Two-Phase Increment of Ca^{2+} Uptake, Intracellular Ca^{2+} Concentration, and Histamine Release Following Antigen Stimulation in Mouse Bone Marrow-Derived Mast Cells (BMMC)

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ABSTRACT—The relationship between the influx of Ca^{2+} into cells or cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and the histamine release following antigen stimulation in mouse bone marrow-derived mast cells (BMMC) was examined, and the results were compared with those from human lung mast cells (HLMC) and rat peritoneal mast cells (RPMC) in some experiments. Anaphylactic histamine release from BMMC as well as HLMC, but not that from RPMC, was dependent on the extracellular Ca^{2+}. When BMMC were challenged by antigen following radioactive $^{45}Ca^{2+}$ addition, two phases of $^{45}Ca^{2+}$ influx into the cells were observed. The first phase, which was initiated and completed within 30 sec and 2 min, respectively, after antigen treatment, appeared to be related to anaphylactic histamine release. The second influx began 30 sec subsequent to the first one and lasted for at least 2 min, and this occurred after the completion of the histamine release; So far, it is not known how this second influx participates in the intracellular event(s). On the other hand, only one sustained elevation of $[Ca^{2+}]_i$ occurred that reached its maximum within 2 min after antigen stimulation. Following stimulation of BMMC with antigen in the absence of Ca^{2+}, Ca^{2+} addition 1 to 5 min later time-dependently enhanced the histamine release, although the release was deteriorated by further extension of Ca^{2+} addition. In contrast, the releasability of HLMC was rapidly decreased. These results indicate that extracellular Ca^{2+} not only is prerequisite for anaphylactic histamine release from BMMC, but also may modulate the release and participate in some intracellular event(s) which has yet to be focused upon.

Keywords: Mast cell, Histamine, Calcium, Anaphylaxis, Desensitization

It is well documented that the elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which results from either release from intracellular Ca^{2+} store sites or influx of Ca^{2+} from the extracellular medium, is a requisite process for the release of histamine from mast cells (MC) irrespective of whether the cells are induced by immunological or non-immunological stimuli (1-6). In general, it has been accepted that protein tyrosine kinase and subsequently phospholipase C (PLC) ($PLC_{\gamma 1}$) prior to the elevation of $[Ca^{2+}]_i$ are activated following antigen stimulation (7-11) and that the activation of the latter cleaves phospholipids into diacylglycerol, which activates protein kinase C (12), and inositol 1,4,5-trisphosphate (IP_3), which induces release of Ca^{2+} from intracellular Ca^{2+} store sites (13, 14). On the other hand, it is still obscure how the Ca channel for Ca^{2+} influx is opened and how influxed Ca^{2+} participates in the process of histamine release.

Rat peritoneal mast cells (RPMC) have been frequently used for the elucidation of mechanisms of histamine release stimulated by various stimuli and assessment of antiallergic drugs, because the cells are easily isolated and highly purified. However, the cells do not require extracellular Ca^{2+} for anaphylactic histamine release (15-17), whereas human lung mast cells (HLMC) (18, 19), guinea pig lung mast cells (20) and mouse peritoneal mast cells (MPMC) (21) do. Thus, there is a species difference of MC in the requirement of extracellular Ca^{2+} for anaphylactic histamine release.

Mouse bone marrow-derived mast cells (BMMC), which were used to first prove that MC are proliferated from multipotential hemopoietic stem cells of the bone

marrow (22), are a good system for investigating the mechanisms of chemical mediator release because these cells are homogeneous (23, 24), can be obtained in a large amount by in vitro culture (24–27), and respond well to immunological stimulation (25–27).

In the present study, we investigated whether extracellular Ca^{2+} is required for and how the influx of Ca^{2+} and elevation of $[Ca^{2+}]_i$ are related to the histamine release in BMMC during anaphylaxis, and the results were compared with those from HLMC and RPMC in some experiments.

MATERIALS AND METHODS

Reagents

Reagents and their sources were as follows: ethylenediaminetetraacetic acid (EDTA) and $CaCl_2 \cdot 2H_2O$ (Nacalai Tesque, Kyoto); bovine serum albumin (BSA, Fr. V, fatty acid-free; Boehringer Mannheim, Tokyo), $^{45}CaCl_2$ (370 GBq/g Ca; New England Nuclear, Boston, MA, USA); fura-2-acetoxymethyl ester (Dojindo Labo., Kamimasuki, Kumamoto); collagenase (Iwaki Glass, Tokyo); deoxyribonuclease I (Carbiochem-Behring, La Jolla, CA, USA); α medium (Flow Labo., Irvine, Scotland); horse serum (Gibco Lab., Grand Island, NY, USA); Percoll (Pharmacia Fine Chem., Uppsala, Sweden); GIT medium (Nihon Pharm., Tokyo); fetal bovine serum (Filtron Pty, Victoria, Australia); NaCl, KCl and NaH_2PO_4 (Suprapur®, Merck, Darmstadt, Germany); gelatin, $MgCl_2 \cdot 6H_2O$, $NaHCO_3$ and glucose (Merck); and Triton X-100 (Wako Pure Chem., Osaka). The other reagents used were the highest grade of commercial products available.

Rat antiserum against dinitrophenylated *Ascaris suum* extracts (DNP-As) was prepared according to the method of Tada and Okumura (28). The antiserum titer was 1 : 256 when assessed by 48-hr passive cutaneous anaphylaxis (29). Mite extracts from *Dermatophagoides farinae* were supplied from Dr. H. Nagai of Gifu Pharmaceutical University.

Animals

Eight-week-old, male BALB/c and BDF_1 mice, and seven-week-old male and ten-week-old female Wistar rats were purchased from Japan SLC, Hamamatsu.

Human lungs

Macroscopically normal portions of surgically resected human lung for carcinoma were used for the experiments as soon as possible.

Culture of BMMC

BMMC were obtained as described elsewhere (27, 30). In brief, bone marrow cells from BALB/c mice were cultured for 5 weeks with α medium supplemented with 20% horse serum, 100 μM 2-mercaptoethanol, 100 μM non-essential amino acids, 60 μg/ml kanamycin sulfate and 10% or 20% conditioned medium from BDF_1 mouse spleen cells. After 5 weeks of culture, purities of the BMMC were more than 95% as assessed by alcian blue staining (31).

Passive sensitization of BMMC

BMMC obtained by 5-week culture were passively sensitized by adding 1/10 volume of the anti-DNP-As rat serum and incubated at 37℃ for 16–24 hr under the BMMC-culture conditions. After the sensitization, BMMC were washed twice with 0.1% BSA-containing Tyrode's solution (composition of Tyrode's solution: 140 mM NaCl, 3.0 mM KCl, 0.5 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 1.8 mM $CaCl_2$ and 5.5 mM glucose, adjusted to pH 7.4 with $NaHCO_3$) and suspended at 10^5, 10^6 or 4×10^6 BMMC/ml with 0.1% BSA-containing and Ca^{2+}-free, 0.1% BSA-containing, or Ca^{2+}-free Tyrode's solution. The passively sensitized BMMC were used throughout the experiments.

Preparation of isolated human lung mast cells (HLMC)

HLMC from the human lung were enzymatically dispersed, purified and cultured by the following procedures: After cutting the tissue into pieces of $1 \times 1 \times 3$ mm in size with a McIlwain tissue chopper, the fragments were suspended with 0.1% BSA-containing and Ca^{2+}-free Tyrode's solution. The suspension was treated with 30 U/ml collagenase and 10 μg/ml deoxyribonuclease I at 37℃ for 20 min, 6 times. The respective dispersed cells by the 2nd to 6th enzyme treatments were washed with 0.1% BSA-containing and Ca^{2+}-free Tyrode's solution, combined together, and cultured for 12 hr with GIT medium containing 10% fetal bovine serum.

The non-adherent and loosely adherent cells on polystyrene culture flasks were harvested and submitted to counter current centrifugation elutriation (Beckman, Fullerton, CA, USA) under conditions similar to those described by Schulman et al. (32) for purification of HLMC. Following culture for 18 hr and suspension with 100% Percoll, the HLMC were further purified by Percoll-discontinuous density centrifugation (33). HLMC that migrated into the 50% and 60% Percoll layers were collected and washed twice with GIT medium.

Passive sensitization of HLMC

HLMC (approximately 10^5 HLMC/ml) were suspended with GIT medium containing 10% fetal bovine serum and again cultured for 20 hr in the presence of mite-sensitive human atopic serum (1/30 volume, radioallergosorbent

test value>30%) for passive sensitization. After culture, the harvested HLMC were washed twice with 0.1% BSA-containing and Ca^{2+}-free Tyrode's solution. Resultant HLMC with purities of 81.8±2.7% (mean±S.E., n=8) were suspended at 2×10^4 HLMC/ml with Ca^{2+}-free Tyrode's solution with or without 0.1% BSA.

Harvest and purification of peritoneal mast cells (RPMC) from passively sensitized rats

Rats were passively sensitized by i.p. injection of 0.2 ml/animal of the anti-DNP-As rat serum. Forty-eight hours later, following anesthesia by inhalation of diethyl ether, the animals were killed by stunning and exsanguinated through the section of the carotid artery. Following i.p. injection with 100 ml/kg of Ca^{2+}-free and 0.1% BSA-, 0.1% gelatin- and 10 U/ml heparin-containing mast cell medium [MCM, composition: 150 mM NaCl, 3.7 mM KCl, 3.0 mM Na_2HPO_4, 3.5 mM KH_2PO_4 and 6.0 mM glucose, adjusted to pH at 6.8 with 5 N NaOH] and gentle massage of the abdomen for 1 min, the peritoneal fluid, including RPMC, was collected. The fluid was gently centrifuged ($50\times g$, 7 min, 4℃, 3 times) to obtain the partially purified mast cells. Then, they were further purified according to the method of Sullivan et al. (34) by centrifugation through a 31.5% BSA layer. RPMC obtained were suspended with 0.1% BSA-containing and Ca^{2+}-free MCM at 10^5 RPMC/ml. The purified RPMC had a purity of 97.7±0.4% (mean±S.E., n=3).

Anaphylactic histamine release

Unless otherwise stated, BMMC (10^5 BMMC/ml) and HLMC (2×10^4 HLMC/ml) in 0.1% BSA-containing and Ca^{2+}-free Tyrode's solution or RPMC (10^5 RPMC/ml) in 0.1% BSA-containing and Ca^{2+}-free MCM were distributed into individual tubes (2 ml/tube) and incubated at 37℃ for 10 min following addition of $CaCl_2$ at final concentrations of 1.8 mM (BMMC and HLMC) or 0.9 mM (RPMC). Histamine release was then performed by stimulation with the antigen (BMMC: 100 ng/ml, HLMC: 5 μg/ml and RPMC: 10 μg/ml) at 37℃ for 20 min (BMMC), 30 min (HLMC) or 10 min (RPMC). After completion of the reaction, they were cooled in ice-water and centrifuged ($1,700\times g$, 10 min, 4℃). Each resultant supernatant was stored at −20℃ until the histamine assay.

For the time course experiments, BMMC and HLMC suspensions were stimulated with the antigen, with the reaction mixture kept at 37℃, and 1- to 3-ml aliquots were withdrawn at defined times. To stop the reaction, each sample was immediately added into tubes containing sufficient EDTA solution to result in a 5 mM final concentration and was centrifuged at $15,000\times g$ for 30 sec at room temperature. The supernatants obtained were stored under the above-described conditions until the histamine assays. The viability of any MC was greater than 95%, as assessed by trypan blue exclusion prior to the experiments.

Assay of Ca^{2+} uptake

To BMMC suspended at 4×10^6 BMMC/ml with 0.1% BSA-containing and Ca^{2+}-free Tyrode's solution was added $CaCl_2$ at the final concentration of 0.05 mM or 1.8 mM (the concentrations including the ^{45}Ca that was subsequently added) and then they were incubated at 37℃ for 8 min followed by the addition of $^{45}Ca^{2+}$ (final radioactivity of 370 or 740 kBq/ml, respectively). Two minutes later, the cells were challenged by 100 ng/ml antigen, with the reaction mixture kept at 37℃, and 100-μl aliquots were withdrawn at defined times. Each sample was immediately added into tubes containing 50 μl of 6 mM EDTA solution to stop the reaction. The aliquots (100 μl) were layered on 300 μl of 18% BSA solution in a microfuge tube ($4\phi\times46$ mm, Assist, Tokyo) and centrifuged at $10,000\times g$ for 1 min at 4℃. The sediment (BMMC pellet) was recovered by freezing the tube at −80℃ and slicing off the bottom of the tube containing the pellet, which was dissolved in 0.5 ml of 10% Triton X-100. The radioactivity in the solution was measured by a scintillation spectrometer (2000CA; Packard Inst., Zurich, Switzerland). The amount of Ca^{2+} uptake into the cells was expressed as nmol/4×10^6 BMMC, which was calculated from the radioactivity. The recovery of antigen-stimulated BMMC from the centrifugation using 18% BSA solution was 103.8±3.6% (mean±S.E., n=3) when assessed by histamine content of the cells.

Assay of histamine

Histamine in the supernatant was assayed fluorometrically by high performance liquid chromatography (HPLC) over a cation exchange column (TSK gel SP-2SW, $4.6\phi\times50$ mm; Toso, Tokyo) as described by Itoh et al. (35) who modified the method of Yamatodani et al. (36). Histamine release (%) was calculated from following formula:

Histamine release (%)=A/B×100

or

Anaphylactic histamine release (%)=(A−C)/B×100

A: Amount of histamine release by antigen

B: Histamine content in the cells

C: Amount of spontaneous histamine release

Assay of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$)

Following addition of 1/250 volume of 1 mM fura-2-acetoxymethyl ester, BMMC (10^6 BMMC/ml) in 0.1% BSA-containing Tyrode's solution were incubated at 37℃ for 45 min. The cells were then washed three times with 0.1% BSA-containing and Ca^{2+}-free Tyrode's solution,

suspended at 4×10^5 BMMC/ml with Ca^{2+}-free Tyrode's solution and loaded into a cell, which was placed in a spectrofluorometer (F-3000; Hitachi, Tokyo) and kept at 37℃. When the fluorescent intensity at 510 nm induced by the excitation wave length of 340 nm became stable, the BMMC were treated with 1.8 mM Ca^{2+} 5 min before or 5 or 10 min after antigen stimulation. The changes of $[Ca^{2+}]_i$ induced by either antigen or Ca^{2+} were recorded as the relative fluorescent intensities at 510 nm produced by excitation at 340 nm.

Statistical analyses

Statistical analyses were performed by one-way analysis of variance (ANOVA). If a significance was detected, individual group differences were evaluated by Bonferoni's multiple test. A probability value (P) less than 0.05 was considered to be statistically significant.

RESULTS

Effect of EDTA on the anaphylactic histamine release from BMMC, HLMC and RPMC

When BMMC, HLMC and RPMC were challenged with antigen, histamine release was obviously enhanced in MC from any species. Treatment with 5 mM EDTA for 20 sec prior to antigen challenge almost completely and completely suppressed the release from BMMC and

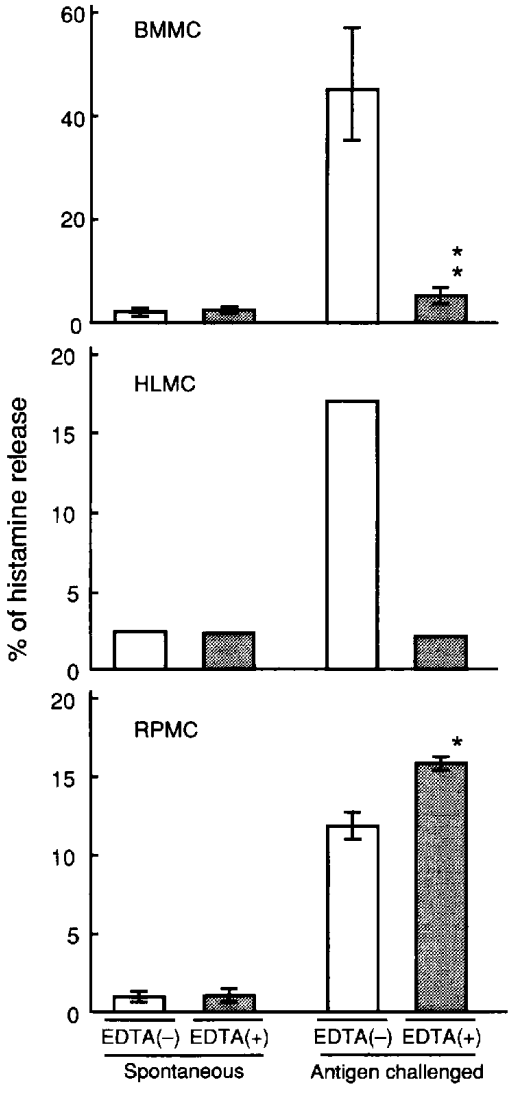


Fig. 1. Effects of ethylenediaminetetraacetic acid (EDTA) on the anaphylactic histamine release from mouse bone marrow-derived mast cells (BMMC), human lung mast cells (HLMC) and rat peritoneal mast cells (RPMC). BMMC (10^5 BMMC/ml) and HLMC (2×10^4 HLMC/ml) in 1.8 mM or RPMC (10^5 RPMC/ml) in 0.9 mM Ca^{2+}-containing medium were treated with (+) or without (−) EDTA at 5 mM final concentration at 37℃ for 20 sec and then were challenged with the antigen for 20 min (BMMC), 30 min (HLMC) or 10 min (RPMC). Each column represents the mean±S.E. of 3 experiments (BMMC and RPMC) or the mean of 2 experiments (HLMC). The histamine contents were 52.6±6.7 ng/10^5 BMMC, 48.2 and 87.1 ng/2×10^4 HLMC and 1,700±40 ng/10^5 RPMC. * and **: statistically significant difference from the respective group stimulated with antigen in the absence of 5 mM EDTA at $P < 0.05$ and 0.01, respectively.

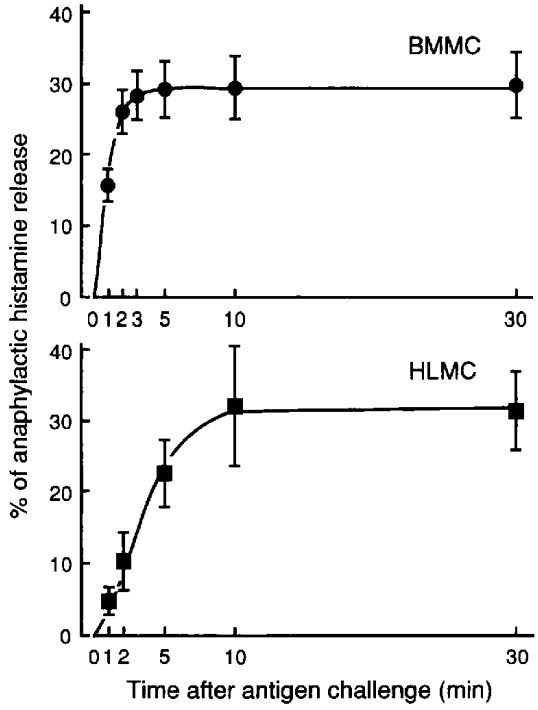


Fig. 2. Time courses of the anaphylactic histamine release from mouse bone marrow-derived mast cells (BMMC) and human lung mast cells (HLMC). BMMC (10^5 BMMC/ml) and HLMC (2×10^4 HLMC/ml) were challenged with the antigen at 37℃ for the indicated time. Each point represents the mean±S.E. of 3 experiments. Histamine contents of BMMC and HLMC were 56.7±4.0 ng/10^5 BMMC and 103.1±6.1 ng/2×10^4 HLMC, respectively.

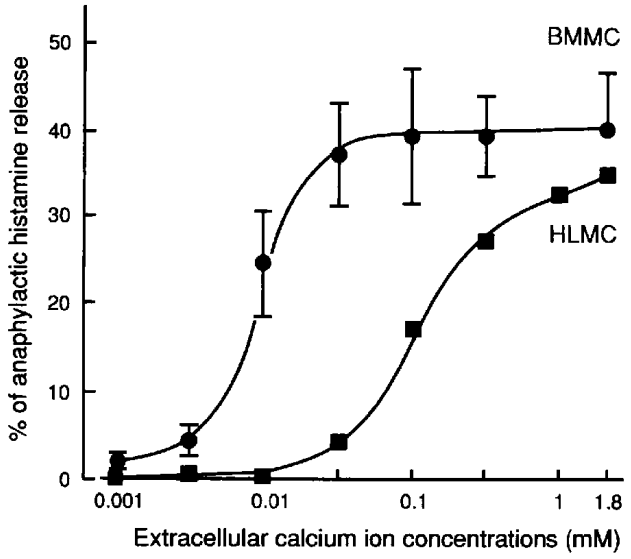


Fig. 3. Influence of extracellular Ca^{2+} concentrations on the anaphylactic histamine release from mouse bone marrow-derived mast cells (BMMC) and human lung mast cells (HLMC). BMMC (10^5 BMMC/ml) and HLMC (2×10^4 HLMC/ml) were suspended in Ca^{2+}-free Tyrode's solution, and then they were challenged with the antigen in the presence of the indicated Ca^{2+} concentrations at 37℃ for 20 min (BMMC) or 30 min (HLMC). $CaCl_2$ was added 10 min before the antigen challenge. Each point represents the mean±S.E. of 4–6 experiments (BMMC) or the mean of 2 experiments (HLMC). The histamine contents were 72.8±11.7 ng/10^5 BMMC and 94.5 and 86.3 ng/2×10^4 HLMC, respectively.

HLMC, respectively, to the spontaneous levels, whereas that from RPMC was not inhibited but rather enhanced (Fig. 1).

Time courses of histamine release from BMMC and HLMC

In BMMC, most of the anaphylactic histamine release occurred within 2 min after the stimulation, reaching maximal levels within 5 min. On the other hand, anaphylactic histamine release from HLMC was somewhat slower than that from BMMC, reaching maximal levels at 10 min. The time for half maximal histamine release from BMMC and HLMC was approximately 1 and 3 min, respectively (Fig. 2).

Influence of extracellular Ca^{2+} on the anaphylactic histamine release from BMMC and HLMC

When BMMC and HLMC, which had been treated with 0.001 to 1.8 mM Ca^{2+} for 10 min, were challenged with antigen, concentration-dependent histamine release was observed in either of the cells. However, Ca^{2+} concentrations required for the release from BMMC were less than one tenth of those required to cause the release from HLMC. The approximate concentration required for the half maximum histamine release in BMMC was 0.01 mM, whereas that in HLMC was 0.1 mM (Fig. 3).

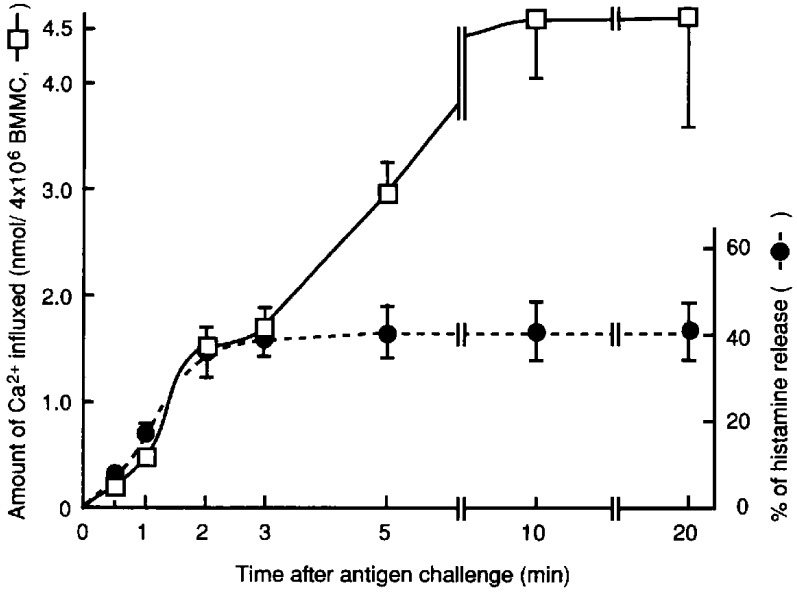


Fig. 4. Time courses of Ca^{2+} uptake into and the anaphylactic histamine release from mouse bone marrow-derived mast cells (BMMC) in the presence of 0.05 mM $CaCl_2$. BMMC (4×10^6 BMMC/ml) were challenged with antigen at 37℃ for the indicated time. Each point represents the mean±S.E. of 5 experiments. Amounts of Ca^{2+} influxed were calculated from the value obtained by subtracting the spontaneous Ca^{2+} uptake from the antigen-induced one at each indicated time. The histamine content was 2,600±240 ng/4×10^6 BMMC.

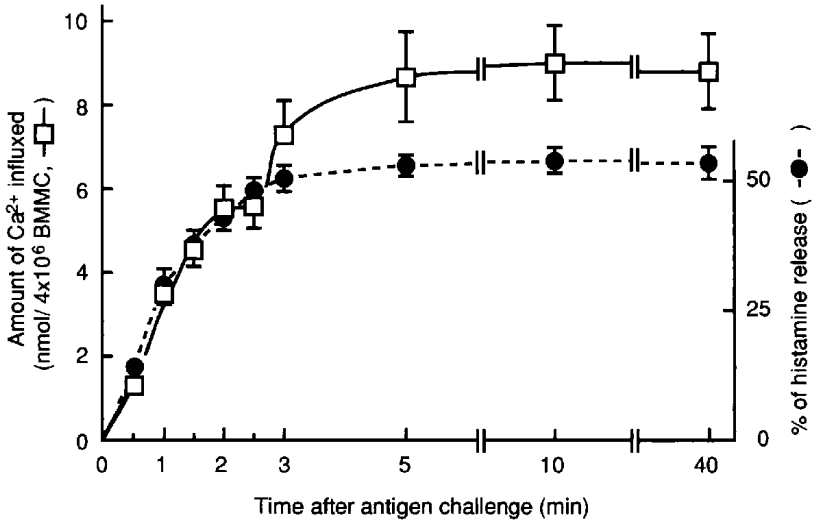
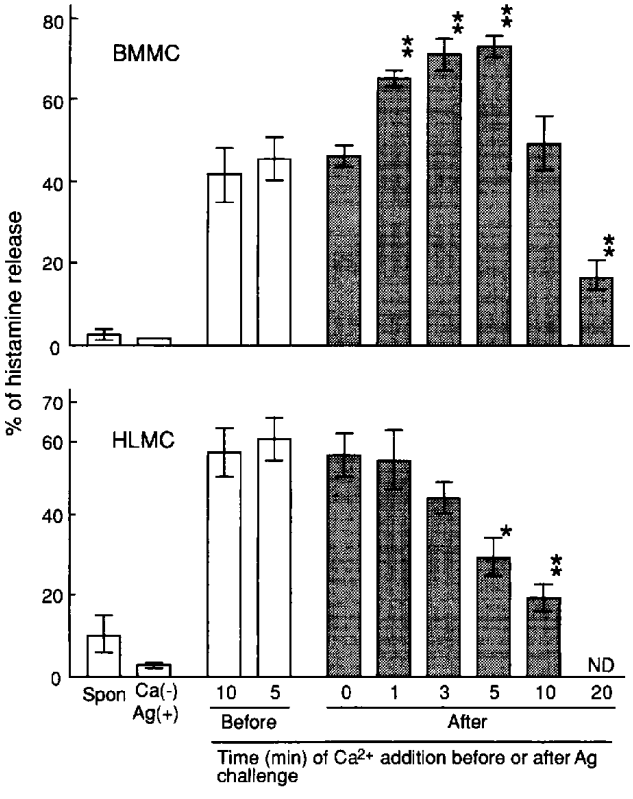


Fig. 5. Time courses of Ca^{2+} uptake into and the anaphylactic histamine release from mouse bone marrow-derived mast cells (BMMC) in the presence of 1.8 mM $CaCl_2$. BMMC (4×10^6 BMMC/ml) were challenged with antigen at 37℃ for the indicated time. Each point represents the mean ± S.E. of 3 experiments. Amounts of Ca^{2+} influxed were calculated from the value obtained by subtracting the spontaneous Ca^{2+} uptake from the antigen-induced one at each indicated time. The histamine content was 3,800 ± 100 ng/4×10^6 BMMC.

Ca^{2+}uptake stimulated with antigen in BMMC

The time course of Ca^{2+} uptake into BMMC was assessed together with that of histamine release from the cells when they were stimulated with antigen in the presence of 0.05 and 1.8 mM Ca^{2+}. Surprisingly, two phases of the uptake were observed with either 0.05 or 1.8 mM Ca^{2+}. The first influx occurred promptly and reached the maximum level at approximately 2 min after the stimulation, at which time the histamine release was almost completed for either 0.05 or 1.8 mM Ca^{2+}. The second increase was initiated 2.5 to 3 min after the stimulation, and lasted for 7 min and 2.5 min in 0.05 and 1.8 mM Ca^{2+}, respectively. Although, at 0.05 mM Ca^{2+}, the amount of Ca^{2+} uptake during the first phase was approximately 1/4 of that at 1.8 mM, the amounts during the second phase were comparable to each other (Figs. 4 and 5).



Effect of Ca^{2+} addition before and after antigen stimulation on the anaphylactic histamine release from BMMC and HLMC

Five- and ten-minute treatments with 1.8 mM Ca^{2+}

Fig. 6. Effect of Ca^{2+} addition before and after antigen stimulation on the anaphylactic histamine release from mouse bone marrow-derived mast cells (BMMC) and human lung mast cells (HLMC). BMMC (10^5 BMMC/ml) and HLMC (2×10^4 HLMC/ml) suspended in Ca^{2+}-free medium were treated with Ca^{2+} at 1.8 mM final concentration at the indicated time prior to or after antigen stimulation. Each point represents the mean ± S.E. of 3 experiments. The histamine contents of BMMC and HLMC were 52.6 ± 6.7 ng/10^5 BMMC and 51.9 ± 4.4 ng/2×10^4 HLMC, respectively. * and **: statistically significant difference from the group treated with Ca^{2+} for 10 min and then challenged with antigen, at $P<0.05$ and 0.01, respectively. Spon: spontaneous histamine release, Ca(−): Ca^{2+}-free, Ag(+): antigen-challenged, ND: not done.

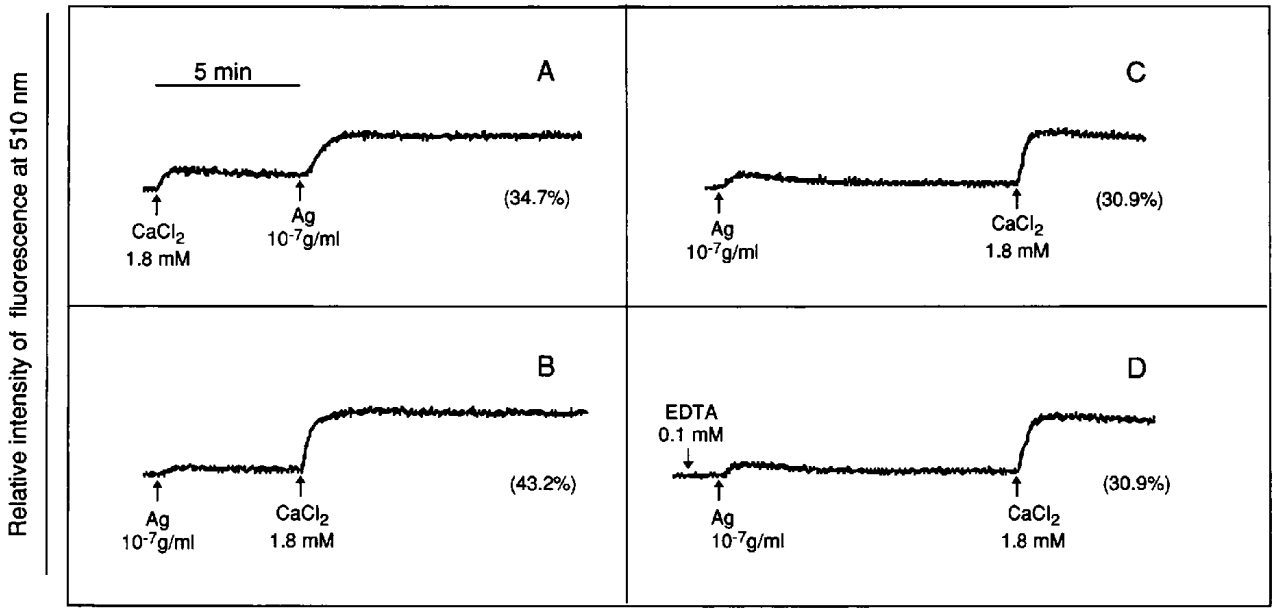


Fig. 7. Antigen-induced changes of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in mouse bone marrow-derived mast cells (BMMC). Fura-2-loaded BMMC (4×10^5 BMMC/ml) in Ca^{2+}-free Tyrode's solution were treated as follows: panel A, treated with Ca^{2+} for 5 min and then challenged with antigen (Ag); panel B, challenged with Ag for 5 min and then treated with Ca^{2+}; panel C, challenged with Ag for 10 min and then treated with Ca^{2+}; and panel D, treated with 0.1 mM ethylenediaminetetraacetic acid (EDTA) for 1 min, stimulated with Ag for 10 min and then treated with Ca^{2+}. Percentages of anaphylactic histamine release from the cells are shown in parentheses. The histamine content was 141.9 ng/4×10^5 BMMC. Two more separate experiments showed similar results.

prior to antigen stimulation induced anaphylactic histamine release similar degrees from either BMMC or HLMC. However, the histamine release was obviously different between both species of MC when Ca^{2+} was added after antigen stimulation: The histamine release from BMMC was time-dependently enhanced by the addition of Ca^{2+} at 1 to 5 min after the stimulation, although further prolongation of the addition decreased the release. On the other hand, the release from HLMC was decreased, without enhancement, and the decrease was dependent on the time between the antigen stimulation and the following Ca^{2+} addition (Fig. 6).

Changes of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by antigen in BMMC

Figure 7 shows the changes of $[Ca^{2+}]_i$ in BMMC when Fura-2-loaded cells in Ca^{2+}-free Tyrode's solution were treated with either antigen or Ca^{2+}. By the treatment with Ca^{2+}, a slight elevation of $[Ca^{2+}]_i$ was observed, which was not linked to histamine release. After 5 min, subsequent addition of antigen induced a rapid increase of $[Ca^{2+}]_i$, which was initiated within 10 sec and reached the maximum level within 2 min, but no further increase was seen, unlike the case in Ca^{2+} uptake into BMMC under similar conditions (Fig. 7A). When the antigen was first added, a slight elevation of $[Ca^{2+}]_i$ was consistently observed without an accompanying histamine release. Further addition of Ca^{2+} 5 and 10 min after antigen stimulation obviously increased $[Ca^{2+}]_i$, inducing anaphylactic histamine release. The degree of $[Ca^{2+}]_i$ elevation by antigen appeared to be lowered to almost the basal level (before antigen stimulation) by prolongation of the treatment time without Ca^{2+} addition (Fig. 7, B and C). When the BMMC pretreated with 0.1 mM EDTA in Ca^{2+}-free Tyrode's solution was stimulated with antigen, rapid $[Ca^{2+}]_i$ elevation, the degree and duration of which were similar to those seen in the BMMC treated without EDTA, was observed (Fig. 7D). Irrespective of whether Ca^{2+} was added before the antigen or vice versa, BMMC well responded to these treatments to release histamine anaphylactically, and the elevation of $[Ca^{2+}]_i$ was maintained for a considerably long time.

DISCUSSION

Anaphylactic histamine release from BMMC was almost but not completely prohibited by the treatment with EDTA for 20 sec prior to the antigen challenge, while that from HLMC was completely inhibited under the same treatment conditions. Compared with HLMC, BMMC required relatively low Ca^{2+} concentrations in the medium for anaphylactic histamine release. In addition to

this, when the cells treated with Ca^{2+}-free medium at least for 1 hr were stimulated with antigen, no histamine release was observed unless Ca^{2+} was subsequently added as shown in the present experiments. Thus, the still remaining portion of the anaphylactic release in the presence of EDTA in BMMC was considered to result from the utilization of a small amount of remaining Ca^{2+} that may be located on or in the cell membrane and can not be chelated with EDTA by such a brief treatment. Therefore, BMMC as well as HLMC can be regarded as cells solely dependent on extracellular Ca^{2+} for the induction of anaphylactic histamine release. In contrast, RPMC are able to release histamine without the requirement of extracellular Ca^{2+}, because these cells anaphylactically liberate a sufficient amount of histamine under the same treatment conditions with EDTA as shown in the present experiments and as reported by other researchers (15–17).

When BMMC were stimulated with antigen in the presence of 0.05 mM or 1.8 mM Ca^{2+}, two phases of Ca^{2+} influx into the cells were recognized in either case. In HLMC, it has been reported that a single phase Ca^{2+} influx, which is prerequisite for histamine release, occurs preceding the release following antigen stimulation (33). Such a time lag was not seen in BMMC. The time course of Ca^{2+} influx into the cells observed in the first phase appeared to be almost the same as that of anaphylactic histamine release, although the reason for the difference between HLMC and BMMC is not clear yet. Nevertheless a similar degree of anaphylactic histamine release was induced in the presence of either 0.05 or 1.8 mM Ca^{2+}; the amounts of Ca^{2+} influxed in 0.05 mM Ca^{2+} was approximately 1/4 of that in 1.8 mM Ca^{2+} in the first phase, strongly suggesting that a small amount of incorporated Ca^{2+} is enough for the histamine release. The influxed Ca^{2+} may be utilized for activation of Type II phospholipase A_2 or myosin light chain kinase, which has been observed to induce histamine release (37, 38). Interestingly enough, the second phase of Ca^{2+} influx, which has never been seen in any other species MC, occurred in BMMC. The duration of the second Ca^{2+} influx differed between low and high concentrations of Ca^{2+} in the medium, but the total amount of the influx was similar to each other. It should be stressed that the second influx did not result in the increase of $[Ca^{2+}]_i$, indicating that almost all of the intracellularly incorporated Ca^{2+} must be effectively utilized for some biochemical event(s), which has not been get identified. Four possibilities for this phenomenon can be proposed: Incorporated Ca^{2+} is used for 1) unknown biochemical event(s), which results in negative feedback regulation of the histamine release because the histamine release apparently ceases coincidently with the increased Ca^{2+} uptake, and the histamine release was enhanced when BMMC were stimulated by the antigen without Ca^{2+} and then followed by the addition of Ca^{2+} 1 to 5 min later as shown in the present experiments; 2) the activation of 5-lipoxygenase, which is an enzyme requiring Ca^{2+} for its activation (39) and is known to form arachidonate 5-lipoxygenase metabolites, e.g., leukotrienes (LTs) (40); 3) supplying a Ca^{2+} store site(s) that is putatively depleted at an early stage following the antigen-antibody reaction on the cell surface; and 4) an intracellular event(s) which has yet to be clarified.

Three kinds of Ca channels are known to be opened by inducers (41): the Ca^{2+}-release-activated channel (CRAC) that is opened when Ca^{2+} in the intracellular store sites is depleted; the Ca channel, that is linked to guanosine triphosphate binding (G) protein; and the Ca channel that is opened by second messengers such as IP_3, or inositol 1,3,4,5-tetrakisphosphate (IP_4) and Ca^{2+} itself. The first phase of Ca^{2+} influx in BMMC can be led through CRAC or the second messenger-opened Ca channel because increased IP_3 within 10 sec after antigen stimulation has been reported in BMMC (4). However, the G protein-linked Ca channel might be ruled out since anaphylactic histamine release may not be caused by the activation of the G protein (42). On the other hand, the second phase of Ca^{2+} influx may be brought about by the opening of the G protein-linked Ca channel, as the majority of biologically active substances, e.g., histamine, LTs and prostaglandin D_2, which may open the Ca channel (43), are supposed to be mostly released when the second Ca^{2+} influx increases. Irrespective of which Ca channel is opened for the first or second Ca^{2+} influx, the Ca channel inducing the second phase of Ca^{2+} influx must be different from that inducing the first phase.

It is generally thought that the absence of Ca^{2+} in the media causes HLMC (18) and MPMC (44, 45) to lose their abilities for anaphylactic histamine release at considerably rapid rates unless Ca^{2+} is added after antigen stimulation. However, as shown in the present experiments, different from HLMC and MPMC, BMMC were resistant to the desensitization for a while: The anaphylactic histamine release was rather enhanced when Ca^{2+} was added 1 to 10 min after the antigen stimulation to BMMC that had been kept in the absence of Ca^{2+}. From these results, it is strongly suggested that the Ca channel opened by antigen stimulation, which probably leads to the first phase of Ca^{2+} influx, is opened for a relatively long time after antigen stimulation in the absence of Ca^{2+}.

In the present experiments, we demonstrated that although extracellular Ca^{2+} is required for the anaphylactic histamine release from both BMMC and HLMC, BMMC show significant differences from HLMC with respect to the following aspects: 1) The pattern of Ca^{2+} incorporation into BMMC was biphasic as contrasted to the

monophasic pattern observed for HLMC; 2) The time courses for anaphylactic histamine release and change of $[Ca^{2+}]_i$ of the BMMC were different from those of HLMC; 3) BMMC were more resistant to densitization than HLMC. From these results, it is strongly suggested that the biochemical process, irrespective of whether it occurs before or after Ca^{2+} influx into the cells, leading to histamine release and/or an unidentified biological event(s) following immunological stimulation in BMMC may be different from that in HLMC.

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