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NADP efficiently inhibits endogenous but not pertussis toxin-catalyzed covalent modification of membrane proteins incubated with NAD

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Incubation of membranes of human erythrocytes and platelets but not of human neutrophils with [^{32}P]NAD leads to covalent modification of various membrane proteins and of added albumin. In membranes of all three cell types, pertussis toxin (PT), in the presence of NAD, specifically labelled a 40 kDa peptide, i.e. the α-subunit of a guanine nucleotide-binding protein. This effect of PT was slightly reduced by NADP, whereas modification of other membrane proteins and of albumin was largely suppressed, independent of whether PT was present or not. Labelling of cytosolic proteins in the presence of NAD was marginal; only in neutrophil cytosol, PT modified a 40 kDa peptide. Membranes of erythrocytes and platelets exhibited NAD-degrading activity, which was inhibited by NADP. The data suggest a high substrate specificity of PT for NAD. Inhibition of endogenous enzymes by NADP may prove useful for the evaluation of PT substrates.

Pertussis toxin; ADP-ribosylation; Guanine nucleotide-binding protein; NADP

1. INTRODUCTION

PT causes covalent modification of peptides of about 40 kDa in membranes of almost all eukaryotic cell types studied including PMNs, RBCs and platelets, presumably by catalyzing ADP-ribosylation of these peptides with NAD being used as a substrate [1]. The peptides modified represent the α-subunits of N-proteins, which are involved in signal transduction across plasma membranes by acting as transducers between cell surface receptors and intracellular messenger-generating enzymes [2] or ion channels [3–6]. In membrane preparations from most tissues including membranes from RBCs and platelets, the PT substrate is most likely identical with the α-subunit of an N-protein termed N_i, which functionally couples inhibitory receptors to adenylyl cyclase [7,8]. In PMN membranes, the main target of PT appears to be the α-subunit of a cell type specific N-protein (N_{PMN} [9,10]), which confers activation of the receptors for chemotactic factors (e.g. formylated peptides, leukotriene B and platelet-activating factor) to various enzymes including phospholipase C [11] and NADPH oxidase [12]. In many tissues, incubation of membrane preparations with [^{32}P]NAD leads to incorporation of radioactivity into a number of peptides. This endogenous activity of membranes blunts the effect of added toxin. Longabaugh et al. [13] recently reported that the efficiency of CT to

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Abbreviations: PMNs, polymorphonuclear neutrophils; RBCs, red blood cells; PT, pertussis toxin; CT, cholera toxin; N-protein, guanine nucleotide-binding protein; BSA, bovine serum albumin (Cohn fraction V); SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography

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ADP-ribosylate the stimulatory N-protein of the adenylyl cyclase complex (N_s) in liver and heart membranes was improved by NADP, which slowed down the degradation of NAD in the reaction mixture but did not compete with NAD for CT-catalyzed ADP-ribosylation of N_s. Using various human blood cells, we provide evidence that in the presence of NAD, endogenous modification of membrane proteins but not of cytosolic proteins is efficiently suppressed by NADP, whereas PT-catalyzed modification of N-protein α-subunits is only slightly inhibited by NADP at high concentrations.

2. MATERIALS AND METHODS

Outdated human erythrocytes, platelet-rich plasma and buffy coat (as a source for PMNs) were provided by the local blood bank. PT was a generous gift from Dr M. Yajima, Kyoto, Japan. NADP was from Boehringer (Mannheim). Sources of other materials and reagents have been described [14].

Plasma membranes and cytosol of PMNs were prepared as in [12]. For complete removal of membranes, the cytosol was recentrifuged for 2 h at 200000 × *g*. Membranes from RBCs were prepared as in [15]. For the preparation of cytosol, isolated RBCs were repeatedly frozen in liquid nitrogen. After centrifugation at 200000 × *g* for 2 h, the supernatant fluid was applied to a DEAE-Sephacel column. Hemoglobin, which did not bind to the resin, was removed by extensive washing. Bound proteins were eluted by 1 M NaCl and concentrated by ultrafiltration, using PM 10 filters (Amicon, Witten, FRG); this preparation is referred to as 'cytosol'. Platelets were isolated as described [16] and disrupted by repeated freezing in liquid nitrogen. Particulate and soluble fractions were separated by 200000 × *g* centrifugation (2 h) and constitute the membraneous and cytosolic preparations, respectively.

PT-catalyzed [^{32}P]ADP-ribosylation was performed as in [17]. Preactivated PT was diluted with BSA-containing buffer to a final concentration of 1.7 μg/ml. Control samples received dilution buffer without the toxin. BSA was 10 μg per assay tube (assay volume 60 μl), and the final NAD concentration was 1 μM with 1–2 μCi [^{32}P]NAD per assay tube. Incubation was for 30 min at 30°C. Variations in the reaction mixture from the basic protocol are given in the figure legends. Prior to SDS-PAGE, proteins were precipitated with acetone and the pellets subsequently washed with trichloroacetic acid and chloroform. SDS-PAGE was performed according to Laemmli [18] as described elsewhere [14]. TLC of nucleotides was performed on polyethyleneimine sheets (Schleicher and Schüll, Dassel, FRG), using 0.1 M LiCl/0.5 M formic acid as the mobile phase [19]. Autoradiography of dried polyacrylamide gels and TLC sheets was performed as described [14]. In some experiments, radiolabelled bands of dried gels were cut out and the radioactivity determined by liquid scintillation counting. [α-^{32}P]ATP was synthesized according to Johnson and Walseth [20] and [^{32}P]NAD as described by Cassel and Pfeuffer [19]. Protein was assayed according to Lowry et al. [21], using BSA as a standard. Results of the experiments shown were typical for those obtained in at least two repeated experiments.

3. RESULTS

In the absence of NADP and PT, there was significant labelling of a number of proteins in

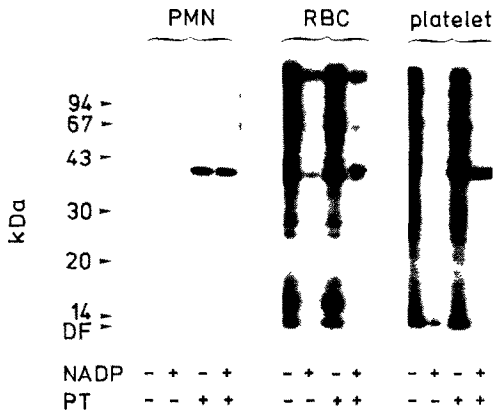

Fig.1. Influence of NADP on endogenous and PT-catalyzed labelling of membrane proteins incubated with [^{32}P]NAD. Membranes of the indicated cell types were incubated with [^{32}P]NAD in the absence and presence of NADP (0.5 mM) and PT. Different amounts of membrane protein were chosen for each cell type to achieve comparable incorporation of radioactivity into the peptides of 40 kDa in the presence of PT (PMNs, 10 μg protein; RBCs, 150 μg; platelets, 100 μg). Shown are autoradiographs of SDS-polyacrylamide gels (12.5%). DF, dye front.

platelet and RBC membranes but not in PMN membranes incubated with [^{32}P]NAD (fig.1). The labelled peptides included one of 67 kDa, most likely representing BSA, a constituent of the reaction mixture and known to be labelled in the presence of [^{32}P]NAD [22]. Endogenous labelling of proteins was strongest in RBC membranes and included peptides with apparent molecular masses larger than 94 kDa (among them presumably spectrin) and a peptide of 39 kDa. The endogenous activity of platelet and RBC membranes was efficiently inhibited by NADP (0.5 mM). In all membrane preparations, PT specifically radiolabelled peptides of about 40 kDa, which are believed to represent the α-subunits of N-proteins. This activity of PT was inhibited very little by NADP. Fig.2 shows a concentration-response curve for NADP. In membranes of platelets and RBCs, NADP at concentrations above 30 μM inhibited labelling of various peptides including those of large apparent molecular masses (>94 kDa) and peptides of 67 kDa; in addition, labelling of peptides of small apparent molecular masses (<20 kDa) and a peptide of 39 kDa was reduced by NADP in RBC membranes; only at concentrations of NADP above 0.3 mM was incorporation into α-subunits slightly reduced in all tested tissues. When increasing amounts of membrane proteins of platelets and RBCs were incubated with PT and [^{32}P]NAD but without NADP, increased incorporation of radioactivity into the α-subunits and other peptides including BSA, which was present at a constant concentration, and into material that moved with the dye front was observed (fig.3). In contrast, only the incorporation into α-subunits paralleled the amount of PMN membrane protein. Labelling of peptides other than the α-subunits was efficiently suppressed by NADP. On the other hand, labelling of α-subunits was only moderately reduced in all tissues and at all amounts of membrane protein employed.

In the soluble fraction of RBCs, very little endogenous activity was detected (fig.4). In contrast, peptides of 39 and 67 kDa were labelled in the absence of PT in cytosol of PMNs and platelets, respectively. The endogenous activity was little affected by NADP at 0.5 mM. Only in PMN cytosol

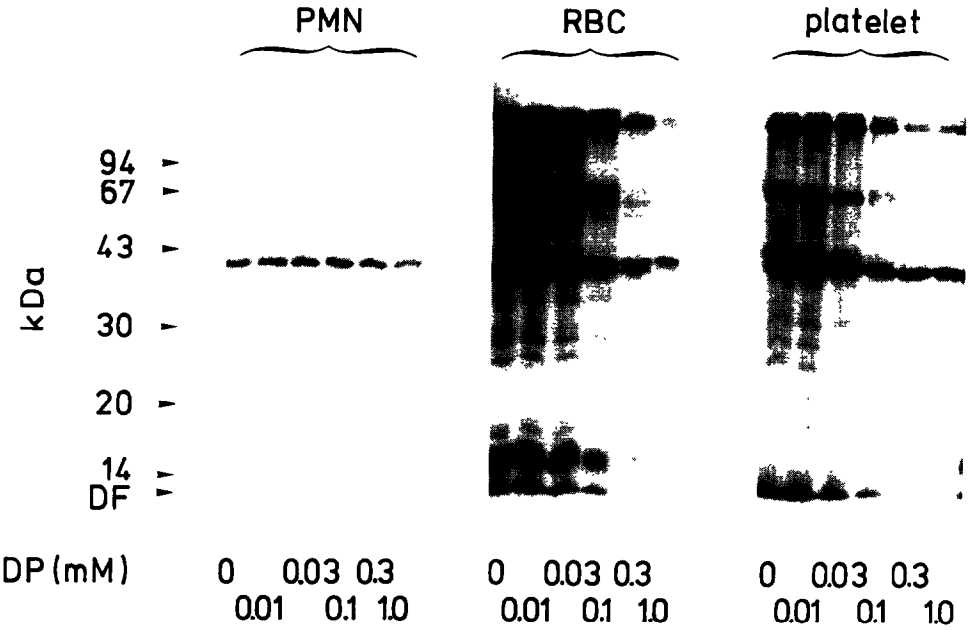

Fig.2. NADP-induced inhibition of covalent modification of membrane proteins incubated with [^{32}P]NAD. Experimental conditions were as described for fig.1, with the exception that all samples received PT and NADP at the indicated concentrations.

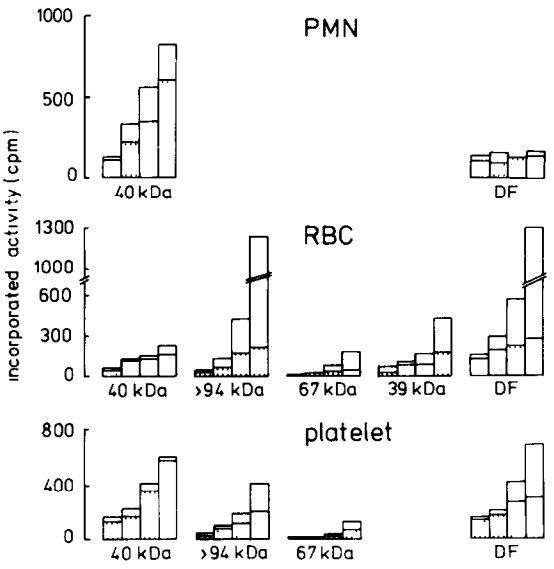

Fig.3. Influence of the amount of membrane protein on endogenous and PT-catalyzed protein modification in the presence of [^{32}P]NAD. Particulate fractions of PMNs, RBCs or platelets were incubated with PT and 1 μM [^{32}P]NAD (about 2 μCi per assay tube) in the absence or presence of NADP (0.5 mM) and subjected to SDS-PAGE. Labelled peptides were identified by autoradiography, excised, and the incorporated activity determined. The amounts of protein per assay tube were 1.3, 2.5, 5 and 10 μg for PMN membranes, 19, 38, 75 and 150 μg for RBC membranes, and 12.5, 25, 50 and 100 μg for platelet membranes (bars from left to right for the indicated molecular masses). Total bars and dotted parts of bars indicate incorporation of radioactivity in the absence and presence of NADP, respectively. In PMN membranes, the activity incorporated into regions other than the 40 kDa region and the dye front was not higher than background activity.

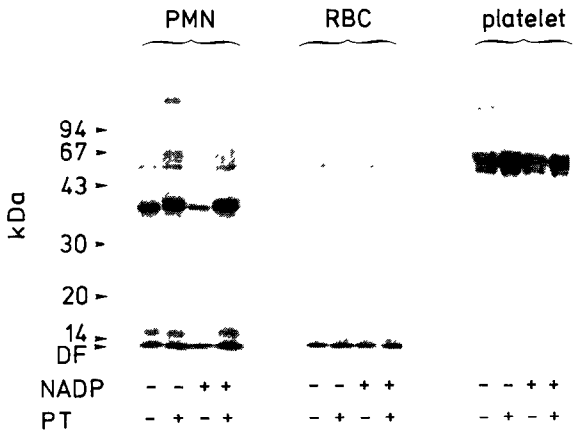

Fig.4. Influence of NADP on endogenous and PT-catalyzed covalent modification of cytosolic proteins incubated with [^{32}P]NAD. Experimental conditions as in fig.1. The amounts of protein employed were 9 μg (PMNs), 11 μg (RBCs) and 12 μg (platelets).

was a PT substrate of 40 kDa clearly detectable. By two-dimensional gel electrophoresis and tryptic peptide maps, this peptide was identical with one of the two PT substrates of PMN plasma membranes, which have indistinguishable apparent molecular masses but differ in charge (unpublished); this cytosolic substrate is, therefore, likely to represent the α-subunit of an N-protein. PT slightly stimulated radiolabelling of peptides of 39 and 67 kDa in PMN and platelet cytosol, respectively. The significance of this PT effect is not clear. In PMN cytosol, the PT-induced incorporation of radioactivity into the peptide of 39 kDa was not or marginally stimulated by the $\beta\gamma$ complex of N-proteins, whereas the incorporation of radioactivity into the 40 kDa peptide was increased more than 10-fold (not shown). This indicates that the peptide of 39 kDa does not interact with the $\beta\gamma$ complex of N-proteins and, therefore, is not related to an N-protein α-subunit.

Incubation of particulate and soluble fractions of the three cell types with [^{32}P]NAD and PT revealed that membranes of erythrocytes and to a lesser extent membranes of platelets contain NAD-degrading enzymes with the major degradation product being ADP-ribose (fig.5); another degradation product exhibited the mobility of P_i. Degradation of NAD was not detectable in PMN membranes and in soluble fractions of the three tested cell types. The NAD degradation in membranes of RBCs and platelets was inhibited by NADP at 0.5 mM. At NADP concentrations lower than 0.1 mM, degradation of NAD was still detectable although reduced in both tissues when protein amounts of 150 μg (RBCs) and 100 μg (platelets) were employed (not shown).

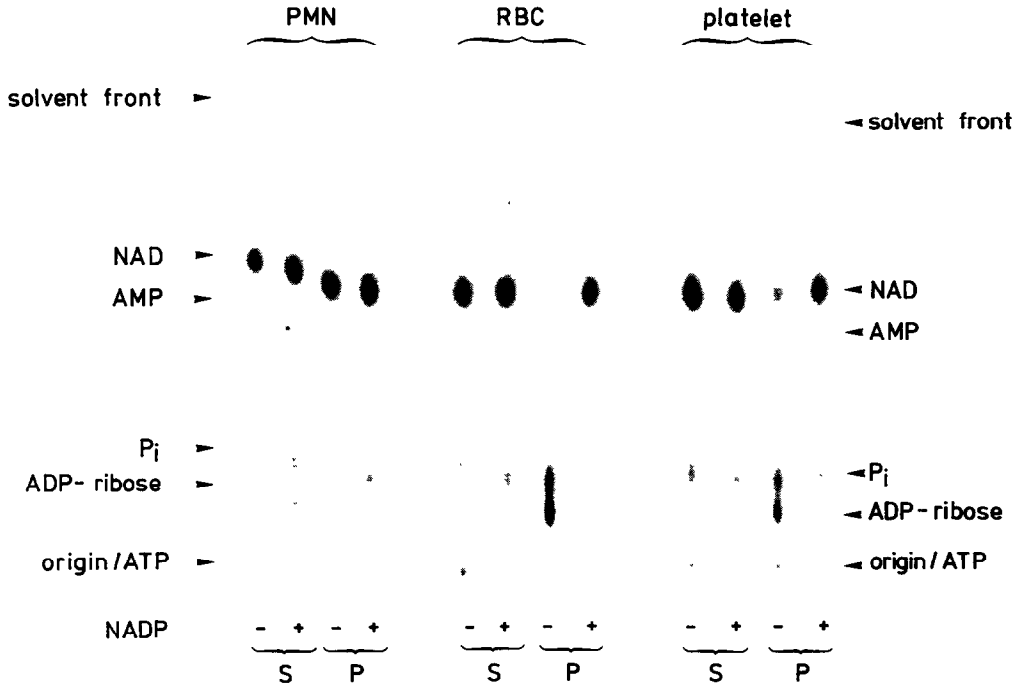

Fig.5. Degradation of [^{32}P]NAD by soluble and particulate fractions of human blood cells in the absence and presence of NADP. The amounts of protein per assay tube were as those in figs 1,4. The various preparations were incubated with PT and [^{32}P]NAD as described in section 2; NADP was 0.5 mM. The incubation was stopped by the addition of 15 μl of 100% trichloroacetic acid. After centrifugation (10000 × g, 30 min at 4°C), 2 μl of the supernatant were diluted 25-fold with 50 mM imidazole buffer (pH 7.0). 1 μl of the diluted supernatants was applied to a TLC sheet. Shown are autoradiographs of developed TLC sheets. PT, cytosolic fractions of all tested tissues and PMN membranes did not possess NAD-degrading activity to an extent that was detectable by TLC. Non-radioactive nucleotides were used as markers and localized by fluorescence. The mobility of phosphate was assumed to be identical with that of the labelled degradation product of boiled [γ-^{32}P]ATP. The fast moving radioactive compound close to the solvent front was not attributable to the markers shown, NADH or NADP. S, soluble fraction; P, particulate fraction.

4. DISCUSSION

The reported data indicate that membrane preparations of RBCs and platelets but not of PMNs contain an activity responsible for covalent modification of various peptides in the presence of NAD. This endogenous activity blunts the effect of exogenous ADP-ribosyltransferases such as PT or CT. This is particularly obvious in membranes of RBCs, where in the presence of [^{32}P]NAD, a number of peptides including one of 39 kDa incorporate activity independently of PT. In all cell types tested, the toxin specifically modifies peptides with an apparent molecular mass of 40 kDa, i.e. the α-subunits of N-proteins.

The coincidence of the inhibitory effect of NADP on protein modification in particulate fractions incubated with NAD on the one hand, and of the inhibitory effect of NADP on NAD degradation on the other, suggest a relationship between the two events. We assume that endogenous NAD glycohydrolases are, at least in part, responsible for NAD degradation, as the major NAD degradation product in RBCs and platelets appears to be ADP-ribose. In analogy to the bacterial toxins, these enzymes may also catalyze ADP-ribosylation of proteins by an inherent ADP-ribosyltransferase activity; alternatively, ADP-ribosylation may occur nonenzymatically once ADP-ribose has been formed [22]. The endogenous NAD-degrading en-

zymes probably utilize NADP besides NAD, as has been reported for microsomal NAD glycohydrolases of Ehrlich ascites cells [23]. The accumulation of phosphate in the NAD-degrading membranes is difficult to explain. The finding that NADP, which presumably inhibits enzymatic breakdown of NAD, also inhibits phosphate accumulation is consistent with the idea that phosphate is derived from an enzymatically formed NAD-degradation product.

The degradation of NAD to ADP-ribose and nicotinamide cannot be accounted for by the NAD glycohydrolase activity of PT [24], as there was no detectable degradation of NAD by PT in the absence or presence of PMN membranes or soluble fractions. In contrast to the endogenous enzymes, the specificity of PT for NAD as its substrate is exceptionally high and its ability to ADP-ribosylate α-subunits is little reduced by a several hundredfold excess of NADP over NAD. The inhibition of PT-catalyzed ADP-ribosylation, which amounted to up to 27% in plasma membranes of PMNs, may be due either to NAD, contaminating the commercially available NADP, or to the fact that NADP is a low-affinity substrate for PT.

The endogenous activity that modifies proteins in the presence of NAD differs from tissue to tissue and is much higher in particulate than soluble fractions. The enzymes responsible for this activity appear to be very active in membranes of RBCs and less active in a crude particulate fraction of platelets; they are not detectable in plasma membranes from PMNs. Whether these differences are due to differences in enzyme activity or substrate availability is not known, although the latter assumption is less likely as labelling of the exogenous substrate, BSA, increased with the amount of membrane protein employed. In any case, the use of NADP as a constituent in assays designed for identification and characterization of PT substrates, i.e. the α-subunits of N-proteins, improves the specific signal considerably. Besides its usefulness for the autoradiographic evaluation of SDS-polyacrylamide gels, the use of NADP will most likely also improve the specificity of the filter assay method [25] and the polyethylene glycol precipitation method [26] for purified PT substrates by inhibiting PT-independent labelling of contaminating proteins and of exogenous proteins that are introduced into the reaction mixture. Finally, it may open up the possibility of using assays based on protein adsorption or protein precipitation for qualitative and quantitative evaluation of PT substrates in membranes and crude membranous extracts.

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