LOCALIZED MAST CELL DEGRANULATION INDUCED BY CONCANAVALIN A-SEPHAROSE BEADS

Implications for the Ca\textsuperscript{2+} Hypothesis of Stimulus-Secretion Coupling

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ABSTRACT

Concanavalin A (Con A) covalently linked to Sepharose 4B beads induced localized degranulation of sensitized rat peritoneal mast cells in regions of contact between beads and cells. This degranulation was Ca\textsuperscript{2+} dependent and was not seen when sensitized mast cells bound to beads conjugated with a nonstimulating lectin, wheat germ agglutinin, or when unsensitized mast cells bound to Con A-Sepharose. The finding that sensitized mast cells which had adhered to Con A-Sepharose beads degranulated in regions of the cell away from the area of bead contact if exposed to soluble Con A excluded the possibility that the localized release was due to a redistribution of the IgE receptors or putative Ca\textsuperscript{2+} channels to the region of bead contact. The results suggest that, if an influx of Ca\textsuperscript{2+} is the mechanism for initiating mast cell degranulation, then the opening of Ca\textsuperscript{2+} channels in the plasma membrane of activated mast cells is a localized event and that Ca\textsuperscript{2+} acts locally within the cell to initiate exocytosis.

KEY WORDS mast cell · concanavalin A · Ca\textsuperscript{2+} · localized degranulations

A general property of many secretory cells is that they require extracellular calcium in addition to the relevant stimulus for the release of the specific materials stored in the secretory granules or vesicles of the cell (7). Secretion occurs by exocytosis and is thought to be initiated by an influx of Ca\textsuperscript{2+} into the cell (12) which is mediated by a transient increase in the Ca\textsuperscript{2+} permeability of the plasma membrane; this allows Ca\textsuperscript{2+} to enter the cell down the steep concentration gradient that exists between the extracellular fluid and the cytosol in all cells (7, 13). The molecular events involved in opening and closing such putative Ca\textsuperscript{2+} channels are largely unknown; nor is it known how Ca\textsuperscript{2+} acts inside the cell to initiate exocytosis.

The mast cell provides a relatively simple and accessible system for studying these early events in triggered secretion. These cells have high-affinity surface receptors for the Fc region of immunoglobulin E (IgE)—so called Fc receptors (2), which enable mast cells to bind IgE from the serum and tissue fluids (3). Thus, peritoneal mast cells obtained from appropriately immunized rats have cytophilic IgE antibodies on their surfaces which serve as receptors for the specific antigen. Such sensitized cells can be stimulated to secrete histamine and 5-hydroxytryptamine (which are...
stored in secretory granules) by exposure to specific antigen (3), anti-Ig antibody (15), or the lectin concanavalin A (Con A) (20). These ligands bind to, and cross-link, the cell surface IgE (16, 20, 22), and degranulation, which begins within seconds, is over within a minute (4, 17, 18). The process is energy dependent (27), requires extracellular \( \text{Ca}^{2+} \) (11), and does not kill the cell (17).

There is increasing evidence that an influx of \( \text{Ca}^{2+} \) initiates exocytosis in mast cells. Extracellular \( \text{Ca}^{2+} \) is a required (11) and sufficient ion for \( \text{Ca}^{2+} \) initiates exocytosis in mast cells. Extracellular \( \text{Ca}^{2+} \) acts locally within the cell to initiate exocytosis.

**MATERIALS AND METHODS**

**Preparation of Mast Cells**

Male Wistar rats were immunized with ovalbumin in pertussis vaccine; 15-30 days later, a cell suspension containing 2-5% mast cells was obtained by peritoneal lavage, and the mast cells were purified to better than 90% by centrifugation through a discontinuous density gradient of human serum albumin (Kabi, A.B. Sweden), as previously described (22). The cells were washed once in 40 ml of \( \text{Ca}^{2+} \)-free Tyrode’s solution (137 mM NaCl; 2.7 mM KCl; 0.4 mM NaH₂PO₄; 1.0 mM MgCl₂; 5.6 mM glucose; 20 mM Hepes adjusted to pH 7.4 with NaOH) and resuspended at 1.5-2 \( \times 10^6 \) cells/ml in \( \text{Ca}^{2+} \)-free Tyrode’s solution.

**Mast Cell Stimulation Using Concanavalin A-Sepharose 4B Beads**

Con A-Sepharose 4B beads (Pharmacia Fine Chemicals, Piscataway, N. J.) were adjusted to a concentration of \( \sim 2 \times 10^4 \) beads/ml, washed five times in 15 ml of \( \text{Ca}^{2+} \)-free Tyrode’s solution and used at a final concentration of \( \sim 10^4 \) beads/ml. Mast cells (\( \sim 10^6 \)ml) and beads were suspended in the presence or absence of 1.8 mM Ca²⁺, centrifuged at 180 g for 5 min at room temperature (or 4°C) to maximize the number of mast cells adhering to beads, and then incubated at 37°C for 2-1/2-10 min. Since phosphatidyl serine (PS) has been shown to enhance the degranulation of stimulated rat mast cells, probably by potentiating the increased membrane permeability to \( \text{Ca}^{2+} \) (9), in some experiments cells were centrifuged with Con A-Sepharose beads in the presence of PS (Sigma Chemical Co., St. Louis, Mo.; 10 \( \mu \)g/ml and EDTA (0.2 mM) and then \( \text{Ca}^{2+} \) was added; in some of these experiments, soluble Con A (Miles, Yeda, 10 \( \mu \)g/ml) or A23187 (Eli Lilly and Co., Indianapolis, Ind., \( 5 \times 10^{-6} \)M) was added after the cells had been at 37°C for 2-1/2 min and the incubation was continued for another 2-1/2 min. In other experiments, wheat germ agglutinin (WGA)-Sepharose 6MB (Pharmacia Fine Chemicals) beads were used as described above at a concentration of 10⁴ beads/ml.

**Electron Microscopy**

After 15 min of incubation at 37°C, suspensions of mast cells and beads were fixed by the addition of 9 ml of cold 3% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.3) for 15 min at 0°C, followed by 60 min at room temperature. After washing, they were postfixed in 0.1 M Na cacodylate-buffered 1% OsO₄ for 1 h at 0°C, washed and resuspended in 0.5% aqueous uranyl acetate for 30 min at 0°C. After a final wash, they were resuspended in 1 drop of 2% agar, centrifuged in the warm, and then cooled. The resultant pellet was cut into small (1-2 mm³) pieces, dehydrated in ethanol followed by propylene oxide, and embedded in Epon 812. Thin sections were cut from blocks previously examined by light microscopy, stained with uranyl acetate followed by lead citrate (unless otherwise stated), and examined in an AE1 6EMB electron microscope at 60 kV.

**Light Microscopy**

Thick (1 \( \mu \)m) sections were cut from Epon 812-embedded blocks and stained with 0.1% Toluidine Blue in 2.5% sodium carbonate. It was found that, once the sections had been flooded with Toluidine Blue, gentle heating over a Bunsen flame greatly increased granule staining. The preparations were examined in a Wild microscope with a \( \times 100 \) bright field objective lens. Cells were scored as locally released when altered granules were only found adjacent to the bead, or within cavities next to the bead. Light micrographs were taken on a Zeiss Ultraphot II with a \( \times 63 \) objective lens.

**RESULTS**

When sensitized mast cells were centrifuged and incubated for 2-1/2-10 min at 37°C with Con A-
Sepharose beads in the presence of 1.8 mM Ca\(^{2+}\), 50-70% of the cells adhering to beads showed degranulation and in 75-100% of these the degranulation was localized to the region of contact between the beads and the cells (Table I, Figs. 2 and 5). Similar results were obtained when cells and beads were centrifuged in PS and EDTA (at room temperature or 4°C) and then incubated in Ca\(^{2+}\) and PS for 2½-5 min at 37°C (Table II). When Ca\(^{2+}\) was omitted during the incubation step, the majority of cells did not degranulate (Table I, Figs. 1 and 4); in those that did, >90% of the degranulation was generalized and not localized to the region of bead-cell contact (Table I). When cells were centrifuged with Con A-Sepharose beads in PS and EDTA and then incubated in the presence of Ca\(^{2+}\) at 37°C for 2½-5 min and then fixed and examined, >75% of the degranulated cells showed localized degranulation. However, when soluble Con A was added after 2½ min of incubation and the cells were fixed after a further 2½ min at 37°C, 70-80% of the bead-adherent cells degranulated and, in this case, >75% of the degranulation was generalized (Table I). When soluble Con A was added after 2½ min of incubation and the cells were fixed after a further 2½ min at 37°C, 70-80% of the bead-adherent cells degranulated and, in this case, >75% of the degranulation was generalized (Table I). The addition of the divalent cation ionophore A23187 in place of the soluble Con A stimulated 70-80% of the cells, which were adherent to the beads, to degranulate in a non-localized manner (data not shown).

Several experiments were done to assess the specificity of the localized degranulation. Soluble WGA did not induce significant histamine secretion in sensitized mast cells, even though large amounts of WGA coupled to fluorescein or ferritin bound to both sensitized and unsensitized mast cells (D. Lawson, C. Fewtrell, and M. C. Raff. Unpublished Observations). When sensitized cells were centrifuged and incubated with WGA-Sepharose 6B beads in the presence of 1.8 mM Ca\(^{2+}\), many cells adhered to the beads but virtually no localized degranulation was seen (Table I); for reasons that are unclear, in several experiments, ~30% of the adherent cells showed some generalized degranulation in the presence or absence of Ca\(^{2+}\) (Table I). Unsensitized mast cells are generally not activated by soluble Con A (19). When such cells were incubated and centrifuged with Con A-Sepharose beads, the proportion of cells adhering to the beads was not appreciably different from that seen with sensitized cells, but <5% of the adherent cells showed localized degranulation (Table I); here again, most of the cells showed generalized degranulation which was Ca\(^{2+}\) dependent (Table I).

Table I

<table>
<thead>
<tr>
<th>Treatment of cells*</th>
<th>Total cells degranulated (mean ± SE)</th>
<th>Distribution of degranulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>Local %</td>
</tr>
<tr>
<td>Con A-Sepharose + Ca(^{2+})</td>
<td>58 ± 9</td>
<td>89 ± 11</td>
</tr>
<tr>
<td>Con A-Sepharose without Ca(^{2+})</td>
<td>13 ± 5</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>WGA-Sepharose + Ca(^{2+})</td>
<td>32 ± 5</td>
<td>2 ± 0.4</td>
</tr>
<tr>
<td>Con A-Sepharose + Ca(^{2+}) (unsensitized cells)</td>
<td>24 ± 1</td>
<td>4 ± 0.6</td>
</tr>
</tbody>
</table>

* Ovalbumin-sensitized cells used except where indicated.
‡ Mean of five experiments ± SE. 50-80 bead-adherent cells assessed in each experiment.

Table II

<table>
<thead>
<tr>
<th>Treatment of cells</th>
<th>Total cells degranulated</th>
<th>Distribution of degranulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>Local %</td>
</tr>
<tr>
<td>Con A-Sepharose + Ca(^{2+}) for 2½ min</td>
<td>44</td>
<td>76</td>
</tr>
<tr>
<td>Con A-Sepharose + Ca(^{2+}) for 5 min</td>
<td>69</td>
<td>80</td>
</tr>
<tr>
<td>Con A-Sepharose + Ca(^{2+}) for 5 min + soluble Con A for final 2½ min</td>
<td>79</td>
<td>21</td>
</tr>
</tbody>
</table>

Cells stimulated in presence of phosphatidyl serine.
FIGURE 1 Mast cells adhering to a Con A-Sepharose bead in the absence of Ca$^{2+}$. No apparent degranulation has occurred. $\times$ 1,200.

FIGURE 2 Mast cells adhering to a Con A-Sepharose bead in the presence of 1.8 mM Ca$^{2+}$ for 10 min at 37°C. Degranulation has occurred and is confined to the region of contact with the bead. $\times$ 1,500.

FIGURE 3 Mast cells adhering to a Con A-Sepharose bead as in Fig. 2 but with the addition of 10 µg/ml of free Con A. Generalized degranulation has occurred in most cells. $\times$ 1,000.

FIGURE 4 Mast cell treated as in Fig. 1. No degranulation has taken place. $\times$ 11,500.
FIGURE 5  Mast cell treated as in Fig. 2, showing localized degranulation in the region of contact with the Con A-Sepharose bead. × 14,250.

FIGURE 6  Mast cell treated as in Fig. 3 showing generalized degranulation. × 11,000.
were similar to those seen when mast cells are activated by soluble ligands (21, 23). The only appreciable difference was that the mast cells were flattened in the region of contact with the beads (Figs. 1–6). When mast cells were centrifuged and incubated with Con A-Sepharose beads in the presence of Ca$^{2+}$, then labeled at 4°C with sheep anti-rat IgG conjugated to ferritin and fixed with glutaraldehyde, the ferritin label was diffusely distributed on the surface of bead-adherent mast cells which had degranulated locally, indicating that the IgE molecules had not all migrated to the area of bead contact.

DISCUSSION

Our results demonstrate that Con A covalently coupled to Sepharose 4B beads induces Ca$^{2+}$-dependent, localized degranulation of sensitized rat peritoneal mast cells in the region of contact between the beads and cells. Two types of experiments indicated that the localized degranulation was due to the binding of mast cell IgE to the Con A-Sepharose: (a) Soluble WGA bound in large amounts to both sensitized and unsensitized mast cells but did not induce histamine secretion, even at concentrations as high as 100 µg/ml. (b) Whereas unsensitized mast cells adhered to Con A-Sepharose beads, they did not show localized degranulation. It has been demonstrated that, while soluble Con A stimulates histamine secretion in sensitized mast cells, it does not do so in unsensitized cells, suggesting that its stimulating activity is due to its binding to surface IgE molecules (20). Consistent with this view is the observation that the binding of monovalent Fab fragments of anti-IgE antibodies to sensitized mast cells inhibits the stimulatory activity of Con A (24).

It is clear that the localized distribution of degranulation was not related to the inability of a mast cell adhering to a bead to degranulate in the non-adherent regions of the cell: when bead-adherent mast cells were incubated under conditions which induced localized degranulation and then exposed for 2'/2-5 min to A23187 or soluble Con A, >75% of the cells showed generalized degranulation. The latter experiment also suggested that all of the IgE on the mast cell surface did not redistribute to the area of contact with the Con A-Sepharose. This point was confirmed by labeling Con A-Sepharose-bound cells with anti-rat IgG-ferritin and finding a generalized distribution of the ferritin molecules on the surface of mast cells showing localized degranulation.

Our findings confirm and extend the light microscopic observations of others demonstrating that the localized application of a variety of mast cell stimulants with a micropipette induces localized degranulation (5, 6, 28, 29). The Ca$^{2+}$-dependence of these reactions was not reported. These previous studies were primarily performed to exclude the possibility that the stimulating ligands had to enter the cell to stimulate secretion; intracellular microinjection of the ligands failed to induce degranulation. Such studies do not formally exclude the possibility that ligand-receptor complexes must be internalized to initiate secretion, although the rapid time course of induction (usually seconds) makes this highly unlikely. Moreover, studies using ferritin coupled Con A and anti-Ig antibodies have shown that internalization of ligand is not required for mast cell stimulation (22). Recently, polymyxin B (26) and 48/80 (14) coupled to Sepharose 4B beads have been shown to stimulate secretion in rat mast cells, but the Ca$^{2+}$-dependence and distribution of the degranulation were not studied. In view of the possibility of ligand leakage from Sepharose beads, in small but effective amounts, one must be cautious in concluding from such studies that ligand or receptor does not have to enter the cell to stimulate degranulation.

Our results and those involving iontophoresis of antigen on to mast cells (29) suggest that the activation of Fc receptors by cross-linking ligands is interpreted locally by the mast cell and not by the cell as a whole. It is possible that in these experiments the cell activation process is generalized but that rapid desensitization occurs in those regions of the cell away from the local stimulus. This seems an unlikely explanation of our results in view of the very slow rate of desensitization observed with soluble Con A (C. Fewtrell and B. D. Gomperts. Unpublished observations). Moreover, when cells and beads were mixed and centrifuged at 4°C in the presence of phosphatidyl serine (PS) to minimize desensitization (9, 25), degranulation was still localized to the region of bead contact.

In view of the accumulating (but still indirect) evidence that an influx of Ca$^{2+}$ initiates exocytosis
in mast cells, the simplest and most likely interpretation of our findings is that Con A-Sepharose beads induced a localized opening of Ca$^{2+}$ channels which was responsible for the localized degranulation. Since adding soluble Con A to bead-adherent cells induced generalized degranulation, it is unlikely that such a postulated localized Ca$^{2+}$ influx could be explained by the redistribution of all of the putative Ca$^{2+}$ channels to the region of bead contact. It seems more likely that, even with soluble ligands, Ca$^{2+}$ channels open locally in the region of membrane where Fc receptors are cross-linked. We cannot exclude the possibility that the Con A beads induced a generalized influx of Ca$^{2+}$ and that some other event(s) necessary for exocytosis restricted the degranulation to the area of bead contact. However, our finding that A23187 was able to induce generalized degranulation in bead adherent cells makes this interpretation much less attractive; it would require that A23187, in addition to its cation carrying properties, be able to signal this “other event(s)” or bypass it.

Although it is unknown how an influx of Ca$^{2+}$ initiates exocytosis, our results make it very unlikely that it activates a secretion mechanism which affects the mast cell as a whole. They also provide further evidence that the diffusion of free Ca$^{2+}$ in the cytosol is restricted, and, since there are relatively few mitochondria in mast cells, it seems likely that there is a mechanism for buffering Ca$^{2+}$ locally, in addition to mitochondrial uptake.

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REFERENCES