Latexin, a carboxypeptidase A inhibitor, is expressed in rat peritoneal mast cells and is associated with granular structures distinct from secretory granules and lysosomes

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Latexin, a protein possessing inhibitory activity against rat carboxypeptidase A1 (CPA1) and CPA2, is expressed in a neuronal subset in the cerebral cortex and cells in other neural and non-neural tissues of rat. Although latexin also inhibits mast-cell CPA (MCCPA), the expression of latexin in rat mast cells has not previously been confirmed. In the present study we examined the expression and subcellular localization of latexin in rat peritoneal mast cells. Western blot and reverse-transcriptase-mediated PCR analyses showed that latexin was contained and expressed in the rat peritoneal mast cells. Immunocytochemically, latexin immunofluorescence was localized on granular structures distinct from MCCPA-, histamine- or cathepsin D-immunopositive granules. Immunoelectron microscopy revealed that latexin was associated with a minority population of granules. The latexin-associated granules were separated from MCCPA-

or histamine-containing granules on a self-generating density gradient of polyvinylpyrrolidone-coated silica-gel particles (Percoll). Treatments with high ionic strength and heparinase released latexin from the granules, suggesting that latexin is non-covalently associated with a heparin-like component of the granules. MCCPA and histamine were released from the mast cells after non-immunological and immunological stimulation with compound 48/80, A23187 and anti-IgE antibody, whereas latexin was not released. These results show that latexin is synthesized in rat peritoneal mast cells and suggest that it is associated with a unique type of intracellular granules distinct from MCCPA- and histamine-containing secretory granules and lysosomes.

Key words: heparin, histamine, protease inhibitor, secretion.

INTRODUCTION

Latexin was first found as an antigen of monoclonal antibody PC3.1, which recognizes a subset of neurons in the rat cerebral cortex, and was identified as a 29 kDa protein on an SDS/polyacrylamide gel [1]. Cloning and sequencing of latexin cDNA showed that latexin protein is composed of 223 amino acid residues and has a molecular mass of 25 581 Da [2]. Immunohistochemical analyses of the rat nervous system established that latexin is expressed specifically in neurons of layers V and VI within the lateral cerebral cortex and also in smaller diameter primary sensory neurons of dorsal root and trigeminal ganglia [1,3]. In contrast, Northern and Western blot analyses on tissue distribution of latexin showed that it is expressed in non-neural structures such as the lung and pancreas as well as in neural tissues [2–6]. However, the cell types of latexin-expressing cells in the non-neural tissues have not been determined.

It has been recently reported that latexin has inhibitory activity against rat carboxypeptidase A1 (CPA1), CPA2 and mast-cell CPA (MCCPA) [6], which are encoded by three different genes [7–9]. Most of the tissues expressing latexin produce CPA1 and either CPA2 or a short CPA2 isoform with modified catalytic activity [6,10] but the expression of latexin in the mast cells is still unknown, although latexin inhibits the activity of MCCPA. In addition, latexin seems to be present in the cytoplasm of the cell

body and axonal fibres within cerebral cortical and primary sensory neurons [1,3,4] but the subcellular localization of latexin has not been investigated in non-neural cells including mast cells.

Mast cells contain not only large amounts of proteases such as mast-cell tryptase and chymase in addition to MCCPA [11] but also inhibitors of chymase and tryptase [12–18]. Human cutaneous mast cells contain α_1 -antitrypsin and α_1 -antichymotrypsin as chymase inhibitors [12–15]. Bovine liver capsule and rat peritoneal mast cells contain respectively bovine pancreatic trypsin inhibitor and trypstatin as tryptase inhibitors [16,17]. Bovine pancreatic trypsin inhibitor is synthesized in bovine mast cells and is localized in the same secretory granules as bovine tryptase [16,19]. Although trypstatin, which is a fragment of the inter- α -trypsin inhibitor light chain, is co-purified with tryptase from the rat mast cells, it is not produced in the mast cells. Because inter- α -trypsin inhibitor light chain, the precursor of trypstatin, is synthesized in the liver and secreted into the serum, it is postulated that the precursor is taken up into mast cell granules from the serum and processed into trypstatin [17,20]. In contrast, there is no information that the inhibitor of MCCPA is contained in mast cells.

Here we describe the expression and subcellular localization of latexin in the peritoneal mast cells of rat. Latexin was contained and expressed in the peritoneal mast cells. In the mast cells, latexin was associated with granular structures distinct from

Abbreviations used: CBB, Coomassie Brilliant Blue; CPA, carboxypeptidase A; MCCPA, mast-cell CPA; RT-PCR, reverse-transcriptase-mediated PCR.

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granules containing MCCPA, histamine and cathepsin D. Treatment of the peritoneal mast cells with compound 48/80, A23187 or anti-IgE serum induced the extracellular secretion of MCCPA and histamine but not of latexin.

EXPERIMENTAL

Materials

Monoclonal antibody PC3.1 against latexin [1] and rabbit anti-(bovine CPA) antibody (Biogenesis, Poole, Dorset, U.K.), were used for immunocytochemistry. For Western blot analysis, rabbit polyclonal antisera directed against latexin and rat MCCPA were prepared by using synthetic peptides of Cys-designed Lys-His-Asn-Ser-Arg-Leu-Pro-Lys-Glu-Ala-Pro-Ala-Glu and Gly-Arg-His-Ser-Tyr-Ala-Lys-Tyr-Asn-Asp-Trp-Asn-Lys, which corresponded to the latexin C-terminal and the MCCPA N-terminal sequences of residues 211–223 and 4–16 respectively [2,21,22]. The sequences were not homologous with any portion of other known proteins [3,21]. The former peptide was provided by R. Kato of our institute and conjugated to keyhole-limpet haemocyanin with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester for the immunization of rabbits [22]. The latter peptide, synthesized as a multiple antigenic peptide system, was obtained from Sawady Technology (Tokyo, Japan) and used for immunization without conjugation. The anti-(latexin C-terminal peptide) serum recognized a single protein band corresponding to latexin with an apparent molecular mass of 29 kDa on a Western blot of mast cells [2,3]. In contrast, Western blot analysis showed that the anti-(MCCPA N-terminal peptide) serum recognized three protein bands with apparent molecular masses of 34, 36 and 38 kDa in the rat peritoneal mast cells. The three proteins were purified by immunoaffinity with the antiserum from an extract of mast cell granules treated with 3 M NaCl [21,23] and the N-terminal sequence of the major 38 kDa protein was determined as Ile-Ala-Gly-Arg-His-Ser-Tyr-Ala-Lys-Tyr-Asn-Asp by amino acid sequence analysis, performed by S. Ichinose and Dr A. Omori of our institute. Similarly, the three proteins were purified by immunoaffinity with the anti-(bovine CPA) antibody from the extract of the mast cell granules and identified as components of MCCPA by Western blot analysis with the former antibody (see legend to Figure 4). These results indicate that the three proteins were identical with components of MCCPA [21].

Rabbit anti-(rat cathepsin D) polyclonal antibody was generously provided by Dr E. Kominami [24]. Rabbit polyclonal antibody against histamine and fluoroprobe-labelled goat polyclonal antibodies against rabbit IgG were purchased from Chemicon. Rabbit anti-(rat IgE) antibody RARa/IgE(Fc) was from Nordic Immunology (Tilburg, The Netherlands). Compound 48/80, A23187, *o*-phthalaldehyde and heparinase I were obtained from Sigma. The heparin-affinity column was from Pierce. Other reagents were of special purity grade and were mainly from Sigma.

Preparation of peritoneal mast cells and serum

The mast cells were prepared from the peritoneal cavity of male Wistar rats (12–15 weeks old) by separation on polyvinylpyrrolidone-coated silica-gel particles (Percoll) [25]. The following steps were performed at 4 °C or at ice-cold temperature unless stated otherwise. The prepared mast cells were resuspended in PBS, pH 7.4, containing 10% (v/v) precolostrum newborn calf serum (Mitsubishi Kagaku Chemical Corp., Tokyo, Japan) to give a cell concentration of $(2\text{--}5) \times 10^6$ cells/ml. The purity of the mast cells was estimated to be more than 90% by the

Toluidine Blue staining method [26]. For the experiment on expression of the latexin gene, fresh mast cells were prepared on a small scale: the cells were isolated from a rat within 65–70 min after the rat had been killed with CO₂ gas; they were finally suspended in Lock's solution. Unless otherwise mentioned, mast cells were prepared on a large scale from five or six rats.

For the preparation of rat serum, 200 µl of blood was collected from the tail vein of a rat after the tail had been cut with a razor; the blood was incubated at 37 °C for 30 min, left at 4 °C overnight and then centrifuged to obtain the serum [22]. The protein concentration of the serum was determined as 65 mg/ml.

Reverse-transcriptase-mediated PCR (RT-PCR)

After the mast cells had been prepared from the peritoneal cavity of a rat, total RNA was prepared by using a RNeasy Total RNA kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's manual. cDNA was synthesized at 37 °C for 60 min from 0.3 µg of total RNA with random primers and Moloney-murine-leukaemia virus reverse transcriptase (RNase H⁻) and was amplified by PCR with sense and anti-sense primers and *Taq* Gold DNA polymerase by using the following programme: 95 °C for 9 min, 59 °C for 30 s and 72 °C for 30 s (1 cycle), 94 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s (35 cycles), 94 °C for 30 s, 59 °C for 30 s and 72 °C for 5 min (1 cycle). The primer sequences used for the amplification of coding regions of latexin [2,27] were 5'-ATCTCTTTGAACTAGGATAGCCCGC-3' (sense primer) and 5'-TCTTCCTCATCTGGGTTCTTGCCGA-3' (anti-sense primer). The primers were obtained from Espec Oligo Service (Tsukuba, Japan).

Preparation of mast cell granules

Mast cell granules were prepared from the rat peritoneal mast cells and separated into two fractions with relative densities of approx. 1.05 and 1.2, by the method of Krüger et al. [28]. The mast cells were suspended in 800 µl of Lock's solution [154 mM NaCl/5.6 mM KCl/2.3 mM CaCl₂/5.6 mM glucose/5.0 mM Hepes buffer (pH 7.4)] and disrupted by sonication at 10 °C for 30 s with a bath-type sonicator (Branson, model-B12). After disrupted cell suspension had been centrifuged at 200 *g* for 5 min to remove cell debris, granules were sedimented from the supernatant of the cell suspension by centrifugation at 2000 *g* for 20 min. The granules, after resuspension in 200 µl of Lock's solution, were layered on 2.5 ml of 80% (v/v) Percoll solution containing 137 mM NaCl, 2.7 mM KCl, 20 mM Pipes, pH 6.8, 5.6 mM glucose and 1 mg/ml BSA, then separated by centrifugation at 28000 *g* for 20 min into two bands close to the top and the bottom on a Percoll self-generating gradient. Each band fraction was collected, washed twice with PBS by centrifugation at 2000 *g* for 20 min and resuspended in 50 µl of PBS.

Immunocytochemistry

The localization of latexin, MCCPA or histamine in the peritoneal mast cells was examined with the immunofluorescence labelling method. The peritoneal mast cells were adsorbed on poly-(D-lysine)-coated glass slides and fixed with 0.1 M phosphate buffer, pH 7.4, containing 4% (w/v) paraformaldehyde, 0.2% picric acid and 0.1% (v/v) glutaraldehyde at 4 °C for 5 min. The adsorbed cells were incubated at 4 °C overnight with PBS containing 5% (v/v) normal goat serum, 0.1% (v/v) Triton X-100 and a cocktail of PC3.1 monoclonal antibody against latexin (13 µg/ml) [1], with rabbit polyclonal antibodies specific for bovine CPA (diluted 1:500), histamine (diluted 1:100) or rat

Figure 1 Presence of latexin protein in peritoneal mast cells

(A) Protein profile of mast cells and serum on SDS/polyacrylamide gel. Proteins of the mast cells (lane 1) and serum (lane 2) (5 μ g of each) were separated by SDS/PAGE and stained with CBB R250. (B) Western blot of latexin in mast cells and serum. The latexin protein in mast cells (lane 1) and serum (lane 2) (10 μ g of each) was detected with anti-(latexin C-terminal peptide) polyclonal antibody. The positions of molecular mass standards are indicated (in kDa) at the left.

cathepsin D (diluted 1:200). After treatment with FITC and rhodamine-conjugated secondary antibody (diluted 1:50) at 23 °C for 2 h, the localization of antigens was analysed with an Axioplan fluorescence microscope equipped with differential interference contrast optics (Carl Zeiss, Oberkochen, Germany) or an MRC600 confocal laser microscope (Bio-Rad).

The ultrastructural localization of latexin in the peritoneal mast cells was examined by the conventional pre-embedding method of immunoelectron microscopy [29]. The mast-cell suspensions were fixed with 0.1 M phosphate buffer, pH 7.4, containing 4% (w/v) paraformaldehyde, 0.1% (v/v) glutaraldehyde and 8% (w/v) sucrose on ice for 90 min. After the cells had been embedded in 1% (w/v) agarose and OCT compound (Lak-Tek), they were cryosectioned and incubated sequentially at 4 °C for 48 h with PC3.1 antibody (130 μ g/ml) at 4 °C for 48 h and horseradish-peroxidase-conjugated goat anti-(mouse IgG) antibody (diluted 1:20) at 4 °C for 48 h. After the sections had been re-fixed with 2.5% (v/v) glutaraldehyde, the bound antibody was detected with 0.5% diaminobenzidine/0.05% (v/v) H_2O_2 and further stained with 0.5% OsO_4 . The stained sections were embedded in Epon 812 resin; ultrathin sections 70 nm in thickness were examined with a JEM-1200EX electron microscope (JEOL, Akishima, Japan).

Reversible dissociation of latexin from low-density granules

For the dissociation of latexin from the low-density granules, the low-density granule fraction suspended in PBS, pH 7.4, was incubated on ice for 1 h with various concentrations of NaCl and centrifuged at 7800 g for 10 min to separate it into precipitate and supernatant. Precipitates were resuspended in the same volume of PBS. For the reassociation of latexin with the residual precipitates, the low-density granule preparation was first treated with 1.5 M NaCl to dissociate latexin from low-density granules and then centrifuged to yield supernatant and precipitate fractions as described above. The supernatant fraction was diluted 1:15 with 10 mM Hepes buffer, pH 7.5, then mixed with the

precipitate fraction and kept overnight on ice. The mixture was centrifuged at 7800 g for 10 min to sediment the reassociated complexes.

Treatment of low-density granules with heparinase

The low-density granule fraction suspended in PBS, pH 7.4, was incubated at 37 °C for 5 h with 10 units of heparinase I [30] and separated into precipitate and supernatant fractions by centrifugation of 7800 g at 4 °C for 10 min.

Release of MCCPA and histamine

Freshly prepared mast cells suspended in 20 μ l of Lock's solution were incubated for 5 min at 26 °C. An equal volume of compound 48/80, A23187 and anti-rat IgE serum, as secretion stimulants, were added to the cell suspension to give final concentrations of 3 μ g/ml and 5 μ M and a 1:20 dilution respectively. After appropriate periods at 26 °C, the cells were cooled on ice and adjusted to 1.0 or 1.5 M NaCl [31]. After the cells had been left for 1 h on ice, they were centrifuged at 200 g for 5 min at 4 °C to separate them into supernatant and cell pellet. The sedimented cells were resuspended in the same volume of Lock's solution as the supernatant. The quantities of MCCPA and latexin were determined in duplicate for 10 μ l of supernatant and cell fraction by semi-quantitative Western blot analyses as described below. For histamine release, the step of NaCl addition was omitted. The stimulant-treated cells were cooled on ice and centrifuged to separate them into supernatant and cell pellet. The sedimented cells were resuspended in Lock's solution containing 0.1% Triton X-100, twice frozen in solid CO_2 /ethanol and thawed to extract residual histamine. The histamine content was determined in duplicate for 2.5 μ l of the supernatant and solubilized cell extract. The histamine concentration was determined by a fluorometric method with *o*-phthalaldehyde [32]. The release ratio was calculated as the ratio of the released amount to the sum of the residual and released amounts [31] and as an average from duplicated experiments.

Purification of recombinant latexin protein

Latexin cDNA was linked to a pET-15b vector whose sequence for a His-tag region was truncated, and introduced into recipient *Escherichia coli* BL21 cells supplied by Novagen. The production of recombinant latexin protein was induced by the addition of isopropyl β -D-thiogalactoside. The recombinant latexin was purified by immunoaffinity with PC3.1 monoclonal antibody from the cells overproducing latexin [2].

Analytical procedures

Protein concentration was determined by the method of Lowry et al. [33], with BSA as standard. SDS/PAGE was performed by the method of Laemmli [34], with 12% (w/v) polyacrylamide gel. Gels were stained with Coomassie Brilliant Blue (CBB) R250 or silver salt with a silver-staining kit of Daiichi Chemical (Tokyo, Japan). Western blotting for latexin and MCCPA was performed in accordance with the laboratory manual of Harlow and Lane, with rabbit anti-(latexin C-terminal peptide) and anti-(MCCPA N-terminal peptide) sera respectively [2,22]. The immunoreactivity of latexin was detected with horseradish-peroxidase-labelled goat anti-(rabbit IgG) antibody by using an enhanced chemiluminescence detection kit (Amersham). For the quantitative analysis of latexin, its immunoreactivity was detected with FITC-labelled donkey anti-(rabbit IgG) antibody and analysed quantitatively with a FluorImager 595 system (Mol-

ecular Dynamics). The immunoreactivity of MCCPA was detected with alkaline-phosphatase-conjugated anti-(rabbit IgG) antibody by using an Attaphos substrate set (Boehringer Mannheim, Mannheim, Germany) and analysed quantitatively with the FluorImager 595 system.

For the detection of DNA fragments of less than 1 kbp, they were separated on 0.5% agarose/1.25% (w/v) Synergel gel (Diversified Biotech, Boston, MA, U.S.A.) and detected by the FluorImager system after being stained with 0.01% Vistra Green for 30 min at 37 °C (Amersham).

RESULTS

Presence and expression of latexin in peritoneal mast cells

Mast cells are widely distributed in various tissues such as the lung, skin, stomach and brain, and in the peritoneal cavity

Figure 2 Expression of latexin gene in peritoneal mast cells

RT-PCR of latexin mRNA. Total RNA was prepared from freshly isolated mast cells and reverse-transcribed with random primers to produce cDNA. PCR products from total RNA (lane 1) and cDNA (lane 2) which were synthesized with sense and anti-sense primers specific for the latexin gene were analysed by agarose-gel electrophoresis. The DNA fragment predicted from RT-PCR of latexin mRNA by using the sense and anti-sense primers specific for the latexin gene is 355 bp in size; it was digested into two fragments of 179 and 176 bp with *EcoRV* and two fragments of 308 and 47 bp with *HincII* [2]. Thus the RT-PCR products of latexin mRNA were digested with the restriction enzymes *EcoRV* (lane 3) and *HincII* (lane 4). The scale at the left shows DNA sizes in base pairs.

[35,36]. Peritoneal mast cells were isolated from the peritoneal lavage fluid of the rat. To determine whether latexin was contained in the mast cells or not, the presence of latexin protein was tested for by Western blot analysis.

Figure 1 shows the protein profile of peritoneal mast cells on a SDS/polyacrylamide gel and the Western blot of latexin in the mast cells. The protein band of latexin with an apparent molecular mass of 29 kDa was detected on the Western blot [1] but the protein band corresponding to latexin was not detected on the CBB-stained polyacrylamide gel. This implies that the peritoneal mast cells contained a small content of latexin protein.

Next we examined whether or not latexin protein was synthesized in the mast cells, because it has been described that trypstatin, mast cell tryptase inhibitor of rat, is synthesized in the liver, secreted into the serum and then taken up from the serum by mast cells [20]. The gene expression of latexin in the mast cells was therefore examined by RT-PCR to assess the biosynthesis of latexin in the mast cells. The peritoneal mast cells were freshly isolated and total RNA was immediately prepared. As shown in Figure 2, the DNA fragment with a predicted size of 355 bp was not amplified with primers for latexin from the total RNA lacking a step of reverse transcription (lane 1) but the DNA fragment was amplified from the mast cell cDNA (lane 2). The identity of the RT-PCR products was confirmed by digestion with restriction enzymes (Figure 2, lanes 3 and 4). These results indicated that latexin mRNA is expressed in the peritoneal mast cells.

Finally we examined whether or not rat serum contained latexin protein (Figure 1). The latexin protein band of 29 kDa was not detected in the serum by Western blotting, suggesting that latexin was not present in the serum above a detection limit.

These results show that latexin protein is contained and biosynthesized in the peritoneal mast cells.

Subcellular localization of latexin in peritoneal mast cells

The peritoneal mast cells were freshly isolated from peritoneal lavage fluid of rat; the subcellular localization of latexin in the peritoneal mast cells was examined immunocytochemically with PC3.1 monoclonal antibody. All cells containing a large number of granular structures were latexin-immunoreactive (Figure 3).

Figure 3 Localization of latexin in rat peritoneal mast cells

(A) Differential interference contrast-microscopic image of the isolated peritoneal mast cells. (B) Immunofluorescence image of latexin in the same area as in (A). Latexin was stained with PC3.1 monoclonal antibody and FITC-conjugated goat anti-(mouse IgG) antibody. Scale bars, 20 μ m.

Figure 4 Subcellular localization of latexin, MCCPA, histamine and cathepsin D in a peritoneal mast cell

(A) Two-colour immunofluorescence images for latexin and each of MCCPA (a–c), histamine (d–f) and cathepsin D (g–i). Latexin (a, d) was stained with PC3.1 monoclonal antibody and rhodamine-conjugated goat anti-(mouse IgG) antibody. MCCPA (b), histamine (e) and cathepsin D (h) were stained with rabbit anti-(bovine CPA) serum, rabbit anti-histamine serum, and rabbit anti-(cathepsin D) antibody respectively, and FITC-conjugated goat anti-(rabbit IgG) antibody. Two images of (a) and (b), (d) and (e) and (g) and (h) in the same optical section (320 nm thickness) of confocal laser microscopy are superimposed in (c), (f) and (i) respectively. Scale bar, 5 μ m. (B) MCCPA protein immunoprecipitated with anti-(bovine CPA) antibody from isolated mast-cell granules (see the Experimental section for details). MCCPA components were detectable as major proteins with silver staining of SDS/PAGE (lane 1) and Western blotting with anti-(MCCPA N-terminal peptide) serum (lane 2). Their total content was estimated densitometrically to be approx. 75%. The arrow indicates MCCPA protein bands. The positions of molecular mass standards are indicated (in kDa) at the left.

The number of Toluidine Blue-stained cells was closely correlated to that of latexin-immunoreactive cells, strongly suggesting that all mast cells were latexin-immunoreactive. The immunoreactivity was localized on clusters of granular structures distributed unevenly in the cytoplasm (Figure 3B). The number of latexin-immunoreactive granular structures per cell ranged from approx. 10 to 20.

Mast cells have a great number of secretory granules containing various preformed chemical mediators such as histamine, proteases, phosphatases and heparin proteoglycan [35]. MCCPA, whose activity is inhibited by latexin, is also a protease mediator [6,35,37]. To examine whether or not the latexin-immunoreactive granular structures in the peritoneal mast cells were identical with MCCPA- or histamine-containing secretory granules, the localization of latexin was compared with that of MCCPA or histamine by two-colour immunofluorescence labelling experiments with PC3.1 monoclonal antibody against latexin, and

polyclonal antibodies against bovine CPA and histamine. Latexin immunoreactivity was localized on a small number of granular structures [Figure 4A(a)], whereas MCCPA immunoreactivity was found on a comparatively large number of granules [Figure 4A(b)]. The two immunoreactivities did not overlap in the same optical section of confocal laser microscopy [Figure 4A(c)], showing that latexin was associated with granular structures distinct from MCCPA-containing granules. Similar results were obtained for latexin- and histamine-immunoreactivities [Figure 4A(d–f)]. The number of latexin-associated granular structures was much smaller than that of histamine-containing granules.

In addition, mast cells contain lysosomes as a type of granule [38–40]. We examined immunocytochemically whether latexin co-localizes to the same vesicles as lysosomal marker by using antibodies against latexin and cathepsin D as a lysosomal marker [24,40]. Cathepsin D immunoreactivity was localized on some granular structures but the immunoreactivity was distributed

Figure 5 Immunoelectron micrograph of the latexin-associated granule in a peritoneal mast cell

Latexin was stained with PC3.1 antibody and horseradish-peroxidase-conjugated goat anti-(mouse IgG) antibody. A latexin-immunoreactive granule is seen to be interposed among granules with no or very weak immunoreactivity. Some portions of granules adjacent to the latexin-immunoreactive granule are also electron-dense, possibly owing to a diffusion artifact of the peroxidase reaction product or latexin molecules during the preparation of the specimen during pre-embedding [29]. The ultrathin section was 70 nm thick. Scale bar, 1 μ m.

differently from the latexin immunoreactivity [Figure 4A(g-i)]. This result showed that the localization of latexin on the granules was distinct from that of lysosomes.

The detailed feature of the latexin-immunoreactive structures in the peritoneal mast cells was examined by immunoelectron microscopy. As shown in Figure 5, a small number of strongly immunopositive granules were interposed among densely packed granules with no, or very weak, immunoreactivity. The sizes of latexin-positive granules were comparable to those of latexin-negative or weak ones. The number of granules in a section containing the cellular nucleus per cell was 0–5 for latexin-positive granules and 47–125 for latexin-negative ones. The total number of granules in 16 sections was 35 latexin-positive and 1474 latexin-negative. Therefore the percentage of latexin-positive granules was 2.3%.

From the above results it was concluded that latexin is associated with a unique type of granule distinct from MCCPA- or histamine-containing granules and lysosomes. In the following sections, the latexin-associated granular structure will be designated the 'latexin granule'.

Separation of latexin granules from MCCPA- or histamine-containing granules

To characterize latexin granules biochemically, mast-cell granules were separated into two types of granule of low and high densities by the method developed by Krüger et al. [28]. The

peritoneal mast cells were sonicated and centrifuged to yield supernatant (S) and primary granule pellet (P) fractions. The P fraction was resuspended and separated into two distinct bands with low (L) and high (H) densities on a self-generating gradient of Percoll.

The protein composition and latexin content of the preparations during the course of separation were analysed by SDS/PAGE and Western blotting respectively. The protein band patterns of whole mast cell, primary granule pellet P and two granule fractions (L and H) were similar except for that of supernatant S (Figure 6A). As shown in Figures 6(A) and 6(B), the protein band of latexin with an apparent molecular mass of 29 kDa was detected by Western blotting but not by CBB staining. The latexin protein in mast cells was recovered mainly in the primary pellet P, and little in the supernatant S. After separation on a Percoll density gradient, latexin was recovered in the L fraction but not in the H fraction (Figure 6B).

It has been reported that sonication treatment for the disruption of mast cells, even if controlled, impairs intact membranes of high-density granules to a certain extent, generating membrane-free granules of low density and varying the yield of high-density granules in preparations [28]. It was therefore possible that sonication for disruption of mast cells might have resulted in the artificial generation of latexin granules with low density. To examine this possibility, mast cells were disrupted by homogenization with a Teflon homogenizer rather than by sonication; the granules were separated into L and H fractions. Latexin was

Figure 6 Separation of granules in peritoneal mast cells

(A, B) The protein composition of granules (A) and the distribution of latexin (B) are displayed by SDS/PAGE and Western blotting respectively. Mast cells (lane 1) were disrupted by sonication and centrifuged into precipitated granule fraction (lane 2) and supernatant (lane 3). The granule fraction was separated on a Percoll density gradient into two fractions of low (lane 4) and high (lane 5) density (see the text for details). Protein amounts of 1 and 10 μ g per preparation were applied for protein composition analysis by silver staining (A) and Western blotting of latexin (B) respectively. (C) MCCPA was detected with Western blotting in intact mast cells (lane 1; 1 μ g of protein) and in granule fractions of low density (lane 2; 0.5 μ g in 10 μ l) and high density (lane 3; 1.5 μ g in 10 μ l). MCCPA was seen also in mast cells (lane 4; 6000 cells in 10 μ l) and supernatant (lane 5; 10 μ l) prepared at 30 min after the addition of 3 μ g/ml compound 48/80 at 26 °C. The positions of molecular mass markers are indicated (in kDa) at the left.

recovered only in the L fraction, not in the H fraction (results not shown), suggesting that latexin granules had a lower density *in situ* irrespective of sonication.

In contrast, a Western blot analysis of the whole mast-cell preparation showed that three protein bands of 34, 36 and 38 kDa detected by SDS/PAGE corresponded to MCCPA components (Figures 6A and 6C). These protein bands were detected in the H and L fractions (Figure 6C). The MCCPA content was greater in the H fraction than in the L fraction, even though the MCCPA content in the two fractions varied between experiments.

These results indicate that native latexin granules are of low density, whereas most MCCPA- or histamine-containing granules are of high density. In addition, results of SDS/PAGE and Western blot analyses suggest that the total content of latexin in a whole mast cell or in low-density granules is much smaller than that of MCCPA in the cell or in high-density granules.

Reversible dissociation of latexin from low-density granules

To assess whether latexin in the latexin granules was associated with granular matrix or was contained in the granules as a soluble protein, the removal of latexin from the granules was first examined under a high salt concentration. After various concentrations of NaCl had been added to the L fraction, the mixture was separated into supernatant and precipitate fractions. Latexin was not detected in the supernatant at 0.5 or 1.0 M NaCl but most of the original latexin content was recovered in the supernatant at 1.5 M NaCl (Figure 7A).

Conversely, it was examined whether once-dissociated latexin could be reassociated with the residual granular matrix. The L fraction was incubated with 1.5 M NaCl and then separated into

supernatant and pellet fractions. The supernatant was diluted to give a final NaCl concentration of approx. 0.1 M and then mixed with the resuspended pellet. After incubation overnight, the mixture was again centrifuged to separate it into the two fractions. Most of the latexin content was recovered in the precipitate fraction (Figure 7B).

These results show that latexin is reversibly dissociated from the low-density granules, suggesting that it is non-covalently associated with a certain kind of granular matrix of the latexin granules.

Treatment of low-density granules with heparinase

Some proteases, such as chymase and MCCPA, are associated with heparin proteoglycan; however, treatment at high ionic strength dissociates those proteases from the macromolecular matrix [31,37]. To examine whether latexin was also bound to a heparin-like component in the latexin granules, the latexin-granule-containing L fraction was treated with heparinase I, purified first by Linker and Hovingh [30], which specifically cleaves the polysaccharide chain of heparin.

The L fraction was treated with or without heparinase I and then separated into supernatant and precipitate fractions. Latexin was detected exclusively in the precipitate when incubated without heparinase but predominantly in the supernatant after treatment with heparinase I (Figure 8), suggesting that latexin molecules were associated with the saccharide portion of a heparin-like substance in the latexin granules.

Next we examined whether or not latexin could be associated with heparin directly, by using recombinant latexin protein and a heparin-immobilizing affinity column. The purified recombinant latexin was applied to the column at 0.1 M NaCl and neutral pH. After washing, latexin was eluted at a higher NaCl

Figure 7 Reversible dissociation of latexin from low-density granules

(A) Dissociation of latexin. The low-density granule fraction (L) was incubated on ice for 1 h at 0.5 M NaCl (lanes 1 and 2), 1.0 M NaCl (lanes 3 and 4) or 1.5 M NaCl (lanes 5 and 6) and then separated into two fractions of precipitate (P) (lanes 1, 3 and 5) and supernatant (S) (lanes 2, 4 and 6). (B) Reassociation of latexin. The L fraction was treated with 1.5 M NaCl and centrifuged to yield S and P (lane 1, S; lane 2, P). After dilution 1:15, S was mixed with P and kept overnight on ice. The mixture was centrifuged to separate it into S (lane 3) and P (lane 4). Latexin was detected by Western blotting. The positions of molecular mass markers are indicated (in kDa) at the left.

Figure 8 Release of latexin from low-density granules by treatment with heparinase

The low-density granule fraction was treated (lanes 3 and 4) or not (lanes 1 and 2) with heparinase I and then separated into precipitate (lanes 1 and 3) and supernatant (lanes 2 and 4) fractions by centrifugation. Latexin was detected by Western blotting.

concentration, approx. 0.7 M (results not shown), implying that latexin was associated directly with heparin.

These results suggest that latexin is associated directly with a heparin-like component of the latexin granules.

Table 1 Comparison in extent of net release of latexin, MCCPA and histamine from mast cells activated with non-immunological and immunological treatments

Mast cells were activated with 3 µg/ml compound 48/80, 5 µM A23187 or anti-IgE antiserum (diluted 1:20) at 26 °C. The amounts of latexin, MCCPA and histamine released at 5 and 30 min after activation were determined as described in the Experimental section. Net release percentage was estimated as the difference in extent of release between no treatment and stimulant treatments between 5 and 30 min. In all cases, no release of latexin, MCCPA or histamine from mast cells without activation treatment was detected within 30 min.

	Net release extent (%)				
	Compound 48/80			A23187 1.5	anti-IgE 1.5
	NaCl (M) ...	0	1.0	1.5	
Latexin		0	0	0	0
MCCPA		—	12–16	16–23	23–34
Histamine		46–48	—	—	—

Activation of mast cells with non-immunological and immunological treatments

Mast cells are activated naturally by complexes of antigen and IgE or artificially by anti-IgE antibody and stimulation reagents, resulting in the degranulation of secretory granules and the release to the extracellular space of preformed chemical mediators stored in the secretory granules [35,41–44]. We examined whether or not latexin was released extracellularly from activated mast cells by using compound 48/80, A23187 and anti-IgE serum, in comparison with the secretion of MCCPA and/or histamine as preformed chemical mediators. It has been described that macromolecular complexes containing MCCPA, heparin proteoglycan and additional proteins are released with mast-cell activation and remain concentrated at the extracellular surface of the activated mast cells. Thus washing the mast cells with 1 M NaCl is necessary to extract the complexes from the cell surface [31]. In contrast, because latexin was dissociated from the latexin granules in the presence of 1.5 M NaCl as described above, treatment of the activated mast cells with 1.5 M NaCl was also examined.

First, the release of histamine and latexin was examined without treatment with NaCl. Activation of the mast cells with compound 48/80 caused the release of 46–48 % of the histamine content in the mast cells within 30 min, whereas no significant release of latexin was detected even 30 min after the stimulation (Table 1). Next, the mast cells were stimulated with compound 48/80 and then washed with 1.0 or 1.5 M NaCl. The activated mast cells released 12–16 % and 16–23 % of the MCCPA contents of the cells in the presence of 1.0 and 1.5 M NaCl respectively within 30 min but did not release latexin even 30 min after activation (Figure 6C and Table 1). Similarly, the mast cells activated with A23187 or anti-IgE serum secreted approx. 30 % of their MCCPA but did not secrete latexin in the presence of 1.5 M NaCl (Table 1).

These results show that, unlike MCCPA and histamine, latexin is not secreted from the mast cells activated by non-immunological and immunological treatments.

DISCUSSION

The present study showed that rat peritoneal mast cells contain latexin, an inhibitor of CPA, and that the latexin protein is biosynthesized in the peritoneal mast cells. It is well known that

mast cells contain many kinds of protease, such as chymase, trypsin and MCCPA, in their secretory granules [11,35,36] and also various protease inhibitors of chymase and trypsin, such as α_1 -antitrypsin, α_1 -antichymotrypsin and trypstatin [12–18]. However, because the presence of an intracellular inhibitor of rat MCCPA has not yet been reported, the present paper is the first to describe that latexin, which has inhibitory activity against MCCPA [6], is produced and contained in rat peritoneal mast cells.

Immunocytochemical and immunoelectron microscopic observations revealed that, in rat peritoneal mast cells, latexin is distributed heterogeneously in the cytoplasm and is associated with a minority population of granules distinct from MCCPA- or histamine-containing granules. Because latexin in neocortical and primary sensory neurons seems to be distributed evenly in the cell body and axonal fibres [1,3,5] and is sometimes observed in the nucleus (K. Takiguchi-Hayashi, unpublished work), the granular localization of latexin in the mast cells might be a unique characteristic of mast cells. In addition, immunohistochemical analyses showed that latexin immunoreactivity was localized on granular structures in dispersed cells situated within the lung mucosa, stomach mucosa and skin connective tissue (K. Takiguchi-Hayashi, unpublished work), suggesting that the immunoreactivity might be derived, at least in part, from mast cells resident within those tissues [2,6,35,36].

The present biochemical analyses defined various properties of the latexin granules. The density of latexin granules was low, whereas that of MCCPA- or histamine-containing granules was high, indicating that the latexin granules are physically distinct from MCCPA- or histamine-containing granules. However, granules containing MCCPA were also collected in low-density granule preparations. The MCCPA content of the low-density granule preparations varied between experiments. It is likely that the sonication of mast cells before granule separation partly impairs the perigranular membranes of the high-density granules but does not dissociate MCCPA, which is still complexed with the heparin matrix [31,37], resulting in low-density granules with a comparatively high content of MCCPA, as described by Krüger et al. [28].

Latexin protein was reversibly dissociated from the granular matrix of the low-density granules with a high concentration of NaCl and was released from the matrix by treatment with heparinase. These results suggest that the latexin granules contain a heparin-like substance to which latexin is non-covalently bound. However, in the mast cells heparin is synthesized in the endoplasmic reticulum and the Golgi and is stored in secretory granules as heparin serglycin proteoglycan [45]. Considering the subcellular localization of heparin, the latexin granules seem to be a kind of secretory granule; however, they are not, as discussed below. Therefore the origin of the heparin-like substance in the non-secretory latexin granules remains to be investigated.

When the mast cells were activated by compound 48/80, A23187 or anti-IgE serum, MCCPA and histamine were released extracellularly; however, latexin release was not detected even after the activated mast cells had been washed with 1.5 M NaCl, at which concentration latexin was dissociated from the latexin granules. It has been described that after mast cell activation, macromolecular complexes containing MCCPA and heparin proteoglycan in the secretory granules remain concentrated at the extracellular surface of the activated cells and is extracted by washing with 1 M NaCl without damage to the integrity of the perigranular or plasma membrane of the activated mast cells [31]. Because the percentage release of MCCPA was not very different between washing with 1.0 and 1.5 M NaCl, the integrity of the plasma membrane of the activated mast cells

seems not to be impaired in the presence of 1.5 M NaCl. Because latexin is dissociated from the latexin granules with 1.5 M NaCl, washing of the activated mast cells with 1.5 M NaCl should allow latexin to be dissociated from latexin granules to the extracellular space, even if these granules remain at the extracellular surface of the activated mast cells. The absence of release of latexin from the activated mast cells in the presence of 1.5 M NaCl shows that latexin is not secreted from the mast cells by activation with the Ca^{2+} ionophore A23187 or anti-IgE antibody as well as compound 48/80. These results strongly suggest that latexin is not secreted from mast cells even under conditions of operation of diverse signalling pathways for mast cell activation.

Although an immunoelectron microscopic study did not determine the presence or absence of a perigranular membrane to the latexin granules because of relatively weak cellular fixation required for the subsequent immunoreaction of latexin, the heparinase treatment released latexin from the granular matrix of the latexin granules described above and, in addition, latexin does not have a signal sequence at its own N-terminus nor a high hydrophobic sequence operating as a membrane-translocating segment in other regions [2]. These findings suggest that the latexin granules might not have a perigranular membrane as a barrier against the penetration of latexin and heparinase.

On the subcellular localization of proteases and their inhibitors in mast cells, bovine trypsin and its inhibitor have been shown to be stored in the same secretory granules [16]. It has been suggested that rat trypsin and trypstatin are in the same secretory granules [17,20]. It has consequently been considered that these protease inhibitors might have a role in the regulation of trypsin activity within the mast cells and in the extracellular medium or as an acute-phase reactant during inflammatory conditions after release by degranulation [16,20]. In contrast, latexin present in the rat peritoneal mast cells is localized in a different granular compartment from that of MCCPA, implying that latexin does not interact with MCCPA in the normal mast cells. It is therefore possible that, when MCCPA is released accidentally into the cytoplasmic space from the secretory granules containing MCCPA, latexin granules complexed with heparin, which are considered to have no perigranular membrane, catch the released MCCPA in the heparin component and inhibit its proteolytic activity by latexin.

We thank M. Ishida for sectioning of rat non-neural tissues, R. Kato for preparation of latexin C-terminal peptide antigen, Dr Y. Kawasaki and T. Saito for technical advice on preparation of rat peritoneal mast cells, Dr A. Omori and S. Ichinose for amino acid sequencing, Y. Matsumura for help with this manuscript, and Dr E. Kominami (Juntendo University School of Medicine, Tokyo, Japan), for the generous gift of anti-(rat cathepsin D) polyclonal antibody.

REFERENCES

- 1 Arimatsu, Y., Miyamoto, M., Nihonmatsu, I., Hirata, K., Uratani, Y., Hatanaka, Y. and Takiguchi-Hayashi, K. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8879–8883
- 2 Hatanaka, Y., Uratani, Y., Takiguchi-Hayashi, K., Omori, A., Sato, K., Miyamoto, M. and Arimatsu, Y. (1994) *Eur. J. Neurosci.* **6**, 973–982
- 3 Takiguchi-Hayashi, K., Sato, M., Sugo, N., Ishida, M., Sato, K., Uratani, Y. and Arimatsu, Y. (1998) *Brain Res.* **801**, 9–20
- 4 Arimatsu, Y., Nihonmatsu, I., Hirata, K. and Takiguchi-Hayashi, K. (1994) *J. Neurosci.* **14**, 2020–2031
- 5 Takiguchi-Hayashi, K. and Arimatsu, Y. (1995) *NeuroReport* **6**, 281–283
- 6 Normant, E., Martres, M. P., Schwartz, J.-C. and Gros, C. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 12225–12229
- 7 Clauser, E., Gardell, S. J., Craik, C. S., MacDonald, R. J. and Rutter, W. J. (1988) *J. Biol. Chem.* **263**, 17837–17845
- 8 Gardell, S. J., Craik, C. S., Clauser, E., Goldsmith, E. J., Stewart, C.-B., Graf, M. and Rutter, W. J. (1988) *J. Biol. Chem.* **263**, 17828–17836
- 9 Lützel-Schwab, C., Pejler, G., Aveskog, M. and Hellman, L. (1997) *J. Exp. Med.* **185**, 13–29

- 10 Normant, E., Gros, C. and Schwartz, J.-C. (1995) *J. Biol. Chem.* **270**, 20543–20549
- 11 Harvima, R. J. and Schwartz, L. B. (1993) in *Immunopharmacology of Mast Cells and Basophils* (Foreman, J. C., ed.), pp. 115–138, Academic Press, Tokyo
- 12 Akiyama, M., Watanabe, Y. and Nishikawa, T. (1991) *Acta Pathol. Jpn.* **41**, 344–349
- 13 Harvima, I. T., Naukkarinen, A., Paukkonen, K., Harvima, R. J., Aalto, M. L., Schwartz, L. B. and Horsmanheimo, M. (1993) *Arch. Dermatol. Res.* **285**, 184–192
- 14 Kaminska, R., Harvima, I. T., Naukkarinen, A., Nilsson, G. and Horsmanheimo, M. (1996) *J. Pathol.* **180**, 434–440
- 15 Harvima, I. T., Haapanen, L., Ackermann, L., Naukkarinen, A., Harvima, R. J. and Horsmanheimo, M. (1999) *Acta Derm. Venereol.* **79**, 98–104
- 16 Fiorucci, L., Erba, F., Falasca, L., Dini, L. and Ascoli, F. (1995) *Biochim. Biophys. Acta* **1243**, 407–413
- 17 Kido, H., Yokogoshi, Y. and Katunuma, N. (1988) *J. Biol. Chem.* **263**, 18104–18107
- 18 Westin, U., Polling, A., Ljungkrantz, I. and Ohlsson, K. (1999) *Biol. Chem.* **380**, 489–493
- 19 Pallaoro, M., Gambacurta, A., Fiorucci, L., Mignogna, G., Barra, D. and Ascoli, F. (1996) *Eur. J. Biochem.* **237**, 100–105
- 20 Itoh, H., Ide, H., Ishikawa, H. and Nawa, Y. (1994) *J. Biol. Chem.* **269**, 3818–3822
- 21 Cole, K. R., Kumar, S., Trong, H. L., Woodbury, R. G., Walsh, K. A. and Neurath, H. (1991) *Biochemistry* **30**, 648–655
- 22 Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
- 23 Uratani, Y. (1992) *J. Biol. Chem.* **267**, 5177–5183
- 24 Muno, D., Ishidoh, K., Ueno, T. and Kominami, E. (1993) *Arch. Biochem. Biophys.* **306**, 103–110
- 25 Koffer, A., Tatham, P. E. R. and Gomperts, B. D. (1990) *J. Cell Biol.* **111**, 919–927
- 26 Schueller, E., Peutsch, M., Bohacek, L. G. and Gupta, R. K. (1967) *Can. J. Med. Technol.* **29**, 137–138
- 27 Miyasaka, N., Hatanaka, Y., Jin, M. and Arimatsu, Y. (1999) *Mol. Brain Res.* **69**, 62–72
- 28 Krüger, P. G., Lagunoff, D. and Wan, H. (1980) *Exp. Cell Res.* **129**, 83–93
- 29 Willson, M. B. and Nakane, P. K. (1978) in *Immunofluorescence and Related Staining Techniques* (Knapp, W., Holubar, K. and Wick, G., eds.), pp. 215–225, Elsevier/North Holland Biomedical Press, Amsterdam
- 30 Linker, A. and Hovingh, P. (1972) *Methods Enzymol.* **28**, 902–911
- 31 Schwartz, L. B., Riedel, C., Schratz, J. J. and Austen, K. F. (1982) *J. Immunol.* **128**, 1128–1133
- 32 Shore, P. A., Burkhalter, A. and Cohn, Jr, V. H. (1959) *J. Pharmacol. Exp. Ther.* **127**, 182–186
- 33 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 34 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 35 Barrett, K. E. and Pearce, F. L. (1993) in *Immunopharmacology of Mast Cells and Basophils* (Foreman, J. C., ed.), pp. 29–42, Academic Press, Tokyo
- 36 Page, C. P. and Minshall, E. (1993) in *Immunopharmacology of Mast Cells and Basophils* (Foreman, J. C., ed.), pp. 181–195, Academic Press, Tokyo
- 37 Everitt, M. T. and Neurath, H. (1980) *FEBS Lett.* **110**, 292–296
- 38 Sannes, P. L., McDonald, J. K., Allen, R. C. and Spicer, S. S. (1979) *J. Histochem. Cytochem.* **27**, 1496–1498
- 39 Jamur, M. C., Vugman, I. and Hand, A. R. (1986) *Cell Tiss. Res.* **244**, 557–563
- 40 Baram, D., Adachi, R., Medalia, O., Tuvim, M., Dickey, B. F., Mekori, Y. A. and Sagi-Eisenberg, R. (1999) *J. Exp. Med.* **189**, 1649–1657
- 41 Röhlich, P., Anderson, P. and Uvnäs, B. (1971) *J. Cell Biol.* **51**, 465–483
- 42 Lawson, D., Raff, M. C., Gomperts, B., Fewtrell, C. and Gilula, N. B. (1977) *J. Cell Biol.* **72**, 242–259
- 43 Morrison, D. C., Roser, J. F., Henson, P. M. and Cochrane, C. G. (1974) *J. Immunol.* **112**, 573–582
- 44 Koibuchi, Y., Ichikawa, A., Nakagawa, M. and Tomita, K. (1985) *Eur. J. Pharmacol.* **115**, 163–170
- 45 Lindall, L., Lidhold, K., Spillmann, D. and Kjellen, L. (1994) *Thrombosis Res.* **75**, 1–32

Received 19 July 1999/23 November 1999; accepted 4 January 2000