CHANGE IN THE CATION DEPENDENCE OF RAT LIVER ADENYLATE CYCLASE AFTER PROTEOLYTIC ACTIVATION

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1. Introduction

We recently reported that rat liver plasma membrane adenylate cyclase activity could be increased after treatment of the membranes by a variety of proteases, with the exception of trypsin [1–3]. This effect was due to an increase in the maximal velocity of the enzyme, and appeared relatively similar for several cyclase activities, basal as well as those stimulated by fluoride, GTP or hormones [1]. Since then, a similar effect has been noted for other tissues and cell lines: ovary [4]; fibroblasts [5,6]; turkey erythrocytes [3].

The cation requirement of most of the adenylate cyclase systems can be satisfied almost equally well by either Mg^{2+} or Mn^{2+} [7]. The true substrate is assumed to be a cation–ATP complex and free cations appear to be required activators acting at a distinct cation binding site [8–10].

Here, we present evidence that the proteolytic activation of rat liver adenylate cyclase activity is apparent only when Mg^{2+} is used as the required cation together with the substrate, ATP, and not in the presence of Mn^{2+}. In view of the current concepts of the molecular anatomy of the adenylate cyclase systems [11], it can be concluded that the activating effect of proteolysis is not exerted directly on the catalytic subunit of the complex enzyme system, but rather on a closely related regulatory (possibly inhibitory) component.

2. Materials and methods

Purified proteolytic enzymes were obtained as follows: thermolysin (protease type X, lot 54 C) prepared from *Bacillus thermoproteolyticus* and subtilisin BPN' (protease type VII, lot 113 C) prepared from *Bacillus amyloliquefaciens* were from Sigma; α-chymotrypsin (lot CDI 8 LK), trypsin (lot TRL 36 C 876), soybean trypsin inhibitor (lot 3570 SI 58 C 584), and papain (lot 37 D 784) were purchased from Worthington. The concentrations of the various enzymes are expressed in terms of μg protein present in the various preparations. Cyclic AMP and creatine phosphate were from Calbiochem. Creatine kinase and ATP were from Boehringer Mannheim. All other chemicals were from Merck, Darmstadt. [α-^{32}P]ATP (22 Ci/mmol) was from Amersham, Radiochemical Centre. Cyclic [8-^{3}H]AMP (10 Ci/mmol) was obtained from the CEA (Saclay).

2.1. *Preparation of rat liver plasma membranes*

Hepatic plasma membranes from female, adrenalectomized [12] Wistar rats were prepared according to [13] up to step 11. Several batches of liver membranes were used in the experiments reported here and gave similar results.

2.2. *Adenylate cyclase assay*

Adenylate cyclase activity (ATP pyrophosphate-lyase (cyclizing) EC 4.6.1.1) was measured as in [14]. The assay mixture contained 0.5 mM [α-^{32}P]ATP ($1–2 \times 10^6$ cpm), 3 mM $MgCl_2$ or 2 mM $MnCl_2$, 1 mM EDTA, 1 mM cyclic AMP, 50 mM Tris–HCl (pH 7.6) an ATP regenerating system consisting of 25 mM phosphocreatine and 1 mg/ml creatine phosphokinase, and 20–25 μg membrane protein in 60 μl final vol. The cation concentrations used were found to be

optimal for the present assay conditions. Variations from this composition are indicated in the legends to figures. Incubation was initiated by addition of the protein and was performed in a shaking waterbath at 30°C. In most experiments reported here, proteases were added at time zero together with the incubation mixture. The reaction was terminated by a modification [1] of the procedure in [15]. Blank values obtained with this procedure were always < 0.005% of the total radioactivity applied. The yield of cyclic AMP eluted from the column was calculated from pre-addition of cyclic [8-^{3}H]AMP (20 000 cpm). Sample counting was performed, after addition of 10 ml Ready-Solve EP (Beckman), in an Intertechnique SL 30 liquid scintillation counter. Protein was estimated by Lowry's procedure using bovine serum albumin as standard. Results are expressed as nmol cyclic AMP formed in 10 min/mg membrane protein at 30°C. The results, obtained from triplicate determinations, agreed within ± 5%.

2.3. *Enzymatic assay of α-chymotrypsin*

Enzyme activity of α-chymotrypsin was measured using benzoyl–Tyr-*p* nitroanilide as substrate, as described in [16].

3. Results

Figure 1 depicts the dose-dependent effect of α-chymotrypsin upon basal and activated (GTP, guanyl-imidodiphosphate, epinephrine + GTP and fluoride) adenylate cyclase activities from rat liver plasma membrane. The left hand panel shows activation of the Mg^{2+}-dependent adenylate cyclase activity with varying amounts of α-chymotrypsin. For all the cyclase activities tested, the maximal effect (1.5–2.5-fold increase) was obtained at 7.5 μg α-chymotrypsin/ml.

In contrast, when adenylate cyclase was assayed in the presence of Mn^{2+}, incubation with α-chymotrypsin had no effect except for a very slight activation of the fluoride and epinephrine + GTP activated forms on the enzyme (fig. 1B). This was not due to an inhibition of the intrinsic proteolytic activity of α-chymotrypsin by Mn^{2+} since the activity of α-chymotrypsin upon an artificial substrate, *N*-benzoyl–L-Tyr-*p*-nitroanilide [16], was the same, whether 3 mM

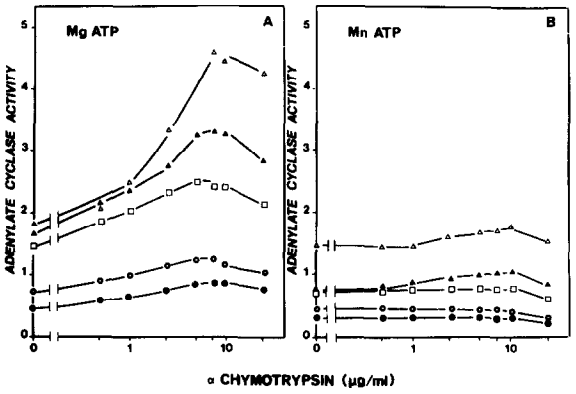

Fig. 1. Effect of α-chymotrypsin on the stimulation of rat liver plasma membrane adenylate cyclase by various effectors. Assay conditions were those in section 2.2 with Mg^{2+} (panel A) or Mn^{2+} (panel B) as the cosubstrate. Membranes (20 μg protein/assay) were incubated in the presence of varying amounts of α-chymotrypsin with no other addition (●), or in the presence of 10 μM GTP (○), 10 μM Gpp(NH)p (□), 50 μM epinephrine plus 10 μM GTP (▲) or 10 mM NaF (△). Activity is expressed in nmol cyclic AMP formed in 10 min/mg membrane protein at 30°C.

Mg^{2+} or 2 mM Mn^{2+} was added to the incubation mixture. Further, α-chymotrypsin, subtilisin, papain and thermolysin all had a biphasic effect upon basal adenylate cyclase activity when Mg–ATP was used as the substrate (fig. 2). They activated adenylate cyclase 2-fold at low concentration, with a maximal effect at 5–10 μg protease/ml (or 15–33 μg protease/mg membrane protein). At high concentration, they became inhibitory; the cyclase activity was even less than the control value when the incubation was performed in the presence of 50 μg/ml subtilisin. In contrast, when Mn–ATP was used as the substrate of the cyclizing reaction, the proteases exerted no activation upon cyclase. Only the inhibitory effect was observed, at high protease concentration (fig. 2).

Fluoride-stimulated cyclase activity, which was slightly sensitive to proteolysis in the presence of Mn^{2+} (fig. 1B), was used to assess the effects of proteolysis in the presence of both Mg^{2+} and Mn^{2+}. As shown in fig. 3A, α-chymotrypsin brought about a 120% increase in the fluoride-stimulated cyclase activity, in the presence of 3 mM Mg^{2+}, 0.5 mM ATP and 1 mM EDTA. At higher concentrations of Mg^{2+} (10 mM), the relative effect of proteolysis led to only

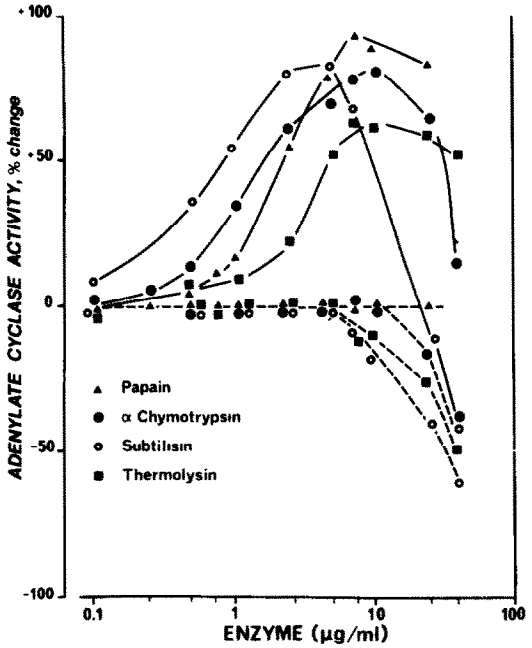

Fig. 2. Effect of various proteases upon the basal rat liver plasma membrane adenylate cyclase. The assays were performed as in section 2.2. Membranes were incubated for 10 min at 30°C in the presence of varying concentrations of subtilisin (○), α-chymotrypsin (●), papain (▲) and thermolysin (■). Adenylate cyclase activity is expressed as % of change as compared to control level assayed in the presence of Mg–ATP (——) (0.4 nmol cyclic AMP formed in 10 min/mg membrane protein), or in the presence of Mn–ATP (- - -) (0.3 nmol cyclic AMP formed in 10 min/mg membrane protein).

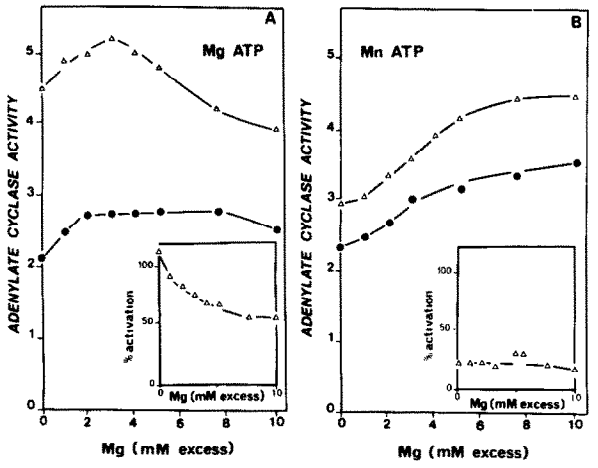

Fig. 3. Effect of Mg^{2+} in excess over Mg–ATP (panel A) or over Mn–ATP (panel B). The assay medium was as in section 2.2. ATP was 0.5 mM, NaF 10 mM. Membrane proteins were incubated in the absence (●) or in the presence (△) of 7.5 µg/ml α-chymotrypsin. The insets represent the percent of activation due to proteolytic agent as compared to the control cyclase activity. Activity is expressed in nmol cyclic AMP formed in 10 min/mg membrane protein.

a 60% increase. When adenylate cyclase was assayed in the presence of Mn^{2+} (fig. 3B), the small relative increase in activity brought about by proteolysis (+ 20%), was not modified by increasing Mg^{2+} from 0–10 mM. Proteolysis did not modify the affinities of the enzyme for either free metal ion [3] or metal–ATP [1]. It involved only a change in the V_{max} of the reaction.

It was, therefore, important to decide whether the Mg^{2+} versus Mn^{2+} sensitivity of the proteolytically-activated cyclase was restricted to the activation process of cyclase, or to the catalytic step itself. This aspect was studied in a 2 step assay procedure wherein adenylate cyclase was first activated for 5 min at 30°C by varying concentration of α-chymotrypsin (from 0.1–20 µg/ml) in the absence of the cyclase assay mixture. The effect of α-chymotrypsin was then blocked by 0.2 mg/ml soybean trypsin inhibitor and adenylate cyclase activity assayed as in section 2.2 in the presence of either Mg–ATP or Mn–ATP. As shown in fig. 4, the activating effect of low concentrations of α-chymotrypsin (⩽ 10 µg/ml) was only visible when the enzyme was tested thereafter in the presence of Mg^{2+}. When the enzyme was assayed in the presence of Mn^{2+}, only the inhibitory effect of α-chymotrypsin could be observed.

4. Discussion

These results clearly indicate that the proteolytically-activated form of rat liver membrane adenylate cyclase can be detected only when the enzyme is assayed in the presence of Mg^{2+}, and not in the presence of Mn^{2+}. In most cyclase systems [7], Mn^{2+} can substitute for Mg^{2+} as cosubstrate; both cations also regulate the cyclase through binding to an additional metal site, distinct from the catalytic site [8–10]. Furthermore,

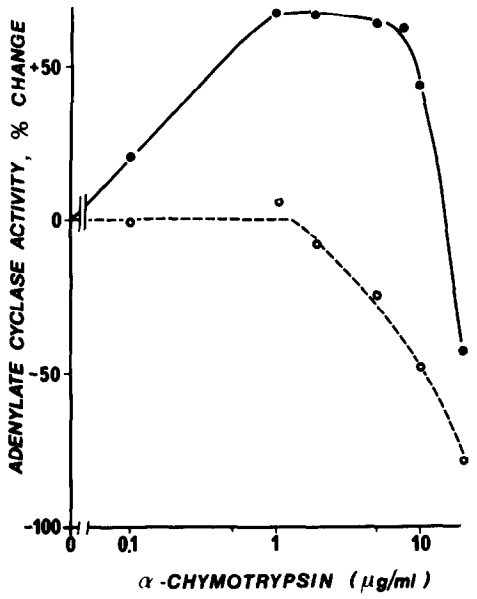

Fig. 4. Pre-activation of adenylate cyclase by proteolysis. Rat liver plasma membranes (2 mg/ml) were preincubated for 5 min at 30°C with various concentrations α-chymotrypsin. The proteolytic effect was stopped by addition of soybean trypsin inhibitor (0.2 mg/ml) for 1 min at 30°C. Aliquots (40 μl) of the mixture were then assayed for adenylate cyclase activity in the presence of Mg–ATP (•) or in the presence of Mn-ATP (○) as in section 2.2. Adenylate cyclase activity is expressed as % of change as compared to control levels (0.45 nmol cyclic AMP formed in 10 min/mg protein in the presence of Mg–ATP, and 0.35 nmol cyclic AMP formed in 10 min/mg protein in the presence of Mn–ATP). Those control levels were not changed by the addition of soybean trypsin inhibitor alone, or when the inhibitor was added before α-chymotrypsin.

Mn^{2+} has been reported to modulate in various ways the effect of many hormonal and nonhormonal agents on adenylate cyclase (reviewed [17]). It is shown [11] in S 49 lymphoma cells and [18] by rat liver, that adenylate cyclase could be freed, by solubilization, of certain regulatory component(s), the 'G/F' or 'N' subunit, believed responsible for the sensitivity of the enzyme to GTP and NaF. In its 'simple' form, cyclase could be assayed only in the presence of Mn^{2+} and not Mg^{2+} [11]. The G/F subunit was presumed therefore, to be able to transform cyclase into a form capable of interacting with its physiological substrate, Mg–ATP. Of particular relevance to the present study is also the fact that high concentrations of Mn^{2+} lead to a loss of hormone and guanine nucleotide sensitivity of the adenylate cyclase from frog erythrocyte membranes, while basal and NaF, remained relatively preserved [17].

Since proteolysis appears, in our experiments, to activate only the form of adenylate cyclase which can be measured in the presence of Mg^{2+}, it is therefore tempting to conclude that it affects regulatory component(s) of the system related to G/F or N subunit and not the catalytic site of the enzyme itself. Moreover, due to our ignorance of the exact molecular nature of the adenylate cyclase complex in the plasma membrane, this possibility is obviously speculative, but would be in agreement with the fact that proteolytic activation of adenylate cyclase was predominantly observed upon the GTP-activated form of the enzyme in cultured fibroblasts [5] and in fat cells [19].

Thus, our data strengthen the recent and numerous reports [20] which suggest an increasingly complex structure for the adenylate cyclase system, and are compatible with a primary effect of proteolysis on regulatory components of the cyclase system, possibly related to the so-called G/F or N factors.

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