ADP-RIBOSYLATION OF SARCOLEMMA MEMBRANE PROTEINS IN THE PRESENCE OF CHOLERA TOXIN AND ITS INFLUENCE ON INSULIN-STIMULATED MEMBRANE PROTEIN KINASE ACTIVITY

O. WALAAS, R. S. HORN, E. LYSTAD and A. ADLER
Institute of Medical Biochemistry, University of Oslo, Oslo, Norway

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1. Introduction

We have reported that insulin stimulates cyclic AMP-independent protein kinase activity in sarcolemma membranes [1]. This insulin effect was enhanced by μM levels of GTP [2]. The suggestion was made that a GTP-binding protein was involved in the hormonal control of this protein kinase. To gain support for this assumption we have investigated protein kinase activity after preincubation of sarcolemma in the presence of cholera toxin and NAD^+. The A_1 fragment of cholera toxin is known to catalyze protein ADP-ribosylation from NAD^+ [3] and the well-known stimulation of adenylated cyclase by cholera toxin has been attributed to ADP-ribosylation of a membrane protein of M_r 42 000 [4,5]. It is indicated that this membrane protein is identical with the GTP-binding protein involved in control of adenylate cyclase activity [6]. Here we demonstrate ADP-ribosylation of a sarcolemma membrane protein of M_r 56 000. This results in inhibition of cyclic AMP-independent protein kinase activity and abolishes the stimulatory effect by insulin on this enzyme.

2. Experimental

[^{32}P]NAD^+ (phosphate in [^{32}P]AMP) 23–33 Ci/mmol was obtained from New England Nuclear and [γ-^{32}P]ATP, 18–20 Ci/mmol from Radiochemical Centre, Amersham. Nucleotides, snake venom phosphodiesterase and other reagents were purchased from Sigma. Cholera toxin was obtained from Schwarz/Mann.

2.1. *Treatment of membranes with cholera toxin and [^{32}P]NAD^+*

The procedure in [7,8] was used with some modifications. Sarcolemma membranes from skeletal muscle were prepared as in [1,2]. Cholera toxin was activated by preincubation of the toxin (200 μg/ml) in 50 mM imidazole buffer (pH 7.4) containing 0.1% ovalbumin and 2 mM DTT for 10 min at 37°C. Membranes (150 μg protein) were incubated in 50 mM imidazole buffer (pH 7.4) containing 18 μM [^{32}P]-NAD^+, 20 μM GTP, 0.1 mM $MgCl_2$ and activated cholera toxin (0–25 μg/ml) for 30 min at 30°C in a total volume of 100 μl. The incubation mixture was diluted with 10 ml imidazole buffer (pH 7.4) containing 2 mM $MgCl_2$ and centrifuged at 60 000 × g for 30 min. After washing, the pellet was suspended in 30 mM Tris buffer containing 3% SDS and was heated for 2 min at 100°C. SDS–polyacrylamide gel electrophoresis was performed after incubation of the sample mixture with 40 mM DTT for 30 min at 37°C as in [1,2]. Radioactive protein bands were detected by cutting the gel into 2 mm slices and counting in a scintillation spectrometer. [^{32}P]Product identification was done according to [9] by precipitation of the ^{32}P-labeled membrane with 5% trichloroacetic acid and digestion with snake venom phosphodiesterase.

2.2. *Determination of protein kinase activity*

Membranes (300 μg protein) were preincubated with 4 mM NAD^+, 20 μM GTP, 0.1 mM $MgCl_2$ and activated cholera toxin (0–10 μg/ml) in 50 mM imidazole buffer (pH 7.4) for 30 min at 30°C. The incubation mixture was diluted with 10 ml cold imidazole buffer containing 2 mM $MgCl_2$ and was then centrifuged at 30 000 rev./min for 30 min. The washed pel-

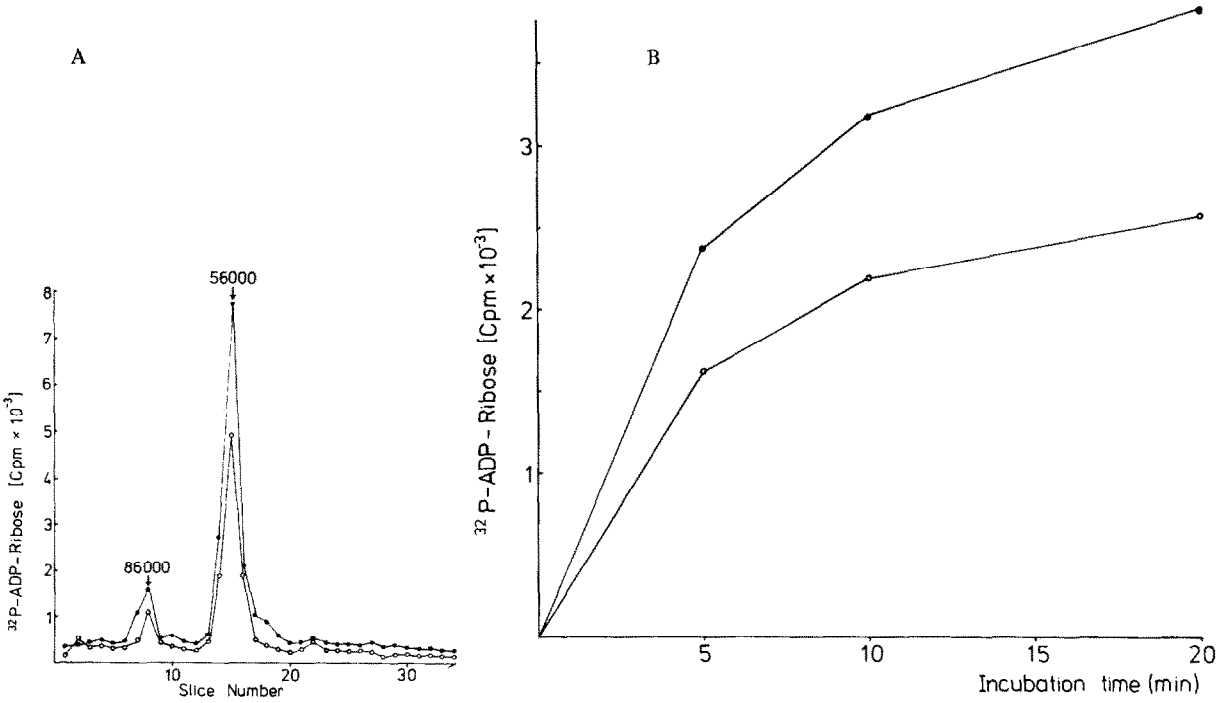

Fig.1. (A) [^{32}P]ADP-ribosylation of sarcolemma membrane proteins. The membranes were incubated with 18 μM [^{32}P]NAD$^+$ in the absence (○) and the presence (●) of cholera toxin (10 μg/ml). The ^{32}P-labeled proteins were separated by SDS–polyacrylamide gel electrophoresis as in section 2. (B) Time course of [^{32}P]ADP ribosylation of M_r 56 000 protein in sarcolemma membranes. Incubation was performed with 10 μM [^{32}P]NAD$^+$ in the absence (○) and the presence (●) of cholera toxin (10 μg/ml).

let was suspended in 50 mM imidazole buffer and aliquots (10 μg protein) were incubated with 50 μM [γ-^{32}P]ATP, 10 mM $MgCl_2$, 1 μM GTP, 50 μg histone 11A without or with insulin (1 mU/ml) for 10 s at 30°C. Details of the procedure for determination of histone [^{32}P]phosphorylation have been reported [2].

3. Results

Incubation of sarcolemma membranes using [^{32}P]-NAD$^+$ as substrate resulted in ^{32}P-labeling of two membrane proteins. By SDS–polyacrylamide gel electrophoresis (fig.1a) it is shown that a protein of M_r 56 000 was preferentially labeled while the other slightly ^{32}P-labeled protein had M_r 86 000. Digestion of the ^{32}P-labeled membranes with snake venom phosphodiesterase yielded 5′-[^{32}P]AMP as the only ^{32}P-labeled nucleotide. This shows that incubation of the membranes with NAD$^+$ results in mono-ADP-ribosylation. The two protein bands are to an appreciable extent ADP-ribosylated by endogenous ADP-ribosyltransferase activity in the membrane. Furthermore, incubation of the membrane with cholera toxin + NAD$^+$ slightly increases the ^{32}P-labeling of the protein M_r 56 000 (table 1). Both in the absence and the presence of cholera toxin maximal ADP-ribosylation was obtained by incubation of the membranes for 20–30 min (fig.1b). As shown in table 2, ^{32}P-labeling of the M_r 56 000 protein was increased by 20 μM GTP or ATP and even more by 20 μM GTP + 5 mM ATP. This effect of nucleotides is in accordance with observation on erythrocyte membranes [8]. Since the sarcolemma membranes rapidly hydrolyze ATP and GTP [2] the effect of 5 mM ATP may attributed to generation of nucleoside triphosphates during incubation.

The effect of endogenous as well as cholera toxin mediated protein ADP-ribosylation on membrane protein kinase activity is shown in table 3. The stimulatory effect of insulin on protein kinase activity is abolished after protein ADP-ribosylation in the absence of cholera toxin while the basal activity is unchanged. If ADP-ribosylation was further increased

Table 1
The effect of cholera toxin on [^{32}P]ADP ribosylation of sarcolemma membrane protein of M_r 56 000

Cholera toxin (μg/ml)	ADP-ribose incorporated (pmol/mg protein)
0	44.5 ± 3.0 (4)
5	48.4 ± 2.1 (4)
10	55.2 ± 4.4 (4)

Incubation system: 20 μM [^{32}P]NAD$^+$, 20 μM GTP, 0.1 mM $MgCl_2$ in 50 mM imidazole buffer (pH 7.4); no. expt in parentheses

by incubation of the membranes in the presence of cholera toxin protein kinase activity was inhibited both in the absence and the presence of insulin. The activity in the presence of insulin was slightly lower than the activity in the absence of the hormone (significant in paired experiments). At 10 μg/ml of cholera toxin protein kinase activity in the presence of insulin was inhibited to 66%. Preincubation of the membranes with cholera toxin in the absence of NAD$^+$ did not have any effect on protein kinase activity.

4. Discussion

In erythrocyte membranes cholera toxin using ^{32}P-labeled NAD$^+$ as substrate specifically labels a protein of M_r 42 000 [4,5]. A protein of the same M_r has been identified as the adenylate cyclase-associated GTP-binding protein [6]. It is strongly indicated that ADP-ribosylation of this protein is responsible for the activation of adenylate cyclase by cholera toxin. This has been explained by inhibition of GTPase activity associated with the protein [10] with subsequent extensive and persistent activation of adenylate cyclase. In plasma membranes from wild-type S49 lymphoma cells cholera toxin catalyzes ADP-ribosylation of a protein of M_r 45 000 and in addition of a protein M_r 52 000–55 000 [11,12]. In liver membranes the guanyl regulatory protein contains 3 different subunits of M_r 52 000, 45 000 and 35 000. By treatment with cholera toxin and [^{32}P]NAD the 2 larger subunits are covalently labeled [13].

As shown in this work on sarcolemma membranes a protein of M_r 56 000 is preferentially ADP-ribosylated while labeling of M_r 42 000–45 000 proteins was absent. The ^{32}P-labeling of the M_r 56 000 membrane protein was dependent upon GTP or ATP. It is, therefore, a possibility that the M_r 56 000 sarcolemma protein represents a GTP-binding protein or a subunit of such a protein in this membrane. Pretreatment of the membranes with NAD$^+$ abolished the GTP-dependent insulin stimulation of the membrane protein kinase. After preincubation in the presence of cholera toxin + NAD$^+$ protein ADP-ribosylation was further increased, and protein kinase activity was strongly inhibited. The specific effect by cholera toxin may be due to ADP-ribosylation of specific sites on the protein. This result is at variance with the increase of adenylate cyclase activity promoted by ADP-ribosylation in the presence of cholera toxin. It is, however, indicated that the mechanism of the toxin effect on adenylate cyclase is rather complex.

Table 2
The effect of GTP and ATP on [^{32}P]ADP-ribosylation of the sarcolemma membrane protein of M_r 56 000

Additions	ADP-ribose incorporated (pmol/mg protein)
NAD$^+$	28.7 ± 1.2 (3)
NAD$^+$ + 20 μM GTP	47.1 ± 2.0 (3)
NAD$^+$ + 20 μM ATP	42.8 ± 2.7 (3)
NAD$^+$ + 20 μM GTP 5 mM ATP	81.1 ± 6.9 (3)

[^{32}P]NAD$^+$ was 18 μM and activated cholera toxin (5 μg/ml) was present during incubation. Details are in section 2; no expt in parentheses

Table 3
Effect of ADP-ribosylation of sarcolemma membrane proteins on endogenous cyclic AMP-independent protein kinase activity

Preincubation additions	Protein kinase activity (as % control) Control	Insulin
None	100 (5) (control)	140 ± 5[a] (3)
NAD$^+$	103 ± 5 (5)	108 ± 6[a] (3)
NAD$^+$ + cholera toxin (5 μg/ml)	94[a] ± 8 (5)	83 ± 5[a] (3)
NAD$^+$ + cholera toxin (10 μg/ml)	64[a] ± 8 (5)	48 ± 9[a] (3)
Cholera toxin (10 μg/ml)	95 ± 2 (3)	–

[a] Significance ($P < 0.01$) *vs* appropriate controls

Procedures were as in section 2; no expt in parentheses

Thus, treatment of the membrane with cholera toxin abolishes the fluoride stimulation of adenylate cyclase [10]. Moreover, it is indicated that the toxin not only inhibits GTPase but also enhances the exchange of guanine nucleotides at the regulatory protein [14]. The existence of separate guanine regulatory components is also indicated, one mediating stimulation and the other inhibition of adenylate cyclase by GTP [15]. Similar complex control mechanisms may be involved in the hormonal control of membrane protein kinase. The inhibition of protein kinase due to ADP-ribosylation of the M_r 56 000 membrane protein gives indirect support for the hypothesis that a nucleotide binding is involved in the control of the activity of this membrane enzyme.

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