Vol. 156, No. 1, 1988
October 14, 1988

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 361-367

DIFFERENT TYPES OF ADP-RIBOSE PROTEIN BONDS FORMED BY BOTULINUM C2 TOXIN, BOTULINUM ADP-RIBOSYLTRANSFERASE C3 AND PERTUSSIS TOXIN

Klaus Aktories[1], Ingo Just[1] and Walter Rosenthal[2]

[1] Rudolf-Buchheim-Institut für Pharmakologie der Justus-Liebig Universität Giessen, Frankfurter Str. 107, D-6300 Giessen, F.R.G.

[2] Institut für Pharmakologie, Freie Universität Berlin, Thielallee 69/73, D-1000 Berlin 33, F.R.G.

Received September 9, 1988

SUMMARY: We attempted to characterize ADP-ribose-amino acid bonds formed by various bacterial toxins. The ADP-ribose-arginine bond formed by botulinum C2 toxin in actin was cleaved with a half-life of about 2 h by treatment with hydroxylamine (0.5 M). In contrast, the ADP-ribose-cysteine bond formed by pertussis toxin in transducin and the ADP-ribose-amino acid linkage formed by botulinum ADP-ribosyltransferase C3 in platelet cytosolic proteins were not affected by hydroxylamine. $HgCl_2$ cleaved the ADP-ribose-amino acid bond formed by pertussis toxin in transducin but not those formed by botulinum C2 toxin or botulinum ADP-ribosyltransferase C3 in actin and platelet cytosolic proteins, respectively. NaOH (0.5 M) cleaved the ADP-ribose-amino acid bonds formed by botulinum C2 toxin and pertussis toxin but not the one formed by botulinum ADP-ribosyltransferase C3. The data indicate that the ADP-ribose bond formed by botulinum ADP-ribosyltransferase C3 differs from those formed by the known bacterial ADP-ribosylating toxins.

Various bacterial toxins such as diphtheria, cholera and pertussis toxin affect the metabolism of the eukaryotic cell by ADP-ribosylation of proteins [1]. These ADP-ribosyltransferases differ in their protein substrate specificity and with respect to the amino acid residue modified by the respective toxin. While diphtheria toxin ADP-ribosylates diphthamide [2], a posttranslationally modified histidine, cholera toxin ADP-ribosylates arginine residues [3], and pertussis toxin modifies cysteine residues [4].

0006-291X/88 $1.50

Recently, it was shown that Clostridium botulinum type C produces two distinct ADP-ribosyltransferases, botulinum C2 toxin and botulinum ADP-ribosyltransferase C3 [5-7]. Whereas botulinum C2 toxin ADP-ribosylates actin in arginine-177 [8], the substrate for botulinum ADP-ribosyltransferase C3 appears to be a GTP-binding protein [9]; the ADP-ribose-amino acid bond of this protein has not yet been characterized.

Hilz and coworkers showed that ADP-ribose-protein bonds can be characterized by their stability towards neutral hydroxylamine [10], dilute alkali [10] and mercury ions [11]. The mono-ADP-ribosylation of a glutamate residue catalyzed by poly-ADP-ribose synthase is characterized by its instability towards neutral hydroxylamine or dilute alkali [10,18]. This type of ADP-ribose bond was, therefore, called hydroxylamine-"labile" (half-life 3-10 min). The ADP-ribose-arginine bond formed by mono-ADP-ribosyltransferase purified from turkey erythrocytes is reportedly more stable against hydroxylamine [19]. Similarly, the ADP-ribose-arginine bond formed by cholera toxin in G-proteins, G_s and transducin, has a half-life of 1-2 h in the presence of hydroxylamine [20].

We studied the stability of the ADP-ribose protein bond formed by botulinum C2 toxin, botulinum ADP-ribosyltransferase C3 and pertussis toxin and report here that the ADP-ribosylation catalyzed by botulinum ADP-ribosyltransferase C3 is more stable than the ADP-ribose-arginine bonds formed by botulinum C2 toxin or the ADP-ribose-cysteine bond formed by pertussis toxin.

MATERIALS AND METHODS

Materials

Botulinum C2 toxin and botulinum ADP-ribosyltransferase C3 were purified as described [12,13]. Transducin was prepared according to Kühn [14]. Pertussis toxin was a gift of Dr. M. Yajima (Kobe, Japan). [^{32}P]NAD was prepared according to Cassel and Pfeuffer [15]. Hydroxylamine-HCl was obtained from Sigma (Deisenhofen, FRG) and $HgCl_2$ from Merck (Darmstadt, FRG). All other reagents were from commercial sources.

Preparation of human platelet membranes and cytosol

Platelets were isolated as described [16] and lysed by freezing and thawing in a hypotonic medium containing 10 mM triethanolamine/HCl (pH 7.5) and 5 mM EDTA. Platelet membranes and cytosol were obtained by centrifugation of the lysate for 15 min at 30.000 xg and kept at -20°C until use.

N-ethylmaleimide treatment of platelet membranes and cytosol

Membranes and cytosol of human platelets were incubated with the indicated concentrations of N-ethylmaleimide for 15 min at 25° C. After termination of the reaction by addition of dithiothreitol (10 mM, final concentration), membranes and cytosol were immediately used in the ADP-ribosylation assay.

ADP-ribosylation assay

ADP-ribosylation was carried out in a medium containing 10 mM thymidine, 0.5 mM ATP, 6 mM $MgCl_2$, 0.1 µM [^{32}P]NAD (about 1 µCi/tube), ADP-ribosylating toxins at the indicated concentrations and 50 mM triethanolamine/HCl (pH 7.5) with about 100-300 µg of platelet membranous or cytosolic protein or 1 µg transducin in a total volume of 200 µl. ADP-ribosylation with pertussis toxin (activated by 20 mM dithiothreitol) was performed in the presence of 0.01% Lubrol PX. After 30 min of incubation at 37° C, reactions were terminated by the addition of 2% SDS and 100 mM HEPES, pH 7.5 (final concentrations each).

Studies on the stability of ADP-ribose-protein bonds with hydroxylamine, NaOH and $HgCl_2$

After termination of the ADP-ribosylation with SDS/HEPES, 50 µl aliquots of the reaction mixture were added to the same volume of 2 mM or 1 M NaCl, 1 M NH_2OH (pH 7.5), 1 N NaOH or 2 mM $HgCl_2$, and incubated for the indicated periods of time at 37° C or as indicated. Thereafter, proteins were precipitated by addition of trichloroacetic acid (20%, final concentration). The formed pellet was washed with diethylether and dissolved in 50 µl of electrophoresis buffer. SDS-PAGE was performed according to Laemmli [17]. Gels were stained with Coomassie Blue, destained and subjected to autoradiography for 24-48 h.

RESULTS AND DISCUSSION

The stability of ADP-ribose bonds formed by botulinum C2 toxin, botulinum ADP-ribosyltransferase C3 and pertussis toxin were studied under denaturing conditions in order to prevent any shielding effects of the native proteins. As is shown in Fig. 1A, in the presence of 0.5 M hydroxylamine (pH 7.5) ^{32}P-ADP-ribose-arginine residues formed by botulinum C2 toxin in actin were degraded by about 50% after an incubabation of 2 h. A similar stability has been reported for ADP-ribose-arginine bonds formed by endogenous ADP-ribosyltransferases and cholera toxin [19,20]. The ADP-ribose-amino acid bond formed by botulinum ADP-ribosyltransferase C3 was not affected by hydroxylamine. In accordance with a previous report [20], pertussis toxin-catalyzed ADP-ribosylation of a cysteine residue in transducin was stable towards treatment with hydroxylamine (Fig. 1B). It was recently reported that the substrate of botulinum ADP-ribosyltransferase C3 is

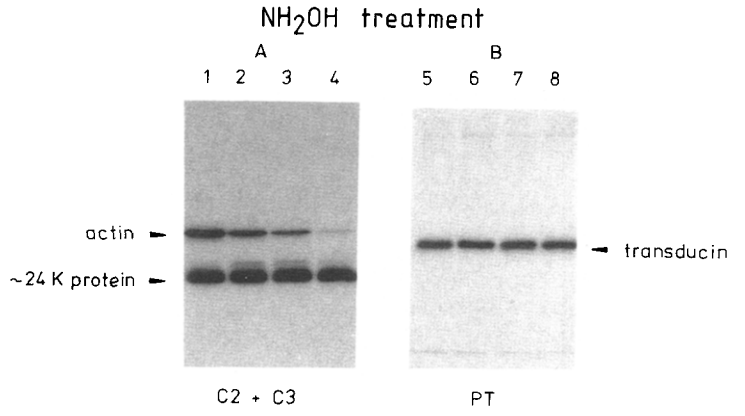
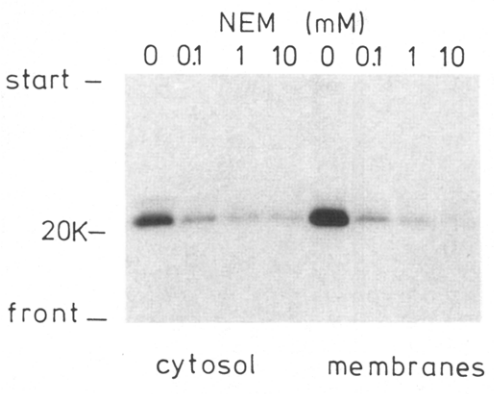
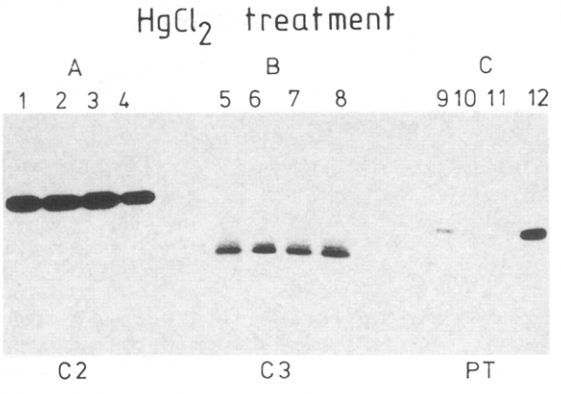


Fig. 1: Stabilities towards NH_2OH of ^{32}P-ADP-ribose-amino acid bonds formed by botulinum C2 toxin, botulinum ADP-ribosyltransferase C3 and pertussis toxin. Platelet cytosol (A) was incubated with [^{32}P]NAD and botulinum C2 toxin (C2, 0.1 µg/ml) and botulinum ADP-ribosyltransferase C3 (C3, 3 µg/ml); purified transducin (B) was incubated with [^{32}P]NAD and pertussis toxin (PT, 20 µg/ml). Thereafter, samples were treated with 0.5 M NaCl for 180 min (lanes 1, 5) or with 0.5 M NH_2OH for 30 min (lanes 2, 6) 60 min (lanes 3, 7), 120 min (lane 8) or 180 min (lane 4). SDS PAGE of samples was performed (15% acrylamide in A, 11% acrylamide in B), and ^{32}P-ADP-ribosylated peptides were analyzed by autoradiography. The M_r values of radiolabelled peptides were 43 kDa (corresponding to the M_r of actin), 22-24 kDa (corresponding to the M_r of GTP-binding proteins ADP-ribosylated by botulinum ADP-ribosyltransferase C3) and 39 kDa (corresponding to the M_r of the α-subunit of transducin) in panels A and B, respectively.

sensitive towards the sulfhydryl-group-alkylating reagent, N-ethylmaleimide, employed at a high concentration (200 mM) [21]. In platelet membranes and cytosol, ADP-ribosylation of 21-24 kDa proteins by ADP-ribosyltransferase C3 was inhibited by N-ethylmaleimide at a concentration as low as 100 µM (Fig. 2) Since pertussis toxin-induced ADP-ribosylation of cysteine residues is also prevented by N-ethylmaleimide at low concentrations [22], one might assume that ADP-ribosyltransferase C3, too, modifies cysteine residues. In order to test this hypothesis, we took advantage of the recent finding that the ADP-ribose-cysteine bond is sensitive to mercury ions [11]. As shown in Fig. 3C, treatment of ADP-ribosylated transducin with 1 mM $HgCl_2$ for 10 min largely removed the ADP-ribose residue; after 1 h of incubation, the loss of ADP-ribose appeared to be complete. Neither the ADP-ribose-arginine bond in actin (Fig. 3A) nor the ADP-



(2)



(3)

Fig. 2: Influence of N-ethylmaleimide on ADP-ribosylation by botulinum ADP-ribosyltransferase C3. Membranes and cytosol of human platelets were pretreated with N-ethylmaleimide (NEM) at the indicated concentrations as described. After incubation with botulinum ADP-ribosyltransferase C3 (3 µg/ml) and [^{32}P]NAD, samples were subjected to SDS PAGE and ^{32}P-ADP-ribosylated peptides were analyzed by autoradiography.

Fig. 3: Stability towards $HgCl_2$ of ^{32}P-ADP-ribose-amino acid bonds formed by botulinum C2 toxin, botulinum ADP-ribosyltransferase C3 and pertussis toxin. Platelet cytosol (A,B) was incubated with [^{32}P]NAD and botulinum C2 toxin (C2, 1 µg/ml) or botulinum ADP-ribosyltransferase C3 (C3, 3 µg/ml); purified transducin (C) was incubated with [^{32}P]NAD and pertussis toxin (PT, 20 µg/ml). Thereafter, samples were treated with 1 mM NaCl for 60 min (lanes 4, 8, 12) or with 1 mM $HgCl_2$ for 10 min (lanes 1, 5, 9), 30 min (lanes 2, 6, 10) and 60 min (lanes 3, 7, 11), respectively. Samples were subjected to SDS PAGE (11% acrylamide), and ^{32}P-ADP-ribosylated peptides were analyzed by autoradiography. The M_r values of the respective ^{32}P-ADP-ribosylated substrates were as in Fig. 1.

ribose-amino acid linkage in the botulinum ADP-ribosyltransferase C3 substrate (Fig. 3B) was affected by mercury ions after incubations for up to 1 h. The high resistence towards dilute alkali of the ADP-ribose-amino acid bond formed by botulinum ADP-ribosyltransferase C3 is depicted in Fig. 4. While treatment with 0.5 M NaOH at 37°C resulted in a time-dependent cleavage of the ADP-ribose-amino acid bonds in actin and in transducin, the ADP-ribose-amino acid bond of the botulinum ADP-ribosyltransferase C3 substrate remained almost unaffected.

Taken together, the data presented indicate that the ADP-ribose-amino acid bond formed by botulinum ADP-ribosyl-

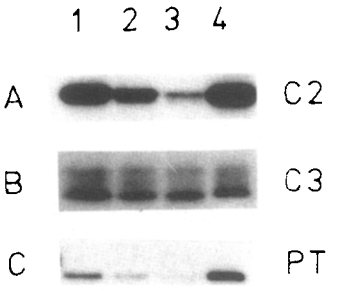


Fig. 4: Stability towards NaOH of ^{32}P-ADP-ribose-amino acid bonds formed by botulinum C2 toxin, botulinum ADP-ribosyltransferase C3 and pertussis toxin. Platelet cytosol (A,B) was incubated with [^{32}P]NAD and botulinum C2 toxin (C2, 1 µg/ml) or botulinum ADP-ribosyltransferase C3 (C3, 3µg/ml); purified transducin (C) was incubated with [^{32}P]NAD and pertussis toxin (PT, 20 µg/ml). Thereafter, samples were treated with 0.5 M NaCl for 60 min (lane 4) or with 0.5 M NaOH for 10 min (lane 1), 30 min (lane 2) and 60 min (lane 3). Samples were subjected to SDS PAGE (11 % acrylamide) and ^{32}P-ADP-ribosylated peptides were analyzed by autoradiography. The M_r values of the respective ^{32}P-ADP-ribosylated substrates were as in Fig. 1.

transferase C3 differs in its stability towards hydroxylamine, mercury ions and dilute alkali from ADP-ribose-amino acid bonds formed by botulinum C2 and pertussis toxins. So far, a similarly stable ADP-ribose-amino acid bond has only been observed in the elongation factor 2, in which diphtheria toxin catalyzes ADP-ribosylation of a diphthamide residue [24]. As diphthamide is an amino acid unique for the elongation factor [1,2] our findings suggest a novel type of ADP-ribose bond formed by botulinum ADP-ribosyltransferase C3.

ACKNOWLEDGEMENTS: The authors are indebted to Dr. M. Yajima for supplying pertussis toxin and to Mrs. M. Laux for excellent technical assistance. This work was supported by grants of the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

REFERENCES

1. Foster, J.W., and Kinney, D.W. (1985) CRC Critical Rev. Microbiol. 11, 273-298.
2. Van Ness, B.G., Howard, J.B., and Bodley, J.W. (1980) J. Biol. Chem. 255, 10710-10716.
3. Moss, J., and Vaughan, M. (1978) Proc. Natl. Acad. Sci. USA 75, 3621-3624.
4. West, R.E., Moss, J., Vaughan, M., Liu, T., and Liu, T.-Y. (1985) J. Biol. Chem. 260, 14428-14430.

5. Aktories, K., Bärmann, M., Chhatwal, G.S., and Presek, P. (1986) TIPS 8, 158-160.
6. Aktories, K., Bärmann, M., Ohishi, I., Tsuyama, S., Jakobs, K.H., and Habermann, E. (1986) Nature (Lond.) 322, 390-392.
7. Aktories, K., Weller, U., and Chhatwal, G.S. (1987) FEBS Lett. 212, 109-113.
8. Vandekerckhove, J., Schering, B., Bärmann, M., and Aktories, K. (1988) J. Biol. Chem. 262, 696-700.
9. Aktories, K., and Frevert, J. (1987) Biochem. J. 247, 363-368.
10. Bredehorst, R., Wielckens, K., Gartemann, A., Lengyel, H., Klapproth, K., and Hilz, H. (1978) Eur. J. Biochem. 92, 129-135.
11. Meyer, T, Koch, R., Fanick, W., and Hilz, H. (1988) Biol. Chem. Hoppe Seyler, in press.
12. Ohishi, I., Iwasaki, W., and Sakaguchi, T. (1980) Infect. Immun. 30, 668-673.
13. Aktories, K., Rösener, S., Blaschke, U., and Chhatwal, G.S. (1988) Eur. J. Biochem. 172, 445-450.
14. Kühn, H. (1980) Nature (Lond.). 283, 587-589.
15. Cassel, D., and Pfeuffer, T. (19787) Proc. Natl. Acad. Sci. USA 75, 2669-2673.
16. Jakobs, K.H., Lasch, P., Minuth, M., Aktories, K., and Schultz, G. (1982)J. Biol. Chem. 257, 2829-2833.
17. Laemmli, U.K. (1970) Nature (Lond.) 227, 680-685.
18. Burzio, L.O. (1982) In: ADP-Ribosylation Reactions (Hayaishi, O., and Ueda, K., eds.) pp. 103-116, Academic Press, New York.
19. Moss, J., Yost, D.A., and Stanley, S.J. (1983) J. Biol. Chem. 258, 6466-6470.
20. Hsia, J.A., Tsai, S.-C., Adamik, R., Yost, D.A., Hewlett, E.L., and Moss, J. (1985) J. Biol. Chem. 260, 16187-16191.
21. Rubin, E.J., Gill, M., Boquet, P., and Popoff, M.R. (1988) Mol. Cell. Biol. 8, 418-426.
22. Aktories, K., Hungerer, K.D., Robbel, L., and Jakobs, K.H. (1984) IUPHAR 9th Intern. Congress of Pharmacology, London, Abstr. Vol. 1655 P.
23. Payne, D.M., Jacobson, E.L., Moss, J., and Jacobson, M.K. (1985) Biochemistry 24, 7540-7549.