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Effects of phosphorylation of inhibitory GTP-binding protein by cyclic AMP-dependent protein kinase on its ADP-ribosylation by pertussis toxin, islet-activating protein

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Pretreatment of rat cardiac myocytes with the β-adrenergic agonist, db-cAMP or forskolin decreased ADP-ribosylation of 40–41 kDa protein by islet-activating protein (IAP) in cell membranes. Addition of activated cyclic AMP-dependent protein kinase (protein kinase A) catalytic subunit and $MgCl_2$ also decreased ADP-ribosylation of 40–41 kDa protein by IAP in cell membranes. The α- and β-subunits of partially purified inhibitory GTP-binding protein (G_i) were both phosphorylated by protein kinase A. The amounts of phosphate incorporated into the subunits of G_i were 0.34 and 0.18 mol/mol protein. These show that phosphorylation of G_i by protein kinase A results in a decrease in its ADP-ribosylation by IAP.

ADP-ribosylation; Islet-activating protein; cyclic AMP-dependent protein kinase; Phosphorylation; GTP-binding protein

1. INTRODUCTION

Inhibitory GTP-binding protein (G_i) consists of three subunits (α, β, γ) with molecular masses of 41, 35 and 10 kDa. Experiments using purified G_i showed that the trimeric structure was essential for ADP-ribosylation of the α-subunit by islet-activating protein (IAP) [1]. Therefore, ADP-ribosylation of G_i (41 kDa) by IAP could reflect the physical states of the GTP-binding proteins.

Purified G_i has been reported to be phosphorylated by protein kinase C [2]. The effects of phosphorylation of G_i (41 kDa) by protein kinase C in human thrombocytes and S49 mouse lymphoma cells have been described [2–4]. Here, we investigated whether activation of protein kinase A affected the ADP-ribosylation of G_i by IAP using myocytes from rat cardiac ventricle and whether G_i was phosphorylated by protein kinase A using partially purified G_i.

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Abbreviations: db-cAMP, dibutyryl cyclic AMP; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate

2. MATERIALS AND METHODS

The following reagents were used: [^{32}P]NAD, [γ-^{32}P]ATP (New England Nuclear), IAP (Kakenyaku Kako, Japan), and cAMP-dependent protein kinase and its catalytic subunit (Sigma). All other materials used were of the highest quality available.

Rat cardiac ventricular myocytes were isolated according to Powell and Twist [5]. Cells were homogenized in a Polytron homogenizer in hypotonic medium containing 10 mM triethanolamine (TEA)-HCl (pH 7.4) and 5 mM EDTA, and the homogenate was centrifuged for 5 min at 10000 × *g*. The pellet was washed twice with hypotonic buffer and used as the preparation of rat cardiac myocyte membranes.

G_i was partially purified from rat brain as in [1].

The reaction medium for ADP-ribosylation consisted of 50 mM TEA-HCl buffer (pH 7.4), 2–5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM ATP, 1 μM [^{32}P]NAD (41.8 Ci/mmol), 10 mM thymidine, IAP (25 μg/ml) and cell membranes (10 μg/tube) or purified G_i (0.4 μg/tube) and the reaction was carried out at 25°C for 90 min.

The reaction mixture for phosphorylation consisted of 50 mM TEA-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM EDTA, 10 μM [γ-^{32}P]ATP (5–10 × 10^5 cpm/nmol), protein kinase A catalytic subunit (5 U/tube) and purified G_i (0.4 μg/tube) or cell membranes (10 μg/tube) and the reaction was carried out at 25°C for 60 min.

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Both reactions were terminated by addition of 20% trichloroacetic acid after the indicated times, the mixtures then being centrifuged at 10000 × g for 5 min. The precipitates were washed three times with 10 mM TEA-HCl buffer (pH 7.4) at 4°C, heated in Laemmli sample buffer for 3 min at 90°C and subjected to electrophoresis in 10–20% linear gradient gels. Gels were then autoradiographed and the area of absorbance of the bands was measured using a chromatoscanner (Shimadzu, CS-930). The radioactivity was also determined by direct counting of the gel in experiments using purified G_i. The amounts of $G_i\alpha$ and $G_i\beta$ were determined by densitometric scanning of the respective protein bands.

3. RESULTS AND DISCUSSION

Under the conditions used, ADP-ribosylation of the 40–41 kDa protein in rat cardiac myocyte membranes was linear for about 60 min, and almost saturated after 90 min. Further additions of IAP or [^{32}P]NAD after 90 min did not increase the extent of ADP-ribosylation of the protein. Thus, ADP-ribosylation of protein in the membranes by IAP appeared to be saturated after 90 min under these experimental conditions. Purified G_i (41 kDa) was also ADP-ribosylated by IAP and compared with the ADP-ribosylated protein in cardiac myocyte membranes. In autoradiograms of the products of ADP-ribosylation by IAP of purified G_i and cardiac myocyte membranes, two bands were observed at similar positions (40–41 kDa), the intensities of both being significantly reduced in preparations obtained in the presence of 100 μM GTPγS. This result suggests that the proteins ADP-ribosylated by IAP in cardiac myocyte membranes included G_i (41 kDa).

Table 1

Effects of activation of cyclic AMP-dependent protein kinase on ADP-ribosylation of inhibitory GTP-binding protein by islet-activating protein in rat cardiac ventricular myocytes or membranes

Pretreatment	Area of absorbance			Percentage of control value
	Expt 1	Expt 2	Expt 3	
(A)				
Control	12395	7301	10019	
l-Isoproterenol	5022	3302	7467	53.4 ± 18.4 (3)
Control	22630	12568	17833	
Dibutyryl cAMP	10474	7812	5225	70.5 ± 13.5 (3)
Forskolin	5164	4211	4065	69.4 ± 16.7 (3)
(B)				
Control	12409	2286	655	
Protein kinase A catalytic subunit	7104	1408	213	50.5 ± 15.7 (3)

Data (densitometric units) and percentage of control value of the area of absorbance of the band of 40–41 kDa protein in the autoradiogram are shown. (A) Rat cardiac ventricular myocytes were preincubated without or with 10 μM l-isoproterenol, 1 mM db-cAMP or 100 μM forskolin for 30 min at 37°C. (B) Membranes of rat myocytes were preincubated without or with protein kinase A catalytic subunit (50–100 U/tube) in the presence of 5 mM $MgCl_2$ and 1 mM ATP for 15 min at 37°C. Their membranes were then ADP-ribosylated with IAP and [^{32}P]NAD under the experimental conditions described in section 2. Means ± SD for the number of independent experiments in parentheses are given

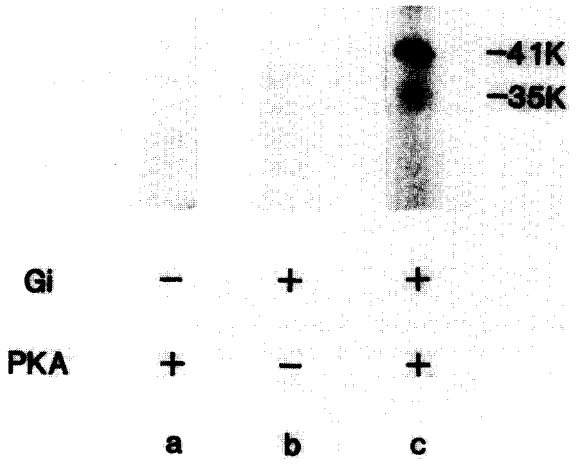

Fig.1. Phosphorylation of purified G_i by protein kinase A. Autoradiograms of gel after SDS-PAGE of phosphorylation products by protein kinase A catalytic subunit in the presence of 5 mM $MgCl_2$ and 1 mM [γ-^{32}P]ATP are shown. Experimental details as described in section 2. Purified G_i (0.4 μg/tube) was phosphorylated with protein kinase A catalytic subunit (5 U/tube) in the presence of 1 mM [γ-^{32}P]ATP in a final reaction volume of 100 μl. Bands containing the α-subunit were cut out and counted to determine the extent of phosphorylation. Results are representative of two or three experiments.

Preincubation of intact ventricular myocytes with l-isoproterenol (10 μM) at 37°C for 30 min significantly decreased ADP-ribosylation of their membranes by IAP by about 47% (table 1). As shown in table 1, pretreatment of myocytes with db-cAMP or forskolin also decreased ADP-ribosylation by IAP by about 29 and 31%, respectively. Thus, an increase in intracellular cAMP level had a similar effect to pretreatment with l-isoproterenol. We subsequently investigated

whether the effect of intracellular cAMP was due to activation of protein kinase A. We found that addition of activated protein kinase A catalytic subunit and MgATP to the membrane also decreased ADP-ribosylation of the 40–41 kDa protein by IAP by about 49% (table 1). These results suggested that phosphorylation of G_i by protein kinase A resulted in a decrease in its ADP-ribosylation by IAP.

Phosphorylation of partially purified G_i (41 kDa) by protein kinase A catalytic subunit was studied. The α- (41 kDa and β-subunits (35 kDa) were both phosphorylated (fig.1). No autophosphorylation of protein kinase A catalytic subunits was observed under these experimental conditions. The amount of ^{32}P incorporated in the presence of protein kinase A reached nearly the maximal level in 20 min and almost saturation in 60 min. The maximal amount of ^{32}P incorporated was about 0.34 and 0.18 mol/mol protein for the α- and β-subunit of G_i, respectively, under these experimental conditions.

Two types of G_i-protein have been purified; IAP-sensitive 41 and 40 kDa G-proteins [6]. The former is G_i (G_i1), which is coupled with adenylate cyclase inhibition [1,6], however, the coupling response of the latter is unknown. Possibly, the 40 kDa G-protein mediates receptor-stimulated phospholipase C activation in an IAP-sensitive manner. In addition, three kinds of cDNA of IAP-sensitive G_i have been reported besides those of G_o and G_t [7]. Therefore, the G_i sample used here may also have contained another type of G_i termed G_i3 [8] and the proteins (40–41 kDa) in membranes of cardiac cells that were ADP-ribosylated by IAP may also have included other kinds of IAP-sensitive G-proteins.

We have shown that purified G_i is a substrate for protein kinase A. The low degree of phosphorylation may be due to the occurrence of intrinsic phosphorylation and/or denaturation of G_i during purification. Improvement in the experimental conditions for phosphorylation may increase the amount of ^{32}P incorporated into G_i-protein.

Since G_i is a good substrate for ADP-ribosylation by IAP only when its three subunits are present as a trimer, a decrease in ADP-ribosylation of G_i by IAP may be due to dissociation of the three subunits of G_i [1]. On the other hand, the nonhydrolyzable guanine nucleotide analog, GTPγS, has been reported to reduce ADP-ribosylation of G_i by IAP without causing dissociation of the subunits of G_i [9]. Further work appears to be necessary to elucidate the molecular mechanism underlying the decrease in ADP-ribosylation by phosphorylation.

In summary, the above results indicate that the phosphorylation of G_i by protein kinase A may modify the state and function of G_i and alter the extent of its ADP-ribosylation by IAP.

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