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INHIBITION BY CROMOGLYCATE OF HISTAMINE RELEASE FROM RAT PERITONEAL MAST CELLS INDUCED BY MIXTURES OF DEXTRAN, PHOSPHATIDYL SERINE AND CALCIUM IONS

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1 Dextran releases histamine from rat peritoneal mast cells in the presence but not in the absence of phosphatidyl serine (PS). With PS (10 μg/ml) present the effect of dextran was concentration-dependent in the range of 0.2-6 mg/ml.

2 PS releases little histamine on its own but mixed with dextran (6 mg/ml) produces a graded effect in the range of 0.3-10 μg/ml.

3 The combination of dextran and PS releases no histamine in a medium containing less than 0.1 mM calcium. As the calcium concentration is increased, release occurs and a maximum is reached at calcium 1 mM; at higher concentrations release falls off sharply.

4 Histamine release by the combination of dextran (6 mg/ml), PS (10 μg/ml) and calcium (1.8 mM) is inhibited by cromoglycate in the same range (1-30 μM) that inhibits anaphylactic histamine release from rat peritoneal cells.

5 Inhibition by cromoglycate occurred with all dextran concentrations tested (0.2-15 mg/ml) but high concentrations of PS (30 and 100 μg/ml) overcame the effect of cromoglycate (2 and 10 μM). Calcium concentrations above 1 mM augmented inhibition by cromoglycate 2 and 10 μM.

Introduction

Injection of dextran into rats produces an anaphylactoid reaction which is characterized by hyperaemia, pruritis and oedema of the face, ears and paws (Voorhees, Baker & Pulaski, 1951). Feldberg & Talesnik (1953) suggested that these symptoms were the result of histamine released locally in areas of the body rich in mast cells but Parratt & West (1957) demonstrated that the effects were due largely to released 5-hydroxytryptamine.

Dias da Silva & Lemos Fernandes (1965) showed that dextran released histamine *in vitro* from rat isolated peritoneal cells and Goth, Adams & Knoohuizen (1971) found that the release of histamine from these cells was greatly augmented when phosphatidyl serine (PS) was also added. This phospholipid also enhances anaphylactic histamine release from this *in vitro* cell preparation (Mongar & Svec, 1972).

The antiallergic drug cromoglycate inhibits the extravasation of albumin-bound dye (blueing reaction) into rat skin produced by dextran (Assem & Richter, 1971) and reduces histamine release into the peritoneal cavity caused by intraperitoneal injection of dextran (Hanahoe, Holliman, Gordan & Wieczorek, 1972).

This paper defines the optimal conditions required to produce histamine release from rat peritoneal cells *in vitro* with mixtures of dextran and PS. The inhibition of this histamine release by cromoglycate in the presence of different concentrations of dextran, PS and calcium is also described.

Part of this work has already been communicated to the British Pharmacological Society (Garland & Mongar, 1973).

Methods

Female Canterbury Ash Wistar rats were used to provide peritoneal mast cells. Each rat was first

anaesthetized with diethyl ether and decapitated and then 10 ml cold saline (154 mM NaCl) containing heparin 100 μg/ml (Boots Pure Drug Co. Ltd.) was injected intraperitoneally. After the peritoneum had been massaged for approximately 1 min, the fluid was withdrawn through a mid-line abdominal incision. The peritoneal washings from at least five rats were pooled and kept on ice until used. The washings were centrifuged at 250 *g* for 6 min, the supernatant was discarded and the cell pellet resuspended in cold Tyrode (3 ml per rat) of the following composition (mM): NaCl 137; $NaHCO_3$ 12; NaH_2PO_4 0.3; KCl 2.7; $MgCl_2$ 1.0; dextrose 5.6. All chemicals were of Analar quality. Normally the $CaCl_2$ concentration was 1.8 mM but in experiments where calcium concentration was varied this was achieved by adding $CaCl_2$ stock solution (BDH 1M) both to the cell suspension and the releasing mixture which had been prepared in calcium-free Tyrode solution. In some experiments where the high calcium concentration used produced a precipitate, the actual activity of free calcium ions was kindly measured by Dr R. Hyde using a liquid membrane calcium ion electrode (Orion Research). Alternatively, the phosphate and bicarbonate in the medium were replaced by HEPES (*N*-2-hydroxyethylpiperazine-*N*-2 ethanesulphonic acid) 12 mM supplied by BDH, which allowed the calcium concentration to be raised to 30 mM without precipitation or change of pH. The cell suspension (0.5 ml aliquots in duplicate) was pre-incubated at 37° C for 2 min before 0.5 ml of a mixture of dextran and/or PS in Tyrode was added. For the inhibitor studies cromoglycate was included in this mixture. Incubation at 37° C was continued for 5 min, maximal release of histamine having been found in this time. The reaction was then stopped by adding 4 ml ice cold Tyrode and each sample was centrifuged at 250 *g* for 6 minutes. The supernatant was decanted, the pellets resuspended in 5 ml Tyrode and boiled for 10 min to release residual histamine.

Released and residual histamine were assayed on the atropinized guinea-pig ileum (Boura, Mongar & Schild, 1954) and the released histamine was expressed as % total and corrected for spontaneous release in Tyrode alone (normally about 2%). The total histamine content ranged between 2-6 μg per 0.5 ml aliquot of cells. Cromoglycate (0.1-1,000 μM) was found to influence neither spontaneous histamine release nor the histamine assay. Disodium cromoglycate was equilibrated with atmospheric moisture when it was converted to the penta-hydrate with a molecular weight of 602.

The dextran which was used had a molecular weight of 110,000 and was supplied as a 6% solution in 0.9% saline (Dextraven, Fisons Ltd.).

PS was supplied by Koch-Light Ltd., and was shown by Mongar & Svec (1972), who carried out an amino acid analysis on hydrolysed material, to contain about 80% serine with traces of other amino acids as impurities.

Results

Conditions for histamine release by dextran

Dextran alone at concentrations up to 15 mg/ml released little more histamine than that released spontaneously, but when mixed with PS 10 μg/ml the release was enhanced greatly and was concentration dependent in the range, dextran 0.2 to 6 mg/ml (Figure 1).

PS in concentrations up to 30 μg/ml had no effect on spontaneous histamine release but 100 and 300 μg/ml caused a small increase in spontaneous release. When mixed with dextran 6 mg/ml, PS produced a concentration-dependent histamine release with a maximum at 10 μg/ml (Figure 2).

Foreman & Mongar (1972a) showed that the potentiation by PS of dextran-induced histamine release from rat peritoneal mast cells required calcium ions. These observations have been confirmed and extended by the present studies in which the dextran (6 mg/ml) and PS (10 μg/ml) concentrations were kept constant whilst the calcium concentration of Tyrode solution used to resuspend the cells was varied from 0.1 to 30 mM. Calcium concentrations up to 10 mM were found not to affect spontaneous histamine release. A graded increase in dextran/PS-induced histamine release was observed up to a peak value with calcium 3 mM. Higher concentrations (10 and 30 mM) depressed release (Table 1a).

In Tyrode solution, calcium concentrations greater than 3 mM produced a slight precipitate, probably of $Ca(HCO_3)_2$, as HCO_3^- is the major binding species at pH 7.8. However, the loss of soluble calcium was small. Measurements with a calcium ion electrode showed that the nominal 10 and 30 mM calcium solutions actually contained 9.7 and 29 mM calcium after applying suitable activity coefficient corrections. A potentially more serious complication was the effect of precipitation on pH. When the calcium concentration of ungassed Tyrode solution was raised to 30 mM, the pH fell from 7.8 to 7.4. This was probably due to calcium ion competing with hydrogen ion for the anionic species; consequently at high calcium concentrations the concentration of free anion species will decrease and the concentration of hydrogen ion will increase. These complications

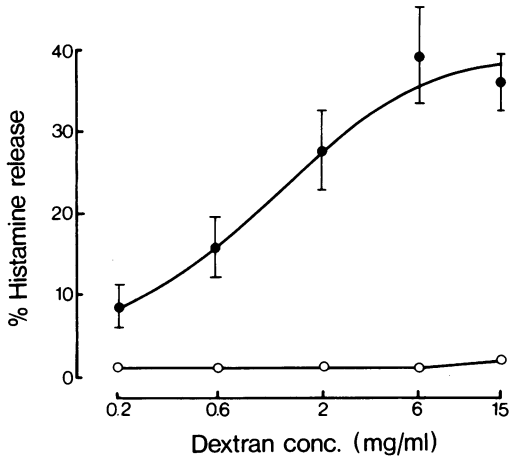

Fig. 1 Effect on histamine release from rat peritoneal mast cells of different concentrations of dextran in Tyrode solution containing calcium 1.8 mM: ○, without phosphatidyl serine (PS) (mean values from two experiments in duplicate); and ●, mixed with PS 10 μg/ml (mean values ±s.e. from three experiments in duplicate). All values have been corrected for spontaneous histamine release in Tyrode.

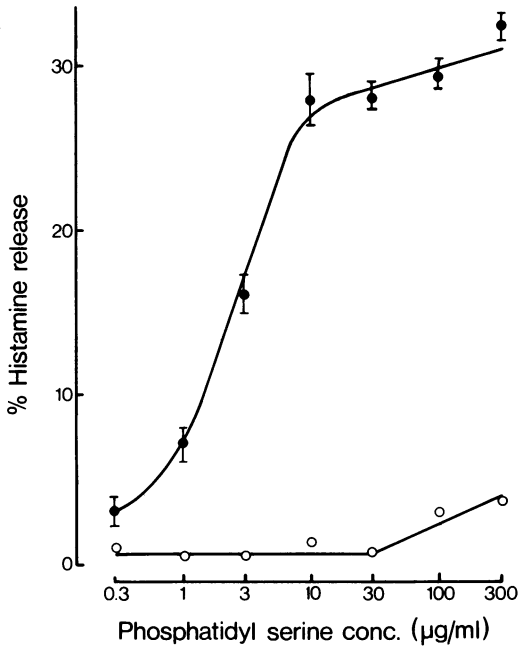

Fig. 2 Effect on histamine release of different concentrations of phosphatidyl serine in Tyrode containing calcium 1.8 mM: ○, without dextran and ●, mixed with dextran 6 mg/ml. All values have been corrected for spontaneous histamine release in Tyrode solution.

were avoided by using an incubation medium in which the buffer was HEPES 12 mM (pH 7.8). This allowed calcium concentration to be raised to 30 mM without precipitation or pH change. The calcium concentration-effect curve produced in HEPES buffered Tyrode reached a peak at 1 mM with depression of histamine release occurring at higher concentrations (Table 1b).

Inhibition by cromoglycate

Near optimal conditions for histamine release (dextran 6 mg/ml and PS 10 μg/ml in Tyrode solution containing calcium at 1.8 mM) were chosen to study the effect of the antiallergic drug cromoglycate. It produced marked inhibition in this system (Table 2) and the concentration-effect curve was almost coincident with that for the previously reported inhibition of anaphylactic histamine release (Kusner, Dubnick & Herzig, 1973; Garland, 1973). These results contrast with those for inhibition of 48/80-induced histamine release which required approximately 100 times more cromoglycate (Figure 3).

The mechanism of inhibition of histamine

Table 1 Histamine release, expressed as % total, by dextran (6 mg/ml) and phosphatidyl serine (10 μg/ml).

	Calcium concentration					
	0.1 mM	*0.3 mM*	*1 mM*	*3 mM*	*10 mM*	*30 mM*
(a) Release	0.5	6.2	31.1	42.0	29.8	14.0
± s.e.	0.5	1.9	2.2	2.2	7.1	2.3
(b) Release	1.35	22.1	51.8	40.1	25.9	5.5
± s.e.	0.1	1.0	0.9	0.9	0.7	0.5

Calcium concentration was varied in: (*a*) bicarbonate-buffered Tyrode (mean of three experiments in duplicate); (*b*) HEPES-buffered Tyrode (mean of five experiments in duplicate). The results are corrected for spontaneous release (2.9% ± 0.26 in (*a*) and 2.4% ± 0.25 in (*b*)).

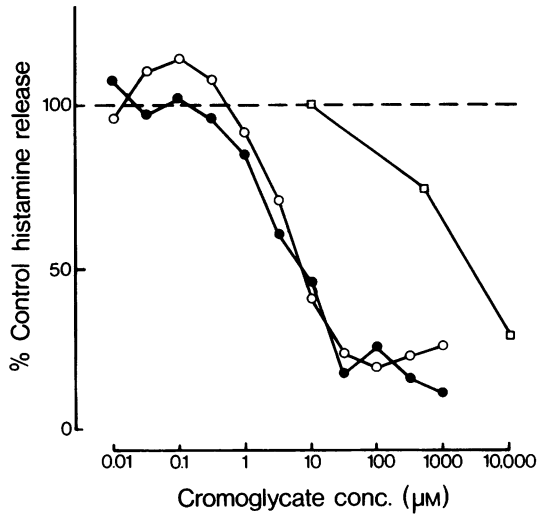

Fig. 3 Comparison of the concentration effect curves for inhibition by cromoglycate of histamine release from rat peritoneal cells induced by: ○, antigen challenge (ovalbumin 1 μg/ml final concentration) of actively sensitized rat peritoneal cells (mean values from six experiments in duplicate) (after Garland, 1973); ●, dextran 6 mg/ml plus phosphatidyl serine 10 μg/ml in bicarbonate-buffered Tyrode containing calcium 1.8 mM (mean values from six experiments in duplicate); □, compound 48/80 (10 mg/ml) which caused 12-70% histamine release in the absence of cromoglycate (mean values from three experiments in duplicate).

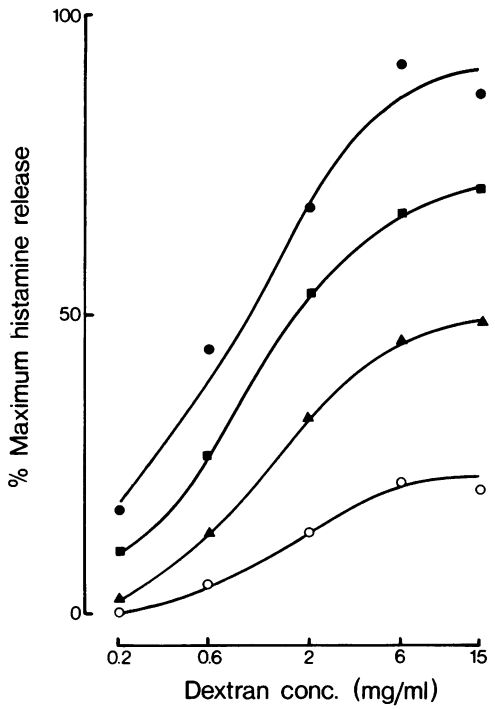

Fig. 4 Percentage histamine release from rat peritoneal mast cells by different concentrations of dextran mixed with phosphatidyl serine 10 μg/ml in Tyrode solution containing calcium 1.8 mM: without inhibitor, ●; and with cromoglycate 2 μM, ■; 10 μM, ▲; 300 μM, ○; included in the mixture. Values are the mean of two experiments (in duplicate) and have been corrected for spontaneous release and expressed as a percentage of the maximum release in each experiment.

release by dextran was investigated by varying in turn the concentration of dextran, PS and calcium in the presence of cromoglycate 2, 10 and 300 μM.

The effect of varying dextran concentration

The concentrations of dextran used were 0.2-15 mg/ml, PS being kept at 10 μg/ml and calcium at 1.8 mM. Progressively increasing inhibition with increasing cromoglycate concentration was observed at each dextran concentration used and inhibition was not surmounted by increasing dextran concentration to 15 mg/ml (Figure 4).

The effect of varying phosphatidyl serine concentration

The PS concentration was varied over the range 0.3-300 μg/ml while dextran (6 mg/ml) and calcium (1.8 mM) were kept constant (Figure 5). The lower concentrations of cromoglycate, 2 and 10 μM, caused the PS concentration response curve to shift to the right. The slope of the control curve

Table 2 Histamine release (mean of six experiments in duplicate) in the presence of 0.01-1,000 μM cromoglycate expressed as % control release (100%) in the absence of inhibitor.

	Cromoglycate concentration (μM)										
	0.01	*0.03*	*0.1*	*0.3*	*1*	*3*	*10*	*30*	*100*	*300*	*1,000*
Release	107	97	101	94.6	84	59.5	42.5	16.2	24.6	14.7	10
± s.e.	3.5	3.1	3.2	3.8	6.4	4.1	5.1	2.5	3.2	3.5	1.4

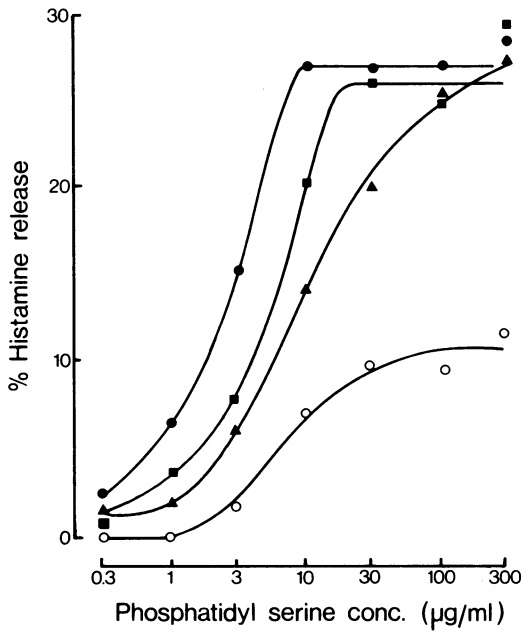

Fig. 5 Percentage histamine release from rat peritoneal mast cells by different concentrations of phosphatidyl serine mixed with dextran 6 mg/ml in Tyrode containing calcium 1.8 M: without inhibitor, •; and with cromoglycate 2 μM, ▪; 10 μM, ▲; 300 μM, ○ included in the mixture.

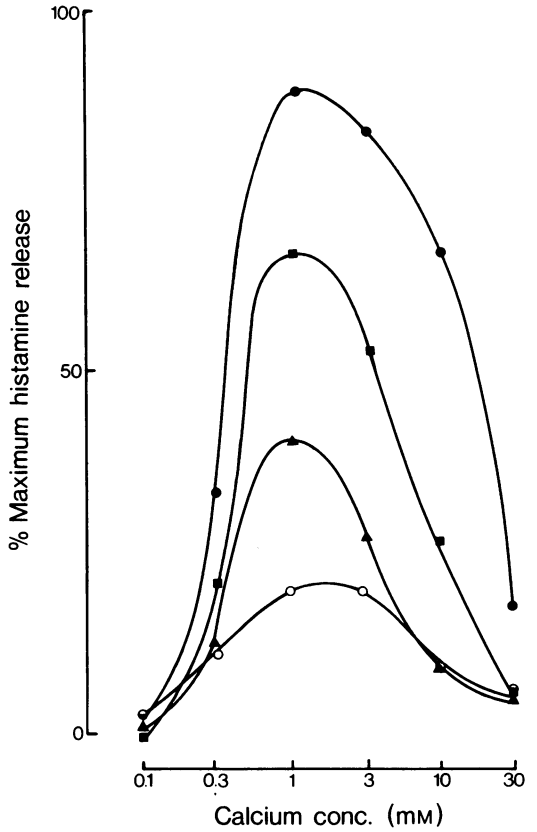

Fig. 6 Percentage histamine release from rat peritoneal mast cells by different calcium concentrations added to mixtures of dextran 6 mg/ml and phosphatidyl serine 10 μg/ml in Tyrode solution: without inhibitor, •; and with cromoglycate 2 μM, ▪; 10 μM, ▲; 300 μM, ○ included in the mixture. Values shown are the mean of five experiments (in duplicate), two with bicarbonate-buffered Tyrode and three with HEPES-buffered Tyrode and are expressed as a percentage of the maximum release in each experiment.

and those obtained in the presence of cromoglycate 2 and 10 μM did not differ significantly (9.7, s.e. 0.4; 10.0, s.e. 0.5; 10.2, s.e. 0.3; respectively). Inhibition of histamine release was clearly overcome by increasing the PS concentration. The higher concentration (300 μM) of cromoglycate depressed both the slope (4.5, s.e. 0.4) and maximum of the control curve.

The effect of varying calcium concentration

Figure 6 shows the effect on histamine release of varying the calcium concentration between 0.1-30 mM without inhibitor and in the presence of cromoglycate 2, 10 and 300 μM. Inhibition by cromoglycate could be detected at calcium concentrations of 0.3 mM and above. When the release in the presence of cromoglycate was expressed as a percentage of the control release at each calcium concentration, it was observed that inhibition by cromoglycate 2 and 10 μM became significantly greater ($P \leqslant 0.05$) as calcium concentration was increased from 1 to 10 and 30 mM (Table 3). Such a relationship was no longer seen when cromoglycate 300 μM was used.

Discussion

These experiments confirm the observations that release of histamine from rat peritoneal cells by dextran requires the addition of PS (Goth & Knoohuizen, 1962, and Goth *et al.*, 1971). Under optimum conditions release was usually greater than 25% of the total histamine present and was reproducible from experiment to experiment (Table 1, Figures 1 and 2). The values for histamine release have not been normalized so the low standard errors shown represent the error both between occasions as well as within individual experiments. The threshold concentration of PS

was less than 1 μg/ml and a maximum effect was obtained at 10 μg/ml (Figure 2). This range is similar to that for augmentation of anaphylactic histamine release in this system (Mongar & Svec, 1972). In contrast, addition of PS was not required for dextran to release histamine *in vitro* from other rat tissues, e.g. skin (Beraldo, Dias da Silva & Lemos Fernandes, 1962) or mesentery (Goth, 1961). Moreover PS does not appreciably augment antigen-induced release from sensitized rat mesentery or lung (Mongar & Svec, 1972). The augmentation by PS is limited to peritoneal cells and the absolute dependence upon added PS is a property peculiar to dextran-induced histamine release.

Dias da Silva & Lemos Fernandes (1965) claimed that, in their experiments, dextran alone, at 37°C, released histamine from such cells and attributed the failure reported by Goth & Knoohuizen (1962) to the low incubation temperature (20-24°C) which they used. The present experiments were all performed at 37°C, while those reported by Baxter (1972), who obtained essentially similar results, were done at 25°C. It seems, therefore, that the failure of dextran alone to release appreciable amounts of histamine from rat isolated mast cells is not attributable to the temperature of the incubation medium.

The augmentation by PS of dextran-induced histamine release was found to be calcium dependent (Foreman & Mongar, 1972a). The present experiments confirm this finding, optimal release being obtained with calcium 1 mM. When the calcium concentration was increased above the physiological range histamine release was inhibited by 28-50% in calcium 10 mM and 67-90% in calcium 30 mM. In this way, histamine release induced by dextran plus PS resembles antigen-induced and ATP-induced histamine release from rat mast cells (Foreman & Mongar, 1972b; Dahlquist, Diamant & Krüger, 1973). One possible explanation of the bell-shaped dose-response curves is that calcium has two sites of action. At one, lower concentrations (0.1-1.0 mM) are essential for anaphylactoid histamine release while at the other increasing concentrations results in increasing interference with a different stage of the release process.

Histamine release from rat peritoneal cells is inhibited by the same concentration of cromoglycate whether the release is caused by dextran plus PS or by an anaphylactic reaction suggesting attack upon a pathway common to the two release mechanisms.

In the dextran system, the agonist causing release may be considered to be dextran, PS or calcium ions; each produces a concentration-dependent effect if the other two components are present at fixed concentrations (compare Figs 1-3) and all three components are necessary for the release. Inhibition of dextran-evoked release by cromoglycate was not overcome by increasing the dextran concentration, the slope and maximum of the concentration-effect curve being progressively depressed by increasing concentrations of cromoglycate, suggesting non-competitive antagonism (Arunlakshana & Schild, 1959). PS had a different effect. The two lower concentrations (2 and 10 μM) caused a parallel shift of the concentration-effect curve and a much higher concentration (300 μM) depressed the slope and the maximum. This result could be explained as a non-competitive interaction between cromoglycate and PS with 'spare' receptors for the phospholipid accounting for the initial parallel shift (Stephenson, 1956).

Inhibition by low concentrations of cromo-

Table 3 % Inhibition of histamine release (mean of five experiments in duplicate ± s.e.) by cromoglycate at different calcium concentrations.

Cromoglycate concentration	*Calcium concentration* 1 mM	3 mM	10 mM	30 mM
2 μM	24.0 ±6.3	36.2* ±5.5	61.5** ±4.9	67.9** ±10.8
10 μM	50.7 ±15.4	68.2* ±10.4	85.8* ±6.7	92.0** ±4.8
300 μM	76.1 ±8.0	76.2^{NS} ±8.5	86.8^{NS} ±4.0	89.0$^{N.S.}$ ±6.0

Inhibition by cromoglycate 2 and 10 μM was found to be significantly greater (by paired *t*-test) in Tyrode containing 3, 10 and 30 mM calcium than in 1 mM calcium, * $P < 0.05$; ** $P < 0.01$. Such an effect was not found (N.S.) when cromoglycate 300 μM was present.

glycate (2 and 10 μM) was augmented by raising the calcium concentration of the incubation medium (Table 3). This augmentation may be the result of an addition of the effects of cromoglycate and calcium ions or it may be an interaction in solution between cromoglycate and calcium ions, or finally it may be that cromoglycate inhibits histamine release by a calcium-requiring mechanism. An indication of a similar effect is given by analysis of the results of Marshall (1972) who used cromoglycate to inhibit 48/80-induced histamine release from a similar cell population.

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