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Proteolytic activation of membrane-bound guanylate cyclase

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Abstract

Membrane-bound guanylate cyclase-A (GC-A), the receptor for atrial natriuretic factor (ANF), has been shown to be regulated by its kinase-like domain. To resolve the nature of this regulation, we measured the effects of various proteases on the activity of guanylate cyclase in rat lung membranes, and on the activity of the bacterial-expressed catalytic domain (GC-c) and on a recombinant peptide composed of both the kinase-like and catalytic domain (GC-kc) of guanylate cyclase. Pronase increased rat guanylate cyclase activity in a biphasic manner with a maximal effect at about 10–20 μg per assay tube. Thermolysin had effects similar to those of pronase on the activity of guanylate cyclase in rat lung membranes. In the case of bacterial-expressed proteins, pronase increased the activity of GC-kc, but not GC-c. These results indicate that GC-A contains an autoinhibitory site on its kinase-like domain, and that removal of the autoinhibitory site by limited proteolysis leads to enzyme activation. GC-A was poorly activated by ANF and ATP after the rat lung membrane was pretreated with pronase, suggesting that ANF/ATP and pronase activate guanylate cyclase through the same mechanism. It is suggested that the binding of ANF and ATP to GC-A may induce a conformational change of the receptor that releases the inhibitory constraint on enzyme activity leading to enzyme activation.

Keywords: Limited proteolysis; Guanylate cyclase; Pronase; Atrial natriuretic factor; cGMP; Autoinhibitory site

1. Introduction

ANF, synthesized by the cardiac atria, brain, and other tissues, produces profound changes in renal and cardiovascular functions, such as diuresis, natriuresis, and hypotension [for review, see Refs. 1 and 2]. One of the ANF receptors has been shown to be GC-A [for review, see Refs. 3–5]. The binding of ANF stimulates GC-A activity leading to the elevation of intracellular cGMP, which mediates most of the biological effects of ANF. It has been shown that maximal activation of GC-A by ANF requires the presence of ATP [6–13]. The effect of ATP on guanylate cyclase activity is due, in part, to nucleotide binding because the hydrolysis-resistant ATP analogs, AMPPNP and AMPPCP, also increase basal and/or ANF-stimulated guanylate cyclase [6–8,11]. The ATP-binding site has been suggested to be located on the kinase-like domain of GC-A [12]. However, other regulatory proteins such as protein kinase may also be involved in guanylate cyclase activation by ATP [7,10,13]. Therefore, the mechanisms of guanylate cyclase activation by ANF remain unclear.

GC-A contains an ANF-binding domain in its extracellular region, and a catalytic domain and a kinase-like domain in its intracellular region, where guanylate cyclase activity resides. It has been shown that deletion of the kinase-like domain of GC-A leads to an increase [14] or decrease [12] in basal enzyme activity. Although the results are opposite, these studies suggest that the kinase-like domain may regulate the active site (catalytic cavity) of guanylate cyclase. Releasing the inhibitory constraint has been shown to be the mechanism that activates many serine/threonine and tyrosine protein kinases such as cAMP- and cGMP-dependent protein kinases, myosin light chain kinase, protein kinase C, PDGF receptor, EGF receptor, and c-src proto-oncogene [15–22]. Binding of the ligand to its

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Abbreviations: ANF, atrial natriuretic factor; cAMP and cGMP, cyclic AMP and cyclic GMP, respectively; DTT dithiothreitol; EGF, epidermal growth factor; GC-A, membrane-bound guanylate cyclase-A; GC-c, the catalytic domain of guanylate cyclase; GC-kc, the kinase-like domain plus the catalytic domain of guanylate cyclase; and PDGF, platelet-derived growth factor.

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receptor releases the inhibitory constraint and leads to enzyme activation. Limited proteolysis can mimic the effects of ligand binding to its receptor and lead to the removal of the inhibitory constraint on enzyme activity. However, whether limited proteolysis can activate guanylate cyclase is controversial in the literature. Activation of rat liver guanylate cyclase by trypsin has been observed by Hanoune and co-workers [23,24], but not by Waldman and co-workers [25]. To examine whether guanylate cyclase is subjected to an inhibitory constraint, we measured the effects of several proteases on the activity of guanylate cyclase in rat lung membranes. We have expressed the GC-c [26] and the GC-kc [27] in *Escherichia coli.* To identify the inhibitory site, we measured the effect of pronase on the activity of the bacterial-expressed GC-c and GC-kc. The results indicated that the active site of guanylate cyclase is constrained by a region on the kinase-like domain.

2. Materials and methods

2.1. Materials

Synthetic ANF (rat ANF, 8–33) was purchased from Peninsula Laboratories, Inc. V8, pepsin, trypsin, pronase, thermolysin, and other common chemicals were purchased from the Sigma Chemical Co.

2.2. Preparation of rat lung membranes

Lungs from Sprague–Dawley rats (male, 200–250 g) were obtained after decapitation. Fresh rat lungs were homogenized at 4° with a Polytron homogenizer (Brinkmann Instruments) in 50 mM Tris buffer (pH 7.6), containing 1 mM EDTA, 1 mM DTT, 0.1% phenylmethylsulfonyl fluoride, phosphoramidon (10 μg/mL), leupeptin (10 μg/mL), pepstatin (10 μg/mL), and 250 mM sucrose. Homogenates were then centrifuged for 30 min at 35,000 *g* at 4°. Pellets were resuspended in the same buffer except that the concentration of EDTA was reduced to 0.1 mM. For the limited proteolysis study, membrane suspensions were centrifuged and resuspended in 50 mM Tris buffer (pH 7.6), containing 250 mM sucrose to remove protease inhibitors.

2.3. Guanylate cyclase assay

Guanylate cyclase was assayed at 37° in the presence of 50 mM Tris (pH 7.6), 2 mM isobutylmethylxanthine, 1 mM GTP, 4 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin, 25 mM creatine phosphate, and 55 U/mL of creatine kinase (135 U/mg protein) in a final volume of 0.1 mL. Reactions were initiated by the addition of the membrane suspension or bacterial-expressed proteins, incubated for 3 or 10 min, and terminated by the addition of 0.5 mL of 50 mM chilled sodium acetate (pH 4.0). Generated cGMP was quantified by radioimmunoassay [7–10,26,27].

2.4. Expression of GC-c and GC-kc in E. coli

Bacteria containing the GC-c [26] or the GC-kc of GC-A (bp 2673–3401) in pQE-9 [27] were grown in LB medium containing 100 μg/mL of ampicillin and 25 μg/mL of kanamycin at 30°. For induction of gene expression, isopropyl β-D-thiogalactopyranoside (200 μg/mL) was added, and bacteria were grown either overnight (for GC-c) or for 6 hr (for GC-kc).

2.5. Preparation of the bacterial extract of GC-c

Bacteria expressing GC-c were pelleted and resuspended in 25 mM Tris–HCl buffer (pH 7.6), containing 250 mM sucrose, 0.5 mM EDTA, and lysozyme (1 mg/mL) at 4°. Thirty minutes later, bacteria were sonicated in the presence of 5 mM DTT, 0.1% phenylmethylsulfonyl fluoride, phosphoramidon (10 μg/mL), and leupeptin (10 μg/mL). The suspension was then centrifuged, and the supernatant was used for assay of guanylate cyclase [26].

2.6. Isolation of inclusion bodies and renaturation of GC-kc

Bacteria expressing GC-kc (600 mL) were pelleted and resuspended in 40 mL of lysis buffer containing 25 mM Tris–HCl (pH 7.6), 0.5 mM EDTA, 250 mM sucrose, and 40 mg lysozyme. Thirty minutes later, 10 mM $MgCl_2$, 1 mM $MnCl_2$, and DNAse 1 (10 μg/mL) were added, and the bacterial suspension was kept on ice for another 30 min. Detergent buffer containing 0.2 M NaCl, 1% (w/v) deoxycholic acid, 1% (v/v) Nonidet P-40, and 2 mM EDTA was then added to the lysate. After a 30-min incubation at 4°, the mixture was centrifuged (5000 *g*, 10 min); the pellet was suspended in 0.5% Triton X-100 for 30 min and centrifuged (5000 *g*). The Triton X-100 extraction procedure was repeated until a tight pellet was obtained. The pellet (the inclusion bodies) was then resuspended in 20 mL Tris–HCl buffer (25 mM, pH 7.6) containing 1 mM EDTA.

For renaturation, the inclusion bodies containing GC-kc were first solubilized in 6 M guanidine–HCl in the presence of 5 mM DTT and 1 mM EDTA for 1 hr at room temperature. The insoluble material was removed by centrifugation. The enzyme activity of the solubilized GC-kc was restored by renaturation in Tris–HCl buffer (pH 7.6; 10 μg/mL), containing 2 mM $MgCl_2$, 1 mM DTT, 0.2 mM cystamine, and 250 mM sucrose [27].

3. Results

3.1. Effects of pronase and thermolysin on basal, ANF-, and ATP-stimulated guanylate cyclase activity in rat lung membranes, and on soluble guanylate cyclase from rat lungs

In rat lung membranes, guanylate cyclase activity was linear for at least 7 min in both the presence and absence of

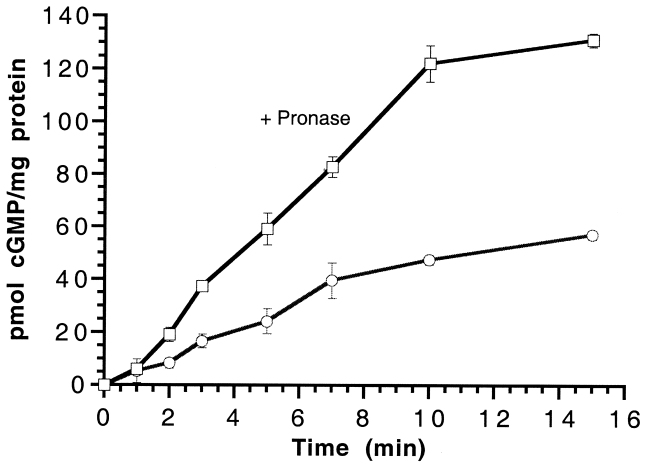

Fig. 1. Time course of pronase-catalyzed guanylate cyclase activation in rat lung membranes. Guanylate cyclase activity was measured in the presence (squares) and absence (circles) of 10 μg pronase. Values are means ± SD of four replicates.

10 μg pronase (Fig. 1). The presence of pronase increased the initial rate of cGMP formation, indicating that pronase treatment increases guanylate cyclase activity. Pronase affected basal and ANF-stimulated guanylate cyclase activity in these membranes with a peak at about 10–20 μg/100 μL (the assay volume) (Fig. 2A). Basal and ANF-stimulated enzyme activity were increased about 3.3- and 2.42-fold, respectively, by 10 μg/100 μL of pronase. Pronase at high concentrations inhibited guanylate cyclase activity, presumably by cleaving its catalytic domain. Although pronase increased basal and ANF-stimulated guanylate cyclase activity, it inhibited ATP-stimulated guanylate cyclase activity in a concentration-dependent manner with an IC_{50} of about 10 μg (Fig. 2B). Guanylate cyclase activity stimulated by both ATP (0.5 mM) and ANF (0.1 μM) was more sensitive to pronase than that stimulated by ATP alone. The IC_{50} of pronase for ANF/ATP-stimulated guanylate cyclase activity was about 2 μg.

Like pronase, thermolysin increased basal and ANF-stimulated guanylate cyclase activity (Fig. 3A), although its effect was less than that of pronase. Thermolysin inhibited 0.5 mM ATP- and ANF/ATP-stimulated guanylate cyclase activity with an IC_{50} at about 0.6 and 0.03 U, respectively (Fig. 3B). In contrast, V8 (up to 2 U per assay tube) and pepsin (up to 50 U per assay tube) had little effect on basal and ANF-stimulated enzyme activity, but inhibited ATP- and ANF/ATP-stimulated enzyme activity by about 30 and 63%, respectively (data not shown).

In the above experiments, the effects of proteases on basal and stimulated guanylate cyclase activity were measured by including proteases in the enzyme assay. To examine whether GC-A can still be activated by ANF and ATP after limited proteolysis, we incubated rat lung membranes with pronase (10 μg per assay tube) at 37° for 3 min, followed by centrifugation to remove the proteases. The pellet was resuspended with the preparation buffer without protease inhibitors and then assayed for basal, ANF-, and ATP-stimulated guanylate cyclase activity. Figure 4 shows that after pronase treatment, GC-A activity was slightly elevated compared with untreated membranes. The enzyme activity could not be robustly stimulated by ANF and/or ATP.

In contrast to its stimulatory effect on membrane-bound GC-A, pronase inhibited the activity of soluble guanylate cyclase from rat lungs (Fig. 5).

3.2. Effects of pronase on the activity of the bacterial-expressed GC-c and the GC-kc of guanylate cyclase

Proteolytic activation of GC-A could be due to the removal of an exogenous inhibitor or an inhibitory region in

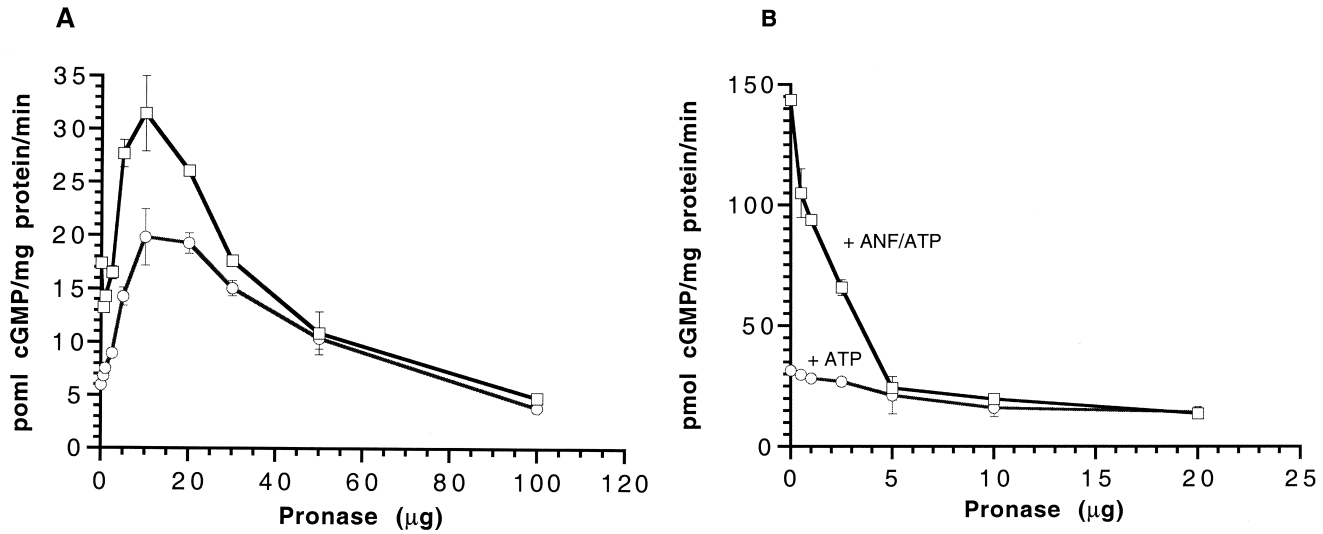

Fig. 2. Effects of pronase on basal, ANF-, and ATP-stimulated guanylate cyclase activity in rat lung membranes. Membranes were incubated (A) with pronase in both the absence (circles) and the presence (squares) of 0.1 μM ANF, or (B) with pronase in the presence of 0.5 mM ATP (circles) or 0.1 μM ANF plus 0.5 mM ATP (squares). Values are means ± SD of four replicates.

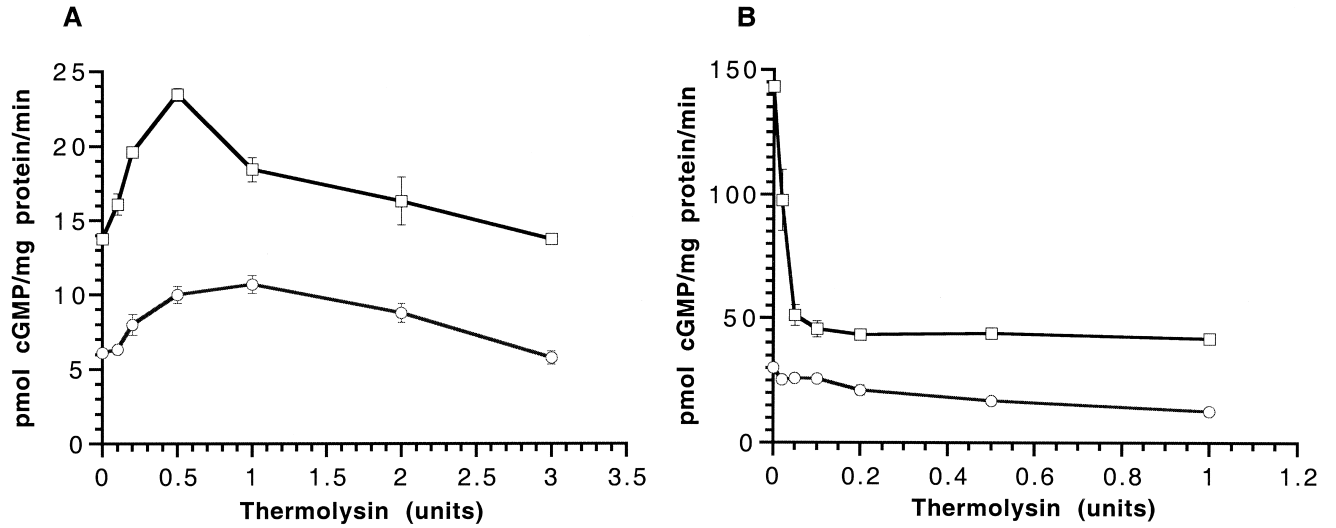

Fig. 3. Effects of thermolysin on basal, ANF-, and ATP-stimulated guanylate cyclase activity in rat lung membranes. Guanylate cyclase activity was measured in membranes incubated with (A) thermolysin in the absence (circles) and the presence (squares) of 0.1 μM ANF, or (B) thermolysin in the presence of 0.5 mM ATP (circles) or 0.1 μM ANF plus 0.5 mM ATP (squares). Values are means ± SD of four replicates.

the GC-A (the autoinhibitory site). To distinguish between these two possibilities, we compared the effects of pronase on bacterial-expressed GC-c and GC-kc. GC-c was present in bacterial inclusion bodies and cytosol. Cytosolic GC-c was catalytically active [26,28,29]. In contrast, GC-kc was only present in bacterial inclusion bodies. Its function could be restored through renaturation in guanidinine–HCl and dilution ([27] and see "Materials and methods").

Figure 6 shows the effects of pronase on the activity of GC-c and GC-kc. As in rat lung membranes, pronase increased GC-kc activity about 3.3-fold. However, it had no effect on the activity of GC-c.

4. Discussion

Deletion mutagenesis studies suggest that the activity of GC-A is either suppressed [14] or stimulated [12] by its kinase-like domain. In this study, we used another approach to resolve this controversy. We measured the effects of limited proteolysis on guanylate cyclase activity in rat lung membranes and in bacterial-expressed GC-c and GC-kc. Proteases such as pronase and thermolysin increased basal guanylate cyclase activity in rat lung membranes. Thus, our limited proteolysis studies indicate that in the unstimulated state, GC-A is under an inhibitory constraint, and that removal of the inhibitory constraint by limited proteolysis leads to enzyme activation. At higher concentrations, both pronase and thermolysin inhibited guanylate cyclase activity, presumably by cleaving the catalytic domain, which is

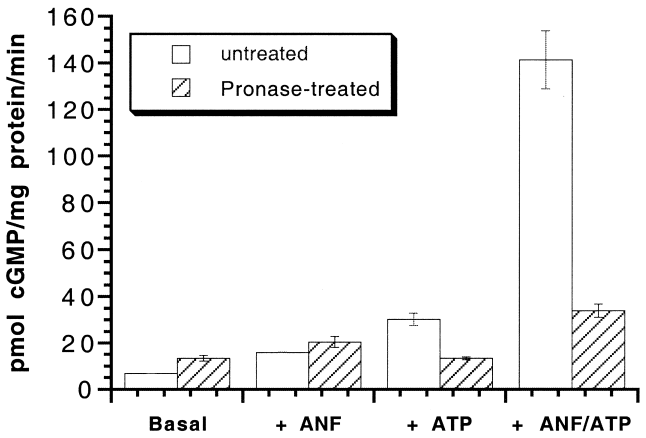

Fig. 4. Failure of GC-A activation by ANF and ATP in pronase-treated membranes. Rat lung membranes were incubated with (hatched columns) and without (open columns) pronase. Guanylate cyclase activity stimulated by ANF and ATP was measured under both conditions. Values are means ± SD of four replicates.

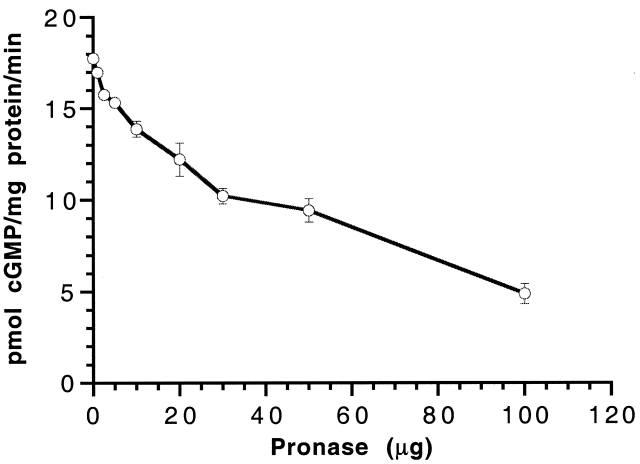

Fig. 5. Effects of pronase on soluble guanylate cyclase. Values are means ± SD of four replicates.

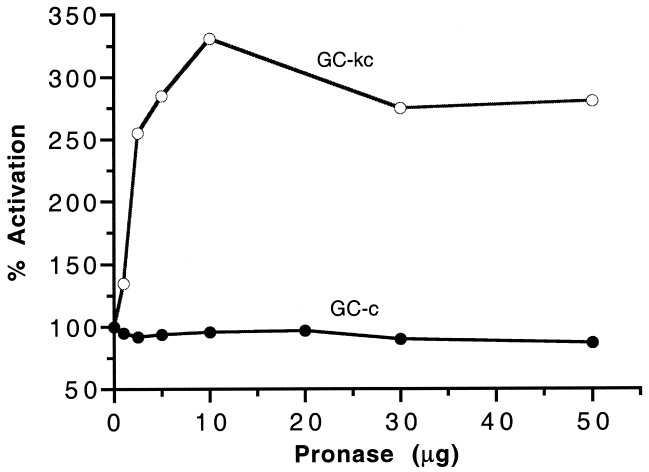

Fig. 6. Effects of pronase on the activity of GC-c and GC-kc. Values are the means of four replicates.

located at the carboxyl terminus of the enzyme. Other proteases such as V8, pepsin, and trypsin did not activate guanylate cyclase, probably because they cleave the residues on the active site of the enzyme. It should be noted that trypsin has been reported to activate guanylate cyclase in rat liver membranes [23,24], although contradictory results have been reported [25]. The discrepancy between these and our results could be due to the difference in sample preparations (crude membranes vs purified recombinant proteins), which might alter the susceptibility of the enzyme to trypsin.

Pronase and thermolysin also increased ANF-stimulated guanylate cyclase activity in rat lung membranes. This enhancement was likely due to elevated catalytic enzyme activity, and not to an increase or decrease of ANF clearance because addition of ANF [4–23]-NH2, the specific ligand for ANF clearance receptor, did not alter the effect of pronase on ANF-stimulated guanylate cyclase activity (data not shown). In contrast to basal and ANF-stimulated guanylate cyclase activity, ATP-stimulated enzyme activity was inhibited by pronase and thermolysin in rat lung membranes. Generally, proteins undergo a conformational change upon ligand binding. It is likely that the binding of ATP to guanylate cyclase induces a conformational change that exposes its active site to proteases. A similar argument may account for the higher sensitivity of ANF/ATP-stimulated guanylate cyclase activity than ATP-stimulated enzyme activity to pronase and thermolysin. The activation of guanylate cyclase by ATP has been found to vary with membrane preparations and with different laboratories. This variation may be due, in part, to the proteolysis of GC-A during membrane preparation because guanylate cyclase activity was not robustly stimulated by ATP and ANF after a brief incubation with pronase (Fig. 4).

The kinase-like domain of GC-A has been suggested to repress [14] or increase [12] the activity of the catalytic domain because deletion of this domain leads to an increase or decrease in basal enzyme activity. One possible reason for this controversy may be due to the fact that crude membranes were used for the studies. Our limited proteolysis studies with rat lung membranes support the hypothesis that GC-A is subject to an inhibitory constraint. However, these studies using the crude membranes could not resolve whether the suppression of guanylate cyclase activity is due to an exogenous inhibitor or to an autoinhibitory site located on the enzyme. To determine the nature of the inhibitory constraint imposed on GC-A, we examined the effects of pronase on the activity of GC-c and GC-kc. Pronase increased the activity of GC-kc, but not GC-c. These results clearly demonstrated that GC-A is constrained by an autoinhibitory site (not by an exogenous inhibitor), and that the autoinhibitory site is located on the kinase-like domain of GC-A. The exact location of the autoinhibitory site on GC-A remains unknown. We are in the process of determining the autoinhibitory site with the site-directed mutagenesis technique.

Activation of an enzyme by releasing the inhibitory constraint exerted by its autoinhibitory site has been shown to be an important mechanism in protein serine/threonine kinases (e.g. cAMP-and cGMP-dependent protein kinases, myosin light chain kinase, calmodulin-dependent kinase, protein kinase C) [15–20], protein tyrosine kinases (e.g. PDGF receptor, EGF receptor, and c-src proto-oncogene) [21,22], and calcineurin [30]. Binding of the ligand releases the inhibitory constraint and leads to enzyme activation. The effects of ligand binding on enzyme activation can be mimicked by limited proteolysis. In this study, we found that limited proteolysis increased guanylate cyclase activity, and that after proteolysis, the enzyme could not be robustly activated by ANF and ATP (Fig. 4). These results suggest that ANF, ATP, and limited proteolysis activate GC-A through the same mechanism (i.e. releasing the inhibitory constraint). Upon binding ANF and ATP, GC-A may undergo a conformational change, because its susceptibility to proteases was different in the presence and absence of ANF/ATP (Fig. 2).

The mechanisms of GC-A activation by ANF and ATP remain to be established. Unlike growth factor receptor such as EGF receptor, which requires receptor dimerization for activation, GC-A exists as a high molecular weight oligomer in the absence of ANF [for review, see Ref. 5]. Furthermore, activation of GC-A by ATP may require the participation of other proteins such as protein kinase and phosphatase [7,13]. However, although the initial and intermediate steps of GC-A activation are not clear, the final step of the enzyme activation is likely to be the release of an inhibitory constraint imposed on the active site of GC-A. It is suggested that the binding of ANF and ATP to GC-A may, directly or indirectly, induce a conformational change that disrupts the interactions between the autoinhibitory site and the active site of GC-A, thereby activating guanylate cyclase activity.

In conclusion, limited proteolysis increased the activity of GC-kc and guanylate cyclase, but not that of GC-c, in rat

lung membranes. These results indicate that in the unstimulated state, GC-A is constrained by an autoinhibitory site on its kinase-like domain. It is likely that binding of ANF and ATP to GC-A increases enzyme activity by releasing an inhibitory constraint.

Acknowledgments

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