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Cholera toxin action on rabbit renal brush-border membranes inhibits phosphate transport

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Cholera toxin was used to enhance ADP-ribosylation of rabbit renal brush-border membranes. Treatment of brush-border membrane sheets with cholera toxin in the presence of NAD resulted in a specific inhibition of the initial phase of Na^+-dependent P_i uptake, compared to controls incubated with NAD alone. The P_i uptake was determined after conversion of the membrane sheets to vesicles. The equilibrium uptake of P_i, the Na^+-independent uptake of P_i, the Na^+-dependent uptake of L-proline and the activities of several brush-border membrane enzymes were not changed. The inhibition of P_i transport was dependent on the presence of both NAD and cholera toxin. Incubation of membrane sheets with [^{3}H]NAD produced acid-stable binding of radioactivity to the membranes and the binding was increased 5-fold by the presence of cholera toxin. The use of [^{32}P]NAD and autoradiography confirmed that the bound radioactivity was associated with several different membrane proteins, and that cholera toxin increased binding to these proteins including three that were not labelled in the absence of the toxin. The specific inhibitory action of cholera toxin on Na^+/P_i cotransport is probably mediated by ADP-ribosylation of membrane proteins, suggesting that the P_i transport system can be regulated by ADP-ribosylation, at least in vitro.

Introduction

Cholera and pertussis toxins possess an ADP-ribosyltransferase activity which transfers the ADP-ribose moiety from NAD to a variety of arginine-containing proteins. Subunit A is the fragment of cholera toxin that has the ADP-ribosyltransferase activity. Cholera toxin exhibits a wide range of substrate specificity. It ADP-ribosylates a variety of proteins including the GTP-binding N_s protein, G-proteins such as transducin and GTP-binding proteins from brain and neutrophils, histone H1, protamine, cytoskeletal proteins and some glycopeptide hormones [8,9]. Cholera toxin also ADP-ribosylates a number of low molecular weight guanidine-containing compounds [11]. ADP-ribosyltransferase activity is present not only in these toxins but also in the cells of eukaryotic tissues [12], including the brush-border membrane of the kidney proximal tubule cell [3,5].

Intraperitoneal injection of rats with nicotinamide increases the NAD content of the renal proximal tubule [1] and is accompanied by specific inhibition of the Na^+-dependent phosphate (P_i) transport system in the renal brush-border membrane [2]. On the basis of these findings we proposed that NAD may serve as an intracellular regulator of the P_i transporter through a mechanism such as ADP-ribosylation [3,4]. The proposal is supported by the in vitro studies of Hammerman et al. [5] who showed that introduction of NAD inside canine brush-border membrane vesicles was accompanied by ADP-ribosylation of the membrane and specific inhibition of Na^+-dependent P_i transport. Other studies in the rat, however, indicated that P_i transport was unchanged by intravesicular NAD and there was little evidence of membrane ribosylation [6].

In an attempt to resolve this controversy we prepared brush-border membranes in a non-vesiculated 'sheet' form [7] in order to ensure that NAD would have free access to the cytosolic surface and to avoid problems associated with making brush border membrane vesicles permeable to NAD. After exposure of the sheets to NAD, the membranes were washed and converted to sealed vesicles and Na^+-dependent P_i transport (Na^+/P_i

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cotransport) was assayed by the standard procedure. Cholera toxin was used to enhance endogenous ADP-ribosylation of the membranes.

Methods

The experimental protocol is summarized in Fig. 1. Brush-border membranes in non-vesiculated sheet form were prepared from rabbit kidney as described previously in great detail [7,13]. All steps were carried out at 0–4°C. The cortex was removed from four kidneys, sliced and placed in 0.5 M sucrose solution. A homogenate (5% w/v) was prepared with five complete strokes of a hand-held loose-fitting Dounce homogenizer followed by two strokes in a rotating Potter-Elvehjem homogenizer. The homogenate was layered over 11 ml of 1.4 M sucrose in a 38 ml centrifuge tube and the interface was lightly stirred. After centrifugation for 60 min at 90000 × *g* in a Beckman SW-27 rotor the interfacial layer and the solution above it were removed and centrifuged for 15 min at 4000 × *g*. The pellet containing brush-border membrane sheets was resuspended in 0.5 M sucrose, using 1 ml/g cortex, and centrifuged again for 5 min at 32000 × *g*. The sheets of brush-border membranes sedimented as a pink loose layer over a dark pellet. The supernatant was removed and used to resuspend the pink layer. The suspension was centrifuged for 10 min at 4000 × *g*. The resulting pellet was resuspended in 0 5 M sucrose using 0.5 ml/g cortex and was centrifuged for 10 min at 4000 × *g*. This was repeated twice except that the first centrifugation was at 2000 × *g* and the second at 1000 × *g*. The purity of the final brush-border membrane pellet was determined by electron microscopy and by assays of brush-border membrane enzymes.

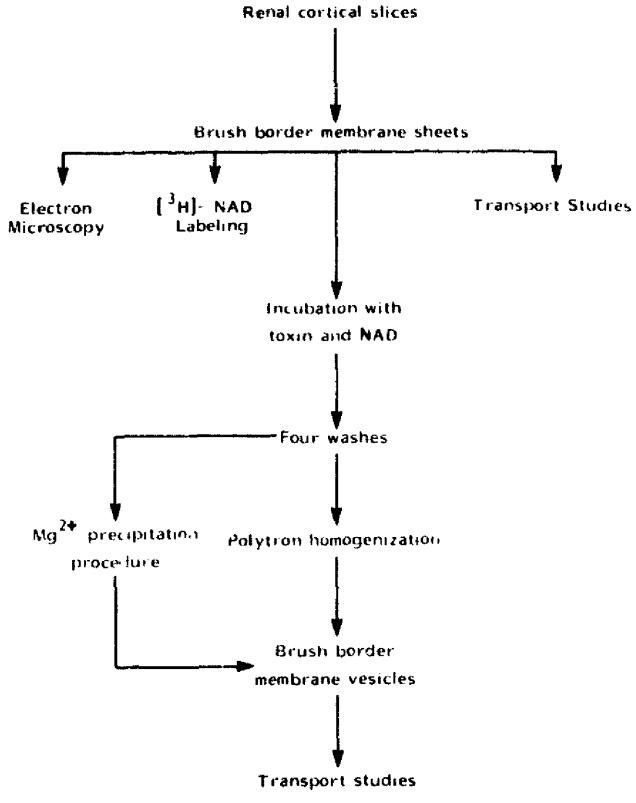

Fig. 1. Outline of the sequence of experiments.

Brush-border membrane pellets were prepared for electron microscopy by fixing for 24 h at 4°C in 2.5% glutaraldehyde in 0.1 M cacodylate-HCl buffer. The pellets were washed with buffer and treated with 1% OsO_4 in 0.1 M cacodylate-HCl buffer for 1 h at 4°C. After washing with buffer, the pellets were dehydrated in a graded series of ethanol solutions, then infiltrated and embedded in Epon 812 resin. This sections were double stained with uranyl acetate and lead citrate.

When used directly for transport studies the membrane suspension was diluted with 5 mM Tris-Hepes (pH 8.5) to adjust the sucrose concentration to 0.3 M. Uptake of solutes such as P_i and L-proline was determined by the rapid filtration procedure used and described previously [2,5,7]. There was 0.13–0.15 mg protein in each uptake tube.

Incubation of brush-border membrane sheets with cholera toxin (A subunit) from *Vibrio cholera* was carried out as follows. The membrane suspension was centrifuged for 10 min at 4000 × *g* and the pellet (7–8 mg membrane protein) was resuspended in ADP-ribosylation buffer which contained 0.5 M sucrose, 0.1 M KH_2PO_4, 20 mM thymidine, 2 mM ATP, 0.2 mM GTP, 20 mM $MgCl_2$, 4 mM EDTA and 1 mM ADP-ribose (pH 7.0). Activated toxin [10] was added to a final concentration of 50–80 μg/ml followed by addition of NAD to a final concentration of 0.3 mM and the mixture (final volume 1.0 ml) was incubated for 20 min at 32–35°C. The membrane sheets were recovered by centrifugation for 10 min at 4000 × *g* and washed four times with 30 ml of 0.5 M sucrose. The final suspension was diluted with 5 mM Tris-Hepes (pH 8.5) to bring the sucrose concentration to 0.3 M. The membrane sheets were converted to vesicles by Polytron homogenization using three 30-s bursts at 30-s intervals [7]. Debris was pelleted by centrifugation for 12 min at 1500 × *g* and brush-border membrane vesicles were recovered by centrifuging the supernatant for 20 min at 45000 × *g*. The membrane pellet was resuspended in the minimum amount of buffer containing 300 mM mannitol and 5 mM Tris-Hepes (pH 8.5) and was used immediately for studies on transport of P_i and L-proline as described above.

Incorporation of ADP-ribose into brush-border membrane sheets was determined in an assay mixture of ADP-ribosylation buffer (see above) containing 7–8 mg membrane protein, 50 μg cholera toxin and 0.5 mM [^{3}H]NAD (3500 cpm/μl). The total volume was 0.3 ml

and the reaction was initiated by addition of the NAD. After incubation for 25 min at 35°C the reaction was stopped by addition of 1.0 ml of 6% ice-cold trichloroacetic acid [3]. In order to correct for trapping of the isotope blanks were included by adding the acid to the reaction mixture prior to addition of the NAD [3]. After standing on ice for 10 min the mixture was centrifuged for 10 min at 9000 × *g* and the supernatant discarded. The membrane pellet was resuspended in 1.5 ml of trichloroacetic acid by sonication and centrifuged as before. After two additional washes the final pellet was resuspended in 0.15 ml of trichloroacetic acid and used for scintillation counting and protein determination. When the membranes were used for gel electrophoresis, the incubation was carried out as described above except that [^{32}P]NAD was used and the reaction was stopped by addition of an equal volume of a solution containing 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% β-mercaptoethanol and 125 mM Tris-HCl (pH 6.8). The sample was prepared for electrophoresis on 7% polyacrylamide gels containing 0.1% SDS, as described by Laemmli [14].

Protein content and the activities of brush-border membrane enzymes and Na^+/K^+-ATPase were determined as described previously [2,3,7]. All experiments were conducted on at least three separate occasions, unless stated otherwise. Control and experimental groups were processed simultaneously in each experiment and significant differences between groups were determined by Student's *t*-test. A value for $P > 0.05$ was considered not significant.

Results

The preparation of a membrane fraction enriched in brush-border membranes was confirmed by assays of typical marker enzymes. The activities of the brush-border enzymes γ-glutamyltranspeptidase, leucine aminopeptidase, and alkaline phosphatase were increased at least 15-fold in the membrane preparations compared to the starting cortical homogenate (Table I). In contrast, the activity of Na^+/K^+-ATPase in the membrane fraction was reduced to 0.81 μmol/h per mg protein compared to a value of 1.63 μmol/h per mg protein in the cortical homogenate, indicating minimal contamination by basolateral membranes.

Electron micrographs of the brush border membrane sheet preparations showed that most of the membranes were in the form of large non-vesiculated fragments (Fig. 2A). Some intact microvilli also were present (Fig. 2B). The absence of a sealed vesicular compartment was confirmed when the P_i transport properties of the preparations were determined. There was no characteristic overshoot in the presence of a Na^+ gradient (Fig. 3), and replacement of Na^+ with K^+ produced no significant change in P_i uptake. These results were not due to inactivation of the P_i transporter because the overshoot and marked Na^+ dependency were restored when the sheets were converted to sealed membrane vesicles (Fig. 3).

TABLE I

Enzyme activities in brush-border membrane sheets

Data are mean ± S.E. from three experiments. BBMS, brush-border membrane sheets. The sheets were incubated with NAD (0.3 mM) and cholera toxin (50–80 μg/ml) in ADP-ribosylation buffer.

	γ-Glutamyl transferase (μmol/h per mg protein)	Leucine aminopeptidase (μmol/h per mg protein)	Alkaline phosphatase (μmol/h per mg protein)
Homogenate	11.5 ± 0.2	3.1 ± 0.5	1.7 ± 0.2
BBMS fraction	180 ± 12	98 ± 11	34 ± 5
BBMS after incubation with cholera toxin and NAD	179 ± 12	93 ± 12	30 ± 5

The morphological and functional data strongly suggest that the incubation medium has unhindered access to the cytosolic surface of the brush border membrane when the membranes are in the non-vesiculated sheet form.

In preliminary studies, brush border membrane sheets were treated with cholera toxin in the absence of NAD, washed and converted to membrane vesicles. Na^+/P_i cotransport by these vesicles was not different compared to control vesicles prepared from sheets not exposed to the toxin. Cholera toxin treatment of sheets also had no effect on the activities of typical brush-border membrane enzymes (Table I). Finally, incubation of suspensions of brush-border membrane vesicles with cholera toxin, followed by four washes, produced no change in Na^+/P_i cotransport. Additional studies showed that incubation of brush-border membrane sheets with NAD alone, in the absence of cholera toxin, produced no inhibition of P_i transport determined after washing and conversion of the sheets to vesicles.

Treatment of membrane sheets with cholera toxin in

TABLE II

Na^+/L-proline uptake by vesicles from toxin-treated sheets

Data are mean ± S.E. from three experiments. Treatment of membrane sheets with toxin and NAD was carried out as in Table I.

	Time (min)	Uptake (pmol/mg protein)
Controls treated with NAD only	0.5	51.4 ± 6.6
	100.0	19.6 ± 3.4
Treated with toxin and NAD	0.5	49.3 ± 14.9
	100.0	17.4 ± 2.9

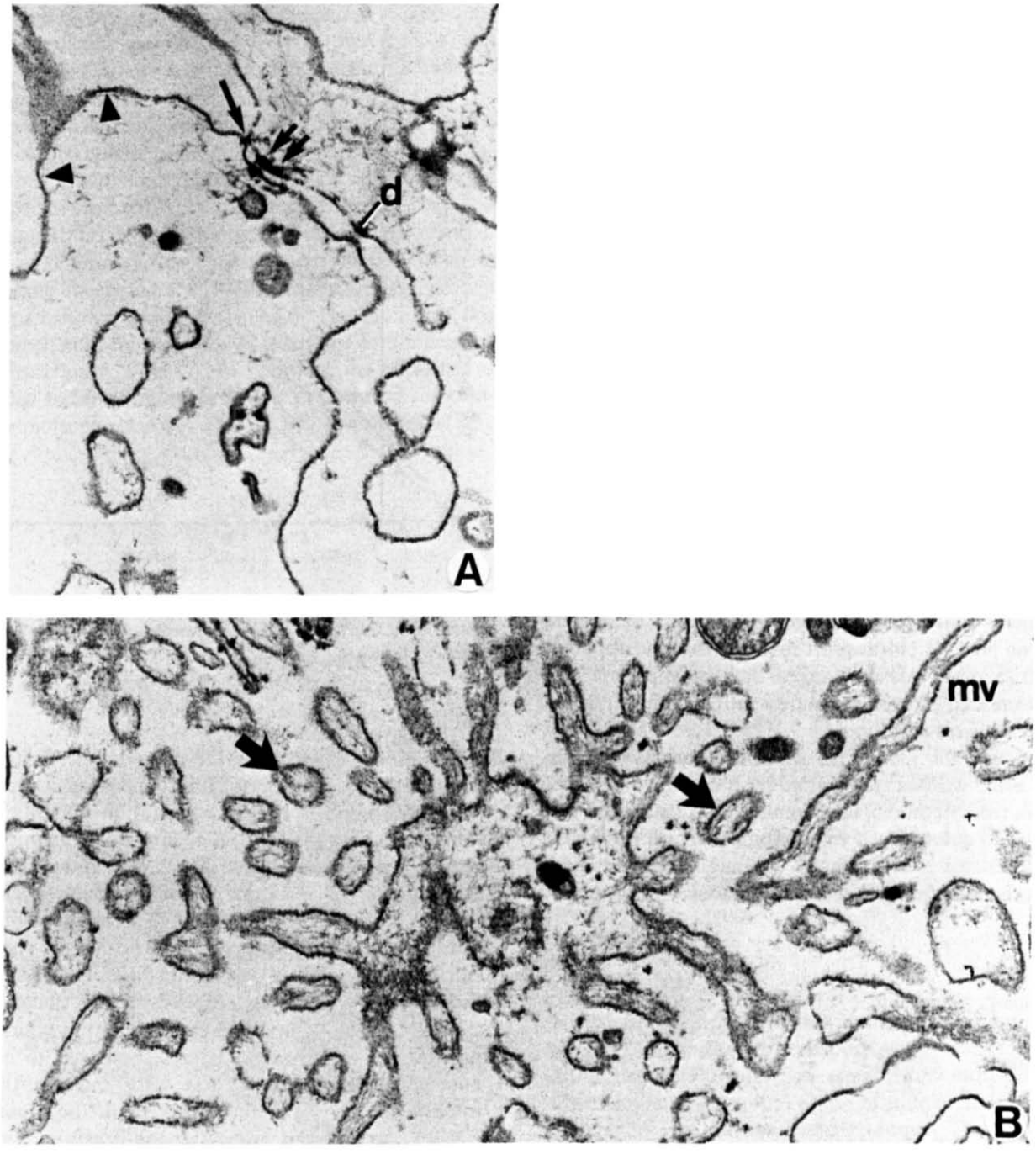

Fig. 2. Transmission electron micrographs of brush-border membrane sheet preparations. Final magnification ×44000. (A) Broad sheets of plasma membranes linked by an intact junctional complex bearing occluding (arrow), adherens (double arrows), and desmosomal (d) components. The cytosolic surface of the membranes is indicated by arrowheads. (B) Several elongated profiles (mv) exhibit ultrastructure suggestive of brush-border microvilli. Numerous vesicle-like structures (arrows) appear to be cross sections of microvilli. Both types of structures contain filamentous material probably derived from the cytoskeletal core of the microvilli.

the presence of NAD produced significant inhibition of the initial phase of Na^+/P_i cotransport at all time points studied. The uptake at 10 s, for example, was inhibited by 38% (Fig. 4). In contrast the uptake at equilibrium (100 min) was not different from controls (Fig. 4). The controls in these studies were brush-border membranes that were incubated with NAD in ADP-ribosylation buffer but in the absence of the toxin. The specificity of cholera toxin action is indicated by the absence of any inhibition of Na^+-independent P_i transport which was 106 ± 4 (mean ± S.E.) in controls compared to 117 ± 11 pmol/mg protein per 30 s after toxin treatment. Na^+/proline cotransport also was not altered by the toxin (Table II). As discussed above, the

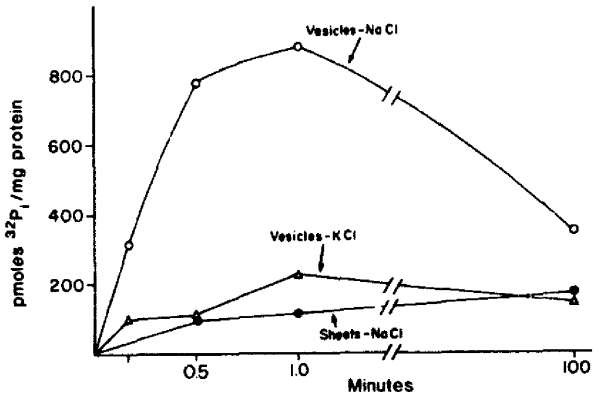

Fig. 3. Time-course of $^{32}P_i$ transport by sheets and vesicles of brush-border membrane. Na^+ gradient-dependent P_i transport was determined both in membrane sheet preparations (●) and after conversion of the sheets to membrane vesicles (○). P_i uptake by membrane vesicles in the presence of a K^+ gradient instead of Na^+ is shown for comparison (△).

inhibition of P_i transport required the presence of both cholera toxin and NAD.

Kinetic analysis of the inhibitory action of cholera toxin on Na^+/P_i cotransport revealed that the apparent K_m (0.25 mM) was unchanged but the apparent V_{max} was decreased to 2.33 compared to 3.03 nmol/mg per 20 s in controls (Fig. 5).

Although the membrane sheet suspensions were diluted and washed during conversion to membrane vesicles (see Methods), additional experiments were carried out to determine if the inhibitory action of cholera toxin persisted after extensive washing. In these experiments the membrane sheets were treated with toxin and

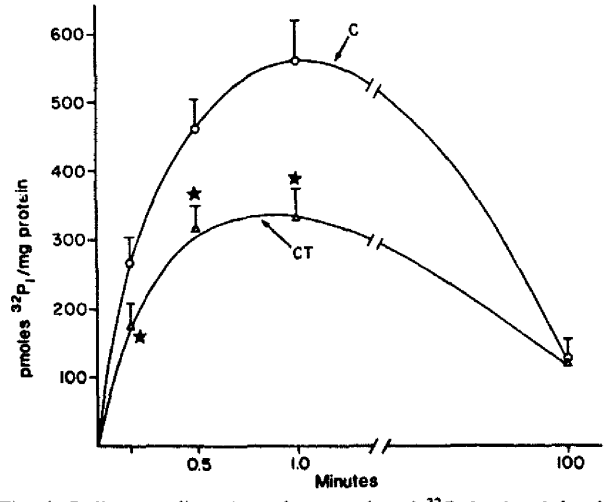

Fig. 4. Sodium gradient-dependent uptake of $^{32}P_i$ by brush-border membrane vesicles prepared from membrane sheets. The brush-border membrane sheets were treated with NAD alone (C, ○) or with both NAD and cholera toxin (CT, △). Data are mean ± S.E. from 4–5 experiments. * indicates significant difference ($P < 0.05$) from controls (C).

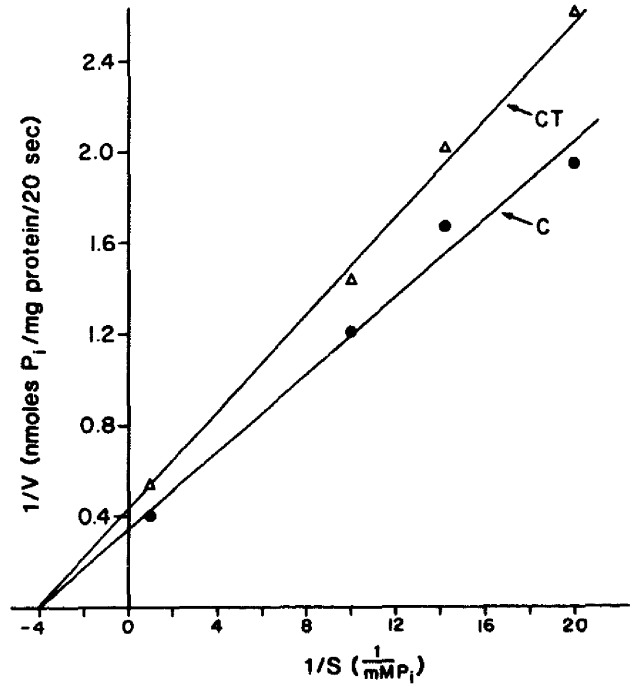

Fig. 5. Double-reciprocal plot of Na^+-dependent uptake of $^{32}P_i$ by brush border membrane vesicles prepared from membrane sheets. The sheets were treated either with NAD alone (C, ●) or with both NAD and cholera toxin (CT, △).

NAD in the standard ADP-ribosylation buffer. The sheets were washed several times and then converted to membrane vesicles by homogenization and Mg^{2+} precipitation (Fig. 1). This procedure is analogous to the second step of the double Mg^{2+} precipitation method used for direct isolation of brush-border membrane vesicles from renal cortex [6]. The vesicles obtained from the sheets by this procedure showed the characteristic overshoot for Na^+/P_i cotransport. The P_i uptake at the 30-s time point was inhibited by 35% (mean of two experiments, each analyzed in triplicate) in vesicles prepared from toxin-treated sheets compared to controls not exposed to toxin. These data indicate that the inhibitory effect of toxin treatment in the presence of NAD was persistent even after the brush-border membranes had been washed many times.

Confirmation that cholera toxin was stimulating ADP-ribosylation of brush-border membrane sheets was obtained with the use of [^{3}H]NAD radiolabelled on the adenine moiety. Membrane sheets in ADP-ribosylation buffer were incubated with [^{3}H]NAD (0.5 mM) in the presence and absence of toxin, and ADP-ribosylation was assessed as acid-stable binding of radioactivity to the membranes, as in previous studies [3]. In the absence of cholera toxin there was a small amount of ADP-ribosylation due most likely to the presence of an ADP-ribosyltransferase in the brush-border membrane [3]. The acid-stable incorporation of radioactivity was 241 ± 102 pmol [^{3}H]NAD/mg protein per 20 min

(mean ± S.E., $n = 3$). This was increased 5-fold to 1250 ± 283 pmol/mg per 20 min ($n = 3$, $P < 0.05$, t-test) when cholera toxin was included in the incubation medium.

The increased incorporation of radioactivity in the presence of cholera toxin was detected also on autoradiograms of SDS-polyacrylamide gels. These studies were performed with [^{32}P]NAD and strongly suggest that membrane proteins were ADP-ribosylated. Endogenous ADP-ribosylation (toxin absent) of brush-border membrane sheets led to ^{32}P-labelling of several protein bands, as shown in Fig. 6 (lane D). The same proteins were labelled in the presence of toxin but with increased intensity (Fig. 6, lane C). In addition, the presence of toxi led to labelling of three new proteins corresponding to molecular masses of 158, 97, and 45 kDa. The amount of protein applied to the gel was reduced for toxin treated membranes because much more radioactivity was incorporated. Comparison of the gel pattern in lanes A and B (Fig. 6) indicates that cholera toxin produced no major changes in the proteins present in the brush-border membrane. A densitometric scan of the autoradiograms in lanes C and D shows more clearly the appearance of three new labelled bands due to the presence of cholera toxin (Fig. 7).

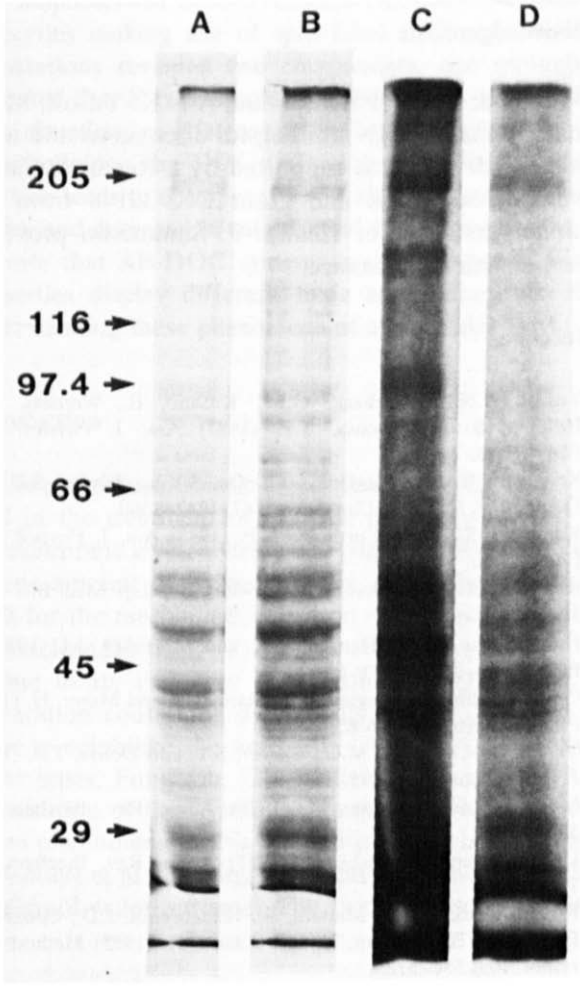

Fig. 6. Bush-border membrane proteins labelled by [^{32}P]NAD in the presence and absence of cholera toxin. The membrane sheets were solubilised and separated by SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie blue (lanes A and B), dried and used for autoradiography (lanes C and D). Lanes B and D are controls (toxin absent), lanes A and C are toxin-treated membrane sheets. Amounts of protein applied to the gel were either 0.123 mg (A and C) or 0.246 mg (B and D). The mobilities of protein standards of known molecular masses (kDa) are shown on the left.

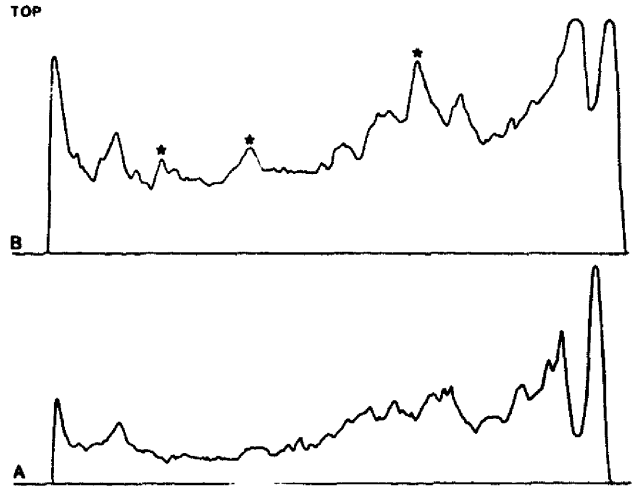

Fig. 7. Densitometric scans of autoradiograms. Panel A represents control membranes (toxin absent) and corresponds to Fig. 6D. Panel B represents toxin-treated membranes and corresponds to Fig. 6C. The top of the gels is on the left side, as indicated. Asterisks (*) mark the position of three specific protein bands in toxin-treated membranes (B) that were not labelled in control membranes (A). From left to right, the mobilities of these bands correspond to molecular masses of 158, 97 and 45 kDa, respectively.

Discussion

Incubation of brush-border membrane sheets with NAD and cholera toxin produced specific inhibition of the Na^+-dependent P_i transport system. There was no difference in P_i uptake at equilibrium indicating that the changes in the initial phase of uptake are not due to differences in intravesicular volume. There was no change in Na^+-independent P_i transport or in the activities of several brush-border membrane enzymes. The absence of inhibition of L-proline transport, another Na^+-dependent process, strongly suggests that the inhibition of P_i transport is due to a direct effect on the P_i transporter rather than to dissipation of the transmembrane Na^+ gradient. The inhibitory effect persisted even after extensive washing of the membrane to remove traces of intact NAD and possible products of NAD hydrolysis.

No inhibition of Na^+-dependent P_i transport occurred when either NAD or toxin was omitted from the incubation medium, suggesting that the inhibition of the Na^+/P_i cotransporter may be due to the use of NAD

for an ADP-ribosylation reaction catalysed by cholera toxin. Furthermore, cholera toxin had no effect when isolated brush border membrane vesicles were incubated with the toxin, and washed prior to measurement of P_i transport. These data indicate that the mechanism of toxin/NAD inhibition of Na^+/P_i cotransport may involve ADP-ribosylation of the cytosolic surface of the brush-border membrane. The same concentration of toxin that produced inhibition of P_i transport also produced a marked stimulation of brush-order membrane ADP-ribosylation, providing further support for the idea that the changes in P_i transport may be mediated by an ADP-ribosylation reaction.

Gel electrophoresis and autoradiography showed that several protein bands are ADP-ribosylated in the presence of ^{32}P-labelled NAD (Figs. 6D and 7A), most likely due to an endogenous ADP-ribosyltransferase present in the brush-border membrane [3]. The presence of cholera toxin increased the amount of radioactivity present in all these bands and, in addition, caused ADP-ribosylation of three new protein bands (Figs. 6C and 7B). Thus, the toxin produced specific changes in the overall ADP-ribosylation pattern which may help explain why Na^+/proline cotransport was not affected (Table II). Since inhibition of P_i transport is observed only in the presence of both NAD and toxin, it is possible that one or more of the bands which is ADP-ribosylated only in the presence of NAD and toxin (Fig. 7B) may be part of the Na^+/P_i cotransport system or may be involved in its regulation.

The observation that incubation of membrane sheets with NAD alone had no effect on P_i transport is not consistent with the findings of Hammerman et al. [5] and may be due, in part, to our use of brush-border membranes from the kidney of the rabbit rather than the dog. The studies of Gmaj et al. [6], who reported that Na^+/P_i cotransport was not inhibited by intravesicular NAD, were carried out on brush-border membranes from the rat. The use of different species may have contributed to the contrasting data from different laboratories. Alternatively, since NAD is a competitive inhibitor of Na^+/P_i cotransport [15], failure to remove NAD or hydrolysis products such as P_i could complicate interpretation of the data on P_i transport. In the present study the brush-border membranes were washed extensively after exposure to NAD and prior to measurement of Na^+/P_i cotransport.

The use of brush-border membrane sheets is a unique approach which allows access to the cytosolic surface of the membrane, and avoids the problems associated with making vesiculated membranes permeable to NAD [5,6]. It appears that the activity of the P_i transporter is uniquely sensitive to modification by an ADP-ribosylation reaction that requires NAD and cholera toxin. The endogenous activity of the brush-border membrane ADP-ribosyltransferase [3] appears to be inadequate to account for the changes in P_i transport, at least in the rabbit, since incubation with NAD alone did not change P_i transport. It is possible that the membrane ADP-ribsosyltransferase is inactivated during the membrane isolation and treatment procedure, or that an essential cofactor is removed by the washing steps. If ADP-ribosylation is an important intracellular mechanism for regulating brush-border P_i transport, an additional intracellular factor with ADP-ribosyltransferase activity may be required to increase the membrane ribosylation, as does cholera toxin in vitro. Finally, the present data do not rule out the possibility that ADP-ribosylation of the external surface of the brush-border membrane may contribute to the changes in P_i transport.

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