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THE INHIBITION BY DISODIUM CROMOGLYCATE *IN VITRO* OF ANAPHYLACTICALLY INDUCED HISTAMINE RELEASE FROM RAT PERITONEAL MAST CELLS

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ABSTRACT

KUSNER, E. J., B. DUBNICK AND D. J. HERZIG: The inhibition by disodium cromoglycate *in vitro* of anaphylactically induced histamine release from rat peritoneal mast cells. J. Pharmacol. Exp. Ther. **184:** 41–46, 1973.

Rat peritoneal mast cells were sensitized *in vitro* with rat reaginic serum against ovalbumin and then were washed and suspended in buffer. Disodium cromoglycate (DSCG) (0.5–20 μM) caused a concentration-dependent inhibition of histamine release when added with antigen; 5 μM produced a 50% inhibition. A 4-fold increase in antigen concentration did not reverse inhibition suggesting that the DSCG did not compete directly with the antigen. Preincubation of sensitized mast cells with DSCG for various times up to 20 minutes before adding antigen resulted in a time dependent loss of inhibition which followed an s-shaped curve independent of initial DSCG concentration. After 20 minutes preincubation with DSCG the extent of reinhibition with fresh DSCG added with antigen was inversely dependent on initial DSCG concentration. Evidence exists to show that loss of inhibition is not due to a change of any factor external to the cell or to the loss of active DSCG from the medium. From the stoichiometry of the inhibition/reinhibition and from the time course of loss of inhibition, it is suggested that DSCG acts indirectly, inducing the formation of an unstable species in mast cells from a precursor present in a limited amount.

Disodium cromoglycate (DSCG) has shown effectiveness in treatment of allergic asthma (Pepys, 1969). This action has been attributed to its ability to interfere with the reagin-induced degranulation and release of histamine as seen with the mast cells in several animal species. In the rat it has been shown to inhibit passive cutaneous anaphylaxis with reaginic antibody by protecting against degranulation of the tissue mast cell (Orr *et al.*, 1970; Morse *et al.*, 1969). It also inhibits histamine release from both perfused intact lungs and lung slices from the guinea pig (Assem and Mongar, 1970), from human and monkey lung fragments, and from peripheral human leucocytes *in vitro* when sensitized with human atopic serum (Assem and Mongar, 1970).

Recently Orr *et al.* (1971) have reported that DSCG inhibits the release of histamine from rat peritoneal cells by compound 48/80 *in vitro*. The degree of inhibition was found to be dependent on the time that the cells were exposed to DSCG prior to the addition of 48/80. Although the histamine release initiated by 48/80 and by anaphylaxis have many features in common, they are

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TABLE 1

The effect of incubation time, DSCG and ovalbumin on histamine release from rat peritoneal mast cells

Addition	Incubation Time	Histamine (±S.E.M.) (N = 4)			
		Extracellular [A]		Cell Residue [B]	Total (A + B)
	min	*μg*	%[a]	*μg*	*μg*
HBSS	1	0.6 ± 0.0	3.1 ± 0.1	17.0 ± 0.5	
	20	0.8 ± 0.0	4.7 ± 0.2	16.7 ± 0.2	
DSCG (20 μM)	1	0.6 ± 0.0	3.2 ± 0.1	16.6 ± 0.3	
	20	0.7 ± 0.0	4.1 ± 0.2	16.5 ± 0.2	
DSCG (50 μM)	1	0.8 ± 0.0	4.2 ± 0.1	19.2 ± 0.2	
	20	1.0 ± 0.0	5.0 ± 0.1	19.2 ± 0.2	
HBSS	5	0.6 ± 0.0	3.2 ± 0.1	17.5 ± 0.3	18.0 ± 0.3
Antigen	5	5.3 ± 0.2	29.3 ± 0.2	12.8 ± 0.4	18.1 ± 0.5
HBSS					16.0 ± 0.0[b]

[a] Percentage of total histamine.

[b] Total measured directly on a boiled aliquot of cell suspension.

not identical (Johnson and Moran, 1971; Goth *et al.*, 1971; Bloom and Chakravarty, 1970). Therefore, we felt it worthwhile to investigate the effects of DSCG *in vitro* on anaphylactic histamine release from rat peritoneal cells. In this report, we provide evidence that: 1) DSCG *in vitro* will inhibit the *in vitro* anaphylactic release of mast cell histamine; 2) that this inhibition is lost if sufficient time elapses between the addition of DSCG and addition of antigen; 3) the mast cell in the presence of DSCG eventually becomes resistant to inhibition by DSCG.

Methods

Antiserum. Male, Sprague-Dawley rats (Charles River COBS), 125–175 g, were injected i.m. with 1 mg of ovalbumin (5 times recrystallized, Pentex, Kankakee, Ill.) in 1 ml of saline. They also received 2 × 10^{10} *Bordetella pertussis* organisms i.p. (Bio 210, Parke-Davis and Company, Detroit, Mich.). Fourteen days later the animals were anesthetized with ether and exsanguinated by cardiac puncture. After clotting overnight at 4°C the serum was removed, centrifuged, pooled and stored at −85°C. This serum was titered by passive cutaneous anaphylaxis (PCA) in the rat after a 4- and a 48-hour latency (Ovary, 1964). The dilution yielding a diameter of approximately 5 mm with a 48-hour latency (reaginic) was usually between 1:40 and 1:80. We demonstrated the reaginic (IgE) nature of the antibody by DEAE-cellulose chromatography, as well as by the heat and sulfhydryl sensitivity of the antiserum (Orange *et al.*, 1970).

Isolation and sensitization of peritoneal mast cells. Hanks' balanced salt solution (HBSS, Gibco, Grand Island, N.Y.) was adjusted to pH 6.9 with 5% (v/v) of 0.1 M phosphate buffer for use in all experiments. Male, Sprague-Dawley rats (Charles River COBS), approximately six weeks old, were decapitated and exsanguinated. Ten milliliters of HBSS with 0.10% human serum albumin (Nutritional Biochemicals Corporation, Cleveland, Ohio) were injected i.p. The peritoneal cavity was then opened and the fluid was aspirated with a plastic Pasteur pipet. The fluid was centrifuged at 350 × *g* for two minutes at 4°C. The cells from six animals were resuspended in a small volume of HBSS and combined. They were recentrifuged and resuspended in 2 ml of rat antiserum with 0.1 mg of heparin. After incubation for two hours at 37°C in a shaking water bath with occasional stirring they were diluted with 1 ml of HBSS and centrifuged. The cells were then suspended in 30 ml of HBSS.

Histamine release and assay. To duplicate 1.8-ml aliquots of the cell suspension 40 μg of ovalbumin and appropriate amounts of DSCG and then sufficient HBSS to bring the volume to 2.0 ml were added. This 1.8-ml aliquot contains about 4 × 10^5 cells and approximately 17 μg of histamine (table 1). After incubation at 37°C for five minutes with agitation, the samples were centrifuged and the supernatant was assayed for histamine by an automated fluorometric method (Kusner and Herzig, 1971). There was essentially no difference in the percent histamine released at antigen concentrations between 0.2 and 200 μg/ml. The total histamine for calculating the percent release was measured in the supernatant of a 1.8-ml sample of cells diluted to 2.0 ml with HBSS, boiled for five minutes and then centrifuged. To show that this truly represented the histamine present after treatment of the cells with ovalbumin or DSCG, we incubated cells and then centrifuged them. The supernatant was assayed for the extracellular histamine and the cells were resuspended in fresh HBSS, boiled and assayed for residual cellular histamine. The sum of the extracellular and residual histamine was found to be the same as that of an aliquot of untreated cells that were boiled and assayed (table 1).

All results were corrected for the "spontaneous" (antigen-independent) release measured in a sample to which was added HBSS in place of ovalbumin. In these experiments it was usually 3 to 5% of the total cell histamine. As seen in table 1, the amount of antigen-independent release did not change with the amount of DSCG present or the length of time of the incubation. As shown by Bloom and Chakravarty (1970) and confirmed by us, the antigen-induced release of

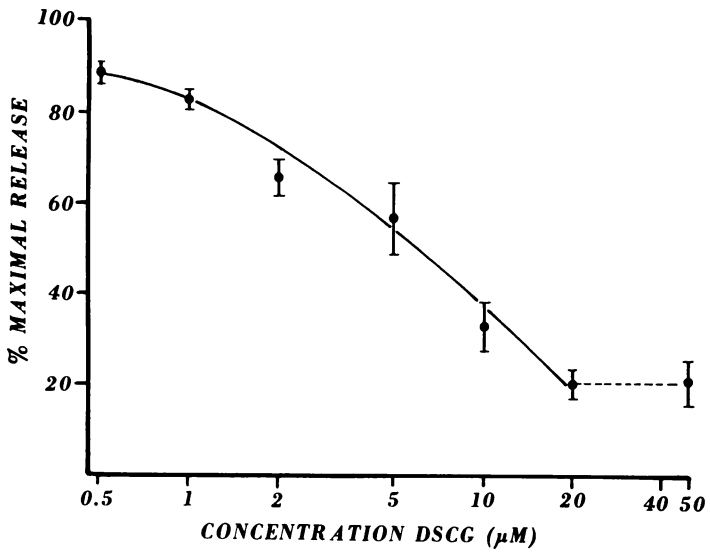

FIG. 1. Effect of various concentrations of DSCG on peritoneal mast cell histamine release. Cells were sensitized and challenged as described under "Methods." The DSCG was added with the antigen in saline. Each value is the average (±S.E.M.) of six duplicate determinations (except for the one at 49 μM) reported as the percentage of control value. The line connecting the points was calculated by the least squares method.

histamine is a rapid process and is complete within 30 seconds at 37°C. Further incubation or additional antigen (with or without fresh HBSS) did not change the amount of histamine released. The maximum that was released under these conditions was never more than 40 to 45% and was generally in the range of 20 to 30% of the total histamine in the cells (see, for example, Bach *et al.*, 1971; Bloom and Chakravarty, 1970). In most of the experiments reported here the normal net release (antigen-dependent less antigen-independent) without DSCG was greater than 20%. If it was not, the experiment was discarded. Because of the variability in this maximum release, the data from each experiment were normalized by expressing the release in the presence of DSCG as a percentage of the normal (antigen-dependent, no DSCG) release.

In all cases the concentrations are expressed as the final concentrations. The molecular weight for DSCG used was 512.4. We showed that DSCG up to 50 μM had no effect on the measurement of known amounts of histamine in the automated fluorometric assay (Kusner and Herzig, 1971).

Results

The effect of DSCG concentration on the release of histamine. The inhibition of release of histamine from rat peritoneal mast cells was related to the DSCG concentration between 0.5 and 19.6 μM when the DSCG was added to the sensitized cells simultaneously with the antigen (fig. 1). The residual ability of the cell to release histamine at a DSCG concentration of 19.6 μM (20.6% of maximal release) was found to be no greater than at a DSCG concentration of 49 μM (21.0%). Thus in this concentration range the inhibitory effect of DSCG reaches a plateau at

TABLE 2

Effect of varying the antigen concentration on DSCG inhibition in vitro from rat peritoneal mast cells of anaphylactic histamine release

Final Antigen Conc.	Final DSCG Conc.	Percent Histamine Release	Percent Inhibition by DSCG (20 μM)
μg/ml	μM		
20	0	20.6 ± 2.8[a]	
20	20	4.7 ± 1.0[b]	77
40	0	20.0 ± 2.2[a]	
40	20	3.6 ± 0.6[b]	80
80	0	21.3 ± 2.9[a]	
80	20	4.0 ± 0.5[b]	81

[a] There was no significant difference among these values by Student's *t* test. Each is the mean ± S.E.M. of four experiments, each run in duplicate.

[b] There was no significant difference among these values by Student's *t* test. Each is the mean ± S.E.M. of four experiments, each run in duplicate.

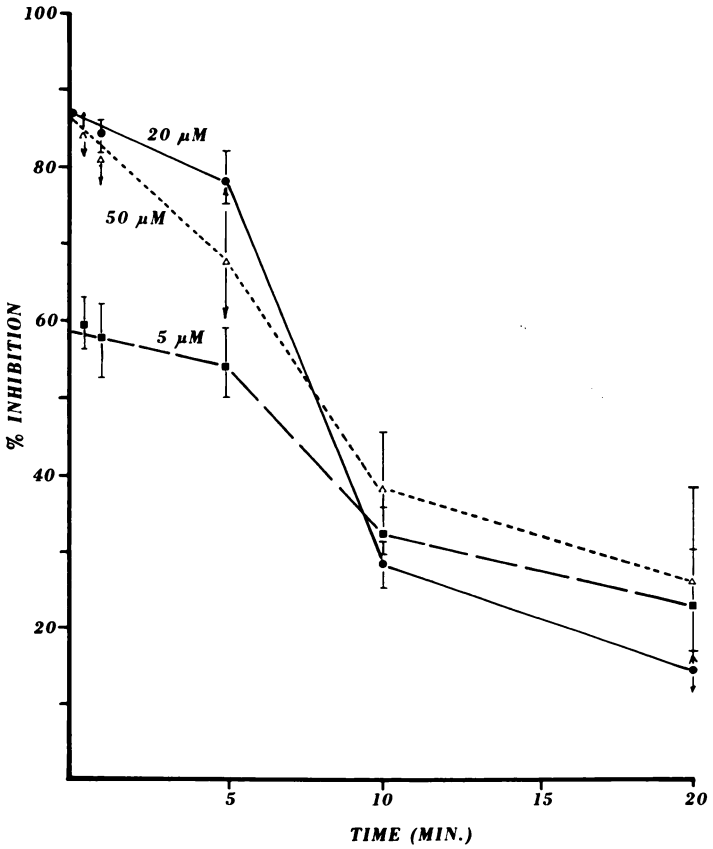

FIG. 2. Effect of preincubation of sensitized peritoneal cells with DSCG for various times before challenge. DSCG was added to the sensitized cell suspension and at various times ovalbumin was added to aliquots and the incubation was continued for five minutes. This was done in the presence of 5, 20 and 50 μM DSCG. Each point on the curve is the mean of three or four duplicate assays (±S.E.M.). ■, 5 μM, ●, 20 μM, △, 50 μM.

about 80% inhibition. However, at 490 μM DSCG, there was 100% inhibition.

Effect of varying the antigen concentration on the inhibition by DSCG. In preliminary experiments we had established that the optimum release of histamine from rat peritoneal cells was obtained when the ovalbumin concentration was 20 μg/ml during the challenge. From the data presented in table 2, it can be seen that increasing the ovalbumin concentration to 40 or 80 μg/ml resulted in no more or less release than at 20 μg/ml. Moreover, DSCG (20 μM) was equally effective in inhibiting the histamine release from the peritoneal cells at all three concentrations of ovalbumin.

Effect of preincubation with DSCG. As can be seen in figure 2, if the sensitized mast cells were incubated for up to 20 minutes with DSCG before adding the ovalbumin there was a loss of DSCG inhibition and a restoration of the sensitivity of the cell to antigen. The reappearance of sensitivity to antigen or reactivation occurred slowly during the first 5 minutes. Between 5 and 10 minutes there was a rapid reactivation of the release process and then between 10 and 20 minutes the rate of reactivation slowed down. Although there was a marked difference between the initial inhibition at 5 μM and that at 20 or 50 μM DSCG, there was little or no difference in the course of the reactivation or in the final level of reactivity at 20 minutes. If the cells were preincubated for one hour with 20 μM DSCG, there was complete restoration of sensitivity to antigen.

Reestablishing DSCG inhibition. To see whether it was possible to reestablish the DSCG inhibition of the histamine release in reactivated mast cells, sensitized peritoneal cells were incubated with various concentrations of DSCG between 1 and 20 μM for 20 minutes, then ovalbumin was added with fresh 20 μM DSCG. Figure

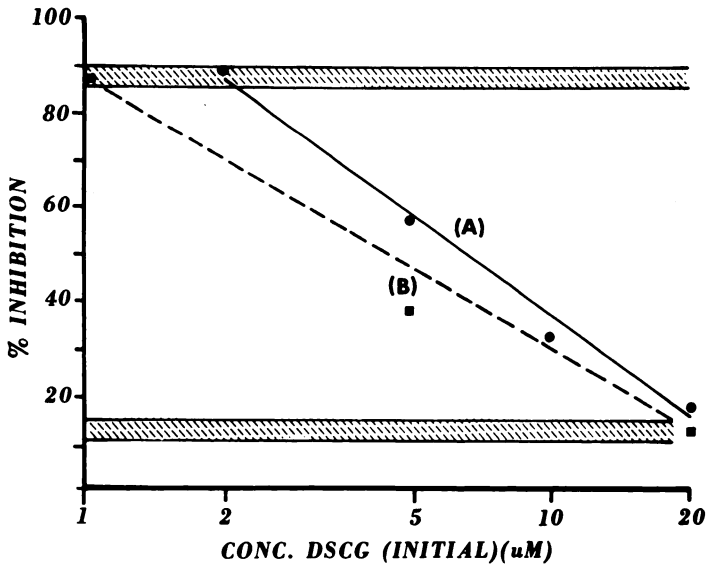

FIG. 3. Effect of preincubation of sensitized cells in various concentrations of DSCG upon subsequent addition of DSCG with the ovalbumin. Sensitized, washed peritoneal cells were incubated with various concentrations of DSCG between 2 and 20 μM for 20 minutes. Then 20 μM DSCG was added with the ovalbumin and the incubation continued for 5 minutes (line A). In another group of experiments the cells were centrifuged after the initial incubation in 1 to 20 μM DSCG for 20 minutes and resuspended in fresh HBSS, and then 20 μM DSCG was added with the ovalbumin (line B). The shaded area at the top of the graph represents the inhibition seen when 20 μM DSCG was added simultaneously with the antigen. The shaded area at the bottom of the graph represents the inhibition seen when the cells were preincubated with 20 μM DSCG for 20 minutes before adding antigen.

3, line A, is a plot of the amount of reinhibition that was achieved under these conditions. One can see that the extent of the reinhibition with a second dose of 20 μM DSCG was inversely related to the amount of DSCG present in the first incubation. If 20 μM DSCG was present in the first incubation, the addition of 40 or 60 μM DSCG with ovalbumin challenge resulted in a reinhibition of only 37 and 33%, respectively.

To see whether the reactivated cells would regain their sensitivity if they were transfered to fresh buffer, the following experiment was done. Mast cells were sensitized and incubated for 20 minutes with various concentrations of DSCG. They were then centrifuged, resuspended in fresh HBSS and challenged in the presence of 20 μM DSCG (fig. 3, line B). There is no statistical difference between lines A and B.

Another experiment was done to determine if the DSCG in the medium after 20 minutes of incubation with mast cells was still able to inhibit cells that had never been exposed to DSCG. The sensitized mast cells were incubated with 10 μM DSCG as above for 20 minutes. The suspension was centrifuged and the cells were discarded. The supernatant with the DSCG was used to suspend fresh, sensitized mast cells and ovalbumin was added immediately. The inhibition was the same in this case as when aliquots of the same cells in buffer were challenged with ovalbumin and 10 μM DSCG.

Attempts were made to identify chemically any changes in the DSCG in the medium after the incubation of sensitized mast cells. By both specific ultraviolet absorption and by fluoresence (Cox *et al.*, 1970), we could see no change in this DSCG from that of DSCG freshly dissolved in HBSS at the same concentration. However, the sensitivity of these techniques is such that it would be hard to identify changes in a small amount of DSCG.

Discussion

As Morse *et al.* (1969) and Orr *et al.* (1970) have shown, DSCG *in vivo* inhibits reaginic release of histamine and slow-reacting substance of anaphylaxis from mast cells. In this report we provide evidence that DSCG will act *in vitro* on the anaphylactic histamine release from rat peritoneal mast cells. As shown in figure 1, the inhibition of reagin-dependent histamine release was dependent on the DSCG concentration between

0.5 and 20 μM. This corresponds approximately to the effective dose range found for inhibition of rat reaginic passive cutaneous anaphylaxis, *i.e.*, between 0.2 and 40 μmol per kg b.wt. when injected i.v. with antigen (Cox *et al.*, 1970; D. J. Herzig and L. J. Robichaud, unpublished observations). Because increasing the concentration of antigen relative to the amount of DSCG did not overcome the inhibition by DSCG, it appears that the effects of DSCG are not on the antigen or directly competitive with it for the same receptor. This is at some variance with the results of Orr *et al.* (1971), who studied the time-dependent inhibition by DSCG of histamine release induced by 48/80 from mast cells, where it was possible to overcome the DSCG inhibition by increasing the 48/80 concentration. This may possibly be explained by the fact that anaphylactic- and 48/80-induced release are different (Johnson and Moran, 1971; Goth *et al.*, 1971; Bloom and Chakravarty, 1970) and that as the 48/80 concentration is increased, the release seems to involve increasing loss of cytoplasmic elements from the cell by a cytotoxic mechanism. However, since it was possible to inhibit histamine release induced either anaphylactically or by 48/80, presumably DSCG acts on the part of the release process that is common to both.

If the sensitized cells were incubated with the DSCG before the ovalbumin was added, then the inhibitory activity of DSCG disappeared and the cells again released histamine upon anaphylactic challenge (fig. 2). Both the rate and the extent of the reactivation were independent of the concentration of DSCG. The data in figure 3 show that after the cells have become reactivated they can be reinhibited by fresh DSCG, but only to an extent inversely proportional to the extent of the original inhibition. Since there was no measurable change in the DSCG in the incubation medium, apparently the effect of DSCG is on or in the cell.

The data presented may be interpreted as: DSCG induces the formation of an inhibitor of histamine release, the concentration of which is dependent on the DSCG concentration. Continued incubation of the cell, even in the presence of DSCG, results in a destruction of this inhibitor. The rate and extent of its destruction is not dependent on the amount of inhibition. The maximum amount of this inhibitor, that can be formed in the cell, is limited, and the addition of fresh DSCG can, therefore, stimulate the elaboration of only that amount of inhibitor from precursor that was not used up initially.

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