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Presence of three pertussis toxin substrates and $G_o\alpha$ immunoreactivity in both plasma and granule membranes of chromaffin cells

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GTP-binding proteins have been proposed to be involved in some secretory processes. *Bordetella pertussis* toxin is known to catalyze ADP-ribosylation of several GTP-binding proteins. In this paper, the subcellular localization of *B. pertussis* toxin substrates has been explored in chromaffin cells of bovine adrenal medulla. With appropriate gel electrophoresis conditions, three ADP-ribosylated substrates of 39, 40 and 41 kDa were detectable in both plasma and granule membranes. The more intense labelling occurred on the 40 kDa component, while the 41 kDa species exhibited electrophoretic mobility similar to that of $G_i\alpha$. Significant immunoreactivity with anti-$G_o\alpha$ antibodies was detected at the level of the 39 kDa faster component. The association of G-proteins with granule and plasma membranes suggests the involvement of these proteins in the exocytotic process or in its regulation.

Chromaffin granule; G-protein; Pertussis toxin; Exocytosis; Secretory granule

1. INTRODUCTION

Among the guanine nucleotide binding proteins, a family of regulatory proteins called G-proteins has been described that transduces extracellular signals to specific intracellular effectors (review [1]). These proteins are heterotrimers, in which the α-subunit binds GTP and express GTPase activity. The β- and γ-subunits are tightly associated and may serve to anchor the complex in the membrane [2]. The identified G-proteins differ mostly in the peptidic sequence of the α-subunits but possess highly homologous β- and perhaps γ-subunits. Some of these G-proteins, for example G_i and G_o, have their α-subunit specifically ADP-ribosylated by *Bordetella pertussis* toxin. G_i mediates adenylate cyclase inhibition [3] while the function of the other protein (G_o) has not yet been elucidated although it is very abundant in brain tissues [4–7].

Several recent observations suggest that G-proteins are involved in the exocytotic process [8,9]. However, these functionally postulated G-proteins have not been fully characterized. In the present work, we have investigated the nature of the G-protein present in adrenal medulla secretory cells by SDS-PAGE electrophoresis. The chromaffin subcellular localization of *B. pertussis* toxin substrates and G_o immunoreactivity was studied in fractions obtained from continuous sucrose density gradients. Three different substrates from the

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Abbreviations: NAD, nicotinamide adenine; GTP, guanosine triphosphate; GTP-γ-S, guanosine 5′-(3-*O*-thio)triphosphate; Gpp(NH)p, guanosine 5′-(β,γ-imido) triphosphate; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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toxin (39, 40 and 41 kDa) were identified; these were specifically ADP-ribosylated by pertussis toxin in both plasma and granule membrane fractions. Immunoblotting indicated that the 39 kDa subunit detected by ADP-ribosylation on the membranes was immunologically similar to the $G_o\alpha$ subunit.

2. MATERIALS AND METHODS

2.1. *Fractionation of the crude chromaffin granule fraction on continuous sucrose density gradients*

The crude chromaffin granule fraction was obtained by centrifuging the low speed $800 \times g$ supernatant at $11500 \times g$ for 20 min [10]. The resulting sediment (crude granule fraction) was suspended in 0.3 M sucrose and layered onto a linear 1.0–2.2 M sucrose gradient. The tube containing the density gradient was then centrifuged in an SW 41 rotor type (Beckman) at $113000 \times g$ for 90 min. Nineteen fractions were separated from top to bottom. The different fractions were assayed for protein, chromogranin A, ADP-ribosylation by pertussis toxin, G_o immunoreactivity and adenylate cyclase activity.

2.2. *Purification of G_o and G_i subunits*

Purification from bovine brain of the mixed preparation of G_o and G_i was performed by successive elution through DEAE Sephacel (Pharmacia), AcA 34 (LKB) and heptylamine-Sepharose columns as described by Sternweis and Robishaw [4]. The pure $G_o\alpha$ subunit was obtained from the G_i and G_o mixture by further separation on heptylamine-Sepharose in the presence of 10 μM $AlCl_3$, 10 mM NaF and 6 mM $MgCl_2$. The G-β-γ entity was obtained from the mixture of G_i and G_o as described by Neer et al. [5]. The purified fraction, $G_o\alpha$, was used for the immunization of the rabbits to raise specific antibodies [11].

2.3. *Pertussis toxin-catalyzed ADP-ribosylation*

Fractions from continuous sucrose density gradients (20–60 μg of protein) or purified G_o and G_i proteins (0.25 μg of protein) were ADP-ribosylated with pertussis toxin (List Biological Laboratories, Campbell, CA, USA) as described by Ribeiro-Neto et al. [12] with minor modifications according to Sternweis and Robishaw [4].

The reaction was stopped by the addition of 2% SDS with 100 μg/ml bovine serum albumin and proteins were precipitated overnight at 4°C with 10% trichloroacetic acid. After centrifugation, 10 min at $10000 \times g$, the pellets were washed twice with ethyl ether, dried and analyzed by SDS-PAGE with or without 100 mM *N*-ethylmaleimide according to Evans et al. [13]. Autoradiograms shown were obtained from dried gels without an intensifying screen.

2.4. *Immunoblotting of SDS-polyacrylamide gels (SDS-PAGE)*

Proteins were subjected to SDS electrophoresis on polyacrylamide gels (10% acrylamide/0.13% bisacrylamide) and then transferred onto nitrocellulose sheets during 18 h at 20 V [14]. The nitrocellulose sheet was then treated and reacted with the antibody specific to the α-subunit from the G_o calf protein. Radioiodinated protein A was used to detect bound antibodies and the dried blots were exposed to Kodak XAR film. Autoradiograms were quantified by scanning densitometry using a Vernon photometric recorder equipped with an automatic integrator (Delsi Enica 10).

2.5. *Other*

Proteins were assayed by the Folin method [15] using bovine serum albumin as standard. Chromogranin A was estimated by an ELISA assay using specific antichromogranin A antibody [16].

3. RESULTS

Sucrose density fractions of chromaffin cells containing either plasma membranes or secretory granule membranes were subjected to ADP-ribosylation by pertussis toxin. On SDS-PAGE using modified Laemmli conditions (fig.1), three ADP-ribosylated bands were detected after autoradiography in both plasma (lane 1) and granule membranes (lane 2). These three pertussis toxin substrates were compared with those purified from calf brain, i.e. pure $G_o\alpha$ (lane 3) and a mixture of $G_o\alpha$ and $G_i\alpha$ (lane 4). Among the three ADP-ribosylated substrates of chromaffin cells, the band with the higher mobility migrates in a manner similar to purified $G_o\alpha$ subunit (39 kDa). The band with the lower mobility migrates like $G_i\alpha$

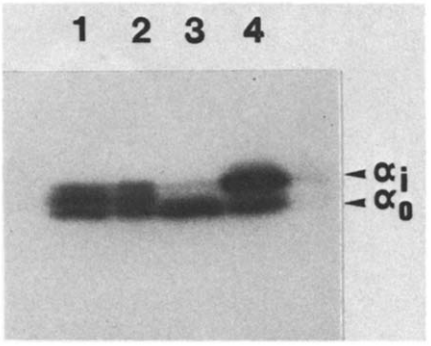

Fig.1. Autoradiogram of pertussis toxin catalyzed ADP-ribosylated proteins after electrophoresis through 10% polyacrylamide gel. Loaded proteins were (lanes): fraction 3 from sucrose density gradient (1); fraction 13 from sucrose density gradient (2); purified G_o protein (3); G_i and G_o mixture (4).

and exhibits a molecular mass of 41 kDa. When compared to the mixture of G_o and G_i, the third band clearly lies between these two well characterized G-proteins. This intermediate band, with an apparent molecular mass of 40 kDa, is the most heavily labelled band in chromaffin cells. We then investigated whether these three pertussis toxin substrates of chromaffin cells are present in all the subcellular fractions collected from a continuous sucrose density gradient. From a crude granule fraction layered on such a gradient, nineteen fractions were collected: chromaffin granules were recovered in fractions 8–18, mitochondria, lysosomes and Golgi cisternae in fractions 4–8 and most remaining plasma membranes in fractions 2 and 3 [17]. Fig.2 shows the presence of the three pertussis toxin substrates in all the fractions, however the intensity of the three ADP-ribosylated bands is very significant in fractions 2 and 3 which contain plasma membranes and also in fractions 12–16 where the bulk of chromaffin granules is mainly recovered. Interestingly, the intermediate band is more intensely labelled than the two others throughout the gradient.

Immunoblotting experiments were carried out to identify further the 39 kDa pertussis toxin substrate using rabbit polyclonal antibodies raised against pure bovine brain $G_o\alpha$ [11]. Fig.3 shows that both plasma and granule membranes display an immunoreactivity with the anti-$G_o\alpha$ antibodies. Electrophoresis of subcellular membrane fractions run in parallel with purified bovine brain $G_i\alpha$ and $G_o\alpha$ subunits confirm the presence in adrenal medulla of a protein immunologically identical to $G_o\alpha$.

It was then assessed whether the $G_o\alpha$-like material follows the distribution of the pertussis substrate on the same sucrose gradient. Thus, proteins in all fractions were electrophoretically separated, transferred and stained with the antibodies, and then quantified by scanning (fig.4). The profile of $G_o\alpha$ immunoreactivity is very similar to that of the pertussis toxin substrates (see fig.2): the highest cross-reactivity was found in fractions 2–3 and 12–16. The presence of the $G_o\alpha$-like subunit on the granule membrane of chromaffin cells is established by the parallelism of the peak of chromogranin A, a specific marker of secretory granules, with that of $G_o\alpha$ immunoreac-

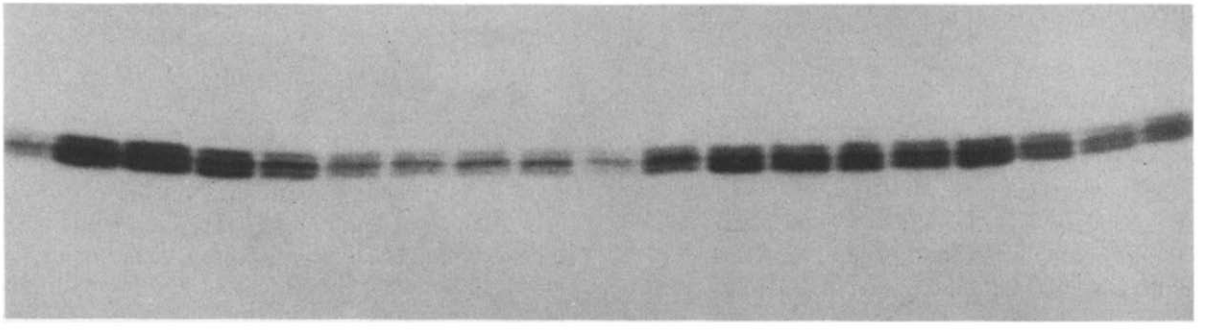

Fig.2. Autoradiogram of pertussis toxin-catalyzed ADP-ribosylated fractions collected from continuous sucrose density gradients layered with the crude chromaffin granule fraction. The fractions were collected and numbered from the top, where most plasma membranes are recovered (fractions 2 and 3), to the bottom where chromaffin granules sedimented (fractions 10–18). All the samples were treated with 100 mM NEM.

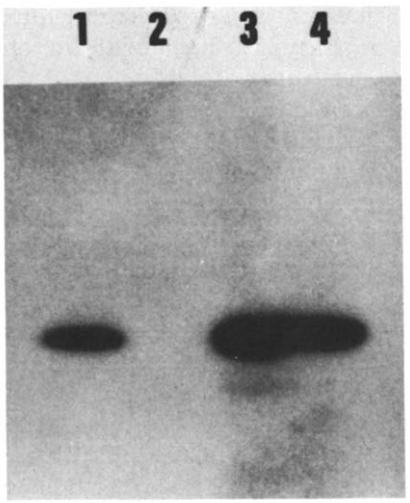

Fig.3. $G_o\alpha$ immunoreactivity in chromaffin cells. Proteins loaded were (lanes): α-subunit from purified G_o (1); purified G_i (2); fraction 3 from sucrose density gradient (3); fraction 13 from sucrose density gradient (4). The samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The blot was probed with antiserum raised against the $G_o\alpha$ subunit and then treated with radioiodinated protein A. The antiserum was used at a 1/100 dilution.

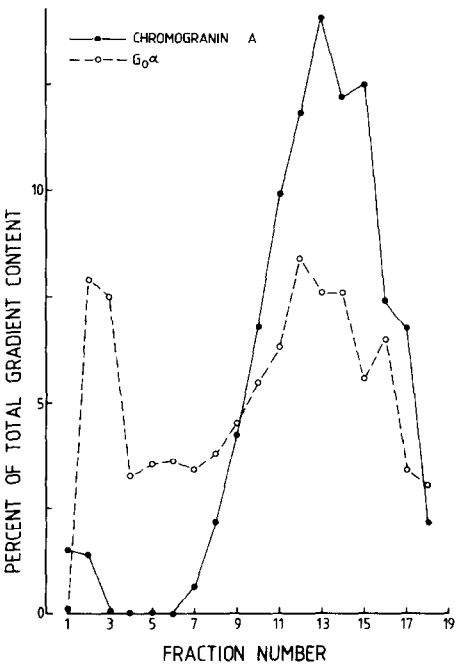

Fig.4. Distribution of studied proteins in a sucrose density gradient after centrifugation of crude chromaffin granule preparations. Data shown are expressed as the percentage of total immunoreactivity recovered on the gradient.

tivity in the fractions from 10 to 18. This observation reinforces the idea that G-proteins are not solely located in plasma membranes. The close $G_o\alpha$ immunoreactivity in plasma and granule membranes seems to exclude a contamination of the granule fraction by plasma membranes.

4. DISCUSSION

Pertussis toxin has been reported to catalyze the ADP-ribosylation of one, two or three peptides depending on the nature of the tissues and also on experimental conditions [5,18–21]. We developed specific conditions in which the three substrates can be easily detected. Firstly, the ratio bisacrylamide/acrylamide (0.13:10) was changed compared to standard Laemmli conditions (0.27:10) in order to obtain the best electrophoretic separation of the α-subunits of the G-proteins. Secondly, to sharpen the bands on SDS-PAGE, samples were treated with NEM after the action of DTT, as described [3,13]. Under such conditions, we demonstrated the coexistence of three G-proteins of 41, 40 and 39 kDa which were specifically ADP-ribosylated by pertussis toxin in adrenal medulla fractions. The 41 kDa component is believed to be G_i, the ubiquitous G-protein that transduces inhibition of adenylate cyclase [3]. The 39 kDa pertussis substrate is assumed to be G_o since the antiserum raised against the G_o protein purified from bovine brain recognized this component. The function of this 39 kDa protein, found in abundance in bovine brain [4,5], has not yet been elucidated. The major substrate of pertussis toxin in chromaffin cells is a 40 kDa component. Such a 40 kDa peptide substrate for pertussis toxin has been found in heart [18], adipocytes [22], neutrophils [21], polymorphonuclear leukocytes [19], monocytes [19] and cell lines U937 [19], glioma C_6 and NG 108-15 [20]. The possible identity between this 40 kDa pertussis toxin substrate and the brain $G_o\alpha$ subunit was investigated. In adipocytes, Rapiejko et al. [23] showed an immunoreactivity with their anti-$G_o\alpha$ antibodies at

39–40 kDa suggesting the presence of $G_o\alpha$ [23]. Conversely, the existence of a 40 kDa pertussis substrate (different from $G_o\alpha$) was ascertained in neutrophils since the large amount of pertussis toxin substrate is immunochemically distinct from previously identified substrates G_i and G_o [21].

In chromaffin cells, we showed the presence of the three pertussis toxin substrates in small amounts in subcellular fractions (4–11) containing mitochondria, lysosomes and Golgi cisternae, while the level of ADP-ribosylation in granule membranes is similar to that of plasma membranes. This indicates that G-proteins are not restricted to plasma membranes, suggesting a possible intracellular role. Actually, redistribution of proteins can never be excluded upon homogenization and subcellular fractionation. However, the possible contamination of granule fractions by plasma membranes appears unlikely since the amount of pertussis toxin substrates in granule membranes is similar to that of plasma membrane fragments.

In mast cells, pertussis toxin has been shown to trigger simultaneous inhibition of inositol phospholipid breakdown, arachidonic acid release and histamine secretion [8]. In addition, GTP analogues can activate the release from permeabilized neutrophils [9]. In chromaffin cells, the involvement of G-proteins has also been reported since (i) GTP-γ-S inhibits Ca^{2+}-dependent exocytosis [24] and, (ii) GppNHp, a non-hydrolyzable analogue of GTP, produces stimulation of the ATP-dependent, Ca^{2+}-independent release of catecholamine [25].

It is not yet known whether the role of G-proteins in specific exocytotic steps cannot be extended to any secretory cells. It is interesting to emphasize that the significant ADP-ribosylation labelling of the 40 kDa G-protein in chromaffin cells is similar to that described in neutrophils [21]. A clear effect of pertussis toxin has not been found in permeabilized chromaffin cells. However the action of pertussis toxin in these intact cells remains to be fully explored.

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