Vol. 160, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1989 Pages 67-71

AN ENDOGENOUS INHIBITOR OF THE ADP-RIBOSYLATION OF GTP-BINDING PROTEINS BY PERTUSSIS TOXIN IS PRESENT IN BOVINE BRAIN

Miki Hara-Yokoyama and Shunsuke Furuyama

Department of Physiology, Nihon University School of Dentistry at Matsudo, 2-870-1 Sakae-cho Nishi, Matsudo, Chiba 271, Japan

Received February 25, 1989

The ADP-ribosylation of GTP-binding proteins (G-proteins) catalyzed by pertussis toxin was inhibited by endogenous inhibitor activity in the membrane extract of bovine brain. Most of the activity appeared in the fractions eluted from a DEAE-Sephacel column by 0.5 M NaCl. The activity was heat-stable and sensitive to pronase K. The results suggest the presence of an endogenous inhibitor of pertussis toxin in bovine brain.

Bacterial toxins interrupt the signal transduction cycle at different steps by the ADP-ribosylation of G-proteins (1-5). For example, pertussis toxin causes the functional uncoupling of G_i from its receptor (6,7). Two lines of evidence also suggest the involvement of such ADP-ribosylation in the modulation of the signal transduction process in cells. One is the identification of an endogenous ADP-ribosyltransferase, comparable in its effects to pertussis toxin, in human erythrocytes (8,9). The other is the finding that rat liver contains endogenous inhibitor activity for pertussis toxin (10). Therefore, both ADP-ribosyltransferase and the ADP-ribosylation inhibitor can theoretically participate in the regulation of G-proteins.

G-proteins are abundant in and can be isolated in pure form from the brain, which makes it a suitable organ to use in investigation of the regulatory mechanisms of G-proteins. We have found that bovine brain contains an endogenous inhibitor of the pertussis toxin-catalyzed ADP-ribosylation of G-proteins.

Abbreviations : G_i, regulatory protein that mediates the inhibition of adenylate cyclase; G_o, a similar GTP-binding protein of unknown function; GTPγS, guanosine 5'-(3-O-thio)triphosphate; SDS, sodium dodecyl sulfate.

0006-291X/89 $1.50

MATERIALS AND METHODS

Preparation of Membrane Extract Brains were obtained from freshly slaughtered cattle and were processed at temperature below 4°C. Membrane was prepared as described by Sternweis et al.(11).

DEAE-Sephacel Column Chromatography First, 100 ml of a 1% cholate extract was obtained from 250 g of bovine brain cerebral tissue. The extract was put on a DEAE-Sephacel column (2.7 x 28 cm) equilibrated with buffer A (20 mM Tris-Cl, pH 8.0, with 1 mM EDTA, 1 mM dithiothreitol, and 1% cholate). Elution was performed with 500 ml of a linear gradient of NaCl (0-250 mM) in buffer A, followed by 500 mM NaCl in buffer A. Fractions of 8 ml were collected.

Assays GTPγS binding activity was measured as described before (11). To measure the pertussis toxin substrate activity, 5 µl of the sample was added to 45 µl of assay mixture A (40 mM Tris-Cl, pH 8.0, 5 mM thymidine, 20 mM dithiothreitol, 1 mM L-α-dimyristoyl phosphatidylcholine, 20 mM isonicotinic acid hydrazide, 5 µM GTP), containing 1 µM [^{32}P]NAD (1-20 Ci/mmol) and different amounts of pertussis toxin. The toxin had been activated by incubation with 50 mM dithiothreitol and 1 mM ATP for 15 min at 40°C. The sample was incubated with the reaction mixture at 40° C for 2 h, and then the radioactivity incorporated by protein was measured as described by Neer et al.(12). To measure the inhibition of the ADP-ribosylation of G-proteins by pertussis toxin, 5 µl of sample was incubated at 40°C for 2 h in 45 µl of assay mixture A, containing 1 µM NAD and 16 µg/ml activated pertussis toxin. Usually, more than 95% of the G-proteins in the sample became ADP-ribosylated with unlabelled NAD. After the addition of 30 µl of assay mixture A containing [^{32}P]NAD (1-20 Ci/mmol) and 30 µg/ml of the G-protein fraction from bovine brain, the reaction mixture was incubated for 2 h more. The radioactivity incorporated into proteins was measured as for the first assay (12). As the G-protein fraction, we used fraction No.40 from the chromatography, since it showed the highest GTPγS binding activity. In the control experiment, 5 µl of buffer A was added.

Gel Electrophoresis An 11% polyacrylamide gel electrophoresis in the presence of SDS was run as described by Laemmli (13). The ratio of acrylamide to N,N'-methylenebis acrylamide in the running gel and in the stacking gel was 33:1 (14) and 37.5:1 (13), respectively. Gels were stained with Coomassie Blue and dyed gels were autoradiographed at -20°C with Kodak Omat film.

RESULTS AND DISCUSSION

Membrane extract of bovine brain prepared with various concentrations of cholate and incubated with [^{32}P]NAD and activated pertussis toxin caused ADP-ribosylation of both the 39-kDa and 41-kDa species (Fig. 1). The extent of ribosylation increased as the concentration of cholate used to extract the membrane increased from 0 to 0.5%; thus the extraction of G_o and G_i was cholate-dependent over this range. However, when the cholate concentration was higher than 0.5%, ADP-ribosylation was actually decreased. This did not seem to be due to inadequate

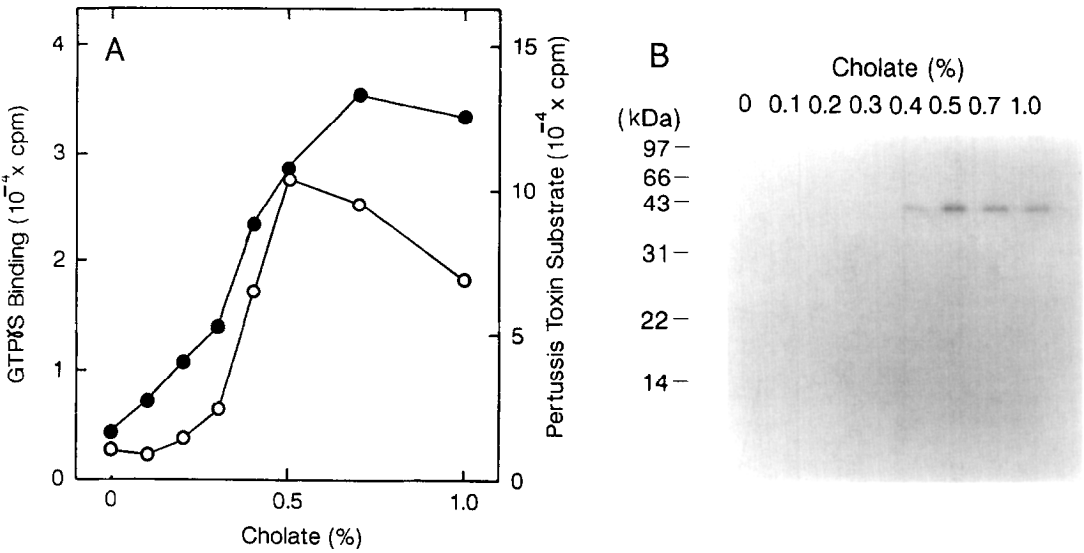

Fig. 1. (A) GTPγS binding activity and pertussis toxin substrate activity of cholate extracts from bovine brain membranes. Cholate was added to each 50 ml of the suspension up to the final concentrations as indicated. The [^{35}S]GTPγS binding activity (●; 5 µl/assay) and pertussis toxin substrate activity (○; 2.5 µl/assay) of the cholate extract were measured. In the measurement of the pertussis toxin substrate activity, the final concentration of pertussis toxin was 2 µg/ml and the final concentration of cholate was adjusted to 0.05%. **(B)[^{32}P]ADP-ribosylation of pertussis toxin substrate in the cholate extract.** The ADP-ribosylation products were treated as described elsewhere (12), separated by electrophoresis, and autoradiographed.

extraction of G_o and G_i, because GTPγS binding did not decrease as the cholate concentration increased up to 1.0%. The results suggested that an inhibitor of the ADP-ribosylation of G-proteins by pertussis toxin was present in the membrane and was extracted by cholate.

We fractionated the inhibitor extracted by 1% cholate on a DEAE-Sephacel column. The pertussis toxin substrate activity appeared as a single peak eluted by 100 mM NaCl; the peak overlapped the major peak of GTPγS binding activity (Fig. 2). Such peak fractions are rich in G_i and G_o (11). The major peak of the inhibitor activity that reduced incorporation of radioactivity catalyzed by pertussis toxin was in the fractions eluted by 0.5 M NaCl. When the reaction product was analyzed by gel electrophoresis following ADP-ribosylation catalyzed by pertussis toxin, incorporation of radioactivity into both 39- and 41-kDa species was shown to be inhibited by addition of the inhibitor fraction (inset in Fig. 2).

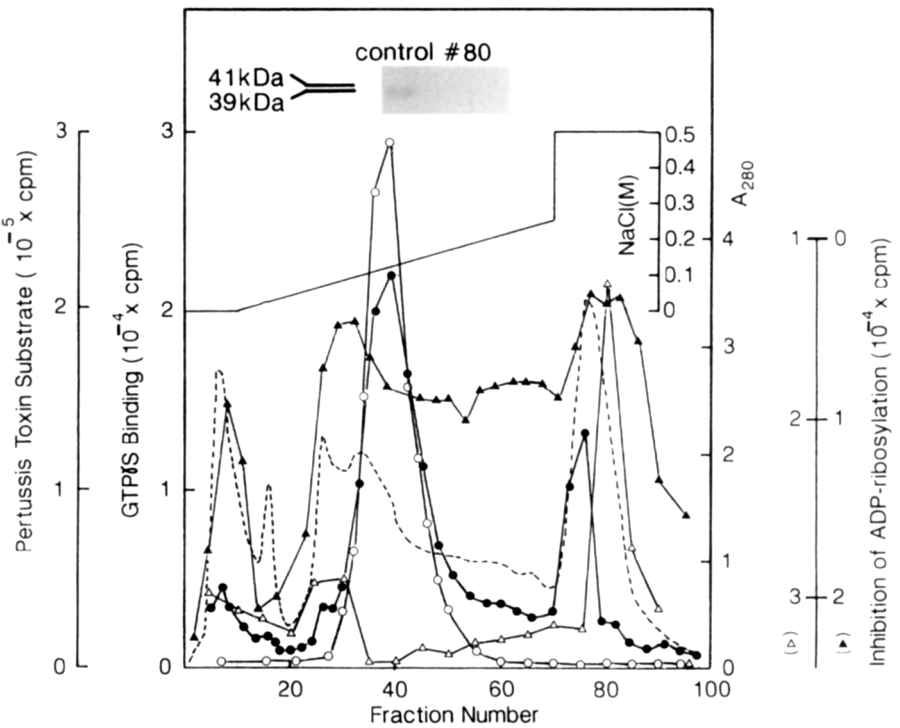

Fig. 2. DEAE-Sephacel column chromatography of bovine brain membrane extract. A 1% cholate extract of bovine brain was fractionated and assayed as described in "Materials and Methods". The final concentration of pertussis toxin in the assay mixture for substrate activity was 9 µg/ml. The radioactivity was plotted in reverse to show the inhibitor activity. [^{35}S]GTPγS binding activity (10 µl/assay), ●; pertussis toxin substrate activity (5 µl/assay), ○; inhibitor activity with 5 µl/assay, ▲ and with 1 µl/assay, △; absorbance at 280 nm (broken line); concentration of NaCl (solid line). (Inset) Inhibition of the [^{32}P]ADP-ribosylation of 41-kDa and 39-kDa species. Here, 5 µl of the DEAE-Sephacel column fraction No.80 or buffer A as the control was incubated first with pertussis toxin and unlabelled NAD and then with G-protein and [^{32}P]NAD as described in "Materials and Methods". Each 20 µl of sample was treated, separated by electrophoresis, and autoradiographed.

Inhibitor activity was stable for at least several months when the fractions were kept at -80°C, and repeated freezing and thawing did not cause any loss of activity. After heat treatment at 100 °C for 5 min, the inhibitor activity remained unchanged (Fig. 3), but it was sensitive to protease treatment for at least 1 h (Fig. 3). Since the inhibitory activity was sensitive to pronase K, this indicated that the inhibitor was proteinic. It may possibly be a peptide, as its activity was quite heat-stable.

The finding of endogenous inhibitor activity in the brain probably indicates the physiological importance of the regulation of G-proteins by endogenous ADP-ribosylation.

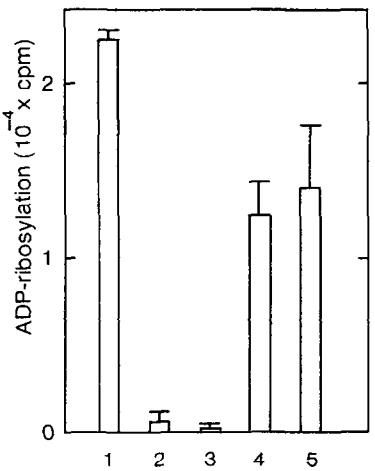

Fig. 3. Effects of heat treatment and pronase K treatment. 100 µl of the inhibitor fraction No. 80 from the chromatography was heated at 100 °C for 5 min. Another 30 µl of the fraction or buffer A was mixed with 5 µl of 1 µg/ml pronase K and incubated at 37 °C for 1 h and 20 µl of soybean trypsin inhibitor (50 mg/ml) was added after incubation. The inhibition by trypsin inhibitor of pronase K activity was confirmed using bovine serum albumin as the substrate (data not shown). The inhibition caused by 5 µl of the heat-treated sample or the sample treated by pronase K was measured as described under "Materials and Methods". Values are means of duplicates + S. D. The lanes are 1 control; 2 inhibitor fraction without treatment; 3 heat-treated inhibitor fraction; 4 buffer A treated with pronase K; 5 inhibitor fraction treated with pronase K.

REFERENCES

1. Cassel, D., and Pfeuffer, T. (1978) Proc. Natl. Acad. Sci. USA 75, 2669-2673.
2. Gill, D. M., and Meren, R. (1978) Proc. Natl. Acad. Sci. USA 75, 3050-3054.
3. Northup, J. K., Sternweis, P. C., Smigel, M. D., Schleifer, L. S., Ross, E. M., and Gilman, A. G. (1980) Proc. Natl. Acad. Sci. USA 77, 6516-6520.
4. Katada, T., and Ui, M. (1982) J. Biol. Chem. 257, 7210-7216.
5. Bokoch, G. M., Katada, T., Northup, J. K., Hewlett, E. L., and Gilman, A. G. (1983) J. Biol. Chem. 258, 2072-2075.
6. Kurose, H., Katada, T., Amano, T., and Ui, M. (1983) J. Biol. Chem. 258, 4870-4875.
7. Hsia, J. A., Moss, J., Hewlett, E. L., and Vaughan, M. (1984) J. Biol. Chem. 259, 1086-1090.
8. Tanuma, S., Kawashima, K., and Endo, H. (1987) J. Biochem. (Tokyo) 101, 821-824.
9. Tanuma, S., Kawashima, K., and Endo, H. (1988) J. Biol. Chem. 263, 5485-5489.
10. Hara-Yokoyama, M., and Furuyama, S. (1988) FEBS Lett. 234, 27-30.
11. Sternweis, P. C., and Robishaw, J. D. (1984) J. Biol. Chem. 259, 13806-13813.
12. Neer, E. J., Lok, J. M., and Wolf, L. G. (1984) J. Biol. Chem. 259, 14222-14229.
13. Laemmli, U. K. (1970) Nature (London) 227, 680-689.
14. Mumby, S., Pang, I.-H., Gilman, A. G., and Sternweis, P. C. (1988) J. Biol. Chem. 263, 2020-2026.