THE DIFFERENTIAL DETERGENT SOLUBILIZATION OF ADENYLATE CYCLASE AND POLYPEPTIDES ADP-RIBOSYLATED WITH CHOLERA TOXIN SUGGESTS AN EXCESS OF G/F PROTEIN RELATIVE TO ADENYLATE CYCLASE IN RAT PANCREATIC PLASMA MEMBRANES

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1. Introduction

Rat pancreatic adenylate cyclase [1] consists of at least 3 components: the catalytical unit, hormone receptors, and G/F nucleotide regulatory protein(s) [2] that, upon interaction with guanine nucleotides, fluoride or cholera toxin, activate or attenuate the catalytical unit. The hydrolysis of GTP by a low K_m GTPase closely associated with G/F [3] is the switch-off mechanism of pancreatic adenylate cyclase activity and its inhibition, after cholera toxin pretreatment in the presence of NAD^+, maintains the system in the active state [4]. Cholera toxin transfers the ADP-ribose moiety of NAD^+ to G/F in several types of plasma membranes [5–13] as well as to G/F purified from liver membranes [14]. Here, we compared the solubilization of ADP-ribosylated G/F protein(s) with that of preactivated adenylate cyclase, by submitting rat pancreatic plasma membranes to various detergents. The low yield of G/F protein(s) solubilized with the non-ionic detergent G 3707 contrasted with a much higher solubilization of adenylate cyclase and suggests a 'spareness' of pancreatic G/F protein(s) relative to adenylate cyclase.

2. Materials and methods

Cholera toxin was from Schwartz-Mann (Orangeburg NY) or from Sigma Chemical Co. (St Louis MO).

Abbreviations: DTT, dithiothreitol; SDS, sodium dodecylsulfate; Tes, *N*-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid; GTPγS, guanosine 5'-*O*-(3-thio)triphosphate

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Sodium cholate and sodium deoxycholate were from Sigma. [α-^{32}P]NAD^+ was obtained from New England Nuclear (Dreieich). The non-ionic detergent G 3707, a lauryl polyoxyethylene with 7 ethylenoxide groups/molecule was from Atlas Chemical Industries (Everberg). The source of the other chemicals was as in [4].

Rat pancreatic plasma membranes were prepared as in [15] and stored in liquid nitrogen until use. Human erythrocyte membranes were prepared within 2 days of blood collection [16]. Membrane proteins were assayed by the Lowry method [17] after centrifuging detergent containing samples and using bovine serum albumin as standard.

2.1. *ADP-ribosylation*

Rat pancreatic plasma membranes were washed by centrifugation [1] and resuspended at 3–5 mg protein/ml in 10 mM Tes–Tris buffer (pH 7.5) containing 30 mM NaCl, 0.1 mM $MgCl_2$ and 0.2 mM DTT. One volume of this membrane suspension was added to an equal volume of the complete cytosol of packed human erythrocytes [4] or of a 'cytosolic factor' (purified as in [18]) and to 3 vol. reaction medium so as to obtain, at final concentration, 25 μM [α-^{32}P]-NAD^+ (2 Ci/mmol), 5 mM ATP, 1 mM GTP, 10 mM phosphocreatine, creatine phosphokinase (10 U/ml), 10 mM thymidine, 1 mM nicotinamide, cholera toxin (20 μg/ml), and the buffer mentioned in the figure legends. ADP-ribosylation was conducted for 30 min at 30°C. The cholera toxin used was pre-activated at 500 μg/ml for 10 min at 37°C with 0.5% SDS, 0.1% ovalbumin, and 20 mM DTT. Control experiments were conducted with an appropriate dilution of this activating buffer lacking the toxin. The labelling of

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washed pancreatic plasma membranes was measured after protein coprecipitation with 0.5 mg bovine serum albumin in the presence of 10% trichloroacetic acid and filtration on glass fiber filters GF/B (Whatman, Maidstone). Nucleotides present in the supernatant of the ADP-ribosylating medium were separated on a silica gel $60F_{254}$ TLC plastic roll (Merck, Darmstadt), developed in 60% ammonium sulfate buffered with 0.1 M sodium phosphate (pH 6.9) enriched with 2% 1-propanol, and detected by UV light and autoradiography.

2.2. *Electrophoresis of membrane proteins*

Labelled membranes were heated for 1 min at 100°C in 10 mM Tris–HCl buffer (pH 8.0) containing 1% SDS, 1 mM EDTA, 1% 2-mercaptoethanol, 20% sucrose, and bromophenol blue as tracking dye. Electrophoresis of the dissolved material was carried out by polyacrylamide-gradient gel in SDS. Linear gradient gels (4–30% acrylamide, 3 mM thick [19]) were prepared as described by Pharmacia Fine Chemicals (Uppsala). The electrophoresis buffer was made of 40 mM Tris, 20 mM glycine, 2 mM EDTA, and 0.2% SDS, adjusted to pH 7.4 with acetic acid. After 20 min at 75 V in the gel electrophoresis apparatus GE 2/4 from Pharmacia, sharp stacking of the sample was observed before entering into the gel. The electrophoresis was then continued for 4 h at 150 V. The gels were stained in 0.1% Coomassie brilliant blue R-250, dried in the gel slab drier GSD-4 from Pharmacia, and exposed for 1–7 days to R of RP X-Omat film using intensifying screens from Kodak (Vilvoorde).

3. Results

The ADP-ribosylation of G/F proteins in membranes from erythrocytes [5,6,10] (but apparently not from hepatocytes [20]) requires the presence of erythrocyte cytosol in addition to cholera toxin. In [4] we used the whole erythrocyte cytosol and cholera toxin to activate adenylate cyclase in pancreatic plasma membranes. Using similar conditions with [α-^{32}P]NAD$^+$, we observed that cytosolic proteins were labelled in addition to plasma membrane proteins (not shown). This was in line with [6]; however, it was difficult to wash out pancreatic plasma membranes of all cytosolic components. Such difficulties have been avoided by replacing the whole erythrocyte cytosol either by a protein cofactor purified 1000-fold from this cytosol [18] or by an acetate or phosphate buffer used at high concentration [8]. With rat pancreatic plasma membranes we found that it was necessary to use the purified cytosolic cofactor together with acetate of phosphate (fig.1(7,8)) to obtain a membrane labelling as efficient as that observed with the whole cytosol (fig.1(6)). Contrasting with the labelling of membrane proteins, that depended strongly upon incubation conditions, the proportion of [α-^{32}P]NAD$^+$ hydrolyzed in parallel was similar under all conditions (~30% after 30 min) and reflected mostly degradation by nonspecific NADase activity.

Autoradiograms of pancreatic plasma membrane proteins revealed the regular presence (fig.1) of 2 major (42 kM_r, 50 kM_r) and 3 minor (20 kM_r, 30 kM_r, 68 kM_r) radioactive bands. A last 23 kM_r radioactive component was labelled when phosphate or acetate buffer was also present at high concentra-

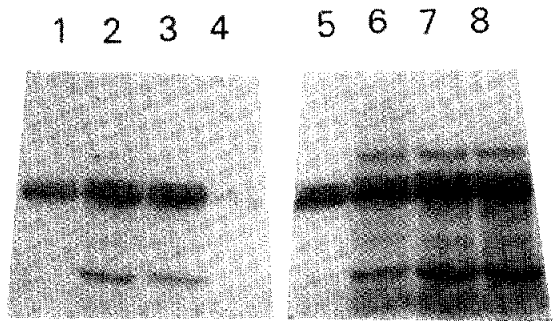

Fig.1. Effects of 4 buffers and cytosolic factor(s) on the electrophoretic and autoradiographic profile of ADP-ribosylated polypeptides of rat pancreatic plasma membranes incubated with [α-^{32}P]NAD$^+$ and cholera toxin. Pancreatic plasma membranes (35 μg) were incubated for 30 min at 30°C in 0.1 ml of the basal labelling medium described in section 2, that contained, inter alia, 20 μg cholera toxin/ml, a buffer, and erythrocyte cytosol or purified cytosolic factor. Washed membranes were solubilized in 0.1 ml SDS-electrophoresis sample buffer. Aliquots of 20 μl were analyzed on 4–30% acrylamide slab gel autoradiography. The ADP-ribosylation of total membrane protein, measured in parallel after trichloroacetic acid precipitation, and expressed as pmol/mg membrane protein, was in channel: (1) 2.0 with 20 mM Tes–Tris (pH 7.4); (2) 4.5 with 250 mM sodium acetate (pH 6.5); (3) 3.9 with 250 mM potassium phosphate (pH 6.9); (4) 0.5 with 250 mM potassium bicarbonate (pH 7.4); (5) 2.8 with 20 mM Tes–Tris (pH 7.4) and purified cytosolic factor; (6) 7.4 with 20 mM Tes–Tris (pH 7.4) and complete erythrocyte cytosol; (7) 9.9 with 250 mM sodium acetate (pH 6.4) and purified cytosolic factor; (8) 10.6 with 250 mM potassium phosphate (pH 6.9) and purified cytosolic factor.

tion in the incubation medium. No labelling of pancreatic plasma membranes was detected in control experiments conducted in the absence of cholera toxin (fig.2A(1)). Based on these preliminary data, further experiments were routinely conducted with the purified cytosolic cofactor from human erythrocytes and a 50 mM phosphate buffer: this moderate buffer concentration allowed a labelling of the 23 kM_r component lower than that obtained with a 250 mM

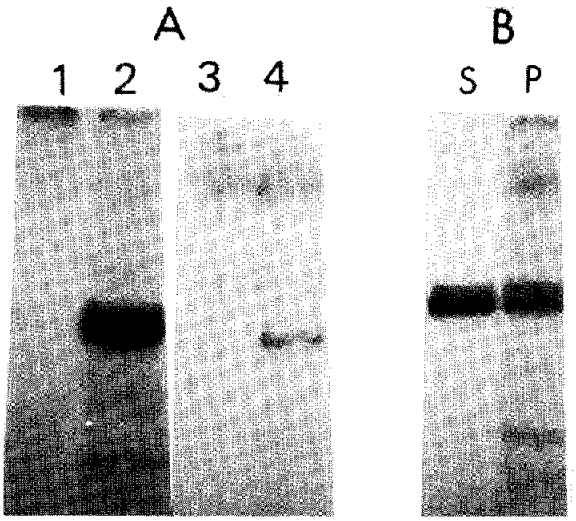

Fig.2. Specificity of cholera toxin-dependent labelling and solubilization with G 3707 of ADP-ribosylated polypeptides of rat pancreatic plasma membranes. (A) Plasma membranes from rat pancreas [1,2] or human erythrocytes [3,4] were labelled in the absence [1,3] or presence [2,4] of 20 μg cholera toxin/ml, as in section 2. The medium contained the purified cytosolic factor and was buffered with 50 mM sodium phosphate (pH 7.4). Washed membranes were solubilized in SDS–electrophoresis sample buffer at ~1 mg protein/ml. ADP-ribosylation of total protein, measured in parallel and expressed as pmol/mg membrane protein, was 7.8 in channel (2), 0.9 in channel (4) and almost zero in channels (1,3). (B) Washed pancreatic plasma membranes labelled in the presence of cholera toxin (fig.2A(2)) were solubilized by homogenization of 0.5 mg membrane protein in 0.25 ml 10 mM Tes–Tris (pH 7.4) and 1% of the non-ionic detergent G 3707. After 1 h at 0°C, the preparation was centrifuged 5×10^{10} rad^2/s in rotor 50 Ti in the L 8-55 Beckmann ultracentrifuge. The collected supernatant (S) contained 12% of total radioactivity. The residual pellet (P) was rehomogenized in 0.25 ml of the same buffer. Aliquots of supernatant (S) and of resuspended pellet (P) were treated with an equal volume of SDS-electrophoresis sample buffer. Before electrophoresis, the material in P was diluted 4 times so that the concentration of labelled protein was equal in P and S. SDS electrophoresis of 50 μl samples was performed on 4–30% gradient gels.

phosphate buffer while maintaining at high level the labelling of 42 kM_r and 50 kM_r polypeptides (compare fig.2A(2) to fig.1(8)).

The M_r-values of 42 000 and 50 000, estimated for the 2 major pancreatic bands, was comparable to that of ADP-ribosylated polypeptides from other G/F oligoproteins [7,12,14] and, further, the mobilities of the 42 kM_r pancreatic component (fig.2A(2)) and of the single 42 kM_r component from human erythrocyte ghosts [8,9], labelled for comparison (fig.2A(4)) were the same.

We next tested the solubility of ADP-ribosylated proteins from pancreatic plasma membranes: the non-ionic detergent G 3707 used at 1% extracted only the 42 kM_r and 50 kM_r labelled bands (fig.2B(S)) and the yield of solubilization was low (9 ± 2%, table 1) so that these components remained the major radioactive bands in the residual particular material (fig.2B(P)). The solubilization yield was not improved in the presence of 5 mM $MgCl_2$ or 2 mM EDTA, and a second extraction with G 3707 yielded very low additional amounts of 42 kM_r and 50 kM_r (1%, not shown). The pattern of ADP-ribosylation and solubilization in 1% G 3707, of membranes pretreated with 10 mM sodium fluoride or with 10 μM GTPγS and 0.3 μM C-terminal octapeptide of cholecystokinin was similar to that of native membranes (not shown). This poor solubilization of 42 kM_r and 50 kM_r polypeptides with G 3707 contrasted with the nearly complete solubilization of adenylate cyclase achieved with G 3707 after preactivation of the same pancreatic plasma membrane preparations with fluoride or cholera toxin: Mg^{2+}- and Mn^{2+}-dependent adenylate cyclase activities were equally soluble (table 1) (the solubility of basal adenylate cyclase could not be tested as this form of the enzyme was labile in the presence of detergent).

The yield of soluble labelled proteins was 3.6-fold higher with 12 mM deoxycholate (32 ± 2%) than with G 3707 (9 ± 2%) and similar with 12 mM cholate (14 ± 4%) (table 1). Like G 3707, deoxycholate solubilized adenylate cyclase almost completely whereas the yield with cholate was lower (table 1). The labelled material extracted with G 3707 and deoxycholate was made of 42 kM_r and 50 kM_r bands only as shown by SDS–polyacrylamide gel electrophoresis. Cholate extracted, in addition, some 30 kM_r labelled material (not shown). Lubrol PX (Sigma) and Lubrol 12A9 (Imperial Chemical Industries, Brussels), 2 non-ionic detergents, when tested at 1%, gave similar solubilization yields of labelled polypeptides as 1% G 3707;

Table 1
Solubilization (%) of ADP-ribosylated proteins and preactivated adenylate cyclase from rat pancreatic plasma membranes

	Radioactive proteins	Adenylate cyclase activity (n = 1)			
Pretreatment	(A) 25 μM [^{32}P]NAD$^+$ + cholera toxin	(B) 10 mM NaF		(C) 0.1 mM [α-^{32}P]NAD$^+$ + cholera toxin[a]	
Cation in adenylate cyclase assay medium		Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
1% G 3707	9 ± 2 (n = 7)	88	86	90	86
12 mM deoxycholate	32 ± 2 (n = 4)	96	98	85	87
12 mM cholate	14 ± 4 (n = 3)	54	48	63	73

[a] The solubilization of radioactive proteins in membranes labelled at 0.1 mM [^{32}P]NAD was 6%, 18% and 8%, with G 3707, deoxycholate and cholate, respectively; i.e., not higher than in (A)

(A) The labelling of membranes with 25 μM [α-^{32}P]NAD$^+$ was performed in the presence of 20 μg cholera toxin/ml as in fig.2

(B) Preactivation of adenylate cyclase with 10 mM NaF for 5 min as in [1]

(C) Intoxication of adenylate cyclase with 20 μg cholera toxin/ml was performed as in [4], except that at 0.1 mM [α-^{32}P]NAD (0.4 Ci/mmol) was used to obtain rapidly a quantitative intoxication of the enzyme

Washed membranes were homogenized at 1–1.5 mg protein/ml in 10 mM Tes–Tris (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.25 M sucrose and the indicated concentration of detergent. After incubation for 1 h at 0°C with occasional stirring, the samples were centrifuged at 5 × 10^{10} rad^2/s in a 50 Ti rotor. The pellets were rehomogenized in the original volume of the same detergent solution. Radioactivity in proteins was determined after trichloroacetic acid precipitation. Adenylate cyclase assays were performed in the presence of either 5 mM $MgCl_2$ or 1 mM $MnCl_2$, in a medium containing 30 mM Tris–HCl (pH 7.4), 0.3 mM [α-^{32}P]-ATP, 1 mM cyclic AMP, 10 mM phosphocreatine, 10 units phosphocreatine kinase/ml, 6 mM NaF, and 5 mg/ml crude phosphatidylcholine from Sigma (addition of phospholipids in excess (w/w) overcame the inhibitory effect of detergent on adenylate cyclase). The enzyme preparation in 0.04 ml was added to 0.08 ml incubation medium and incubated for 15 min at 30°C. Cyclic [^{32}P]AMP was isolated as in [26]

digitonin at 0.5% was 2 times less efficient and 0.5% β-D-octylglucopyranoside (Sigma Chemical Co.) was completely inefficient.

It is noteworthy that the poor extractability (9 ± 2%) of pancreatic labelled proteins with G 3707 contrasted with the higher (59 ± 4%, n = 5) solubilization of labelled human erythrocyte proteins that we observed with G 3707, in line with the high solubility of the 42 kM_r polypeptide of erythrocyte documented with other non-ionic detergents [8].

4. Discussion

The labelling with [^{32}P]NAD$^+$ of proteins in rat pancreatic plasma membranes required the presence of cholera toxin and the analysis of ADP-ribosylated polypeptides by SDS–polyacrylamide gradient gel electrophoresis showed 2 major 42 kM_r and 50 kM_r polypeptides and a few minor labelled peptides. The labelled polypeptides 42 kM_r and 50 kM_r were the only one to be solubilized by the non-ionic detergent G 3707 (fig.2B(S)). The same detergent solubilized adenylate cyclase easily in a form that involved the presence of G/F protein(s) [21,22] as MgATP was readily converted into cyclic AMP (table 1). This indicates that polypeptides 42 kM_r and 50 kM_r were the only radioactive species contributing to G/F in pancreatic plasma membranes. The same conclusion has been attained for polypeptides 45 kM_r and 52–53 kM_r in membranes from S49 lymphoma and HTCA cells [7], and polypeptides 42 kM_r and 47 kM_r from fibroblasts [23]. Similarly, polypeptides 45 kM_r and 55 kM_r are the only one to be ADP-ribosylated by cholera toxin in G/F from rat liver membranes, a 130 kM_r oligoprotein made of 3 polypeptides: 30 kM_r, 45 kM_r and 55 kM_r [14]. In contrast, there is only one ADP-ribosylated polypeptide in pigeon [5,6] and human [8,9] erythrocyte ghosts and in thymus [12]

and, in our hands, the electrophoretic mobility of the single ADP-ribosylated polypeptide from human erythrocyte was identical to that of the 42 kM_r pancreatic polypeptide, both polypeptides running immediately in front of an ovalbumin 43 kM_r standard.

G/F oligoproteins extracted from various sources with non-ionic detergents are all capable to reconstitute the Mg^{2+}-dependent activity of adenylate cyclase from *cyc*$^-$ cell line [21] despite differences in peptide map and in the number of labelled polypeptides (suggesting similarity but not identity among this class of polypeptides [11]). On this basis, the present pancreatic G/F oligoprotein is likely to have similar properties.

An important consideration in interpreting our data is that G/F proteins located in plasma membranes are stalked intrinsic proteins [8,11] that are as easily extracted with non-ionic detergents whether combined or not combined with the catalytical unit of adenylate cyclase in the native state. This is the case for G/F proteins from pigeon erythrocyte, rabbit liver, and S49 lymphoma cells that are part of an adenylate cyclase system and for G/F proteins in membranes of human erythrocyte and HTC4 cells that are devoid of adenylate cyclase [8,21]. A necessary coexistence of G/F and adenylate cyclase, is also ruled out by the presence of G/F in soluble form in the cytosol of 4 rat tissues [24]. It was of interest, therefore, to observe that the solubility of pancreatic G/F in G 3707 was low, when compared to that of the activated pancreatic adenylate cyclase (table 1). This might indicate that it was mostly that part of G/F associated with adenylate cyclase that was solubilized and, hence, capable to activate the dissolved enzyme. Another major part of G/F remained insoluble in residual rat pancreatic plasma membranes and was presumably tightly associated with other effector systems (such as cytoskeletal membrane proteins [25]) that resisted solubilization.

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