PROTEOLYSIS ACTIVATES ADENYLATE CYCLASE IN RAT LIVER AND AC⁻ LYMPHOMA CELL INDEPENDENTLY OF THE GUANINE NUCLEOTIDE REGULATORY SITE

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1. Introduction

Adenylate cyclase activity is normally under the control of modulating factors such as various hormones, neutrotransmitters, cations and adenosine. Guanine nucleotides seem to be necessary for the expression of stimulatory as well as inhibitory regulations, probably via binding to two distinct nucleotides binding proteins (N_s and N_i, respectively) [1–3]. Mild proteolysis can also produce an activation of the adenylate cyclase of rat liver plasma membranes [4–7], cultured fibroblasts [8,9], kidney cells [10], rat ovarian membranes [11,12] and rat cerebral cortical membranes [13]. In some cases, the stimulatory effect of proteolysis was more marked, or uniquely observed, in the presence of GTP [8–10,14].

Here, we demonstrate that proteolysis activates cyclase in rat liver plasma membrane and in the AC⁻ mutant of the S49 lymphoma cell line, independently of the GTP regulatory component (which will be refered to as N).

2. Experimental

Dulbecco-Vogt minimum essential medium was obtained from the NIH media unit. Heat-treated horse serum, glutamine, and penicillin–streptomycin were purchased from GIBCO. Purified α-chymotrypsin (lot 6485415) was from Worthington. Soybean trypsin inhibitor, creatine phosphate, creatine kinase, ATP synthesized from adenosine, DNase I and cholera toxin were from Sigma. [α-^{32}P]ATP (30 Ci/mmol) was from Amersham; cyclic [8-^{3}H]AMP (13 Ci/mmol) was obtained from CEA (Saclay).

The AC⁻ clone of S49 murine lymphoma cells (94.15.1) was kindly provided by Dr Henry Bourne (Dept. Pharmacol. UCSF). The cells were propagated in Dulbecco-Vogt medium containing 10% heat-treated horse serum essentially as in [15]. Cells were harvested at 2–3 × 10^6 cells/ml.

2.1. *Plasma membrane preparation*

Liver plasma membranes were prepared from female, Wistar rats (~100 g body wt) following [16] up to step 11. The purified membrane preparations were suspended in 1 mM $NaHCO_3$ and stored up to 6 weeks in liquid nitrogen without any loss of adenylate cyclase activity. Several batches of liver membranes were used in the experiments reported here; similar results were obtained for all of them.

The AC⁻ plasma membranes were prepared as follows: Cells were harvested with the use of a De la Vall cream separator at 1800 ml/min flowrate. DNase was added to the pellet at 0.4 mg/ml final conc. in the presence of 0.4 mM $MgCl_2$ and 0.04 mM $CaCl_2$. The disaggregated cells were washed as in [17]. The cells (at 1.5 × 10^8 cells/ml) were equilibrated at 4°C for 1 h with 400 lb/in^2 of nitrogen, then lysed by decompression [17]. This procedure gave >95% lysis by the trypan blue dye-exclusion test. Plasma membranes were isolated as in [17] and stored in liquid nitrogen prior to use.

2.2. *Adenylate cyclase assay*

Adenylate cyclase activity was measured as in [4] for rat liver plasma membranes or [2] for AC⁻ membranes. The assay medium for rat liver plasma membranes contained 0.5 mM [α-^{32}P]ATP (10^6 cpm), 3 mM $MgCl_2$, 1 mM EDTA, 1 mM cyclic AMP, 50 mM Tris–

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HCl (pH 7.6) and ATP regenerating system, consisting of 25 mM phosphocreatine and 1 mg/ml of creatine phosphokinase, and 20 μg membrane protein in 60 μl final vol. Incubations were carried out for 10 min at 30°C. The reaction was terminated by a modification [4] of the procedure in [18]. The yield was calculated from previous addition of cyclic [8-^{3}H]AMP. Results are expressed as nmol cyclic AMP formed in 10 min/mg protein at 30°C. The results, obtained from triplicate determinations, agreed within ±5%. The assay medium for AC^- membranes contained 0.1 mM [α-^{32}P]ATP (5 × 10^6 cpm), 2 mM $MgCl_2$, 25 μM cyclic AMP, 5 mM creatine phosphate, creatine kinase at 3.3 units/ml, 30 mM Tris–HCl (pH 7.6). Reactions were initiated by the addition of 15 μg membrane protein in 100 μl final vol. Incubations were done for 15 min at 30°C. The cAMP produced was assayed as in [19]. Results are expressed as pmol cAMP formed . 15 min^{-1} . mg $protein^{-1}$ at 30°C. Protein was estimated as in [20] using bovine serum albumin as standard.

2.3. *Treatment with cholera toxin*

Rat liver plasma membranes (100 μg/ml) were incubated with 50 mM Tris–HCl (pH 7.6), 1 mM dithiothreitol, 1 mM NAD, 1 mM ATP, 3 mg protein/ml liver cytosol (105 000 × *g* supernatant), and 100 μg/ml cholera toxin pre-incubated for 30 min at 37°C with 20 mM dithiothreitol. After 20 min at 30°C, the samples were diluted 20-fold in cold 50 mM Tris–HCl (pH 7.6) and centrifuged at 20 000 rev./min for 15 min at 4°C. The supernatant fluid was removed and the pellets were resuspended in 50 mM Tris–HCl (pH 7.6). Control membranes were obtained after the same pretreatment, in the absence of cholera toxin.

3. Results

Addition of 7 μg α-chymotrypsin/ml incubation medium (i.e., 21 μg protease/mg membrane protein) to rat liver plasma membranes markedly enhanced by up to 100% adenylate cyclase activity assayed in the absence or in the presence of NaF, Gpp(NH)p, or GTP (fig.1). The affinity of the cyclase system for either effector was not modified by the presence of α-chymotrypsin in the assay medium. Identical results (not shown) were observed when the membranes were first pretreated by α-chymotrypsin prior to assay of cyclase activity. Moreover, a similar effect of α-chymotrypsin was observed when the membranes were first preincubated with GTP or Gpp(NH)p (fig.2). This effect was maximal (2-fold

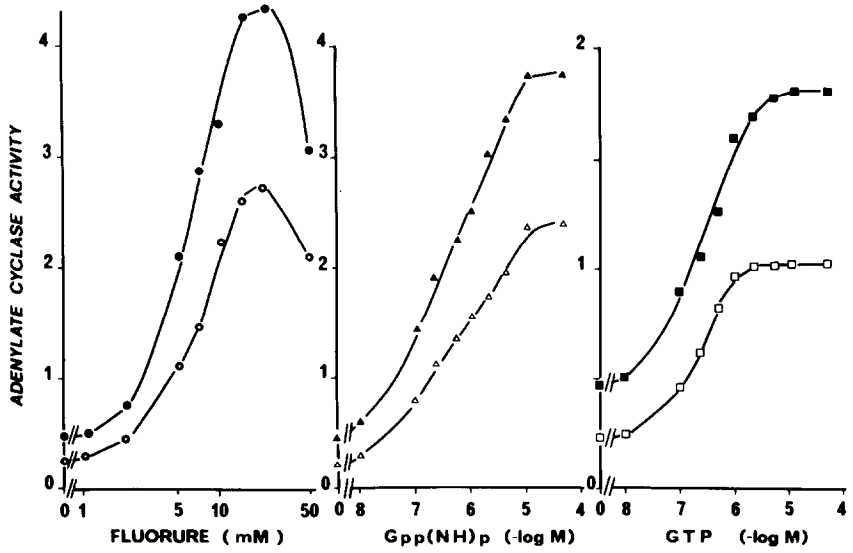

Fig.1. Effect of α-chymotrypsin on stimulation of adenylate cyclase by NaF, Gpp(NH)p or GTP. The assays were performed as in section 2 in the presence of varying concentrations of fluoride (○,●), Gpp(NH)p (△,▲) or GTP (□,■), with (closed symbols) or without (open symbols) 7 μg/ml α-chymotrypsin for 11 μg membrane protein in the assay medium. Activity is expressed as nmol cyclic AMP formed . 10 min^{-1} . mg membrane $protein^{-1}$ at 30°C.

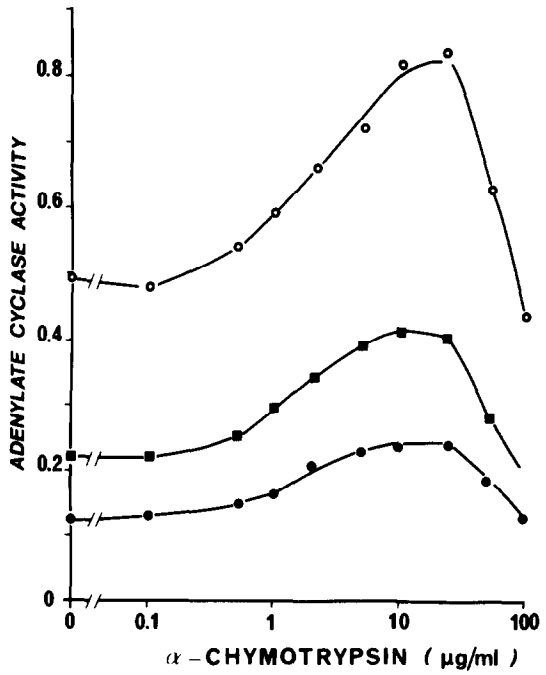

Fig.2. Effect of α-chymotrypsin after preactivation of adenylate cyclase by guanine nucleotides. Membranes (0.5 mg/ml were preincubated 10 min at 30°C with GTP (5×10^{-5} M) (■), 15 min at 30°C with Gpp(NH)p (5×10^{6} M) (○) or 15 min at 30°C with 50 mM Tris–HCl (pH 7.6) (●) then incubated with various concentrations of α-chymotrypsin. Adenylate cyclase activity is expressed as nmol cyclic AMP formed . mg protein^{-1} . 10 min^{-1} at 30°C.

increase) with 10 μg α-chymotrypsin/ml assay medium. These results are in agreement with data obtained with crude collagenase in the same system [4], which indicated that all cyclase activities (basal or stimulated by fluoride, GTP, glucagon or epinephrine) were similarly enhanced by the proteolytic action of crude collagenase. Taken together, these results demonstrate that, in rat liver plasma membranes, proteolysis activates cyclase regardless of the presence or absence of any specific activator.

This finding was confirmed by experiments in which the effect of cholera toxin, which is supposed to act via inhibition of a GTPase activity [21], was examined, in combination with that of proteolysis. Membranes were first preincubated in the presence or absence of 100 μg/ml cholera toxin, as in section 2. They were then incubated in the presence of increasing amounts of α-chymotrypsin in the assay medium. Preincubation with cholera toxin led to a 5–7-fold increase in the basal activity of cyclase (fig.3). Yet, the stimulating effect of α-chymotrypsin was similar (2–3-fold increase) whether or not the membranes were preactivated by cholera toxin. The complete independence of action of α-chymotrypsin and cholera toxin was further confirmed by the reverse experiment. Membranes were first pretreated with various amounts of α-chymotrypsin, then treated or not by cholera toxin, and finally assayed in the absence (fig.4A) or presence of GTP or fluoride (fig.4B). As shown in fig.4A, the same relative stimulating effect of cholera toxin upon control cyclase could be observed, whatever the concentration of α-chymotrypsin used in the preincubation step. However, the final activity obtained

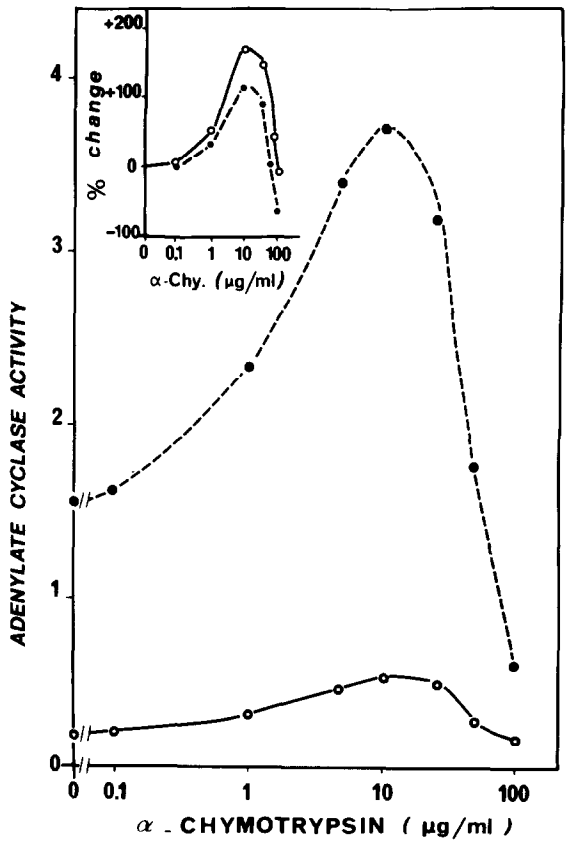

Fig.3. Effect of α-chymotrypsin on adenylate cyclase activity after preactivation by cholera toxin. Membranes (100 μg/ml) were preincubated in the presence (●) or in the absence (○) of 100 μg/ml cholera toxin as in section 2. Pretreated membranes were then incubated in the presence of various amounts of α-chymotrypsin in the assay medium. Activity is expressed as nmol cyclic AMP formed . 10 min^{-1} . mg membrane protein^{-1} at 30°C. The inset represents the % change due to proteolysis as compared to the control cyclase activity.

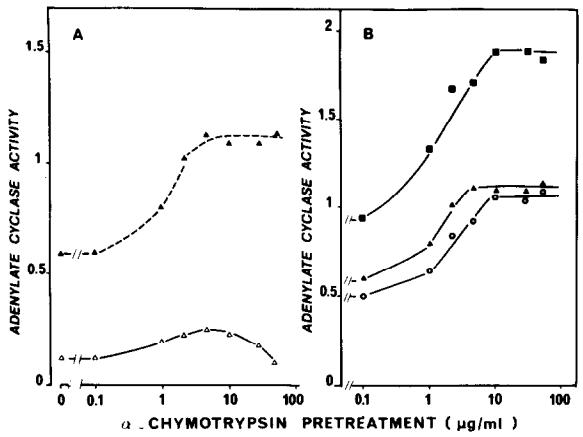

Fig.4. Effect of cholera toxin after pretreatment of liver membranes with α-chymotrypsin. Membranes (4 mg/ml) were preincubated for 5 min at 30°C with various concentrations of protease. The proteolytic effect was stopped by further incubation with soybean trypsin inhibitor (0.4 mg/ml final conc.), for 2 min at 30°C. Aliquots (300 μl) of the mixture were then incubated in the presence or absence of cholera toxin as in section 2. After washing, the resuspended pellets were finally assayed in the presence of various activators. (A) compares basal activity in the absence (△) or in the presence (▲) of cholera toxin. (B) compares basal activity (▲) to NaF stimulated (○) or GTP stimulated (■) adenylate cyclase after cholera toxin.

was lower than that of the previous experiment. This is probably due to the more complex and longer experimental protocol used. After activation with cholera toxin, adenylate cyclase could still be stimulated by GTP, but was no longer sensitive to NaF (fig.4B, and [21]). It should be noted that the effect

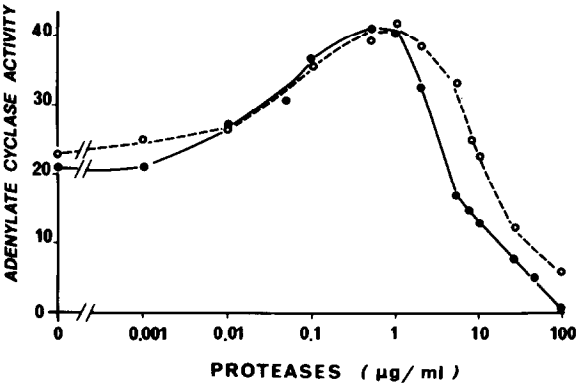

Fig.5. Effect of proteases on the adenylate cyclase activity of AC⁻ S49 lymphoma cell membrane. The assays were performed as in section 2 in the presence of varying concentrations of α-chymotrypsin (○) or papain (●) in the assay medium. Activities are expressed as pmol cAMP formed . 15 min^{-1} . mg $protein^{-1}$ at 30°C.

of α-chymotrypsin is usually biphasic. In this experiment, ⩽10 μg α-chymotrypsin/ml activated the control cyclase whereas higher concentrations inhibited. This biphasic effect was no longer observed when the preactivated cyclase was assayed after further stimulation with cholera toxin.

The AC⁻ mutant of the S49 lymphoma cell line possesses an adenylate cyclase activity which cannot be stimulated by fluoride, hormones, guanine nucleotides, or cholera toxin. Moreover, this mutant has been shown to lack the N component that binds GTP, at least in a functional form [22–25]. This cyclase activity can be assayed in the presence of Mn^{2+}-ATP. As shown in fig.5, the Mn^{2+}-dependent adenylate cyclase of the AC⁻ mutant was stimulated by α-chymotrypsin and papain. The effects were biphasic and maximal stimulation (2-fold increase) was obtained for 1 μg protease/ml assay mixture (i.e., 7 μg protease/mg membrane protein). The protease-activated enzyme did not become sensitive to any stimulating factor (not shown). The very small cyclase activity observed when tested in the presence of Mg^{2+}-ATP can also be activated by proteolysis (not shown). These results differ from those obtained with rat liver adenylate cyclase, which can only be activated by proteolysis in the presence of Mg^{2+}-ATP [6].

4. Discussion

These data demonstrate that proteolytic activation of adenylate cyclase can occur independently of the GTP binding subunit N, at least in plasma membranes from rat liver and from the AC⁻ mutant of the S49 lymphoma cell line. The evidence for this is as follows:

(1) Proteolytic treatment of rat liver plasma membrane enhanced adenylate cyclase activity regardless of the presence or absence of fluoride, hormone or guanine nucleotides;
(2) It stimulated cyclase in the presence of Gpp(NH)p as well as GTP, thus eliminating a possible effect on a GTPase activity;
(3) α-Chymotrypsin and cholera toxin activated cyclase in an additive manner, whatever the order of addition. This indicates that they act independently of each other;
(4) The AC⁻ mutant of the S49 lymphoma cell line is devoid of functional N subunit, yet the adenylate cyclase could be activated by proteolysis.

These data contrast with those obtained in other

systems, namely adipose tissue [4], fibroblasts [8,9], kidney [10], for which the activating effect induced by proteolysis was predominant when GTP was added in the assay medium. A common feature of all the above mentioned systems is the fact that GTP exerts an inhibitory effect upon cyclase (see also [26] for adipose tissue) and that this effect appears to be specifically relieved by proteolysis. In particular, the proteolytic treatment of NRK cells in [27] was accompanied by a decrease in a 46 000 dalton membrane protein, which could possibly be related to GTP function. Noteworthy is the fact that the liver cyclase is not inhibited by guanine nucleotides.

It appears, therefore, that proteolytic activation of adenylate cyclase can occur through at least two independent mechanisms. One mechanism might be related to destruction of a GTP-dependent inhibitory process which, at least in studies of the adipocyte cyclase system [14,26], is separate from the process that causes stimulation of cyclase by GTP. The second mechanism shown in this study, is activation by modification of the catalytic unit or a protein closely associated with this unit. Clearly it is not the GTP-regulatory component in the liver. The site of proteolytic action might either be an inhibitory component, or an inactive precursor converted by proteolysis into an activator of adenylate cyclase. Preliminary experiments on rat liver have eliminated the possibility that the hypothetical inhibitory component could be the sites through which calcium and adenosine [28] inhibit adenylate cyclase; the dose-dependent inhibition of adenylate cyclase by these agents was not modified when liver cyclase was preactivated by proteolysis.

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