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Carboxypeptidase A-catalyzed direct conversion of leukotriene C_4 to leukotriene F_4

Pallu Reddanna,[a] K. Sandeep Prabhu,[b] Jay Whelan,[c] and C. Channa Reddy[b,*]

[a] *College of Life Sciences, University of Hyderabad, Hyderabad 500134, India*

[b] *Department of Veterinary Science and The Center for Molecular Toxicology and Carcinogenesis, The Pennsylvania State University, University Park, PA 16802, USA*

[c] *Department of Nutrition, University of Tennessee, Knoxville, TN 37996, USA*

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Abstract

Leukotrienes (LTs) are 5-lipoxygenase (5-LO)-derived arachidonic metabolites that constitute a potent set of lipid mediators produced by inflammatory cells. Leukotriene A_4, a labile allylic epoxide formed from arachidonic acid by dual 5-LO activity, is the precursor for LTB_4 and LTC_4 synthesis. LTC_4 is further transformed enzymatically by the sequential action of γ-glutamyltranspeptidase and dipeptidase to LTD_4 and LTE_4, respectively. In this report, we present evidence that bovine pancreatic carboxypeptidase A (CPA), which shares significant sequence homology with CPA in mast cell granules, catalyzes the conversion of LTC_4 to LTF_4 via the hydrolysis of an amide bond. The identity of CPA-catalyzed LTC_4 hydrolysis product as LTF_4 was confirmed by several analytical criteria, including enzymatic conversion to conjugated tetraene by soybean LO, conversion to LTE_4 by γ-glutamyltranspeptidase, cochromatography with the standard LTF_4 and positive-ion fast-atom bombardment mass spectral analysis. Thus, it appears that the physiological significance of this single-step transformation may point toward a major cellular homeostatic mechanism of metabolizing LTC_4, a potent bronco- and vasoconstrictor, to a less potent form of cysteinyl LTs.

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Keywords: Cysteinyl leukotrienes; Leukotriene F_4; Carboxypeptidase A

Cysteinyl leukotrienes (LTs)[1] are a family of bioactive compounds that have been historically associated with the pathogenesis of asthma. They are primarily produced during inflammatory and immunological responses by the effector cells such as mast cells and basophils [1–4]. An intrinsic dual lipoxygenase activity associated with 5-lipoxygenase is responsible for the two-step conversion of arachidonic acid to LTA_4, an unstable epoxide intermediate [5–7]. Leukotriene A_4 is transformed further into either LTB_4 catalyzed by LTA_4 hydrolase or LTC_4 catalyzed by a subclass of glutathione *S*-transferases [1]. Leukotriene C_4 is the parent compound from which all other sulfidopeptide LTs are derived; LTD_4 and LTE_4 are known to be produced by the sequential action of γ-glutamyltranspeptidase and a dipeptidase on LTC_4 (Fig. 1) [8]. It is believed that LTC_4, LTD_4, and, to a lesser extent, LTE_4 of the 5,6-cysteinyl LT series constitute the previously known "slow-reacting substance of anaphylaxis," termed for its slow and sustained abilities to contract smooth muscle [1]. Previously Denis et al. [9] have reported that a γ-glutamyl derivative of LTE_4, designated as LTF_4, exerts a pulmonary effect. However, the precise reaction pathway by which LTF_4 is generated has remained enigmatic. It generally assumed that LTF_4 is formed from LTE_4 by the action of

* Corresponding author. Fax: 1-814-863-6140.
E-mail address: ccr1@psu.edu (C. Channa Reddy).

[1] *Abbreviations used:* LTA_4, 5*S*-*trans*-5,6-oxido-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid; LTB_4, 5*S*,12*R*-dihydroxy-6*Z*,8*E*,10*E*,14*Z*-eicosatetraenoic acid; LTC_4, 5*S*-hydroxy-6*R*-(*S*-glutathionyl)-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid; LTF_4, 5*S*-hydroxy-6*R*-(*S*-γ-glutamylcysteinyl)-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid; +FAB, fast-atom bombardment (positive ion); RP, reverse-phase; CPA, corboxypeptidase A; CPB, carboxypeptidase B; ELA, elastase; LO, lipoxygenase.

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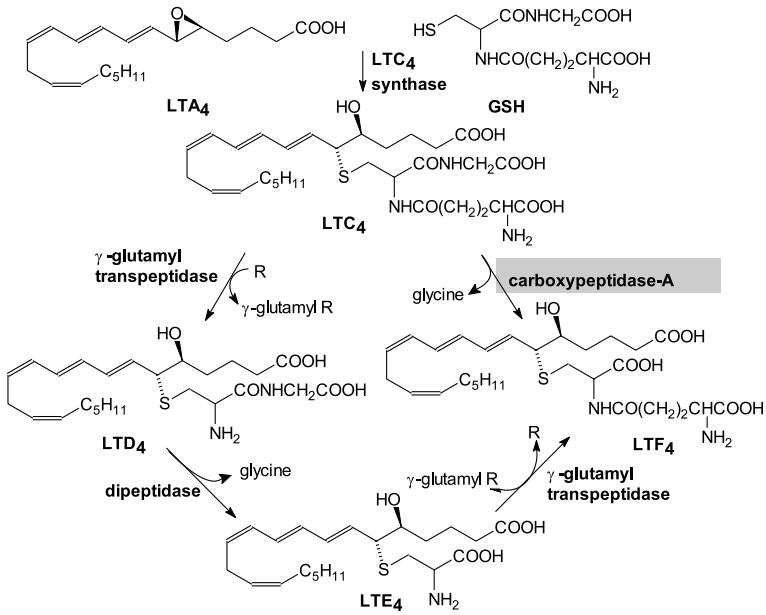

Fig. 1. Enzymatic pathways involved in cysteinyl LT biosynthesis. The highlighted gray box indicates the new enzymatic pathway shown to be catalyzed by CPA.

γ-glutamyltranspeptidase in the presence of GSH [10,11]. Bernstrom and Hammarström [11] have ruled out a direct enzymatic transformation of LTC_4 to LTF_4 by the dipeptidase due to the substrate specificity of the dipeptidase.

Mast cells, derived from hematopoietic progenitor cells, complete their differentiation and maturation in the connective tissues or in the serosa-lined peritoneal cavities [2]. Studies have shown that mast cells also represent a potential source of a vast array of cytokines, growth factors, and chemokines [2]. Interestingly, mast cells play a major role as effector cells in asthma and allergic disorders via IgG- and/or IgE-dependent mechanisms to release significant amounts of carboxypeptidases and other proteases [2]. In addition, other mediators such as histamines, proteoglycans, leukotrienes, and prostaglandins are produced by the mast cells to cause neutrophil recruitment and enhanced vascular permeability [2]. Mast cells synthesize and store in their secretory granules large amounts of serine proteases including carboxypeptidase A (CPA) [12]. However, the role of mast cell proteases in inflammatory and/or immunological responses is not clearly understood. In the present study, we examined the ability of bovine pancreatic CPA, which shares significant sequence homology with rat mast cell CPA, particularly with respect to the conservation of functional amino acid residues in the active site [13], to catalyze the direct conversion of LTC_4 to LTF_4. This is the first study demonstrating that CPA, a zinc-containing aromatic protease, can efficiently transform LTC_4 to LTF_4 via its amidase activity.

Materials and methods

Chemicals

Glutathione, arachidonic acid, γ-glutamyltranspeptidase, bovine pancreatic carboxypeptidase A (2× crystallized), porcine pancreatic carboxypeptidase B (CPB; EC 3.4.17.2), porcine pancreatic elastase (ELA; EC 3.4.21.36), soybean 15-lipoxygenase, and potato carboxypeptidase inhibitor protein (Product No. C0279) were purchased from Sigma (St. Louis, MO). [2-^{3}H]glycine-labeled GSH was purchased from DuPont-NEN (Boston, MA). HPLC-grade solvents were products of J. T. Baker (Phillipsburg, NJ). Sep-Pak C_{18} solid-phase extraction cartridges were from Waters (Milford, MA).

Preparation of various LTs

5,6-LTA_4 methyl ester was prepared from 5(*S*)-HPETE methyl ester by an improved biomimetic method; 5,6-LTC methyl ester was chemically synthesized from 5,6-LTA and purified by RP-HPLC as previously reported by our laboratory [14]. 5,6-LTC_4 free acid was generated by alkaline hydrolysis of the methyl ester with 0.1 N LiOH in ethanol for 3 h. [^{3}H]5,6-LTC_4 free acid was chemically prepared from 5,6-LTA_4 methyl ester as described above using [2-^{3}H]glycine–glutathione (10 μCi/μmol). Standard 5,6-LTE_4 free acid was similarly synthesized chemically from 5,6-LTA_4 methyl ester [15]. Standard 5,6-LTF_4 free acid was enzymatically synthesized from 5,6-LTE_4 free acid by the action of γ-glutamyltranspeptidase as described earlier [11].

Assay conditions for the conversion of 5,6-LTC_4 to 5,6-LTF_4 catalyzed by CPA, CPB, and ELA

The CPA reaction mixture (1.0 ml) contained 25 mM Tris–HCl, pH 7.5, 0.5 M KCl, 106 units (U) of CPA (specific activity toward hippuryl-L-phenylalanine = 53 U/mg protein), and 125 μM 5,6-LTC_4 in 100 μl ethanol. The reaction was started by the addition of LTC_4 and incubated at 25 °C for 15 min. The reaction was arrested by the addition of acetic acid (final concentration of 0.2 N) and the reaction products were partially purified by a Sep-Pak C_{18} cartridge. For partial conversion of LTC_4 to LTF_4, the reaction was terminated at 1 min of incubation. In reactions involving CPA inhibitor, the reaction conditions were identical to those described above except for the addition of 2 mg CPA inhibitor per 1.0 ml reaction. The CPB-catalyzed conversion of LTC_4 to LTF_4 was performed in 25 mM Tris–HCl buffer, pH 7.65, containing 0.1 M NaCl; the ELA-catalyzed peptidolysis of LTC_4 to LTF_4 was performed in 0.1 M Tris–HCl buffer, pH 8.0, containing 0.15 M NaCl. The reaction mixture in each case contained 100 U of enzyme. All other conditions were identical to those mentioned for CPA.

Conversion of 5,6-LTF_4 to 5,6-LTE_4 catalyzed by γ-glutamyltranspeptidase

The γ-glutamyltranspeptidase reaction mixture (2.0 ml) contained, 0.1 M Tris–HCl, pH 8.0, 5 mM glycylglycine, 20 U of γ-glutamyltranspeptidase (specific activity = 5.4 U/mg protein), and 125 μM 5,6-LTF_4 in 50 μl of ethanol. The reaction was started by the addition of LTF_4 and incubated at 25 °C for 15 min. The reaction was arrested by the addition of acetic acid (final conc. of 0.2 N) and the reaction products were partially purified by solid-phase extraction on a Sep-Pak C_{18} cartridge.

Conversion of 5,6-LTF_4 to a conjugated tetraene

The conversion of 5,6-LTF_4 to a conjugated tetraene, catalyzed by soybean LO, was monitored over time by UV absorption spectroscopy using a Beckman DU-7 scanning spectrophotometer. The reaction mixture consisted of 0.15 M potassium phosphate buffer, pH 9.0, 10 U of soybean LO, and 125 μM LTF_4.

Analytical procedures

Various reaction mixtures were extracted by passage through Sep-Pak C_{18} cartridges preequilibrated with methanol, and the LTs were eluted with methanol. RP-HPLC was performed on the extracts using a Rainin C_{18} column (10 mm × 250 mm) with methanol:water:acetic acid (700:300:0.08 v/v/v) as solvent system and the compounds were eluted at the rate of 6 ml/min. The effluent was monitored at 280 nm and compounds were identified by comparison of retention times to those of synthesized standards. Individual peaks were subjected to cochromatography with authentic standards.

FAB-MS/MS analysis of CPA-catalyzed LTC_4 hydrolysis product

5,6-LTF_4 preparations from CPA-catalyzed hydrolysis of 5,6-LTC_4 were purified by reversed-phase HPLC as described above. The fractions corresponding to 5,6-LTF_4 (Fig. 2; peaks II) were collected, dried, and subjected to +FAB-MS/MS analysis on a VG ZAB-SEQ (VG Analytical, Manchester, UK) hybrid mass spectrometer as described [16,17].

Results

Conversion of LTC_4 to LTF_4 catalyzed by CPA

Purified bovine pancreatic CPA transformed 5,6-LTC_4 readily to a product eluting after LTC_4 peak on reverse-phase HPLC (Fig. 2C). The transformation proceeded to near completion after 15 min (Fig. 2D). There was no conversion of LTC_4 to peak II when the reaction was carried out in the presence of CPA inhibitor (Fig. 2B). It was confirmed by cochromatography that the retention time of peak II corresponded to that of the standard 5,6-LTF_4 (Fig. 2E). Upon γ-glutamyltranspeptidase treatment using glycylglycine as an acceptor molecule, peak II was converted into LTE_4, as confirmed by cochromatography of peak III with standard LTE_4 (Fig. 2F). Peak II exhibited a UV spectrum (Fig. 2D; inset) indistinguishable from the spectrum of LTC_4, indicating the retention of conjugated triene geometry of the product in peak II. When the product from peak II was incubated with soybean LO, a typical conjugated tetraene spectral shift occurred in a time-dependent manner (Fig. 3; spectra B and C), similar to the one originally described for LTC_4 [18]. The absorption maximum for the conjugated tetraene was 292 nm with lesser maxima at 308 and 323 nm as compared to the absorption maximum of 280 nm for the parent LTF_4 substrate. The spectral data suggest that peak II has retained double bonds at the same positions and with the same geometry as those in the precursor LTC_4 (7,9-*trans*-11,14-*cis*).

The identity of peak II as 5,6-LTF_4 was further confirmed with [2-^{3}H-glycine]5,6-LTC_4 as substrate for CPA. When the products of this reaction were passed through a C_{18} Sep-Pak cartridge, the aqueous effluent contained the major fraction of radioactivity (67%), indicating the hydrolysis of [2-^{3}H]glycine from [^{3}H]5,6-LTC_4. Finally, peak II was confirmed as LTF_4 based on

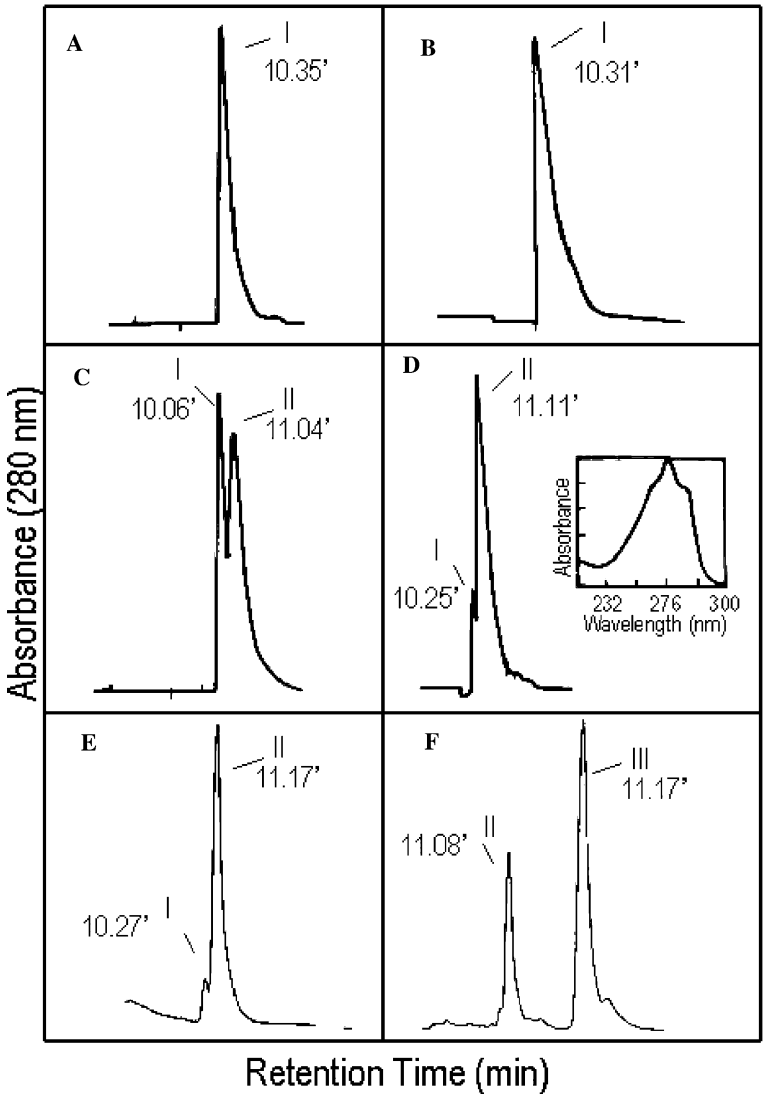

Fig. 2. Conversion of LTC_4 to LTF_4 catalyzed by CPA. Reverse-phase HPLC of CPA and LTC_4 reaction products: (A) LTC_4 in buffer without enzyme as control; (B) with enzyme in the presence of CPA inhibitor; (C) formation of LTF_4 after 1 min of CPA addition to represent partial conversion; (D) increase in LTF_4 synthesis at the end of 15 min of incubation with CPA; (E) peak II cochromatographed with standard 5,6-LTF_4; (F) peak II incubated with γ-glutamyltranspeptidase and glycylglycine coeluting with standard LTE_4. Peaks I, II, and III represent LTC_4, LTF_4, and LTE_4, respectively. All other conditions are as mentioned under Materials and methods. Inset: UV spectrum of peak II showing the characteristic of conjugated triene. Numbers near peaks indicate retention times of LTs (in minutes). Data shown are representative of $n = 3$.

+FAB at m/z 569.3 $[M + H]^+$, which exhibited a typical fragmentation pattern of ions, m/z 319 and 301, expected for standard LTF_4 free acid (Fig. 4).

Specificity of CPA

Of the various LTC_4 isomers tested, 5,6-LTC_4 free acid was found to be the best substrate for CPA. The enzyme showed negligible activity with 14,15-LTC_4 free acid and 14,15-LTC_4 methyl ester as substrates, suggesting positional specificity (data not shown). The methyl ester of 5,6-LTC_4 was also found to be a substrate for CPA; however, the activity was approximately 30% that of the free acid (data not shown). This observation suggests that a free carboxyl group on the fatty acid molecule is a requirement for CPA-catalyzed conversion of LTC_4 to LTF_4. Neither CPB nor ELA were able to catalyze the conversion of 5,6-LTC_4 to 5,6-LTF_4. In each case, the chromatogram was similar to that in Fig. 2A.

Discussion

In the present study, a new enzymatic pathway for the formation of 5,6-LTF_4 directly from 5,6-LTC_4, where CPA catalyzes the conversion of 5,6-LTC_4 to 5,6-LTF_4, has been identified. Other related proteases such as CPB

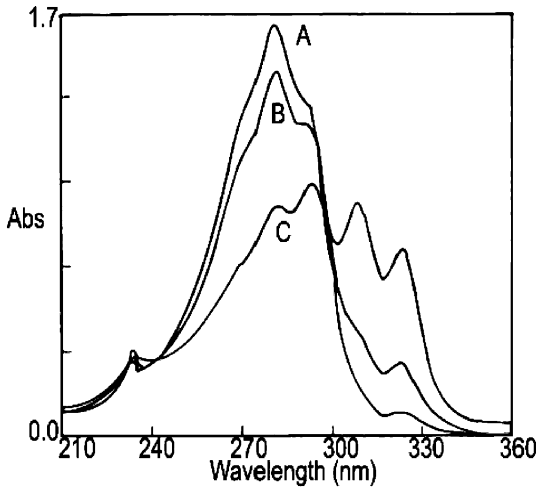

Fig. 3. UV spectra showing the time-dependent oxidation of LTF_4 (peak II) by soybean 15-LO. Spectra A, B, and C represent $t = 0$, $t = 1$, and $t = 15$ min post-15-LO reaction, respectively. The reaction conditions are as mentioned under Materials and methods.

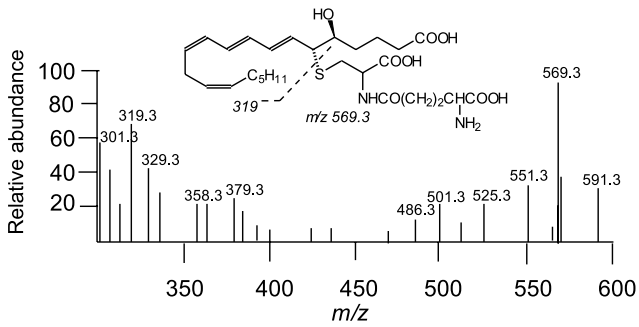

Fig. 4. Collision-induced dissociation of the $[M–H]^+$ cations of LTF_4 (peak II) at m/z 569.3.

and ELA, which catalyze the hydrolysis of amide bonds adjacent to basic amino acids and smaller uncharged side chains, respectively, were unable to catalyze the hydrolysis of 5,6-LTC_4. When 5,6-LTC_4 was incubated with bovine pancreatic CPA, it was rapidly converted to a less polar product (Fig. 2, peak II), which has been identified as 5,6-LTF_4 by (a) HPLC cochromatography with the standard 5,6-LTF_4, (b) conversion to 5,6-LTE_4 by γ-glutamyltranspeptidase, (c) recovering unlabeled peak II from reactions of [2-^{3}H]glycine-labeled 5,6-LTC_4 with CPA, (d) +FAB analysis of peak II, and (e) retention of original stereo isomerism of the double bonds in LTF_4 as determined by the soybean 15-LO reaction. All these data conclusively demonstrate that the CPA catalyzed the conversion of 5,6-LTC_4 to 5,6-LTF_4.

It is interesting to note that the 5,6-LTC_4 methyl ester is a relatively poor substrate, which indicates the preference of the enzyme for the more polar compounds and the hydrophilic nature of the active site. The important residues for binding and catalysis in the carboxypeptidase A active site include Glu-270, Arg-145, Arg-71, Tyr-248, and the zinc-bound water molecule [19]. Of these active site residues, the most functionally enigmatic center appears to be the zinc-bound water molecule, which confers a hydrophilic nature to the active site. This might be responsible for the specificity of the enzyme toward more polar free acids as substrates compared to their corresponding methyl esters. Furthermore, an interesting finding of the present study is that 5,6-LTC_4 but not 14,15-LTC_4 is a good substrate for CPA, suggesting that the positions of glutathionyl and hydroxyl functional moieties in the fatty acid substrate may provide snug fits for the substrate within the various subsites in the active site of the protease [20]. The dynamics of substrate binding at each of the subsites of substrate recognition in the active site could also play major roles in the preferential hydrolysis of the scissile bond in 5,6-LTC_4. Preliminary studies with 5,6-LTD_4, which contains cysteinyl glycine and lacks the cysteinyl aminoglutamyl residue, indicated that only CPB and ELA catalyzed its hydrolysis to 5,6-LTE_4, albeit with very low efficiency, while, CPA did not catalyze the reaction (data not shown). These results also suggest that the presence of a glutamyl group in 5,6-LTC_4 may be essential for the specificity of the CPA-catalyzed reaction.

The physiological significance of the conversion of 5,6-LTC_4 to 5,6-LTF_4 by CPA remains to be established. However, it is very well known that 5,6-LTC_4 is a highly potent broncho- and vasoconstrictor and the literature is replete with evidence that it mediates various hypersensitivity and inflammatory processes [21–25]. Leukotriene F_4, on the other hand, has been reported to be the least potent cysteinyl LT; therefore, CPA-catalyzed transformation of 5,6-LTC_4 to 5,6-LTF_4 may represent a cellular homeostatic mechanism for eliminating these highly reactive lipid mediators produced in the connective and peripheral tissue by the mast cells. In fact, compared to LTC_4, D_4, and E_4, the LTF_4 has been shown to be a relatively weak agonist of guinea pig ileal smooth muscle contraction [26]. Furthermore, in the anesthetized pig, LTC_4, D_4, and E_4 increased hind limb perfusion pressure, while, LTF_4 decreased perfusion pressure suggesting that LTF_4 may be a less potent form of cysteinyl LTs [27].

Carboxypeptidase present in mast cell granules has been postulated to be involved in the tissue inflammatory processes [28,29]. It is conceivable that CPA may be involved in the metabolism of 5,6-LTC_4, which is produced during inflammatory responses, to 5,6-LTF_4, a less potent mediator. Thus, it will be interesting to undertake an investigation to analyze the metabolism of 5,6-LTC_4 and carboxypeptidase activity levels during inflammatory responses of immune cells. The results of this study may open a potential window to analyze the role of various mast cell proteases such as 1–10, which

have been cloned and overexpressed from rat mast cells [12], for their ability to metabolize very potent LTC_4 into less reactive LTF_4. Such studies to understand the physiological significance of the CPA-catalyzed conversion of LTC_4 to LTF_4 are currently underway in our laboratory.

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