PROTEOLYTIC ACTIVATION OF ADENYLATE CYCLASE FROM RAT-LIVER PLASMA MEMBRANES

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1. Introduction

We have recently demonstrated [1] that the adenylate cyclase (EC 4.6.1.1) system of rat-liver plasma membranes is stimulated by a proteolytic contaminant from commercial preparations of crude collagenase (from *Clostridium histolyticum*). This factor, which is endowed with a proteolytic activity other than collagenase itself or clostripain, promoted a 2-fold increase in the basal activity as well as in the activities stimulated by catecholamines, glucagon, fluoride or GTP. This effect was due to an increase in the maximal velocity of the cyclizing reaction without modification in the K_m of the enzyme either for its substrate MgATP or for the stimulating agents. Treatment of plasma membranes by this factor resulted in direct and irreversible stimulation of the adenylate cyclase system and did not modify other membranous enzymic activities (such as ATPases or 5'-nucleotidase). Moreover, the morphological structure of the membranes remained unchanged [1].

In the present work, we demonstrate that this activating property is not restricted to the bacterial factor previously studied, but is mimicked by several highly purified proteinases of varied origin. We report results of studies using several different proteinases: microbial metalloenzymes (EC 3.4.24.4) such as thermolysin and pronase, SH-proteinases such as papain (EC 3.4.22.2), and serine proteinases such as alpha-chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), elastase (EC 3.4.21.11) and subtilisin BPN' (EC 3.4.21.14). We propose that mild proteolysis represents a new type of adenylate cyclase activation which might be linked either to an increase in the catalytic constant of the adenylate cyclase system, or to an unmasking of new catalytic sites.

Abbreviations: SDS, sodium dodecyl sulfate; cyclic AMP, cyclic adenosine 3',5'-monophosphate; EDTA, ethylene diamine tetraacetic acid

2. Experimental procedure

2.1. *Materials*

Purified proteolytic enzymes were obtained as follows: thermolysin (protease type X, lot 54C) prepared from *Bacillus thermoproteolyticus*, subtilisin BPN' (protease type VII, lot 113C) prepared from *Bacillus amyloliquefaciens* and pronase (protease type VI, lot 122C) prepared from *Streptomyces griseus*, were all from Sigma; elastase (lot ESFF 55E563), papain (lot PAP 34S857) and trypsin (lot TRL 36C876) were from Worthington and alpha-chymotrypsin (lot 6485415) was from Boehringer. Crude collagenase from *Clostridium histolyticum* (lot CLS 2KD) was purchased from Worthington. The concentrations of the various enzymes are expressed in terms of micrograms of protein present in the various preparations. Cyclic AMP and creatine phosphate were from Calbiochem. Nucleotides (disodium salts), bovine serum albumin (fraction V) and (*R*)-(-)-epinephrine bitartrate were obtained from Sigma. Creatine kinase was purchased from Boehringer. [α-^{32}P] ATP (21.5 Ci/mmol) was from New England Nuclear, and cyclic [8-^{3}H] AMP (13 Ci/mmol) was obtained from the CEA (Saclay, France).

2.2. *Methods*

2.2.1.Preparation of plasma membranes

Female, adrenalectomized, Wistar rats were used [2]. Hepatic plasma membranes were prepared according to the procedure devised by Neville up to step 11 [3]. Several batches of liver membranes were used in the experiments reported here; similar results were obtained with all of them.

2.2.2.Adenylate cyclase assay

The assay medium contained 0.5 mM [α-^{32}P] ATP (10^6 cpm), 3 mM $MgCl_2$ (except when otherwise indicated), 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 20–30 μg membrane protein in final vol. 60 μl. Incubation and sample counting were performed as previously described [4]. 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 protein at 30°C. The results, obtained from triplicate determinations, agreed within ± 5%.

2.2.3.SDS–Polyacrylamide electrophoresis

SDS–Polyacrylamide gels (10% monomer) were prepared, electrophorized, and stained according to the method of Laemmli [5]. Membranes were mixed with the 'sample buffer', which contained (final concentrations): 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol. The proteins were completely dissolved by boiling for 2 min. The running sample volume was 50 μl and contained 100 μg membrane protein.

3. Results

The various proteinases were added to the incubation medium at the beginning of the cyclizing reaction and their effect upon cyclic AMP formation was assessed during a 10 min incubation. Figure 1 shows the effect of increasing concentrations of papain, alpha-chymotrypsin, subtilisin and pronase upon the fluoride-sensitive adenylate cyclase. The effect appeared two-fold: activation at low concentrations, inhibition at high concentrations. Activation was half-maximal for 2–5 μg alpha-chymotrypsin, papain and subtilisin/

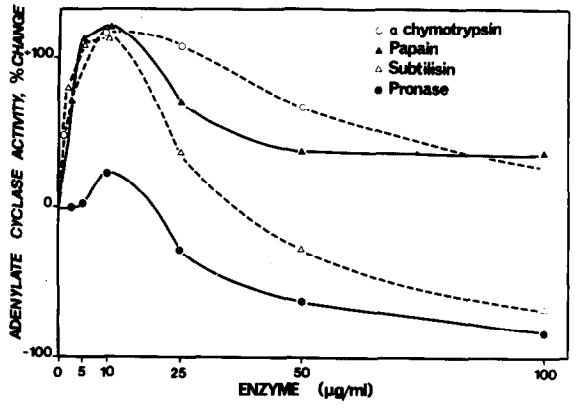

Fig.1. Effect of various purified proteinases upon the fluoride-sensitive adenylate cyclase. Assay conditions were those described under 'Experimental Procedure'. Membranes were incubated for 10 min at 30°C with 10 mM NaF and in the presence of varying concentrations of (○) alpha-chymotrypsin, (▲) papain, (△) subtilisin and (●) pronase. Adenylate cyclase activity is expressed as percent change as compared to control level (1.6 nmol cyclic AMP formed/10 min/mg membrane protein).

ml assay mixture and maximal (2-fold increase) from 10 μg/ml of each of these enzymes. Pronase also exhibited its maximal activation at 10 μg/ml; this activation however, did not exceed 20%. For all these enzymes, an inhibitory effect was observed at concentrations higher than 10–15 μg/ml; inhibition was complete with 100 μg/ml of subtilisin or pronase.

Figure 2 depicts the effect of increasing amounts

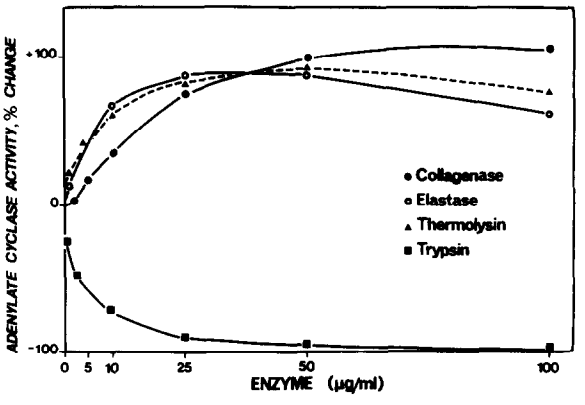

Fig.2. Effect of various proteinases upon the fluoride-sensitive adenylate cyclase. The assays were performed as described in the legend to fig.1, in the presence of varying amounts of (○) elastase, (▲) thermolysin, (●) collagenase and (■) trypsin.

of elastase, thermolysin and trypsin as compared with that of crude collagenase (see also ref.[1]). Half-maximal activation was observed at 10 μg/ml elastase and thermolysin and maximal activation occurred at 25 μg/ml. No inhibition was observed at higher concentrations of these proteinases. In contrast, trypsin was inhibitory, even at very low concentrations (0.5 μg/ml). Half-maximal inhibition was observed for 2–5 μg/ml trypsin, while complete inhibition of cyclase activity was observed at 50 μg/ml of this enzyme. An important degradative effect was also observed during incubation (10 min) in the presence of papain, subtilisin and alpha-chymotrypsin at concentrations higher than 10 μg/ml. This decrease in activation at the highest concentrations used was not observed for elastase and thermolysin. Thus, in subsequent kinetic and electrophoretic experiments, we used purified elastase from hog pancreas which was apparently not inhibitory at high concentration and migrated in a single band in SDS–polyacrylamide gel.

The velocity of cyclic AMP accumulation, (measured during consecutive 2 min periods) was studied as a function of time after the addition of elastase (fig.3).

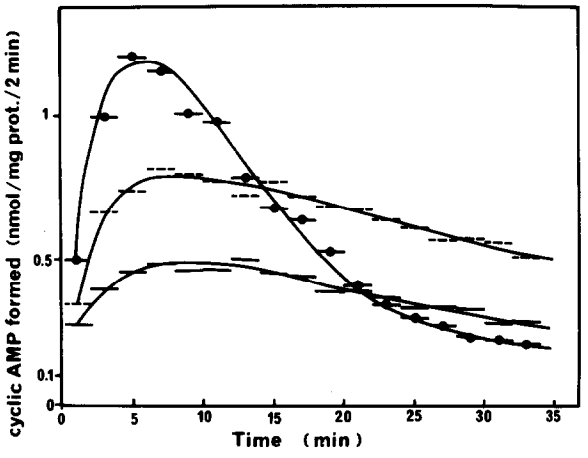

Fig.3. Time-course of adenylate cyclase activation by elastase. The assay medium was as described under Experimental Procedure except that it did not contain labeled [α-^{32}P]ATP. Time 0 of incubation was marked by the mixing of plasma membranes and cyclase assay medium containing 0 (——), 5 (---) or 50 (-•-) μg/ml elastase; both solutions were previously equilibrated at 30°C for 2 min. At the indicated time, aliquots of the assay medium were added to [α-^{32}P]ATP and the cyclase assay was performed for an additional 2 min period at 30°C. Adenylate cyclase activity is expressed as nmol cyclic AMP formed 2 min/mg membrane protein.

Addition of fluoride alone evoked a gradual increase in the velocity which was maximal after 6 min incubation. The velocity then remained constant, corresponding to a linear accumulation of cyclic AMP between 6 min and 18 min; subsequently the velocity decreased slowly. Addition of 5 μg/ml elastase promoted a striking acceleration in the cyclizing reaction, without changing the general pattern of the curve. Addition of 50 μg/ml elastase promoted more than a 2-fold increase in the velocity, which was maximal (1.2 nmol cyclic AMP formed/mg membrane protein in two min) between 4 min and 6 min of incubation. The velocity then decreased and fell to the level obtained with fluoride alone after 20 min incubation; this possibly resulted from the degradative action of elastase upon the catalytic site itself.

As previously shown with collagenase [1], proteolysis

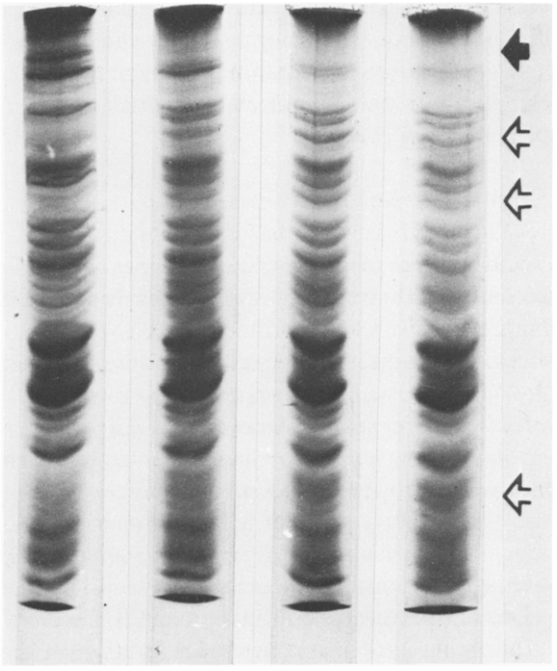

Fig.4. SDS–Polyacrylamide electrophoresis of rat-liver plasma membranes. Membranes (4 mg/ml) were preincubated in the presence of elastase (0.1 mg/ml) for 0, 15, 30 and 60 s (left to right). Reaction was stopped by the addition of the 'sample buffer' and by immediate boiling. Solubilized membranes (100 μg/50 μl) were allowed to run as described under Experimental Procedure. Origin was at top, anode at bottom. Solid arrow represents bands which have disappeared; open arrows represent bands which have appeared after elastase treatment.

appears to specifically stimulate adenylate cyclase system [1]. Thus, the question arises as to whether a change occurred in the structure of one or several proteins of the plasma membranes. We therefore studied the effect of low concentrations of elastase upon the electrophoretic pattern obtained from the membranes in SDS–polyacrylamide gels. Membranes, untreated or treated with 0.1 mg/ml elastase for 0, 15, 30, or 60 s, were electrophorized on SDS–polyacrylamide gels (fig.4). Normal, untreated membranes exhibited a usual proteic pattern [6], comprising 25–30 different bands. Addition of elastase, for incubation period as brief as 15 s, evoked a disappearance of two bands (indicated by solid arrow in fig.4) in the high molecular mass region (200 000–250 000 daltons) and the appearance of new proteins (indicated by open arrows) in the region of 100 000 daltons molecular mass. The effect of elastase upon the proteic pattern of the membranes paralleled adenylate cyclase activation with respect to the concentration range and the time course of the proteolytic action. Such high molecular weight proteins were insensitive to tryptic action during the same period.

4. Discussion

In the present report, we provide evidence that the adenylate cyclase system of rat-liver plasma membranes is highly sensitive to mild proteolysis. A 2–3-fold increase in activity was observed with various purified proteinases, but with no clear specificity towards a proteic sequence: serine esterase was as potent as metallo-enzymes or SH-proteinases in activating adenylate cyclase. However, the activating process seems to involve the cleavage of an intracatenary peptide bond. Exopeptidases which split-off free amino groups which are either N-terminal (like leucine aminopeptidase) or C-terminal (like carboxypeptidase A and B) are unable to activate the cyclase at concentrations ranging from 1–100 μg/ml (data not shown).

The nature of the proteolytic action of elastase upon the membranes was biphasic (fig.3). The increased reaction velocity observed during the first minutes of incubation was probably due to the unmasking of new catalytic sites. The decrease observed thereafter, at the highest concentrations, was probably due to the degradative action of this enzyme upon the catalytic site itself.

Proteolytic removal of some specific high molecular weight membrane proteins has been reported; as an example the Large External Transformation Sensitive (LETS: mol. wt 210 000–270 000) proteins are highly sensitive to trypsin [7] and papain [8]. Although the effect of elastase (fig.4) also appeared to be mainly exerted upon proteins of high molecular weight, the phenomenon described here is probably not related to the loss of LETS proteins: trypsin was absolutely ineffective in activating cyclase and loss of LETS proteins has never been correlated with a direct increase in cyclic AMP level [9,10].

In conclusion, we report here that several proteolytic enzymes are able to activate adenylate cyclase in a manner similar to that previously reported for crude collagenase from *Clostridium histolyticum* [1]. This as yet indescribed proteolytic activating process of adenylate cyclase system is most probably due to the unmasking of new catalytic sites, and appears related to structural modifications of one (or more) membrane proteins. The physiological relevance of such an activation may be of great importance if we consider that many circulating proteins as well as membranous ones [11] possess proteolytic activity, and may thus be involved in the modulation of the 'activable state' of target cells or tissues by unmasking new cyclase catalytic sites.

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