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Enzymatic activation of autotaxin by divalent cations without EF-hand loop region involvement

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Abstract

Autotaxin (ATX) is a recently described member of the nucleotide pyrophosphatase/phosphodiesterase (NPP) family of proteins with potent tumor cell motility-stimulating activity. Like other NPPs, ATX is a glycoprotein with peptide sequences homologous to the catalytic site of bovine intestinal alkaline phosphodiesterase (PDE) and the loop region of an EF-hand motif. The PDE active site of ATX has been associated with the motility-stimulating activity of ATX. In this study, we examined the roles of the EF-hand loop region and of divalent cations on the enzymatic activities of ATX. Ca^{2+} or Mg^{2+} was each demonstrated to increase the PDE activity of ATX in a concentration-dependent manner, whereas incubation of ATX with chelating agents abolished this activity, indicating a requirement for divalent cations. Non-linear regression analysis of enzyme kinetic data indicated that addition of these divalent cations increases reaction velocity predominantly through an effect on $V_{max.}$ Three mutant proteins, Ala^{740}-, Ala^{742}-, and Ala^{751}-ATX, in the EF-hand loop region of ATX had enzymatic activity comparable to that of the wild-type protein. A deletion mutation of the entire loop region resulted in slightly reduced PDE activity but normal motility-stimulating activity. However, the PDE activity of this same deletion mutant remained sensitive to augmentation by cations, strongly implying that cations exert their effect by interactions outside of the EF-hand loop region.

Keywords: Autotaxin (ATX); Tumor cell motility; Type I phosphodiesterase (PDE); EF-hand loop; Mutagenesis

1. Introduction

ATX, a 125-kDa glycoprotein, was initially isolated from the culture supernatants of a human melanoma cell line (A2058) [1]. It has been shown to stimulate random and directed motility of human tumor cells at high picomolar to low nanomolar concentrations. The locomotory response to ATX was demonstrated to be sensitive to pretreatment of the cells with pertussis toxin, indicating that a G protein is involved in the signal transduction pathway [2]. Cloning and sequencing of ATX [3,4] have revealed homology with a family of NPPs, which include the ectoprotein PC-1 [5], rat brain PD-Iα [6], and rat neural differentiation antigen ($gp130^{RB\ 13-6}$) [7]. The deduced amino acid sequence of these proteins revealed that each has two adjacent somatomedin B domains, a PDE active site, and an EF-hand loop region. A recent study showed that the PDE catalytic site of ATX is necessary for its tumor cell motility-stimulating activity [8].

The role of divalent cations in the NPPs is a subject of particular interest because of the presence of a partial EF-hand motif within these molecules. Belli *et al.* [9] suggested that divalent cations stabilize the conformation of PC-1. Divalent cations were also found to protect solubilized PC-1 against thermal denaturation and proteolysis. Rebbe *et al.* [10] found that the PDE activity of PC-1 is decreased by chelating agents, and Oda *et al.* [11] found that this activity is stimulated by adding divalent cations. In addition to affecting PDE activity, divalent cations were also found to stimulate, whereas chelating agents inhibited the autophos-

Abbreviations: ATX, autotaxin; NPP, nucleotide pyrophosphatase and phosphodiesterase; PDE, phosphodiesterase; PCR, polymerase chain reaction; and DMEM, Dulbecco's modified Eagle's medium.

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phorylation of PC-1 [11]. In this manuscript, we characterize the role of the EF-hand loop region and the effect of divalent cations on the PDE activity of ATX.

2. Materials and methods

2.1. Materials

DEAE-dextran, EDTA, EGTA, and *p*-nitrophenyl TMP were obtained from the Sigma Chemical Co. The 48-well microchemotaxis chambers and the polyvinylpyrrolidone-free polycarbonate membranes were purchased from NeuroProbe.

2.2. Mutagenesis of the EF-hand loop of ATX

A 2.8-kb pair DNA fragment (T9S2A), encoding the entire 863-amino acid peptide of teratocarcinoma ATX, was constructed in pcDNA3 (Invitrogen Co.), as described previously, to produce pcDNATX [8]. The deletion mutant of the EF-hand loop of ATX was constructed by using PCR and DNA cloning procedures as described previously [12]. The site-directed mutants of the EF-hand loop of ATX were made by overlap extension PCR [13]. Each mutant plasmid was sequenced to confirm the presence of the mutation and the fidelity of the PCR amplification. COS-1 cells were transiently transfected with pcDNATX or with mutant plasmid using the DEAE-dextran method [14]. Transfected cells were allowed to recover overnight in complete medium [DMEM supplemented with 2 mM glutamine, 100 U/mL of penicillin and 100 μg/mL of streptomycin, and 10% (v/v) heat-inactivated fetal bovine serum]; then the medium was changed to DMEM containing 0.1 mg/mL of bovine serum albumin. After 48 hr, the medium was harvested and concentrated using a Centriprep-30 ultrafiltration device (Amicon). The control for all experiments was COS-1 cells, transfected with the empty (pcDNA3) vector. The concentrated supernatants were partially purified by lectin affinity chromatography utilizing concanavalin A-agarose (Vector Laboratories), as described previously in detail [8].

2.3. Assay for type I PDE activity

The 5′-nucleotide PDE activity was measured using the colorimetric method as described previously in detail [15]. Samples (20 μL) were added to 80 μL of 50 mM HEPES (pH 7.3) containing 5 mM *p*-nitrophenyl TMP (Sigma). After incubation at 37° for 90 min, the reactions were terminated by the addition of 0.1 N NaOH (900 μL). The reaction product was quantified by reading the absorbance at 410 nm.

Enzyme kinetics were assayed by varying the substrate (*p*-nitrophenyl TMP) concentration with or without a constant concentration of Mg^{2+} (10 mM) and measuring product formation after a 20-min incubation at 37°. Linear regression analysis of the (410 nm) absorbance values for known concentrations of *p*-nitrophenol yielded the following simple formula for calculating product formation:

$$(A_{410}) \times 64 = \text{nmol } p\text{-nitrophenol}$$

Reaction rates (nanomoles/minute) could then be calculated by dividing this number by 20 min. Non-linear regression analyses of the kinetic data with calculation of V_{max} and K_m were performed utilizing the statistical software package Prism 3.0 for Mac (GraphPad Software, Inc.).

2.4. Western blot analysis

Protein samples were separated by SDS–PAGE in a Tris/glycine buffer system using prepared 8–16% gradient gels (NOVEX) and transferred to Immobilon membranes for western blot analysis as described previously [8]. Immunoblots utilized affinity-purified anti-ATX 102 peptide (1:500) as primary antibody and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (1:50,000) as secondary antibody (Pierce). The blot was treated with Enhanced Chemiluminescence (ECL) reagents using the manufacturer's protocol (Amersham Life Sciences), exposed to Hyperfilm-ECL for 0.5 to 5 min, and then developed in an X-Omat film developer. The band density was analyzed utilizing EagleSight Software v. 3.2 (Stratagene).

2.5. Cell motility assay

Motility assays were performed in triplicate using a 48-well microchemotaxis chamber as described previously in detail [1]. Eight micron pore filters (Nucleopore) were coated with 0.01% gelatin in 0.1% acetic acid overnight and used in these modified Boyden chamber motility assays. Filters were fixed and stained using Diff-Quik reagents (American Scientific Products). Chemotaxis was quantified densitometrically using EagleSight Software v. 3.2 (Stratagene) for data analysis.

3. Results

3.1. Effect of chelating agents on PDE activity of ATX

ATX, initially isolated as an agent that stimulates tumor cell motility, was later determined to be a member of the NPP family of ecto-exo enzymes. Since then, it has been shown that the tumor cell motility-stimulating activity of ATX is closely linked to its PDE catalytic site [8]. Previous studies have found that the enzyme activities of PC-1 are obliterated by chelating agents. We, therefore, preincubated partially purified ATX with EDTA or EGTA and assayed the effect on PDE activity. As shown in Fig. 1, each of the chelating agents decreased the PDE activity of ATX in a concentration-dependent manner. This activity was abol-

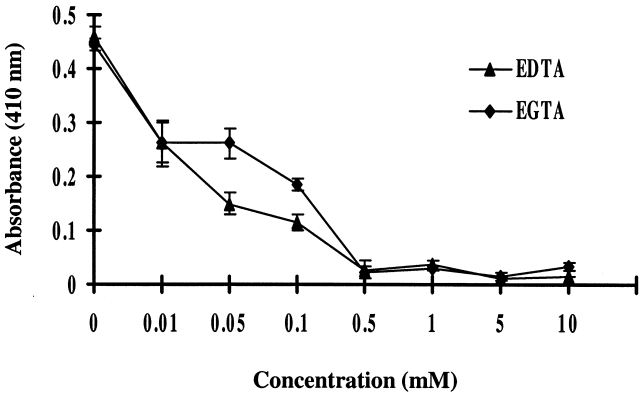


Fig. 1. Effect of chelating agents on PDE activity of ATX. Partially purified ATX was preincubated with EDTA or EGTA for 30 min at 37°, and its PDE activity was assayed as described in "Materials and methods." Data (means ± SD) shown are typically representative of four separate experiments.

ished at concentrations of 0.5 mM for both chelating agents. These data strongly suggest that the PDE activity of ATX requires the presence of divalent cations.

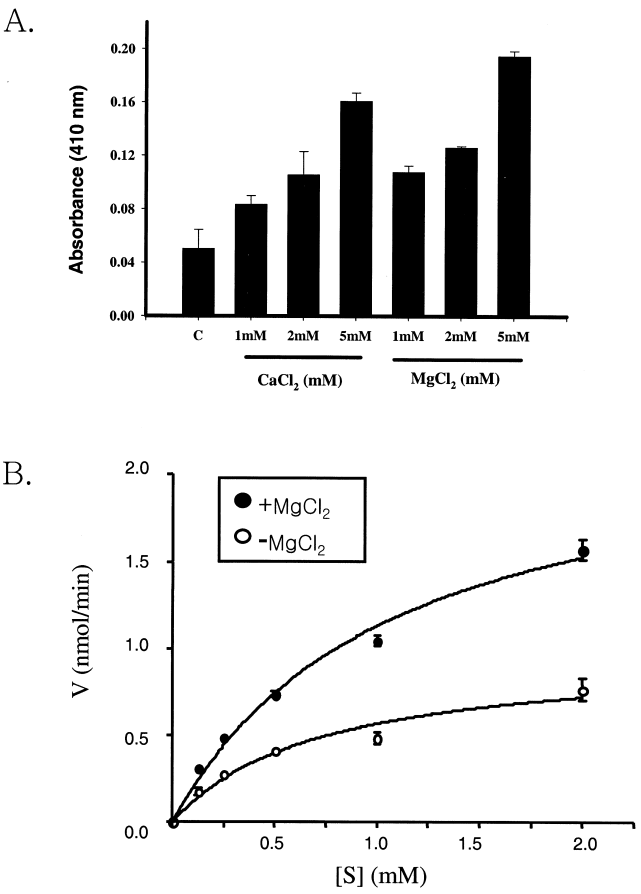
3.2. Effect of divalent cations on PDE activity of ATX

To confirm that divalent cations affect the NPP activity of ATX, we next measured this activity in the presence of increasing concentrations of Ca^{2+} or Mg^{2+}. We found that the addition of either cation augmented the measured PDE activity of ATX in a concentration-dependent manner (Fig. 2A). At 2 mM $CaCl_2$ and $MgCl_2$, this PDE activity of ATX was increased 110 and 151%, respectively, above control (C) levels. The stimulation of PDE activity by cations was not simply the result of an increase in ionic strength since the inclusion of NaCl at concentrations up to 300 mM had no effect on the PDE activity of purified ATX [16].

We next compared the kinetics of the PDE reaction with or without the addition of (10 mM) Mg^{2+} to the reaction mixture (Fig. 2B). Non-linear regression analysis of the substrate concentration versus the reaction velocity data revealed a small increase (48%) in K_m (0.73 ± 0.18 mM without Mg^{2+} compared to 1.08 ± 0.16 mM with Mg^{2+}); however, this difference was not statistically significant. There was a much larger increase (140%) in V_{max} (0.98 ± 0.11 nmol/min without Mg^{2+} and 2.35 ± 0.17 nmol/min with Mg^{2+}) that was statistically significant at $P < 0.05$. One interpretation of these data is that Mg^{2+} does not change the affinity of the enzymatic site for its substrate.

3.3. Effect of EF-hand loop mutations on the enzymatic activities of ATX

Having shown that the NPP activity of ATX is enhanced by the addition of cations to the reaction, we next sought to determine whether the EF-hand loop region of ATX was required for this effect. We constructed three site-directed



	Km (mM)	Vmax (nmol/min)
-$MgCl_2$	0.73 ± 0.18	0.98 ± 0.11
+$MgCl_2$	1.08 ± 0.16	2.35 ± 0.17

Fig. 2. Effect of divalent cations on PDE activity of ATX. (A) PDE activity of partially purified ATX assayed at various concentrations of $CaCl_2$ or $MgCl_2$. (B) Non-linear regression analysis of PDE activity of ATX in the presence or absence of 10 mM $MgCl_2$. Data (means ± SD) shown are typically representative of four separate experiments.

point mutants and a deletion mutant within the EF-hand loop of ATX (Fig. 3). Site-directed mutant proteins, D740A, D742A, and D751A, as well as a deletion mutant of the entire EF-hand region (EF), were expressed in and secreted by COS-1 cells. The conditioned medium from each mutant cell line was partially purified through a concanvalin-A agarose column, and then compared by immunoblot analysis (Fig. 4A). As seen in Fig. 4B, the point-mutated proteins had only slight differences in their PDE activity from wild-type ATX. Specifically, D740A, D742A, and D751A mutants had 90, 81, and 98%, respectively, of the PDE activity of wild-type ATX. In addition, these mutants had almost identical tumor cell motility-stimulating activities compared with wild-type ATX (Fig. 4C). When we deleted the entire EF-hand loop region, the resultant mutant protein had 73% of the PDE activity of wild-type ATX. This same EF mutant

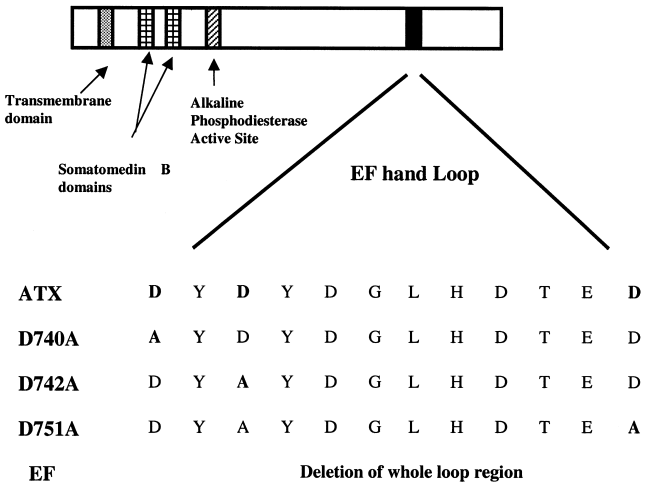


Fig. 3. Peptide sequence of ATX and mutants around the EF-hand loop region.

had identical tumor cell motility-stimulating activity as wild-type ATX. Although there was a slight reduction in the PDE activity of some mutants, our results suggest that the EF-hand loop region of ATX is not necessary for tumor cell motility-stimulating activities.

3.4. Effect of divalent cations on the PDE activity of the EF-hand loop region deleted ATX mutant

We compared the effects of divalent cations on the PDE activity of the EF-hand loop deletion mutant (EF in Fig. 3) with that of wild-type ATX. Increasing concentrations of divalent cations increased the PDE activity of the deletion mutant in a manner that was virtually identical to their effect on wild-type ATX (Fig. 5). Therefore, the EF-hand loop region of ATX is not required for the augmentation of PDE enzymatic activity by divalent cations.

4. Discussion

The deduced amino acid sequence of ATX has revealed that it contains a peptide that is very close to the consensus sequence for an EF-hand loop region. This strongly acidic peptide is thought to provide the actual Ca^{2+} binding site for calmodulin, calcineurin B, troponin C, the large subunit of calpain, and many other calcium-binding proteins [17]. However, what role this EF-hand-like motif might play in the enzymatic or tumor cell motility-stimulating activities of ATX was unknown. In this study, we demonstrated that divalent cations enhance the PDE activity of ATX. We produced a series of point mutations in the EF-hand region of ATX as well as a deletion mutation of the entire loop region. Utilizing these mutants, we demonstrated that the ATX EF-hand region is not necessary for either its PDE or motility-stimulating activities. Furthermore, the full deletion mutant remained sensitive to enhancement of its PDE activity by the addition of divalent cations.

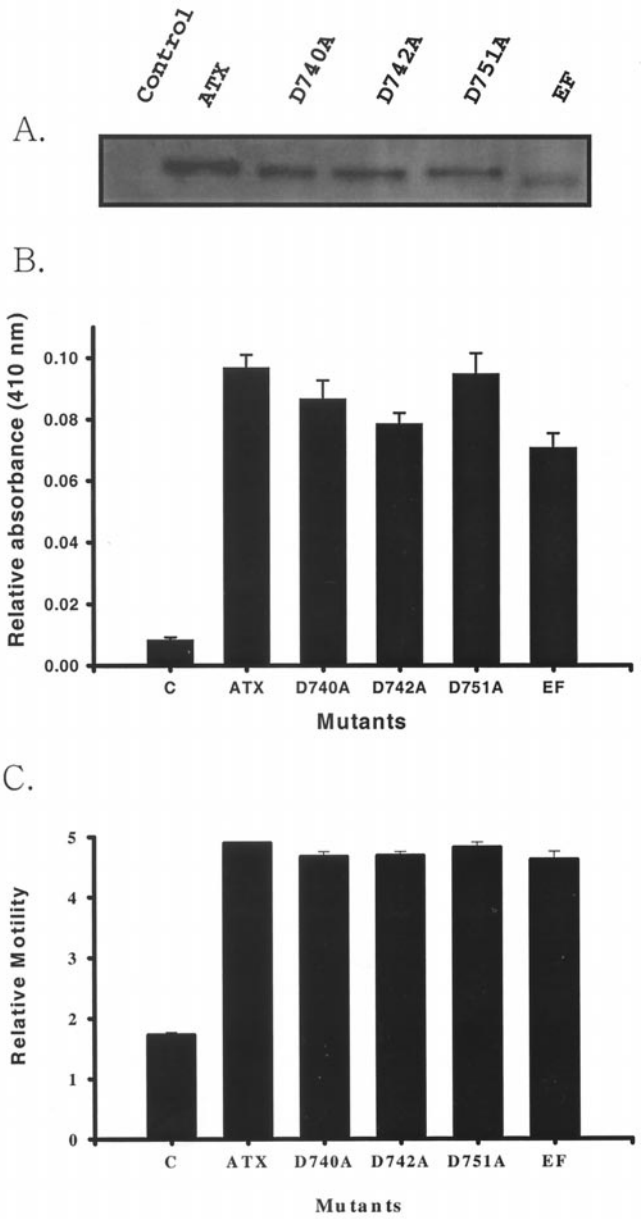


Fig. 4. Effect of site-directed and deletion mutations in the EF-hand loop region of ATX on PDE activities and on tumor cell motility-stimulating capacity of the resulting mutant rATXs. (A) Immunoblot of the mutant recombinant proteins secreted by COS-1 cells after transfection with vectors containing wild-type or mutant ATX cDNA. (B) PDE activity was quantitated by measuring hydrolysis of the substrate, *p*-nitrophenol-TMP, for 20 min. (C) The partially purified and concentrated mutant rATXs were utilized as chemoattractants in simultaneous motility assays. Supernatants from COS-1 cells that had been transfected by empty plasmid vector served as the control. The results are normalized to their relative activities based on the band intensity of the immunoblot. The results are shown as the relative activities compared with the band intensity of the immunoblot. Data are the means ± SD from triplicate samples and are typically representative of two separate experiments.

Rebbe *et al.* [10] reported that the PDE activity of the NPP, PC-1, was decreased in the presence of EDTA. Oda *et*

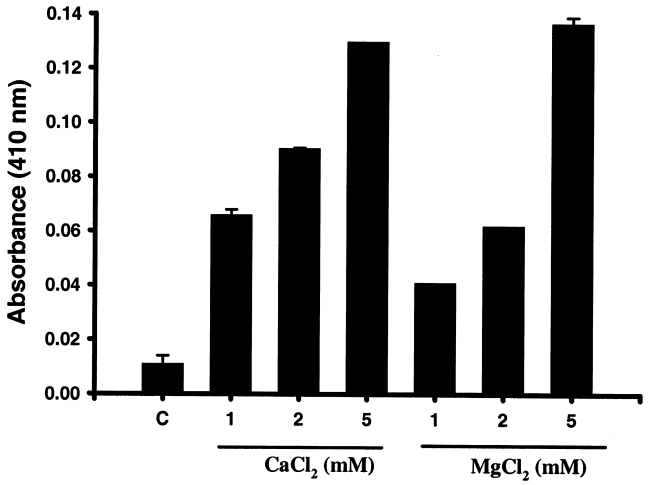


Fig. 5. Effect of divalent cations on the PDE activity of the EF-hand loop region deleted ATX mutant. PDE activity was measured as described in "Materials and methods." Data are the means ± SD from triplicate samples and are typically representative of two separate experiments.

al. [11], utilizing the bovine homolog of PC-1, MAFP, found that divalent cations activated PDE reactivity in a concentration-dependent manner. We found similar effects for ATX, although at lower cation concentrations. This concentration-dependent effect of cations appears to elevate the V_{max} rather than the binding affinity of the substrate. Possible interpretations of these data are that Mg^{2+} increases the efficiency of hydrolysis or the release of the second leaving group (thymidylate) in a kind of ping-pong reaction mechanism.

A typical EF-hand motif includes an acidic loop region between a pair of alpha helices to give a characteristic helix-loop-helix structure [18,19]. In these motifs, the oxygen groups of the highly conserved aspartic acid residues bind the calcium ions [20], although variant mechanisms of binding are known to exist. Many high-affinity calcium-binding proteins contain two or more of these helix-loop-helix motifs, which are thought to act cooperatively to increase their affinity for Ca^{2+} [16]. However, other members of the NPP family have an isolated EF-hand loop region that is highly homologous to that of ATX. Belli *et al.* [9] showed that Ca^{2+} inhibited the binding of EF-hand-specific antibodies to PC-1, suggesting that Ca^{2+} may bind on or near the EF-hand portion of the PC-1 molecule. Very recently, Andoh *et al.* [19] introduced a series of mutations into the EF-hand loop region of B10 and found that the resultant lysates had much reduced PDE activity, although the expression levels were not reported. This work suggested that the EF-hand might play a role in the enzymatic activity of B10. Our data indicate that ATX may be somewhat different, in this regard, from other members of this family. Although its PDE activity is augmented by divalent cations, this effect does not appear to depend on an intact EF-hand loop region.

In fact, we do not know where Ca^{2+} binds or how it acts to increase the PDE activity of ATX. A possible interaction between Ca^{2+} and ATX may be at or near Thr^{210} of the protein, which has been shown to be essential for its PDE activity [8,17]. We have also demonstrated that a threonine residue of ATX is autophosphorylated and dephosphorylated by the intrinsic enzymatic activities of the protein [15]. Ca^{2+} is known to bind to phosphoryl residues. Additionally, recent studies have focused on the role of certain proteins that function as Ca^{2+} receptors [21]. These can transduce signals in such cells as parathyroid cells [22]. In the present work, the kinetic analysis suggests that the stimulation of PDE activity by divalent cations results from an effect on a rate-limiting step other than substrate binding, perhaps hydrolysis or release of the second leaving group, although other explanations are possible including the production of a preferred conformation for activation.

In summary, we have described two major findings for ATX. First, divalent cations increase the PDE activity of ATX. Neither the PDE activity nor its enhancement by divalent cations depends upon an intact EF-hand loop motif found within the ATX molecule. Thus, the divalent cations may affect the enzymatic activities of ATX through an as yet undefined mechanism. Second, the chemoattractant stimulatory activity of ATX is not dependent upon the EF-hand loop region. Since ATX has PDE activities, which may be involved in its motogenic properties, it would be interesting to extend these studies by characterizing the binding of Ca^{2+} and Mg^{2+} to the protein.

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