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EFFECT OF *BORDETELLA PERTUSSIS* TOXIN ON ADP-RIBOSYLATION OF MEMBRANE PROTEINS, ADENYLATE CYCLASE ACTIVITY AND INSULIN RELEASE IN RAT PANCREATIC ISLETS

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SUMMARY: Exposure of rat pancreatic islet membranes to [α-^{32}P]-NAD^+ in the presence of *Bordetella Pertussis* toxin (islet-activating protein) reveals the ADP-ribosylation of a peptide with a Mr close to 41 kDa, which corresponds to the α-subunit of the guanine nucleotide regulatory protein Ni. Islets removed from rats pretreated with the *Bordetella Pertussis* toxin display a specific increase in adenylate cyclase responsiveness to GTP and are characterized by a resistance to the inhibitory action of α_2-adrenergic agonists upon either adenylate cyclase activity or glucose-induced insulin release.

The dual hormonal control of adenylate cyclase through specific receptors involves two guanyl nucleotide regulatory proteins, Ns and Ni, which mediate, respectively, the stimulation and inhibition of adenylate cyclase activity (1-3). We have recently documented the presence of Ns in rat pancreatic islets by measuring the ADP-ribosylation of the two α_s-subunits of Ns in islet membranes exposed to [α-^{32}P]NAD^+ in the presence of cholera toxin (Svoboda et al., submitted for publication). In other tissues, the Ni regulatory protein is composed of an α_i-subunit (Mr 39-41 kDa), which is specifically ADP-ribosylated by *Bordetella Pertussis* toxin, and a β-subunit (Mr 35 kDa), which is apparently identical to the β-subunit of Ns (1). In the present study, we have investigated the ADP-ribosylation of membrane proteins, the activity of adenylate cyclase and the release of insulin in pancreatic islets exposed to the *Bordetella Pertussis* toxin.

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MATERIALS AND METHODS

The *Bordetella Pertussis* toxin (islet-activating protein) was purified to homogeneity from the supernatant of *Bordetella Pertussis* cultures by a modification (Svoboda et al., manuscript in preparation) of methods described elsewhere (4, 5). [α-^{32}P]-NAD^+ was purchased from New England Nuclear (Du Pont de Nemours, Brussels, Belgium).

For the study of ADP-ribosylation of membrane proteins in islets, groups of 500 or more islets were isolated by the collagenase method (6) and sonicated, membranes being collected by centrifugation (30 min at 100,000 g). Membrane proteins (10-20 µg/sample) were incubated in centrifugation tubes (maximal content : 0.175 ml) fitting rotor 42.2 Ti (Beckman, Palo Alto, Ca, USA) for 60 min at 30°C in 0.150 ml (final volume) of a medium containing 80 mM potassium phosphate buffer (pH 7.4), 1 mM ATP, 0.1 mM GTP, 10 mM thymidine, 1 mM ADP-ribose, 1 mM nicotinamide, 10 mM phosphocreatine, 10 units/ml creatine phosphokinase, 10 µM [α-^{32}P]NAD^+ (10 Ci/mmol) and 8 µg/ml purified preactivated toxin. The toxin (80 µg/ml) was preactivated by a 10 min incubation at 37°C in the presence of 50 mM dithiothreitol in 100 mM Tris-glycine buffer (pH 8.3). This toxin activation medium was diluted 10-fold in the ADP-ribosylation medium. ADP-ribosylation was terminated by cooling at 0°C and centrifugation (20 min at 40,000 rpm). The membranes were washed twice by resuspending the pellet in 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl and 1 mM EDTA, and centrifugation (20 min at 40,000 rpm). The final pellet was dissolved in 0.05 ml of electrophoresis sample buffer consisting of 0.125 M Tris-Cl (pH 6.8), 5 % SDS and 5 % 2-mercaptoethanol, heated for 1 min at 100°C and eventually applied on the gel. Polyacrylamide gel electrophoresis was carried out on 200 x 180 x 0.7 mm slab gels (12.5 % and 5 % of polyacrylamide for separating and stacking gels, respectively) in the GE 2/4 LS apparatus of Pharmacia (Uppsala, Sweden). The discontinuous Laemmli buffer system was used with buffer concentrations twice as high as those of the original method (7). Electrophoresis was conducted for 16 h at 90 V. The gels were stained with Coomassie brilliant blue R-250, dried in the gel slab drier GSD-4 from Pharmacia, and exposed for 3 days at -70°C to S X-Omat film using Kodak intensifying screens. Gel calibration was made with a kit of standard proteins from Pharmacia consisting of phosphorylase b (Mr 94 kDa), bovine serum albumin (Mr 67 kDa), ovalbumin (Mr 45 kDa), carbonic anhydrase (Mr 30 kDa), trypsin inhibitor (Mr 20 kDa) and α-lactalbumin (Mr 14.4 kDa).

The analysis of the supernatants of the ADP-ribosylating medium was performed by TLC on silica gel 60 F 254 plates (Merck, Darmstadt, FRG). TLC were developed in 60 % ammonium sulfate buffered with 0.1 M sodium phosphate buffer (pH 6.9) enriched with 2 % 1-propanol. Plate autoradiographies revealed that, after a 60 min incubation period, more than 90 % of the radioactivity in the medium remained as [α-^{32}P]NAD^+.

In order to study the effect of *Bordetella Pertussis* toxin upon islet adenylate cyclase activity or insulin release, rats (284 $\pm$ 8 g body weight; n = 21) received a single intraperitoneal injection of the toxin (3.0 µg) diluted in saline (0.2 ml). Whereas the body weight of control rats increased steadily (+ 4.7 $\pm$ 1.4 g/day, n = 6), the animals injected with the toxin failed to gain weight over the first 48 hours following the administration of the toxin (- 1.1 $\pm$ 1.6 g/day, n = 10), and then lost weight over the ensuing 3-4 days (- 5.0 $\pm$ 1.6 g/day, n = 5). The methods used to measure insulin release from intact

islets (8) and adenylate cyclase activity in islet homogenates (9, 10) are described in detail elsewhere. The incubation media used to measure insulin release contained ascorbic acid (0.5 mM) to minimize the oxidation of epinephrine. For the assay of adenylate cyclase, groups of 500 islets were homogenized in 0.8 ml of a Tris-HCl buffer (25 mM; pH 7.6) containing $MgCl_2$ (5.0 mM), EGTA (1.5 mM), dithiothreitol (3.0 mM) and bovine albumin (0.5 mg/ml). After a first centrifugation (5 min, 800 g, 4°C) to remove nuclei and cell debris, the enzyme activity was measured either in the supernatant crude homogenate or in a particulate fraction obtained by a further centrifugation for 20 min at 4°C and 12,000 g (10). The assay medium always contained, in addition to the reagents listed elsewhere (10), 200 mM NaCl.

All results, including those mentioned above, are expressed as the mean (± SEM) together with the number of individual observations (n) and statistical significance of differences between mean values, as assessed by Student's t-test.

RESULTS

Pancreatic islet membranes were ADP-ribosylated in the presence of purified *Bordetella Pertussis* toxin. Over a 60 min incubation at 30°C, 20-25 pmol ADP-ribose were incorporated per mg membrane protein, as judged by TCA precipitation and filtration. No labelling was detected in the absence of the toxin. Electrophoretic and autoradiographic analysis showed the presence of one single radioactive species with a Mr close to 41 kDa in the islet membrane pellet (Fig. 1, right lane).

Treatment *in vivo* with the toxin for 2-5 days caused three significant changes in the activity of islet adenylate cyclase. First, the basal activity was slightly decreased. Thus, relative to the paired control value, the basal activity in toxin-treated animals averaged 77.5 ± 9.7 % and 70.0 ± 7.2 % (n = 3-4) in the particulate fraction and crude homogenate, respectively. On the contrary, the response to GTP, GTPγS or NaF, relative to paired basal value, tended to be higher in toxin-treated than control animals (Table 1). Second, in the particulate fraction, the GTP-induced increment in reaction velocity, when expressed relative to the paired value for the GTPγS-induced increment in velocity, was higher in toxin-treated than control animals.

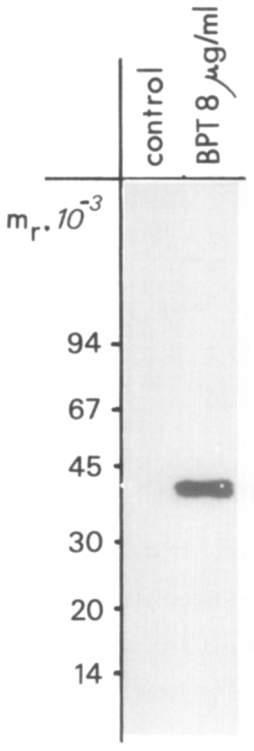

Fig. 1. Autoradiographic analysis of islet membranes ADP-ribosylated in the absence (control) or presence of *Bordetella Pertussis* toxin (BPT). The position of molecular weight standards is indicated on the left side of the figure. This experiment is representative of 3 similar experiments made with different membrane preparations.

Thus, expressed in such a way, the GTP/GTPγS ratio averaged in control rats no more than 65.9 ± 10.8 % ($P < 0.01$) of the paired value found in toxin-treated animals. This indicates that treatment with the toxin increased preferentially the response to GTP, relative to that evoked by GTPγS. Last, in the crude homogenate, clonidine caused a modest but highly significant inhibition ($P < 0.01$) of adenylate cyclase activity in control rats, whilst failing to affect the reaction velocity in toxin-treated animals. It should be stressed that, as judged from quadruplicate measurements performed in each crude homogenate either in the absence or presence of clonidine, the inhibitory action of the α_2-adrenergic agonist upon enzyme activity was always signi-

Table 1. Adenylate cyclase activity in pancreatic islets from control and toxin-treated rats

Cellular Fraction	Agent (μM)	Control	Toxin-treated
	Basal velocity (amol/min per islet equivalent)		
Particulate fraction	-	181 ± 59*	123 ± 33
Crude homogenate	-	515 ± 123	332 ± 64
	Modified velocity (% of paired basal value)		
Particulate fraction	GTP (10)	133 ± 9	159 ± 9
Particulate fraction	GTPγS (10)	303 ± 24	390 ± 37
Particulate fraction	NaF (10^4)	419 ± 13	478 ± 45
Crude homogenate	Clonidine (10)	85 ± 2	97 ± 3

*Mean values (± SEM) are derived from triplicate or quadruplicate measurements performed in each of 3-4 separate experiments; in each individual experiment, islets were prepared from the pancreases of 3 control and 3 toxin-treated rats (killed 2-5 days after injection of the toxin).

ficant in control rats (n = 4) and not so in 3 out of 4 groups of toxin-treated animals. In the crude homogenate, the relative extent of the inhibitory action of clonidine upon adenylate cyclase activity was little affected by the addition of GTP (10-100 μM) to the assay medium (data not shown), a situation possibly attributable to contamination by endogenous GTP.

The mean rate of insulin release evoked by D-glucose (16.7 mM) was not vastly different in control and toxin-treated animals (Table 2). In islets from control rats, epinephrine abolished glucose-stimulated insulin release, the secretory rate being not significantly different from basal value. Treatment with the toxin caused a time-related suppression of the inhibitory action of epinephrine. Thus, when the rats were killed 20 hours after the injection of the toxin, the inhibitory effect of epinephrine, although still detectable, was much less marked than in control animals : it amounted to 34.7 instead of 90.4 percent. When the animals were killed 2 days after injection of the toxin, no ef-

Table 2. Effect of epinephrine upon insulin release (μU/islet per 60 min) evoked by D-glucose (16.7 mM) in pancreatic islets from control and toxin-treated rats

Animals	No epinephrine	Epinephrine (0.4 μM)	P
Control	301.1 ± 18.2 (24)*	29.0 ± 9.3 (24)	< 0.001
Toxin-treated (1 day)	312.2 ± 34.8 (11)	204.0 ± 18.1 (11)	< 0.02
(2 days)	337.8 ± 10.5 (12)	333.6 ± 24.1 (12)	> 0.8

*Mean values (± SEM) are shown together with the number of individual observations. In each group, the islets were derived from 3-6 rats.

fect of epinephrine upon glucose-stimulated insulin release could anymore be observed.

DISCUSSION

In the pancreatic B-cell, adrenergic agents inhibit insulin secretion by activating α_2-adrenoceptors (11-13). The suppression of insulin release appears attributable, in part at least, to inhibition of adenylate cyclase, as judged from data obtained both in crude islet homogenates or intact islets (14). The present results confirm that the inhibition of insulin release by epinephrine is abolished after pretreatment with *Bordetella Pertussis* toxin (15), and also confirm (16) that this pretreatment leads to typical changes in the responsiveness of adenylate cyclase, mainly a resistance to the inhibitory action of α_2-agonist and an increased sensitivity to GTP. The molecular weight and toxin-dependency of ADP-ribosylation of islet membrane proteins, as documented in the present study,further indicate the presence in islet cell membranes of the α-subunit of the Ni regulatory protein. Incidentally, ADP-ribosylation of trichloroacetic-insoluble material in intact islets was recently reported (17),but no information was provided on either the effect of microbial toxins upon ADP-ribosylation or the molecular weight

of labelled peptides. The results of the present study indicate that islet cells, like other cell types (1-3) are equipped with suitable receptor-transducer systems for the informational transfer of an input hormonal signal to an output catalytic response.

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