Novel GTP-binding proteins in plasma membranes and zymogen granule membranes from rat pancreas and in pancreatic AR 4-2J cell membranes

Monique Lambert, Ngoc-Diem Bui and J. Christophe

Department of Biochemistry and Nutrition, Medical School, Université Libre de Bruxelles, 115, Boulevard de Waterloo, B-1000 Brussels, Belgium

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Photoaffinity labelling with [α-^{32}P]GTP allowed to detect a 54 kDa GTP-binding protein in rat pancreatic plasma membranes and in pancreatic AR 4-2J cell membranes. Like the 42 and 48 kDa $G_s\alpha$ subunits and the 41 kDa $G_i\alpha$ subunit, this protein was absent from zymogen granule membranes. Contrastingly, a new 28 kDa GTP-binding protein (detected by [α-^{32}P]GTP binding on immobilized proteins) and a 25 kDa protein (ADP-ribosylated by botulinum toxin D) were found in all three membrane preparations. This is to our knowledge the first report on GTP-binding proteins in zymogen granule membranes.

GTP-binding protein; ADP-ribosylation; Zymogen granule membrane; (Rat pancreas); (Rat pancreatic acinar cell line AR 4-2J)

1. INTRODUCTION

Pancreatic exocrine secretion can be stimulated by two groups of secretagogues. Ca^{2+}-mobilizing secretagogues, like CCK, cholinergic agents and bombesin, stimulate PIP_2 breakdown, producing the second messengers DAG and 1,4,5-IP_3 acting in synergy to activate protein kinase C. The coupling of cholinergic and CCK receptors to phospholipase C is mediated by a G-protein distinct from G_s and G_i but as yet unidentified [1]. CCK can also stimulate PC hydrolysis by phospholipase D, contributing to DAG formation [2]; to our knowledge, the role of a G-protein in the coupling of CCK receptors to phospholipase D has not yet been investigated.

The related peptides VIP, secretin and helodermin, the second group of secretagogues, stimulate pancreatic exocrine secretion through activation of adenylate cyclase. The coupling of their receptors with adenylate cyclase is mediated by G_s, which was identified in rat pancreatic plasma membranes: two forms of α_s subunit [3] are detected by ADP-ribosylation in the presence of CT. Low-affinity CCK receptors, whose occupancy correlates with the downstroke of the dose–response curve for enzyme secretion, are also coupled through G_s with adenylate cyclase in the rat pancreas [4,5]. Pancreatic adenylate cyclase is submitted to a negative regulation through somatostatin receptors [6] coupled to adenylate cyclase through G_i [7] already identified by ADP-ribosylation in the presence of PT [8].

In vitro interactions of ZG with isolated pancreatic plasma membranes are modulated by GTPγS but not by Ca^{2+}, suggesting the existence of an additional G-protein G_E, involved in exocytosis, in ZG and/or plasma membranes [9].

The aim of the present work was to identify novel G-proteins including small ras-like G-proteins in plasma membranes and ZG membranes from rat pancreas as well as in membranes from the pancreatic acinar cell line AR 4-2J.

2. MATERIALS AND METHODS

Nicotinamide adenine dinucleotide [adenylate ^{32}P] (10–50 Ci/mmol) and guanosine 5′-trisphosphate [α-^{32}P] (<600 Ci/mmol) were purchased from New England Nuclear (Dupont de Nemours, Brussels, Belgium). CT was from Sigma (St. Louis, MO, USA) and BTD from Wako (Sopar-Biochem, Brussels, Belgium). PT was prepared by M. Svoboda in our department [4].

Semipurified rat pancreatic plasma membranes were prepared as in Ref. [10]. ZG membranes prepared as described in Ref. [11] were suspended in PBS, then kept frozen until use. Preparation of crude membranes from AR 4-2J cells was as in Ref. [12].

Membrane [^{32}P]ADP-ribosylation with CT or PT was conducted as described [8], the reaction terminated by cooling and centrifuga-

Correspondence address: M. Lambert, Department of Biochemistry and Nutrition, Medical School, Université Libre de Bruxelles, 115, Boulevard de Waterloo, B-1000 Brussels, Belgium

Abbreviations: G-protein, GTP-binding protein; PIP_2, phosphatidylinositol-4,5-bisphosphate; 1,4,5-IP_3, inositol 1,4,5-trisphosphate; DAG, 1,2-diacylglycerol; PC, phosphatidylcholine; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); CCK, cholecystokinin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffer saline (10 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl); PT, *Bordetella pertussis* toxin; CT, cholera toxin; BTD, botulinum toxin D; BSA, bovine serum albumin; ZG, zymogen granule

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tion, and pellets were washed twice with cold PBS before solubilization and electrophoresis.

BTD was thiol-preactivated for 10 min at 30°C with 10 mM dithiothreitol. For ADP-ribosylation by BTD 50–200 μg membrane protein was incubated 60 min at 30°C in 75 μl of 10 mM Tris-HCl (pH 7.6), 10 mM thymidine, 0.1 mM GTP, 1 mM ATP, 0.4 mM $MgCl_2$, 1 mM ADP-ribose, 1 mM nicotinamide, 10 mM creatine phosphate, 10 U/ml creatine kinase, 10 μM nicotinamide adenine dinucleotide [adenylate-^{32}P] (5–10 μCi/tube), 4 mM dithiothreitol and 80 μg/ml preactivated toxin. The reaction was stopped by cooling and centrifugation, pellets were washed with cold PBS and solubilized for electrophoresis.

Crosslinking of [α-^{32}P]GTP on membrane protein was conducted as described by others [13]. 50–150 μg proteins were incubated for 10 min at 4°C in 50 μl of 20 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 100 mM NaCl, 2 mM $MgCl_2$, 10 μM ATP, 0.1 mg/ml BSA and 0.5 μM [α-^{32}P]GTP (600 Ci/mmol); the mixture maintained at 0°C was then irradiated for 7 min at a distance of 15 cm with a UV-IR HP 3116 Philips mercury lamp. After UV irradiation, 10 μl of 4 mM GTP and 750 μl PBS were added to each tube. Samples were centrifuged and pellets were washed once more with cold PBS and dissolved before electrophoresis.

SDS-PAGE of ADP-ribosylated and UV-crosslinked membranes was performed with 12% homogeneous polyacrylamide slab gels (180 × 160 × 1.4 mm) [12].

Before [α-^{32}P]GTP binding on immobilized proteins, SDS-PAGE (on 80 × 80 × 0.7 mm slab gels made of 16% polyacrylamide) and electroblotting on nitrocellulose were conducted as in Ref. [14]. For [α-^{32}P]GTP binding on immobilized membrane proteins, nitrocellulose blots were incubated and autoradiographed as in Ref. [15].

3. RESULTS

3.1. *Crosslinking of [α-^{32}P]GTP on membrane proteins*

[α-^{32}P]GTP was crosslinked to a main 54 kDa band in pancreatic plasma membranes as well as in crude AR 4-2J cell membranes (Fig. 1). Crosslinking specificity was shown by low residual labelling when the incubation was conducted in the presence of 10 μM unlabelled GTP. Minor radioactive bands, of 69 kDa in pancreatic plasma membranes and 32 kDa in both membrane preparations, were also detected. In contrast, ZG membranes appeared to be devoid of proteins detectable by GTP crosslinking (Fig. 1).

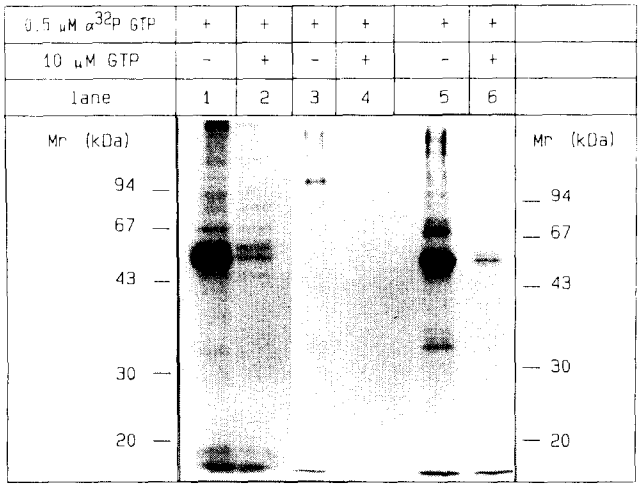

Fig. 1. Photoaffinity labelling of membrane proteins with [α-^{32}P]GTP. Three membrane preparations were crosslinked to [α-^{32}P]GTP and submitted to SDS-PAGE and autoradiography. Lanes 1, 2: AR 4-2J cell membranes; lanes 3, 4: pancreatic ZG membranes; lanes 5, 6: pancreatic plasma membranes. This experiment was representative of three others.

3.2. *ADP-ribosylation in the presence of bacterial toxins*

The two forms of $G_s\alpha$ (42 and 48 kDa) previously detected in rat pancreatic plasma membranes and AR

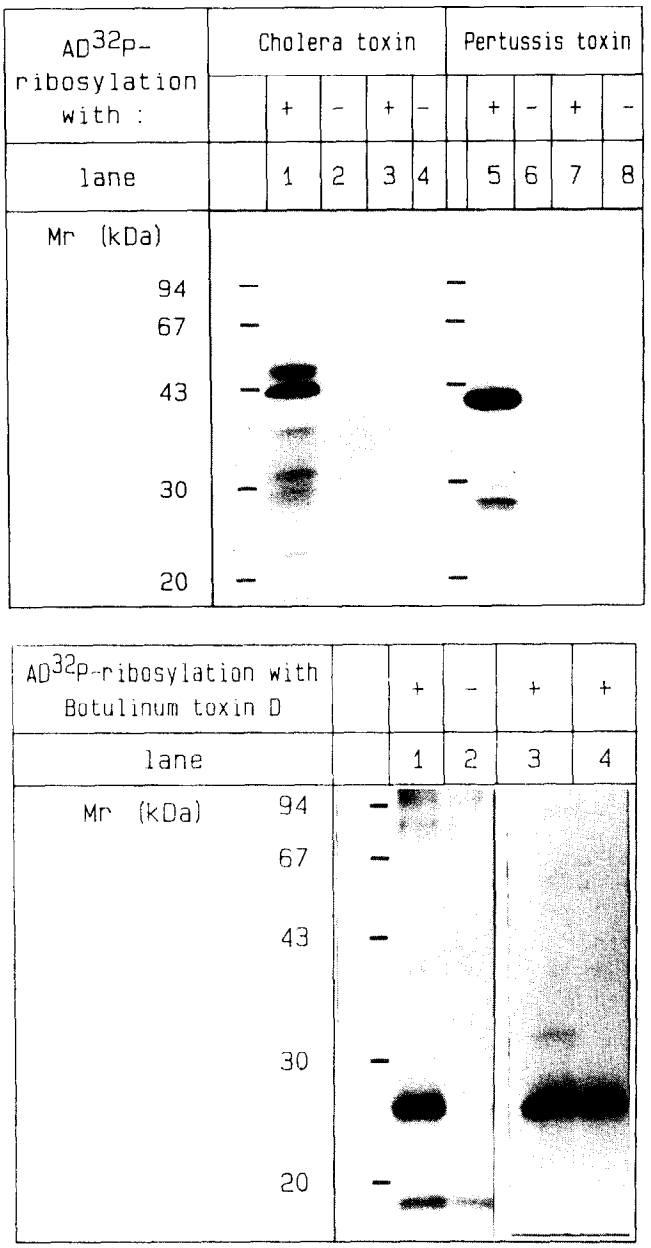

Fig. 2. ADP-ribosylation of membrane proteins by bacterial toxins. (A) Lanes 1, 2, 5, 6: pancreatic plasma membranes; lanes 3, 4, 7, 8: ZG membranes. (B) Lanes 1, 2: AR 4-2J cell membranes; lane 3: pancreatic plasma membranes; lane 4: pancreatic ZG membranes. This experiment was representative of three others.

4-2J membranes [3,12] by ADP-ribosylation in the presence of CT seemed to be absent from ZG membranes (Fig. 2A). In the presence of PT, a 41 kDa protein assumed to be $G_i\alpha$ was ADP-ribosylated in pancreatic plasma membranes and AR 4-2J membranes [8,12]; a very weak radioactive band of 41 kDa was also seen in ZG membranes (Fig. 2A) but this could be due to a slight contamination by plasma membranes rather than to an intrinsic ZG membrane protein.

Incubation of all three membrane preparations with BTD in the presence of [α-^{32}P]NAD resulted in the labelling of a 25 kDa protein (Fig. 2B) and, contrasting with $G_s\alpha$ and $G_i\alpha$, the substrate of BTD appeared to be at least as abundant in ZG membranes as in plasma membranes or AR 4-2J crude membranes.

3.3. *[α-^{32}P]GTP binding on immobilized membrane proteins*

[α-^{32}P]GTP was specifically bound on a 28 kDa protein in pancreatic plasma membranes, AR 4-2J crude membranes and ZG membranes (Fig. 3). The high affinity of this binding was shown by the absence of bound radioactivity after blot incubation in the presence of 1 μM unlabelled GTP (Fig. 3, lanes 1–3).

4. DISCUSSION

We used different techniques, each with its own specificity, to identify G-proteins. ADP-ribosylation in the presence of CT or PT detects the α subunit of the heterotrimeric proteins G_s, G_i, and G_o, involved in receptor signalling; however, other heterotrimeric G proteins are not substrate for CT or PT [16]. In our system, ADP-ribosylation in the presence of CT or PT confirmed the presence of two $G_s\alpha$ (42 and 48 kDa) and one $G_i\alpha$ (41 kDa) in pancreatic plasma membranes and AR 4-2J cell membranes. By contrast, these G-proteins were virtually absent from ZG membranes. This distribution is compatible with the role of G_s and G_i in the coupling of hormone receptors with adenylate cyclase.

The ADP-ribosyl transferase activity of our BTD preparation was probably due to a C_3 toxin contaminant known to use low kDa ras-like G-proteins as substrate [17]. In our hands, a 25 kDa protein was ADP-ribosylated by BTD in rat pancreatic plasma and ZG membranes, and in AR 4-2J cell membranes. In two out of nine experiments, weakly radioactive bands of 22 and 27 kDa were also detectable. Crude membranes from mouse pancreas were previously found to contain a 21 kDa substrate for BTD [18].

Incubation of native membranes with [α-^{32}P]GTP followed by UV irradiation resulted in photoaffinity labelling of a 54 kDa GTP-binding protein: this new G protein was present in pancreatic plasma membranes and AR 4-2J cell membranes but not in ZG membranes. Other weakly radioactive bands could be seen but none had the same mobility in SDS-PAGE as α_s or α_i subunits. In some autoradiographs, the 54 kDa band appeared as a doublet with distinct affinities for GTP.

Small G-proteins, unlike the α subunit of heterotrimeric G-proteins, retain their capacity to bind GTP after SDS-PAGE and blotting on nitrocellulose [19]. Using this approach we detected a 28 kDa G-protein in rat pancreatic plasma membranes, ZG membranes, and AR 4-2J cell membranes. Although not run in identical polyacrylamide gels, we think that the 28 kDa protein detected by this technique was distinct from the 25 kDa BT substrate and from small 22–25 kDa G-proteins detected by [α-^{32}P]GTP blot-overlay assay in the Golgi and rough endoplasmic reticulum of canine pancreas [20].

The function of these new G-proteins is unknown but some are likely to be involved in protein transport between intracellular compartments along the secretory pathway [21] and it is tempting to postulate a role in intracellular transport and/or exocytosis for the small 28 kDa G-protein primarily localized in ZG membranes.

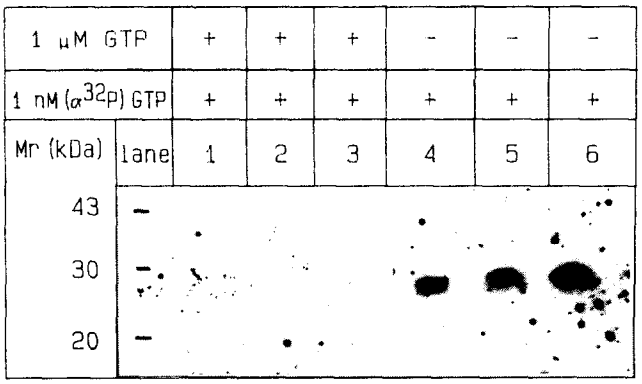

Fig. 3. Binding of [α-^{32}P]GTP on immobilized membrane proteins. Membrane proteins were submitted to SDS-PAGE followed by electrotransfer on nitrocellulose and blots were incubated as described in section 2. Lanes 1, 6: pancreatic plasma membranes; lanes 2, 5: pancreatic ZG membranes; lanes 3, 4: AR 4-2J cell membranes; lanes 1, 2, 3: non-specific binding. This experiment was representative of five others.

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