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CARBOXYPEPTIDASE A IN MOUSE MAST CELLS

Identification, Characterization, and Use as a Differentiation Marker[1]

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By using a conventional spectrophotometric assay with hippuryl-L-phenylalanine as the substrate, 10^6 BALB/c mouse serosal mast cells possessed 1.5 ± 0.43 U (mean ± SE, n = 5, range = 0.48 to 2.5) of carboxypeptidase A activity, while T cell factor-dependent, mouse bone marrow-derived mast cells (BMMC) had barely detectable levels of 0.01 ± 0.001 U/10^6 cells (mean ± SE, n = 3). In order to characterize the carboxypeptidase A present in the BMMC, a sensitive assay was developed that used angiotensin I as the substrate and reverse phase-high performance liquid chromatography to separate and quantify production of the cleavage product des-leu-angiotensin I. Using this assay, mouse BMMC carboxypeptidase A had a neutral to basic pH optimum and hydrolyzed angiotensin I with a K_m of 0.78 mM. The antigen-induced net percent release of carboxypeptidase A from IgE-sensitized BMMC was proportional to that of the secretory granule component β-hexosaminidase which indicates a secretory granule location for the exopeptidase. As defined by exclusion during Sepharose CL-2B chromatography, carboxypeptidase A was exocytosed as a >1 × 10^7 m.w. complex bound to proteoglycans. Because BMMC cocultured with mouse skin-derived 3T3 fibroblasts are known to undergo an increase in histamine content and biosynthesis of ^{35}S-labeled heparin proteoglycans, carboxypeptidase A activity was measured during BMMC/fibroblast coculture for 0 to 28 days. The carboxypeptidase A activity increased progressively during 28 days of co-culture from 0.004 ± 0.002 U/10^6 starting BMMC (mean ± SE, n = 3) to 0.36 ± 0.10 U/10^6 co-cultured mast cells. These findings indicate that carboxypeptidase A, a neutral protease, is exocytosed from the secretory granules of mouse mast cells bound to proteoglycan and is increased during the in vitro differentiation of mouse BMMC from mucosal-like mast cells to serosal-like mast cells.

The secretory granules of rat serosal mast cells contain substantial amounts of an exopeptidase with carboxypeptidase A-like preference for aromatic and branched-chain aliphatic carboxyl-terminal residues (1–3). The rat serosal mast cell carboxypeptidase A is distinct from rat pancreatic carboxypeptidase A in that the mast cell enzyme contains 12 more lysine residues/molecule than the pancreatic enzyme (3, 4). In exhibiting a neutral to basic rather than an acidic pH optimum, this mast cell carboxypeptidase A is also distinct from the carboxypeptidase in the lysozomal vesicles of rat liver and human fibroblasts (5). Rat serosal mast cell carboxypeptidase A is Zn^{2+}-dependent and has a m.w. of 35,000. Because of an isoelectric point of more than 9, it remains ionically linked to heparin proteoglycans after exocytosis from the secretory granules (6, 7). Rat mast cell protease I (RMCP I)[3], a distinct intragranular neutral protease with chymotrypsin-like substrate specificity, is also released in a macromolecular complex with heparin proteoglycan and carboxypeptidase A from serosal mast cells (7, 8). After RMCP I cleavage of a putative extracellular substrate at leucine and aromatic amino acid residues, the carboxypeptidase A would be expected to remove the newly exposed carboxyl-terminal amino acid.

Because of the limited availability of in vivo differentiated mast cells from the mouse, little is known about their neutral proteases. Thus, unlike the rat, proteases have not been used to distinguish T cell factor-dependent mucosal mast cells from T cell factor-independent connective tissue mast cells (9, 10). T cell-dependent, mucosal-like mast cells (11) have been derived in vitro from mouse bone marrow progenitors (BMMC) (12) in the presence of interleukin 3. The secretory granules of 10^6 BMMC contain ~2 μg of highly sulfated proteoglycans and ~2 μg of basically charged serine endopeptidases that are enzymatically active at neutral pH (13, 14). The serine proteases and the proteoglycans are exocytosed together in macromolecular complexes (14). When cocultured with fibroblasts, mouse BMMC become more like serosal mast cells in that they counterstain with safranin, increase their histamine content, and increase their synthesis of heparin proteoglycans relative to chondroitin sulfate E proteoglycans (15).

In the present study, carboxypeptidase A activity in mouse BMMC has been characterized with regard to pH optimum, K_m, secretory granule location, and release in

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[3] Abbreviations used in this paper: RMCP I, rat mast cell protease I; BMMC, bone marrow-derived mast cell; DFP, diisopropylflurophosphate; HBSS$^-$, Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution; RP-HPLC, reverse phase-high performance liquid chromatography.

macromolecular complexes with secretory granule proteoglycans. Upon coculture of BMMC with fibroblasts for 28 days, the carboxypeptidase A activity of the mast cells increased approximately 90-fold to a level ~25% of that found in BALB/c mouse serosal mast cells. This finding supports the importance of the fibroblast microenvironment in regulating the amount of a neutral protease that is packaged in the secretory granules of mouse mast cells.

MATERIALS AND METHODS

Preparation of mouse and rat serosal mast cells. Serosal mast cells were obtained from BALB/c mice and Sprague-Dawley rats (The Jackson Laboratory, Bar Harbor, ME) by peritoneal lavage as previously described (16). Tyrode's buffer containing 0.1% (w/v) gelatin was injected into the peritoneal cavity of each animal, and the peritoneal eluate was aspirated. After centrifugation at 200 × G for 10 min, the cells were resuspended in 1 ml of the Tyrode's buffer and were layered on top of 2 ml of 22% (w/v) metrizamide (Nyegaard and Co., Oslo, Norway) in Tyrode's buffer. The serosal mast cells were concentrated by sedimentation at 200 × G for 15 min, washed twice in Tyrode's buffer, counted, and stained with toluidine blue (17). The resulting serosal mast cells of 90 to 96% purity were resuspended in Tyrode's buffer at 1×10^6 cells^6/ml, sonicated (Branson Sonifier, 10 pulses, setting 7, 50% duty cycle), and stored frozen at −20°C until used.

Culture of mouse BMMC in the presence and absence of mouse fibroblasts. Bone marrow cells from BALB/c mice were cultured for 3 to 6 wk in 50% enriched medium (RPMI 1640 containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (GIBCO, Grand Island, NY)) and 50% (v/v) WEHI-3- (American Type Culture Collection, Rockville, MD) conditioned medium (12).

Co-culture of BMMC and fibroblasts was performed as previously described (15). Swiss albino mouse skin-derived 3T3 fibroblasts (1×10^4) (American Type Culture Collection) were seeded into 35-mm culture dishes and maintained until confluence occurred (7 to 10 days). BMMC (3 to 4×10^5 cells suspended in 2 ml of 50%-enriched medium/50% WEHI-3-conditioned medium) were seeded into culture dishes containing 3 to 4×10^5 fibroblasts. The culture medium was changed every 2 days, and any nonadherent cells were discarded. At 1-wk intervals, duplicate plates of the cocultures were washed twice with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution ($HBSS^-$) and treated with 0.05% trypsin (GIBCO) in $HBSS^-$ for 10 min at 37°C. The dispersed cells from one of the plates were stained with toluidine blue to quantify the number of mast cells (15) and those from the duplicate plate were washed three times in $HBSS^-$ and sonicated as described above for serosal mast cells. In one experiment, the dispersed cocultured cells were resuspended in 0.5 ml of Tyrode's buffer containing 0.1% gelatin, layered on top of a discontinuous gradient consisting of 2 ml each of 10, 13, 18, and 22% (w/v) metrizamide, and centrifuged at 1000 × G for 45 min at room temperature. Mast cells and fibroblasts of >99% purity were obtained from the 18/22% and 10/13% metrizamide interfaces, respectively, and were separately washed, resuspended, and sonicated in $HBSS^-$.

Activation of mouse BMMC and analysis of exocytosed products. BMMC were activated with 0.5 μM calcium ionophore A23187 (Calbiochem, La Jolla, CA) at 1×10^7 cells/ml in the modified Tyrode's buffer without gelatin for 20 min at 37°C as previously described (18). Alternatively, 1.5×10^7 BMMC were sensitized for 1 hr at 37°C with 150 μg of mouse monoclonal anti-2,4-dinitrophenyl (DNP) IgE (19). The IgE-sensitized BMMC were then washed once in modified Tyrode's buffer without gelatin and resuspended at 2.5×10^6 cells/ml in the same buffer. Samples (0.2 ml) of cells were challenged for 10 min at 37°C with 0 to 40 ng of DNP conjugated to bovine serum albumin in 50 μl of the Tyrode's buffer (20, 21). After immunologic or calcium ionophore activation, the mast cells were pelleted by centrifugation at 200 × G for 10 min at room temperature. The supernatants were removed, and the cell pellets were resuspended in modified Tyrode's buffer without gelatin and sonicated. Cell viability was routinely monitored by following the cellular exclusion of Trypan blue and the release of the cytosol marker lactate dehydrogenase (22). The two granule markers, histamine (23) and β-hexosaminidase (24), were measured as described. Percent release of cellular constituents was calculated as the amount of the substance in the supernatant divided by the sum of the amount of the substance in the supernatant and the cell pellet. The net percent release was calculated by subtracting percent spontaneous release from percent agonist-induced release.

Carboxypeptidase A determination. Samples containing sonicates of 2.5 to 5.0×10^4 serosal mast cells or 1 to 2×10^6 BMMC were incubated at 22°C with 2 ml of 1 mM hippuryl-L-phenylalanine (Sigma Chemical Co., St. Louis, MO) in 0.5 M NaCl, 0.02 M Tris, pH 7.2, and the absorbance was measured at 254 nm (25). One unit of carboxypeptidase A was defined as that amount of enzyme that cleaved 1 μmol of hippuryl-L-phenylalanine/min. By incubating sonicates with hippuryl-L-arginine and hippuryl-L-lysine (Sigma), mast cells were also examined for carboxypeptidase B activity (26).

In order to reduce the number of mast cells required for each carboxypeptidase A assay, we used angiotensin I as the substrate and reverse phase-high performance liquid chromatography (RP-HPLC) to follow conversion of angiotensin I to des-leu-angiotensin I (27, 28). This assay was developed by using purified bovine pancreatic carboxypeptidase A (Sigma) dissolved in 0.15 M NaCl, 0.02 M Tris, pH 7.2. A total of 50 μl of pancreatic carboxypeptidase A (0.001 U) was incubated at 37°C with 200 μl of 1 mM angiotensin I (Peninsula Labs, Belmont, CA) in the 0.02 M Tris buffer. Samples (20 μl) were withdrawn and mixed with 0.2% (v/v) trifluoroacetic acid in water; one-half of each of these mixtures was injected onto the RP-HPLC column. As assessed by RP-HPLC, there was linear production over a 30-min period of a single digestion product with a retention time of 16 min, along with a corresponding decrease in the substrate that possessed a retention time of 23 min. Amino acid analysis was performed according to the method of Hirs (29) and revealed that the composition of the digestion product was that of des-leu-angiotensin I. For carboxypeptidase A determinations, 50-μl samples of mast cell-derived supernatants or whole mast cell sonicates were mixed with 200 μl of the digestion buffer containing 1 mM angiotensin I. After a 15-min incubation at 37°C, the reaction was stopped by the addition of 250 μl of 0.2% (v/v) trifluoroacetic acid in water. Loss of angiotensin I and generation of the degradation product was monitored by RP-HPLC and amino acid analysis. Carboxypeptidase A activity was quantified in arbitrary units based on the amount of des-leu-angiotensin I that was formed as determined by measuring the area under the peak that eluted at 16 min. The RP-HPLC column was standardized each day with a sample of the pancreatic carboxypeptidase A digest of angiotensin I. On any particular day of analysis the retention time of the des-leu-angiotensin I varied by less than 1 min.

RP-HPLC was performed on a Rainin system (Rainin, Woburn, MA) equipped with an Apple IIc computer and a Milton-Roy (Riviera Beach, FL) variable wavelength spectrophotometer set to 210 nm. Pump control and spectrophotometer output were monitored using Rainin software. A Vydac (Hesperia, CA) 218TPS column (250 × 4.6 mm) containing octadecylsilyl packing with 5-μm porous particles was used in conjunction with a 25 × 4.6 mm pre-column containing 10 μm octadecylsilyl packing. Solvent A consisted of water/trifluoroacetic acid (100/0.1, v/v), and solvent B consisted of water/acetonitrile/trifluoroacetic acid (60/40/0.1, v/v/v). The solvent program for the resolution of des-leu-angiotensin I was isocratic for 5 min with 60% solvent A and 40% solvent B followed by a linear gradient to 10% solvent A and 90% solvent B over 20 min; the flow was 1 ml/min.

Because of the presence of endopeptidases that degraded angiotensin I, a separate RP-HPLC carboxypeptidase assay was developed for the BMMC/fibroblast coculture samples. One mM hippuryl-L-phenylalanine was used as the substrate, and the 0.02 M Tris buffer was adjusted to pH 9 in order to eliminate any contribution of the 3T3 fibroblast-derived carboxypeptidase which has a pH optimum of ≤5 with this substrate (5) (W.E. Serafin, unpublished observations). This assay was developed by incubating bovine pancreatic carboxypeptidase A (0.001 U) in 50 μl of $HBSS^-$ at 37°C with 200 μl of the 1 mM hippuryl-L-phenylalanine solution. At 5-min intervals for up to 30 min, 20-μl aliquots were withdrawn, mixed with 20 μl of 0.2% trifluoroacetic acid, and subjected to RP-HPLC. With increasing time of incubation, there was linear production of two digestion products with retention times on the RP-HPLC column of 1.8 and 2.6 min. These retention times corresponded to those of authentic L-phenylalanine and hippuric acid, respectively. For RP-HPLC, the digests were applied to a 150 × 4.6 mm octadecylsilyl column and eluted isocratically with 65% solvent A and 35% solvent B at 1.5 ml/min; absorbance was monitored at 210 nm. After every 10 injections, undigested bound hippuryl-L-phenylalanine was removed from the RP-HPLC column by washing with 100% solvent B for 10 min. Phenylalanine was quantified by measuring the area under the peak on the chromatogram. Conversion of area units to micromoles was accomplished by comparison to a standard curve that was generated by the injection of 0 to 3 nmol of authentic L-phenylalanine. A total of 1 U of carboxypeptidase A was designated as the amount of enzyme that cleaved hippuryl-L-phenylalanine to produce 1 μmol of phenylalanine/min. For analysis of the mast cell/fibroblast co-culture samples, 50-μl sonicates representing 1×10^4 mast cells were mixed with 200 μl of the pH 9 digestion buffer containing 1 mM

hippuryl-L-phenylalanine. After a 15-min incubation at 37°C, the reaction was stopped by the addition of 250 μl of 0.2% (v/v) trifluoroacetic acid in water, and 20-μl samples were injected onto the RP-HPLC column for product quantitation.

Characterization of mouse BMMC carboxypeptidase A. The carboxypeptidase of mouse BMMC was characterized by susceptibility to inhibition by the carboxypeptidase A inhibitor derived from potatoes (30) (Sigma), the divalent cation chelating agents 1,10-phenanthroline (3) and EDTA, and the serine protease inhibitor diisopropylfluorophosphate (DFP). A total of 250 μl of supernatant from 2.5 × 10^6 calcium ionophore-activated BMMC was incubated at 37°C for 1 hr with an equal volume of 0.15 M NaCl, 0.2 M Tris, pH 7.2, buffer alone or containing 0.3 to 300 μM potato carboxypeptidase A inhibitor, 2 mM 1,10-phenanthroline, 10 mM EDTA, or 2 mM DFP. To calculate inhibition of enzyme activity, the amount of des-leu-angiotensin I produced by the 50-μl samples of the inhibitor-treated supernatant was divided by the amount of des-leu-angiotensin I produced by the untreated supernatant.

The pH optimum for the mouse BMMC carboxypeptidase was determined for both the angiotensin I and hippuryl-L-phenylalanine substrates over the pH range of 5 to 11 using a 0.15 M NaCl, 0.2 M 2-(*N*-morpholino) ethanesulfonic acid buffer adjusted to pH 5, 6, or 7; a 0.15 M NaCl, 0.2 M Tris buffer adjusted to pH 7, 8, or 9; and a 0.15 M NaCl, 0.2 M glycine buffer adjusted to pH 9, 10, or 11. For each assay, 10 μl of calcium ionophore-activated BMMC supernatant were preincubated for 5 min at 37°C with 20 μl of the buffer. A total of 10 μl of 0.77 mM angiotensin I or 1 mM hippuryl-L-phenylalanine was added, and the samples were incubated at 37°C for 15 min. The reactions were stopped by the addition of 100 μl of 0.1% trifluoroacetic acid, and the digests were assessed by RP-HPLC. For kinetic studies of the BMMC carboxypeptidase A, 0.22 to 3.7 mM angiotensin I or hippuryl-L-phenylalanine were dissolved in 0.15 M NaCl, 0.02 M Tris, pH 7.2. After prewarming at 37°C, 85 μl of substrate were added to 15 μl of BMMC supernatant, and the reactions were allowed to proceed for 2 min at 37°C. Reactions were stopped by the addition of 100 μl of 0.2% trifluoroacetic acid, and the amounts of digestion products were determined by RP-HPLC. The K_m values for the hydrolysis of angiotensin I and hippuryl-L-phenylalanine were calculated by use of double reciprocal plots (31).

Gel filtration chromatography. BMMC (3 to 7 × 10^7) were incubated for 17 hr with 60 ml of culture medium containing 1 mCi of [^{35}S]sulfate (4000 Ci/mmol, New England Nuclear, Boston, MA). Radiolabeled BMMC were processed and activated with calcium ionophore as described above for unlabeled cells. A total of 3 ml of supernatant from 3 × 10^7 activated BMMC were applied at 4°C to an 80- × 0.8-cm Sepharaose CL-2B column (Pharmacia Fine Chemicals, Piscataway, NJ) that had been equilibrated with 0.15 M NaCl, 0.01 M Tris, pH 7.2; 1-ml fractions were collected. The ^{35}S-labeled macromolecules in the collected fractions were quantified by liquid scintillation counting of 50-μl samples of the eluate. Carboxypeptidase A activity was assessed by incubating 100 μl of each column fraction with angiotensin I. In one experiment using unlabeled BMMC, the Sepharose CL-2B filtration profile of the serine proteases was determined by incubating 200-μl samples of the column fractions with 5 μCi of [1,3-^{3}H]DFP (4 Ci/mmol, Amersham Corp., Arlington Heights, IL) in 200 μl of 0.15 M NaCl, 0.2 M Tris, pH 7.2. After a 1-hr incubation at 37°C, the resulting [^{3}H]DFP-labeled proteins were separated from unbound radioactivity by Sephadex G-25/PD-10 (Pharmacia) gel filtration chromatography (14) using 4 M NaCl containing 0.05% gelatin as the eluant. The radioactivity eluting in the void volume of the Sephadex G-25 column resulted from the presence of [^{3}H]DFP-labeled serine proteases (13, 14). The void and total volumes of the Sepharose CL-2B and Sephadex G-25 columns were determined by use of blue Dextran 2000 (Pharmacia) and [^{35}S]sulfate, respectively.

RESULTS

Identification of carboxypeptidase A in the secretory granules of mouse mast cells. As determined by the conventional spectrophotometric assay, sonicates of 10^6 BALB/c mouse and Sprague-Dawley rat serosal mast cells contained 1.5 ± 0.43 U (mean ± SE, n = 5, range = 0.48 to 2.5) and 1.0 ± 0.1 U (n = 3), respectively, of carboxypeptidase A activity. However, sonicates of 10^6 mouse BMMC possessed extremely low levels of carboxypeptidase A of 0.01 ± 0.001 U (mean ± SE, n = 3). No carboxypeptidase B activity was detected in BMMC or serosal mast cells.

Because the spectrophotometric assay for carboxypeptidase A required large numbers of BMMC, a more sensitive assay was developed that was based on the RP-HPLC resolution of the conversion of angiotensin I to des-leu-angiotensin I. Degradation of angiotensin I by proteases present in the supernatants of calcium ionophore-activated BMMC occurred in a linear manner relative to the amount of supernatant. A single digestion product (Fig. 1*B*) that possessed a retention time on RP-HPLC identical to that of the des-leu-angiotensin I standard was found. Amino acid analysis of this peptide (Table I) revealed one less leucine residue than that in angiotensin I, thereby confirming that des-leu-angiotensin I was the degradation product. Whereas carboxypeptidase A activity was barely detectable by the spectrophotometric assay of sonicates of 1 × 10^6 BMMC, carboxypeptidase A was readily demonstrated with 2.5 × 10^4 BMMC when the angiotensin I cleavage/RP-HPLC assay was used.

After sensitization with mouse monoclonal anti-DNP IgE, BMMC were challenged with a range of doses of DNP-bovine serum albumin in order to determine the relation-

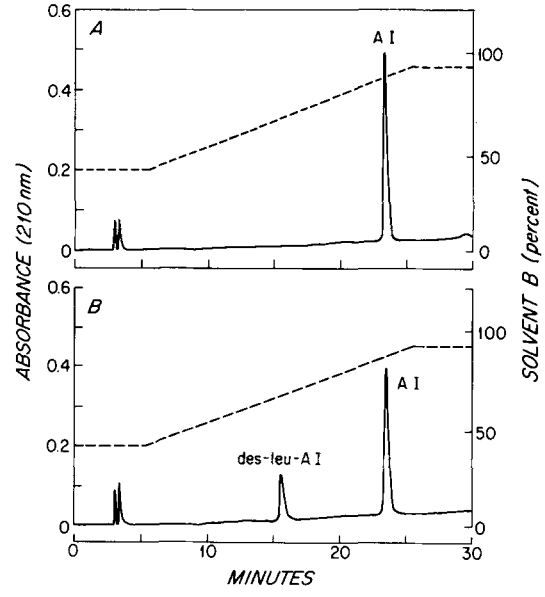

Figure 1. RP-HPLC analysis of the angiotensin I digestion product produced by the enzymes present in the supernatants of 2.5 × 10^4 calcium ionophore-activated mouse BMMC. Samples were incubated with angiotensin I for 0 (*A*) and 30 min (*B*), and the digestion products were separated by RP-HPLC. The retention time for angiotensin I (*AI*) is indicated. The des-leu-angiotensin I (*des-leu-AI*) standard, generated by pancreatic carboxypeptidase A degradation of angiotensin I, eluted at 16 min. The gradient profile (---) is shown.

TABLE I

Amino acid composition of the peptide generated by BMMC enzymatic degradation of angiotensin I[a]

Amino Acid	Amount (nmol)	Integer
Asp	3.96	1
Pro	4.01	1
Val	3.57	1
Ile	3.31	1
Tyr	3.53	1
Phe	4.01	1
His	7.31	2
Arg	3.82	1
All others	≤0.12	0

[a] The amino acid sequence of angiotensin I is Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu. The loss of one leucine residue indicates that the sequence of the unknown peptide recovered from the RP-HPLC column is Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His.

ship of carboxypeptidase A exocytosis to that of known constituents of the secretory granule. Samples of the supernatants and sonicated cell pellets from three separate experiments were assessed for carboxypeptidase A activity by incubation with angiotensin angiotensin I. Linear regression analysis revealed that the net percent release of carboxypeptidase A was proportional to that of β-hexosaminidase. A slope of 0.97, a y-intercept of 0.5%, and a correlation coefficient of 0.92 (n = 19) were obtained (Fig. 2). In one experiment in which the exocytosis of the carboxypeptidase A was compared with that of histamine, the five data points lay on the same line as that for exocytosed β-hexosaminidase (Fig. 2). The proportional release of histamine to that of β-hexosaminidase, as defined by linear regression analysis, has been reported previously for BMMC (20).

Characterization of mouse BMMC carboxypeptidase A. Digestion of the angiotensin I substrate by the BMMC-derived carboxypeptidase was inhibited in a dose-dependent fashion by the carboxypeptidase A inhibitor derived from potatoes (Fig. 3). Upon incubation with 1.6 and 150 μM concentrations of the potato inhibitor, the BMMC carboxypeptidase A was 50 and 100% inactivated, respectively. Degradation of angiotensin I was totally inhibited when the supernatant from calcium ionophore-activated BMMC was incubated with 2 mM phenanthroline or 10 mM EDTA, but was unaffected by incubation with 2 mM DFP.

When cleavage of angiotensin I by the carboxypeptidase present in the supernatants of calcium ionophore-activated BMMC was assessed over the pH range of 5 to 11, a broad pH optimum of 7.0 to 8.5 was obtained (data not shown). As assessed by double reciprocal plots with a representative experiment shown in Figure 4, the K_m for degradation of angiotensin I was 0.78 ± 0.09 mM (mean ± SE, n = 3).

Exocytosis from mouse BMMC of carboxypeptidase A in a macromolecular complex. When ^{35}S-labeled mouse BMMC were activated with 0.5 μM calcium ionophore and the resulting supernatant was applied to a Sepharose CL-2B gel filtration column, 65% of the recovered carboxypeptidase A activity and 7% of the ^{35}S-labeled proteoglycans co-eluted in the excluded volume of the column as a macromolecular complex with a m.w. of >10^7 (Fig. 5*A*). The remaining carboxypeptidase A activity eluted in a broad peak with K_{av} = 0.77. The majority of the ^{35}S-labeled proteoglycans eluted with a K_{av} of 0.69 indicating that the m.w. was approximately 200,000 which is similar to that reported for uncomplexed chondroitin sulfate E proteoglycans (14). In three experiments, including that depicted in Figure 5, 66 ± 4.0% (mean ± SE) of the exocytosed carboxypeptidase A and 6.7 ± 1.6% of the exocytosed ^{35}S-labeled proteoglycans coeluted in the excluded volume of the Sepharose CL-2B column. In one experiment in which BMMC were not ^{35}S-labeled before activation, the Sepharose CL-2B chromatography column fractions were assessed for both [^{3}H]DFP-binding proteins and carboxypeptidase A activity (Fig. 5*B*). In this experiment, 84% of the carboxypeptidase A activity and 79% of the [^{3}H]DFP-binding proteins filtered in the excluded volume of the column.

Effect of co-culture of mouse BMMC with fibroblasts on the carboxypeptidase A content of the mast cells. Replicate co-cultures of BMMC with mouse skin-derived

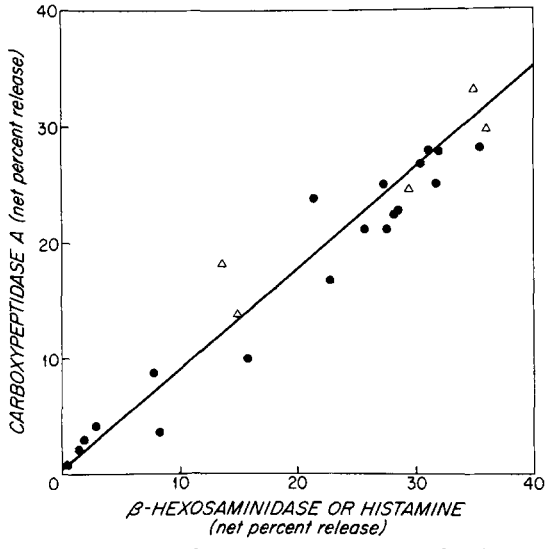

Figure 2. Comparison of the net percent releases of carboxypeptidase A with that of β-hexosaminidase and histamine from immunologically activated mouse BMMC. The best line-fit for the release of carboxypeptidase A is compared with that of β-hexosaminidase (●) (n = 19). The net percent release of the cytosolic marker lactate dehydrogenase was 3.6 ± 2.4 (mean ± SD, n = 19) in these experiments. In a separate experiment, the release of carboxypeptidase A was compared with that of histamine (△).

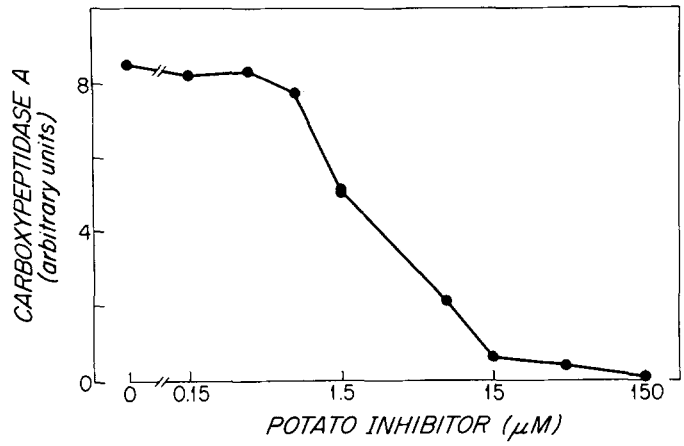

Figure 3. Dose response of the potato carboxypeptidase A inhibitor on the digestion of angiotensin I by the enzymes present in the supernatant of calcium ionophore-activated mouse BMMC. After a preincubation of the mouse BMMC supernatant with 0.15 to 150 μM potato inhibitor, the proteases in the supernatant were analyzed for their ability to generate des-leu-angiotensin I (●—●).

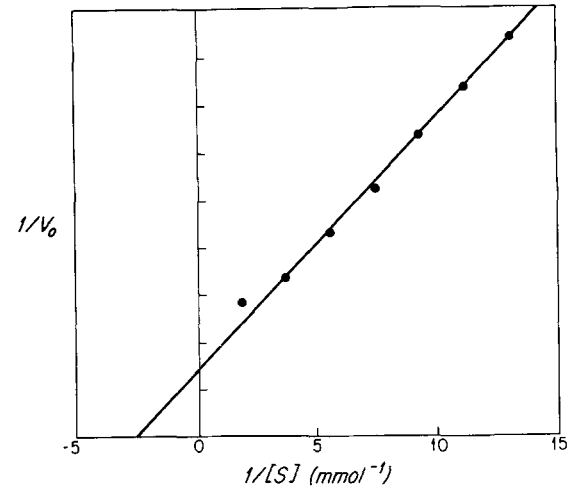

Figure 4. Kinetics of degradation of angiotensin I to des-leu-angiotensin I by the carboxypeptidase A in the supernatant of calcium ionophore activated mouse BMMC. V_o and [*S*] are the initial rate and substrate concentration, respectively.

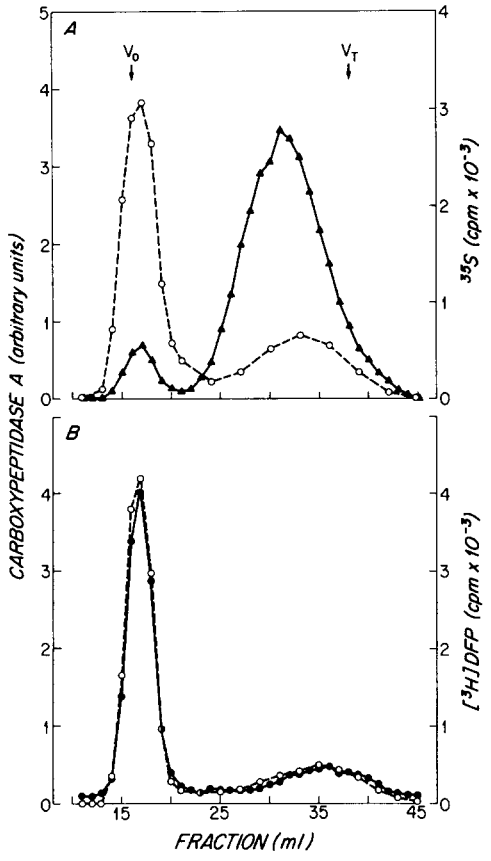

Figure 5. Sepharose CL-2B gel filtration chromatography of the supernatants of calcium ionophore-activated mouse BMMC. *A*, Fractions were assessed for carboxypeptidase A activity (O---O) and ^{35}S-labeled proteoglycans (▲——▲). The recoveries of the applied carboxypeptidase A and ^{35}S-labeled proteoglycans were 78 and 80%, respectively. *B*, In a separate experiment, fractions were assessed for carboxypeptidase A (O---O) and [^{3}H]DFP-binding proteins (●——●). The recoveries of the applied carboxypeptidase A and [^{3}H]DFP-binding proteins were 79 and 90%, respectively.

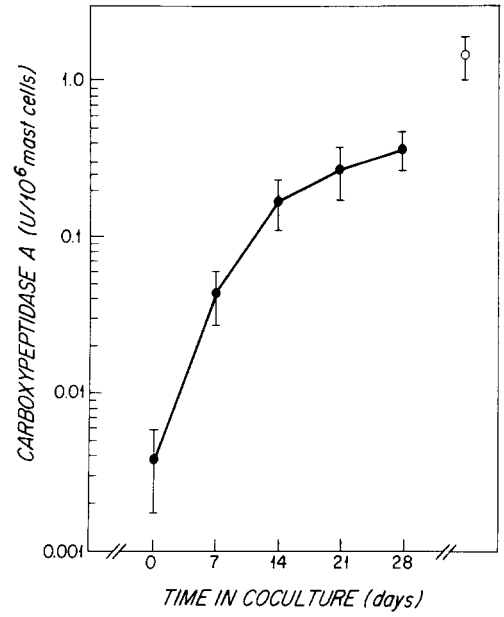

Figure 6. The effects of co-culture of BMMC with fibroblasts on the levels of carboxypeptidase A in the mast cells (●——●). Carboxypeptidase A activity was determined by the hippuryl-L-phenylalanine/RP-HPLC assay performed at pH 9 at 37°C. Each value (●) is the mean ± SE for three experiments. For comparison, the level of carboxypeptidase A (mean ± SE, n = 5) in mouse serosal mast cells is also displayed (O). This value is comparable to that obtained with the conventional spectrophotometric assay performed at pH 7.2 at 22°C.

3T3 fibroblasts were maintained for 0 to 28 days; at 1-wk intervals individual cocultures were analyzed for carboxypeptidase A activity. Because of the presence in some of these samples of proteases that degraded angiotensin I, carboxypeptidase A was assayed by release of phenylalanine from hippuryl-L-phenylalanine. Resolution of the reaction products by RP-HPLC was used to increase sensitivity. With this assay, the carboxypeptidase A that was exocytosed from calcium ionophore-activated BMMC had a pH optimum of 7 to 8 (data not shown) and a K_m of 0.21 mM (n = 1). In three 28-day co-culture experiments, the level of carboxypeptidase A in 10^6 mouse BMMC increased from a starting value of 0.004 ± 0.002 U (mean ± SE) to 0.36 ± 0.10 U (Fig. 6). In these same three co-cultures, starting BMMC were entirely safranin-negative; after 28 days of co-culture, 46 ± 3% (mean ± SE) of the mast cells were converted to predominantly safranin-positive (>50% safranin-positive granules). In the one co-culture assessed, histamine increased from 0.2 μg/10^6 starting BMMC to 1.2 μg/10^6 mast cells co-cultured for 28 days. Undetectable amounts of carboxypeptidase A (less than 5×10^{-4} U/10^6 cells) were present in starting fibroblasts and fibroblasts that had been maintained in parallel culture for 28 days in 50% WEHI-3-conditioned medium but in the absence of mast cells. Similarly, co-cultured fibroblasts that had been separated from BMMC by trypsinization and metrizamide gradient centrifugation did not have detectable amounts of carboxypeptidase A. BALB/c mouse serosal mast cells possessed 1.4 ± 0.44 U of carboxypeptidase/10^6 cells (mean ± SE, n = 5) when analyzed for carboxypeptidase A activity by this second RP-HPLC technique.

DISCUSSION

As assessed by a direct spectrophotometric measurement of the cleavage of hippuryl-L-phenylalanine, BALB/c mouse serosal mast cells contained 1.5 ± 0.43 U (mean ± SE, n = 5, range = 0.48 to 2.5) of carboxypeptidase A/10^6 cells which was somewhat greater than that measured in Sprague-Dawley rat serosal mast cells. In contrast, the amount of carboxypeptidase A present in 10^6 mouse BMMC (0.01 ± 0.001 U; mean ± SE, n = 3) was almost below detection using the conventional spectrophotometric assay. In order to reduce the number of mast cells required per assay, RP-HPLC was used to quantify the cleavage of angiotensin I to des-leu-angiotensin I (Fig. 1; Table I) and the cleavage of hippuryl-L-phenylalanine to phenylalanine.

As determined by pH optimum, K_m (Fig. 4), and susceptibility to inhibition by both divalent cation-chelating agents and the carboxypeptidase A inhibitor derived from potatoes (Fig. 3), the mouse BMMC-derived carboxypeptidase A is similar to the carboxypeptidase A that is present in the secretory granules of rat serosal mast cells (5, 6). Because the BMMC carboxypeptidase was exocytosed from immunologically stimulated cells in a linear relationship with the secretory granule marker β-hexosaminidase with a correlation coefficient of 0.92 (Fig. 2), it was concluded that the exopeptidase was located in the secretory granules. Approximately 70% of the carboxypeptidase A that was exocytosed was bound to intragranular proteoglycans in macromolecular complexes with m.w. > 10^7 (Fig. 5*A*). Furthermore, in this study (Fig. 5*B*) and a previous report (14), it was demonstrated that the BMMC secretory granule serine proteases are also

exocytosed in the macromolecular complexes with proteoglycans. We have previously shown that this macromolecular complex contains both chondroitin sulfate E and heparin proteoglycans (14). It is postulated that the presence of such complexes allows the exopeptidases to remain in close proximity to the endopeptidases after exocytosis from the cell thereby facilitating sequential endopeptidase/exopeptidase cleavages of peptide substrates. Fixation of these neutral proteases to proteoglycans may also prevent inactivation of the proteases as has been described for heparin proteoglycan regulation of the enzymatic activity of RMCP I (32) and tryptase (33). The preferential organization of the mouse mast cell carboxypeptidase A in the macromolecular complex may also limit its access to large m.w. substrates, as has been found for RMCP I (32, 34).

Because carboxypeptidase A was present in extremely small amounts in the mucosal mast cell-like BMMC and was present in large amounts in the mouse serosal mast cells, it was considered possible that this enzyme could be used as a differentiation marker of mouse mast cells. Upon co-culture with the 3T3 fibroblasts, mouse BMMC increased their carboxypeptidase A content approximately 90-fold (Fig. 6) to a level more similar to that in serosal mast cells. These findings complement those of a previous study (15) in which it was observed that the fibroblast microenvironment increased histamine content and ^{35}S-heparin biosynthesis and converted the staining characteristics of mast cells from safranin-negative to safranin-positive. Thus, each of the three major classes of constituents in the secretory granules of mast cells, namely amines, proteoglycans, and neutral proteases, has been shown to change when the mouse BMMC differentiates from a mucosal-like to a serosal-like mast cell upon co-culture with fibroblasts.

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