INCREASE IN Mg^{2+} AFFINITY AS A POSSIBLE MECHANISM OF CHYMOTRYPTIC ACTIVATION OF ADENYLATE CYCLASE IN RAT HEART MEMBRANES

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(Received February 27, 1985)

Chymotryptic activation of adenylate cyclase from rat heart was examined with respect to guanosine triphosphate (GTP), adenosine triphosphate (ATP) and Mg^{2+} concentrations. The activation was inhibited by aprotinin. In the absence or presence of GTP, chymotrypsin stimulated the cyclase at the same ratio. GTP exerted stimulatory (up to 0.1 mM) and inhibitory (at 1 mM) effects on the cyclase. Both effects were not affected by chymotrypsin. ATP at low concentrations increased the cyclase activity dose dependently and at high concentrations decreased the cyclase activity. Chymotrypsin retarded the appearance of the decreasing phase. This effect of chymotrypsin was similar to that of increasing the concentration of Mg^{2+} in the reaction mixture. According to double-reciprocal plots of Mg^{2+} concentrations and the cyclase activity, chymotrypsin diminished K_m for Mg^{2+} with little effect on the V_{max} . Hill plots of the same data showed that the Hill coefficient was about 1 and was not altered by chymotrypsin. These results suggest that chymotryptic activation of adenylate cyclase is mainly due to increasing the affinity for Mg^{2+} of the system.

Keywords —adenylate cyclase; chymotrypsin; magnesium ion affinity; rat heart

INTRODUCTION

It has been shown by a number of laboratories that mild proteolysis stimulates adenylate cyclase.^{1–3)} In some reports, the stimulations were due to inactivation of the inhibitory pathway,^{4–6)} while in others, multiple sites of actions of proteases were involved.^{7,8)} Generally, proteases stimulate the cyclase by acting at the inner face of the plasma membranes⁹⁾ but the precise mechanism is still unclear. On the other hand, adenylate cyclase is known to be regulated by guanosine triphosphate (GTP) and Mg^{2+} .¹⁰⁾ In this paper, the feature of chymotryptic activation of adenylate cyclase in rat heart membranes was examined in relation to these regulators.

MATERIALS AND METHODS

Reagents — Reagents used were adenosine triphosphate (ATP, Nakarai), dithiothreitol (Wako), 3-isobutyl-1-methyl xanthine (Al-

drich), GTP (Boehringer Mannheim), phosphocreatine, phosphocreatine kinase and aprotinin (Sigma) and α -chymotrypsin (Miles).

Preparation of Membrane Adenylate Cyclase — Male Wistar rats (180–200 g) were decapitated and the hearts were washed in cold 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM dithiothreitol, and homogenized in 10 ml of the same buffer with a teflon-glass homogenizer. The homogenate was filtered through 3-layers of gauze, and the filtrate was centrifuged at $3000 \times g$ for 15 min at 4 °C. The pellet was washed three times by centrifugation as mentioned above. Proteins were measured by the method of Lowry *et al.*¹¹⁾ using bovine serum albumin as the standard.

Adenylate Cyclase Assay — The standard assay medium contained 50 mM Tris-HCl (pH 7.4); 1 mM 3-isobutyl-1-methylxanthine; 2.5 mM ATP; 5mM $MgCl_2$; 1 mM dithiothreitol; 5

mM phosphocreatine and 20 units of phosphocreatine kinase to make a final volume of 600 μ l. Reactions were initiated by the addition of membranes to give a final concentration of 100 μ g protein/ml. Incubations were carried out for 10 min at 37 °C and were terminated by heating in boiling water for 2 min. Adenosine 3', 5'-cyclic monophosphate (cyclic AMP) was measured by an enzyme immunoassay.¹²⁾ Student's *t*-test was used to determine statistical significances between control and test values.

RESULTS

Adenylate cyclase activity was linear with time of incubation for 40 min in the absence and for 20 min in the presence of 10 μ g/ml chymotrypsin (Fig. 1). The cyclase was activated by 10 μ g/ml chymotrypsin at all the membrane concentration studied, 12.5–400 μ g protein/ml, nearly in the same ratio. Chymotrypsin activated the cyclase about 3-fold. This activation was inhibited by aprotinin without change in basal activity (Table I). Chymotrypsin enhanced the cyclase activity dose-dependently at the range of 0.1–10 μ g/ml (Fig. 2). While at 100 μ g/ml, the cyclase was inhibited, probably due to the destruction of catalytic component by chymotrypsin. Furthermore, the cyclase was clearly activated by chymotrypsin in the absence of GTP, although isoproterenol which is well known to require GTP for activation, had no effect under the same condition (data not shown). Chymotryptic activation was not affected by 10 μ M GTP, since the ratio of chymotrypsin-treated activity to control activity was similar in the ab-

sence and presence of GTP (Table II). Figure 3 shows the biphasic effects of GTP on adenylate cyclase. Maximal stimulation was evoked by 10–100 μ M GTP both in the absence and pre-

FIG. 1. Time Course of Basal and Chymotrypsin-Stimulated Adenylate Cyclase Activity in Rat Heart Membranes

The activity was measured in the absence (○) or presence (●) of 10 μ g/ml chymotrypsin added to the standard assay medium containing 10 μ M GTP. Results was expressed as a relative value to the activity obtained from 40 min incubation in the presence of 10 μ g/ml chymotrypsin. a) $p < 0.01$, b) $p < 0.05$ ($n = 3$).

TABLE I. Effects of Aprotinin on Activation of Adenylate Cyclase by Chymotrypsin

	Aprotinin (KIU/ml)			
	0	11.7	35.1	117
Basal	125.0 $\pm$ 16.2	121.3 $\pm$ 13.4	113.8 $\pm$ 13.3	117.5 $\pm$ 15.1
Chymotrypsin (10 μ g/ml)	275.0 $\pm$ 23.8 ^{a)}	263.3 $\pm$ 38.1 ^{b)}	227.5 $\pm$ 27.8 ^{b)}	145.0 $\pm$ 13.1

a) $p < 0.01$. b) $p < 0.05$. The activities were presented as pmol/mg protein/10 min. Each value shows the mean $\pm$ S.E. of 4 experiments.

sence of chymotrypsin. It should be noted that the inhibitory phase of 1 mM GTP was not altered by chymotrypsin. Figure 4 shows the double-reciprocal plots of ATP concentrations (6.25 μ M–2.5 mM) and adenylate cyclase activity. Plots that were nearly linear were obtained at ATP concentrations below 100 μ M; however, at higher concentrations, the cyclase activity de-

creased. When the reactions were carried out in the presence of chymotrypsin, the decreasing phase of the high level of ATP was considerably attenuated and large differences in the cyclase activities with and without chymotrypsin re-

FIG. 2. *Effects of Chymotrypsin on Adenylate Cyclase Activity*

The activity was measured in the absence (○) or Presence (●) of 100 μ M GTP. a) $p < 0.01$, b) $p < 0.05$ ($n=6$).

FIG. 3. *Effects of GTP on Adenylate Cyclase Activity*

The activity was measured in the absence (○) or presence of 1 μ g/ml (▲) and 10 μ g/ml (●) chymotrypsin. ($n=5$).

TABLE II. *Effects of GTP on Chymotryptic Activation of Adenylate Cyclase in Rat Heart Membranes*

	GTP no addition				GTP 10 μ M			
	0	0.1	1.0	10.0	0	0.1	1.0	10.0
Chymotrypsin								
Adenylate cyclase								
activity	41.8	49.3	57.8	135.0	80.8	85.6	130.0	265.0
Activity ratio	1.0	1.2	1.4	3.2	1.0	1.1	1.6	3.3

The activity was measured in the absence or presence of 10 μ M GTP and the indicated concentrations of chymotrypsin (μ g/ml). Cyclic AMP formed was expressed as pmol/mg protein/10 min. Activity ratio indicates relative value to each control activity. Each value shows the mean of 4 experiments.

FIG. 4. *Double-Reciprocal Plots of Various Concentrations of ATP and Adenylate Cyclase Activity*
The activity was measured in the absence (○) or presence (●) of 10 μ g/ml chymotrypsin. 10 μ M GTP was contained in each case.

FIG. 5. *Effects of ATP on Adenylate Cyclase Activity*

The activity was measured in the absence (open symbols) or presence (closed symbols) of 10 μ g/ml chymotrypsin. Mg^{2+} concentrations in the mediums are 1 mM (Δ , $\blacktriangle$), 5 mM ($\circ$, $\bullet$) and 10 mM ($\square$, $\blacksquare$). 10 μ M GTP was contained in each case. ($n=4$).

sulted. At low concentrations of ATP, the differences between them were very small. However, chymotrypsin did not inactivate the mechanism of the decreasing phase since, in the presence of maximal concentration of ATP used, the cyclase activity also decreased (Fig. 5). The appearance of the decreasing phase was retarded by chymotrypsin or higher concentration of Mg^{2+} or both. These findings suggest that chymotryptic activation has relevance to ATP and Mg^{2+} . Figure 6 shows the cyclase activity at vari-

FIG. 6. *Effects of Mg^{2+} on Adenylate Cyclase Activity*

The activity was measured in the absence (○) or presence (●) of 10 μ g/ml chymotrypsin. 200 μ M ATP and 10 μ M GTP were contained in each case. Mg^{2+} concentrations were calculated as a free form, not complexed with ATP. The stability constant for ATP used is 16600 M^{-1} .¹⁸⁾ ($n=8$).

ous concentrations of Mg^{2+} . The K_m value for Mg^{2+} was diminished, 7.2 to 2.8 mM, by chymotrypsin but V_{max} changed slightly, 556 to 645 pmol/mg protein/10 min. Figure 7 shows Hill plots of the same data. Hill coefficients obtained in the absence and presence of chymotrypsin were 0.93 and 0.98, respectively. Chymotrypsin did not affect cooperativity of Mg^{2+} binding to the cyclase system.

DISCUSSION

Adenylate cyclase was activated by chymotrypsin and the activation was inhibited by aprotinin, a protease inhibitor naturally occurring in bovine mast cell.¹⁵⁾ This result is in agreement with other observations in that the zymogens of proteases failed to activate the cyclase¹⁶⁾ and that other protease inhibitors blocked the activation.^{3,16)} Therefore, chymotryptic activation of the cyclase appears to be due to mild proteoly-

sis. Chymotryptic activation of the cyclase was seen even without GTP in the reaction mixture and was contrasted with the activation by isoproterenol. GTP exerted biphasic action on the cyclase, stimulation at low and inhibition at high concentration. Both phases were chymotrypsin-insensitive. These observations suggest that GTP is not involved in chymotryptic activation as reported elsewhere.⁷⁾ The influence of the concentrations of ATP on the cyclase activity were also biphasic, increasing activity at low and decreasing at high concentration. The decreasing phase was attenuated by chymotrypsin. The attenuation was not due to inactivating the mechanism of the decreasing phase since ATP at the highest concentration used inhibited the cyclase again. However, chymotryptic activation was seen only at high level of ATP. The retardation of the decreasing phase was caused by not only chymotrypsin but also by higher concentrations of Mg^{2+} , suggesting that Mg^{2+} is relevant to chymotryptic activation. Mg^{2+} is thought to function in a complex with ATP as substrate and to interact with metal sites of the cyclase system following activation.^{13,17-20)} Therefore, the decreasing phase at a high level of ATP might be due to the increase in a putative potent inhibitor, "free" ATP,^{13,14)} namely ATP not complexed with Mg^{2+} , or due to the decrease in Mg^{2+} concentration for interaction with the metal site.^{18,20)} In either case, it is clear that higher activity of the cyclase can be attained by increasing the concentration or enhancing utility of Mg^{2+} . Chymotrypsin enhanced the affinity for Mg^{2+} and resulted in higher activity of the cyclase. In addition, chymotrypsin did not affect the V_{max} greatly with respect to Mg^{2+} concentration. These data are in contrast to the action of isoproterenol²²⁾ and glucagon,¹⁷⁾ whose effects were accompanied with an increasing V_{max} as well as a decreasing K_m for Mg^{2+} . Therefore, chymotrypsin is one of the unique activators of adenylate cyclase. In this study, it was shown that mild proteolysis by chymotrypsin activates adenylate cyclase in rat heart membranes by increasing affinity for Mg^{2+} .

FIG. 7. Hill Plots of Mg^{2+} and Adenylate Cyclase Activity

The plots were calculated from the data presented in Fig. 6.

Acknowledgement The author wishes to thank Professor K. Hattori for useful comments on the manuscript.

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