Guanine nucleotide binding protein involved in muscarinic responses in the pig coronary artery is insensitive to islet-activating protein

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In an attempt to identify the nature of guanine nucleotide binding protein(s) (G-protein) involved in the acetylcholine (ACh)-induced (muscarinic) response of pig coronary-artery smooth muscle, we studied the effect of ADP-ribosylation of specific membrane protein(s) catalysed by islet-activating protein (IAP; pertussis toxin). The ACh-stimulated and guanine nucleotide-dependent activities of phosphatidylinositol 4,5-bisphosphate (PIP₂) phosphodiesterase (PDE), assessed by the production of inositol 1,4,5-trisphosphate (IP₃) from exogenously applied PIP₂, were not modified, in either IAP-treated or non-treated cell homogenates used as the enzyme source. In intact tissues, pretreatment with up to 100 ng of IAP/ml inhibited neither the ACh-induced decrease in the amount of inositol phospholipids nor the increase in the amounts of phosphatidic acid and of inositol phosphates. IAP treatment increased the amount of cyclic AMP accumulated by isoprenaline. These observations suggest that G-protein which couples the muscarinic receptor to PIP₂-PDE is insensitive to IAP. Such being the case, the nature of this protein(s) probably differs from that required for the regulation of adenylate cyclase activities (N_i or G_i).

INTRODUCTION

The agonist-induced stimulation of phosphatidylinositol 4,5-bisphosphate (PIP₂) hydrolysis has been given increasing attention, as two types of second messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol, are produced by hydrolysis (Berridge & Irvine, 1984; Nishizuka, 1984). In contrast with studies on the function of IP₃ or protein kinase C through diacylglycerol, little is known of mechanisms by which receptor occupation induces the hydrolysis of PIP₂. Reports on work with mast cells (Gomperts, 1983), platelets (Haslam & Davidson, 1984), neutrophils (Cockcroft & Gomperts, 1985), salivary glands (Litosh *et al.*, 1985) or hepatocytes (Wallace & Fain, 1985) indicated that a guanine nucleotide binding protein(s) (G-protein) is involved in coupling specific receptors to the phosphodiesterase (PDE or phospholipase C) causing PIP₂ hydrolysis. This suggestion was based on observations that, in membrane preparations or permeabilized cells, GTP and its unhydrolysable analogues (but not GDP and its analogues, which competitively inhibit the GTP-evoked effects) stimulated the PIP₂ hydrolysis and elicited specific functions, for example 5-hydroxytryptamine (serotonin) secretion in platelets (Haslam & Davidson, 1984).

Nakamura & Ui (1985) reported that treatment of rat peritoneal mast cells with islet-activating protein (IAP; pertussis toxin) for 2 h blocked PIP₂ hydrolysis and histamine secretion, as induced by compound 48/80. These inhibitory effects induced by IAP correlated well with the IAP-catalysed ADP-ribosylation of the 41 kDa protein, which may be the same as, or closely related to,

the α subunit of N_i (or G_i), the inhibitory G-protein of the adenylate cyclase system (Ui, 1984), as shown by its *M_r* and inhibition of ADP-ribosylation in the presence of guanosine 5'-[γ -thio]triphosphate (GTP[S]). Essentially the same observations were made with neutrophils stimulated with chemotactic peptides by Ui's group (Okajima *et al.*, 1985) and others (Smith *et al.*, 1985). Therefore they concluded that the G-protein involved in the receptor-stimulated PIP₂ hydrolysis is the IAP substrate N_i, at least in mast cells and neutrophils.

In a previous study (Sasaguri *et al.*, 1985), we suggested that a G-protein is involved in the muscarinic stimulation of PIP₂ breakdown in the homogenate from the pig coronary-arterial smooth muscle. The purpose of the present study was to clarify the nature of this G-protein. For this purpose, we examined the effect of IAP treatment on PIP₂-PDE activity, using the cell homogenate as the enzyme source and exogenous ³²P-labelled PIP₂ as the substrate. In addition, in intact cells, ACh-induced hydrolysis of inositol phospholipids, accumulation of inositol phosphates, and mechanical responses were also examined, in both control (non-treated) and IAP-treated muscle tissues.

MATERIALS AND METHODS

Preparation of cell homogenate and ADP-ribosylation by IAP

Coronary arteries were excised from pig hearts obtained at a local abattoir within 30 min after slaughter. Adventitia and adherent connective tissue were carefully removed under a binocular microscope,

Abbreviations used: PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; PA, phosphatidic acid; IP₃, inositol 1,4,5-trisphosphate; IP₂, inositol 1,4-bisphosphate; IP, inositol monophosphate; PDE, phosphodiesterase; G-protein, guanine nucleotide binding protein; IAP, islet-activating protein; GTP[S], guanosine 5'-[γ -thio]triphosphate; ACh, acetylcholine.

and the endothelium was carefully ablated with cotton-wool.

Muscle strips (about 1.5 g wet wt.) were homogenized with a Polytron PT10 (3 × 30 s at half-maximal speed) in solution containing 0.1 M-KCl, 20 mM-Tris/maleate buffer (pH 6.8), 5 mM-MgCl₂, 1 mM-EGTA, 1 mM-dithiothreitol and 1 mM-di-isopropyl fluorophosphate. Tris/maleate buffer was prepared as described by Sasaguri *et al.* (1985). The crude homogenate was centrifuged at 500 g for 5 min, and the resultant supernatant was used as a cell homogenate. The particulate fraction (100 000 g pellet) was prepared as described by Sasaguri *et al.* (1985). Protein concentration was determined by the method of Lowry *et al.* (1951). The homogenate or particulate fraction (2 mg of protein/ml) was ADP-ribosylated by incubation in a solution containing 0.1 M-KCl, 20 mM-Tris/maleate buffer (pH 6.8), 2 mM-MgCl₂, 1 mM-ATP, 20 mM-thymidine, 10 μM-NAD⁺ and 100 μg of IAP/ml for 20 min at 30 °C. The IAP-treated sample served for measurement of PIP₂-PDE activity, as described below. In the control sample, IAP was omitted from the incubation medium. When the ADP-ribosylation of proteins during IAP treatment was examined (for Fig. 2), non-radioactive NAD⁺ in the above mixture was replaced by 10 μM-[adenylate-³²P]NAD⁺ (sp. radioactivity 6.9 Ci/mmol). The IAP-treated samples were dissolved in Laemmli (1970) sample buffer containing 1% SDS and boiled in water for 3 min. The incorporation of ³²P into the membrane proteins was determined by separation of the proteins by electrophoresis on an 8% -polyacrylamide gel (Laemmli, 1970), followed by autoradiography for 18 h.

PIP₂-PDE assay

Unlabelled PIP₂ (sodium form; dissolved in water) and [³²P]PIP₂ (dissolved in chloroform) were prepared as described by Sasaguri *et al.* (1985). After evaporation of chloroform, [³²P]PIP₂ was mixed with unlabelled PIP₂ to give a specific radioactivity of 100 c.p.m./nmol and then sonicated to become homogeneous. The control or IAP-treated homogenate of arterial smooth muscle (protein concn. 0.2 mg/ml), prepared as described above, was incubated in the assay medium (1 ml) containing 0.1 M-KCl, 20 mM-Tris/maleate buffer (pH 6.8), 1 mM-MgCl₂, 0.14 μM-Ca²⁺ (0.12 mM-CaCl₂ plus 1 mM-EGTA; Sasaguri *et al.*, 1985), 10 μM-GTP[S], 100 μM-[³²P]PIP₂ and 10 μM- eserine, with or without 10 μM-acetylcholine (ACh), at 37 °C for indicated times. The reaction was terminated by addition of 1 ml of 10% (w/v) trichloroacetic acid. Procedures used for the assay of [³²P]IP₃ production were the same as described by Sasaguri *et al.* (1985).

Assay of inositol phospholipids and phosphatidic acid

Muscle strips (about 40 mg wet wt.) prepared as described above were radiolabelled in phosphate-free Krebs-Ringer solution (pH 7.4), containing 40 μCi of [³²P]P_i (sp. radioactivity 30–40 Ci/mmol)/ml, at 37 °C for 2 h, and bubbled with O₂/CO₂ (19:1). IAP (100 ng/ml) was then added to the medium, and the incubation continued for an additional 3 h. The strips were then washed three times with a solution containing 20 mM-Hepes/Tris buffer (pH 7.4), 130 mM-NaCl, 5 mM-KCl, 1 mM-MgCl₂, 1.5 mM-CaCl₂, 1 mM-KH₂PO₄ and 5.5 mM-glucose, and then stimulated with 10 μM-ACh in 500 μl of the solution for various times (10–120 s). Hepes/Tris

buffer was made by adjusting approx. 0.2 M-Hepes to pH 7.4 at 37 °C with Tris and diluting to 20 mM. The reaction was halted by adding 1.8 ml of ice-cold chloroform/methanol/12 M-HCl (50:100:1, by vol.). The strips were then homogenized with a glass homogenizer. Crude lipid extract in the solvent was chromatographed on a silica gel 60 plate (Merck), as described by Billah & Lapetina (1982). The plate was then autoradiographed on Sakura X-ray film for 12–15 h. The fractions corresponding to PI, PIP, PIP₂ and PA on the plate were scraped off, and counted for radioactivity in a liquid-scintillation counter.

Assay of inositol phosphates

The muscle strip prepared as described above was labelled with 5 μCi of *myo*-[2-³H(n)]inositol (sp. radioactivity 15.8 Ci/mmol)/ml in Krebs-Ringer solution (pH 7.4) at 37 °C for 6 h, bubbled with O₂/CO₂ (19:1). For the final 3 h, IAP (100 ng/ml) was added to the medium. After washing three times with solution containing 20 mM-Hepes/Tris buffer (pH 7.4), 120 mM-NaCl, 10 mM-LiCl, 5 mM-KCl, 1 mM-MgCl₂, 1.5 mM-CaCl₂, 1 mM-KH₂PO₄ and 5.5 mM-glucose, the strip was stimulated with 10 μM-ACh in 1 ml of the solution for various times (10–120 s). The reaction was halted by adding 1 ml of 10% trichloroacetic acid. The tissue was then homogenized, and after centrifugation at 1000 g for 10 min the acid-soluble supernatant was treated with diethyl ether (3 × 10 ml) to remove the trichloroacetic acid. After neutralization with NaOH, the supernatant was applied to a column containing 1 ml of Dowex 1 (formate form; 50–100 mesh) anion-exchange resin, for an analysis of inositol phosphates (Sasaguri *et al.*, 1985).

Cyclic AMP assay

Muscle strips (about 20 mg wet wt.) were incubated in Krebs-Ringer solution, with or without 100 ng of IAP/ml, for 3 h. After washing with solution containing 20 mM-Hepes/Tris buffer (pH 7.4), 130 mM-NaCl, 5 mM-KCl, 1 mM-MgCl₂, 1.5 mM-CaCl₂, 1 mM-KH₂PO₄ and 5.5 mM-glucose, the strip was stimulated with various concentrations of L-isoprenaline in 1 ml of the same solution at 37 °C for 3 min. The reaction was halted by immersing the muscle strip into liquid N₂. The concentration of cyclic AMP of the extract was measured with a radioimmunoassay kit (Yamasa Shoyu, Tokyo, Japan).

Recordings of mechanical activity of the muscles

To record tension of the muscle cells, the vessel was carefully teased apart with jeweller's forceps, opened along the longitudinal direction, and circular cut strips (50–75 μm in width, 300 μm in length and 20 μm in thickness) were prepared, in Krebs-Ringer solution. The preparation was set up in a small chamber (0.9 ml) through which the test solution was superfused rapidly by jetting from one end and by siphoning off simultaneously with a water pump from the other end. Both ends of the preparation were fixed between pieces of Scotch double stick tape (3 M Co., St Paul, MN, U.S.A.), and isometric tension was recorded with a strain-gauge transducer (U-gauge; Shinko Co., Tokyo, Japan). The tissue was superfused with solution containing 20 mM-Hepes/Tris buffer (pH 7.4), 130 mM-NaCl, 5 mM-KCl, 1 mM-MgCl₂, 1.5 mM-CaCl₂, 1 mM-KH₂PO₄,

Fig. 1. Effects of various concentrations of GTP[S] on the activity of PIP₂-PDE for production of IP₃ in the particulate fraction

The particulate fraction (0.5 mg of protein/ml) was incubated with [³²P]PIP₂ (100 μM) in a solution containing 0.1 M-KCl, 20 mM-Tris/maleate buffer (pH 6.8), 1 mM-MgCl₂, 0.14 μM free Ca²⁺, the indicated concentration of GTP[S], and 10 μM- eserine: ○, 10 μM-ACh; ●, no ACh. Vertical bars indicate S.E.M. (n = 5).

5.5 mM-glucose and various concentrations of IAP, at 32 °C for 4–12 h. CaCl₂ was omitted and 2 mM-EGTA was added to make the Ca²⁺-free buffer. The above solution containing 10 μM-ACh was applied to induce contraction. NaCl (130 mM) was replaced with an equimolar concentration of KCl to obtain the K⁺-induced contraction (Itoh *et al.*, 1982).

Chemicals

IAP was a gift from Professor M. Ui, Hokkaido University, Japan. [adenylate-³²P]NAD⁺ and myo-[2-

³H(n)]inositol were purchased from New England Nuclear. GTP[S] was from Boehringer Mannheim. Other compounds, including ACh, eserine, NAD⁺, ATP, thymidine, and 1-isoprenaline, were from Sigma.

RESULTS

Effect of GTP[S] on PIP₂-PDE activity in the particulate fraction of the coronary artery

The effects of ACh with GTP[S] on the PIP₂-PDE activity were examined by using the particulate fraction of the coronary artery (Fig. 1). In the presence of 1 mM-MgCl₂, GTP[S] in a concentration-dependent manner produced IP₃. However, in the presence of ACh, the dose-response curve for GTP[S] activation of the PIP₂-PDE was shifted to the left. At concentrations over 100 μM, GTP[S] alone stimulated the PIP₂-PDE activity, and at 1 mM, IP₃ was produced maximally, irrespective of the presence of 10 μM-ACh. These results indicate that guanine nucleotides may regulate ACh-induced PIP₂ hydrolysis, the effects of which may be exerted through a G-protein located in the plasma membrane. The effect of GTP[S] was also observed with the particulate fraction, as with the homogenate (Sasaguri *et al.*, 1985). However, the activity of PIP₂-PDE in the particulate fraction was much lower than that in the homogenate, i.e. 1.4 nmol of IP₃ was produced by incubating PIP₂ with 0.5 mg of the homogenate protein for 2 min, whereas only 0.28 nmol was produced with the same concentration of proteins in the particulate fraction. Thus we used the homogenate fraction to examine whether the G-protein involved in the ACh-stimulated PIP₂ hydrolysis in the pig coronary artery is sensitive to IAP.

Effect of IAP-treated homogenates on PIP₂-PDE activity in smooth muscles of the coronary artery

Fig. 2(a) shows an autoradiogram of the electrophoretic pattern of the homogenate and particulate fractions (treated with IAP for 20 min) on SDS/polyacrylamide

Fig. 2. ADP-ribosylation of the membrane proteins by IAP

The homogenate or the particulate fraction (2 mg of protein/ml) was incubated in a medium containing 0.1 M-KCl, 20 mM-Tris/maleate buffer (pH 6.8), 2 mM-MgCl₂, 1 mM-ATP, 20 mM-thymidine, 10 μM-[adenylate-³²P]NAD⁺, and various concentrations of IAP at 30 °C for 20 min. (a) Autoradiogram of SDS/polyacrylamide gel. Lanes 1 and 2, homogenate; lanes 3 and 4, the particulate fraction; lanes 1 and 3, no IAP; lanes 2 and 4, +100 μg of IAP/ml. (b) Dependence on IAP concentration of ADP-ribosylation of 41 kDa protein in the cell homogenate. The radioactivities of 41 kDa protein separated by SDS/polyacrylamide-gel electrophoresis are shown as percentages of the activity obtained in the treatment with 100 ng of IAP/ml. Bars show S.E.M. (n = 3).

gel. The native IAP, but not the A-protomer of IAP (an active ADP-ribosylating enzyme), could catalyse the ADP-ribosylation of the 41 kDa protein of the homogenate (lane 2). The protein ADP-ribosylated by IAP may be the α -subunit of N_i , an inhibitory regulatory protein of adenylate cyclase located in the membrane fraction, because the same protein was also observed in the particulate (lane 4), but not in the cytosol fraction (results not shown). The 41 kDa protein appeared to be maximally ribosylated in the presence of 50–100 μ g of native IAP/ml (Fig. 2b).

The activity of the PIP_2 -PDE was then examined in the homogenate after treatment with 100 μ g of IAP/ml. As shown in Fig. 3(a), in the presence of 1 mM- $MgCl_2$, 0.14 μ M free Ca^{2+} and when using 10 μ M-GTP[S], there

Fig. 3. Effect of IAP treatment of the homogenate on ACh- or GTP[S]-induced activation of PIP_2 -PDE

(a) The homogenate (0.2 mg/ml) was incubated with [^{32}P] PIP_2 (100 μ M) in a solution containing 0.1 M-KCl, 20 mM-Tris/maleate buffer (pH 6.8), 1 mM- $MgCl_2$, 0.14 μ M free Ca^{2+} , 10 μ M-GTP[S], 10 μ M-eserine, and with (○, ●) or without (□, ■) application of 10 μ M-ACh. (b) The incubation conditions were the same as those in (a), except that the concentration of GTP[S] was 100 μ M (○, ●) or zero (△, ▲), and eserine and ACh were omitted. ○, □, △, Non-treated homogenate; ●, ■, ▲, IAP-treated homogenate. Bars show S.E.M. (n = 5).

was no apparent production of IP_3 . However, when 10 μ M-ACh was added, PIP_2 -PDE was activated, leading to IP_3 production. The amount of IP_3 was seen to increase linearly with time up to 3 min, reaching a plateau at 15–20 min. There was no difference in the IP_3 production, activated by ACh, between the IAP-treated and control preparations of the homogenate. Fig. 3(b) shows the time course of IP_3 production induced by 100 μ M-GTP[S]. Again, no difference between the IAP-treated and control samples was observed while measuring GTP[S]-stimulated IP_3 production.

These results indicate that a G-protein which is insensitive to ADP-ribosylation catalysed by IAP may be involved in the ACh- or GTP[S]-induced activation of PIP_2 -PDE.

Effects of IAP treatment of muscle cells on ACh-induced changes in the amount of inositol phospholipids, phosphatidic acid or inositol phosphates

Our tentative conclusion, that G-protein involved in the ACh-induced PIP_2 hydrolysis in the coronary artery is not an IAP substrate, was confirmed by examining the amount of inositol phospholipids and of inositol phosphates in the intact muscles stimulated with ACh.

Fig. 4 shows ACh-induced changes in the amount of inositol phospholipids, in both IAP-treated and non-treated smooth muscles of the coronary artery. Application of 10 μ M-ACh decreased the amount of PIP_2 , within 10 s, and similar results were observed on the IAP-treated muscle preparation. For example, at 10 s after application of ACh, the amount of PIP_2 decreased to $48 \pm 7\%$ of the control, in non-treated muscle, and to $42 \pm 6\%$ in IAP-treated muscle. The amount of PIP also decreased within 10 s, in both samples ($63 \pm 8\%$ in IAP-treated and $58 \pm 7\%$ in non-treated muscle, at 10 s). The decrease in PI was less than that of PIP_2 or PIP ($86 \pm 6\%$ in IAP-treated and $88 \pm 6\%$ in non-treated muscle, at 120 s). The PA content gradually increased and was not affected by IAP treatment ($180 \pm 17\%$ in IAP-treated and $202 \pm 18\%$ in non-treated muscle, at 120 s). We also observed the effect of IAP treatment on the production of inositol phosphates in the water-soluble fraction of [3H]inositol-labelled muscle stimulated with ACh. LiCl (10 mM) was added to the reaction medium to inhibit the inositol monophosphate (IP) and inositol 1,4-bisphosphate (IP_2) phosphatases (Storey *et al.*, 1984). As shown in Fig. 5(a), application of 10 μ M-ACh rapidly increased the amount of IP_3 , giving a maximal increase at 10 s, followed by a gradual decline. There was no significant difference between the IAP-treated and non-treated muscle ($155 \pm 11\%$ in IAP-treated and $143 \pm 13\%$ in non-treated muscle, at 10 s). Total inositol phosphate, comprising IP_3 , IP_2 and IP, was gradually increased up to 120 s after application of ACh, with no difference between IAP-treated and non-treated muscles ($163 \pm 10\%$ in IAP-treated and $157 \pm 12\%$ in non-treated muscle, at 120 s) (Fig. 5b). The gradual decrease in the accumulated IP_3 during the prolonged incubation may be due to degradation by IP_3 phosphatase, which is not inhibited by Li^+ (Storey *et al.*, 1984; Sasaguri *et al.*, 1985).

As shown in Table 1, the production of cyclic AMP by isoprenaline was enhanced by the treatment with IAP, with maximum effect being observed at 100 ng of IAP/ml. The enhancement of β -adrenergic-stimulated cyclic AMP accumulation, in the IAP-treated muscle,

Fig. 4. Effect of IAP treatment on hydrolysis of inositol phospholipids induced by ACh in intact smooth muscles

³²P-labelled arterial muscle incubated in the absence (○, □) or presence (●, ■) of 100 μg of IAP/ml for 3 h was stimulated by 10 μM-ACh at zero time. The amounts of inositol phospholipids and PA were determined by the assay method described in the Materials and methods section. (a) PIP₂; (b) PIP; (c) PI; (d) PA. ○, ●, 10 μM-ACh; □, ■, no ACh. The amount of each lipid before the stimulation was normalized as 100% [non-treated: PIP₂, 65154 ± 6492; PIP, 10476 ± 963; PI, 35112 ± 4221; PA, 12936 ± 759 (c.p.m./mg of protein); IAP-treated: PIP₂, 49554 ± 4003; PIP, 8160 ± 824; PI, 34092 ± 4107; PA, 11208 ± 823 (c.p.m./mg of protein)]. Vertical bars indicate S.E.M. (n = 5).

may reflect elimination of the functioning of N_i by IAP treatment. Incubation of intact muscles for 3 h with IAP seemed adequate to eliminate the physiological functions of N_i. The application of 10 μM-ACh for 3 min did not alter the cellular cyclic AMP content in both IAP-treated and non-treated muscles (4.9 ± 0.4 in non-treated and 4.8 ± 0.6 pmol/mg of protein in IAP-treated muscle).

Effect of IAP treatment on the mechanical responses induced by ACh

Fig. 6(a) shows the mechanical responses in control and IAP-treated muscle, as induced by either high K⁺ or ACh, with or without external Ca²⁺. High-K⁺ (135 mM)-induced contraction was completely blocked by the addition of 0.1 μM-nifedipine, a potent Ca²⁺-channel blocker, and was not observed in the absence of external Ca²⁺ (results not shown), thereby indicating that this contraction is caused by a voltage-dependent Ca²⁺ influx (Kuriyama *et al.* 1982). On the other hand, the contraction evoked by 10 μM-ACh was much larger than that induced by high K⁺ (compare A-2 with A-1 in Fig. 6) and was only slightly decreased in the absence of external Ca²⁺ (compare A-3 with A-2). The contraction evoked by ACh in the absence of Ca²⁺ showed characteristics of the shortening of duration of tension rather than a decrease in the maximal amplitude. Since the application of ACh to this tissue modified neither the membrane potential nor the membrane resistance (Ito

et al., 1979) and the ACh-induced contraction was not inhibited by Ca²⁺-channel blockers (Itoh *et al.*, 1984), the contraction induced by ACh is probably mainly due to mobilization of stored Ca²⁺. The receptor-operated Ca²⁺ influx may also contribute to the continuation of the tension in the presence of external Ca²⁺ (Itoh *et al.*, 1984).

When the muscle strips were preincubated with IAP in concentrations over 30 ng/ml at 35 °C for 3 h, the ACh-induced contraction was slightly inhibited (see B-2, B-3, C-2 and C-3, Fig. 6), but the high-K⁺-induced contraction was not affected (see B-1 and C-1). Fig. 6(b) shows a plot of the maximal tension in muscles treated with various doses of IAP, relative to that in the control (non-treated) sample. The ACh-induced tension was decreased to 78 or 68% in muscle treated with 100 ng of IAP/ml and 92 or 86% in muscle treated with 30 ng of IAP/ml in the presence or absence of external Ca²⁺ respectively.

DISCUSSION

In the present study using the homogenate or particulate fraction of the pig coronary artery, native IAP catalysed the ADP-ribosylation of 41 kDa protein, even in the absence of a preactivated A-protomer. Similar observations were made with C6 glioma cells by Tamura *et al.* (1982) and Katada *et al.* (1983). These may

Fig. 5. Effects of IAP treatment on accumulation of inositol phosphates induced by ACh in intact smooth muscles

[³H]Inositol-labelled arterial muscle, not treated (○, □) or treated with 100 ng of IAP/ml for 3 h (●, ■) was stimulated by 10 μ M-ACh at zero time, in the presence of 10 mM-LiCl. The assay method for inositol phosphates in the water-soluble fraction was described in the Materials and methods section. (a) IP₃; (b) total inositol phosphates, including IP₃, IP₂ and IP. ○, ●, 10 μ M-ACh; □, ■, no ACh. The amounts of these inositol phosphates before stimulation by ACh were normalized as 100% [non-treated: IP₃, 39.5 $\pm$ 3.1; total inositol phosphates, 231.5 $\pm$ 32.2 (c.p.m./mg of protein); IAP-treated: IP₃, 50.5 $\pm$ 4.7; total inositol phosphates, 301.0 $\pm$ 26.9 (c.p.m./mg of protein)]. Bars indicate S.E.M. (n = 5).

be due to the close association of the processing enzymes which activate the A-protomer with the particulate fraction.

In the homogenate from coronary artery, although the 41 kDa protein (probably α -subunit of N_i) was maximally ADP-ribosylated by preincubation with IAP, IP₃ was produced to the same extent as observed in the control sample from exogenously added PIP₂ on addition of ACh and a relatively low dose of GTP[S] (10 μ M). A relatively high dose (100 μ M) of GTP[S] itself (without addition of ACh) led to the production of IP₃, with no difference between IAP-treated or non-treated homo-

Table 1. Enhancement of the accumulation of cyclic AMP stimulated by isoprenaline after treatment with IAP

Cyclic AMP contents in non-treated or IAP-treated muscles were measured 3 min after stimulation by isoprenaline. The procedure is described in the Materials and methods section. Results are means $\pm$ S.E.M. (n = 5).

	Cyclic AMP (pmol/mg of protein)	
	Non-treated	IAP-treated
Basal	4.8 $\pm$ 0.5	4.6 $\pm$ 0.7
0.1 μ M-Isoprenaline	8.5 $\pm$ 0.6	9.6 $\pm$ 0.7
1 μ M-Isoprenaline	10.6 $\pm$ 1.3	16.6 $\pm$ 1.6
10 μ M-Isoprenaline	13.7 $\pm$ 1.6	23.0 $\pm$ 1.7

genes being observed. The lack of effect of ADP-ribosylation of N_i by IAP treatment on PIP₂-PDE activity in the cell-free system was confirmed with intact muscle tissues. Enhancement of β -agonist-stimulated cyclic AMP accumulation by IAP treatment would (albeit indirectly) indicate that N_i was ADP-ribosylated, and consequently that the function of N_i was abolished during the pretreatment of intact muscles with IAP, although no appreciable change in cyclic AMP concentrations was observed in unstimulated IAP-treated muscles, as was the case in C6 glioma cells (Katada *et al.*, 1982). The decrease in PIP₂ or the production of IP₃ observed in [³²P]- or [³H]-inositol-labelled muscle stimulated with ACh, respectively, was not affected by prior incubation with IAP. Why the ³²P radioactivities of PIP₂ and PIP were diminished by approx. 20% after IAP treatment (see the legend of Fig. 4) is unknown. However, IAP may inhibit PI and PIP kinase, because the ³²P radioactivity of PI was not altered by IAP treatment. These results, taken together with those obtained with the cell-free system, strongly suggest that a G-protein, which is insensitive to IAP, is involved in the muscarinic stimulation of PIP₂ breakdown in arterial smooth muscles.

Data are accumulating to indicate that N_i is not the G-protein that mediates inositol phospholipid hydrolysis in at least the majority of cell types. Masters *et al.* (1985) reported that muscarinic-stimulation-induced PIP₂ breakdown and Ca²⁺ mobilization in cultured chick heart cells and 1321 N1 human astrocytoma cells was not inhibited by IAP treatment, although the muscarinic inhibition of β -adrenergic-agonist-induced cyclic AMP accumulation was attenuated by IAP treatment. Martin *et al.* (1985) obtained confirming evidence that a G-protein other than N_i was responsible for muscarinic stimulation in 1321 N1 cells, i.e. the treatment of membrane preparations with N-ethylmaleimide, which inactivated N_i by alkylation, did not affect the extent of guanine nucleotide-sensitive high-affinity binding of carbachol to muscarinic receptors. Furthermore, Murayama & Ui (1985) reported that the release of inositol from mouse 3T3 fibroblasts, stimulated by thrombin, bradykinin, platelet-activating factor or PA, was not inhibited by prior incubation with IAP, whereas the Ca²⁺ influx and resulting arachidonate release were markedly inhibited. Moreover, the prostaglandin-induced breakdown of PI was not susceptible to IAP in NG 108-15 cells (Kurose & Ui, 1985), and bradykinin-stimulated GTPase

Fig. 6. Effects of IAP treatment on the K⁺-induced and ACh-induced contractions evoked in arterial smooth muscles

(a) Contractions recorded from non-treated muscles (A), or from muscles treated with 30 ng of IAP/ml (B), or 100 ng of IAP/ml treated (C), induced by 135 mM-K⁺ (A-1, B-1, C-1), or by 10 μM-ACh in the presence (A-2, B-2, C-2) or the absence (A-3, B-3, C-3) of external Ca²⁺. (b) Effects of various concentrations of IAP on the maximum amplitude of contractions evoked by 135 mM-K⁺ (Δ), or by 10 μM-ACh in the presence (○) or the absence (●) of external Ca²⁺. Relative amplitudes of the contraction evoked by high K⁺ or ACh in the non-treated muscles were normalized as 1.0. Bars indicate S.E.M. (*n* = 5).

activity in the membrane preparation of NG 108-15 cells was not eliminated by prior incubation with IAP, although the muscarinic receptor may be coupled with N₁ (Grandt *et al.*, 1986). Houslay *et al.* (1986) reported that platelet-activating factor and U44069, which stimulate inositol phospholipid metabolism in human platelets, activate a GTPase which is distinct from N_s (a stimulatory G-protein of the adenylate cyclase system; Ui, 1984) and N₁, using cholera toxin and IAP respectively. Therefore whether or not G-protein involved

in signal transduction by Ca²⁺-mobilizing agonists is an IAP substrate seems to differ with the tissue and agonists. Further studies are required to identify the G-protein.

The mechanical responses in muscles are reflections of the changes in the myoplasmic free Ca²⁺ concentration (Kuriyama *et al.*, 1982). The increase in the myoplasmic free [Ca²⁺], as induced by ACh in the presence of external Ca²⁺, is mainly due to mobilization from Ca²⁺ stores. The receptor-operated Ca²⁺ influx may also contribute, but to a lesser extent, as the ACh-induced contraction was slightly larger and continued longer than was observed in the absence of Ca²⁺ in the solution. The treatment of muscle with IAP may not modify the mechanical machinery, since the high-K⁺-induced contraction was not altered by IAP treatment. However, the ACh-induced contraction was moderately inhibited by IAP treatment, with or without external Ca²⁺, thereby indicating that the myoplasmic Ca²⁺-increasing process is inhibited. It seems possible that the receptor-operated Ca²⁺ channel is closely associated with the IAP substrate N₁, because the continuation of the ACh-induced contraction in the presence of external Ca²⁺ was abolished in IAP-treated muscles. Such may be the case in neutrophils. The IAP treatment of neutrophils completely eliminated the chemotactic-peptide-stimulated Ca²⁺ influx (Okajima & Ui, 1984), probably caused by the opening of receptor-operated Ca²⁺ channels (Reuter, 1983). Another explanation for mechanisms underlying decreases in the contraction may be involvement of accumulation of cyclic AMP by IAP treatment, because cyclic AMP will inhibit the contraction (Itoh *et al.*, 1985). However, the cellular content of cyclic AMP remained unchanged in the IAP-treated muscle, in the absence or presence of ACh. Further experiments on the mechanism underlying the inhibitory action of IAP on the agonist-induced contraction are required.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. We thank M. Ohara for reading the manuscript.

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Received 28 April 1986/16 June 1986; accepted 1 July 1986