An Initial Signal of Activation of Rat Peritoneal Mast Cells Stimulated by *Datura stramonium* Agglutinin: A Confocal Fluorescence Microscopic Analysis of Intracellular Calcium Ion and Cytoskeletal Assembly

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ABSTRACT A confocal fluorescence microscope using fluo-3 and 9-(dicyanovinyl)-julolidine (DCVJ) was used to study the mast cell activation by the N-acetyl glucosamine oligomer specific lectin *Datura stramonium* agglutinin (DSA) and inhibition by antagonist lectins having affinity to N-acetyl glucosamine (GlcNAc). DSA induced a transient increase in intracellular free calcium concentration ([Ca²⁺]) followed by cytoskeletal disassembly and reassembly in rat peritoneal mast cells. These changes induced by DSA resulted in histamine release. The time course of fluorescence intensity in mast cells loaded with fluo-3 or DCVJ and activated by DSA resembled those activated by the basic polymer compound 48/80. Inhibition of [Ca²⁺] rise by antagonist lectins was responsible for the inhibition of cytoskeletal assembly and the consequent histamine release induced by DSA. At the level of the individual cell, a mast cell stimulated by DSA responds in an all-or-none fashion. DSA possibly induced intracellular calcium mobilization and cytoskeletal change by recognizing the GlcNAc-oligomer residues of specific glycoproteins of mast cells.

Keywords: Mast cell, Intracellular calcium ion, Cytoskeletal assembly, Confocal fluorescence microscope, *Datura stramonium* agglutinin

GlcNAc oligomer specific lectin DSA is a useful tool for examining the pertussis toxin-sensitive mechanisms of histamine release from rat peritoneal mast cells, as previously described (1). The GlcNAc-specific lectins WGA and STA were antagonists of DSA and compound 48/80. They may occupy sugar residues of glycoproteins that serve as binding sites for DSA, which overlap one of the binding sites of compound 48/80, prevent access of DSA and compound 48/80 to the binding sites and inhibit the histamine release induced by DSA and compound 48/80 (1–3). Con A, however, did not interfere with the interactions and did not inhibit the histamine release, since it recognizes high-mannose sugar-residues but not GlcNAc oligomer (1, 3).

DSA releases histamine (1), but it is not clear whether the increase in [Ca²⁺], is one of the initial signals triggering histamine release or whether the histamine releasing mechanisms are coupled with changing cytoskeletal assembly. Confocal fluorescence microscopy, a new technology, revealed important information about cell activation. Real time changes in the fluorescence of indicator dyes and fluorescent molecular rotors in living cells can be measured. In this study, we used the calcium ion indicator fluo-3 and the fluorescent molecular rotor DCVJ. Fluo-3 is a useful dye, but intracellular calcium ion concentration can not be calculated from its fluorescence. The intensity of DCVJ fluorescence increased with the increasing cytoskeletal assembly of actin and tubulin molecules, and cytochalasin D inhibited the increase in the DCVJ fluorescence intensity in activated mast cells (4). We also examined the mechanisms of action of antagonist lectins (1) using real time measurement. To clarify the mechanisms of the DSA-induced histamine release, confocal fluorescence microscopic imaging using Con A and the antagonist lectins WGA and STA was carried out.

Abbreviations used are: GlcNAc, N-acetyl glucosamine; DSA, *Datura stramonium* agglutinin; WGA, wheat germ agglutinin; STA, *Solanum tuberosum* agglutinin; Con A, concanavalin A; [Ca²⁺], intracellular free calcium concentration; Fluo-3, 1-(2-amino-5-[(2,7-dichloro-6-hydroxy-3-oxo-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-N,N',N''-tetraacetic acid; DCVJ, 9-(dicyanovinyl)-julolidine.
MATERIALS AND METHODS

Chemicals

DCVJ was prepared from julolidine by the method of Kuder et al. (5). Fluo-3-AM, the pentaacetoxyethyl ester of fluo-3, was purchased from Dojindo Lab. (Kumamoto). Compound 48/80 was from Sigma Chemical Co. (St. Louis, MO, USA). DSA, WGA and Con A were purchased from the Honen Corp. (Tokyo). STA was from EY Laboratories (San Mateo, CA, USA). HEPES-buffered Tyrode solution had the following composition: 137 mM NaCl, 2.7 mM KCl, 12 mM HEPES, 1 mM MgCl₂, 0.3 mM CaCl₂, 5.6 mM dextrose and 0.03% BSA, pH 7.4.

Preparation of purified mast cells

Mast cells from the peritoneal cavity of male Sprague-Dawley rats weighing 350 g were purified with Percoll (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). Percoll (1.8 ml) was diluted with 10 x HEPES-buffered Tyrode solution without dextrose and BSA (0.2 ml), and the pH of the mixture was adjusted to 7.4. A suspension of mixed peritoneal cells (1 ml) was layered onto Percoll (2 ml) in a test tube and then centrifuged at 400 x g for 20 min at 4 °C. Mast cells precipitated at the bottom of the test tube after centrifugation. The purified mast cells were washed twice with HEPES-buffered Tyrode solution to remove the Percoll by centrifugation at 400 x g for 10 min at 4 °C. The purity of the mast cells in the final preparation was more than 95% as determined by toluidine blue-staining. The viability of the mast cells was more than 90% from trypan blue extrusion.

Confocal fluorescence microscope

Confocal fluorescence microscopic images of fluo-3-loaded mast cells and DCVJ-loaded mast cells (10⁵ cells in 0.4 ml of HEPES-buffered Tyrode solution/one observation chamber) were taken under a confocal fluorescence microscope system (MRC-600; Bio-Rad, Richmond, CA, USA) with an inverted epifluorescence microscope (TMD-EFO microscope; Nikon, Tokyo) every 10 sec. The purified mast cells were transferred to an observation chamber and then allowed to settle for more than 30 min in a CO₂ incubator. For calcium measurement, the cells after incubation were treated with 10 μM fluo-3-AM for 20 min and then with or without WGA, STA or Con A (200 μg/ml) for a further 10 min in the presence of fluo-3-AM at 37 °C. Before the measurement, the fluo-3-loaded mast cells were washed once with HEPES-buffered Tyrode solution to remove unreacted fluo-3-AM and resuspended in HEPES-buffered Tyrode solution with and without WGA, STA or Con A (200 μg/ml). To measure the cytoskeletal assembly, the cells were incubated in the presence and absence of WGA, STA or Con A (200 μg/ml) for 10 min at 37 °C and then treated with 2.5 μg/ml DCVJ for 1 min at 37 °C. DCVJ-loaded mast cells were not washed before measurement, because DCVJ in extracellular solution did not have fluorescence. DCVJ- or fluo-3-loaded mast cells were activated by DSA (20 or 100 μg/ml) or compound 48/80 (5 μg/ml). Fluo-3- or DCVJ-loaded mast cells were excited at 488 nm by an argon ion laser, and the fluorescence emission was observed above 515 nm. The temperature of the observation chamber was kept constant at 37 °C. We calculated the relative fluorescence intensity of all of fluo-3-loaded mast cells under a confocal fluorescence microscope and counted the number of activated mast cells. We repeated the same experiment at least three times using different samples of pooled cells.

Assay of histamine release from rat peritoneal mast cells

A 0.5-ml sample of cell suspension (2 x 10⁶ cells) in HEPES-buffered Tyrode solution was incubated with and without WGA or STA (200 μg/ml) for 10 min at 37 °C. The cells were then incubated with DSA (20 μg/ml or 100 μg/ml) or compound 48/80 (5 μg/ml) for 10 min at 37 °C. Ice-cold HEPES-buffered Tyrode solution (2.3 ml) was added at the indicated time to terminate the reaction, and the reaction mixture was centrifuged at 1,600 x g for 10 min at 4 °C. Histamine in the supernatant was determined using the fluorometric assay of Shore et al. (6). Histamine release was calculated as a percentage of the total cell content. Values for histamine release are given as the means ± S.E. for several replicate experiments on different samples of pooled cells. The spontaneous histamine release in the absence of DSA or compound 48/80 for 10 min at 37 °C was 3.4 ± 3.3% (n = 10). No appreciable release of histamine for 10 min at 37 °C was observed in the presence of WGA and STA under our conditions. Con A (200 mg/ml) was incubated with the mast cells in the absence of phosphatidylserine for 10 min at 37 °C. The mast cells were desensitized to Con A, although no appreciable histamine release was observed. After desensitization, DSA or compound 48/80 was added to the reaction mixture. The inhibitory effects of the lectins were calculated by the following equation:

\[
\text{% Inhibition} = 100 - \frac{\text{histamine release with WGA, STA or Con A - spontaneous release}}{\text{histamine release without WGA, STA or Con A - spontaneous release}} \times 100
\]

Statistical analysis

The statistical significance was evaluated by the unpaired Student’s t-test, with P < 0.05 being regarded as significant.
RESULTS

Histamine release and confocal fluorescence microscopic imaging of the mast cells

The fluorescence intensity in individual fluo-3- or DCVJ-loaded mast cells increased when the cells were activated by DSA (20 or 100 µg/ml) or compound 48/80 (5 µg/ml). We observed the activation of all of the cells after addition of DSA or compound 48/80 under a confocal fluorescence microscope. According to the fluorescence changes in fluo-3- and DCVJ-loaded mast cells in the presence of various concentrations of DSA, DSA (20 µg/ml) was sufficient to induce remarkable changes in the fluorescence increase. Figure 1 (A and B) shows the time-course of the fluorescence intensity in individual fluo-3- or DCVJ-loaded mast cells activated by DSA (20 µg/ml). When fluo-3-loaded mast cells were activated by DSA (20 µg/ml), fluo-3-intensity rapidly increased as net DCVJ fluorescence decreased or remained unchanged. By 50 sec, the fluorescence of fluo-3 decreased as that of DCVJ increased. The change in the fluo-3 fluorescence was transient and that of DCVJ was not. These changes induced by DSA (20 µg/ml) resembled those induced by DSA (100 µg/ml) (data not shown) and compound 48/80 (5 µg/ml).

Fig. 1. Typical time course of fluorescence-intensity in fluo-3- and DCVJ-loaded mast cells activated by DSA or compound 48/80 at 37°C. A, fluorescence-intensity changes in four fluo-3-loaded mast cells activated by DSA (20 µg/ml); B, fluorescence-intensity changes in four DCVJ-loaded mast cells activated by DSA (20 µg/ml); C, fluorescence-intensity changes in four fluo-3-loaded mast cells activated by compound 48/80 (5 µg/ml); D, fluorescence-intensity changes in four DCVJ-loaded mast cells activated by compound 48/80 (5 µg/ml).
Transient decrease of net DCVJ fluorescence induced by compound 48/80 (Fig. 1D) was remarkable.

Figure 2 shows typical confocal fluorescence microscopy images of DCVJ-loaded mast cells activated by DSA (100 μg/ml), which released 72.0±4.4% (n=6) of the total cell histamine in 10 min at 37°C. DCVJ fluorescence gradually increased from the cell surface to the center of the cell. Using DSA (20 μg/ml) or compound 48/80 (5 μg/ml), we observed similar changes in the confocal fluorescence microscopy images of DCVJ-loaded mast cells (data not shown).

Figure 3 shows the time-course of histamine release induced by DSA (20 μg/ml) and compound 48/80 (5 μg/ml). DSA and compound 48/80 released histamine as rapidly as [Ca^{2+}] increased.

Effects of WGA, STA and Con A on the histamine release and confocal fluorescence microscopic imaging of the mast cells stimulated by DSA

WGA, STA and Con A (200 μg/ml) did not induce any appreciable histamine release from mast cells (1), or any fluorescence changes in fluo-3- or DCVJ-loaded mast cells. Table 1 shows the effects of a 10-min-incubation with WGA, STA and Con A (200 μg/ml) on the histamine release induced by DSA (20 μg/ml). In the absence of WGA, STA or Con A, DSA (20 μg/ml) released

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Fig. 2. Typical confocal fluorescence microscopy images of DCVJ-loaded mast cells after the addition of DSA (100 μg/ml). Sequential fluorescence images of the mast cells stimulated by DSA are shown from the top row to bottom row, left to right. Fluorescence images were collected every 10 sec at 37°C, except for the last one, which corresponded to 3 min after stimulation.

Fig. 3. Time course of histamine release from mast cells after the addition of DSA and compound 48/80. A 0.5-ml sample of cell suspension (2×10⁶ cells) in HEPES-buffered Tyrode solution was incubated with DSA (20 μg/ml) at 37°C. Ice-cold HEPES-buffered Tyrode solution (2.3 ml) was added at the indicated time to terminate the reaction, and the reaction mixture was centrifuged at 1,600 x g for 10 min at 4°C. Histamine release was calculated as a percentage of the total cell content. Values for histamine release are given as the means±S.E. for 6 experiments on different samples of pooled cells. Circles, histamine release % induced by DSA (20 μg/ml); squares, histamine release % induced by compound 48/80 (5 μg/ml); triangles, spontaneous histamine release % in the absence of DSA and compound 48/80.
Table 1. Effects of WGA, STA and Con A on the histamine release induced by DSA

| Lectins (200 µg/ml) | Inhibitory effects on the histamine release induced by DSA (20 µg/ml) | % Inhibition |
|--------------------|-------------------------------------------------------------|-------------|
| WGA                | 83.8±3.1*** (n=4)                                           |             |
| STA                | 38.0±9.9** (n=5)                                             |             |
| Con A              | 9.5±2.8 (n=5)                                                |             |

A 0.5-ml sample of cell suspension (2×10^6 cells) in HEPES-buffered Tyrode solution was incubated with or without WGA, STA or Con A (200 µg/ml) for 10 min at 37°C. The cells were then incubated with DSA (20 µg/ml) for a further 10 min at 37°C. The control value of the histamine release percentages induced by DSA (20 µg/ml) in the absence of WGA, STA or Con A was 49.7±4.6% (n=6). The spontaneous histamine release was 2.9±0.4% (n=6). Values for inhibition of the histamine release are given as the means ± S.E. for several replicate experiments on different samples of pooled cells. ***P<0.001, **P<0.01.

Figure 4 shows the average time-course of fluorescence-intensity changes in four fluo-3-loaded mast cells activated by DSA (20 µg/ml) in the presence of WGA, STA and Con A (200 µg/ml). WGA (200 µg/ml) inhibited the increase in the intensity of fluo-3 induced by DSA (20 µg/ml) (Fig. 4C). In the presence of WGA (200 µg/ml), the average increase in fluo-3 fluorescence intensity induced by DSA (20 µg/ml) was less than 40 (relative fluorescence intensity), indicating that an increase in intensity about 40 (relative fluorescence intensity) was not enough to induce cell activation. The inhibition of the [Ca^{2+}] increase was responsible for the inhibitory effect of WGA on cytoskeletal reassembly, and the histamine 49.7±4.6% (n=6) of the total histamine content from mast cells within 10 min at 37°C. WGA (200 µg/ml) proved to potently inhibit DSA-induced histamine release, the percentage of inhibition being 83.8±3.1% (n=4) (Table 1). STA (200 µg/ml) significantly inhibited the histamine release, but Con A (200 µg/ml) did not.

Fig. 4. Effects of WGA, STA and Con A on the fluorescence-intensity changes in fluo-3-loaded mast cells after the addition of DSA. The purified mast cells were incubated with fluo-3-AM for 20 min and then with and without WGA, STA or Con A (200 µg/ml) for a further 10 min in the presence of fluo-3-AM at 37°C in a CO₂ incubator. Before the measurement, the reaction solution was replaced with fresh HEPES-buffered Tyrode solution with or without WGA, STA or Con A (200 µg/ml). Confocal fluorescence microscopy images in fluo-3-loaded mast cells were taken every 10 sec after addition of DSA (20 µg/ml) at 37°C. The curves represent the average changes of fluo-3 fluorescence intensities in four cells. A, Control; B, Con A (200 µg/ml); C, WGA (200 µg/ml); D, STA (200 µg/ml).
release induced by DSA. DSA (20 µg/ml) increased the average fluo-3 intensities in the presence of STA (200 µg/ml) (Fig. 4D). Therefore, we calculated the relative fluorescence intensity of all of the fluo-3-loaded mast cells under a confocal fluorescence microscope and counted the number of activated mast cells. Fluo-3-loaded mast cells in which the difference in the fluorescence intensity between before and after DSA addition was more than 40 (relative fluorescence intensity) was regarded as activated.

The numbers of mast cells activated by DSA (20 µg/ml) in the presence of STA (200 µg/ml) were significantly reduced to 86.5±3.0% (26.3±3.9 cells × 3 experiments, P <0.02) of the control value in the absence of STA. If the threshold was defined at 50 (relative fluorescence intensity), the number of mast cells activated by DSA in the presence of STA was 77.0±6.2% (26.3±3.9 cells × 3 experiments, P <0.05) which correlated well with the STA inhibition of DSA-induced histamine release. These results indicated that STA (200 µg/ml) decreased the number of activated cells and subsequently inhibited the histamine release. On the other hand, Con A (200 µg/ml) did not inhibit the histamine release induced by DSA (20 µg/ml) and did not reduce the intensities in fluo-3-loaded mast cells activated by DSA (20 µg/ml) (Table 1 and Fig. 4B).

DISCUSSION

We observed real time changes in [Ca²⁺]i and cytoskeletal assembly using fluo-3 and DCVJ, respectively, by means of confocal fluorescence microscopy in living individual mast cells. The rapid increase in fluo-3 fluorescence intensity was an initial signal induced by DSA followed by a decrease and then an increase in net DCVJ fluorescence intensity, suggesting that the rapid increase in [Ca²⁺], was followed by cytoskeletal disassembly and consequent reassembly (Fig. 1, A and B). Because of the former, DCVJ fluorescence increased with a short lag time. It is noteworthy that compound 48/80 (5 µg/ml) and DSA (20 µg/ml) induced similar changes in the confocal fluorescence microscope images of fluo-3- and DCVJ-loaded mast cells (Fig. 1). The time-course profiles in Fig. 1 (C and D) were consistent with previous results in which Furuno et al. (4) used 12.5 µg/ml compound 48/80 in the presence of 1 mM CaCl₂. In chromaffin cells, disassembly of the cytoskeleton seemed to be needed for the secretory granules to move to the plasma membrane. Gelsolin or scinderin, which are calcium-dependent actin filament severing proteins, may disrupt actin networks (7, 8). Unlike cytoskeletal assembly, there was no lag time in histamine release induced by DSA (20 µg/ml) and compound 48/80 (5 µg/ml) in Figs. 1 and 3. This discrepancy arose from the difference between the assay systems; confocal measurement is a real-time observation, but the histamine releasing assay (Fig. 3) is not. It is probable that in the 50 sec after stimulation, we observed net disassembly of the cytoskeleton because of weak assembly and potent disassembly of the cytoskeleton. The weak assembly accompanied with potent disassembly may be enough to induce histamine release. Net assembly of cytoskeleton was observed after 50 sec, since assembly increased concomitantly as disassembly decreased. Tasaka et al. (9) reported that cytoskeletal assembly certainly resulted in the histamine release, but the histamine release did not result in cytoskeletal assembly, because an inhibitor of cytoskeletal assembly, cytochalasin D, inhibited the histamine release.

It is noteworthy that the distribution of the fluorescence increase in a fluo-3-loaded mast cell after addition of DSA differed from that in a DCVJ-loaded mast cell. Fluo-3 fluorescence first increased at the center of the cell, suggesting an increase in [Ca²⁺], at the nucleus. Katagiri et al. (10) also reported an increase in [Ca²⁺], at the nucleus of the mast cells activated by compound 48/80 (10 µg/ml). On the other hand, DCVJ fluorescence gradually increased from the cell surface to the center of the cell in the presence of DSA (Fig. 2). The time-course of activation in mast cells induced by DSA differed from that in RBL-2H3 cells induced by antigens, as reported previously by Nakato et al. (11); DSA (20 µg/ml) induced a rapid increase in [Ca²⁺], and rapid histamine release in a mast cell (Figs. 1A, 1B and 3), whereas antigens induced slow increase in [Ca²⁺], and slow histamine release in a RBL-2H3 cell. The initial signal of mast cell activation induced by DSA was similar to that by compound 48/80.

WGA and STA (200 µg/ml) inhibited the histamine release induced by DSA (20 µg/ml) (Table 1). The profiles of the fluorescence emitted from a fluo-3-loaded mast cell in the presence of both DSA (20 µg/ml) and WGA (200 µg/ml) differed from each other. WGA (200 µg/ml) inhibited the increase in [Ca²⁺], induced by DSA (20 µg/ml), indicating that this resulted in the inhibition of cytoskeletal assembly and consequent histamine release. Finally WGA decreased the number of cells activated by DSA. Con A (200 µg/ml) did not inhibit the increase in [Ca²⁺], induced by DSA (20 µg/ml) and did not decrease the number of activated cells. STA (200 µg/ml) decreased the number of the cells activated by DSA (20 µg/ml). When the threshold was defined at 50 (relative fluorescence intensity), the number of the cells activated by DSA and STA was correlated with the histamine release induced by DSA in the presence of STA. The decrease in the number of activated cells resulted in STA inhibition of the histamine release induced by DSA. Inhibition by WGA and STA (200 µg/ml) suggested that the amount of released histamine seemed to depend on the
number of activated cells. Activation of a mast cell by DSA may occur individually and may be an all-or-none response of each cell with a threshold for intracellular calcium sensitivity, like the activation of RBL-2H3 by calcium ionophore described by Hide et al. (12).

The inhibitory mechanisms of WGA and STA on DSA-induced histamine release were quite different from those by cytchalasin D, which inhibited the increase in DCVJ fluorescence intensity, but not that in fluo-3 fluorescence in mast cells activated by compound 48/80 (12.5 μg/ml) (4). WGA and STA (200 μg/ml) by themselves did not increase the intensity of the fluorescence of DCVJ-loaded mast cells. At present, no conclusion can be made about whether cross-linkage of the membrane glycoproteins by WGA and STA were responsible for inhibition of cytoskeletal assembly. Con A cross-linked FcERI and released histamine from rat mast cells in the presence of phosphatidylinerine and 1 mM calcium (13). WGA cross-linked FcERI and inhibited mast cell activation induced by antigens (14). After the desensitization induced by Con A, intracellular calcium mobilization induced by DSA was active enough to induce the histamine release.

In the presence of extracellular Ca$^{2+}$, the increase in [Ca$^{2+}$], induced by compound 48/80 depended on both Ca$^{2+}$ released from the intracellular Ca$^{2+}$ store (10) and influx of extracellular Ca$^{2+}$ (15). As we observed the Ca$^{2+}$ mobilization under confocal fluorescence microscopy in the presence of 0.3 mM extracellular Ca$^{2+}$, the source for the [Ca$^{2+}$] rise may be the extracellular as well as the stored Ca$^{2+}$.

In this study, we confirmed that WGA and STA competed with DSA on rat peritoneal mast cells by means of a confocal fluorescence microscopic study and found that DSA stimulated a mast cell in an all-or-none manner.

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