DISODIUM CROMOGLYCATE INHIBITION OF SUBSTANCE P-INDUCED HISTAMINE SECRETION IS CALCIUM DEPENDENT

Masahiro NISHIBORI and Kiyomi SAEKI
Department of Pharmacology, Okayama University Medical School,
2-5-1 Shikata-cho, Okayama 700, Japan
Accepted July 15, 1983

Abstract—Histamine secretion was induced by substance P, in a dose-dependent manner, from rat peritoneal mast cells, both in the presence and absence of Ca²⁺ in the medium, and disodium cromoglycate (DSCG) produced a dose-dependent inhibition of the histamine secretion in Ca²⁺-containing medium. However, in the absence of Ca²⁺, DSCG was ineffective or had a far weaker activity. Mg²⁺, Sr²⁺ and Ba²⁺ were ineffective in restoring the DSCG activity when added to medium devoid of divalent metal ions. Therefore, extracellular Ca²⁺ seems to be a specific requirement for the binding of DSCG to its "receptors" on the mast cell surface or some steps in the DSCG action.

Disodium cromoglycate (DSCG) (1, 2) is an antiallergic drug widely prescribed for the treatment of bronchial asthma. It effectively inhibits histamine release (secretion) from rat peritoneal mast cells as induced by the binding of a specific antigen to cell-bound IgE antibody (3–5) as well as by a variety of chemical substances such as dextran (5), compound 48/80 (3, 6–8), substance P (8) and neurotensin (8). Possible mechanisms of the action of DSCG include: blocking of the influx of Ca²⁺ into mast cells (6, 9), inhibition of phosphodiesterase (10, 11) and regulation of phosphorylation of mast cell protein (12).

As Ca²⁺ may play a role of intracellular mediator in the stimulus-secretion coupling in a variety of cells including mast cells (13), further studies on the effects of DSCG on changes produced by histamine-secreting stimuli in the dynamic state of Ca²⁺ in mast cells are warranted. Investigations of the possible influence of Ca²⁺ on the inhibitory effect of DSCG on histamine secretion may also be useful for a better comprehension of the mode of action of this drug. Ennis et al. (7, 14) observed that DSCG inhibited histamine secretion from rat mast cells induced by different stimuli, irrespective of the presence or absence of extracellular Ca²⁺; while Mazurek et al. (15) reported that DSCG covalently conjugated to fluorescent polyacrylamide, polyglutaraldehyde beads bound to rat peritoneal mast cells only in the presence of extracellular Ca²⁺, and these DSCG-bead conjugates inhibited anaphylactic histamine secretion in the presence of extracellular Ca²⁺.

The substance P-induced histamine secretion from rat mast cells apparently does not require extracellular Ca²⁺. Moreover, this reaction is highly sensitive to the inhibitory actions of DSCG in Ca²⁺-containing medium (8). We have now compared the activities of DSCG on substance P-induced histamine secretion from rat peritoneal mast cells, both in the presence and absence of extracellular Ca²⁺.

Materials and Methods

Animals: Male Sprague-Dawley rats aged 10–14 weeks and weighing 350–490 g
(Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were used.

Chemicals and drugs: Substance P triacetate was obtained from the Protein Research Foundation (Minoh, Japan); disodium cromoglycate (DSCG) was from Fujisawa Pharmaceutical Co. (Osaka, Japan); bovine serum albumin (fraction V) was from Armour Pharmaceutical Co. (Kankakee, IL).

Histamine release from rat peritoneal mast cells: Unless otherwise specified, phosphate-buffered saline (PBS) of the following composition was used: NaCl, 154 mM; KCl, 2.7 mM; CaCl₂, 0.9 mM; Na₂HPO₄-KH₂PO₄ buffer, pH 7.1, 6.7 mM; glucose, 5.6 mM; bovine serum albumin, 0.05%. The suspensions of rat peritoneal cells containing 5–8% mast cells were prepared as described previously (8). Before the addition of DSCG, the suspended cells were preincubated for 5 min at 37°C. Substance P was added to the cell suspension 10 sec after the addition of DSCG, and the incubation was continued for a further 5 min at the same temperature. In experiments to test the effect of divalent metal ions, Ca²⁺-free PBS (PBS without added Ca²⁺) was used for collecting and subsequently suspending the peritoneal cells. CaCl₂, MgCl₂, SrCl₂ or BaCl₂ was added to the cell suspension at the start of the preincubation. After incubation, each tube was immediately transferred to an ice bath, and the preparation was centrifuged at 650×g for 10 min at 0°C. The histamine contents of both the supernatant and the precipitate were determined. Siliconized glassware was used throughout. Histamine release was assayed in duplicate samples.

Determination of histamine: The histamine content of each sample was determined fluorometrically by the method of Shore et al. (16), omitting the extraction procedures with organic solvents, as described by Loeffler et al. (17). After fluorofore formation, 2 M citric acid instead of 3 N HCl was used as the acidifying agent (18). DSCG and substance P did not interfere to any significant extent with the histamine assay. The percentage of histamine release was calculated using the following equation: histamine release (%) = \((\text{histamine content of the supernatant}) \times 100/[(\text{histamine content of the supernatant}) + (\text{histamine content of the precipitate})]\). Percent inhibition of histamine release was calculated as follows: % inhibition = \(((% \text{ release from the non-treated cells}) - (% \text{ release from the treated cells})) \times 100/(% \text{ release from the non-treated cells}).

Results
Substance P induced a release of histamine from rat peritoneal mast cells, in a dose-dependent manner, both in the presence and absence of extracellular Ca²⁺ (Fig. 1A and B). In the presence of Ca²⁺, DSCG (10⁻⁵ M) significantly inhibited the release induced by substance P (10⁻⁶–2×10⁻⁵ M). DSCG produced a rightward but non-parallel shift of the dose-response curve (Fig. 1A). Contrary to the effectiveness in the presence of Ca²⁺, DSCG (10⁻⁵ M) was ineffective in inhibiting the release induced by substance P, at concentrations of 10⁻⁶–5×10⁻⁵ M (Fig. 1B). Ca²⁺ at concentrations of 0.2 mM and above significantly inhibited the histamine release induced by substance P (10⁻⁵ M) (Fig. 2). Spontaneous histamine release at a Ca²⁺ concentration of 1.8 mM was significantly lower than that in the absence of Ca²⁺. In all the following experiments, 10⁻⁵ M substance P was used.

When the usual PBS was used throughout all the experimental procedures, DSCG produced a marked and significant inhibition of the substance P-induced histamine release at concentrations of 10⁻⁵ M and above (Fig. 3). DSCG was effective in inhibiting the histamine release, even when the peritoneal cells were at first collected and suspended in
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Fig. 1. Dose-response curves for the substance P-induced histamine release from rat peritoneal mast cells in the presence • and absence ○ of DSCG (10⁻⁵ M). A: ordinary PBS and B: Ca²⁺-free PBS. The results are the means±S.E.M. of 4 experiments on different pools of cells. Significantly different from the corresponding value in the absence of DSCG as determined by Student's unpaired t-test: *P<0.05, **P<0.005, ***P<0.001.

Ca²⁺-free PBS and Ca²⁺ (0.9 mM) was added to the medium at the start of the preincubation. The histamine release in the absence of DSCG (control) was somewhat higher in the former experimental condition. However, DSCG (10⁻⁶–10⁻³ M) produced much the same degree of inhibition of histamine release under both these conditions.

On the contrary, the inhibitory activity of DSCG in Ca²⁺-free medium was far weaker than that in Ca²⁺-containing medium. When Ca²⁺-free PBS was used throughout, DSCG was ineffective at concentrations up to 10⁻⁵ M. The release was inhibited at 10⁻⁴ and 10⁻³ M in the absence of Ca²⁺, but the activities in these concentrations were less than one third of those observed in the presence of Ca²⁺.

When substance P was tested in three different media containing 0.9 mM Mg²⁺, 0.9 mM Sr²⁺ and 0.9 mM Ba²⁺, respectively, no significant differences in the histamine releasing activity of substance P (10⁻⁵ M) were observed compared with the activity in the medium lacking divalent metal ions (Table 1). However, Ca²⁺ at the same concen-
Fig. 3. Effect of Ca\(^{2+}\) on the inhibition by DSCG of the histamine release induced by substance P from rat peritoneal mast cells. After the preincubation in the presence (0.9 mM) or absence of Ca\(^{2+}\), DSCG and substance P (10\(^{-5}\) M) were added to the cell suspension at an interval of 10 sec. The symbols used are as follows: □ Ca\(^{2+}\) present in the medium throughout all the experimental procedures, Δ Ca\(^{2+}\) absent throughout, ○ Ca\(^{2+}\) added to the medium at the beginning of the preincubation. The results are the means±S.E.M. of 4 experiments on different pools of cells corrected for spontaneous release. In all of the 3 sets of experiments, spontaneous histamine release was in the range of 2.7–8.1%. Significantly different from the corresponding control value in the absence of DSCG as determined by Student's unpaired t-test: *P<0.05, **P<0.01, ***P<0.005, ****P<0.00.

Table 1. Effect of divalent metal ions on the inhibition by DSCG of the histamine release induced by substance P from rat peritoneal mast cells

<table>
<thead>
<tr>
<th>Drugs</th>
<th>% Histamine release in the presence (0.9 mM) of different divalent metal ions</th>
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<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>Substance P</td>
<td>40.2±2.9</td>
</tr>
<tr>
<td>Substance P+DSCG</td>
<td>(-1.7±5.8)</td>
</tr>
</tbody>
</table>

DSCG (10\(^{-5}\) M) and substance P (10\(^{-5}\) M) were added at 10 sec intervals. The results are the means±S.E.M. of 4 experiments on different pools of cells. The figures in parentheses represent % inhibition of histamine release. The spontaneous release (%) in the medium containing no added divalent metal ions was 6.1±1.1; and the releases in the presence of Mg\(^{2+}\), Ca\(^{2+}\), Sr\(^{2+}\) and Ba\(^{2+}\) were 4.0±0.7, 2.8±0.4, 3.5±1.0 and 3.3±0.8, respectively. DSCG had no significant effect on spontaneous releases. The results in the table have been corrected for the respective spontaneous releases. *P<0.02 compared with the corresponding value in the absence of divalent metal ion (unpaired t-test). \(^{b}\)P<0.005 compared with the control value in the absence of DSCG (unpaired t-test). \(^{c}\)P<0.02 compared with the control value (paired t-test).

Discussion

Anaphylactic histamine secretion from rat mast cells (19, 20) as well as the histamine secretion induced by chemical substances such as dextran (21) and α-chymotrypsin (22) require the presence of extracellular Ca\(^{2+}\) for an optimal response. On the other hand, a polyamine such as compound 48/80 (22–24) and a peptide such as somatostatin (25) produce a marked histamine secretion, even in the absence of extracellular Ca\(^{2+}\). However, Ca\(^{2+}\) is essential for the stimulus-secretion coupling of these substances. In
these cases, the Ca^{2+} mobilized from binding sites in mast cells seems to play an intracellular mediator role (25-28).

The histamine release-inducing activity of substance P on rat mast cells was first reported in 1973 (29). Recently, substance P was shown to produce a marked histamine release, irrespective of the presence or absence of extracellular Ca^{2+} (8). Rat mast cells incubated with 2.5 mM EDTA in Ca^{2+}-free medium for 4 hr at 37°C are not responsive to substance P; however, the responsiveness can be largely restored by adding Ca^{2+} (0.9 mM) shortly before the substance P challenge (Ikeda and Saeki, unpublished observation). Therefore, cell-bound Ca^{2+} seems to be mobilized and utilized for the release response induced by substance P. Such is the case with regard to responses to compound 48/80 and somatostatin.

In the present study, the substance P-induced histamine release was inhibited by Ca^{2+} at concentrations of 0.2 mM and above. A similar inhibitory effect of Ca^{2+} was also observed by Fewtrell et al. (30). It is likely that Ca^{2+}-influx is induced by substance P when the Ca^{2+} stores in mast cells have been depleted by preincubation with chelating agents and that extracellular Ca^{2+} exerts an inhibitory rather than an enhancing influence on the histamine release when substance P is applied to mast cells with sufficient amounts of stored Ca^{2+}.

The histamine release induced by substance P in the presence of extracellular Ca^{2+} has pH and temperature optima and depends on the energy supply (8). When tested in glucose-free medium, the histamine release induced by substance P (10^{-5} M) in the absence of extracellular Ca^{2+} is abolished by antimycin A (1 \mu M). The responsiveness to substance P suppressed by antimycin A can be restored by the addition of glucose (5.6 mM). Therefore, substance P induces a secretory response in mast cells in the absence as well as in the presence of extracellular Ca^{2+}.

It has been reported that the degree of inhibition by DSCG of the histamine secretion depends on the intensity of the control response (3, 7). In the present study, DSCG (10^{-5} M) had a significant inhibitory effect on the histamine secretion induced by substance P (10^{-6}-2 \times 10^{-5} M) in Ca^{2+}-containing medium. However, in Ca^{2+}-free medium, DSCG was ineffective for the same degree of secretion as the response in the presence of Ca^{2+}. Thus, the inhibitory effect of DSCG depends on extracellular Ca^{2+}, apart from the dependence on the intensity of the control response. The addition of Ca^{2+} to the cell suspension at the start of preincubation restored the inhibitory effect of DSCG. The view that DSCG may block the Ca^{2+}-influx seems to be inconsistent with the inhibitory effect of extracellular Ca^{2+} on the substance P-induced histamine secretion. Consequently, the present results suggest that DSCG inhibits the secretion through a Ca^{2+}-dependent process(es), but probably not by the blocking of Ca^{2+}-influx.

Ennis et al. (7, 14) observed that DSCG was an effective inhibitor of various histamine-secreting responses of rat mast cell, irrespective of their requirement for extracellular Ca^{2+}, and that DSCG suppressed the responses independent of the extracellular Ca^{2+} both in the presence and absence of Ca^{2+} in the medium. At present we have no clear explanation for the inconsistencies between our results and those reported by Ennis et al. (7, 14).

Mazurek et al. (15, 31) showed that Ca^{2+}, Mg^{2+}, Sr^{2+} and Ba^{2+} all form complexes with DSCG in a solvent of low polarity and that DSCG binds to the mast cell surface in the presence but not absence of extracellular Ca^{2+}. The binding of DSCG to the mast cell surface in the presence of extracellular Ca^{2+}
may be indispensable as the first step in the drug action. In the present experiments, only Ca$^{2+}$ was effective in restoring the drug activity lost in the absence of divalent metal ions. On the other hand, a DSCG analogue, 6,7-dihydro-6,8,8,10-tetramethyl-8H-pyrano[3,2-g]-chromon-2-carboxylic acid, inhibited the substance P-induced histamine release even in the absence of Ca$^{2+}$ to some extent, and Ba$^{2+}$ and Sr$^{2+}$ could substitute for Ca$^{2+}$ (Tsutsumi et al., unpublished observation). Therefore, among the divalent metal ions tested, Ca$^{2+}$ may be selectively required for the binding of DSCG to the specific binding sites ("receptors") on the mast cell surface or some steps in the DSCG action.

Acknowledgement: This study was supported in part by grant 56570077 from the Ministry of Education, Science and Culture, Japan. We thank M. Ohara of Kyushu University for comments on the manuscript.

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