Biochimica et Biophysica Acta, 732 (1983) 110–116
Elsevier

BBA 71716

NAD^+-INDUCED INHIBITION OF PHOSPHATE TRANSPORT IN CANINE RENAL BRUSH-BORDER MEMBRANES

MEDIATION THROUGH A PROCESS OTHER THAN OR IN ADDITION TO NAD^+ HYDROLYSIS

MARC R. HAMMERMAN, VALERIE M. CORPUS and JEREMIAH J. MORRISSEY

Metabolism and Renal Divisions, Department of Internal Medicine, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110 (U.S.A.)

(Received November 9th, 1982)
(Revised manuscript received March 1st, 1983)

Key words: Phosphate transport; ADP ribosylation; NAD^+ hydrolysis; (Brush-border membrane)

We previously demonstrated inhibition of Na^+-dependent $^{32}P_i$ transport in canine renal brush-border membranes in association with NAD^+-induced ADP ribosylation of membrane protein(s) and postulated that NAD^+ inhibits P_i transport across the brush-border membrane via ADP ribosylation. Recently it was shown that incubation of rat brush-border membrane with NAD^+ resulted in release of P_i which was prevented by EDTA. It was proposed that NAD^+-mediated inhibition of $^{32}P_i$ transport might occur through this mechanism. To determine whether NAD^+ inhibited $^{32}P_i$ transport by a mechanism other than or in addition to release of P_i, we compared Na^+-dependent $^{32}P_i$ counterflow in brush-border membrane equilibrated with P_i or with P_i generated from NAD^+. Release of P_i from NAD^+ incubated with brush-border membrane was confirmed. The increased uptake of $^{32}P_i$ which was demonstrated in brush-border membrane equilibrated with P_i was not measured when intravesicular P_i was generated from a concentration of NAD^+ which effected ADP-ribosylation of brush border membranes (100 μM NAD^+). In contrast, increased uptake of $^{32}P_i$ was demonstrated when intravesicular P_i was generated from 1 μM NAD^+ which did not effect ADP ribosylation. Mg^{2+}-dependent ADP ribosylation of brush-border membrane incubated with NAD^+ was demonstrated which persisted during the time interval of $^{32}P_i$ uptake measurements. Our findings are compatible with the hypothesis that NAD^+-induced ADP ribosylation of brush-border membrane protein(s) results in inhibition of P_i transport across the membrane in vivo. EDTA may act to prevent this inhibition in brush-border membrane by chelation of Mg^{2+} and decreased ADP ribosylation.

Introduction

It has been shown that addition of NAD^+ to isolated suspensions of renal brush-border membrane vesicles from rat [1] or dog [2] resulted in decreased Na^+-dependent $^{32}P_i$ transport in the vesicles. Berndt et al. [3] demonstrated that PTH infusion in thyroparathyroidectomized rats increased renal cortical $NAD^+/NADH$ ratios and proposed that intracellular NAD^+ mediates a phosphaturic effect of PTH through an 'interaction' with the brush-border membrane in vivo. Kempson [4] reported preliminary evidence of NAD^+-induced ADP ribosylation of brush-border membranes isolated from rat kidney. We have since demonstrated NAD^+-induced ADP ribosy-

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PTH, parathyroid hormone; SDS, sodium dodecyl sulfate.

lation of a specific protein band (M_r 62000) demonstrable on SDS-polyacrylamide gels of isolated canine renal brush-border membrane suspensions associated with inhibition of Na^+-dependent $^{32}P_i$ transport [2]. These observations suggested that a phosphaturic effect of PTH may be mediated via NAD^+-induced ADP ribosylation of the renal cortical cellular brush-border membrane.

Recently Tenenhouse and Chu [5] have demonstrated hydrolysis of NAD^+ by brush-border membranes isolated from rat kidney resulting in the appearance of 1 mol of adenosine and 2 mol of P_i from each mol of NAD^+. Incubation of membranes with EDTA in addition to NAD^+ prevented the release of P_i and prevented the inhibition of Na^+-dependent $^{32}P_i$ transport in brush-border vesicles which was demonstrated in the absence of EDTA. The investigators proposed that it was the P_i liberated from NAD^+ and not NAD^+ itself which inhibited Na^+-dependent $^{32}P_i$ transport in isolated brush-border vesicles.

Because of the importance of defining the mechanism by which NAD^+ inhibits Na^+-dependent $^{32}P_i$ transport in isolated brush-border vesicles in terms of our understanding of the mediation of phosphaturic effects of PTH, we have conducted the present studies. We demonstrated that NAD^+ is metabolized by isolated canine brush-border membranes such that P_i is released. However, NAD^+ inhibited Na^+-dependent $^{32}P_i$ transport in brush-border vesicles via a mechanism other than or in addition to liberation of P_i from NAD^+. We suggest that this mechanism is ADP ribosylation of the brush-border membrane. ADP ribosylation of brush-border membrane protein(s) (M_r 62000) was a Mg^{2+}-dependent process. Thus the absence of a demonstrable decrease of $^{32}P_i$ transport in brush border membranes incubated with NAD^+ and EDTA could be explained, at least in part, by chelation of Mg^{2+} and resultant inhibition of ADP ribosylation.

Materials and Methods

Isolation of brush-border membrane vesicles and ADP ribosylation of membranes. Mongrel dogs were used in the present studies. Nephrectomies were performed as previously described [6].

Brush-border membrane vesicles were isolated by a $MgCl_2$ (unless otherwise noted) or $CaCl_2$ precipitation technique which has been described previously [2,6,7]. ADP ribosylation of brush-border membranes was effected as before [2]. NAD^+ was added to membrane vesicles in a solution hypotonic relative to that in which the vesicles were suspended so as to induce rupture of membrane vesicles and allow NAD^+ access to the intravesicular space.

SDS-polyacrylamide gel electrophoresis and high-performance liquid chromatography. SDS-polyacrylamide gel electrophoresis was carried out as described in previous studies [8]. Autoradiograms of SDS-polyacrylamide gels containing ^{32}P were developed after 7–10 days. All SDS-polyacrylamide gel experiments were performed on at least three separate occasions. SDS-polyacrylamide gels were calibrated for molecular weights as before [8].

High-performance liquid chromatography of supernatants from trichloroacetic acid precipitated, brush border membranes incubated with [*adenylate*-^{32}P]NAD^+ was performed using methodology detailed previously [7], except a Partisil SAX anion exchange column (Whatman) was utilized using Buffer A (10 mM KH_2PO_4, pH 3.77) and Buffer B (0.25 M KH_2PO_4 + 0.5 M KCl, pH 3.60) as described by Matsumoto et al. [9]. A 1.5 min equilibration with Buffer A was found to be necessary to effect a separation between NAD^+ and AMP.

Measurement of phosphate uptake in membrane vesicles. Phosphate uptake in brush border membrane vesicles was measured by a Millipore filtration technique previously described [8]. All incubations were carried out with freshly prepared membrane vesicles and all uptake determinations were performed in triplicate. Results are expressed as the mean ± S.E. Differences between means were analyzed using Dunnett's Multiple Comparison Procedure [10].

Uptakes of $^{32}P_i$ at 15 s were used as estimates of initial rates of transport. A steady state of $^{32}P_i$ uptake was reached by 90 min [7].

For experiments comparing $^{32}P_i$ uptake in brush-border vesicles equilibrated with P_i, adenosine, nicotinamide and ribose, or with P_i generated from NAD^+ (counterflow studies), 50 μl of (a) 200 or 2 μM P_i (Tris salt), 100 or 1 μM adenosine, 100 or 1 μM nicotinamide, 100 or 1 μM D-ribose, 10

mM $MgCl_2$, 10 mM KCl, 5 mM Hepes-Tris, pH 8, or (b) 100 or 1 μM NAD^+, 10 mM $MgCl_2$, 10 mM KCl, 5 mM Hepes-Tris, pH 8, or (c) 10 mM $MgCl_2$, 10 mM KCl, 5 mM Hepes-Tris, pH 8 were added to brush-border membranes (200–300 μg protein) suspended in 240 mM mannitol, 10 mM $MgCl_2$, 10 mM KCl, 5 mM Hepes-Tris, pH 8 in a manner previously described [2]. The resulting mixture was incubated for 120 min at 30°C (30 min to allow generation of P_i from NAD^+ plus 90 min to allow equilibration of P_i). The uptakes of ^{32}P in brush-border vesicles incubated with 200 μM or 2 μM $^{32}P_i$ or 100 μM or 1 μM [*adenylate*-^{32}P]NAD^+ in this manner measured by Millipore filtration were maximal after 120 min of incubation suggesting that equilibration with $^{32}P_i$ had occurred. We have previously shown that $^{32}P_i$ uptake in brush-border membrane vesicles from dog kidney incubated at 20°C in the absence of Na^+ reached a steady state after 90 min of incubation [6]. In the present studies following the 120 min incubation the uptake of 40 μM or 0.4 μM $^{32}P_i$ was measured over time in the presence of a 100 mM NaCl gradient as described before [2,6].

Protein concentrations of brush-border membrane suspensions were determined by the technique of Lowry et al. [11] using bovine serum albumin as a reference protein.

Measurement of phosphate concentrations in supernatants of brush-border suspensions incubated with NAD$^+$. Brush-border membrane suspensions (200–300 μg protein/10 μl) were incubated with NAD^+ at 30°C for 30 min as for ADP ribosylation. Following incubation the suspensions were immediately centrifuged at 4°C at 35 000 × *g* in a Sorvall RC5B Centrifuge for 20 min (DuPont Instruments, Newtown, CT, U.S.A.) using a SS-34 rotor. Phosphate present in the resulting supernatants was measured using a modification of the method of Taussky and Shorr [12]. Five hundred μl of 1% (w/v) $(NH_4)_6Mo_7O_{24}$, and 5% (w/v) $FeSO_4$ in 1 M H_2SO_4 were added to 500 μl of supernatant and the mixture was incubated at 37°C for 2 min. Absorbance was measured at 740 nm using a Zeiss M4QIII Spectrophotometer and P_i concentrations were calculated from a standard curve constructed using known P_i concentrations measured in the same manner.

Materials. [^{32}P]Orthophosphoric acid (carrier free), and [*adenylate*-^{32}P]NAD^+ (610–800 Ci/mmol) were obtained from New England Nuclear, Boston, MA, U.S.A. β-NAD^+ was obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. Other chemicals were of the highest purity available from commercial sources.

Results

Metabolism of NAD$^+$ by brush-border membranes

High-performance liquid chromatography was conducted using ethyl ether extracted supernatants from trichloroacetic acid precipitated brush border membranes incubated at 30°C with 100 μM [*adenylate*-^{32}P]NAD^+. Radioactivity eluted primarily with NAD^+ and ADPribose standards after 0.5 minute of incubation, but primarily with the P_i

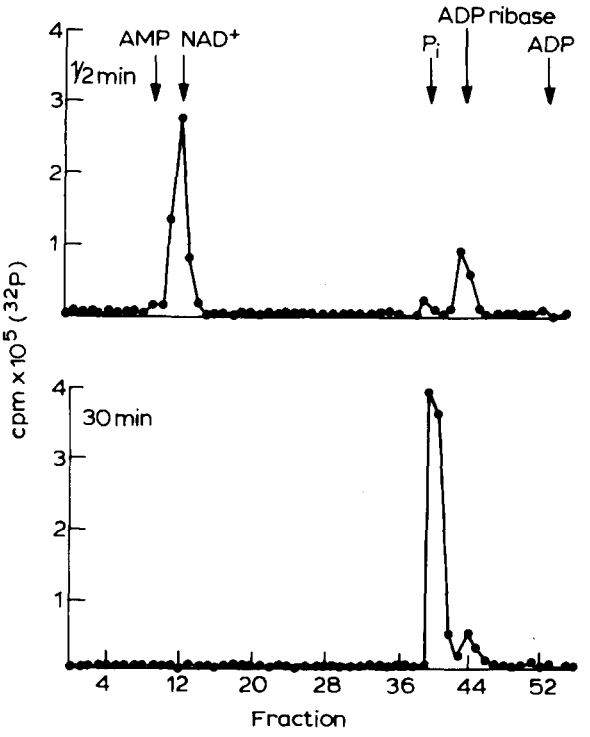

Fig. 1. High-performance liquid chromatography of supernatants from brush-border membranes incubated with [*adenylate*-^{32}P]NAD^+. Supernatants were collected from brush-border suspensions incubated with 100 μM [*adenylate*-^{32}P]NAD^+ for 0.5 min or 30 min. Recoveries of injected radioactivity were 85% for both 0.5 min and 30 min incubations. Fractions were collected every 0.5 min. The positions of unlabelled standards are indicated. Standards were injected with each run. Results are from a representative of three experiments.

standard after 30 min of incubation (Fig. 1). This finding demonstrated that [*adenylate*-^{32}P]NAD^+ was metabolized when incubated with brush border membranes such that $^{32}P_i$ was released. In order to determine whether 2 mol of P_i had been released from each 1 mol of NAD^+ incubated with brush-border membranes under these conditions we measured [P_i] in supernatants of six separate brush border membrane suspensions incubated at 30°C with 100 μM NAD^+ for 30 min. The mean [P_i] was 187 ± 12 μM (not significantly different from 200 μM, Student's *t*-test). These observations are consistent with the findings of Tenenhouse and Chu [5] who demonstrated release of 2 mol of P_i per 1 mol of NAD^+ incubated with rat renal brush border membranes. The [P_i] resulting from incubation of brush-border membranes for 30 min at 30°C with 1 μM NAD^+ was 2 ± 0.3 μM.

Inhibition of phosphate transport in brush-border membranes incubated with NAD$^+$

In order to determine whether NAD^+ inhibits $^{32}P_i$ transport in brush-border membranes via a mechanism other than or in addition to metabolism of NAD^+ and resultant release of P_i, we compared uptakes of $^{32}P_i$ in suspensions of brush-border membranes equilibrated with (a) P_i, adenosine, nicotinamide, and D-ribose, (b) NAD^+ and (c) no additions.

In this manner we compared the enhancement of Na^+-dependent $^{32}P_i$ transport in brush-border membrane vesicles measured at early times of incubation in the presence of intravesicular P_i resulting from equilibration of membranes with P_i or with P_i generated from NAD^+ (counterflow) [6,13]. Two sets of P_i, adenosine, nicotinamide and D-ribose, and NAD^+ concentrations were used in these studies: (a) 200 μM P_i, 100 μM adenosine, 100 μM nicotinamide, 100 μM D-ribose or 100 μM NAD^+, and (b) 2 μM P_i, 1 μM adenosine, 1 μM nicotinamide, 1 μM D-ribose or 1 μM NAD^+. In this manner we measured Na^+-dependent $^{32}P_i$ transport in brush-border vesicles incubated with NAD^+ under conditions where we had previously shown that ADP ribosylation of a 62 kDa protein band would occur (100 μM NAD^+) or would not occur (1 μM NAD^+) [2]. We demonstrated enhancement of 40 μM and 0.4 μM $^{32}P_i$ transport reflected in increased $^{32}P_i$ uptakes measured at t = 15 s and 1 min in membranes equilibrated with 200 μM or 2 μM P_i compared to ^{32}P uptakes measured in the absence of P_i equilibration (Table

TABLE I

THE EFFECT OF NAD^+ ON Na^+-DEPENDENT $^{32}P_i$ TRANSPORT IN BRUSH-BORDER VESICLES EQUILIBRATED WITH P_i

Brush-border vesicles were equilibrated with 2 or 200 μM P_i by incubation with 2 or 200 μM P_i, 1 or 100 μM adenosine, 1 or 100 μM nicotinamide, and 1 or 100 μM D-ribose; or with 1 or 100 μM NAD^+. Na^+-gradient dependent uptakes of 0.4 μM or 40 μM $^{32}P_i$ were measured following 15 s, 1 min, and 90 min of incubation of equilibrated vesicles and compared to uptakes of 0.4 μM or 40 μM $^{32}P_i$ in the absence of P_i equilibration. Each value is the mean ± S.E.

Conditions	P_i uptake (pmol/mg protein)		
	15 s	1 min	90 min
Uptake of 0.4 μM $^{32}P_i$ (n = 3 separate experiments)			
No equilibration	11.9 ± 0.5	18.4 ± 1.1	1.8 ± 0.06
Equilibration with 2 μM P_i	13.9 ± 0.1 [a]	26.1 ± 1.6 [a]	1.8 ± 0.1
Equilibration with P_i from 1 μM NAD^+	13.7 ± 0.3 [b]	26.1 ± 1.5 [b]	1.7 ± 0.1
Uptake of 40 μM $^{32}P_i$ (n = 5 experiments)			
No equilibration	700 ± 58	1278 ± 93	189 ± 37
Equilibration with 200 μM P_i	851 ± 98 [a]	1441 ± 73 [a]	212 ± 41
Equilibration with 100 μM NAD^+	714 ± 65 [c]	1276 ± 86 [c]	192 ± 41

[a] Equilibration with P_i > No equilibration, $P < 0.05$.
[b] Equilibration with P_i from NAD^+ > No equilibration, $P < 0.05$.
[c] Equilibration with P_i from NAD^+ > No equilibration, P = n.s. (not significant).

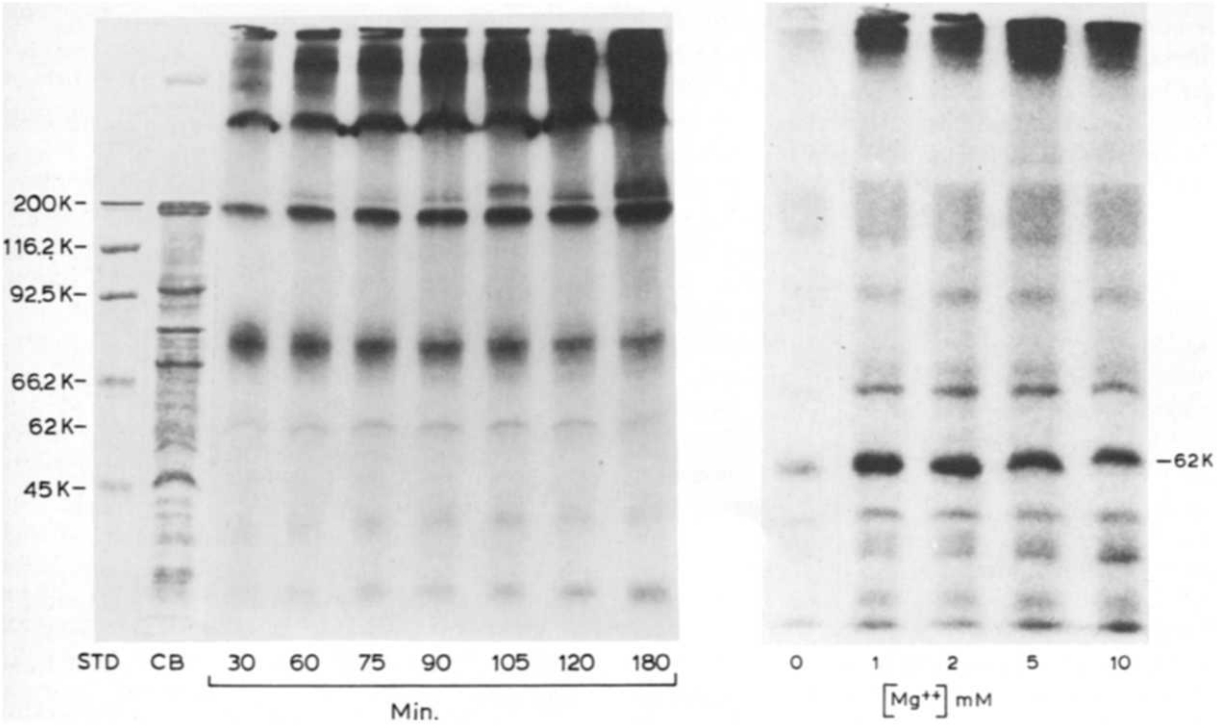

Fig. 2. SDS-polyacrylamide gel electrophoresis and autoradiography of ADP-ribosylated brush-border membrane proteins. Shown is an SDS-polyacrylamide gel electrophoresis of proteins of known molecular weight (STD), an electrophoresis of a brush-border membrane suspensions stained with Coomassie blue (CB), and autoradiograms of brush-border suspensions incubated with 100 μM [*adenylate*-^{32}P]NAD$^+$ for varying time periods. The regions in the vicinity of M_r 200000, 116200, 92500, 66200, 62000 and 45000 are indicated. Samples were subjected to electrophoresis in adjacent slots on the same gel. Results are from a representative experiment. Identical quantities of protein and ^{32}P were added to each slot.

Fig. 3. Autoradiograms of SDS-polyacrylamide gels of ADP-ribosylated brush-border membranes. Membranes prepared by $CaCl_2$ precipitation were incubated for 30 min with 100 μM [*adenylate*-^{32}P]NAD$^+$ in the presence of varying [Mg^{2+}]. The region in the vicinity of M_r 62000 is indicated. Samples were subjected to electrophoresis as in Fig. 2 and are from a representative experiment.

I). In addition, we demonstrated enhancement of 0.4 μM $^{32}P_i$ transport when P_i originated from metabolism of 1 μM NAD$^+$. This finding provided evidence that P_i released from metabolism of NAD$^+$ gained access into the intravesicular space following a 90 min incubation such that ^{32}P counterflow could be demonstrated. In contrast, enhancement of 40 μM $^{32}P_i$ transport was not measured when P_i originated from 100 μM NAD$^+$. Uptakes of P_i measured at steady state did not differ significantly among groups. Thus Na^+ dependent P_i transport as relfected by $^{32}P_i$ counterflow was inhibited by 100 μM NAD$^+$ but not by 1 μM NAD$^+$. This inhibition was effected via a mechanism other than release of P_i from NAD$^+$.

ADP ribosylation of brush-border membrane protiens including the 62 kDa band, was demonstrated in autoradiograms of SDS-polyacrylamide gels of brush-border suspensions following as long as 180 min of incubation with 100 μM NAD$^+$ (Fig. 2). Thus ADP-ribosylation represents a possible mechanism for the inhibition of Na^+-dependent $^{32}P_i$ transport in brush border vesicles incubated with 100 μM NAD$^+$ for 120 min.

The effect of [Mg^{2+}] on ADP ribosylation of brush-border membranes

Brush-border membranes prepared by $CaCl_2$ precipitation were incubated with 100 μM [*adenylate*-^{32}P]NAD$^+$ for 30 min in the presence

of varying concentrations of $MgCl_2$ and ADP ribosylation of the 62 kDa protein band was measured (Fig. 3). When [$MgCl_2$] was less than 10 mM, it was replaced isosmotically with KCl. [^{32}P]ADP ribose incorporation in the 62 kDa band was increased by 1 mM, 2 mM, 5 mM, and 10 mM Mg^{2+} by 24 ± 5%, 44 ± 11%, 57 ± 18% and 45 ± 5%, respectively, compared to incorporation measured in the absence of Mg^{2+} ($n = 4$). The addition of 1 mM EDTA to membranes incubated with [*adenylate*-^{32}P]NAD^+ in the absence of Mg^{2+} did not change [^{32}P]ADPribose incorporation in the 62 kDa band (not shown).

Discussion

These studies confirm the observations of Tenenhouse and Chu [5] regarding the liberation of P_i from NAD^+ incubated with renal brush-border membranes. Thus, metabolism of NAD^+ by brush-border membranes should be considered in the interpretation of experiments measuring $^{32}P_i$ transport in membranes incubated with NAD^+. It is likely that at least a portion of the NAD^+-associated inhibition of Na^+-dependent $^{32}P_i$ transport that we reported in a previous publication [2] resulted from release of P_i from NAD^+. The present experiments measuring $^{32}P_i$ counterflow were designed to determine the effect of NAD^+ on $^{32}P_i$ transport under conditions whereby NAD^+ and P_i released from NAD^+ might influence $^{32}P_i$ transport in opposite ways (P_i acting to stimulate $^{32}P_i$ transport and NAD^+ acting to inhibit $^{32}P_i$ transport). $^{32}P_i$ counterflow was inhibited by NAD^+ which was present in a concentration that we have shown effected ADP ribosylation of the brush-border membranes (100 μM NAD^+), but not by NAD^+ present in a concentration that did not effect ADP ribosylation (1 μM NAD^+). These results show that 100 μM NAD^+ inhibited Na^+-dependent $^{32}P_i$ uptake in brush border vesicles via a mechanism other than or in addition to release of P_i resulting from metabolism of NAD^+. We have shown that uptake of $^{32}P_i$ in brush border vesicles from canine kidney consists of a component of $^{32}P_i$ transport into an intravesicular space and a component of $^{32}P_i$ binding to brush-border vesicles [6]. The demonstration that 100 μM NAD^+ inhibited counterflow of $^{32}P_i$ strongly suggests that the component of $^{32}P_i$ uptake which was inhibited by NAD^+ represented the transport component of $^{32}P_i$ uptake rather than the binding component [6]. We interpret these findings as consistent with 100 μM NAD^+ mediated inhibition of Na^+-dependent $^{32}P_i$ transport in isolated brush border membrane vesicles being effected via ADP ribosylation of the membranes.

Kempson has reported that ADP ribosylation of rat renal brush-border membranes is a Mg^{2+}-dependent process [4]. Our data show enhanced ADP ribosylation of the 62 kDa protein band in the presence of Mg^{2+}. These results suggest an alternative explanation to the one proposed by Tenenhouse and Chu to explain the absence of NAD^+-mediated inhibition of $^{32}P_i$ transport in brush-border vesicles observed in the presence of EDTA. It is possible that EDTA acted to inhibit ADP ribosylation of membrane proteins through chelation of Mg^{2+} which was used in the preparation of brush-border membranes from rat kidneys, and prevented NAD^+-mediated inhibition of Na^+-dependent $^{32}P_i$ transport at least in part via a resultant inhibition of ADP ribosylation.

We have proposed that phosphaturic effects of PTH are mediated via cAMP-dependent phosphorylation and independently via ADP ribosylation of a 62 kDa protein in the dog renal brush border membrane [2]. In the present studies it is of interest that [^{32}P]ADP ribosylation of the 62 kDa protein band in autoradiograms of SDS-polyacrylamide gels of brush-border suspensions was demonstrated following as long as 180 min of incubation with [*adenylate*-^{32}P]NAD^+. This finding is in contrast to cAMP-dependent ^{32}P-phosphorylation of the 62 kDa protein band which was markedly reduced following 5 min of incubation with [γ-^{32}P]ATP and cAMP and no longer demonstrable following 30 min of incubation [7]. Since NAD^+ is degraded during incubation with brush-border membranes, the persistence of [^{32}P]ADP ribosylation of the protein band cannot be attributed to persistence of [*adenylate*-^{32}P]NAD^+ in brush-border suspensions in contrast to metabolism of ATP and cAMP [7]. One explanation for this finding would be that the enzymes required for the removal of ADPribose from brush-border membrane proteins are not present in the brush-border membrane as is the phos-

phoprotein phosphatase which dephosphorylates the 62 kDa protein band. It is possible, for example that a cytoplasmic protein which would not be present in brush-border membrane suspensions acts to remove ADPribose from the 62 kDa protein in vivo and thus might serve to reverse the phosphaturic effect of PTH. Such an enzyme has been reported to exist in rat liver cytosol [14]. Alternatively, ADP ribosylation of the 62 kDa protein could be an irreversible process in vivo as may be the case for ADP ribosylations of other proteins [15] and restoration of the preexisting state of renal P_i reabsorption might require new protein synthesis. Such a mechanism for reversal of the effects of ADP ribosylation would be consistent with our proposal that PTH-induced phosphaturia effected by ADP ribosylation mediates long-term need to excrete P_i and results from prolonged elevations of PTH [2]. Rapid reversal of such phosphaturia would not generally be required, and synthesis of new membrane protein could conceivably occur rapidly enough to allow such a reversal within a time frame so as to be noninjurious to the organism.

Acknowledgements

We wish to acknowledge the typing skills of Ms. Lynn Wesselmann. M.R.H. was supported by United States Public Health Service Grant RO1 AM27600 from the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD). J.J.M. was supported by Program Project Grant AM09976 from NIAMDD.

References

1 Kempson, S.A., Colon-Otero, G., Lise Ou, S.-Y., Turner, S.T. and Dousa, T.P. (1981) J. Clin. Invest. 67, 1347–1360
2 Hammerman, M.R., Hansen, V.A. and Morrissey, J.J. (1982) J. Biol. Chem. 257, 12380–12386
3 Berndt, T.J., Knox, F.G., Kempson, S.A. and Dousa, T.P. (1981) Endocrinology 108, 2005–2007
4 Kempson, S.A. (1982) Clin. Res. 30, 78A
5 Tenenhouse, H.S. and Chu, Y.L. (1982) Biochem. J. 204, 635–638
6 Hammerman, M.R., Karl, I.E. and Hruska, K.A. (1980) Biochim. Biophys. Acta 603, 322–335
7 Hammerman, M.R., Hansen, V.A. and Morrissey, J.J. (1983) Biochim. Biophys. Acta 755, 10–16
8 Hammerman, M.R. and Hruska, K.A. (1982) J. Biol. Chem. 257, 992–999
9 Matsumoto, S.S., Raivio, K.O. and Seegmiller, J.E. (1979) J. Biol. Chem. 254, 8956–8962
10 Dunnett, C.W. (1964) Biometrics 20, 482–491
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
12 Taussky, H.E. and Shorr, E. (1953) J. Biol. Chem. 202, 675–685
13 Rosenberg, T. and Wilbrandt, W. (1957) J. Gen. Physiol. 41, 289–296
14 Okayama, H., Honda, M. and Hayaishi, O. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2254–2257
15 Brady, R.O. and Fishman, P.H. 91979) Adv. Enzymol. 50, 303–323