Volume 222, number 1, 51–55 FEB 05123 September 1987

G-proteins in *Torpedo marmorata* electric organ

Differential distribution in pre- and post-synaptic membranes and synaptic vesicles

Madeleine Toutant, Joël Bockaert, Vincent Homburger and Bruno Rouot

Centre CNRS-INSERM de Pharmacologie-Endocrinologie, rue de la Cardonille, 34094 Montpellier Cédex, France

Received 5 August 1987

The nature of the G-proteins present in the pre- and post-synaptic plasma membranes and in the synaptic vesicles of cholinergic nerve terminals purified from the *Torpedo* electric organ was investigated. In pre- and post-synaptic plasma membranes, *Bordetella* pertussis toxin, known to catalyze the ADP-ribosylation of the α-subunit of several G-proteins, labels two substrates at 41 and 39 kDa. The 39 kDa subunit detected by ADP-ribosylation in the synaptic plasma membrane fractions was immunologically similar to the G_o α-subunit purified from calf brain. In contrast to bovine chromaffin cell granules, no G-protein could be detected in *Torpedo* synaptic vesicles either by ADP-ribosylation or by immunoblotting.

G-protein; Pertussis toxin; Pre-synaptic membrane; Synaptic vesicle

1. INTRODUCTION

Guanine nucleotide binding regulatory proteins (G-proteins) are involved in many transducing systems. G-proteins are heterotrimers (α, β, γ) in which the α-subunit binds and hydrolyzes GTP and can in many instances be ADP-ribosylated by specific toxins such as *Bordetella* pertussis toxin. In cells from neural origin, three distinct types of G-proteins have been reported to be ADP-ribosylated by pertussis toxin [1–3]. These three substrates differ by the apparent molecular mass in SDS-PAGE of their α-subunit (39, 40 and 41 kDa) while their β-subunit (35–36 kDa) and γ-subunit (8 kDa) are considered to be identical.

Correspondence address: M. Toutant, CCIPE, rue de la Cardonille, 34094 Montpellier Cédex, France

Abbreviations: NAD, nicotinamide adenine; GTP, guanosine triphosphate; GTP-γ-S, guanosine 5'-(3-*O*-thio)triphosphate; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PSPM, pre-synaptic plasma membrane

The 41 kDa component is believed to be part of G_i, the ubiquitous G-protein that transduces inhibition of adenylate cyclase [4]. The 39 kDa pertussis substrate, very abundant in the brain could be involved in the modulation of ionic channels as shown for opiate receptors on the voltage-dependent calcium channels [5]. The pertussis toxin substrate with an apparent molecular mass of 40 kDa has also been detected in other tissues like C_6 glioma cells, rat fat cells, rabbit heart, and neutrophils [6–9]. Such a 40 kDa substrate has been recently purified from porcine brain [10] and from neutrophils [11]. However, the identity between these two proteins remains to be demonstrated. It has been suggested that a 40 kDa subunit might be coupled to phospholipase C in certain secretory cell types such as neutrophils and mast cells. Recently we also evidenced beside the 41 and 39 kDa, a pertussis toxin substrate at 40 kDa in chromaffin cells. Interestingly, all pertussis toxin substrates evidenced in the chromaffin plasma membranes were also present in the membranes of chromaffin granules [2]. This prompted

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

us to study the nature of the G-proteins present in another well studied neurotransmitter release system: the cholinergic nerve terminals from the *Torpedo* electric organ. Only two substrates (41 and 39 kDa) were observed in the pre-synaptic plasma membrane. The 39 kDa subunit detected by ADP-ribosylation on the pre-synaptic plasma membrane was immunologically similar to the G_o α-subunit. In synaptic vesicles, no G-protein could be detected either by immunoblotting or by ADP-ribosylation.

2. MATERIALS AND METHODS

2.1. *Purification of PSPM and synaptic vesicles*

Pure fractions of cholinergic synaptic vesicles can be prepared from the *Torpedo* electric organ [12]. In the present work, synaptic vesicles are purified as described by Diebler and Lazereg [13]. Fractions of high purity were obtained after two successive centrifugations in sucrose gradients. The second centrifugation entailed a flotation gradient after equilibration of the synaptic vesicles in 2 M sucrose. PSPMs were purified directly from frozen electric organs according to the large scale procedure developed by Morel et al. [14]. Fraction of plasma membranes of higher density was collected at the 1.0–1.2 M sucrose interface. It contains plasma membranes of post-synaptic cells [14].

2.2. *Purification of G-proteins*

Purification of G-proteins from bovine brain membranes was performed by successive elution through DEAE Sephacel (Pharmacia), AcA 34 (LKB) and heptylamine-Sepharose columns. Pure G_o α-subunit, enriched G_i and G_o fractions were obtained as described [15].

2.3. *Pertussis toxin-catalyzed ADP-ribosylation*

Fractions containing PSPM, synaptic vesicles and post-synaptic membranes (20 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 [2]. The samples were analyzed by SDS-PAGE with 100 mM NEM according to [16].

2.4. *Immunoblotting of SDS-PAGE*

Proteins were subjected to SDS electrophoresis on polyacrylamide gels (10% acrylamide/0.13% bisacrylamide) and then transferred onto nitrocellulose sheets for 18 h at 20 V [17]. The nitrocellulose sheet was then treated and reacted with the antibody specific to the α-subunit from the G_o calf protein. A peroxidase-conjugated goat anti-rabbit immunoglobulin preparation (Institut Pasteur Production) was used as a second antibody.

3. RESULTS

The incubation of PSPM isolated from the

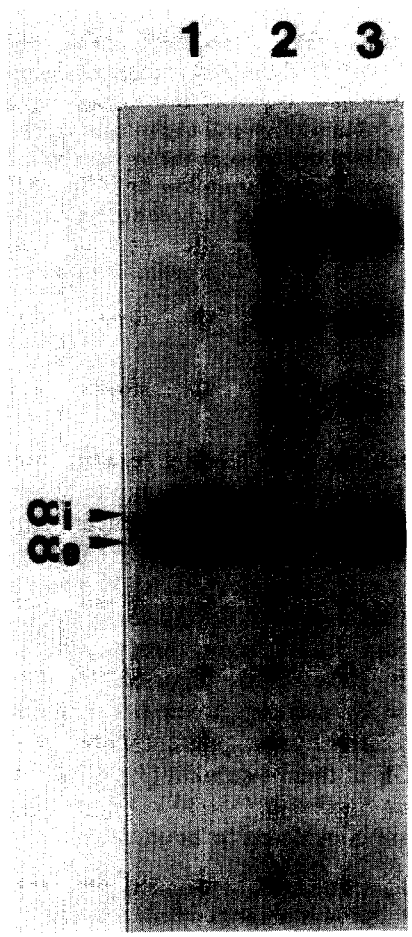

Fig.1. Autoradiogram of ADP-ribosylated proteins with [α-^{32}P]NAD in the presence (lane 1 and 3) or in absence (lane 2) of pertussis toxin. Loaded proteins submitted to electrophoresis through 10% polyacrylamide-SDS gel were: purified mixture of calf brain G_o and G_i proteins (lane 1), the PSPM fraction (lane 2 and 3).

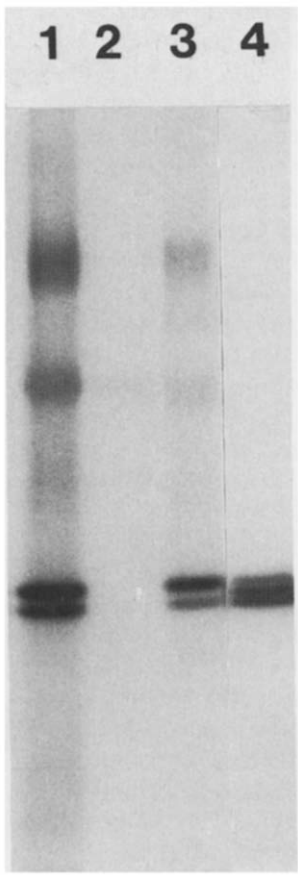

Fig.2. Comparison of pertussis toxin substrates in subcellular fractions purified from the *Torpedo* electric organ and from bovine chromaffin cells. For each fraction 20 μg of protein were ADP-ribosylated with pertussis toxin. Loaded proteins were PSPM (lane 1), synaptic vesicles (lane 2), post-synaptic membranes (lane 3), chromaffin granules (lane 4). The latter fraction was run in parallel with the others but a good resolution for all the fractions was obtained with different exposition times. 72 h for lanes 1, 2 and 3, and 24 h for lane 4.

Torpedo electric organ, with [^{32}P]NAD in the presence of pertussis toxin, resulted in the incorporation of radioactivity into two proteins (fig.1). The labeled bands of the PSPM fraction (lane 3) were compared, after SDS-PAGE (using modified Laemmli conditions) and autoradiography, to those of a mixture of $G_i\alpha$ and $G_o\alpha$ purified from calf brain (lane 1). Fig.1 clearly shows that the major ADP-ribosylated substrate migrates in a manner similar to $G_i\alpha$ (41 kDa) while the other exhibits

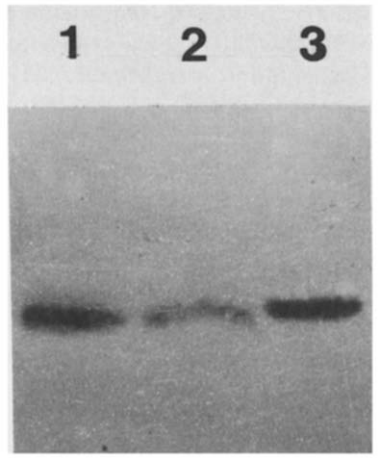

Fig.3. Immunoblot analysis of α_0-subunit present in *Torpedo* electric organ fractions. Proteins loaded were (lanes): purified mixture of G_o and G_i proteins from calf brain (1); the post-synaptic fraction (2); the PSPM fraction (3). The samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose. The blot was probed with an antiserum raised against the $G_o\alpha$-subunit of calf brain. This antiserum was used at a 1/100 dilution.

a molecular mass of 39 kDa identical to that of $G_o\alpha$. This ADP-ribosylation was shown to be toxin dependent since in the absence of toxin, no ADP-ribosylation occurred in PSPM at these molecular masses (lane 2). The labeling at higher molecular masses (lane 2 and 3) probably corresponded to endogenous ADP-ribosylations as previously suggested by Lester et al. [18]. Interestingly, no intermediate band at 40 kDa could be detected by ADP-ribosylation.

We investigated whether the two pertussis toxin substrates observed in PSPM fractions were present in synaptic vesicles and post-synaptic membranes of the *Torpedo marmorata* electric organ. In synaptic vesicles purified by a step of flotation density gradient [13], we did not detect any substrate ADP-ribosylated by pertussis toxin (fig.2, lanes 1 and 2) although the amount of proteins in samples 1, 2, 3 and 4 were equivalent. In post-synaptic membranes, two ADP-ribosylated peptides were also evidenced, the most intensely radiolabeled component had an apparent molecular mass of 41 kDa, the minor band exhibited a molecular mass of 39 kDa (lane 3). Thus, the number of pertussis toxin substrates in the

Torpedo electric organ differs from the three substrates of chromaffin cells evidenced both in plasma [2] and granule membranes (fig.2, lane 4).

To further identify the 39 kDa pertussis toxin substrate immunoblotting experiments were carried out using rabbit polyclonal antibodies raised against pure bovine brain $G_o\alpha$ [15]. Fig.3 (lanes 2 and 3) shows that this component present in PSPM and post-synaptic membrane fractions cross-reacts with anti-$G_o\alpha$ antibodies as does a mixture of purified bovine brain G_i and $G_o\alpha$-subunits (lane 1). This confirms the presence in the *T. marmorata* electric organ, of a protein immunologically identical to $G_o\alpha$. In agreement with pertussis toxin experiments no cross reactivity was detected in synaptic vesicles (not shown).

4. DISCUSSION

In chromaffin cells we have recently reported the presence of three pertussis toxin substrates at 39, 40 and 41 kDa in both plasma and granules membranes [2]. In the present work, we have investigated the nature of pertussis toxin substrates present in different subcellular fractions from the *T. marmorata* electric organ. This tissue offers the advantage of possessing an abundant and homogeneous innervation and has proven to be a useful model to study the cholinergic neuromuscular transmission.

Using pertussis toxin we were unable to detect any G-proteins in the synaptic vesicles purified from the *Torpedo* electric organ while two peptides were ADP-ribosylated in the PSPM fraction and post-synaptic membranes. Thus, the nature and the distribution of G-proteins identified in *Torpedo* electric organ subcellular fractions differ from those previously observed in chromaffin cells. Synaptic vesicles from the *Torpedo* electric organ are known to possess a very low number of proteins as compared to the multiplicity of protein species present in plasma membrane [19]. Several authors [20,21] suggested that the vesicular membrane represents essentially a simplified version of the pre-synaptic plasma membrane since upon gel electrophoresis all vesicle proteins are detected in the plasma membranes, but not vice versa. In fact about ten proteins can be detected by two-dimensional gel electrophoresis in *Torpedo* synaptic vesicles [22], while one hundred proteins are present in chromaffin granules [23,24].

In the PSPM fraction from the *Torpedo* electric organ two peptides are ADP-ribosylated by pertussis toxin. The major substrate of pertussis toxin is a 41 kDa component but its unexpected abundance in electric organ suggests that this 41 kDa substrate might play another role beside the inhibition of cyclase. For example, in mammalian heart, the involvement of a G-protein with an α-subunit of 41 kDa has been evidenced in the coupling between muscarinic receptors and potassium channels [25]. Such a function might be suggested for the 41 kDa substrate of PSPM since muscarinic receptors of electric organ synaptosomes could be involved in the regulation of acetylcholine release [26]. 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. This G_o protein, very abundant in mammalian brain, is probably involved in the functional coupling of some receptors such as opiate receptors to neuronal voltage-dependent calcium channels [5].

The electroplaque assimilated to a non-differentiated muscle cell also displays two ADP-ribosylated proteins at 41 and 39 kDa. However, the ratio of 39/41 kDa is lower in plasma membranes of this post-synaptic cell than in PSPM. Although surprising, in a non-nervous tissue, the relatively large amount of the 39 kDa substrate cannot solely be attributed to pre-synaptic contamination since neuronal tissue represented less than 10% of the post-synaptic fraction.

The 40 kDa pertussis toxin substrate, widely distributed in cells and tissues from different species, is believed to couple some receptors to polyphosphoinositide breakdown [27,28]. At variance with observations in many tissues such as secretory cells [27,28], this substrate could not be detected in the *Torpedo* electric organ. This might indicate the absence of such a transducing function in this organ. Alternatively, it should be considered that, in the electric organ, the G-protein coupled to phospholipase C (if any) is, like in brain, insensitive to pertussis toxin [29]. Finally, the lack of a 40 kDa substrate of pertussis toxin may be due to factors that limit ADP-ribosylation of this peptide as it has been suggested in the case of brain membranes [6].

In conclusion, we demonstrated the presence of

two ADP-ribosylated substrates in the PSPM fraction of the *Torpedo* electric organ, while no G-protein could be detected in the synaptic vesicles. The significance of the presence of a 39 kDa pertussis substrate in non-nervous cells (post-synaptic fraction) remains to be explored. The main difference with chromaffin cells is the absence in the *Torpedo* electric organ of a 40 kDa pertussis toxin substrate. This may be related to differences in the regulation of neurotransmitter release in these two neurosecretory systems.

ACKNOWLEDGEMENTS

The subcellular fractions from the *Torpedo marmorata* electric organ were kindly provided by Drs N. Morel, M.F. Diebler and M. Israel. The chromaffin granules were purified by Dr D. Aunis. They are gratefully acknowledged for these gifts and for critical reading of this manuscript. We thank Katia Alarcon-Gottofrey for typing the manuscript.

REFERENCES

[1] Neer, E.J., Lok, J.M. and Wolf, L. (1984) J. Biol. Chem. 259, 14222–14229.
[2] Toutant, M., Aunis, D., Bockaert, J., Homburger, V. and Rouot, B. (1987) FEBS Lett. 215, 339–344.
[3] Brabet, P., Dumuis, A., Sebben, M., Pantaloni, C., Bockaert, J. and Homburger, V. (1987) J. Neurosci., in press.
[4] Katada, T. and Ui, M. (1982) J. Biol. Chem. 257, 7210–7216.
[5] Hescheler, J., Rosenthal, W., Trautwein, W. and Schultz, G. (1987) Nature 325, 445–447.
[6] Gierschik, P., Falloon, J., Milligan, G., Pines, V.M., Gallin, J.I. and Spiegel, A. (1986) J. Biol. Chem. 261, 8058–8062.
[7] Milligan, G., Gierschik, P., Spiegel, A.M. and Klee, W.A. (1986) FEBS Lett. 195, 225–230.
[8] Rapiejko, P.J., Northup, J.K., Evans, T., Brown, J.E. and Malbon, C.C. (1986) Biochem. J. 240, 35–40.
[9] Malbon, C.C., Maugons, T.J. and Watkins, D. (1985) Biochem. Biophys. Res. Commun. 128, 809–815.
[10] Katada, T., Oinuma, M., Kusakabe, K. and Ui, M. (1987) FEBS Lett. 213, 353–358.
[11] Gierschik, P., Sidiropoulos, D., Spiegel, A. and Jakobs, K.H. (1987) Eur. J. Biochem. 165, 185–194.
[12] Israël, M., Gautron, J. and Lesbats, B. (1968) CR Acad. Sci. (Paris) 226, 273–275.
[13] Diebler, M.F. and Lazereg, S. (1985) J. Neurochem. 44, 1633–1641.
[14] Morel, N., Marsal, J., Manaranche, R., Lazereg, S., Mazie, J.C. and Israël, M. (1985) J. Cell. Biol. 101, 1757–1762.
[15] Homburger, V., Brabet, P., Audigier, Y., Pantaloni, C., Bockaert, J. and Rouot, B. (1987) Mol. Pharmacol. 34, 313–319.
[16] Evans, T., Brown, M.L., Fraser, E.D. and Northup, J.K. (1986) J. Biol. Chem. 261, 7052–7059.
[17] Burnette, W.N. (1981) Anal. Biochem. 112, 195–203.
[18] Lester, H.A., Steer, M.L. and Michaelson, D.M. (1982) J. Neurochem. 38, 1080–1086.
[19] Stadler, H. and Tashiro, T. (1979) Eur. J. Biochem. 101, 171–178.
[20] Morgan, J.P., Zanetta, J.P., Breckenridge, W.C., Vincendon, G. and Gombos, G. (1973) Brain Res. 62, 405–411.
[21] Goodrum, J.F. and Tanuka, R. (1978) Neurochem. Res. 3, 599–617.
[22] Kelly, R.B. and Hooper, J.E. (1982) in: The Secretory Granule (Poisner, A.M. and Trifaro, J.M. eds) pp.81–118, Elsevier, Amsterdam.
[23] Bader, M.F. and Aunis, D. (1983) Neuroscience 8, 165–181.
[24] Aunis, D. and Perrin, D. (1984) J. Neurochem. 42, 1558–1569.
[25] Yatani, A., Codina, J., Brown, A.M. and Birnbaumer, L. (1987) Science 235, 207–211.
[26] Michaelson, D.M., Avissar, S., Kloog, Y. and Sokolosky, M. (1979) Proc. Natl. Acad. Sci. USA 76, 6336–6340.
[27] Nukamura, T. and Ui, M. (1985) J. Biol. Chem. 260, 3584–3593.
[28] Smith, C.D., Lane, B.C., Kusaka, I., Verghese, M.W. and Snyderman, R. (1985) J. Biol. Chem. 260, 5875–5878.
[29] Evans, T., Martin, M.W., Hughes, A.R. and Harden, T.K. (1984) Mol. Pharmacol. 27, 32–37.