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Effects of Calcium and Phosphatidyl Serine in Rat Mast Cell Reaction to Dextran (39376)

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Dextran releases histamine from normal rat mast cells in a reaction that resembles anaphylaxis (1-4). The reaction requires Ca^{2+} in the medium. Using Sprague-Dawley rat mast cells we found that exogenous Ca^{2+} was necessary to initiate the reaction (5), in contrast to its requirement only in the final stage of anaphylactic histamine release from human leukocytes (6). Substantial release from mast cells of most rat strains by dextran or antigen also requires the addition of phosphatidyl serine (PS), as shown by Goth *et al.* (2) and confirmed by others (3, 7, 8). Release from Wistar rat cells does not require added PS (9), however, nor does the human leukocyte reaction (6). The mechanism of histamine release evidently varies in some degree, depending on the type of cells and the releasing agent employed. The present study was made to explore further the roles of Ca^{2+} and PS in histamine release from Sprague-Dawley rat mast cells by dextran.

Methods and materials. Mixed peritoneal cells were obtained from 300-g male Sprague-Dawley rats as previously described (3, 10). The peritoneal cavity was irrigated with 15 ml of normal buffer, or (sometimes, if some of the cells were to be used in low-Ca solutions) with Ca-free buffer, with 5 μg/ml of heparin. The normal buffer was Krebs-Ringer phosphate, pH 7.0, with 1.0 m*M* Ca^{2+}, no Mg^{2+}, and 1 mg/ml of crystalline bovine albumin. For each experiment, the irrigating solution from two or three rats was collected in an ice-cold tube. The cells were sedimented in one or more portions, and resuspended in buffer solution(s) (15 ml/donor rat), without heparin. Replicate 1.5-ml samples[1] of the pooled cells were pipetted into siliconized tubes, into final volumes of 2.5 ml of the desired solutions, and equilibrated for 10 min at 25°. Histamine release from the cells was induced by adding dextran, together with a small amount of PS, in 1.0 ml of the appropriate buffer solution, and incubating for 15 min at 25° (10). Final concentration of dextran (Pharmacia Fine Chemicals, average mol wt 2×10^6) was 1.0 mg/ml, and of PS (Nutritional Biochemicals Corp) 7 μg/ml, except when stated otherwise. PS was added to the control tubes without dextran, and was used in all experiments unless there is a statement to the contrary. In a few experiments, the cells were obtained from rats that had been immunized with egg albumin (EA) and pertussis vaccine (12), and EA 10^{-4} mg/ml (with PS) instead of dextran was used as the releasing agent. Except for the differences in treatment being studied, all cell samples in each experiment were treated alike. Release from duplicate samples by dextran differed, on average, by only $2 \pm 2\%$ of the respective release values ($n = 27$). Histamine determinations were made fluorometrically (16) as previously described (3). Degree of histamine release was assessed by dividing the amount released by the amount released plus the amount retained in the cells.

Results. Effects of Ca^{2+} on histamine

[1] Counts on samples of mixed peritoneal cell suspensions (11) showed about 10^5 mast cells per 1.5 ml, and histamine content was about 2 μg/1.5 ml. The number of cells used per tube was not critical, however, as diluting the suspension 10-fold did not affect the degree of histamine release by dextran (3). Neither did adding 3×10^{-6} *M* histamine with the dextran (3) or adding dextran in the form of the supernatant solution from a previous release reaction (12) affect release. Ficoll- or albumin-gradient fractionation (13) often left the mast cells poorly responsive (3), not corrected by adding back the other cells. Therefore, after it had been established that virtually all the histamine was in the mast cells (3), and that pure mast cells responded in the same manner as unfractionated cells (7, 13-15), we used the mixed cells without fractionation.

release. Dextran released 0 ± 2% of the histamine from cells that had been transferred to Ca-free medium, compared with 46 ± 8% in medium containing 1.0 m*M* Ca^{2+} (mean ± SD, $n = 4$). For comparison, net release values obtained with cells from immunized rats by using the specific antigen EA as the releasing agent, were 13 ± 11% with Ca-free buffer and 31 ± 19% with 1.0 m*M* Ca^{2+} ($n = 4$). Thus, only the release by dextran was completely dependent on exogenous Ca^{2+}.[2] The levels of Ca^{2+} (1.0 m*M*) and PS 10 μg/ml) used in these experiments had been determined to give approximately maximal release under the experimental conditions. Changing the pH inversely affected the required level of Ca^{2+}. Ca^{2+} levels of 0.1 m*M* or less were completely ineffective in supporting release by dextran. EDTA was not used in conjunction with the Ca-free medium.

Ca^{2+} and cell desensitization. The decrease in period-2 histamine release by dextran (with 1 m*M* Ca^{2+}) due to the cells having been exposed to dextran (instead of buffer) in the presence of various levels of Ca^{2+} in period 1, is shown in Table I (last column). The decrease, reflecting the degree of cell desensitization to dextran, was slight when period-1 Ca^{2+} level was 0.1 m*M* or less. With 0.3 m*M* Ca^{2+} in period 1, desensitization was considerable but incomplete, and with 1.0 m*M* Ca^{2+} it was virtually complete as previously shown (5).

Influence of Ca^{2+} on cell integrity. Simply placing the cells for 15–30 min in Ca-free medium at 25° caused them to leak abnormal amounts of histamine (14 ± 5%, compared with 2 ± 1% in normal medium, $n = 13$), and they could not be returned completely to their original state of responsiveness by adding back Ca^{2+}. Table I shows that equilibration of the cells with low-Ca buffers in period 1 caused them to release less histamine after Ca^{2+} had been restored (period 2, first column). In three additional experiments, the mean reduction in release due to prior exposure of the cells to Ca-free medium at 25° was 35% of respective release values. Effects of exposure to low-Ca solutions at 37° were greater than at 25°; effects at 0° were relatively slight.

TABLE I. EFFECT OF Ca^{2+} CONCENTRATION ON HISTAMINE RELEASE AND CELL DESENSITIZATION BY DEXTRAN (1 mg/ml).[a]

Period 1		Period 2: Ca^{2+} 1.0 m*M*	
		Histamine release by dextran (net)	
Ca^{2+} Concn (m*M*)	Histamine release by dextran (net) Percentage of cell histamine	After buffer in period 1 Percentage of cell histamine	Decrease due to dextran in period 1[b] Percentage of cell histamine
0	1	11	0
0.01	0 (1)[c]	13 (15)	2 (1)
0.03	0 (0)	13 (18)	0 (3)
0.10	0 (1)	18 (24)	1 (3)
0.30	21 (32)	24 (32)	18 (26)
1.00	60 (39)	28	28

[a] Replicate samples of pooled cells were equilibrated with buffers with various Ca^{2+} levels. Then representative samples were treated with dextran (for 10 min at 25°) in period 1. Net histamine release by dextran (over that by the corresponding buffer alone) was assessed from determinations on the supernatant solutions. The same cell samples (whether exposed only to buffers or to dextran solutions in period 1) were washed twice with cold Ca-free buffer, equilibrated with 25° 1.0 m*M* Ca^{2+} buffer, and challenged (for 15 min) with dextran in period 2. PS used in all tubes.

[b] Compared with values in column immediately to the left.

[c] Results of a second experiment are shown in parentheses.

Time required to remove effect of Ca^{2+}. Duplicate cell samples that had been harvested in low-Ca buffer and equilibrated with buffers of various Ca^{2+} levels were treated, respectively, with dextran dissolved in the same buffer used for the cells and with dextran in Ca-free buffer. The additions of dextran in Ca-free buffer diluted the Ca^{2+} in the cell medium by 40%, and in each case released the amount of histamine expected with the final (diluted) Ca^{2+} level, rather than that expected with the original level, as shown in Fig. 1. Similarly, with cells harvested in 1.0 m*M* Ca^{2+}, and using a volume of dextran that gave 60% dilution (not shown), the Ca^{2+}-dilution was in large de-

[2] We have recently compared the Ca^{2+} and PS dependence of histamine release from Sprague–Dawley rat mast cells by the following agents: dextran, antigen, anti-IgE, concanavalin A, the ionophore A23187, somatostatin, compound 48/80, protamine, and polylysine. Dependence of the different agents on both Ca^{2+} and PS in the medium differed greatly, but only dextran was completely dependent on exogenous Ca^{2+}.

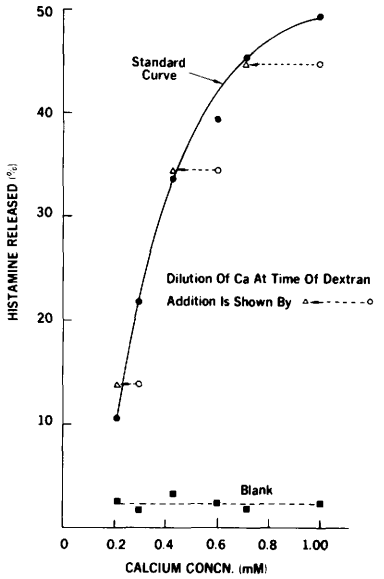

FIG. 1. Effect of diluting Ca^{2+} in cell medium at time of adding dextran. Ca-free dextran solution was added to cell suspensions containing three different levels of Ca^{2+}, and the resulting histamine release in each case is plotted vs the original Ca^{2+} level of the medium (○), and vs the Ca^{2+} level after the Ca-free dextran addition (△). In each case, the plot using the final Ca^{2+} levels falls very close to the standard curve (determined by adding dextran solution containing the same Ca^{2+} level as the cell suspension in each case). The blank values (■) show release by buffer alone.

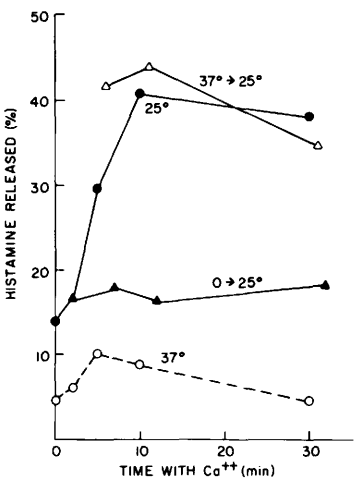

FIG. 2. Effect of preincubation time of cells with 1.0 mM Ca^{2+} on histamine release by dextran. The cells had been in Ca-free buffer at 25° for 15–30 min. Results are shown for preincubation of the cells with Ca^{2+} and challenge with dextran at 25° (-●-); preincubation and challenge at 37° (-○-); preincubation at 0° and challenge after 2 min at 25° (-▲-); and preincubation at 37° and challenge after 2 min at 25° (-△-). Mean results of three 25°-experiments and of four 37°-experiments are shown. Individual results in each group were similar in trend.

gree (usually about 75%) reflected in the resulting decrease in histamine release. Even when cells were concentrated in a small volume of 1.0 m*M* Ca^{2+} buffer and treated with Ca-free dextran in relatively large volume, so that the Ca^{2+} level of the medium was thereby reduced below 0.1 m*M* (a level at which no release occurs from equilibrated cells), virtually no net release occurred (2 expts.). Since histamine release by dextran at 25° starts within 30 sec and is largely completed in 2 min (12), these results show that dilution of Ca^{2+} in the medium promptly altered cell responsiveness almost to the full extent predicted by the degree of dilution (in contrast to the slowness of restoration of the Ca^{2+} effect considered below).

Time required to restore Ca^{2+} effect. Cells that had been in Ca-free buffer for 15–30 min at 25° released relatively small amounts of histamine when Ca^{2+}, 1 m*M*, and dextran were added to them simultaneously (Fig. 2, zero time). A period of preincubation of the cells with the Ca^{2+} was required, before addition of dextran, in order for dextran to produce maximal release. At 25°, a 10-min preincubation was required (Fig. 2). If the cells were preincubated with Ca^{2+} at 0°, and then changed back to 25° for 2 min before challenge with dextran, there was virtually no increase in histamine release as preincubation time increased. When preincubation was at 37°, maximal release at 37° was reached after only about 5 min, although release was small, as always at 37° (10). If the 37°-preincubated cells were changed back to 25° for 2 min before dextran was added, release was greater, and the earlier peak observed at 37° was in part maintained. Thus, a significant part of the effect of Ca^{2+}, depended on the Ca^{2+} reaching, by a temperature-dependent process, cell sites that were not immediately available.

Effects of other cations. In Ca-free medium, Sr^{2+}, Ba^{2+}, and Mg^{2+} in concentrations up to 10 m*M* each consistently failed to replace Ca^{2+} in supporting histamine release by dextran (Table II). Spontaneous

TABLE II. EFFECT OF VARIOUS DIVALENT CATIONS ON HISTAMINE RELEASE FROM THE CELLS BY DEXTRAN.[a]

Cation added	Concn (m*M*)	Histamine release by dextran (net)[b] (% of cell histamine)
None		1 ± 0
SR^{2+}	1.0	1 ± 0
	3.0	0 ± 0
	10.0	1 ± 0
Ba^{2+}	1.0	1 ± 1
	3.0	1 ± 0
	10.0	0 ± 0
Mg^{2+}	1.0	0 ± 1
	3.0	1 ± 1
	10.0	−1 ± 2
Ca^{2+}	1.0	23 ± 15
	3.0	14 ± 10
	10.0	6 ± 3

[a] The solutions were Ca-free except where Ca^{2+} is shown. PS concn 10 μg/ml. Tris-HCl, 18 m*M*, was substituted for phosphate of normal buffer. Cations were added as chlorides. Cells were preincubated with salt solutions for 10 min before dextran was added.

[b] Excess of release by dextran over that by the corresponding salt solution alone, ± SD. Mean results of five experiments, each made with replicate samples of pooled cells, are shown.

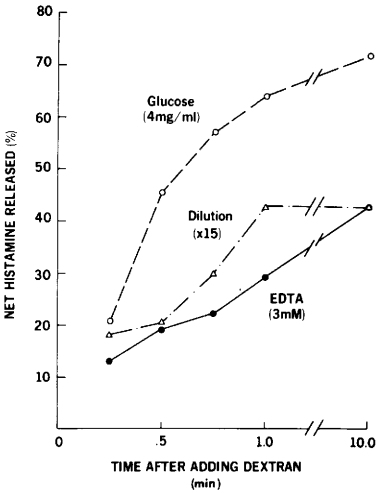

FIG. 3. Reduction in histamine release by dextran due to addition of EDTA or glucose, or to 15-fold dilution of cell suspension with normal buffer. Different cell preparations were used in the three experiments. Additions were made at the indicated times after addition of dextran, and histamine release was determined after 15-min incubation with dextran.

release of histamine into the medium (without dextran) was unaffected or decreased by the cation additions. When used in conjunction with various Ca^{2+} levels, Mg^{2+} 1.0 m*M* was without effect or somewhat inhibitory to release by dextran (not shown). Mn^{2+}, 0.1–1.0 m*M*, did not support release by dextran without Ca^{2+}, and strongly inhibited the release by dextran with Ca^{2+}. Na^{+} and K^{+} were not required for release, as satisfactory release was obtained in 0.18 *M* Tris-HCl buffer with no other cations present except Ca^{2+}. Ouabain, 10^{-5}–10^{-3} *M*, was without effect on release by dextran in normal buffer and in K-free buffer.

Stoppage of release by EDTA or glucose, or by dilution of cell medium. As shown in Fig. 3, adding EDTA (3 m*M*) can stop histamine release (in normal buffer) short of completion, if the EDTA is added to the cells within 1 min after the addition of dextran. Early addition of glucose (4 mg/ml) also diminished the amount of histamine released, as did 15-fold dilution of the cell-dextran mixture with normal buffer. The same results were obtained when the added solutions contained 10 μg/ml of PS, showing that dilution of PS was not the basis of the stoppage.

Effect of PS on histamine release. PS was used in the experiments discussed thus far in concn of 7–10 μg/ml, which was nearly as effective as much higher levels. PS alone did not release histamine. It increased release by dextran from 7 ± 5% up to 52 ± 10% (n = 8), as expected from previous results. We found the effect of PS to be virtually the same, whether it was added to the cells 10 min before dextran or together with the dextran (49% vs 50%, n = 3). Likewise, adding the PS 30–45 min before dextran did not further increase release. Thus, the PS effect was very rapidly established, reasoned as follows. Without PS, dextran releases little histamine, but still rapidly desensitizes the cells (by about 50% in 2 min) (12, 17). Therefore, dextran added with PS would have released much less histamine than dextran added 10 min or longer after PS, if PS had not become effective very rapidly.

Washing the cells twice with buffer after adding PS but before adding dextran, decreased release by 50–90% (compared with release from similarly washed cells to which PS was added after washing, rather than before). This indicated that some of the cell-associated PS could be washed away. We attempted to substitute phosphatidyl glyc-

erol (which does not seem to have been tested) for PS in the release reaction, but found it without definite effect in concns up to 28 μg/ml.

Interaction of Ca^{2+} and PS. As Ca^{2+} concn was varied from 0.2 m*M* up to 3 m*M* in different tubes, histamine release by dextran increased to a maximum and then declined, whether or not PS was used. Use of PS (7–14 μg/ml) in the experiments increased release, as already noted, but also shifted the peak of release to higher Ca^{2+} levels. Thus, whereas release without PS was greater at 0.5 m*M* than at 1.0 m*M* Ca^{2+} in five of six experiments (mean of 11% vs 9%, $p = 0.15$), release with PS was greater at 1.0 m*M* Ca^{2+} in all cases (mean of 56% at 0.5 m*M* vs 60% at 1.0 m*M*, $p = 0.03$), suggesting that the addition of PS increased the level of Ca^{2+} required to give maximum release. Binding of Ca^{2+} by the PS (mole for mole) would not have significantly changed the Ca^{2+} level in the medium.

Discussion. This study showed that histamine release from Sprague–Dawley rat mast cells by dextran was completely dependent on exogenous Ca^{2+} in the medium, in contrast to anaphylactic release from the same cells. Others have also noted the incomplete dependence of anaphylactic release on Ca^{2+} in the medium (18). We demonstrated that the degree of cell desensitization by dextran increased progressively as Ca^{2+} in the medium increased, in amplification of our previous study (5). In the virtual absence of Ca^{2+} in the medium, spontaneous leakage of histamine from the cells increased, and the release response could not be entirely restored to normal by adding back Ca^{2+}.

We found no effective substitute for Ca^{2+} in the release reaction, in contrast to the finding of Foreman and Mongar (19, 20) that Sr^{2+} (1–10 m*M*) and Ba^{2+} (5–10 m*M*) considerably increased release from their cells by antigen without Ca^{2+}. Also, we found that Sr^{2+} and Ba^{2+} (and Mg^{2+}) usually decreased (rather than increased) spontaneous histamine leakage. Whether these differences in results are due to differences in rat strain or releasing agent, or to subtle differences in techniques, is not clear.

PS enhances release from rat mast cells by increasing the rate of release, rather than by prolonging the duration of release (7, 8) by decreasing the rate of desensitization (12, 17). Although histamine release and cell desensitization by dextran are completely dependent on Ca^{2+} in the medium, release in small degree and desensitization at the usual rate occur without PS (12, 17). Several workers (2, 8, 15) have postulated that PS enhances release by activating Na^+, K^+-ATPase, although they found, as we did in this study, that ouabain did not affect the release (8, 17).

We previously suggested that (in addition to other actions of Ca^{2+}) Ca^{2+} and PS might act together at the cell surface, as the dextran receptor function in Sprague–Dawley cells seemed to depend on exogenous Ca^{2+} (5). Others have considered a similar mechanism in anaphylactic release from rat mast cells (19). Possibly the present observations support this concept. Although Ca^{2+} required 10 min to become maximally effective in supporting release when added back to Ca-deficient cells (and its immediate effect was small), dilution of Ca^{2+} in the cell medium was promptly and substantially reflected in a decrease in release by dextran. Likewise, addition of PS to the cell medium was immediately fully effective in increasing release. Thus, both Ca^{2+} and PS produced obligatory effects at quickly accessible cell sites. Interaction of the two factors was shown by their synergism, and probably by an effect of PS on the Ca^{2+} level required for maximum release by dextran. With cells that do not require PS for release, the effects of Ca^{2+} may differ from those observed here (21).

The delayed and incomplete restoration of histamine release associated with the readdition of Ca^{2+} to Ca-deficient Sprague–Dawley cells, complicates experiments involving exposure of cells to Ca-free solutions, such as the present experiments, and our previous studies of cell activation and desensitization without Ca^{2+} (5). However, other investigators, using cells from other sources, do not seem to have encountered these problems (6, 17, 18).

The presently observed stoppage of dextran-induced histamine release by EDTA is not surprising (except perhaps for the promptness of the effect), assuming that Ca^{2+} is required in the final stage of the release, as it is in the human leukocyte-

antigen reaction (6, 22). The stoppage by adding glucose (presumably due to competitive inhibition of dextran binding (1)) and by diluting the cells (probably due to the reduction in dextran concn) was unexpected, however, by analogy with the human leukocyte reaction, as that reaction proceeds in the absence of the releasing agent once the cells have been activated (6).

Summary. Histamine release from Sprague–Dawley rat mast cells by dextran was completely inhibited by the absence of exogenous Ca^{2+} (in contrast to release from the same cells by antigen). Also, spontaneous leakage of histamine from the cells increased in the absence of Ca^{2+}, and cell responsiveness was not completely restored by readding Ca^{2+}. We found no effective substitute for Ca^{2+} in the release reaction. Ca^{2+} was not maximally effective immediately when added back to Ca-deficient cells, but almost the full effect of diluting Ca^{2+} in the medium (which decreased release) and of adding PS (which increased release) were very rapidly established, suggesting that both Ca^{2+} and PS might act (in part) at superficial cell sites. Release from activated cells could be stopped short by adding glucose or by diluting the cell-dextran mixture with normal buffer, as well as by adding EDTA, which deserves further study.

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