Adenylate cyclase inhibition and GTPase stimulation by somatostatin in S49 lymphoma cyc⁻ variants are prevented by islet-activating protein

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cyc⁻-Variants of S49 lymphoma cells are defective in the stimulatory guanine nucleotide site of the adenylate cyclase but contain an inhibitory site. Treatment of cyc⁻ cells with islet-activating protein (IAP), which causes ADP-ribosylation of an M_r 40000 polypeptide in cyc⁻ membranes, abolishes adenylate cyclase inhibition by GTP and the peptide hormone, somatostatin, but not that induced by GTPγS. Furthermore, somatostatin-induced stimulation of GTP hydrolysis is lost. Thus, the data indicate that IAP interferes with the adenylate cyclase system by an action at the inhibitory guanine nucleotide site.

*Islet-activating protein (*Bordetella pertussis *toxin) Adenylate cyclase GTPase Somatostatin Inhibitory guanine nucleotide site*

1. INTRODUCTION

cyc⁻-Variants of S49 lymphoma cells are deficient in the guanine nucleotide-binding regulatory site (N_s) mediating adenylate cyclase stimulation by hormones, guanine nucleotides and cholera toxin [1]. However, the cyc⁻ adenylate cyclase can be inhibited by GTP and particularly by stable GTP analogs such as guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) and guanyl 5′-yl-imidodiphosphate [2,3]. This finding suggested that the N_s-deficient cyc⁻ membranes contain a guanine nucleotide regulatory site (N_i) mediating adenylate cyclase inhibition. The evidence for a N_i component in cyc⁻ membranes has been corroborated by the finding that the cyc⁻ adenylate cyclase is inhibited by the peptide hormone, somatostatin [4]. Similarly as described for hormonal inhibition in various 'complete' cellular systems [5,6], the somatostatin-induced inhibition of cyc⁻ adenylate cyclase is a GTP-dependent process. Furthermore, somatostatin increases GTP hydrolysis in cyc⁻ membranes due to stimulation of a high affinity GTPase [4].

Islet-activating protein (IAP), a *Bordetella pertussis* toxin, catalyzes ADP-ribosylation of an M_r 41000 polypeptide in various cellular systems [7–9]. The substrate for ADP-ribosylation by IAP is apparently composed of M_r 41000 and 35000 subunits and is able to bind guanine nucleotides [10]. Since after IAP treatment the adenylate cyclase inhibition by hormones and GTP was impaired, it has been suggested that IAP by its ADP-ribosylating activity somehow blocks the function of N_i [9]. S49 Lymphoma cyc⁻ variants apparently containing only N_i but not N_s are, thus, an ideal system for testing the question of IAPs action in the adenylate cyclase system. We report here that IAP treatment of cyc⁻ cells prevents somatostatin-induced adenylate cyclase inhibition and GTPase stimulation but not adenylate cyclase inhibition induced by the stable GTP analog, GTPγS.

2. MATERIALS AND METHODS

2.1. *Materials*

IAP was purified to apparent homogeneity on hydroxyapatite columns and haptoglobulin–

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Sepharose columns according to [11] from the supernatant of *Bordetella pertussis* suspensions kindly provided by Drs L. Robbel and F. Blackkolb (Behringwerke, Marburg). Forskolin was donated by Dr H. Metzger (Hoechst AG, Frankfurt). Somatostatin and cholera toxin were obtained from Sigma. [α-^{32}P]NAD (50 Ci/mmol) and [^{14}C]methylated protein mixture as weight markers for gel electrophoresis were from Amersham-Buchler. Other materials were as in [3,4].

2.2. *IAP treatment of S49 lymphoma cyc$^-$ cells*

S49 Lymphoma cyc$^-$ variants were grown in Dulbecco's modified Eagle's medium. Where indicated, the culture medium was fortified with 20 ng IAP/ml to provide IAP-treated cells; the vehicle used for dissolving IAP [11] was added for preparation of control cells. After 24 h treatment, membranes of IAP-treated and control cells were prepared as in [3]. Preparation of membranes of wild-type S49 lymphoma cells was done in an identical manner.

2.3. *Radiolabelling*

Membranes of control cyc$^-$ variants and wild-type cells (~250 μg protein/tube) were incubated without and with 25 μg IAP/ml, 300 μg/ml of cholera toxin (preactivated for 10 min at 37°C with 25 mM dithiothreitol) or their combination for 30 min at 37°C in a reaction buffer containing 1 μM [α-^{32}P]NAD (5–10 μCi/tube), 1 mM ATP, 1 mM GTP, 5 mM $MgCl_2$, 10 mM thymidine, 10 mM arginine, 5 mM creatine phosphate, 0.4 mg creatine kinase/ml and 50 mM triethanolamine–HCl (pH 7.4) in 220 μl final vol. Thereafter, the membranes were pelleted and washed twice with 1 ml 10 mM triethanolamine–HCl (pH 7.4). Radiolabelled membranes were dissolved in 50 μl of a gel sample buffer (4% sodium dodecyl sulfate, 0.002% bromphenol blue, 23% glycerol, 10% 2-mercaptoethanol, 62.5 mM Tris–HCl, pH 6.8) and heated for 5 min at 100°C. Aliquots (25 μl) were subjected to dodecyl sulfate–polyacrylamide gel electrophoresis [12] on slab gels (1.5 mm thick), which consisted of a 8% separating gel and a 3% stacking gel. After electrophoresis, gels were stained with Coomassie brilliant blue, destained, dried and exposed to Kodak X-Omat AR-film for 48–96 h at −80°C.

2.4. *Adenylate cyclase and GTPase assays*

Adenylate cyclase activity was determined as in [4] with 50 μM [α-^{32}P]ATP (~0.4 μCi/tube), 100 μM $MnCl_2$, 0.1 mM cyclic AMP, 1 mM 3-isobutyl-1-methylxanthine, 5 mM creatine phosphate, 0.4 mg creatine kinase/ml, 1 mg bacitracin/ml, 2 mg bovine serum albumin/ml, 100 μM forskolin and 50 mM triethanolamine–HCl (pH 7.4) in 100 μl total vol. Reactions were initiated by the addition of cyc$^-$ membranes (20–30 μg protein/tube) and conducted for 10 min at 30°C. Cyclic AMP formed was isolated as in [13]. GTPase activity was determined as in [4] with 0.5 μM [γ-^{32}P]GTP (~0.1 μCi/tube), 3 mM $MgCl_2$, 1 mM EDTA, 0.1 mM ATP, 1 mM adenyl 5′-yl-imidodiphosphate, 0.1 mM cyclic AMP, 1 mM 3-isobutyl-1-methylxanthine, 5 mM creatine phosphate, 0.4 mg creatine kinase/ml, 1 mg bacitracin/ml, 2 mg bovine serum albumin/ml and 50 mM triethanolamine–HCl (pH 7.4). Incubation with cyc$^-$ membranes (5–10 μg protein/tube) was for 10 min at 30°C. Specific, low K_m GTPase activity was determined as in [4].

3. RESULTS

As described in [1], cholera toxin radiolabelled, in the presence of [α-^{32}P]NAD, two polypeptides with M_r 43000 and 50000 in wild-type but not in cyc$^-$ membranes (fig.1). In contrast, radiolabelling by IAP of a polypeptide with M_r 40000 was observed in both wild-type and control cyc$^-$ membranes. Cholera toxin and IAP caused additional radiolabelling of polypeptides with M_r 21000 and 27000, respectively, which appear to be auto-ADP-ribosylation products of subunits of the toxins [10]. Thus, IAP radiolabels a polypeptide in cyc$^-$ membranes and the radiolabelling pattern is similar to that observed in various other cellular systems [7–10]. Therefore, it was studied whether treatment of cyc$^-$ cells affects the adenylate cyclase and GTPase regulations by somatostatin.

In the presence of 3 μM GTP, somatostatin caused a concentration-dependent decrease in 100 μM forskolin-stimulated adenylate cyclase activity in membranes of control cyc$^-$ variants (fig.2). The inhibition was half-maximal at 1–3 nM and maximal inhibition by ~20% was observed at 30 nM somatostatin. In contrast, in membranes of IAP-treated cells, somatostatin, up

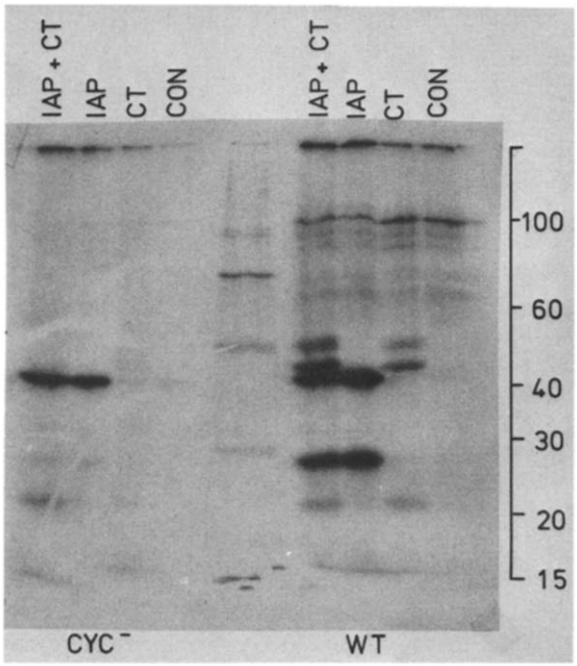

Fig.1. Polyacrylamide gel analysis of radioactive products resulting from IAP or cholera toxin-catalyzed ADP-ribosylation of control cyc⁻ and wild-type S49 lymphoma membranes. Membranes of untreated cyc⁻ variants (CYC⁻, left) and wild-type S49 lymphoma cells (WT, right) were incubated without (CON) and with cholera toxin (CT), IAP or both as indicated in the presence of [α-^{32}P]NAD. Thus labelled membranes were submitted to electrophoresis and autoradiography as in section 2. ^{14}C-Labelled M_r-markers (phosphorylase *b*, 92500; bovine serum albumin, 69000; ovalbumin, 46000; carbonic anhydrase, 30000; lysozyme, 14300) are shown in the middle lanes.

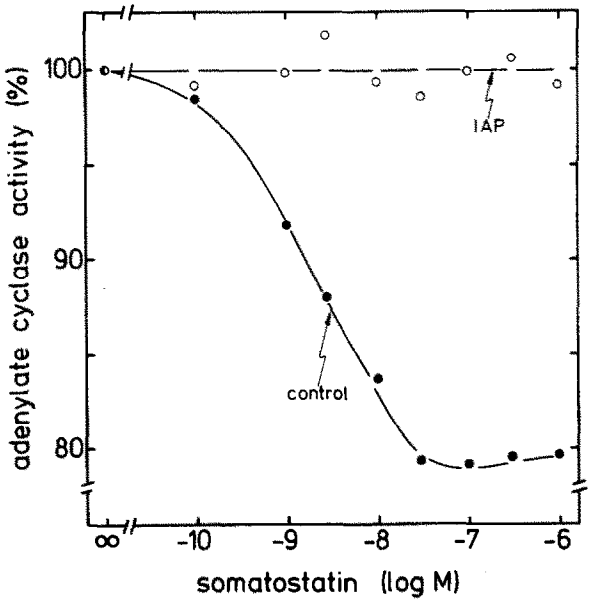

Fig.2. Influence of somatostatin on adenylate cyclase activity in membranes of control and IAP-treated cyc⁻ cells. Adenylate cyclase activity was determined in membranes of control and IAP-treated cyc⁻ cells without and with somatostatin at the indicated concentrations. Forskolin (100 μM) and GTP (3 μM) were present under each condition. Enzyme activity is given as % of control activity measured without somatostatin. Similar data were obtained in 3 separate expt.

to 1 μM, had no effect on adenylate cyclase activity stimulated by forskolin. Similar data were obtained when the effect of somatostatin was studied on the high affinity GTPase activity. In membranes of control cyc⁻ cells, somatostatin stimulated the high affinity GTPase by up to about 35% (fig.3). Half-maximal and maximal stimulations of the GTPase were observed at concentrations of the hormone similar to those required for adenylate cyclase inhibition. Again, in membranes of IAP-treated cyc⁻ cells, somatostatin had no effect on the high affinity GTPase activity.

The cyc⁻ adenylate cyclase is not only inhibited by somatostatin. As shown before [2,3], the en-

Table 1

Influence of IAP on cyc⁻ adenylate cyclase inhibition by guanine nucleotides

Additions	Adenylate cyclase (pmol cAMP.min^{-1}.mg protein^{-1})	
	Control	IAP
None	152 ± 5	142 ± 6
GTP (10 μM)	130 ± 4	140 ± 4
GTPγS (0.1 μM)	98 ± 4	94 ± 5
GTP + GTPγS	127 ± 6	140 ± 5

Adenylate cyclase activity was determined in membranes of control and IAP-treated cyc⁻ variants without and with GTP and GTPγS as indicated after a 10 min preincubation period with all reagents present except for labelled ATP. Forskolin (100 μM) was present under each condition. Mean ± SEM of triplicates are given

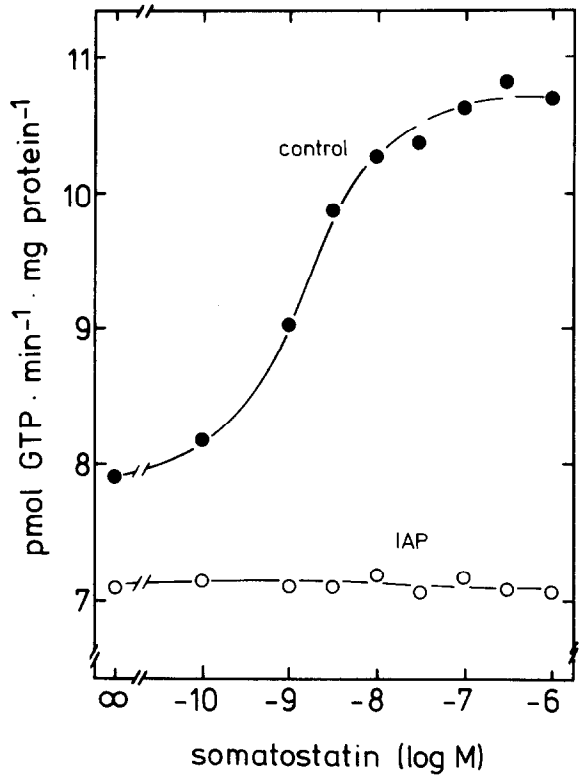

Fig.3. Influence of somatostatin on high affinity GTPase activity in membranes of control and IAP-treated cyc⁻ cells. GTPase activity was determined in membranes of control and IAP-treated cyc⁻ cells without and with somatostatin at the indicated concentrations. High affinity GTPase activity estimated as in [4] is given on the ordinate. Similar data were obtained in 3 separate expt.

zyme can also be inhibited by GTP and by stable GTP analogs. Therefore, it was studied whether IAP treatment affects the enzyme inhibition by these guanine nucleotides. In control membranes, GTPγS (0.1 μM) decreased the forskolin-stimulated activity by ~35% (table 1). GTP, which is less potent and less efficient than GTPγS [2,3], caused a decrease in activity by only ~15% at 10 μM. In membranes of IAP-treated cells, the small inhibition induced by GTP was abolished. However, the inhibition induced by GTPγS was largely unaffected in IAP-treated membranes. Furthermore, GTP antagonized the GTPγS-induced inhibition both in membranes of control and IAP-treated cells. Similar data with regard to radiolabelling by IAP and to the effects of IAP on cyc⁻ adenylate cyclase inhibition by GTP and a stable GTP analog have been reported [14].

4. DISCUSSION

Membranes of cyc⁻ variants of S49 lymphoma cells are deficient in the stimulatory guanine nucleotide site, N_s [1], but these membranes apparently contain the inhibitory guanine nucleotide site, N_i [2–4]. This site appears to mediate cyc⁻ adenylate cyclase inhibition by GTP, stable GTP analogs and the peptide hormone, somatostatin. The *Bordetella pertussis* toxin, IAP, which has been shown to cause an ADP-ribosylation of an M_r 41000 polypeptide in various membrane systems [7–10], also radiolabels in the presence of [α-^{32}P]NAD a polypeptide with a similar M_r in cyc⁻ membranes [14] (fig.1). Functional studies performed with IAP-treated N_s- and N_i-containing cells suggested that the toxin causes a loss of receptor-mediated and GTP-dependent adenylate cyclase inhibition by affecting the involved coupling component, N_i [9]. Therefore, the influence of IAP was studied on adenylate cyclase inhibition by GTP, the stable GTP analog, GTPγS, and by somatostatin in N_s-deficient cyc⁻ membranes and on somatostatin-induced stimulation of a high affinity GTPase in these membranes.

These data show that treatment of cyc⁻ cells with IAP abolishes the GTP-dependent, somatostatin-induced adenylate cyclase inhibition (fig.2). Furthermore, IAP treatment prevented the hormone-induced stimulation of a high affinity GTPase in cyc⁻ membranes (fig.3). In membranes of neuroblastoma × glioma hybrid cells [15] and rat adipocytes [16], hormonal stimulation of the GTPase was also abolished after IAP treatment. Thus, IAP by its ADP-ribosylating activity appears to induce a loss of the function of N_i. Similar to cholera toxin, causing an inhibition of N_s-associated GTPase stimulation [17], IAP appears to exert its effect by inhibition of N_i-associated GTP hydrolysis. In contrast to cholera toxin, however, which amplifies the effects of GTP and stimulatory hormones at N_s [17], the IAP-induced inhibition of GTP hydrolysis at N_i was not accompanied by an increased inhibition of adenylate cyclase by GTP or an inhibitory hormone. On the contrary, IAP abolished their effects. These data suggest that a GTP hydrolysis step is involved in

N_i-mediated adenylate cyclase inhibition. However, the cyc$^-$ adenylate cyclase inhibition induced by stable GTP analogs was not affected by the IAP treatment [14] (table 1).

There are several possibilities for explanation of these apparently contradictory results:

(i) Stable GTP analogs may induce cyc$^-$ adenylate cyclase inhibition by a mechanism not involving N_i, which mediates GTP and somatostatin-induced inhibition. However, the data reported so far suggest that stable GTP analogs and GTP interact at one regulatory site [2,3].

(ii) IAP treatment may prevent the binding of GTP but not that of GTPγS at N_i. This possibility could explain both the loss of adenylate cyclase inhibition by GTP and somatostatin and the loss of GTPase stimulation by the hormone. However, the IAP treatment did not abolish the competitive type of interaction between GTP and GTPγS (table 1), suggesting that the binding of GTP is not affected by IAP.

(iii) Whereas GTP hydrolysis is somehow required for N_i-mediated adenylate cyclase inhibition by GTP and somatostatin, stable GTP analogs may induce a conformational alteration of N_i, which is not affected by IAP and which leads to a persistent adenylate cyclase inhibition. It is not yet clear what the role of GTP hydrolysis is in N_i-mediated adenylate cyclase inhibition. This GTP hydrolysis may provide free energy for driving a cycle, but it may also represent a rapid phosphorylation-dephosphorylation reaction involved in GTP and hormone-induced adenylate cyclase inhibition.

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