Volume 213, number 2, 353-358 FEB 04521 March 1987

A new GTP-binding protein in brain tissues serving as the specific substrate of islet-activating protein, pertussis toxin

Toshiaki Katada, Masayuki Oinuma, Kayoko Kusakabe and Michio Ui+

Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

Received 19 December 1986; revised version received 13 January 1987

A new GTP-binding protein serving as the specific substrate of islet-activating protein (IAP), pertussis toxin, was purified from porcine brain membranes as an $\alpha\beta\gamma$-heterotrimeric structure. The α-subunit of the purified protein ($\alpha_{40}\beta\gamma$) had a molecular mass of 40 kDa and differed from that of G_i ($\alpha_{41}\beta\gamma$) or G_o ($\alpha_{39}\beta\gamma$) previously purified from brain tissues. The fragmentation patterns of limited tryptic digestion and immunological cross-reactivities among the three α were different from one another. However, the $\beta\gamma$-subunit resolved from the three IAP substrates similarly inhibited a membrane-bound adenylate cyclase and their β-subunits were immunologically indistinguishable from one another. Thus, the $\alpha_{40}\beta\gamma$ is a new IAP substrate protein different from G_i or G_o, in the α-subunit only.

GTP-binding protein; Islet-activating protein; (Pertussis toxin)

1. INTRODUCTION

A family of structurally homologous and membrane-associated GTP-binding proteins is present in a variety of vertebrate cells. Functionally identified and characterized members of this family include the stimulatory (G_s) and inhibitory (G_i) GTP-binding proteins of the hormone-sensitive adenylate cyclase system [1] and the transducin (T) of vertebrate retina that communicates between light activation of rhodopsin and stimulation of cyclic GMP-dependent phosphodiesterase [2]. These proteins have been purified from several sources as heterotrimers with an $\alpha\beta\gamma$-subunit structure. The α-subunits of G_s, G_i and T have different molecular masses, viz. 45, 41 and 39 kDa, respectively, and represent the active species in the regulation of the target enzymes [1]. A fourth member of this homologous family, termed G_o, has recently been purified from brain tissues [3–6], although its function remains unknown. The α of G_o (α_o), the molecular mass of which is 39 kDa, as well as the α of G_i (α_i) contain a guanine nucleotide-binding site and a site for IAP-catalyzed ADP-ribosylation. The β-subunits (36 or 35 kDa) of the four proteins seem to be indistinguishable from each other, and the smallest γ has been reported to have a molecular mass of about 8 kDa [1].

Several lines of evidence have been provided for a novel role of some GTP-binding proteins in Ca^{2+}-related signal transduction [7]. In fact, IAP substrate proteins including G_i and G_o have been found to couple certain types of receptors to polyphosphoinositide breakdown in rat mast cells [8], guinea pig neutrophils [9,10] and differentiated human leukemic (HL-60) cells [11]. Moreover, it was reported that muscarinic receptors were linked to K^+ channels in an IAP-susceptible fashion in embryonic chick atrial cells

Correspondence address: T. Katada, Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

+ Present address: Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo 113, Japan

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

[12,13]. These reports prompted us to investigate the heterogeneities of GTP-binding proteins serving as the specific substrate of IAP, since multiple IAP substrates seemed to have multiple functions in receptor-mediated signal transduction. Here, we show that there is a novel IAP substrate in addition to G_i and G_o in brain tissues. The three IAP substrates are purified separately and studied for biological and immunochemical properties.

2. MATERIALS AND METHODS

2.1. *Purification of IAP substrates from porcine brain membranes*

The procedures employed to purify three IAP substrates from porcine brain membranes are based on the methods developed to purify G_i and G_o from rat brain [6] with the following modification. After the three chromatographies with DEAE-Sephacel, Ultrogel AcA-34 and DEAE-Toyopearl 650(S), an aliquot (15 nmol) of the fractions containing IAP substrate activity was diluted with 4 vols Tris-HCl (pH 8.0)/1 mM NaEDTA/1 mM dithiothreitol (TED) containing 0.7% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps) and then applied to a Mono Q HR5/5 column which had been equilibrated with TED/0.7% Chaps/125 mM NaCl. The column was washed and eluted at a flow rate of 1 ml/min with the following series of NaCl gradients using a Pharmacia FPLC system; 125 mM for 5 min; 125–250 mM over 30 min; 250–1000 mM over 5 min. The eluate was collected in fractions of 1 ml. Fig.1A details the activities measured in the eluted fractions. This chromatography resulted in a partial separation of three peaks of IAP-substrate and guanosine 5′-*O*-(thio)triphosphate (GTPγS)-binding activities; the first peak (I, fractions 13–15), second peak (II, fractions 17–19) and third minor peak (III, fractions 20–22). Each peak was diluted with an equal volume of TED/0.7% Chaps and further purified by rechromatography on the Mono Q column under the same conditions. Thus, three IAP substrates were purified to almost homogeneity (fig.1B). Resolution of each IAP substrate to GTPγS-bound α- and βγ-subunits was performed by high-performance gel filtration using a TSK-3000 SW column as in [14]. The polypeptide composition of the purified proteins was analyzed

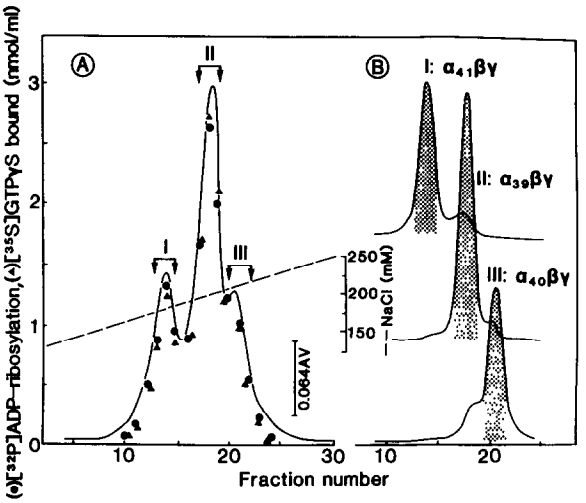

Fig.1. Mono Q chromatography of IAP substrate proteins from porcine brain membranes. (A) IAP substrate-rich fractions were applied to Mono Q and eluted as described in section 2. Aliquots (10 μl) of fractions were assayed for IAP-substrate (●) and ^{35}S-labeled GTPγS-binding (▲) activities. The absorbance at 280 nm of the eluted protein was also monitored (——). (B) Each peak (I, II and III in A) from the Mono Q was further purified by rechromatography on Mono Q under the same conditions.

by SDS-polyacrylamide gel electrophoresis and is shown in fig.2.

2.2. *Assay of activities*

IAP substrate protein was identified by its abilities to be ^{32}P-labeled, ADP-ribosylation in the presence of IAP and ^{32}P-labeled NAD and to bind ^{35}S-labeled GTPγS as in [6,14]. Electrophoresis of polypeptides through SDS-polyacrylamide gels and the treatment of the samples with *N*-ethylmaleimide are described in [3,6]. Adenylate cyclase activity of human platelet membranes was estimated as in [6,15].

2.3. *Preparation of affinity-purified antibodies against α- and βγ-subunits of IAP substrates and immunoblot procedures*

Three groups (each consisting of one or two) of rabbits were injected intradermally with purified rat brain α_i, α_o or βγ in Freund's complete adjuvant (2 ml, 100–200 μg protein per animal), followed by three injections with half as much antigens and at intervals of 2–3 weeks. Animals were

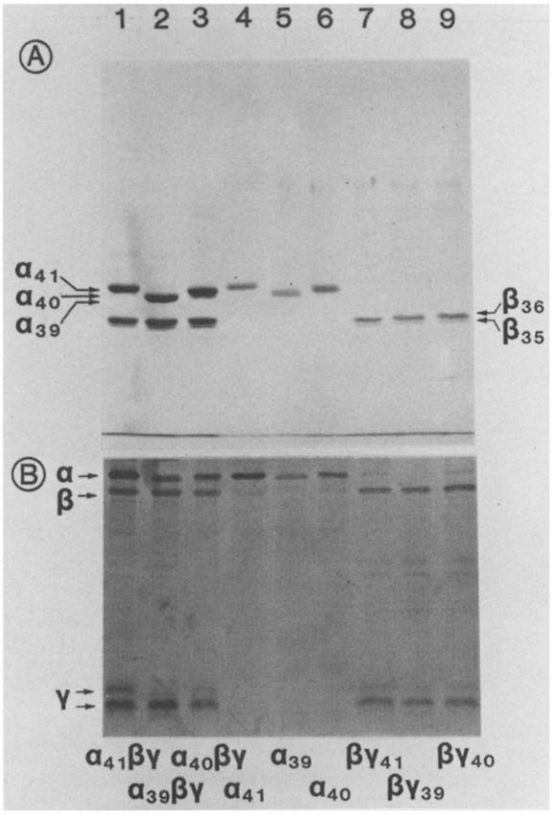

Fig.2. SDS-polyacrylamide gel electrophoresis of IAP substrates purified from porcine brain membranes. Proteins were subjected to SDS-polyacrylamide gel (10% for A and 15% for B) electrophoresis and stained with either Coomassie blue (A) or silver (B) as described in section 2. Lanes: (1,2,3) peak I, II and III in fig.1B, respectively (1 μg for A and 0.2 μg for B); (4,5,6) α-subunits resolved from peak I, II and III, respectively (0.2 μg for A and 0.1 μg for B); (7,8,9) $\beta\gamma$-subunits resolved from peak I, II and III, respectively (0.2 μg for A and 0.1 μg for B).

bled 2 weeks after the final injection. For affinity purification of antisera, 1–2 mg purified α_i, α_o or $\beta\gamma$ from rat brain was coupled to activated CH-Sepharose 4B (Pharmacia) to a final concentration of ~0.5 mg protein per ml gel. Each whole serum or the 40% $(NH_4)_2SO_4$-precipitated fraction was passed through the cognate subunit-immobilized Sepharose, and after extensive washing with 0.1 M $NaHCO_3$/0.5 M NaCl (pH 8.3), specifically bound antibodies were eluted with 0.2 M glycine-HCl (pH 2.5). The eluate was immediately adjusted to pH 7–8 and filtered through Sephadex G-25 (fine) in phosphate-buffered saline containing 0.05% NaN_3. Immunoblot analysis is described in [16].

2.4. *Tryptic digestion of ^{32}P-labeled ADP-ribosylated IAP substrates*

Three IAP substrates purified from porcine brain were incubated at 30°C for 30 min in a reaction mixture containing IAP and ^{32}P-labeled NAD (5000 cpm/pmol) as in [6]. After the incubation, aliquots (500 μl) of the mixture were filtered through a 10 ml column of Sephadex G-25 (fine) in TED/0.05% Lubrol-PX. The radiolabeled IAP substrates (approx. 20 μg/ml), which emerged in the void volume from the columns, were digested at 30°C with 2 μg/ml of tosylphenylalanyl chloromethyl ketone-treated β-trypsin in the above buffer. At the indicated times, aliquots (25 μl) of the reaction mixture were removed and mixed with 5 μl soybean trypsin inhibitor (50 μg/ml) containing 10% SDS. The samples were treated with *N*-ethylmaleimide [3] and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography.

3. RESULTS AND DISCUSSION

In [6], we purified two $\alpha\beta\gamma$-trimeric proteins (G_i and G_o) with IAP-substrate and GTPγS-binding activities from rat brain membranes. They differed from each other in α only; the molecular mass of the two α was 41 kDa (α_i) and 39 kDa (α_o) and α_i could inhibit the adenylate cyclase catalyst, whereas α_o could not [14]. In the course of further studies, however, we found that IAP substrates in brain tissues were more heterogeneous. There were three peaks of IAP-substrate and GTPγS-binding activities after Mono Q column chromatography of IAP substrate-rich fractions from porcine brain membranes (fig.1). Thus, we obtained three $\alpha\beta\gamma$-heterotrimeric IAP substrate proteins (fig.2). These heterogeneous IAP substrates are referred to as $\alpha_{41}\beta\gamma$, $\alpha_{39}\beta\gamma$ and $\alpha_{40}\beta\gamma$, respectively, based on the molecular masses of their α-subunits. The three α were the target of IAP-catalyzed ADP-ribosylation (see figs 3,4) and contained the sites of GTPγS binding (see fig.5). The order of the contents of these proteins was $\alpha_{39}\beta\gamma > \alpha_{41}\beta\gamma > \alpha_{40}\beta\gamma$ in a number of preparations from porcine and rat (not shown) brain membranes.

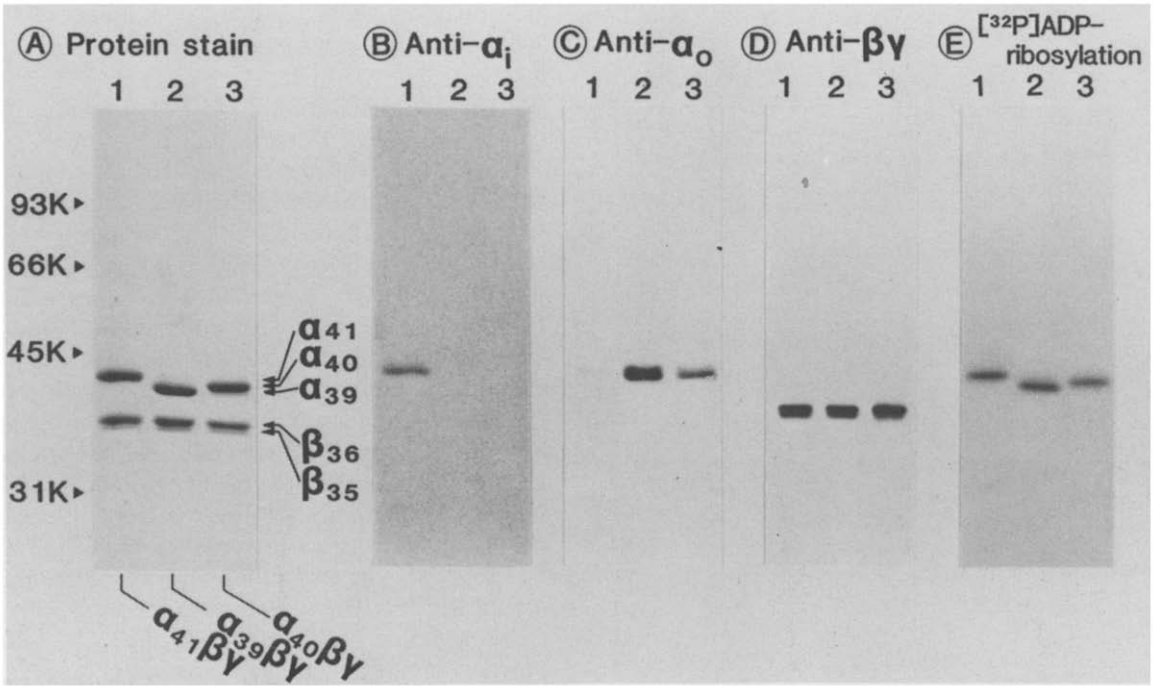

Fig.3. Cross-reaction of porcine brain IAP substrates with affinity-purified antibodies specific for the α and $\beta\gamma$ of rat brain IAP substrates. Three IAP substrates (1 μg) purified from porcine brain were subjected to SDS-polyacrylamide gel (10%) electrophoresis and analyzed for immunoblot as described in section 2. Lanes: (1,2,3) peak I ($\alpha_{41}\beta\gamma$), II ($\alpha_{39}\beta\gamma$) and III ($\alpha_{40}\beta\gamma$) in fig.1B, respectively. (A) Coomassie blue-stained gels, (B) anti-rat α_i antibody, (C) anti-rat α_o antibody, (D) anti-rat $\beta\gamma$ antibody, (E) ^{32}P-labeled ADP-ribosylated α.

As shown in fig.3, antibody raised against rat brain α_i (anti-α_i) reacted with the α_{41} of porcine brain $\alpha_{41}\beta\gamma$. On the other hand, antibody raised against rat brain α_o (anti-α_o) reacted with the α_{39} of porcine brain $\alpha_{39}\beta\gamma$. Thus, the $\alpha_{41}\beta\gamma$ and $\alpha_{39}\beta\gamma$ purified in the present study are the same entities as G_i and G_o, respectively, purified from rat brain [6]. However, the porcine brain $\alpha_{40}\beta\gamma$ was a new IAP substrate protein different from G_i or G_o, since neither anti-α_i nor anti-α_o cross-reacted with the α_{40}. Antibody raised against rat brain $\beta\gamma$ reacted with all the β (36/35 kDa doublet) of the porcine brain IAP substrates, suggesting that the β of the three IAP substrates are the same polypeptide or at least share a common antigenic determinant(s).

Differences among the three α of porcine brain IAP substrates were further supported by the results of the limited tryptic digestion of [^{32}P]ADP-ribosylated IAP substrates (fig.4). The limited fragmentation patterns of the α in the presence of 20 μM $AlCl_3$, 6 mM $MgCl_2$ and 10 mM NaF (AMF) were quite different from those in its absence, confirming our previous results that there was a conformational change in α in the presence of AMF [6]. The ^{32}P-labeled ADP-ribose of the three α was transferred exclusively to the 38–37 kDa fragment when the radiolabeled IAP substrates were digested in the presence of AMF (fig.4B). In the absence of AMF, the majority of the radioactive ADP-ribose was located in the 17–15 kDa fragment (fig.4A). The proteolytic fragments containing ADP-ribose arising from the three α were not identical to one another, although there was a general similarity of the fragmentation patterns among the three α in the presence or absence of AMF.

Fig.5 shows the effects of the porcine brain IAP substrates and their constituent subunits on the adenylate cyclase activity of human platelet membranes. All of the three GTPγS-bound IAP substrates were potent inhibitors of the adenylate

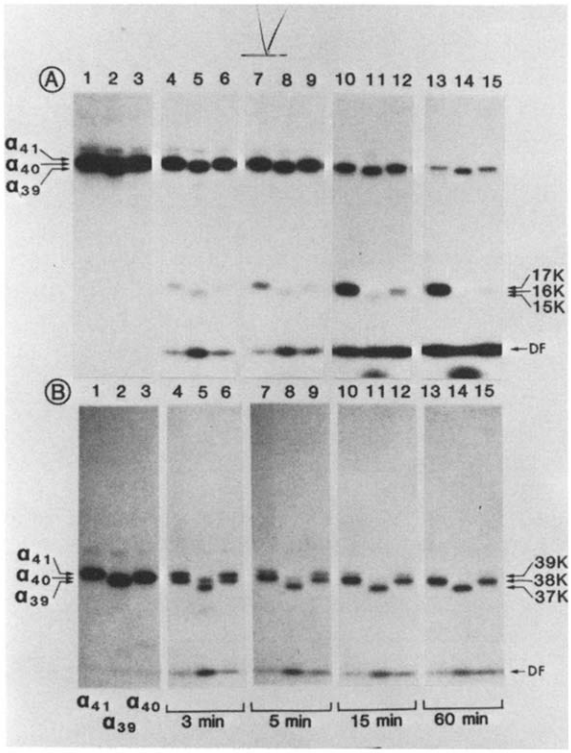

Fig.4. Limited tryptic digestion of ADP-ribosylated porcine brain IAP substrates. Three IAP substrates purified from porcine brain were ^{32}P-labeled ADP-ribosylated and then digested with trypsin in the presence (B) or absence (A) of AMF as described in section 2. At the indicated times, aliquots of the digestion mixture were removed and subjected to SDS-polyacrylamide gel (15% for A and 10% for B) electrophoresis and autoradiography. Lanes: (1,4,7,10,13) peak I ($\alpha_{41}\beta\gamma$) in fig.1B; (2,5,8,11,14) peak II ($\alpha_{39}\beta\gamma$) in fig.1B; (3,6,9,12,15) peak III ($\alpha_{40}\beta\gamma$) in fig.1B; DF, dye front.

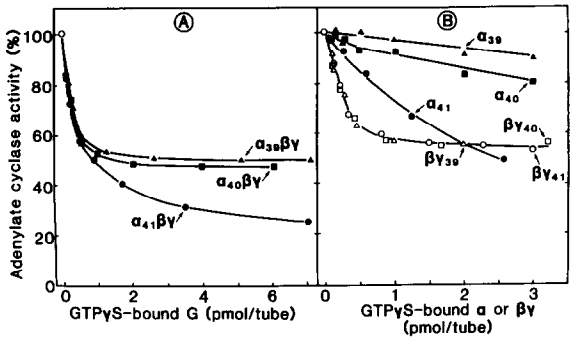

Fig.5. Effects of porcine brain IAP substrates and their constituent α- and $\beta\gamma$-subunits on the adenylate cyclase activity of human platelet membranes. (A) The three IAP substrates (100 μg) were incubated at 30°C for 60 min in 500 μl TED/0.7% Chaps/25 mM $MgCl_2$ containing 5 μM ^{35}S-labeled GTPγS (1000 cpm/pmol) and then filtered through a 10 ml column of Sephadex G-25 in TED (with 0.1 mM EDTA)/1 mM $MgCl_2$/0.7% Chaps. 10-μl aliquots of the GTPγS-bound IAP substrates, which emerged in the void volume from the column, were incubated at 30°C for 30 min with 90 μl of a reaction mixture containing 30 μg of human platelet membranes and 10 μM forskolin. The adenylate cyclase activities were assayed as described in section 2. (B) The GTPγS-bound α and $\beta\gamma$ were obtained from each IAP substrate as described in section 2 and assayed for adenylate cyclase activity as in A. The values are expressed as percentages of the control value obtained without purified proteins (10 μl of a buffer solution used for the G-25 or TSK column elution), which was 420 and 405 pmol cAMP formed/tube for A and B, respectively.

cyclase (fig.5A). Differences were noted, however, in that the inhibition caused by GTPγS-bound $\alpha_{39}\beta\gamma$ or $\alpha_{40}\beta\gamma$ appeared to be saturable at 20 nM (2 pmol/assay tube), while the inhibitory action of $\alpha_{41}\beta\gamma$ was more potent; the degree of the inhibition by GTPγS-bound $\alpha_{41}\beta\gamma$ was apparently larger than that by GTPγS-bound $\alpha_{39}\beta\gamma$ or $\alpha_{40}\beta\gamma$. In previous papers [6,14,15], we have shown that the incubation of G_i (or G_o) with GTPγS results in dissociation into GTPγS-bound α and $\beta\gamma$. Therefore, the effects of GTPγS-bound α and $\beta\gamma$ resolved from each porcine brain IAP substrates were further examined. The $\beta\gamma$ resolved from $\alpha_{41}\beta\gamma$ or $\alpha_{39}\beta\gamma$ was a potent inhibitor of the cyclase (fig.5B), confirming the previous results [6]. The $\beta\gamma$ resolved from the new IAP substrate, $\alpha_{40}\beta\gamma$, also inhibited the cyclase activity in a fashion similar to that from $\alpha_{41}\beta\gamma$ or $\alpha_{39}\beta\gamma$. In contrast to the action of $\beta\gamma$, only the GTPγS-bound α_{41} effectively inhibited the cyclase activity. However, the inhibition by the α_{41} did not saturate at 25 nM; this species inhibited adenylate cyclase with lower apparent affinity than did the resolved $\beta\gamma$. Slight inhibition caused by high concentrations of GTPγS-bound α_{39} or α_{40} would be explained by contamination of these preparations with a few percent of $\beta\gamma$.

In this report, we have purified three IAP substrates from porcine brain membranes. One of them, $\alpha_{40}\beta\gamma$, was a novel IAP substrate protein; the $\alpha_{40}\beta\gamma$ differed from $G_i(\alpha_{41}\beta\gamma)$ or G_o ($\alpha_{39}\beta\gamma$) in

the α only, based on the following observations. (i) The electrophoretic mobilities through SDS-polyacrylamide gels among the three α were not identical to one another either before or after IAP-catalyzed ADP-ribosylation (figs 2,3). (ii) Limited proteolysis of the ADP-ribosylated α with trypsin yielded product formation which were different between α_{40} and α_{41} or α_{39} (fig.4). (iii) No cross-reaction was observed between α_{40} and the affinity-purified antibodies raised against α_i or α_o (fig.3). (iv) Only GTPγS-bound α_{41} could effectively inhibit membrane-bound adenylate cyclase, whereas GTPγS-bound α_{40} or α_{39} could not (fig.5B). (v) The $\beta\gamma$ resolved from the three IAP substrates inhibited the adenylate cyclase in a fashion similar to one another (fig.5B). (vi) The three β were recognized by affinity-purified antibody raised against the $\beta\gamma$ of rat brain IAP substrates (fig.3).

What is the physiological role of the new IAP substrates ($\alpha_{40}\beta\gamma$)? Since $\beta\gamma$ seems to be an important component responsible for adenylate cyclase inhibition, $\alpha_{40}\beta\gamma$ must be as good a communicator as G_i and G_o in receptor-linked inhibition of the cyclase. Nevertheless, it is tempting to speculate that α_{40} (and α_{39}) is capable of interacting with an enzyme system other than adenylate cyclase, since recent studies have revealed that prior exposure of cells to IAP prevents the cell responses to receptor stimulation in a cAMP-independent manner (see section 1).

ACKNOWLEDGEMENTS

This work was supported by research grants from the Scientific Research Fund of the Ministry of Education, Science, and Culture, Japan.

REFERENCES

[1] Gilman, A.G. (1986) Trends Neurosci. 9, 460–463.
[2] Fung, B.K.-K., Hurley, J.G. and Stryer, L. (1981) Proc. Natl. Acad. Sci. USA 78, 152–156.
[3] Sternweis, P.C. and Robishaw, J.D. (1984) J. Biol. Chem. 259, 13806–13813.
[4] Neer, E.J., Lok, J.M. and Wolf, L.G. (1984) J. Biol. Chem. 259, 14222–14229.
[5] Milligan, G. and Klee, W.A. (1985) J. Biol. Chem. 260, 2057–2063.
[6] Katada, T., Oinuma, M. and Ui, M. (1986) J. Biol. Chem. 261, 8182–8191.
[7] Ui, M. (1984) Trends Pharmacol. Sci. 5, 277–279.
[8] Nakamura, T. and Ui, M. (1985) J. Biol. Chem. 260, 3584–3593.
[9] Okajima, F. and Ui, M. (1984) J. Biol. Chem. 259, 13863–13871.
[10] Okajima, F., Katada, T. and Ui, M. (1985) J. Biol. Chem. 260, 6761–6768.
[11] Kikuchi, A., Kozawa, O., Kaibuchi, K., Katada, T., Ui, M. and Takai, Y. (1986) J. Biol. Chem. 261, 11558–11562.
[12] Pfaffinger, P.J., Martin, J.M., Hunter, D.D., Nathanson, N.M. and Hille, B. (1985) Nature 317, 536–538.
[13] Martin, J.M., Hunter, D.D. and Nathanson, N.M. (1985) Biochemistry 24, 7521–7525.
[14] Katada, T., Oinuma, M. and Ui, M. (1986) J. Biol. Chem. 261, 5215–5221.
[15] Katada, T., Bokoch, G.M., Northup, J.K., Ui, M. and Gilman, A.G. (1984) J. Biol. Chem. 259, 3568–3577.
[16] Oinuma, M., Katada, T., Yokosawa, H. and Ui, M. (1986) FEBS Lett. 207, 28–34.