Isoform-dependent activation of adenylyl cyclase by proteolysis

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Abstract Recent findings have suggested that the cellular proteolytic system plays a major role in the regulation of various intra- and extra-cellular signaling. It was previously shown that proteolytic treatment of adenylyl cyclase leads to the activation of this enzyme. We demonstrate that this activation occurs in an adenylyl cyclase isoform-dependent manner. The type II isoform was strongly activated (~500%), the type III isoform was modestly activated (~30%), and the type V isoform was inhibited by trypsin. Activation of type II adenylyl cyclase occurred in trypsin dose- and time-dependent manners and was blocked by a trypsin inhibitor in a dose-dependent manner. Other proteases, such as thrombin and plasminogen, similarly activated the type II isoform, but not the others. Our data suggest that proteolytic activation is an isoform- and thus cell type-dependent mechanism of altering adenylyl cyclase catalytic activity.

Key words: Adenylyl cyclase; Isoform; Proteolysis; Trypsin; Activation

1. Introduction

A variety of agents are known to regulate the catalytic activity of adenylyl cyclase [1,2]. Many hormones and neurotransmitters activate adenylyl cyclase through the cell surface receptors and thereby the stimulatory G-protein, Gsα. Sodium fluoride and guanine nucleotide analogs activate adenylyl cyclase by activating Gsα. Forskolin directly interacts with adenylyl cyclase and enhances its catalytic activity. Post-translational modification of adenylyl cyclase such as phosphorylation by protein kinase C also enhances cAMP production. Changes in the cation concentration, such as Mg and Mn, are yet another mechanism of regulating the catalytic activity of this enzyme.

Besides these mechanisms, it has been known that adenylyl cyclase is activated by proteolysis. The adenylyl cyclase system of rat liver plasma membranes was originally found to be stimulated by proteases contained in bacterial collagenase preparations [3]. This observation was extended by numerous other findings that demonstrated the activation of adenylyl cyclase by a variety of proteinases including trypsin, chymotrypsin, plasmin, and thrombin [4–6]. However, the effect of proteases and the degree of stimulation, as well as the site of interaction, were not consistent among studies using different tissues and proteases [4–6].

Recent molecular cloning studies in the past 5 years revealed that adenylyl cyclase consists of multiple isoforms [2]. Although they all share the common structure, i.e., a module of six transmembrane spans linked to a large cytoplasmic domain that is tandemly repeated, these isoforms have distinct amino acid sequences, biochemical properties, and tissue distribution. Recent studies have also suggested that the cellular proteolytic system plays a major role in the regulation of various intra- and extra-cellular signaling, including cAMP signaling [7]. In the present work, we use adenylyl cyclase isoforms overexpressed in insect cells to show that proteolytic activation of adenylyl cyclase is not a common property but one that belongs only to certain isoforms of adenylyl cyclase.

2. Materials and methods

2.1. Materials

Proteases (trypsin, plasminogen, and thrombin) and trypsin inhibitor (ovomucoid from chicken egg white) were purchased from Sigma (St. Louis, MO).

2.2. Overexpression of recombinant adenylyl cyclase isoforms

Overexpression of types II and V adenylyl cyclase in insect cells was accomplished with the pBluBac system (Invitrogen, San Diego, CA) as previously described [8–10]. For type III adenylyl cyclase, a *Bam*HI–*Hin*dIII cDNA fragment was subcloned into a pBluBacHisB vector, and a high-titer virus stock solution was prepared as previously described [8]. Type II and III adenylyl cyclase cDNA clones were kindly provided by Dr. R. Reed (Johns Hopkins University).

2.3. Insect cell membrane preparations

Types II, III, and V adenylyl cyclase were overexpressed in High Five (H5) insect cells as previously described [8]. In brief, 60 h after infection, insect cells were washed twice with ice-cold phosphate-buffered saline and homogenized in a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, and 200 mM sucrose in the presence or absence of a protease inhibitor mixture (10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 50 U of egg white trypsin inhibitor, 20 μg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone, 20 μg/ml 1-chloro-3-tosylamido-7-amino-2-heptanone, and 2 μg/ml aprotinin). Cells were disrupted with a sonicator or Polytron and centrifuged at $500 \times g$ for 10 min at 4°C. The supernatants were further centrifuged at $100\,000 \times g$ for 30 min at 4°C. The resultant pellets were resuspended in the same buffer without EGTA. The crude membrane preparations were stored at −70°C until use.

2.4. Adenylyl cyclase assay

Adenylyl cyclase activity was measured as previously described [11]. In brief, 4 μg of the crude membrane preparations from insect cells overexpressing adenylyl cyclase isoforms was incubated in a buffer containing 20 mM Hepes (pH 8.0), 5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM dithiothreitol, 0.1 mM ATP, 0.1 mM cAMP, 1 mM creatine phosphate, 8 U/ml creatine phosphokinase, and [^{32}P]αATP (≈1 μCi/assay tube). Trypsin and other proteases were added to the reaction mixture when the assay was started. The reaction mixture was incubated for 20 min at 30°C. cAMP was separated by the method of Salomon [12]. Protein concentration was determined by the method of Bradford [13].

3. Results

3.1. Activation of type II adenylyl cyclase by trypsin

When the reaction mixture contained trypsin, the forskolin-stimulated catalytic activity was altered in trypsin concentration- and adenylyl cyclase isoform-dependent manners (Fig.

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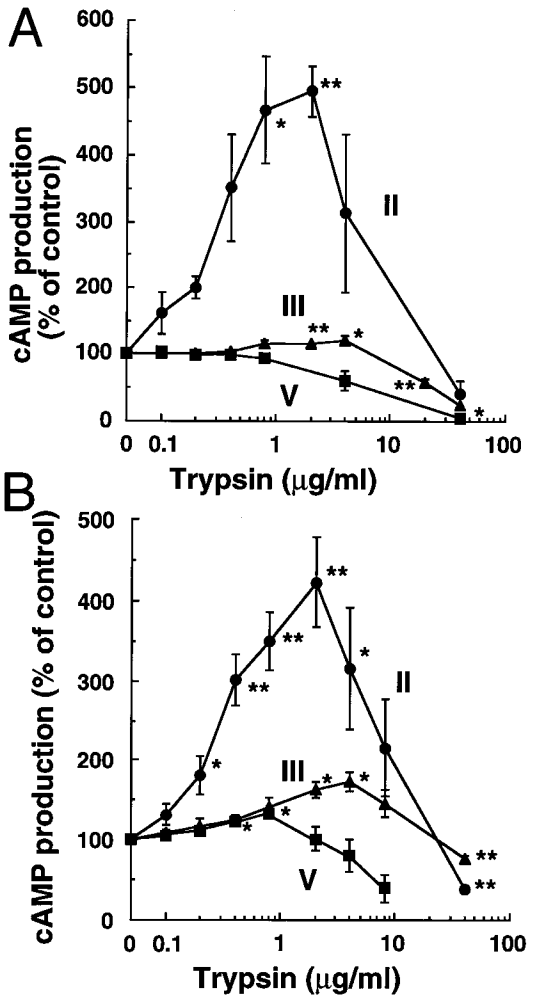

Fig. 1. Effects of trypsin on type II, III, and V adenylyl cyclase catalytic activity. A: Effects of trypsin on forskolin-stimulated activity. B: Effects of trypsin on basal activity. Adenylyl cyclase assays were performed in the presence (A) and absence (B) of 100 μM forskolin with an increasing concentration of trypsin. ●, type II adenylyl cyclase; ▲, type III adenylyl cyclase; ■, type V adenylyl cyclase. Means ± SEM from 3 to 6 independent assays are shown. $^{*}P < 0.05$; $^{**}P < 0.01$ differences form the values without trypsin. In the absence of trypsin, the forskolin-stimulated activity was 11.4 ± 1.5 nmol/20 min/mg for type II, 6.24 ± 0.77 nmol/20 min/mg for type III, and 25.2 ± 2.03 nmol/20 min/mg for type V. The basal activity was 1.62 ± 0.11 nmol/20 min/mg for type II, 0.27 ± 0.02 nmol/20 min/mg for type III, and 1.38 ± 0.24 nmol/20 min/mg for type V.

1A). At low concentrations (0.1–2 μg/ml), trypsin strongly (~500%) activated the type II isoform, modestly (~20%) activated the type III isoform, and scarcely activated the type V isoform. At high concentrations, trypsin inhibited all three isoforms. A similar pattern of alteration was observed when assays were performed without forskolin (Fig. 1B). Thus, the effect of trypsin was biphasic, and the degree of activation was dependent upon the isoform of adenylyl cyclase.

3.2. Time-course of the activation of the type II isoform

The activation of the type II isoform by trypsin was time-dependent (Fig. 2). As early as 5 min after initiation of the reaction, trypsin (2 μg/ml) significantly increased the catalytic activity of type II adenylyl cyclase compared with the activity

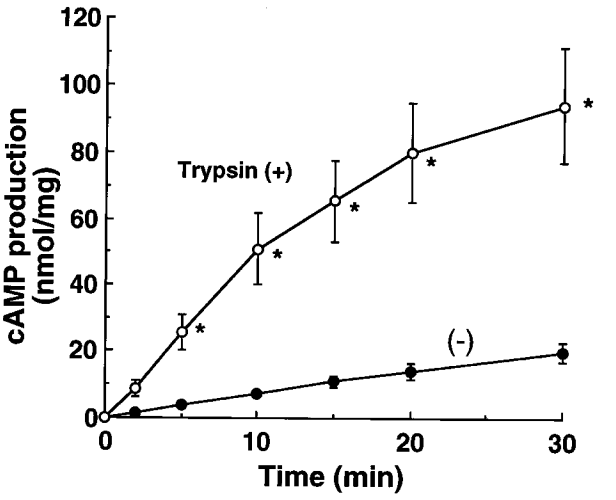

Fig. 2. Effect of trypsin inhibitor on type II adenylyl cyclase activation by trypsin. Adenylyl cyclase assays were performed in the absence (●) or presence (○) of trypsin (0.4 μg/ml) with an increasing concentration of a trypsin inhibitor (ovomucoid from egg white). Means ± SEM from 3 independent assays are shown. $^{*}P < 0.05$ for differences between the values with and without trypsin.

without trypsin. The rate of cAMP production was constant in the absence of trypsin for 30 min; however, the rate gradually decreased over 30 min in the presence of trypsin.

3.3. Effect of trypsin inhibitor

Stimulation of type II adenylyl cyclase by trypsin was negated in the presence of a trypsin inhibitor. Ovomucoid from chicken egg white, a trypsin inhibitor, in the reaction mixture prevented the activation by trypsin in a dose-dependent manner (Fig. 3).

3.4. Kinetic analysis

Activation of type II adenylyl cyclase by trypsin was accompanied by changes in kinetic parameters. The V_{max} value

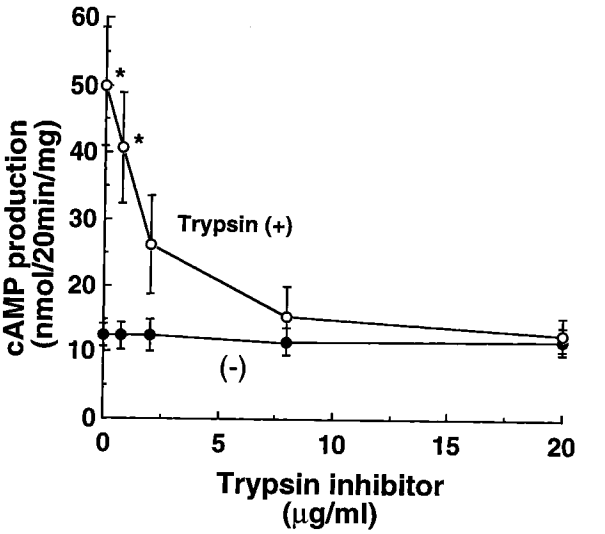

Fig. 3. Time-course of the activation of type II adenylyl cyclase by trypsin. Adenylyl cyclase assays were performed in the absence (●) or presence (○) of trypsin (2 μg/ml) for the indicated time. Means ± SEM from 4 independent assays are shown. $^{*}P < 0.05$ for differences between the values with and without trypsin.

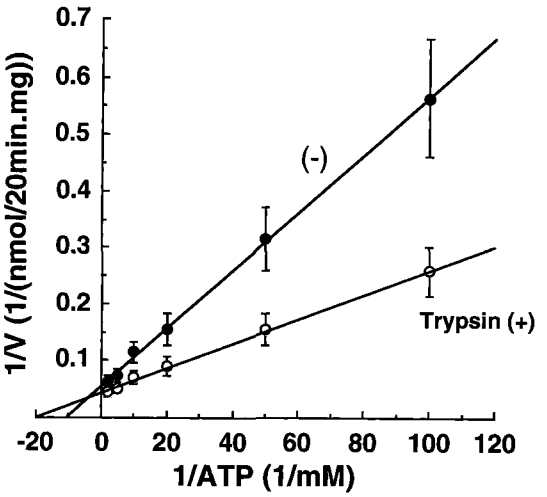

Fig. 4. Kinetic analysis (Lineweaver-Burk plot) of the activation of type II adenylyl cyclase by trypsin. Four micrograms of crude membranes overexpressing type II adenylyl cyclase were incubated in the absence (●) and presence (○) of trypsin (0.4 μg/ml) for 5 min at 30°C. After 5 min incubation, the reaction was stopped by the addition of 2 μg of a trypsin inhibitor from egg white. Adenylyl cyclase assays were performed in the presence of an increasing concentration of ATP for 15 min at 30°C. V_{max}: 0.97±0.17 to 1.24±0.24 nmol/min/mg ($n=3$), n.s. K_m: 93.7±5.6 to 50.7±4.1 μM ($n=3$), $P<0.05$

was increased by 26% while the K_m value decreased by 46%, although only the latter change was statistically significantly (Fig. 4).

3.5. *Effects of plasmin and thrombin*

When plasmin (0–400 μg/ml) and thrombin (0–500 U/ml) were part of the reaction mixture, they mimicked the effect of trypsin. They altered the forskolin-stimulated catalytic activity of adenylyl cyclase in protease concentration- and adenylyl cyclase isoform-dependent manners (Fig. 5). At low concentrations, these proteases strongly activated the type II isoform (500–700%), while the other isoforms were either inhibited or unaffected. At high concentrations, the stimulation of type II adenylyl cyclase was weak.

4. Discussion

Our data show that proteolytic activation of adenylyl cyclase occurs in an isoform-dependent manner. In particular, the type II isoform was potently activated. Type II adenylyl cyclase belongs to a subgroup consisting of types II, IV, and VII, which are widely distributed in many, but not all, tissues [14–16]. Type III adenylyl cyclase, a major olfactory isoform, was poorly activated [17]. Type V adenylyl cyclase, a member of the cardiac subgroup, was resistant to proteolytic activation [18]. The effect of other serine proteases, such as plasmin and thrombin, was similar to that of trypsin among these isoforms. The fact that proteolytic activation by trypsin occurred only in a subset of adenylyl cyclase isoforms may explain the inconsistent results in past studies using different tissues. Trypsin stimulated adenylyl cyclase from ovarian tissues [5,19] and fibroblasts [20,21] but had no effect on or was inhibitory to adenylyl cyclase from liver and erythrocytes [4,22]. We now know that each tissue expresses a distinct mixture of adenylyl cyclase isoforms.

We do not know the exact mechanism that specifically activates type II adenylyl cyclase. It may unmask a new catalytic site(s) by removing a putative inhibitory domain within this enzyme through peptide band hydrolysis. Alternatively, it may modify the conformation of this isoform. We and others have recently demonstrated that type II adenylyl cyclase catalytic activity is sensitive to conformational changes [23,24].

The cellular proteolytic system is now known to be involved in the regulation of various intra- and extra-cellular signaling [7]. The role of proteolysis may vary from the degradation of disused proteins to the formation and activation of a specific class of enzymes and hormones. A cascaded proteolytic reac-

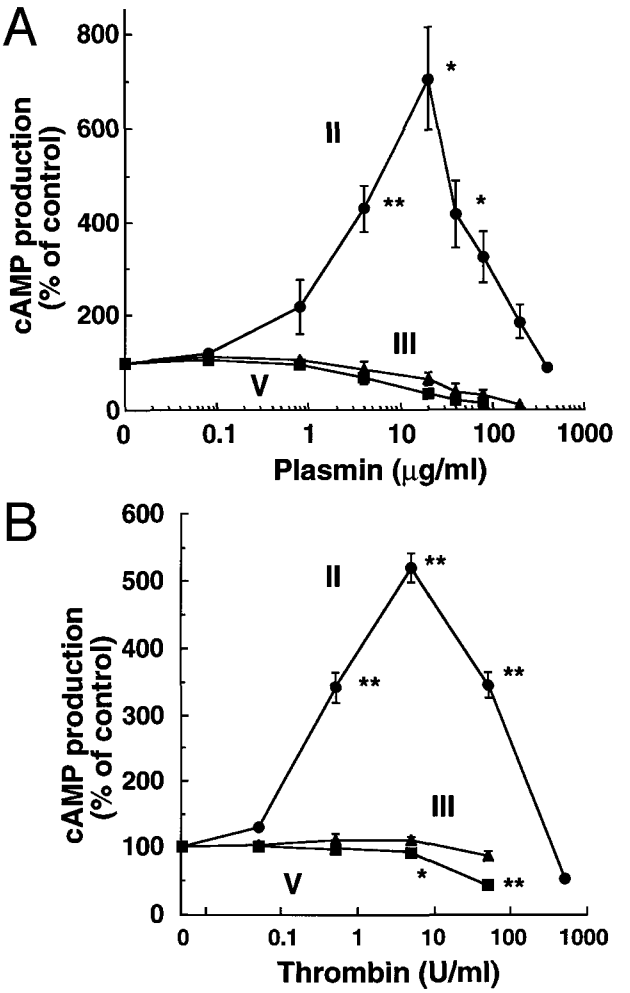

Fig. 5. Effects of plasmin and thrombin on adenylyl cyclase isoforms (types II, III, and V). A: Effect of plasmin. Adenylyl cyclase assays were performed in the presence of 100 μM forskolin with an increasing concentration of plasmin. Means±SEM from 3 or 4 independent assays are shown. $^*P<0.05$; $^{**}P<0.01$ for differences from the values without plasmin. In the absence of plasmin, the forskolin-stimulated activity was 15.9±1.9 nmol/20 min/mg for type II, 6.48±0.59 nmol/20 min/mg for type III, and 29.6±2.4 nmol/20 min/mg for type V. B: Effect of thrombin. Adenylyl cyclase assays were performed in the presence of 100 μM forskolin with an increasing concentration of thrombin. Means±SEM from 3 or 4 independent assays are shown. $^*P<0.05$; $^{**}P<0.01$ for differences from the values without thrombin. In the absence of thrombin, the forskolin-stimulated activity was 15.7±2.3 nmol/20 min/mg for type II, 5.97±0.62 nmol/20 min/mg for type III, and 32.3±1.6 nmol/20 min/mg for type V. ●, type II adenylyl cyclase; ▲, type III adenylyl cyclase; ■, type V adenylyl cyclase.

tion triggered by extracellular stimuli may also result in the control of cell proliferation and differentiation as well as in the programmed cell death. The cAMP signaling system is known to regulate intracellular proteolytic activity. cAMP accelerates the proteolytic processing of proenkephalin to enkephalin [25]. cAMP activates elastase in T cells [26]. cAMP enhances the transcription of granzyme B, a protease from cytotoxic T lymphocytes that is known to play a major role in T lymphocyte-mediated apoptosis [27]. cAMP is also known as an inducer of apoptosis in certain cell types [28,29].

With these recent findings, it may not be surprising that the cAMP signaling is regulated through proteolysis. LH-stimulated cAMP production and subsequent steroidogenesis in Leydig cells requires proteolysis [30]. β-agonist-induced activation of calpain in alveolar epithelial type II cells leads to proteolytic activation of cAMP-dependent protein kinase [31]. Similar proteolytic activation of protein kinase A is also shown in aplasia [32]. Furthermore, regulation of a second messenger producing enzyme by the proteolytic system has been demonstrated: phospholipase C, a diacyl glycerol-producing enzyme, is activated through proteolysis by calpain in an agonist-induced manner [33]. Although we do not know the exact mechanism of adenylyl cyclase activation through proteolysis in vivo, our findings suggest that this activation occurs in an isoform-dependent manner and thus in selected cell types that express proteolysis-sensitive adenylyl cyclase isoforms. Certainly, further studies are needed to elucidate this issue.

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