Volume 234, number 1, 27–30 FEB 05984 July 1988

Endogenous inhibitor of the ADP-ribosylation of (a) G-protein(s) as catalyzed by pertussis toxin is present in rat liver

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Received 12 April 1988; revised version received 29 April 1988

The inhibitor activity of the ADP-ribosylation of (a) G-protein(s) as catalyzed by pertussis toxin was found in the membrane extract of rat liver. The inhibitor activity was found in the fractions of DEAE-Sephacel column chromatography at 50–120 mM NaCl. The inhibitor activity is not due to the degradation of NAD nor to the reverse reaction of pertussis toxin (removal of incorporated ADP-ribose). The present result suggests the presence of an endogenous inhibitor of the ADP-ribosylation reaction of (a) G-protein(s).

Endogenous inhibitor; ADP-ribosylation; Pertussis toxin; (Rat liver)

1. INTRODUCTION

Pertussis toxin specifically catalyzes the mono-ADP ribosylation of the cysteine residue of several GTP-binding proteins (G_i, G_o, transducin, etc.) [1–5] and leads to the functional uncoupling with the receptors. Thus, pertussis toxin has been widely used as a powerful tool to demonstrate the involvement of such toxin-sensitive G-proteins in the physiological responses of the eukaryotic cells. Recently, the comparable ADP-ribosyltransferase activity to pertussis toxin has been found endogenously in eukaryotic cell [6] and has been considered to regulate the signal transduction mechanism in vivo. However, if the endogenous ADP-ribosyltransferase constitutively modifies the function of G-proteins, it will cause the loss of the responses for the receptor stimulation. Thus, to elucidate the physiological importance of the endogenous ADP-ribosylation activity, it is necessary to identify its endogenous inhibitor or activator.

In this context, we have started to investigate the endogenous inhibitor activity of the ADP-ribosylation of the G-protein(s) as catalyzed by pertussis toxin. The present study shows the presence of such inhibitor activity in the membrane extract of rat liver that is distinct from NAD degradation activity and also from removal activity of the incorporated ADP-ribose.

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2. MATERIALS AND METHODS

Liver was taken from two adult male Sprague-Dawley rats (200–300 g). Membrane was prepared essentially according to [2] and suspended in 50 ml of buffer A (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1% sodium cholate) and stirred on ice for 60 min. The suspension was centrifuged at 150000 × *g* for 60 min. The supernatant (40 ml) was used as membrane extract in the following step. The assay mixture for the inhibitor activity contained all the components for the ADP-ribosylation of G-proteins as catalyzed by pertussis toxin. 50 μl of the mixture contained final concentrations of the following: 40 mM Tris-HCl, pH 8.0, 5 mM thymidine, 20 mM dithiothreitol, 0.5 mM L-α-dimyristoyl phosphatidylcholine, 20 mM isonicotinic acid hydrazide, 5 μM GTP, 1 μM [^{32}P]NAD (1–20 Ci/mmol), 300 μg/ml of partially purified G-protein fraction from bovine brain [2], and 8 μg/ml of pertussis toxin (Seikagakyu Kogyo Co.) previously activated by 50 mM dithiothreitol, 1 mM ATP for 15 min at 30°C. To measure the inhibitor activity, 5 μl of sample was added to 50 μl of the assay mixture at the beginning of the reaction. As for the control experiment, the whole assay mixture was used without adding any other component. After incubation at 30°C, the reaction was terminated by

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00145793/88/$3.50

the addition of 0.5 ml of 4% sodium dodecyl sulfate (SDS). The radioactivity incorporated to the protein fraction was measured according to [2].

3. RESULTS

3.1. *Inhibitor activity of the ADP-ribosylation of (a) G-protein(s) as catalyzed by pertussis toxin*

Membrane extract of rat liver was applied to a DEAE-Sephacel column (2 × 30 cm) equilibrated with buffer A. The radioactivity incorporated by pertussis toxin from [^{32}P]NAD to the G-protein fraction was measured in the presence of each eluted fraction. As shown in fig.1, fractions 45 to 55 contained inhibitor activity of the ADP-ribosylation and were used in the following experiments. The reaction product was analyzed by 12.5% SDS-polyacrylamide gel electrophoresis according to Laemmli [7] and its autoradiogram is shown in fig.2. By the addition of the DEAE-Sephacel fraction, the ADP-ribosylation of about 40 kDa species was completely inhibited. This indicates that the DEAE-Sephacel fraction inhibits the ADP-ribosylation of α-subunit of G-protein as catalyzed by pertussis toxin.

3.2. *Inhibitor activity is not due to the degradation of NAD*

It is well known that rat liver cytosol is a rich source of phosphodiesterase that degradates NAD as well as DNA and RNA [8]. In addition, Neer et al. [9] reported that the ADP-ribosylation as catalyzed by cholera toxin was inhibited by the presence of NAD-glycohydrolase activity in bovine brain. Therefore, to examine the degradation activity of NAD in DEAE-Sephacel fraction, the assay mixture of the ADP-ribosylation was analyzed by paper chromatography. The degradation activity of NAD will convert the ^{32}P-labelled species from [α-^{32}P]NAD to [α-^{32}P]AMP in the case of phosphodiesterase or to [α-^{32}P]ADP-ribose in the case of NAD-glycohydrolase. As shown in fig.3, there was no apparent difference between the chromatographic patterns obtained in the presence and absence of the DEAE-Sephacel fraction. The major ^{32}P-labelled species in the reaction mixture after the incubation was found to be NAD in both cases. Therefore the inhibitor activity in the fractions as eluted from DEAE-Sephacel cannot be explained by the degradation of NAD.

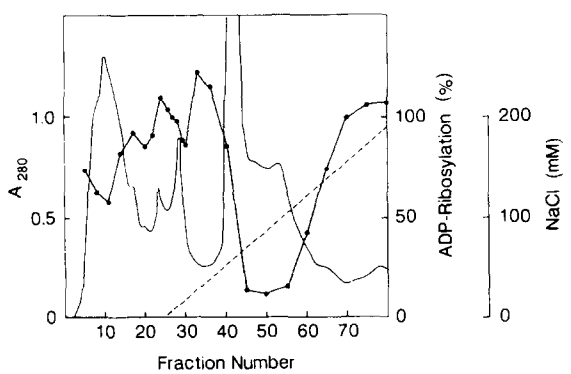

Fig.1. DEAE-Sephacel column chromatography of rat liver membrane extract. Elution was performed with 150 ml of buffer A and then with 500 ml of a linear gradient of NaCl (from 0 to 300 mM). Fractions of 6 ml were collected. The radioactivity as incorporated in the ADP-ribosylation reaction for 3 h is shown by the relative value to that obtained in the control experiment (●). Absorbance at 280 nm is shown with a solid line and the concentration of NaCl with a broken line.

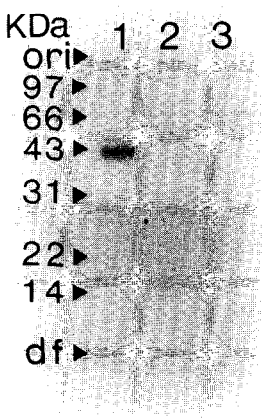

Fig.2. Autoradiogram of SDS-polyacrylamide gel electrophoresis of the ^{32}P-labeled ADP-ribosylation product in the absence (1) and presence (2,3) of the DEAE-Sephacel fraction. In lane 3, G-protein fraction was omitted from the assay mixture.

3.3. *Inhibitor activity is not mediated by the removal of incorporated ADP-ribose*

Fig.4 shows the time course of the ADP-ribosylation as catalyzed by pertussis toxin. In the presence of the DEAE-Sephacel fraction, the plateau level was lowered, while the time required for obtaining the plateau level was almost un-

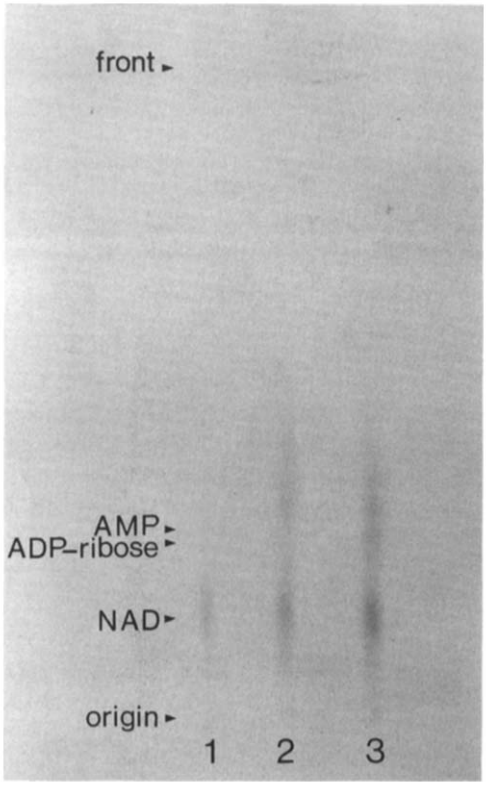

Fig.3. Autoradiogram of paper chromatography of [α-^{32}P]NAD in the assay mixture of ADP-ribosylation before (1) and after (2) incubation, and after incubation in the presence of the DEAE-Sephacel fraction (3). The solvent system was 1 M ammonium acetate (pH 5.0)/95% ethanol (3:7, v/v) [11].

changed. If the ADP-ribosyltransferase activity of pertussis toxin is diminished by the inhibitor activity, the time required for the plateau level will be increased. Thus it is suggested that the inhibitor activity in the DEAE-Sephacel fraction does not affect the pertussis toxin activity. The lowered plateau level can be explained in two different ways. One is the removal of incorporated ADP-ribose from G-protein(s) and the other is the decrease in the substrate concentration. To examine the former possibility, the DEAE-Sephacel fraction was added after the ADP-ribosylation reaction took place (fig.5). The specific activity of NAD was reduced as low as 1/1000, so that the effect of the further ADP-ribosylation was negligible. The results showed that the DEAE-Sephacel fraction had no effect on the incorporated radioactivity of the ADP-ribosylated protein. Therefore,

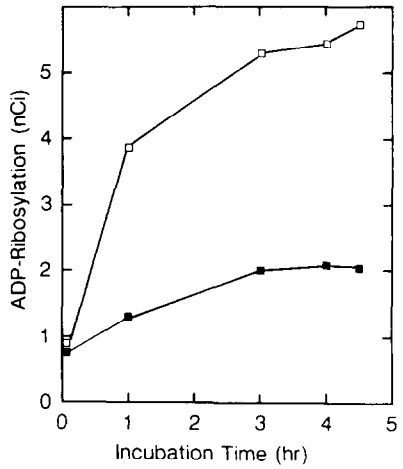

Fig.4. ADP-ribosylation time course in the absence (□) and presence (■) of the DEAE-Sephacel fraction. The incorporated radioactivity per 50 μl of the assay mixture is shown.

the lowered plateau level is not due to the removal of ADP-ribose. Thus the inhibitor activity is probably mediated by the decrease in substrate concentration. As described previously, the inhibitor activity is not due to the degradation of NAD. Therefore we assume that the target of the inhibitor activity is (a) G-protein(s). Further investigation is necessary to elucidate the precise mechanism of the inhibition.

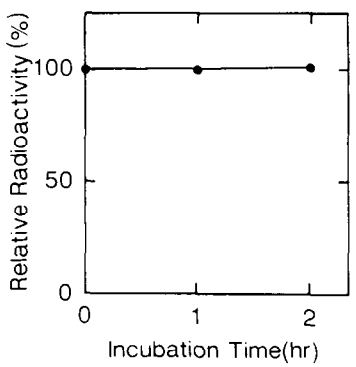

Fig.5. Effect of the DEAE-Sephacel fraction on the incorporated radioactivity by pertussis toxin. The ADP-ribosylation assay mixture containing bovine G-protein fraction was previously incubated for 3 h and then the DEAE-Sephacel fraction was added together with NAD to a final concentration of 1 mM. The radioactivity at $T = 0$ is taken as 100%.

4. DISCUSSION

In the present study, we have found the inhibitor activity of the ADP-ribosylation of (a) G-protein(s) as catalyzed by pertussis toxin from the membrane extract of rat liver. The present study suggests the presence of an endogenous inhibitor of the cellular ADP-ribosyltransferase in rat liver. The ADP-ribosylation activity comparable to pertussis toxin in rat liver was previously proposed by Itoh et al. [11]. The physiological roles of the endogenous ADP-ribosylation will be elucidated by the combined study of endogenous ADP-ribosyltransferase and its endogenous inhibitor.

As mentioned in section 1, the sensitivity to pertussis toxin has been widely used to classify the physiological responses. If the response was not affected by the pertussis toxin treatment in vivo, it was concluded that the response was not mediated by the pertussis toxin-sensitive G-protein. This conclusion is based on the assumption that there is no endogenous inhibitor of pertussis toxin. However, as reported here, we have shown the presence of the endogenous inhibitor activity of pertussis toxin. Accordingly, it is possible that some pertussis toxin-sensitive G-proteins are involved in the physiological responses without being modified by the pertussis toxin treatment.

The purification and the characterization of the inhibitor found in the present study are now in progress in our laboratory. It is expected that the endogenous inhibitor is one of the members that regulate the signal transduction mechanism.

Acknowledgements: This work was supported in part by a Grant-in-Aid for Scientific Research (no.62771501) from the Ministry of Education, Science and Culture of Japan and by a grant from Nihon University School of Dentistry at Matsudo.

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