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EFFECT OF DISODIUM CROMOGLYCATE ON PHOSPHOLIPASE A ACTIVITY

STUDIES WITH EGG YOLK AND ISOLATED MAST CELL SYSTEMS

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Abstract—The proposal that phospholipase A may be involved in the non-immunological release of histamine from mast cells has been investigated using disodium cromoglycate and simple models consisting of egg yolk emulsions or isolated mast cell systems. The results appear to exclude a role for phospholipase A in histamine release from mast cells. Disodium cromoglycate at concentrations between 10^{-7} and 10^{-3} M did not appear to effect the activity of purified snake venom phospholipase A in the egg yolk emulsion substrate system. The enzyme was completely inhibited in the same system by 5×10^{-4} M EDTA. Purified snake venom phospholipase A alone did not cause significant release of histamine from isolated rat peritoneal mast cells. Although phospholipase A and chymotrypsin acted synergistically to release histamine from mast cells, disodium cromoglycate (10^{-6}–10^{-3} M) did not significantly effect this process. In addition, lysis of mast cells by lysolecithin was not inhibited by 10^{-6}–10^{-3} M disodium cromoglycate.

DISODIUM cromoglycate (DSCG), the disodium salt of 1,3-bis (2-carboxychromon-5-yloxy)-2-hydroxypropane, blocks the reagin-mediated release of spasmogens following challenge of allergic individuals.[1] Most previous studies with DSCG have been based on patients or experimental models exhibiting this immunologically-triggered, or "extrinsic", type of allergy. However, evidence is accumulating that DSCG is also effective in the treatment of patients with exercise induced asthma[2] and some with "intrinsic" asthma; i.e. apparently non-immunologically-triggered asthma.[3] Recent experiments with animal models believed to be applicable to humans have provided evidence consistent with the suggestion that DSCG does not prevent combination of cell-bound antibody with antigen. Rather, DSCG appears to inhibit events occurring subsequent to this union but prior to release of spasmogens.[4,5] It seemed appropriate, therefore, to study the action of DSCG in a relatively simple biological system in which the release of pharmacological mediators such as histamine is triggered by non-immunological means.

Mast cells contain substantial quantities of spasmogens and are probably involved in the allergic reaction. Presumably, their involvement is independent of the type of asthma ultimately displayed; i.e. "extrinsic" or "intrinsic". Therefore, mast cells would appear to offer a suitable model for studies of some aspects of the allergic process. They occur both as integral components of various tissues, including lung, and as free cells and may be studied in either context. We have chosen to employ free, purified mast cells as a model because they represent the simplest biological system

likely to retain the necessary relevant characteristics for studying the mode of action of DSCG.

The possible involvement of phospholipase A (EC 3.1.1.4 phosphatide acylhydrolase) in the release of histamine-containing granules from mast cells was suggested in a report by Högberg and Uvnäs.[6] Orr and Cox[7] carried this work further and showed a parallel inhibition by DSCG of histamine release and mast cell degranulation when subcutaneous tissue of rats was treated with phospholipase A or a reaginic antibody/antigen system. We have considered the phospholipase A/mast cell system as a model for the release of spasmogens occurring in "intrinsic" type asthma. The present report includes studies of the effect of DSCG in this system and in the chymotrypsin and chymotrypsin/phospholipase A-mediated release of histamine from isolated mast cells. Additional experiments are described for the effect of DSCG on the lysolecithin-mediated release of histamine from isolated mast cells and the phospholipase A-catalyzed hydrolysis of egg yolk lecithin. In general, DSCG appeared to have little, if any, effect on phospholipase A activity in these systems.

MATERIALS AND METHODS

Purified phospholipase A from *Crotalus terrificus terrificus* venom (Kontrol Nr. 6339405 and 6339406) was purchased from Boehringer Corp. (London). According to the suppliers, the enzyme contained no detectable kallikrein activity. The enzyme concentration was 1 mg/ml and possessed an activity of 200 units/mg (see suppliers specifications). The solvent was 50% (v/v) aqueous glycerol.

Disodium cromoglycate was supplied by Fisons Limited—Pharmaceutical Division (Lot No. D3/54/10). The molecular weight of the tetrahydrate is 584. The ultraviolet spectral characteristics of DSCG show maxima at 238 and 326 nm with absorbancies ($E^{1\%}_{1\,cm}$) of 600 and 163, respectively. Chymotrypsin (4x crystallized, 9–11,000 ATEE units/mg), EDTA and histamine acid phosphate were purchased from British Drug Houses Ltd. Lysolecithin was obtained from Koch–Light.

Substrate for phospholipase A consisted of a 10% emulsion (v/v) of fresh hens egg yolk in 0·2 M NaCl prepared fresh daily using an all-glass homogenizer. Appropriate concentrations of DSCG and EDTA were prepared in distilled water. Reaction rates at pH 7·4 and 37° were measured using a Radiometer Model TT11, SBR2c titrimeter in the pH-stat mode. The manufacturer's microsystem, including water-jacketed thermostat, served for the reaction vessel. Adjustment of initial pH to 7·4 was accomplished by adding 10 mM NaOH with an Agla syringe. The titrant during the reaction was 5 mM NaOH. Reaction mixtures were prepared by mixing 1 ml of substrate and 1 ml of DSCG, EDTA, or distilled water in the reaction vessel. Reactions were started by addition of 5 μl of enzyme. Reaction velocities were measured from the initial slopes of the base uptake curves which resulted after addition of enzyme and were defined as μmoles of OH^- consumed/min.

Mast cells were prepared from exsanguinated 250-g male Ash/F rats previously killed by stunning and severance of the spinal cord. The peritoneal cavities were washed with buffered salt solution (150 mM NaCl, 2·7 mM KCl, 0·9 mM $CaCl_2$, 3·0 mM $Na_2HPO_4 \cdot 2H_2O$, 3·5 mM KH_2PO_4, 5·6 mM dextrose and 0·1% bovine serum albumin)[8] and the recovered cells fractionated on a Ficoll gradient.[9] Siliconized glassware was used in all manipulations involving intact mast cells. The mast cells were

finally suspended in buffered salt solution and an aliquot stained with toluidine blue and counted. The viability of each preparation was judged by visual inspection at this point. Preparations were discarded if less than approximately 90 per cent of the cells were intact. Mast cell concentrations were usually adjusted to $1–3 \times 10^5$ cells/ml.

Histamine was measured fluorimetrically with *o*-phthalaldehyde according to the modification of Anton and Sayre.[10] The extraction procedure described by these authors was omitted in the present investigation because reasonable results could be obtained without it. The "reasonableness" of results in this case was based on the histamine content found per million mast cells. A value of 225 ± 50 nmoles/10^6 cells was taken as the "standard" value. Measurements of histamine content in 0·3 ml samples were accomplished by adding, with stirring, 0·6 ml of 0·167 M NaOH and 0·1 ml of 1·0% *o*-phthalaldehyde in absolute methanol. After 4·0 min, 0·1 ml of 2·5 M citric acid was added and the fluorescence measured in a Baird-Atomic "Fluorospec". The excitation wavelength was 355 nm and the fluorescence was read at 445 nm. Standards consisted of 0·3 ml samples of 0–1·0 nmole histamine acid phosphate in buffered salt solution. Suppression of fluorescence by DSCG was found at concentrations greater than 10^{-4} M. Therefore, separate blanks containing appropriate DSCG concentrations were included for those samples containing DSCG at greater than 10^{-4} M concentrations.

EXPERIMENTAL

Effect of disodium cromoglycate and ethylenediaminetetraacetic acid on phospholipase A activity

The effect of DSCG and EDTA on phospholipase A activity when the enzyme acts on egg yolk lecithin is shown in Fig. 1. The effect of EDTA was included as a positive

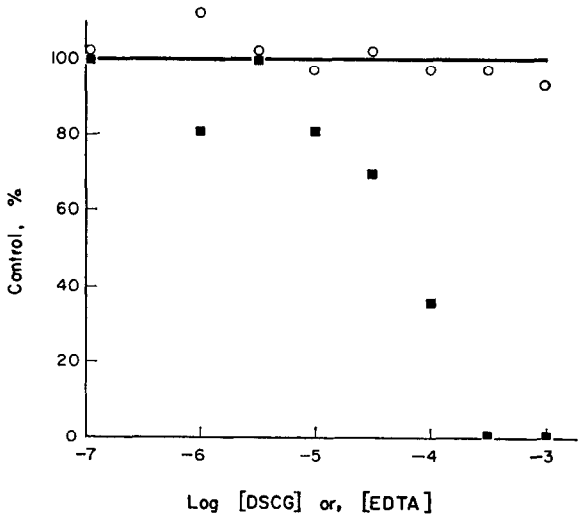

FIG. 1. Dose–response curves for the influence of DSCG (○) and EDTA (■) on the phospholipase A-catalyzed hydrolysis of egg yolk lecithin. Reaction rates were measured with a pH-stat at pH 7·4 and 37°.

control. DSCG was without effect under the conditions of this experiment. EDTA inhibited phospholipase A, presumably by removing Ca^{2+} (but see ref. 11). Experiments utilizing a range of substrate concentrations (0·2–20%, v/v) with and without supplemental Ca^{2+} gave the same results except that higher concentrations of EDTA were required to show inhibition when Ca^{2+} was added. The additional EDTA required corresponded to the amount of Ca^{2+} added.

Release of histamine from isolated rat peritoneal mast cells by phospholipase A

The ability of phospholipase A to cause release of histamine from isolated peritoneal mast cells was determined by incubating 0·5 ml of mast cell suspension with 50, 100, or 200 μl of enzyme solution in a total volume of 1 ml. The remaining volume consisted of 0·1 M tris-HCl, pH 7·8. The final pH was 7·8. The components were incubated for 10 min at 37° before addition of enzyme and the reaction permitted to proceed for 30 min at this temperature. The control included the appropriate volume of 50% (v/v) glycerol in place of the enzyme. Cells and supernatant were separated by centrifugation at 270 *g* for 10 min, 0·7–0·8 ml of supernatant removed with a Pasteur pipette and the cells drained by inversion of the tubes over absorbent tissue. One ml of distilled water was added to the cells for lysis, following which the cell debris was removed by re-centrifugation. Duplicate 0·3 ml samples of supernatant and cell lyzate were analysed for histamine. Histamine release was defined as the ratio of histamine in the supernatants to total histamine. The results are reported in Table 1.

Release of histamine from isolated rat peritoneal mast cells by chymotrypsin

The action of chymotrypsin on isolated peritoneal mast cells was determined in an experiment similar to that for phospholipase A, except that DSCG was also included. All reagents except the mast cell suspension were prepared in 0·1 M tris-HCl, pH 7·8. Reaction mixtures consisted of 0·5 ml of mast cell suspension in buffered salt solution, 0·25 ml of DSCG solution (or buffer for controls), 0·2 ml of buffer, and 50 μl of chymotrypsin (6 mg/ml). Temperature equilibration, time of incubation, collection of samples and histamine analyses were as described above. The results are presented in Table 2.

TABLE 1. EFFECT OF PHOSPHOLIPASE A ON THE RELEASE OF HISTAMINE FROM ISOLATED RAT PERITONEAL MAST CELLS

Experiment No.	μl of enzyme	% Net histamine release
1	50	8
2	50	7
3	100	2
4	100	6
5	200	6
6	200	6

The reaction mixtures consisted of 0·5 ml of mast cell suspension in buffered salt solution, the indicated volume of enzyme and 0·1 M tris-HCl, pH 7·8 to 1·0 ml total volume. Reactions proceeded for 30 min at 37°. Histamine in supernatants and precipitates was determined fluorimetrically using *o*-phthalaldehyde.

TABLE 2. EFFECT OF DISODIUM CROMOGLYCATE ON THE CHYMOTRYPSIN-MEDIATED RELEASE OF HISTAMINE FROM ISOLATED RAT PERITONEAL MAST CELLS

Additions	% Net histamine release
Chymotrypsin	47
+ 10^{-3} M DSCG	37
+ 3×10^{-4} M DSCG	42
+ 10^{-4} M DSCG	42
+ 3×10^{-5} M DSCG	48
+ 10^{-5} M DSCG	44
+ 3×10^{-6} M DSCG	46
+ 10^{-6} M DSCG	48

The reaction mixtures consisted of 0·5 ml of mast cell suspension in buffered salt solution, 0·25 ml DSCG in tris-HCl buffer, pH 7·8 (or buffer for controls), 0·2 ml tris-HCl, pH 7·8 and 50 μl chymotrypsin (6 mg/ml). Reactions proceeded for 30 min at 37°. Histamine in supernatants and precipitates was determined fluorimetrically using *o*-phthalaldehyde.

Release of histamine from isolated rat peritoneal mast cells by phospholipase A plus chymotrypsin

Phospholipase A and chymotrypsin presumably act synergistically in causing the release of histamine from isolated mast cells.[12] The effect of DSCG on this system was ascertained by incubating 0·5 ml of mast cells with 0·2 ml of phospholipase A (or 0·1 ml of phospholipase A plus 0·1 ml of 0·1 M tris buffer, pH 7·8), 50 μl of chymotrypsin (6 mg/ml), and 0·25 ml of selected concentrations of DSCG. All reagents other

TABLE 3. EFFECT OF DISODIUM CROMOGLYCATE ON THE PHOSPHOLIPASE A PLUS CHYMOTRYPSIN-MEDIATED RELEASE OF HISTAMINE FROM ISOLATED RAT PERITONEAL MAST CELLS

	% Net histamine release	
Additions	0·1 ml PL − A	0·2 ml PL − A
Phospholipase A	6	2
Chymotrypsin	14	17
Chymotrypsin + Phospholipase A	20	49
+ 10^{-3} M DSCG	25	40
+ 3×10^{-4} M DSCG	19	31
+ 10^{-4} M DSCG	22	39
+ 3×10^{-5} M DSCG	23	32
+ 10^{-5} M DSCG	24	34
+ 3×10^{-6} M DSCG	21	36
+ 10^{-6} M DSCG	25	31

The reaction mixtures consisted of 0·5 ml of mast cell suspension in buffered salt solution, 0·1 ml phospholipase A + 0·1 ml of 0·1 M tris-HCl, pH 7·8 or 0·2 ml phospholipase A, 50 μl chymotrypsin (6 mg/ml), and 0·25 ml DSCG. All reagents other than the mast cell suspension and phospholipase A were prepared in 0·1 M tris-HCl, pH 7·8. This buffer was substituted for chymotrypsin and DSCG solutions in the corresponding controls. Fifty percent aqueous glycerol was substituted for phospholipase A in the appropriate controls. Reaction conditions and histamine assays were as specified in the text.

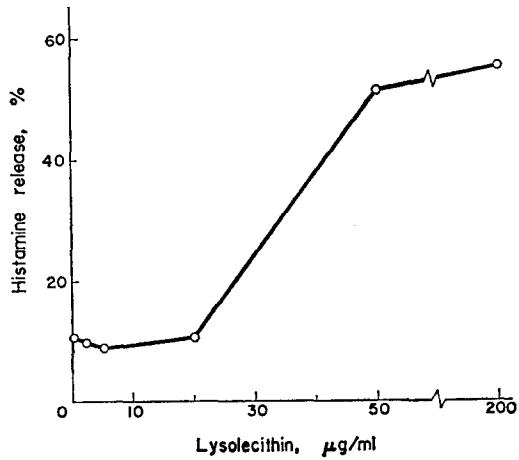

FIG. 2. Dose–response curves for the release of histamine from isolated rat peritoneal mast cells by lysolecithin. Conditions of experiment are given in the text and Table 4.

than mast cells and phospholipase A were prepared in 0·1 M tris buffer, pH 7·8. This buffer was substituted for chymotrypsin and DSCG solutions in the corresponding controls. The results are shown in Table 3.

Effect of DSCG on the lysolecithin-mediated release of histamine from isolated rat peritoneal mast cells

A dose–response curve for the release of histamine from isolated rat peritoneal mast cells by lysolecithin (Fig. 2) was determined in order to select an appropriate concentration of this reagent for the subsequent experiment. A lysolecithin concentration of 50 μg/ml was chosen. To test the effect of DSCG on this system, 0·5 ml of

TABLE 4. EFFECT OF DISODIUM CROMOGLYCATE ON THE LYSOLECITHIN-MEDIATED RELEASE OF HISTAMINE FROM ISOLATED RAT PERITONEAL MAST CELLS

Additions	% Net histamine release
Lysolecithin	96
+ 10^{-3} M DSCG	86
+ 3×10^{-4} M DSCG	92
+ 10^{-4} M DSCG	93
+ 3×10^{-5} M DSCG	93
+ 10^{-5} M DSCG	93
+ 3×10^{-6} M DSCG	94
+ 10^{-6} M DSCG	97

The reaction mixtures consisted of 0·5 ml of mast cell suspension in buffered salt solution, 0·1 ml lysolecithin (500 μg/ml), 0·25 ml DSCG or buffer, and 0·15 ml buffer. All reagents were prepared in buffered salt solution (buffer). Reactions proceeded for 30 min at 37°. Histamine in supernatants and precipitates were determined fluorimetrically using *o*-phthalaldehyde (see text).

mast cells in buffered salt solution was incubated with 0·1 ml of lysolecithin (500 μg/ml), 0·25 ml of various concentrations of DSCG or buffered salt solution, and 0·15 ml of buffered salt solution. The conditions of incubation, sample preparation and histamine assay were as described above. The results are presented in Table 4.

RESULTS AND DISCUSSION

Phospholipase A has been implicated in the mechanism of release of histamine from mast cells by several studies, including the demonstration that mast cells possess the enzyme.[13] Although earlier reports of the effect of phospholipase A on histamine release from mast cells may have included the effects of contaminating enzymes or factors, subsequent work has established that phospholipase A itself is capable of releasing histamine from tissue-embedded mast cells.[7] In addition, DSCG has been shown to inhibit this process to an extent similar to its effect on the antigen-mediated release of histamine.[7]

Further investigation into the mechanism of action of DSCG would seem to include a study of the effect of the compound on phospholipase A itself. Therefore, we determined the dose–response curve for DSCG in a simple enzyme substrate system. The substrate chosen was an egg yolk emulsion. Ideally perhaps, purified lecithin should have been selected as the substrate for this experiment. However, the very limited solubility of lecithin in aqueous solvents on the one hand and the insolubility of DSCG in lipophilic solvents on the other dictated use of the egg yolk system. The dose–response curve for DSCG in this system is presented in Fig. 1. The absence of a well-defined effect shows that DSCG probably does not influence the phospholipase A- mediated histamine release from tissue-embedded mast cells by inhibition of the enzyme *per se*. The concentration range of DSCG used in these experiments included those normally encountered clinically and rose to values encompassing the range applicable for enzyme inhibitors generally. The inclusion of the positive EDTA control demonstrated that the pH-stat method was capable of detecting inhibition of the enzyme if it had occurred during the experiments with DSCG.

Failure to demonstrate an effect of DSCG on phospholipase A activity in the simple enzyme–substrate system led us to examine the effect of the enzyme in a purified mast cell system. Release of histamine from isolated mast cells caused by the action of phospholipase A was studied because this system represents a stage of intermediate complexity between the enzyme acting on a simple substrate and its action on subcutaneous connective tissue-embedded mast cells. The results of experiments utilizing several concentrations of phospholipase A are reported in Table 1. In all cases, only very slight release of histamine above control levels was found. Therefore, it was not possible to investigate the influence of DSCG at this point.

According to Amundsen *et al.*,[12] phospholipase A enhances the action of chymotrypsin in causing the release of histamine from mast cells. Accordingly, an action of phospholipase A on mast cells might be detected by this device and the effect of DSCG, if any, evaluated at this level of biological organization. Table 2 records the results of incubating mast cells with chymotrypsin and shows that DSCG did not inhibit the action of this enzyme. Additional experiments with chymotrypsin in simple substrate systems also showed an absence of an effect by DSCG.

The combined effect of phospholipase A and chymotrypsin acting on isolated mast

cells is shown in Table 3. Two different concentrations of phospholipase A were used. In both cases, the enzymes appeared to act synergistically as reported by Amundsen *et al.*[12] although the synergism was not so pronounced at the lower phospholipase A concentration. In neither case did DSCG appear to effect this result significantly. This finding again showed that phospholipase A itself is not inhibited by DSCG, including the case where it is acting on the mast cell as substrate.

To reconcile the results reported here with those concerning the action of phospholipase A on subcutaneous tissue-embedded mast cells, it may be suggested that the enzyme is effective only secondarily; i.e. it produces an agent which in turn causes histamine release. Examples of these agents may be lysolecithin or fatty acids produced from tissue phospholipids. Alternatively, DSCG may act by a mechanism not directly involving the enzyme; e.g. by causing generalized membrane stabilization or modification of cell wall substrate accessibility. The experiments with lysolecithin were based on the first of these proposals and reports that it does cause histamine release from mast cells.[6,14] The dose–response curve for the release of histamine from mast cells by lysolecithin (Fig. 2) was determined in order to select an appropriate concentration of the reagent for subsequent experiments with DSCG. A concentration of 50 μg/ml of lysolecithin was chosen for the DSCG experiments. The dose–response data (Table 4) show that DSCG did not inhibit the lysolecithin-mediated release of histamine from isolated mast cells. Unfortunately, these negative results are of little value in evaluating the validity of the proposed mechanism of action of DSCG. Further work designed to investigate these proposals is in progress.

Recent *in vivo* experiments with young male rats have shown that chronic subcutaneous administration of DSCG (5 and 50 mg/kg, 6 days/week, 12 weeks) does not alter phospholipase A activities and phospholipid concentrations and compositions of aorta and liver.[15] Furthermore, addition of DSCG at three concentrations (0·1, 0·5 and 1·0 mg/ml) to liver homogenates of normal rats did not significantly affect the activity of hepatic phospholipase toward either lecithin or phosphatidyl ethanolamine.[15]

We recognise that the absence of an effect by DSCG on the activity of *Crotalus* phospholipase A may merely reflect an insensitivity of this particular enzyme. But, while the results reported above do not exclude the possibility that phospholipase A may be involved in the physiological release of histamine from mast cells, they do tend to argue against the direct action of DSCG on this enzyme as being the sensitive step in this process.

From recent evidence it is known that prostaglandin E is released in rat skin by the action of phospholipase A[16] and that prostaglandin E is capable of disrupting mast cells with the release of histamine.[17] It seems reasonable to postulate, therefore, that rather than inhibiting the enzyme, DSCG may stabilize the mast cell membrane against the triggering action of prostaglandin E. This would explain the absence of activity of DSCG against phospholipase A itself while inhibiting the mast cell disruption due to the enzyme in rat subcutaneous connective tissue.

It must be concluded that further work is necessary to establish what role phospholipase A might have in histamine release from mast cells and how this relates to the action of such agents as lysolecithin or prostaglandin E.

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