

Short communication

Possible mechanisms of the concentration-dependent action of substance P to induce histamine release from rat peritoneal mast cells and the effect of extracellular calcium on mast-cell activation

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Rat peritoneal mast cells purified on a Percoll gradient were loaded with the fluorescent Ca^{2+} indicator fura-2 and were challenged with different concentrations of substance P (SP), and intracellular calcium concentrations ($[\text{Ca}^{2+}]_i$) were measured by a spectrofluorometric assay. SP at 5×10^{-6} mol/l and 10^{-5} mol/l caused a significant histamine release with a significant increase in $[\text{Ca}^{2+}]_i$ in a dose-dependent manner. However, SP at 10^{-8} – 10^{-6} mol/l did not induce either histamine release or increase in $[\text{Ca}^{2+}]_i$. Extracellular calcium at 0.9 mM inhibited the histamine release with a significant reduction of $[\text{Ca}^{2+}]_i$ compared with that of the cells in a nominally calcium-free condition. These results indicate that the action of SP on rat mast cells relies upon $[\text{Ca}^{2+}]_i$ to induce histamine release.

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Histamine release induced by substance P (SP) from isolated rat mast cells was first reported by Johnson & Erdös (1) and has been subsequently confirmed by others (2, 3). Structural requirements for histamine release from the cells by SP have been suggested (3, 4), and it is important to point out that the concentrations of SP required to activate histamine release from rat mast cells are about 1000-fold greater than those which stimulate the smooth muscle of the gastrointestinal tract or stimulate secretion from the parotid gland (5). It has been shown that the presence of calcium in the extracellular medium in concentrations greater than 0.1 mM inhibits the response to SP (3). However, the mechanisms of these effects are still uncertain. In the present study, we have identified some new aspects of the action of SP on rat peritoneal mast cells by the measurements of intracellular calcium concentrations ($[\text{Ca}^{2+}]_i$).

Material and methods

Drugs and suppliers

The drugs and their suppliers were as follows: bovine serum albumin (BSA), SP, toluidine blue, heparin, ethylene glycol-bis-(β -aminoethyl ether) N,N'-tetraacetic acid (EGTA) (Sigma Chemical Co., St Louis, MO, USA); Percoll (Pharmacia, Uppsala, Sweden); fura-2/AM (Dojindo Laboratories, Kumamoto, Japan); and others (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

Rat mast-cell preparations

Rat peritoneal mast cells were obtained by peritoneal lavage of male Sprague-Dawley rats (250–300 g, Japan SLC, Inc., Shizuoka, Japan) in amounts of about 1×10^6 mast cells/rat, as described previously (6, 7). The lavage medium (BSSA) was

a heparinized (10 U/ml) balanced salt solution (BSS; 4.0 mM Na_2HPO_4 , 2.7 mM KH_2PO_4 , 150 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl_2 , pH 7.2) containing 0.046% (w/v) BSA. Mast cells in this mixture were purified on a Percoll gradient (8) and were more than 93% pure by toluidine blue staining, and the viability of the mast cells was shown to be more than 98% in the trypan blue exclusion test.

Histamine release from mast cells

Unless otherwise indicated, purified mast cells were washed twice with BSSA and resuspended in BSSA. Mast cells, $0.6\text{--}0.8 \times 10^6$ in 0.9 ml of the medium, were incubated at 37°C for 1 min, and 0.1 ml of SP solution in the concentration required was added. The mixture was further incubated at 37°C for 5 min, a procedure which was shown to be optimal for histamine release based on preliminary studies under various conditions of time and temperature. The reaction was stopped by the addition of 1 ml cold BSSA and immediately cooling in ice for 10 min. The incubation mixture was centrifuged at 70 g for 10 min at 4°C , and the supernatant solutions were obtained for histamine assay.

In some experiments, purified mast cells were washed twice with BSSA prepared without calcium and resuspended in calcium-free BSSA solution containing 1 mM EGTA. Then, histamine release from mast cells was also investigated.

SP was dissolved in 0.1% (w/v) acetic acid to give concentrated stocks of 1 mmol/l. The stock was stored at -25°C in aliquots sufficient for one experiment. For each experiment, the aliquot was thawed, neutralized with NaOH, and diluted to the required concentrations in BSSA or BSSA prepared without calcium before the experiment. The small quantities of sodium acetate induced no histamine release from the mast cells.

The amount of histamine in the supernatant solution and residual histamine in the cell pellet were measured with a fluorometric assay (9). Perchloric acid was added to both the supernatant solution and the cell pellet at a final concentration of 1% (w/v), and the proteins were removed by centrifugation at 2000 g for 10 min at 4°C . Experiments were performed in duplicate, and data on the amount of histamine released in the supernatant solution were expressed as the percentage of histamine release, which was calculated by the amount of histamine in the cell supernatant divided by that both in the supernatant and in the cell pellet. Differences between duplicates were less than 8%.

Changes in $[\text{Ca}^{2+}]_i$

Mast cells were suspended in 3 μM fura-2/AM solution dissolved in BSSA and incubated at 37°C for 15 min. The mast cells were then washed twice with BSSA and resuspended in it. In some experiments, purified mast cells in BSSA prepared without calcium were suspended in 3 μM fura-2/AM solution dissolved in the salt solution and incubated as above. The cells were then washed twice with BSSA prepared without calcium and resuspended in calcium-free BSSA solution containing 1 mM EGTA.

Thereafter, mast cells, $1.0\text{--}1.5 \times 10^6$ in 0.6 ml of the medium were prewarmed in a cuvet for 10 min at 37°C with stirring, and then 0.066 ml of SP solution was added. The cells were maintained at 37°C by a circulating water bath and continually stirred by a small magnetic stirrer to ensure rapid and complete dispersal of the agonist. The fluorescence of fura-2 was measured in a Hitachi F-2000 spectrofluorometer, as described previously (10), with excitations at 340 nm and 380 nm and with emission at 510 nm, a Ca^{2+} -dye dissociation constant, K_D , of 224 nM being used. Calibrations of the fura-2 signals were calculated from the measurements of the ratio of fluorescence intensities, as described before (11), by individually calibrating each run. The ratios of maximum and minimum fluorescence were determined by the addition of 0.1% (v/v) Triton X-100 in the presence of 0.9 mM free Ca^{2+} and in the presence of 2 mM EGTA (pH 9.3), respectively. Cell autofluorescence, determined with cells not loaded with fura-2, was subtracted from the traces before $[\text{Ca}^{2+}]_i$ was calculated. The measurements were taken for 300 s.

Statistical analysis

Each point illustrated in each figure represents the mean and standard error of the mean of five experiments. Statistical analyses were performed with the analysis of variance (ANOVA) followed by Dunnett's multiple comparison test or Duncan's multiple range test. *P* values less than 0.05 were considered statistically significant.

Results

The amount of histamine release into the supernatant solutions from mast cells incubated with 10^{-5} mol/l SP was investigated serially. Histamine released from the cells at 30 s, and 1, 5 and 10 min after the stimulation were 34.1 ± 5.3 (mean $\pm$ SEM), 40.3 ± 5.8 , 42.0 ± 5.5 , and $41.9 \pm 6.3\%$, respectively. When mast cells were incubated with different concentrations of SP at $10^{-8}\text{--}10^{-5}$ mol/l for 5 min,

histamine was released into the supernatant solution by 5×10^{-6} mol/l and 10^{-5} mol/l SP that caused 23.3 ± 2.9 and $39.0 \pm 3.2\%$ histamine release, respectively, amounts which were significantly greater than those of spontaneous release as a control ($P < 0.01$). However, no significant histamine release by SP was observed at 10^{-8} – 10^{-6} mol/l (Fig. 1A). Lactic dehydrogenase release into the supernatant solutions from the mast cells incubated with 5×10^{-6} mol/l and 10^{-5} mol/l SP for 5 min was less than 3% above background, indicating that SP at these concentrations induced histamine release from the mast cells without cytotoxicity.

Next, the cells loaded with the fluorescent Ca^{2+} indicator fura-2 were exposed to different concen-

trations of SP at 10^{-8} – 10^{-5} mol/l, and the fluorescence of fura-2 was measured by a spectrofluorometer. Resting $[\text{Ca}^{2+}]_i$ of the mast cells was 26.8 ± 1.4 nM. When fura-2-loaded rat mast cells were exposed to 10^{-5} mol/l SP, the fluorescence intensity derived from the fura-2-Ca complex increased promptly, reaching the maximum at 7 s, and decreasing gradually to the basal level after 15 s (Fig. 2). The maximal increase in $[\text{Ca}^{2+}]_i$ from the resting level induced by 5×10^{-6} mol/l and 10^{-5} mol/l SP were 74.5 ± 5.0 and 143.4 ± 6.3 nM, respectively, an increase which were significantly greater than that produced with the medium as a control ($P < 0.01$) (Fig. 1B).

As the last part of the present study, $[\text{Ca}^{2+}]_i$ was investigated in rat mast cells stimulated by 10^{-5} mol/l SP in the presence of 0.9 mM calcium or in a nominally calcium-free medium containing 1 mM EGTA. The maximal increases in $[\text{Ca}^{2+}]_i$ from the resting level in the mast cells activated by 10^{-5} mol/l SP in the presence or absence of calcium were 150.7 ± 9.0 nM and 267.3 ± 40.3 nM, respectively, and they are significantly larger than that of the cells produced by the medium as a control (26.6 ± 1.7 nM) ($P < 0.01$). The maximal increase in $[\text{Ca}^{2+}]_i$ in mast cells stimulated by 10^{-5} mol/l SP in a nominally calcium-free medium containing 1 mM EGTA was significantly higher than that by SP in the presence of 0.9 mM calcium ($P < 0.01$) (Fig. 3).

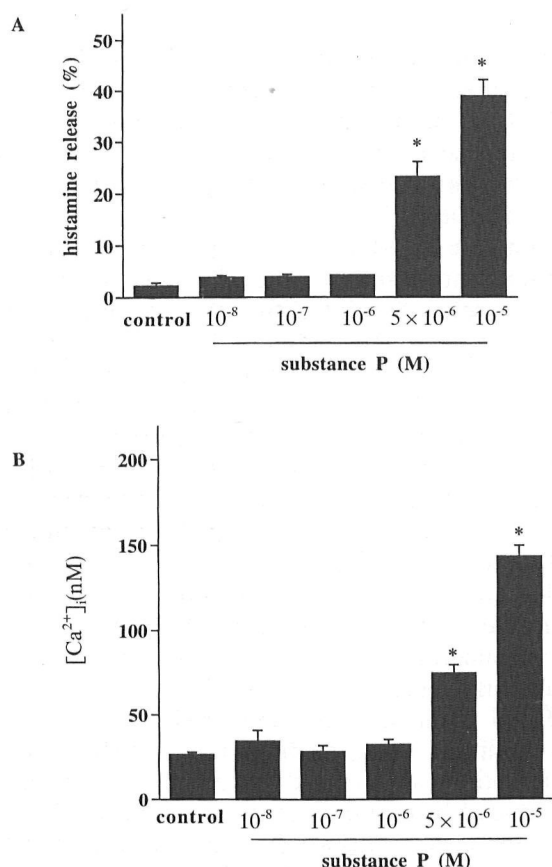


Fig. 1. Substance P (SP)-induced histamine release from rat peritoneal mast cells and maximal increase in intracellular calcium concentrations ($[\text{Ca}^{2+}]_i$) in SP-activated mast cells. A) Purified rat mast cells in 0.9 mM calcium-containing salt solution were incubated with different concentrations of SP for 5 min, and amount of histamine released in supernatant solution was measured, as described in Material and methods. Total histamine was $19.08 \pm 0.60 \times 10^{-7}$ g/ml. B) Rat mast cells in 0.9 mM calcium-containing salt solutions were loaded with fluorescent Ca^{2+} indicator fura-2 and exposed to different concentrations of SP, and then $[\text{Ca}^{2+}]_i$ was measured, as described in Material and methods. Each column represents mean $\pm$ SEM of five experiments. * $P < 0.01$, significantly different from control group (Dunnett's multiple comparison test).

Discussion

Several compounds have been shown to release histamine from rat mast cells even in a calcium-free medium, and SP is one of the typical agents causing histamine release in such a condition (3). Although it has been shown that the threshold concentration at which SP becomes active to release histamine from mast cells is considerably higher than the concentrations of SP required to induce smooth-muscle contraction (3), the mechanism of histamine release from rat mast cells caused by SP has not yet been fully elucidated. In the present study, 5×10^{-6} mol/l and 10^{-5} mol/l SP caused a significant histamine release from rat mast cells, supporting the data reported from other laboratories (3, 12). When rat mast cells loaded with the fluorescent Ca^{2+} indicator fura-2 were exposed to different concentrations of SP, a marked increase of fura-2 fluorescence was noticed with 5×10^{-6} mol/l and 10^{-5} mol/l SP in a dose-dependent manner. In fact, using another fluorescent Ca^{2+} indicator, quin-2, Tasaka et al. (13) showed that 2×10^{-6} mol/l SP induced calcium release from intracellular calcium stores in rat mast cells, and Mousli et al. (12) reported that incubation of rat mast cells with

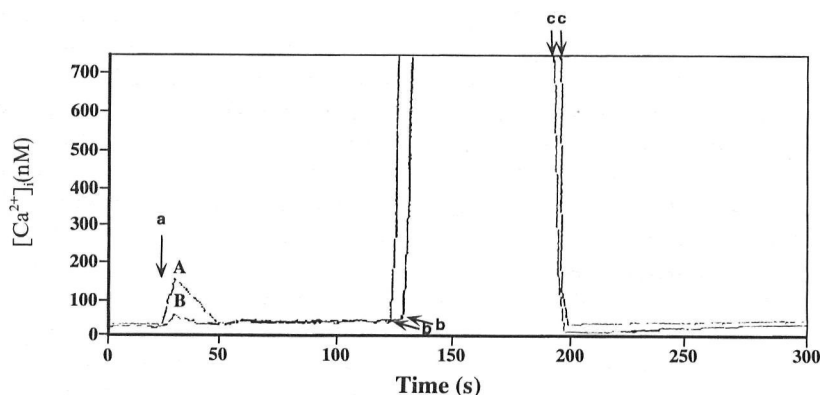


Fig. 2. Time courses of $[Ca^{2+}]_i$ in rat peritoneal mast cells. Mast cells were loaded with fluorescence Ca^{2+} indicator fura-2, and exposed to 10^{-5} mol/l SP (A) or medium (B) in which SP was dissolved as described in the text. Arrows a, b, and c indicate where SP or medium, Triton X-100, and EGTA were added, respectively, and fluorescence of fura-2 was measured serially in spectrofluorometer, as described in text.

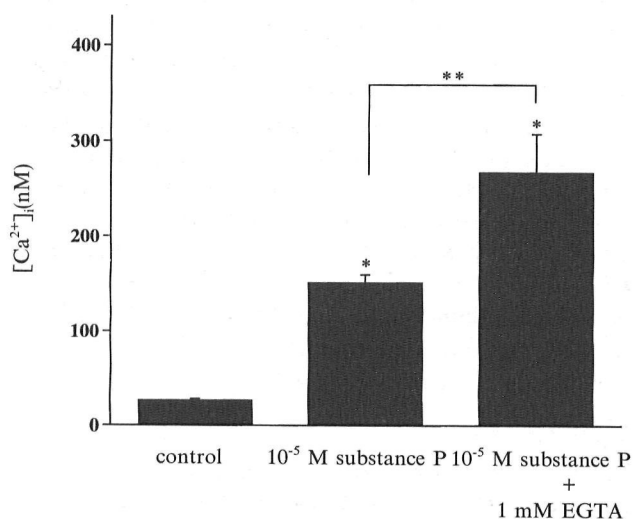


Fig. 3. Maximal increase in $[Ca^{2+}]_i$ in rat mast cells stimulated by 10^{-5} mol/l SP in presence or absence of calcium. Purified mast cells were suspended in 0.9 mM calcium-containing salt solution or in nominally calcium-free medium in presence of 1 mM EGTA, as described in Material and methods. Each column represents mean $\pm$ SEM of five experiments. Mean values of histamine released in supernatant solution from SP-activated mast cells in presence or absence of calcium were 40.5% and 47.2%, respectively. * $P < 0.01$, significantly different from control group; ** $P < 0.01$, significantly different between groups as indicated (Duncan's multiple range test).

10^{-5} mol/l SP resulted in a transient stimulation of phospholipid breakdown with an increase in inositol triphosphate, which may serve as a messenger of calcium mobilization from intracellular calcium stores. Since it is known that changes in the fluorescence intensity reflect $[Ca^{2+}]_i$, the present findings, taken together with the previous observation (12, 13), indicate that SP at concentrations of 5×10^{-6} and 10^{-5} mol/l is potent enough to increase $[Ca^{2+}]_i$ to induce histamine release from rat mast cells.

Although the presence of calcium in the extracellular medium in concentrations greater than 0.1 mM has been shown to inhibit SP-induced histamine release from mast cells (3), the mechanism still remains uncertain. In the present study, mast cells stimulated by 10^{-5} mol/l SP in a nominally calcium-free medium containing 1 mM EGTA released more histamine with the increase in $[Ca^{2+}]_i$ than the cells stimulated by SP in the presence of 0.9 mM calcium. We could not directly demonstrate the mechanisms of the action of extracellular calcium. However, one possible interpretation is that calcium ions in the mast-cell membrane may prevent SP binding to the cells and interfere with the effect of SP on the mobilization of intracellular calcium. Preincubation of the cells in calcium-free medium could be sufficient to remove calcium and to allow increased SP binding and hence increased histamine release. It is also clear that SP relies upon the mobilization of internal stores of calcium to induce histamine release, and these stores may be modified by incubation and washing in calcium-free medium. These findings indicate that further investigations are needed on the role of calcium in SP-induced histamine release from mast cells.

In conclusion, this study demonstrates that the action of SP relies upon $[Ca^{2+}]_i$ to induce histamine release from rat peritoneal mast cells.

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