Evidence for G proteins in rat parotid plasma membranes and secretory granule membranes

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G proteins were identified in rat parotid plasma membrane-enriched fractions and in two populations of isolated secretory granule membrane fractions. Both [³²P]ADP-ribosylation analysis with bacterial toxins and immunoblot analysis with crude and affinity-purified antisera specific for α subunits of G proteins were utilized. Pertussis toxin catalysed the ADP-ribosylation of a 41 kDa substrate in the plasma membrane fraction and both secretory granule membrane fractions. Cholera toxin catalysed the ADP-ribosylation of two substrates with molecular masses of 44 kDa and 48 kDa in the plasma membrane fraction but not in the secretory granule fractions. However, these substrates were detected in the secretory granule fractions when recombinant ADP-ribosylating factor was present in the assay medium. Immunoblot analysis of rat parotid membrane fractions using both affinity-purified and crude antisera revealed strong immunoreactivity of these membranes with anti-G_{sa}, -G_{ia1/2} and -G_{ia3} sera. In contrast G_{sa} was the major substrate found in both of the secretory granule fractions. Granule membrane fractions also reacted moderately with anti-G_{ia3} antiserum, and weakly with anti-G_{ia1/2} and -G_{oa} sera. The results demonstrate that the parotid gland membranes express a number of G proteins. The presence of G proteins in secretory granule membranes suggests that they may play a direct role in regulating exocytosis in exocrine glands.

INTRODUCTION

Agonists stimulate secretion via two major signal transduction pathways in exocrine parotid glands; one involves stimulation of adenylate cyclase and cyclic AMP formation, and the other involves phosphoinositide turnover and the generation of Ins-(1,4,5)P₃, Ca²⁺ and diacylglycerol (Bdolah & Schramm, 1965; Watson *et al.*, 1979; Aub & Putney, 1984). G proteins couple hormonal stimuli to these second messenger systems in a variety of tissues, including the salivary glands (Gilman, 1984; Taylor *et al.*, 1986). These proteins are heterotrimers comprised of α , β and γ subunits. A family of these G proteins has been recognized (Gilman, 1984) which includes transducin, G_i, G_s and G_o, and at least nine genes have been identified that encode distinct α subunits (Lochrie & Simon, 1988). The α subunits include G_{i1}, G_{i2}, G_{i3}, G_o and G_s, and are the sites for ADP-ribosylation by pertussis toxin and cholera toxins (Milligan, 1988). Substrates for ADP-ribosylation have been reported to be present in membrane fractions from many tissues, and more recently antisera to the α subunit proteins have been prepared and utilized to identify and localize G proteins in a host of tissue types (Milligan, 1988). Substrates have been located in the plasma membrane, cytosol and secretory granule membranes. Identification of G proteins in secretory granules, however, has not been widely examined. G_{oa} and G_{ia} have been identified in granule membranes from chromaffin and human neutrophil cells (Toutant *et al.*, 1987; Rotrosen *et al.*, 1988). Volpp *et al.* (1989) also identified a 39 kDa pertussis-toxin substrate in the human neutrophil specific granule-enriched fraction; cholera toxin substrates, however, were only found in the plasma membrane. Little information is available regarding the identification and localization of G proteins in exocrine glands. In rat pancreas, immunocytochemistry revealed that G_o is present in islet cells but not in acinar or ductal cells (Terashima *et al.*, 1987). Schnepfel *et al.*

(1990) utilized both ADP-ribosylation and immunoblot analysis to show that a number of G proteins are expressed in purified rat pancreatic acinar plasma membranes.

Relatively little data are also available concerning G protein occurrence and function in salivary glands. It has been shown that a pertussis toxin-insensitive G protein, G_p (Laniyonu *et al.*, 1988), is coupled to phosphoinositide turnover in rat parotid and submandibular glands (Taylor *et al.*, 1986; Fleming *et al.*, 1989), but the identity of this protein remains unknown. In rat submandibular gland, Fleming *et al.* (1989) demonstrated that a pertussis toxin-sensitive G protein is involved in the muscarinic inhibition of cyclic AMP metabolism; however, this G protein was not characterized. More recently, Ambudkar *et al.* (1990) provided direct evidence, by ADP-ribosylation studies, that G_i is present in the rat parotid gland but is not involved in Ca²⁺ mobilization. G_s has also been identified in both rat pancreatic and submandibular acinar cell membranes by cholera toxin-catalysed [³²P]ADP-ribosylation (Schnepfel *et al.*, 1990; Ahmad *et al.*, 1990) and shown to be involved in the regulation of cyclic AMP (Spearman *et al.*, 1983). To date, however, there have been no published reports in which heterotrimeric G proteins have been identified in exocrine gland secretory granules, although small GTP-binding proteins have been identified in rat pancreatic acinar cell secretory granule membranes (Padfield & Jamieson, 1991).

In the present study, we have identified G proteins in rat plasma-membrane enriched fractions and in two populations of secretory granule membranes i.e. light and heavy, by two methods: pertussis and cholera toxin-catalysed [³²P]ADP-ribosylation, and immunoblot analysis using crude and affinity-purified G protein antisera generated against specific α peptide subunits. Results indicate that rat parotid plasma membranes contain G_s, G_{i1} and/or G_{i2} and G_{i3}, and that G_s is the major G protein of rat parotid secretory granule membranes.

Abbreviations used: ARF, ADP ribosylating factor; DAB, diaminobenzidine; INH, isotonic acid hydrazide; 3-APAD, 3-acetylpyridine adenine dinucleotide; DMPC, dimyristoyl-L- α -phosphatidylcholine.

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MATERIALS AND METHODS

Materials

Materials were obtained as follows. ADP-ribose, ATP, arginine (free base), GTP, NAD⁺, thymidine, isonicotinic acid hydrazide (INH), 3-acetylpyridine adenine dinucleotide (3-APAD), dimyristoyl-L- α -phosphatidylcholine (DMPC), sodium cholate, cell composition marker enzymes and related reagents, normal rabbit sera and diaminobenzidine (DAB) were from Sigma (St. Louis, MO, U.S.A.); Percoll was from Pharmacia (Uppsala, Sweden); pertussis toxin and cholera toxin A subunit were from List Biological Laboratories Inc. (Campbell, CA, U.S.A.); renografin-60 was from E. R. Squibb (New Brunswick, NJ, U.S.A.); dithiothreitol, Tween-20, Coomassie Brilliant Blue 250, SDS/PAGE chemicals and both non-biotinylated and biotinylated low-molecular mass protein standards [rabbit muscle phosphorylase *b* (97 kDa), BSA (66.2 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21.5 kDa)] were from Bio-Rad (Richmond, CA, U.S.A.); Pelikan fountain ink for fountain pens was from Pelikan AG (Hanover, Germany); SDS/PAGE and Western blot apparatus including mini-cell, pre-cast 10% Tris-glycine mini-gels, blotting transfer module, nitrocellulose filters and gel dryer were from Novex (Encinitas, CA, U.S.A.); Medcast/Araldite 502 was from Ted Pella, Inc. (Tustin, CA, U.S.A.); [³²P]NAD⁺ di(triethylammonium) salt [800 Ci (29.6 TBq) mmol], and the crude rabbit antisera AS/7, EC/2, GC/2 and RM/1, raised by immunization with synthetic decapeptides corresponding to G_{1 α 1/2}, G_{1 α 3}, G_{0 α} and G_{s α} respectively, were obtained from DuPont-New England Nuclear (Boston, MA, U.S.A.). The affinity-purified antisera AS/115, EC/54, GO/85 and RM/122, raised against specific peptides representing G_{1 α 1/2}, G_{1 α 3}, G_{0 α} and G_{s α} respectively, were generously supplied by Dr. Allen Spiegel, NIIDDK/NIH Bethesda, MD, U.S.A. Purified recombinant G_{1 α 1}, G_{1 α 3}, G_{0 α} and G_{s α} were generously provided by Dr. Maurine Linder and Dr. Alfred Gilman, University of Texas, Dallas, TX, U.S.A. Adrenal cortical membrane was obtained from Dr. Ron McFarland, University of Washington, Seattle, WA, U.S.A. Human purified recombinant ADP-ribosylation factor (ARF) was generously provided by Dr. Richard Kahn, National Cancer Institute, NIH, Bethesda, MD, U.S.A. All other chemicals were of analytical reagent grade.

Preparation of rat parotid plasma membranes and secretory granule membranes

Plasma membranes were prepared by the procedure of Arvan *et al.* (1983), with slight modification. Briefly, the parotid glands were removed from fasted male Sprague-Dawley rats weighing 200–250 g. The glands were minced and homogenized using a loose-fitting glass-Teflon homogenizer, and a 250 g supernatant and pellet fractions were prepared as described previously (Iversen *et al.*, 1985). The supernatant was used to prepare the secretory granules, while the pellet, containing approx. 50% of the plasma membrane marker (results not shown), was used to prepare the membrane fraction. The 250 g pellet was resuspended in Arvan's solution A (0.5 mM-MgCl₂, 1 mM-NaHCO₃, pH 7.4) and spun for 30 s at 70 g. The supernatant was saved and the pellet was rehomogenized and spun. This step was repeated once more and the pellets were discarded. The three supernatants were combined and diluted with solution B (0.5 mM-MgCl₂, 1 mM-NaHCO₃, 0.7 mM-EDTA, pH 7.4). The supernatants were filtered through 4 layers of cheesecloth and centrifuged at 825 g for 15 min. The pellet was rehomogenized with three strokes in a glass-Teflon homogenizer, diluted, layered above 5 ml of 0.3 M-

sucrose in solution B and spun for 15 min at 825 g. The pellet was then resuspended in 1.38 M-sucrose in solution B, placed in centrifuge tubes, overlaid with 0.3 M-sucrose in solution B and centrifuged at 82 500 g for 2 h. The plasma membrane sheets float to the 0.3–1.38 M interface, while other particulate material forms a pellet. The interface band was collected, supplemented with EDTA to give a final concentration of 1.2 mM, diluted to 0.35 M-sucrose with solution C (0.5 mM-MgCl₂, 1 mM-NaHCO₃, 1.7 mM-EDTA, pH 7) and dispersed with three strokes of a tight-fitting homogenizer. The sample was then spun at 110 000 g for 30 min. The plasma membranes were obtained in the pellet.

Light and heavy secretory granules were isolated as described previously by our laboratory (Iversen *et al.*, 1985). A 250 g supernatant of a homogenate of parotid glands was layered over 30% renografin and centrifuged at 64 000 g. The more mature granules (heavy fraction) were obtained in the pellet. The less mature granules (light fraction) remained suspended in the renografin. A pellet of light granules was obtained by diluting the renografin 1:2 (v/v) with 0.3 M-sucrose homogenization medium and centrifuging. The purity of intact secretory granules was determined using the following cell composition marker enzymes: K⁺-dependent *p*-nitrophenyl phosphatase for plasma membranes (Arvan & Castle, 1982); UDP-galactosyltransferase for Golgi (Kim *et al.*, 1971); monoamine oxidase for mitochondrial outer membranes (Edelstein *et al.*, 1978); cytochrome *c* oxidase for mitochondrial inner membranes (Yonetani & Ray, 1965); glucose-6-phosphatase for endoplasmic reticulum (Swanson, 1950; Kallner, 1975); and β -N-acetyl-D-glucosamidase for lysosomal contamination (Findlay *et al.*, 1958). Granules were lysed overnight and granule membranes were separated from contents as described by Robinovitch *et al.* (1980). Granule membranes were washed twice with hypo-osmotic medium and then twice with iso-osmotic buffer as described by Robinovitch *et al.* (1980) to remove any residual granule content contamination.

Preparation of purified rough endoplasmic reticulum membranes

Membranes were prepared as described by Bayerdörffer *et al.* (1984) for rat pancreas.

Electron microscopy

The pellet of endoplasmic reticulum membranes was resuspended in mannitol buffer and placed in a Microfuge tube with an equal volume of chilled 5% glutaraldehyde in 0.1 M-sodium cacodylate buffer (pH 7.4). After overnight fixation at 4 °C, the membranes were spun down at 16 000 g for 30 min. The supernatant was drawn off and 1% Bacto-Agar was carefully layered on to the pellet. The agar plug containing the membrane pellet was washed in sodium cacodylate buffer, post-fixed in 1% osmium tetroxide in cacodylate buffer for 1 h at 4 °C, dehydrated in a graded series of alcohols, put through three changes of propylene oxide, and embedded in Medcast/Araldite 502. Thin sections were cut, stained with uranyl acetate and lead citrate, and viewed in a Philips 410 electron microscope.

ADP-ribosylation

ADP-ribosylation of membrane proteins by pertussis toxin and cholera toxin (A subunit) were performed as described by Gill & Woolkalis (1988) and Johnson *et al.* (1978) with modifications. Pertussis toxin was pre-activated by incubating equal volumes of pertussis toxin (0.1 μ g/ml) in 20 mM-sodium phosphate buffer, pH 7.0, containing 100 mM-NaCl and 20 mM-dithiothreitol for 30 min at 37 °C. The reaction mixtures for both pertussis and cholera toxins contained, in a final volume of 100 μ l and at pH 7.3, 50 mM-potassium phosphate, 20 mM-NaCl, 10 mM-thymidine and 10 mM-dithiothreitol. Additional reactants for ribosylation with pertussis toxin (2 μ g) were 1 mM-ATP,

10 mM-EDTA and 0.1 % Triton X-100, and with cholera toxin (5 μ g) were 0.1 mM-GTP, 20 mM-arginine and 5 mM-ADP-ribose. The reaction was initiated by the addition of [32 P]NAD $^{+}$ (10 μ M, 55000–76000 c.p.m.). After a 60 min incubation at 37 °C for pertussis toxin and at 30 °C for cholera toxin, the reaction was stopped by diluting the samples with 1 ml of ice-cold 100 mM-NaCl/10 mM-potassium phosphate buffer, pH 7.3.

ARF-dependent cholera toxin-catalysed ADP-ribosylation was assayed according to methods of Weiss *et al.* (1989), with modifications. Purified recombinant ARF was pre-activated with GTP in the presence of Mg $^{2+}$ (Bobak *et al.*, 1990). ARF was incubated at a final concentration of 0.1 mg/ml with 3 mM-DMPC, 0.2 % sodium cholate, 400 nM-GTP, 6 mM-MgCl $_2$ and 1 mM-EDTA in a 10 mM-potassium phosphate buffer, pH 7.3, for 2 h at 30 °C. Additional reactants for ADP-ribosylation by cholera toxin (10 μ g) with activated ARF (1 μ g) included 1 mM-EDTA, 0.1 % sodium cholate, 3 mM-DMPC (suspended by sonication) and 20 mM-INH. The reaction was initiated by the addition of [32 P]NAD $^{+}$ (10 mM; 96000 c.p.m.) and 0.9 mM-3-APAD, and was carried out as described above. NADase inhibitors (INH and 3-APAD) were included (Gill & Woolkalis, 1988). The membranes containing ADP-ribosylated proteins were separated from non-reacted [32 P]NAD $^{+}$ by centrifugation at 48000 g for 30 min at 4 °C and solubilized in SDS (denaturing, reducing) sample buffer. Radiolabelled proteins were resolved by SDS/PAGE according to Laemmli (1970), in 1.5 mm thick pre-cast 10 % Tris-glycine mini-gels. Gels were run for 2.5 h at 4 °C at a constant 125 V. Low-molecular-mass standard proteins were run in parallel with samples. The gels were stained for 4 h in 0.1 % Coomassie Blue R-250, 50 % methanol, 10 % acetic acid and 4 % glycerol, destained overnight in the above solution without dye, equilibrated in 50 % methanol/5 % glycerol solution for 30 min, and air-dried. Autoradiograms were developed after variable exposure of the dried gel to Kodak XAR 5 film with DuPont Cronex intensifying screens at –80 °C. The migration distance and area of 32 P imaging of labelled protein was quantified by scanning densitometry using a LKB 2222-010 Ultrosan XL laser spectrophotometric scanner with integrator and 2400 Gelscan XL software. The apparent molecular masses of substrates for each toxin were estimated by eye from a curve generated by plotting the migration distance of standard proteins transposed from gel to film against their molecular mass (Hames & Rickwood, 1981).

Immunoblotting

Purified recombinant G $_{\alpha}$ proteins, membrane proteins and biotinylated low-molecular-mass standard proteins were separated electrophoretically as described above and transferred to 0.45 μ m-pore-size nitrocellulose blotting filters in 12 mM-Tris/96 mM-glycine buffer, pH 8.3, containing 20 % methanol for 1 h with constant current of 275 mA at 4 °C. Transferred proteins were detected either by India ink staining of nitrocellulose filters (Hancock & Tsang, 1983) or by Coomassie Blue staining of gels. The nitrocellulose blots were blocked with 0.1 % (v/v) Tween-20 in Tris-buffered saline (100 mM-Tris, 0.9 % NaCl, pH 7.5) for 30 min. This procedure and all subsequent ones were run at room temperature and with gentle agitation. The nitrocellulose blots were cut into strips of approx. 20 cm 2 and transferred to shallow acrylic plastic troughs containing 10 ml of diluted primary antiserum (crude or affinity-purified) in blocking buffer and incubated for 30 min. Antibody binding was detected colorimetrically by the avidin/biotinylated horseradish peroxidase immunoassay using the Vectastain ABC kit with biotinylated goat anti-(rabbit IgG) as second antibody and DAB with cobalt ion enhancement as colour substrate (Harlow &

Lane, 1988). After four 5 min washes with blocking buffer, 10 ml of an approx. 5 μ g/ml solution (equivalent to one drop of reagent) of biotinylated anti-(rabbit IgG) second antibody was added per strip. After a 30 min incubation the strips were washed four times for 5 min each in blocking buffer. The washed strips were then incubated with avidin/biotinylated horseradish peroxidase complex solution, prepared as recommended by the manufacturer, for 30 min, washed again as above, and given a final rinse with blocking buffer without Tween-20. Colour development occurred within 30 s and the reaction was stopped by washing strips with water. The migration distance and area of bound antibody associated with each antigen band was quantified by scanning densitometry as described above. Proteins were assayed by the Folin method of Hartree (1972) using BSA as standard.

RESULTS

Determination of membrane fraction purity

Secretory granule fraction purity was assayed using the intact granule preparation rather than the secretory granule membrane preparations *per se*, because of the low yields of membrane obtained after the lysing and washing procedures used. The greatest contamination of granules was by endoplasmic reticulum fragments, which represented approx. one-half of the specific activities found in the 250 g supernatant fraction for both heavy and light secretory granules respectively (Table 1). Contamination of granules by mitochondrial inner and outer membranes was also high, but only for the light granule fractions. Plasma membranes were prepared according to the method of Arvan *et al.* (1983), who determined that 0.4 % or less of total homogenate activity for each of the above markers was recovered in the plasma membrane fraction. Although there were no secretory granule markers available, Arvan *et al.* (1983) estimated granule contamination by measuring the extent of absorption of

Table 1. Marker enzyme analysis of contaminating organelles in secretory granule preparations

Enzyme activities are expressed as a percentage of the total found in the 250 g supernatant and are given as the mean $\pm$ S.E.M. for three preparations of granules. The specific activities (per mg of granule protein) relative to that of the 250 g supernatant are given in parentheses. Enzyme activities in the 250 g supernatant were: K $^{+}$ -dependent *p*-nitrophenol phosphatase, 16.9 nmol $\cdot$ min $^{-1}\cdot$ mg $^{-1}$; UDP-galactosyltransferase, 51.8 pmol $\cdot$ min $^{-1}\cdot$ mg $^{-1}$; monoamine oxidase, 213 pmol $\cdot$ min $^{-1}\cdot$ mg $^{-1}$; cytochrome *c* oxidase, 0.0248 min $^{-1}\cdot$ mg $^{-1}$ (first order rate constant); β -*N*-acetyl-D-glucosaminidase, 16.4 nmol $\cdot$ min $^{-1}\cdot$ mg $^{-1}$; glucose-6-phosphatase, 7.4 nmol of P $_i$ $\cdot$ min $^{-1}\cdot$ mg $^{-1}$. Enzyme assays were performed as indicated in Materials and methods section

Marker	250 g supernatant	Crude light granules	Heavy granules
Glucose-6-phosphatase	100 (1)	2.29 $\pm$ 0.80 (0.50)	4.33 $\pm$ 0.44 (0.35)
K $^{+}$ -dependent- <i>p</i> -nitrophenol phosphatase	100 (1)	0.57 $\pm$ 0.03 (0.11)	0.72 $\pm$ 0.20 (0.06)
UDP-galactosyl-transferase	100 (1)	0.38 $\pm$ 0.03 (0.07)	0.63 $\pm$ 0.06 (0.05)
Monoamine oxidase	100 (1)	2.81 $\pm$ 0.74 (0.55)	1.92 $\pm$ 0.60 (0.15)
Cytochrome <i>c</i> oxidase	100 (1)	3.12 $\pm$ 0.67 (0.58)	1.89 $\pm$ 0.36 (0.15)
β - <i>N</i> -Acetyl-D-glucosaminidase	100 (1)	2.06 $\pm$ 0.22 (0.40)	1.99 $\pm$ 0.18 (0.16)

Fig. 1. Autoradiograms of pertussis toxin-catalysed ADP-ribosylation of membrane proteins of rat parotid gland and bovine adrenal cortex

(a) Adrenal cortical membrane (AD), light (LT) and heavy (HY) secretory granule membranes and plasma membranes (PM) (16 μ g each) were incubated with 32 P-labelled NAD $^{+}$ in the absence (lanes 1, 3, 5 and 7) and presence (lanes 2, 4, 6 and 8) of pertussis toxin. After labelling, membrane proteins (approx. 3 μ g) were separated by SDS/PAGE and analysed by autoradiography after a 7 day exposure. (b) Plasma membrane and light and heavy secretory granule membranes were incubated with 32 P]NAD $^{+}$ in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of pertussis toxin. After labelling, membrane proteins of plasma membrane, light and heavy granule fractions (approx. 5, 4 and 3 μ g respectively) were separated by SDS/PAGE and analysed by autoradiography after a 2 day exposure as described in the Materials and methods section.

radioactive secretory proteins to plasma membranes. Only 0.02 % of the label was found in the plasmalemmal material.

Identification of pertussis and cholera toxin substrates by ADP-ribosylation

Fractions of rat parotid plasma membranes and secretory granule membranes were incubated in the presence and absence of pertussis or cholera toxins and 32 P]NAD $^{+}$. Membrane proteins were separated on 10 % polyacrylamide gels by SDS/PAGE, and the radiolabelled substrates were identified by autoradiography as described in the Materials and methods section. As shown in Fig. 1, pertussis toxin catalysed the ADP-ribosylation of a single protein band with a molecular mass of 41 kDa in plasma membranes, both light and heavy granule membranes, and adrenal cortical membranes which were used as a control.

Fig. 2. Autoradiogram of cholera toxin-catalysed ADP-ribosylation of membrane proteins of the rat parotid gland

Light (LT) and heavy (HY) secretory granule membranes and plasma membranes (PM) were incubated with 32 P]NAD $^{+}$ in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of activated cholera toxin. After labelling, membrane proteins (approx. 3 μ g) were separated by SDS/PAGE and analysed by autoradiography after a 5 day exposure, as described in the Materials and methods section.

Labelling of the plasma membranes and granule membranes was specific; no proteins were labelled in controls without pertussis toxin. Film exposures of shorter duration failed to resolve more than one band for variable gel loads of membrane protein (Fig. 1b). Densitometry measurements, however, corrected for the variable gel loads of protein, indicated that the plasma membrane labelling was greater than that observed for light and heavy secretory granule membranes. The weak pertussis toxin labelling of a 30 kDa band observed on SDS/PAGE (10 % gels) for each membrane fraction, and the labelling at the Bromophenol Blue dye front (approx. 27 kDa) appear to be products of pertussis toxin auto-ADP-ribosylation. Experiments initially performed in the absence of membrane resulted in a similar labelling at 30 kDa and a second broad banding between 24 and 28 kDa (results not shown).

Cholera toxin catalysed the ADP-ribosylation of two protein bands in the plasma membrane having molecular masses of 44 kDa and 48 kDa, although the 48 kDa band was somewhat diffuse (Fig. 2). No labelling was observed for either of the secretory granule membrane fractions under identical assay conditions. Even a 5-fold increase in the gel load of granule membrane protein failed to result in detectable cholera toxin-dependent ADP-ribosylation (Fig. 3). Similarly, the use of NADase inhibitors had no effect, suggesting the absence of significant NAD $^{+}$ hydrolase activity. In other experiments ARF, previously shown to activate cholera toxin-catalysed ADP-ribosylation of purified G $_{\alpha s}$ (Kahn & Gilman, 1984), was examined for its ability to enhance cholera toxin-catalysed ADP-ribosylation of secretory granule membranes. When activated ARF was added to a reaction mixture containing the light secretory granule membrane fraction, ADP-ribosylation of 44 kDa and 48 kDa proteins was detected (Fig. 3). Although heavy secretory granules were not examined, similar results would be expected since immunoblot data with G $_{\alpha s}$ -specific antisera showed no apparent differences between the granule membranes (see Fig. 6a). No radiolabelled peptides were detected in control membranes without cholera toxin and ARF, showing the specificity of the 32 P incorporation. In preliminary studies with adrenal cortical

Fig. 3. Autoradiogram of cholera toxin-catalysed ADP-ribosylation of membrane proteins from light secretory granules in the presence of purified recombinant human ARF

Membranes (18 μ g of protein) were incubated in the presence of [32 P]NAD $^{+}$ with (+) and without (–) cholera toxin (CT) and ARF. After labelling, membrane proteins (approx. 15 μ g) were separated by SDS/PAGE and analysed by autoradiography after a 4 day exposure, as described in the Materials and methods section.

membranes, ARF alone did not produce an effect (results not shown), which is consistent with its role as cofactor. An intense labelling of a small cholera toxin band (approx. 25 kDa) represents auto-ADP-ribosylation of the toxin A peptide which is enhanced by ARF (Tsai *et al.*, 1988). Similar results were obtained in the absence of membranes (results not shown).

Detection of G α subunits by immunoblot analysis

In initial experiments the specificity of antisera for α subunits of purified recombinant G $_{s\alpha}$, G $_{o\alpha}$, G $_{i1\alpha}$ and G $_{i3\alpha}$ proteins (10 ng each) was determined; both crude and affinity-purified antisera were examined. It was felt that this comparison would be useful in

Fig. 5. India ink stain of total protein transferred to nitrocellulose filter (a), and immunoblot analysis of membrane proteins of rat parotid gland reactive to normal rabbit serum (b)

Plasma membranes (PM) and light (LT) and heavy (HY) secretory granule membranes (8 μ g of each) and standard molecular mass non-biotinylated proteins (NP) or biotinylated proteins (BP) were separated by SDS/PAGE and transferred to nitrocellulose filters. The filter was stained with India ink (a) or incubated with normal rabbit serum diluted 1:1000 and immunoassayed as described in the Materials and methods section (b).

light of the commercial availability of the crude antisera. As shown in Fig. 4(a), affinity-purified anti-G $_{s\alpha}$ serum RM/122 recognized both high- (G $_{s\alpha}$ -L) and low- (G $_{s\alpha}$ -S) molecular-mass G $_{s\alpha}$ proteins. It did not cross-react with purified G $_{i\alpha1}$, G $_{i\alpha2}$ or G $_{o\alpha}$ present in 2-fold excess. Identical results were obtained with crude antiserum (results not shown). Affinity-purified anti-G $_{o\alpha}$ serum GO/85 immunoreacted with the purified G $_{o\alpha}$ protein, but also cross-reacted with purified G $_{i\alpha3}$ and G $_{i\alpha1/\alpha2}$ peptides present in 2-fold excess (Fig. 4a). In contrast, crude anti-G $_{o\alpha}$ serum GC/2 immunoreacted only with the purified G $_{o\alpha}$ peptide, as previously reported (Spiegel, 1990) (results not shown). As shown in Fig.

Fig. 4. Specificity of anti-G-protein sera by immunoblot analysis

Purified G α subunits of G $_{s\alpha}$, G $_{o\alpha}$, G $_{i\alpha1/\alpha2}$ and G $_{i\alpha3}$ were diluted in SDS reducing buffer and applied to 10 % polyacrylamide gels. The proteins were resolved by SDS/PAGE and transferred electrophoretically to nitrocellulose filters. The filters were incubated with (a) affinity-purified antisera RM/122 (anti-G $_{s\alpha}$) and GO/85 (anti-G $_{o\alpha}$) diluted 1:1000; (b) affinity-purified antisera AS/115 (anti-G $_{i\alpha1/\alpha2}$; diluted 1:250) and EC/54 (anti-G $_{i\alpha3}$; diluted 1:1000); and (c) normal rabbit serum diluted 1:1000. The immunoassay was carried out as described in the Materials and methods section.

Fig. 6. Immunochemical detection of $G_{\alpha s}$ and $G_{\alpha o}$ subunits in membranes of the rat parotid gland

Plasma membranes (PM) and light (LT) and heavy (HY) secretory granule membranes (8 μ g of each), purified recombinant G_{α} proteins ($G_{\alpha s}$ and $G_{\alpha o}$, 10 ng of each) and standard molecular mass biotinylated proteins (BP) were separated by SDS/PAGE and transferred to nitrocellulose filters. The filters were incubated with (a) affinity-purified antiserum RM/122 and crude antiserum RM/1 (both anti- $G_{\alpha s}$ -S/L; diluted 1:1000) and (b) affinity-purified antiserum GO/85 (diluted 1:250) and crude antiserum GC/2 (diluted 1:1000) (both anti- $G_{\alpha o}$). The immunoassay was carried out as described in the Materials and methods section.

Fig. 7. Immunochemical detection of $G_{\alpha 1/2}$ and $G_{\alpha 3}$ subunits in membranes of the rat parotid gland

Plasma membranes (PM) and light (LT) and heavy (HY) secretory granule membranes (8 μ g of each), purified recombinant G_{α} proteins ($G_{\alpha 1/2}$ and $G_{\alpha 3}$, 10 ng of each) and standard molecular mass biotinylated proteins (BP) were separated by SDS/PAGE and transferred to nitrocellulose filters. The filters were incubated with (a) affinity-purified antiserum EC/54 (diluted 1:250) and crude antiserum EC/2 (diluted 1:1000) (both anti- $G_{\alpha 3}$), (b) affinity-purified antiserum AS/115 (diluted 1:250) and crude antiserum AS/7 (diluted 1:1000) (both anti- $G_{\alpha 1/2}$). The immunoassay was carried out as described in the Materials and methods section.

4(b), affinity-purified anti- $G_{\alpha 1/2}$ serum AS/115 reacted strongly with $G_{\alpha 1}$, and also weakly with $G_{\alpha 3}$ present in 2-fold excess. Affinity-purified anti- $G_{\alpha 3}$ serum EC/54 was strongly reactive to $G_{\alpha 3}$ and also weakly reactive to both $G_{\alpha 1}$ and $G_{\alpha o}$ proteins present in 2-fold excess. The cross-reactivity of crude antisera was the same as that observed with affinity-purified antisera (results not shown). Results are in agreement with those reported by Simonds *et al.* (1989). None of the purified G_{α} proteins (10 ng each) immunoreacted with normal rabbit serum (Fig. 4c).

Membrane proteins transferred to nitrocellulose filters and representative of those analysed immunochemically were visualized by India ink staining (Fig. 5a). Light and heavy granule membrane proteins appeared to differ both qualitatively and quantitatively, providing additional support for two populations of granules as reported previously (Iversen *et al.*, 1985). A peptide band of 53–55 kDa, which stained strongly with India

ink in both granule membrane fractions, represented about 16% of the total protein. This protein is likely to be amylase, since its relative mobility in SDS/PAGE approximates that of a 56.7 kDa protein reported to be amylase in rat parotid gland by Robnovitch & Sreebny (1972).

Weak but discrete bands of immunoreactivity were seen in all membrane preparations for normal rabbit sera (Fig. 5b). These bands were also seen in all membrane preparations for crude antisera, and in the plasma membrane fraction for affinity-purified antisera (Figs. 6 and 7), suggesting that rabbit sera contained naturally occurring antibodies which are cross-reactive to membrane antigens. Background immunoreactivity was not observed with secondary antibody alone.

To identify G proteins in parotid membrane fractions, peptides immunoreactive to both affinity-purified and crude antisera were compared with the specific reactions of purified recombinant G_{α}

Fig. 8. Assessment of contamination by endoplasmic reticulum

(a) Electron micrograph of purified rough endoplasmic reticulum vesicles from rat parotid gland. Rough endoplasmic reticulum vesicles were prepared as described previously (Bayerdörffer *et al.*, 1984). (b) Immunoblot analysis of the $G_{\alpha s}$ subunit. Rough endoplasmic reticulum (ER), plasma membranes (PM), light secretory granule membranes (LT) (7.5 μ g each), purified recombinant $G_{\alpha s}$ (5 ng) and standard molecular mass biotinylated proteins (BP) were separated by SDS/PAGE (12% gels) and transferred to nitrocellulose filters. The filters were incubated with affinity-purified antiserum diluted 1:1000, and the immunoassay was carried out as described in the Materials and methods section.

peptides electrophoresed in parallel. The apparent molecular masses of the peptides of purified α subunits of $G_{s\alpha}$ -S, $G_{s\alpha}$ -L, G_o , G_{i1} and G_{i3} were estimated as described in the Materials and methods section and found to be 43, 47, 40, 41 and 41 kDa, respectively (Figs. 6 and 7). The strength of immunoreactivity of equivalent amounts of purified $G_{s\alpha}$ -L and $G_{s\alpha}$ -S with anti- G_s was comparable (Figs. 4a and 6a). Plasma membranes and both light and heavy secretory granule membranes contained peptides of 43 kDa and 47 kDa which reacted strongly with affinity-purified RM/122 and crude RM/1 (anti- $G_{s\alpha}$) antisera (Fig. 6a). In plasma membranes, these peptides showed equal immunoreactivity. However, in the granule membranes antibody binding was strongest for the 47 kDa peptide; the 43 kDa peptide bound approximately half as much. The additional bands found in the secretory granule membranes, i.e. of molecular masses of approx. 40 kDa and 49 kDa (Fig. 6a) resulted from the use of crude anti- $G_{s\alpha}$ sera.

Plasma membranes also expressed a 40 kDa peptide that reacted strongly to affinity-purified antisera generated against

$G_{o\alpha}$, G_{i23} and $G_{i21/22}$ (Figs. 6b, 7a and 7b). Similar results were obtained with crude antisera, except that immunoreactivity to anti- $G_{o\alpha}$ sera was weak. A peptide of 40 kDa was also present in both secretory granule membrane fractions which was barely detectable with both affinity-purified and crude anti- $G_{o\alpha}$ (Fig. 6b), weakly reactive with anti- $G_{i21/22}$ and moderately reactive with anti- G_{i23} (Figs. 7a and 7b). It was noted that crude and purified antisera generated against $G_{o\alpha}$ (Fig. 6b) also reacted with a 59 kDa peptide which was not reactive with normal rabbit sera. The nature of this peptide is presently unknown.

Because both light and heavy secretory granules contained a relatively high level of contamination by endoplasmic reticulum, further studies were conducted to establish whether the predominant substrate, G_s , found in both granule fractions was due to the presence of endoplasmic reticulum in these fractions. This was of concern, as Audigier *et al.* (1988) and Nigam (1990) reported that G proteins are present in the rough endoplasmic reticulum. Purified endoplasmic reticulum membranes were prepared as described by Bayerdörffer *et al.* (1984) and, as shown in Fig. 8(a), consisted of vesicles that were primarily coated with ribosomes. Immunoblot analysis (Fig. 8b) of the endoplasmic reticulum fraction revealed that anti- $G_{s\alpha}$ sera immunoreacted with 43 kDa and 46 kDa peptides, but the reactivity was weak compared with that obtained with the secretory granule fractions.

DISCUSSION

Studies to identify G proteins in exocrine glands have, thus far, been few. In this paper, we have identified several G proteins in both a rat parotid plasma membrane-enriched fraction and secretory granule membranes (light and heavy) by toxin-catalysed [32 P]ADP-ribosylation, and by immunoblot analysis. Our [32 P]ADP-ribosylation studies revealed a pertussis toxin substrate of approx. 41 kDa in rat plasma membranes, and in both light and heavy secretory granule membrane fractions. We were, however, unable to detect more than one peptide band in our membrane preparations, despite the fact that immunoblots revealed the presence of more than one pertussis toxin substrate. Failure to observe more than one pertussis toxin substrate in plasma membranes and granule membranes may be related to our experimental conditions, since we also failed to detect more than one substrate (41 kDa) in adrenal cortical membranes known to express several pertussis toxin substrates, i.e. G_{i2} and $G_{o\alpha}$. Toutant *et al.* (1987), however, were able to detect ADP-ribosylation of several proteins in chromaffin cell membranes when a ratio of bisacrylamide/acrylamide (0.13:10) was used to obtain electrophoretic patterns instead of the standard Laemmli conditions utilized in our studies.

ADP-ribosylation of plasma membranes by cholera toxin revealed labelling of two peptide bands of 44 kDa and 48 kDa. Under experimental conditions which were successful for ADP-ribosylation of plasma membranes, i.e. the absence of added ARF and NADase inhibitors, we could not detect a cholera toxin substrate in secretory granule membranes. However, ADP-ribosylation of $G_{s\alpha}$, i.e. 44 kDa and 48 kDa peptides, in granule membranes was observed in the presence of ARF. The results would suggest either that secretory granule membranes contain no or insufficient amount of ARF to catalyse ADP-ribosylation by cholera toxin, or that if ARF is present on the secretory granule it is removed when granules are disrupted prior to preparing membranes. The immunoblot findings indicate that G_s protein is present in secretory granules (Fig. 6).

Immunoblot analysis revealed clear differences in the distribution of G_{α} subunits between plasma membranes and secretory granule membranes when either affinity-purified or crude antisera were used. Plasma membranes were highly immuno-

reactive with all of the antisera tested. The strong immunoreactivity of plasma membranes with affinity-purified anti- G_{α} serum is likely due to cross-reactivity noted between this antiserum and anti- $G_{\alpha 3}$ and - $G_{\alpha 1/\alpha 2}$ antisera. Crude anti- G_{α} serum, which did not cross-react with other G_{α} subunits, reacted weakly with a 40 kDa peptide. These findings indicate that G_o is present in low levels. On the other hand, both secretory granule membrane fractions immunoreacted strongly with anti- $G_{\alpha s}$, moderately with anti- $G_{\alpha 3}$ and barely with anti- $G_{\alpha 1/\alpha 2}$ and - $G_{\alpha o}$. The results were similar regardless of whether affinity-purified or crude antisera were used. It was noted, however, that a greater number of non-specific bands were immunodetected with the crude anti- G_s sera. This finding should alert investigators to potential problems of data interpretation when using crude antisera.

The present results clearly show that G_s is the major G protein of the secretory granule membrane. The strong immunoreactivity of both plasma membranes and secretory granule membranes with anti- $G_{\alpha s}$ sera indicates that the immunoreactivity of the secretory granule proteins is not likely to be due to contamination by the plasma membrane. Data presented in Figs. 8(a) and 8(b) also suggest that the strong immunoreactivity of secretory granule membranes with anti- $G_{\alpha s}$ is not simply due to contamination by endoplasmic reticulum, since the immunoreactivity of the endoplasmic reticulum fraction with anti- $G_{\alpha s}$ was weak in comparison with that observed for secretory granule fractions. The presence of a G_s substrate in the endoplasmic reticulum fraction most likely is related to the presence of plasma membranes in this fraction (Bayerdörffer *et al.*, 1984). It is also unlikely that mitochondria account for the G_s signal, since only the light granule membrane showed a significant level of mitochondrial contamination whereas the G_s signal was strong and equal in both granule fractions. The weaker immunoreactivity of the secretory granules with antisera generated against $G_{\alpha 1/\alpha 2}$, $G_{\alpha 3}$ and G_o is less easily interpreted. Given the strength of immunoreactivity, it may reflect cross-reactivity between the antisera or contamination by plasma membranes. No apparent differences in antisera reactivity were noted between the secretory granule fractions. The fact that secretory granule membranes were utilized rather than intact secretory granules suggests that G protein α subunits are part of the membrane rather than of the secretory granule contents. Also, it was previously noted that the two secretory granule fractions utilized in the present study, i.e. light and heavy granules, evidently represent different stages of maturation (Iversen *et al.*, 1985). Moreover, the India ink protein staining results indicate that the membranes of the two secretory granule fractions differ in protein composition. Thus, while there are evidently maturation-related changes in the protein content of secretory granule membranes, these changes do not involve the G proteins.

Of interest was the finding that both affinity-purified and crude antisera, which immunoreacted with the 41 kDa recombinant purified $G_{\alpha 3}$ and $G_{\alpha 1/\alpha 2}$ peptides, also immunoreacted with a peptide of lower molecular mass, i.e. 40 kDa, in both plasma membranes and secretory granule membranes. This difference in the migration of purified peptides and membrane peptides was not apparent with either anti- $G_{\alpha o}$ or anti- $G_{\alpha s}$ sera. It is not likely that the 40 kDa peptide found in rat parotid membranes is G_o , as anti- $G_{\alpha 1/\alpha 2}$ sera did not cross-react with G_o , and anti- $G_{\alpha 3}$ only weakly cross-reacted with G_o (Fig. 4b).

The functional significance of the presence of G_{α} subunits, and in particular $G_{\alpha s}$, in rat parotid secretory granules is also not clear. Rotrosen *et al.* (1988) demonstrated that G_i can translocate from secretory granule membranes to plasma membranes in neutrophils and may represent a mechanism by which cells regulate receptor activity. Whether a similar phenomenon occurs in exocrine cells is not known. GTP-binding proteins may also

control various steps of the exocytotic process, as has been noted in yeast (Goud *et al.*, 1988). The presence of G proteins in rat parotid secretory granules suggests that they may also play a role in exocytosis. Evidence has been obtained that anion-exchange pathways for Cl^- exist in rat parotid secretory granules (Goddard *et al.*, 1989), and Cl^- and K^+ conductances have also been described in isolated secretory granules (DeLisle & Hopfer, 1986; Gasser *et al.*, 1988). Gasser *et al.* (1988) obtained evidence that granule membrane conductance is activated by pretreatment with secretagogues *in vivo*, and they postulated that the Cl^- conductance present in secretory granule membranes is inserted into the luminal membrane during exocytosis. Thevenod *et al.* (1990) reported that GTP and guanosine 5'-[γ -thio]triphosphate decreased Cl^- conductance in both pancreatic and parotid secretory granules, and suggested that G proteins localized in the granule membrane could modulate gating of the Cl^- transporter. Thus our finding that G_s , and possibly G_i , proteins are present in rat secretory granule membranes is consistent with the idea that the G proteins may regulate ion channels, but this hypothesis awaits further experimentation.

In summary, the data presented provide direct evidence of the identity of G proteins in rat parotid membranes. The data show that G_s , $G_{\alpha 1/\alpha 2}$ and $G_{\alpha 3}$ are present in rat parotid plasma membranes, whereas the major G protein in the secretory granule membrane is G_s . To our knowledge this is the first report demonstrating that cholera toxin substrates are present in secretory granule membranes. The finding that G proteins are present in secretory granule membranes suggests that they may be involved in the process of exocytosis.

We thank Dr. Alfred Gilman, Dr. Maureen Linder, Dr. Allen Spiegel and Dr. Richard Kahn for the purified $G\alpha$ peptides, antisera and ARF. We also thank Colleen McKay, Leanne Hutt, Carol Belton and Audrey Wass for their assistance. This work was supported by grant DE05249 from the National Institute of Dental Research.

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Received 16 September 1991/23 January 1991; accepted 4 February 1992