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MECHANISM OF STIMULATION OF ADP-RIBOSYLTRANSFERASE IN THE RENAL BRUSH-BORDER MEMBRANE BY EDTA

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In the presence of NAD$^+$, renal brush-border membranes are mono-ADP-ribosylated by an endogenous ADP-ribosyltransferase. The reaction is inhibited by arginine methyl ester and is markedly stimulated by EDTA. Stimulation by EDTA is likely due, at least in part, to EDTA preventing the destruction of intact NAD$^+$ by other enzymes in the brush-border membrane.

Cytosolic NAD^+ may play a role in the intracellular mechanism for regulation of Na^+-dependent transport of P_i across the renal brush-border membrane [1]. There are specific binding sites for NAD^+ on renal brush-border membranes [2], and a possible mechanism by which NAD^+ may interact with and modify the P_i transport system is by ADP-ribosylation of brush-border membrane proteins. When isolated brush-border membranes are incubated with NAD^+ the membranes are mono-ADP-ribosylated by an endogenous ADP-ribosyltransferase [3,4], and ADP-ribosylation of the cytoplasmic surface of brush-border membranes is accompanied by specific inhibition of Na^+-dependent transport of P_i [3]. We reported previously [4] that the ADP-ribosyltransferase in renal brush-border membranes was markedly stimulated by EDTA in the concentration range 0.3–20.0 mM, with half-maximal stimulation of 19-fold at 1.0 mM EDTA. A mechanism which may account, in part, for this stimulatory action of EDTA has been elucidated and is described in this report.

In order to rupture sealed membrane vesicles, the brush-border membranes used in these studies were frozen at −20°C and, after thawing, were incubated in hypotonic reaction mixtures. Brush-border membranes were prepared from rat renal cortex by the calcium precipitation procedure [1,2,4,5]. The purity of the membrane preparations, assessed by measurement of marker enzymes, was comparable to that reported in previous studies on rats [1,4,5]. The isolated membranes were resuspended in 300 mM mannitol, 5 mM Tris (pH adjusted to 8.0 with Hepes), and were stored frozen at −20°C for up to two weeks. ADP-ribosyltransferase activity in renal brush-border membranes was assayed by a modification of the original procedure [4]. Samples of membrane suspensions were preincubated for 20 min at 37°C in 5 mM Tris-Hepes, pH 8.0, containing 1 mM $MgCl_2$ and the appropriate test agent(s). The reaction was initiated by addition of [*adenylate*-^{32}P]NAD^+ to a final concentration of 0.48 mM [4] and, unless stated otherwise, the standard incubation time was 20 min at 37°C. In the presence of 5 mM EDTA the reaction rate was linear in the range 0.04–0.16

Abbreviations: p[NH]ppG, guanosine 5′-[β,γ-imino]triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

mg brush-border membrane protein. The final volume of the incubation medium was 0.2 ml and the reaction was terminated by addition of 2.0 ml of ice-cold 10% (w/v) trichloroacetic acid. After centrifugation at 6000 × *g* for 10 min the supernatant was removed by aspiration and the acid-insoluble pellet was washed three times by resuspension in 10% (w/v) trichloroacetic acid, using brief (5 s) sonication to ensure complete dispersion of the pellet, followed by centrifugation. The final pellet was solubilized in 0.1 M NaOH for liquid scintillation counting. As in previous studies [4], trapping of radioactivity in the pellets was assessed in control incubations in which trichloroacetic acid was added to the reaction mixture prior to addition of [*adenylate*-^{32}P]NAD$^+$. The activity of ADP-ribosyltransferase was calculated after correcting the experimental values by subtraction of radioactivity incorporated in the controls. All other procedures were as described previously [1,4,5].

In this series of studies, the presence of EDTA at 5 mM stimulated the activity of ADP-ribosyltransferase in renal brush-border membranes by 33-fold (Table I) in close agreement to what was observed previously [4]. At both 20°C and 37°C, the EDTA-stimulated ADP-ribosylation of brush-border membrane increased linearly with incubation times up to 40 min (Fig. 1). The temperature dependence of the reaction is illustrated by the finding of higher activity at 37°C compared to 20°C for all the tested times (Fig. 1). On the basis of these observations an incubation time of 20 min at 37°C was chosen for all future studies.

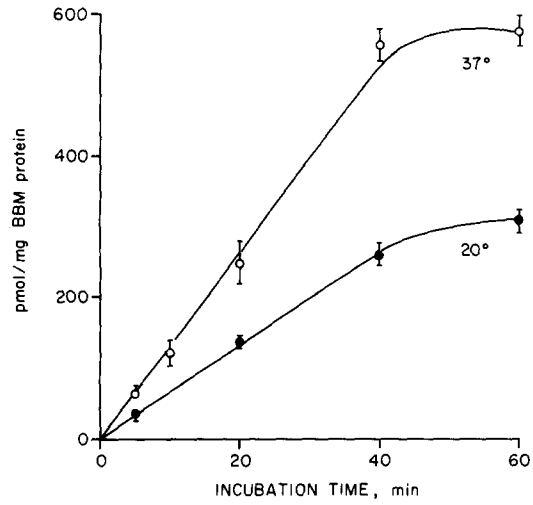

Fig. 1. Time and temperature dependence of ADP-ribosyltransferase activity in isolated renal brush-border membrane (BBM). The incubation medium contained 5 mM EDTA and 0.12 mg membrane protein. See text for full details. The data are the mean ± S.E. of measurements in triplicate.

Additional evidence for the presence of ADP-ribosyltransferase in renal brush-border membranes is provided by the observation that ADP-ribosylation was inhibited in a concentration dependent manner by arginine methyl ester (Fig. 2), a known inhibitor of mono-ADP-ribosylation re-

TABLE I

EFFECT OF VARIOUS AGENTS ON THE ACTIVITY OF ADP-RIBOSYLTRANSFERASE IN ISOLATED RENAL BRUSH-BORDER MEMBRANES

Results are the mean ± S.E. of three or four experiments.

Test agents (final concentrations)	ADP-ribosyltransferase (pmol/mg protein per h)
None	36 ± 5
5 mM EDTA	1183 ± 99
5 mM EDTA + 0.3 M arginine methyl ester	261 ± 8 [a]
5 mM EDTA + 0.3 M lysine	177 ± 7 [a]
5 mM EDTA + 0.3 M histidine	235 ± 10 [a]
5 mM p[NH]ppG	248 ± 10
5 mM p[NH]ppG + 5 mM EDTA	558 ± 16 [a]

[a] Significantly different compared to the activity in the presence of EDTA alone ($P < 0.01$, group *t*-test).

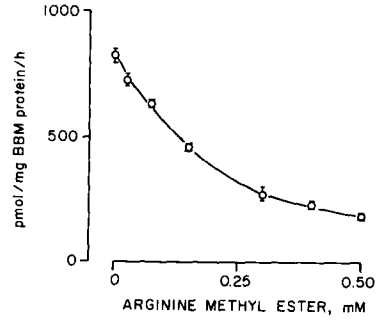

Fig. 2. Inhibition of ADP-ribosyltransferase activity in renal brush-border membranes (BBM) by arginine methyl ester. The incubation medium contained 5 mM EDTA, 0.15 mg membrane protein, and the indicated concentrations of arginine methyl ester. The data are the mean ± S.E. of measurements in triplicate.

actions [6–8]. Arginine methyl ester (and arginine) inhibits ADP-ribosylation of acceptors because it serves as an alternative acceptor and competes for the ADP-ribose liberated by the transferase [6,7]. This had led to the suggestion that certain ADP-ribosyltransferases specifically catalyze ADP-ribosylation of guanidium groups [6,8]. The brush-border transferase may be less specific in its requirement for acceptors since both lysine and histidine, which lack a guanidinium group, are as effective as arginine methyl ester in inhibiting ADP-ribosylation of brush-border membranes (Table I).

The marked stimulation of brush-border ADP-ribosyltransferase by EDTA is unlikely due to removal of inhibitory cations by chelation with EDTA. EDTA stimulates the transferase at concentrations up to 18 mM [4], which is far in excess of what would be required to remove any traces of Ca^{2+} remaining from the membrane isolation procedure. Further, we showed previously [4] that the enzyme activity is not affected by Mg^{2+}. An alternative explanation is suggested by the recent reports [9,10] that NAD^+ is degraded during incubation with isolated brush-border membranes, and that the presence of EDTA inhibits this process and maintains the NAD^+ intact [9]. EDTA, by maintaining the supply of intact NAD^+, may stimulate indirectly the brush-border ADP-ribosyltransferase activity because only intact NAD^+ can serve as a substrate for ADP-ribosyltransferases [4]. When the effect of EDTA on the rate of NAD^+ degradation by isolated membranes was tested, the results confirm that EDTA inhibits this process (Fig. 3). The inhibition is concentration dependent and half-maximal inhibition occurs at 1.0 mM (Fig. 3). This concentration of EDTA also causes half-maximal stimulation of the ADP-ribosyltransferase [4].

Additional support for the suggestion that EDTA acts primarily by maintaining substrate availability is provided by studies with p[NH]ppG. The experiments were initiated because GTP may activate certain ADP-ribosylation reactions [11,12]. p[NH]ppG, a non-hydrolysable analog of GTP, stimulated ADP-ribosylation of brush-border membranes, and the stimulation was concentration dependent over the range 1–50 mM with a maximal increase of almost 8-fold (Fig. 4). Although

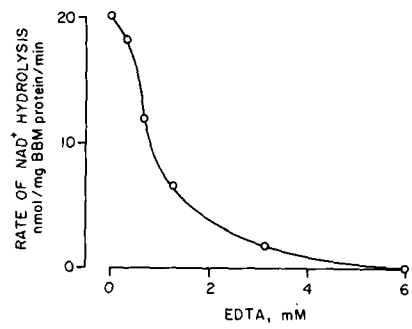

Fig. 3. Effect of EDTA on rate of disappearance of NAD^+ during incubation with renal brush-border membranes. The incubation medium was 50 mM sodium phosphate buffer (pH 8.0) containing 0.32 mg membrane protein, 0.4 mM NAD^+, and EDTA at the indicated concentrations. After 10 min at 37°C the reaction was terminated by addition of ice-cold perchloric acid to a final concentration of 4% (w/v), followed by centrifugation for 5 min at 13000 × *g*. The supernatant was removed and the pH was adjusted to 7.0 by addition of 1 M K_2HPO_4 and 3 M KOH [1]. After centrifugation as before, the amount of NAD^+ in the neutalized supernatant was determined by fluorescence measurement after reduction to NADH by alcohol dehydrogenase [1]. Total hydrolysis of NAD^+ at each EDTA concentration was determined by comparison with the amount of NAD^+ present in blanks in which perchloric acid was added to the reaction mixture prior to addition of NAD^+. Preliminary experiments established that the rate of NAD^+ hydrolysis was linear with respect to both time and protein. The results are the mean values from two experiments.

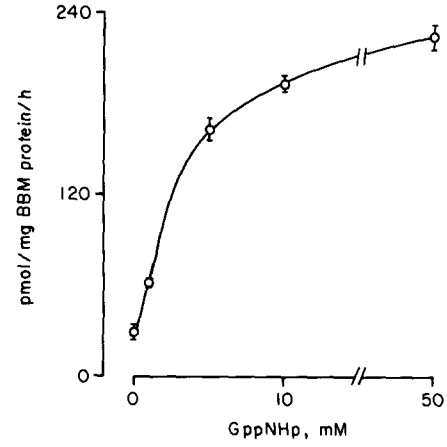

Fig. 4. Stimulation of ADP-ribosyltransferase activity in renal brush-border membranes (BBM) by guanosine 5′-[β,γ-imino]-triphosphate, p[NH]ppG. The incubation medium contained 0.16 mg membrane protein and the indicated concentrations of p[NH]ppG. The data are the mean ± S.E. of measurements in triplicate.

millimolar levels of GTP stimulate slightly the erythrocyte transferase [12], these concentrations are far in excess of that required to activate ADP-ribosylation catalyzed by cholera toxin [11]. This suggests that the p[NH]ppG-induced stimulation of the brush-border transferase may be due to non-specific effects of high concentrations of p[NH]ppG. When EDTA and p[NH]ppG were tested in combination, at concentrations producing sub-maximal stimulation, the stimulatory effects on brush border ADP-ribosylation were not additive (Table I) indicating that a common mechanism may be involved. On the basis of these results we determined whether p[NH]ppG, like EDTA, inhibited NAD^+ degradation by isolated membranes, using the procedure described in the legend to Fig. 3. In the absence of p[NH]ppG the rate of disappearance of NAD^+ during incubation with brush-border membranes was 27.6 ± 0.4 nmol/mg membrane protein per min, compared to 8.7 ± 0.5 (mean $\pm$ S.E.) nmol/mg membrane protein per min in the presence of 10 mM p[NH]ppG. This represents 68% inhibition and was the maximum achieved over the concentration range (1–50 mM) tested. These observations strongly suggest that p[NH]ppG, at these relatively high concentrations, stimulates the brush-border ADP-ribosyltransferase primarily by maintaining the availability of intact NAD^+ substrate.

In conclusion, the previously reported stimulation of brush-border ADP-ribosyltransferase by EDTA has been confirmed. It is suggested that this stimulation occurs primarily because EDTA inhibits NAD^+ breakdown by other brush-border enzymes. The stimulation is not a specific property of EDTA, the studies with p[NH]ppG suggest that any maneuver which suppresses NAD degradation may produce increased ADP-ribosylation of the brush-border membrane. This may have important implications for the control, albeit indirect, of ADP-ribosylation of brush-border membranes in vivo. Finally, the rapid rate of NAD^+ degradation by isolated membranes may contribute to the apparent absence of ADP-ribosyltransferase activity reported recently for rabbit brush border membranes [13].

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