancreatic granules (13,14,16). It is known, for example, that $G_{s\alpha}$ can affect other types of ion channels (27). Gasser et al, (14) provided evidence that the Cl^- conductance of isolated ZGs is activated by pretreatment with secretogogues in vivo and postulated that the resulting influx of osmotically active molecules into ZGs and concomitant swelling may be important for the final incorporation of the granule membrane into the PM. Thus, the assumption has been that granule lysis is the key mechanism in exocytosis (14). While swelling and widening of the fusion pore may contribute to the efficiency of exocytotic swelling or potentiate the release of granule contents, this assumption neglects consideration of the probable role of docking types of mechanisms at the PM. Given that SNARE proteins have recently been found in SGM fractions from exocrine cells (28,29, Watson et al., unpublished data), it is not unreasonable to propose that $G_{s\alpha}$ may interact with these proteins as part of a pre-exocytotic complex providing a molecular trigger for docking or targeting. Heterotrimeric G-proteins, such as G_s, could provide an additional level of regulation or specificity to this process as suggested by Denker et al., (30).

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