EFFECTS OF COMPOUND 48/80, CHYMOTRYPSIN AND ANTI-SERUM ON ISOLATED MAST CELLS UNDER AEROBIC AND ANAEROBIC CONDITIONS

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The anaphylactic histamine release from guinea-pig and rat lung tissues was completely or largely depressed by oxygen lack, respiratory inhibitors and uncouplers of oxidative phosphorylation when the incubation medium was devoid of glucose (1-7). Similar inhibition has been found of compound 48/80-induced histamine release from rat lung tissue and of degranulation of rat mesentery mast cells (6, 8, 9). Since these inhibitions were largely overcome by the presence of glucose (4-9), and phlorizin (10) and 2-deoxyglucose (7) inhibited these glucose-dependent release of histamine, glycolysis should be considered to take part in supplying the energy required for these processes. Chymotrypsin is also known to cause histamine release (11) and degranulation of mast cells (11, 12), and the latter action is also blocked by anoxia and glucose has a preventive effect on this block.

There are a number of works demonstrating the histamine-releasing or degranulating effect of compound 48/80 and some other agents including anti-serum (13) on isolated mast cells or mixed cell suspension taken from rat peritoneal fluid. However, the observations relevant to glucose dependency are all based on the experiments with excised tissues but not with isolated cells, because an anaerobic experiment with isolated mast cells seems to have not yet been successful. Thus, Keller and Beeger (14) reported that the introduction of nitrogen gas into the cell suspension resulted in a complete destruction of mast cells; Moran *et al.* (15) were unable to demonstrate an inhibition of 48/80-induced amine release from mixed peritoneal cells by incubating in their supposedly oxygen-free atmosphere in the absence of glucose.

In the present experiments, with isolated mast cells, the glucose-dependent degranulation by compound 48/80, α -chymotrypsin and anti-serum is demonstrated under nitrogen anaerobiosis with success, and the effects of some inhibitors including phlorizin and 2-deoxyglucose are studied. Observations in aerobiosis are also described.

METHODS AND MATERIALS

Isolation of mast cells: Rats of both sexes weighing 150-300 g were bled by severance of carotid arteries. Five milliliters of buffered physiological solution [NaCl 154 mM,

KCl 2.7 mM, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.9 mM and phosphate buffer pH 7.0 ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 67 mM and KH_2PO_4 67 mM) 10% v/v] containing 0.1% w/v bovine serum albumin (BSA) were injected into peritoneal cavity and gentle massage of the gut through the abdominal wall was performed for 90 seconds. The abdominal wall was then incised along the midventral line and the fluid from the abdominal cavity collected with pipette. This was carefully layered over 2 ml of an ice-cold 44% buffered solution of BSA and centrifuged at $110 \times g$ for 20 minutes. The albumin solution was then removed through a syringe needle, avoiding contamination with interface cells, diluted to 6 ml with the buffered solution and centrifuged at $250 \times g$ for 5 minutes. The deposit in the bottom of the tube was washed by repeating once more the last operation, finally resuspended in the buffered solution and left standing in ice water until used. This suspension contained mast cells more than 90%, practically all of the cells being well preserved.

Experiment on isolated mast cells in aerobiosis: In general experiments, 0.9 ml of mast cell suspensions containing 50,000–100,000 cells was incubated for 15 minutes before addition of 0.1 ml of degranulating agent, compound 48/80, α -chymotrypsin or anti-rat serum—the total volume of 1 ml—in open glass tubes. Incubation was continued for 15 minutes for compound 48/80 and 30 minutes for the other two agents. Immediately after the incubation, the mast cells were fixed with 4% formaldehyde and transferred onto a cover-glass pasted on the bottom side of a round holed slide-glass. Morphological changes of mast cells were observed using an invert-type phase contrast microscope (Olympus PMB, $480\times$) and the percentage of degranulated mast cells was determined. When the effects of metabolic inhibitors were studied, the mast cell suspensions were preincubated with them for 15 minutes. In each experiment control tubes served for counting percentage of spontaneously degranulated mast cells.

Experiment on isolated mast cells in anaerobiosis: After the trial of available techniques, the following method was found to be most suitable for the present purpose. 1.7–1.8 ml of the buffered physiological solution not containing BSA were incubated in a Carrel's flask in which a small piece of yellow phosphorus was present. Through a rubber stopper of the flask two syringe needles were thrust and through a longer needle—the other served as outlet—nitrogen gas was bubbled for at least 15 minutes. After the needle for gassing was withdrawn above the surface of the solution, 0.1 ml of mast cell suspension (about 100,000 cells) was added *via* the needle. Thereafter nitrogen gas was allowed to flush through the space above the surface of the solution. Degranulating agent was then introduced *via* the needle to give a required concentration and a total volume of 2.0 ml. Formaldehyde fixative was introduced by the same way. This technique was successful in making an anaerobic system without mechanical damage to the mast cells.

Glucose, when tested, was present in the incubation medium from the beginning of the incubation. But, in some experiments, the mast cell suspensions were preincubated with metabolic inhibitors for 15 minutes in the absence of glucose in air phase, thereafter 0.1 ml of the suspension was added *via* the needle to the already nitrogenated

incubation medium containing glucose and the inhibitors. Further procedures for incubation and determination of the percentage of degranulated mast cells were the same as described above.

Experiment on mast cells in mesentery pieces: In the aerobic experiments, the technique of incubation for mesentery pieces was the same as used for the suspension of isolated mast cells. After the incubation the mesentery pieces were fixed in a 4% lead subacetate in 50% ethanol with 1% acetic acid for 24 hours and stained with 0.5% toluidine blue for 20-30 minutes, and then transferred through ethanol (3 changes) and xylene (2 changes) and mounted on slides. Usually three pieces of mesentery were used in each test. The procedure used for microscopic determination of percentage of degranulated mast cells was the same as that described by Norton (16) and Kawamoto (17).

In anaerobic experiments, again the same Carrel's flasks were used as for the anaerobic experiments with isolated mast cells. But, in this case the mesentery pieces were present in the flask from the beginning of incubation and nitrogen gas was bubbled through the incubation fluid for at least 15 minutes at a constant speed of 4 liters per hour. Thereafter the needle for gassing was withdrawn so that it was just above the surface of the incubation fluid. Yellow phosphorus was not used. In all these experiments controls also ran parallel.

Histamine assay: Histamine was assayed on the atropinized (atropine sulfate 1.5×10^{-7} M) guinea-pig ileum, and specificity was demonstrated by mepyramine block (mepyramine maleate 1.2×10^{-7} M). The histamine content of isolated mast cells or cell fragments was determined as follows: A few drops of distilled water were added to the cell precipitate separated by centrifugation, and after freezing and thawing, the broken cells were extracted with buffered solution. This was then tested directly for its histamine content. Histamine values are given as the base.

Bovine serum albumin (BSA): Since preliminary experiment showed that a commercial BSA (Fraction V, Armour) is too acidic in a high concentration and affords a deleterious effect on the responsiveness of mast cells isolated with it, albumin was fractionated from fresh bovine serum with $(\text{NH}_4)_2\text{SO}_4$ by a modification of McMeekin's method (18, 19). Bovine serum was diluted with equal volume of water in a beaker placed on a magnetic stirrer and added with $(\text{NH}_4)_2\text{SO}_4$ through a cellophane bag until the concentration was 2.05 M. The precipitated globulin was removed by filtration and the filtrate containing albumin fraction was adjusted to the original volume with 2.05 M $(\text{NH}_4)_2\text{SO}_4$ and the concentration of $(\text{NH}_4)_2\text{SO}_4$ increased to 3.0 M by adding the required amount of $(\text{NH}_4)_2\text{SO}_4$ through a cellophane membrane. The precipitate formed between 2.05 and 3.0 M $(\text{NH}_4)_2\text{SO}_4$ was dissolved in water, dialyzed in running water to remove $(\text{NH}_4)_2\text{SO}_4$ and lyophilized for use. The BSA sample contained small amounts of α - and β -globulin but not γ -globulin, and a 44% solution in physiological buffer showed pH 6.3 giving satisfactory result for the present purpose.

Rabbit anti-rat serum: Anti-rat serum was obtained from rabbits which had received nine intravenous injections of 1 ml rat serum every other day and exsanguinated 10

days after the last injection. The serum was kept frozen until used.

Compound 48/80 was kindly supplied by Burrough Wellcome & Co., Tuckahoe, New York. α -Chymotrypsin (bovine pancreas, cryst. A grade) and 2-deoxyglucose (A grade) were obtained from California Corporation for Biochemical Research, phlorizin from Mann Research Laboratories, and ouabain (*g*-strophanthin, cryst.) and glucose (D-glucose) from E. Merck AG. Other chemicals used were obtained from standard commercial sources.

RESULTS

1. Properties of isolated mast cells

Isolation of biologically intact mast cells has been achieved by a differential centrifugation of rat peritoneal fluid over a concentrated solution of high molecular substances, i.e. a fraction of appropriate molecular weight of 'Ficoll' (a polysaccharide of high molecular weight, AB Pharmacia, Uppsala) (20) and BSA (14, 21-23). Keller and Beeger (14) reported that mast cells lost a considerable amount of histamine and heparin when isolated with concentrated BSA solution, in contrast to only a little loss with 'Ficoll' solution, in the same procedure, although there was no evidence for the difference of the responsiveness of mast cells.

The mast cells isolated by the present method employing a BSA preparation of a low acidity contained $24.1 \pm 2.7 \mu\text{g}$ histamine per 10^6 cells. This was almost the same

FIG. 1. The preservation effect of BSA on the responsiveness of isolated mast cells to the degranulating action of compound 48/80 ($0.5 \mu\text{g/ml}$). Percentages of degranulation are shown of the mast cells preserved at 20°C for indicated periods of time in the buffered medium, with (●—●) and without (○—○) BSA (0.1%). Spontaneous degranulation in the presence (●---●) and in the absence (○---○) of BSA is also indicated. Abscissa: time of preservation after isolation. Ordinate: degranulated mast cells in % of the total.

FIG. 2. Dose-response curves for compound 48/80-induced degranulation of isolated mast cells which have been preserved at 20°C for 4 hours in the presence (●—●) and in the absence (○—○) of BSA (0.1%). Mast cells were incubated with compound 48/80 in the glucose-free buffered medium under air. The spontaneous degranulation deducted.

value as determined on mast cells, not isolated, in rat peritoneal cell suspension, and within the range of values reported by other workers (15, 24-28). This indicates that histamine did not leak out of mast cells in any substantial amount during the present isolation procedure. The content of histamine in mast cells was not decreased at least for six hours, when they were suspended in the buffered medium containing 0.1% of BSA and placed in ice water. The mast cells preserved in this way degranulated in response to such a low concentration of compound 48/80 as 0.1 $\mu\text{g}/\text{ml}$ at 37°C, the same vulnerability of the mast cells isolated with 'Ficoll' solution (20). When the medium was devoid of BSA, the responsiveness to compound 48/80 of the mast cells rapidly decreased at room temperature (20°C) although at 0°C did not change for at least six hours. This is in a striking contrast to intact responsiveness of mast cells preserved in the medium containing 0.1% of BSA at 20°C (Figs. 1 and 2). Therefore, isolated mast cells were preserved in the BSA containing medium at 0°C before the experiments.

FIG. 3. Histamine release and degranulation of isolated mast cells elicited by compound 48/80. Histamine release computed in % of total histamine content. Mast cells were incubated with compound 48/80 in the glucose-free buffered medium under air.

Degranulation by compound 48/80 of isolated mast cells showed a dose-response curve similar to that of histamine release, though histamine release was less marked than degranulation in terms of percentage (Fig. 3). This suggests that these phenomena are two different aspects of the same reaction of mast cells, since incompletely affected mast cells may not lose all histamine contained.

2. Degranulation of mast cells under aerobic condition

a. Time-courses and features of degranulation due to compound 48/80, chymotrypsin and anti-serum

The mast cell suspension was maintained at 37°C in an incubator attached to the microscope. When compound 48/80 was added to a concentration of 0.5 $\mu\text{g}/\text{ml}$,

within a few seconds mast cells became swollen and vacuoles appeared. The cytoplasm of mast cell seemed as if it were bubbling, many vacuoles being formed successively and fused each other. With such an alteration in cytoplasm, granules became easily distinguishable from each other, and conspicuous extrusion of these granules out of the margin of the cell ultimately occurred. The cell membrane was not affected and its continuity well retained, so far as could be observed. A part of the extruded granules adhered to the cell membrane, and the others dispersed into the surrounding medium with Brownian movement. On extruding, the granules enlarged. The whole process of the reaction in one mast cell was a rapid process lasting only a few seconds. The

observation for a considerably long period of time demonstrated that the reaction was all or none in character. A few mast cells remained always unaffected even in a high concentration of compound 48/80. A smaller percentage of mast cells was affected by falling concentration of compound 48/80. The process of degranulation was rapid, independently of the concentrations of the degranulating agent. Degranulated mast cells were easily distinguishable from those unaffected ones by the adherence of extruded granules on the cell membrane and by peculiar alterations of cytoplasm. The feature of the cells was almost unchanged after the fixation with 4% formaldehyde.

There were some differences between degranulation of mast cells by α -chymotrypsin or anti-serum and that by compound 48/80. The changes of mast cells by α -chymotrypsin and anti-serum in a high concentration ended rather rapidly, but in a low concentration there was a considerably long lag phase before the reaction was evoked, although the reaction of individual cells, when once being elicited, proceeded very rapidly and ended in a short while. Therefore, individual cells became degranulated successively in an explosive manner. The morphological changes caused by α -chymotrypsin were similar to those by compound 48/80, but clearly distinct from those by anti-serum. By anti-serum the formation of vacuoles was much more prominent and almost all the space in cytoplasm was occupied by large vacuoles. Furthermore, there could often be observed one or two blister-like configurations protruding beyond the margin of the cell. Finally, the cells whose nucleus was clearly visible in transparent cytoplasm became surrounded by the extruded granules adhering to each other. The granules extruded by anti-serum were larger than those by other agents; this was another effect peculiar to anti-serum. Fig. 4 shows the time-courses of degranulation of mast cells by these

FIG. 4. Time-course curves for degranulation of isolated mast cells induced by compound 48/80, α -chymotrypsin and anti-serum. Mast cells were incubated with these degranulating agents under air in the glucose-free medium (O_2) and under nitrogen in the glucose (5.6 mM) containing medium (N_2). Abscissa represents incubation time. A_1 : 48/80 0.5 $\mu\text{g}/\text{ml}$ (O_2); A_2 : 48/80 0.2 $\mu\text{g}/\text{ml}$ (O_2); a : 48/80 0.5 $\mu\text{g}/\text{ml}$ (N_2); B_1 : α -chymotrypsin 1,000 $\mu\text{g}/\text{ml}$ (O_2); B_2 : α -chymotrypsin 100 $\mu\text{g}/\text{ml}$ (O_2); b : α -chymotrypsin 100 $\mu\text{g}/\text{ml}$ (N_2); C_1 : anti-serum 50% (O_2); C_2 : anti-serum 10% (O_2); c : anti-serum 10% (N_2).

FIG. 5. Dose-response curves for degranulation of isolated mast cells induced by compound 48/80 (●—●), α -chymotrypsin (○—○) and anti-serum (×—×) under air in the glucose-free medium.

Influences of temperature and pH on histamine release from the mast cells induced by compound 48/80 were already described by other workers (28). Concerning degranulation of isolated mast cells by anti-serum, the influence of temperature has also been studied (13), while that of pH has not. Degranulation of mesentery mast cells by α -

FIG. 6. Effect of temperature on degranulating actions of compound 48/80, α -chymotrypsin and anti-serum under air in the glucose-free medium. ●—●: compound 48/80 0.5 μ g/ml; ○—○: α -chymotrypsin 100 μ g/ml; ×—×: anti-serum 10%; ○—○: spontaneous degranulation.

three agents in different concentrations. In view of different processes of degranulation, mast cells were incubated for 15 minutes with compound 48/80, and for 30 minutes with α -chymotrypsin or anti-serum before fixation.

Fig. 5 illustrates the dose-response curves of degranulation of mast cells by these three agents. The concentration of compound 48/80 required to cause submaximum effect was 0.5 μ g/ml, whereas those of α -chymotrypsin and anti-serum were 100 μ g/ml and 5% v/v respectively. Mast cells were not all affected by considerably high doses of any of these agents.

b. Effects of temperature and pH

FIG. 7. Effect of pH on degranulating actions of compound 48/80, α -chymotrypsin and anti-serum under air in the glucose-free medium. ●—●: compound 48/80 0.5 μ g/ml; ○—○: α -chymotrypsin 100 μ g/ml; ×—×: anti-serum 10%; ○—○: spontaneous degranulation.

chymotrypsin was dependent on pH (12), whereas on isolated mast cells the action of α -chymotrypsin has not been investigated in these respects.

In the present experiments on isolated mast cells, degranulating action of all of these agents was dependent on temperature and pH (Figs. 6 and 7). The optimum temperature required for the action of these agents was approximately 37°C. The inhibitory effect of low temperature was reversible, the sensitivity to these agents being fully restored on reincubation of the cells at 37°C, whereas the arrest of the reaction at 45°C was irreversible. The temperature effect on the action of anti-serum was somewhat different from that on the action of α -chymotrypsin in that the reaction caused by anti-serum was almost completely inhibited at 20°C, whereas the action of α -chymotrypsin was still more remarkable at the same temperature. An optimum pH for the action of α -chymotrypsin and anti-serum was approximately at 7.0. An optimum pH for the action of compound 48/80 was between 7.0 and 7.5. This is slightly more alkaline range than that reported by other workers (28).

It may be concluded that these phenomena of temperature and pH dependency of the degranulating action of these agents on isolated mast cells are fundamentally the same as those found in anaphylactic release of histamine from sensitized guinea-pig and rat lung tissues (29-34), and in compound 48/80-induced degranulation of mast cells in rat mesenteries (31, 32).

c. Effects of Ca^{2+} and Mg^{2+}

Influences of Ca^{2+} and Mg^{2+} on degranulation of isolated mast cells induced by compound 48/80, α -chymotrypsin and by anti-serum were studied and compared with those on degranulation of mesentery mast cells. Buffered solution used for isolation and washing of mast cells and for incubation medium were free of Ca^{2+} and Mg^{2+} . When anti-serum was tested, ethylenediamine-tetraacetate (EDTA) (1 mM) was added to chelate Ca^{2+} contained in the serum.

As shown in Fig. 8, degranulating actions of α -chymotrypsin (100 $\mu\text{g}/\text{ml}$) and anti-serum (10%) on both isolated mast cells and mesentery mast cells were clearly inhibited in the absence of Ca^{2+} . However, the presence of Ca^{2+} was not a prerequisite for degranulation by compound 48/80 of isolated mast cells, whereas it was

FIG. 8. Effect of Ca^{2+} on degranulating actions of compound 48/80 (0.5 $\mu\text{g}/\text{ml}$), α -chymotrypsin (100 $\mu\text{g}/\text{ml}$) and anti-serum (10%) on mesentery mast cells (A) and isolated mast cells (B), under air in the glucose-free medium. Unshaded blocks represent degranulation of mast cells in the presence of CaCl_2 (1 mM), and shaded blocks that in the absence of CaCl_2 . Dots represent individual values in separate experiments. When anti-serum was tested in the absence of Ca^{2+} , EDTA (1 mM) was previously added to the medium in order to chelate Ca^{2+} contained in the anti-serum. Spontaneous degranulation (below 5% in each experiment) deducted from all values.

essential for that of mesentery mast cells by the same agent. Similar finding of the effect of Ca lack was reported of histamine release by compound 48/80 from isolated mast cells (28), and from mast cells in peritoneal mixed cells (27). The presence of Mg^{2+} in the incubation medium was not essential for the degranulating action of any of these agents.

d. Effects of respiratory inhibitors and uncouplers of oxidative phosphorylation

Isolated mast cells were incubated in the buffered medium with or without glucose (5.6 mM). KCN to give a final concentration of 10^{-3} M, iodoacetate 10^{-3} M or 2,4-dinitrophenol (DNP) 10^{-4} M were introduced in the buffered medium 15 minutes before the addition of degranulating agents. Anti-serum used for this experiment was dialyzed to remove glucose as far as possible.

As shown in Fig. 9, degranulating actions of compound 48/80, α -chymotrypsin and anti-serum were all completely or largely blocked by these metabolic inhibitors when the medium was devoid of glucose. This inhibition by KCN or DNP was completely counteracted by the presence of glucose in the medium, while the inhibitory action of iodoacetate remained unaffected. These results are in agreement with the findings reported by other workers in tissue level experiments.

FIG. 9. Effect of respiratory inhibitors and uncoupler of oxidative phosphorylation on degranulating actions of compound 48/80 (0.5 μ g/ml), α -chymotrypsin (100 μ g/ml) and anti-serum (10%) under air. The presence and absence of glucose (5.6 mM) in the incubation medium represented as 'Glucose' and 'No glucose'.

1: Control, without inhibitor; 2: with DNP (10^{-4} M); 3: with KCN (10^{-3} M); 4: with iodoacetate (10^{-3} M).

Dots represent individual values in separate experiments. Spontaneous degranulation (below 5% in each experiment) deducted from all values. As for anti-serum see text.

3. Degranulation of mast cells under anaerobic condition

a. Influence of glucose

It has been found that under anaerobic condition degranulation of rat mesentery mast cells *in situ* and histamine release from rat tissues by compound 48/80 do not occur at all in the absence of glucose (8, 9). Similar findings were reported of anaphy-

TABLE 1. Effect of glucose on degranulation of isolated mast cells induced by compound 48/80 (0.5 $\mu\text{g}/\text{ml}$), α -chymotrypsin (100 $\mu\text{g}/\text{ml}$) and anti-serum (10%), under air (O_2) and under nitrogen gas phase (N_2). Figures in parentheses indicate the number of experiments.

Gas phase	Addition to medium	Degranulated mast cells (% , mean $\pm$ S.E.)		
		Compound 48/80	α -Chymotrypsin	Anti-serum
O_2	—	93 $\pm$ 1.7 (4)	94 $\pm$ 0.9 (4)	95 $\pm$ 0.4 (4)
"	Glucose, 5.6 mM	94 $\pm$ 1.3 (4)	95 $\pm$ 0.5 (4)	94 $\pm$ 1.3 (4)
N_2	—	5 $\pm$ 1.2 (8)	4 $\pm$ 0.7 (4)	84 $\pm$ 3.2 (4)
"	Glucose, 5.6 mM	77 $\pm$ 3.5 (8)	80 $\pm$ 4.9 (4)	86 $\pm$ 1.6 (4)

lactic release of histamine from guinea-pig lung tissue (1, 3-7), and more recently of degranulation by α -chymotrypsin of rat mesentery mast cells (12).

As shown in Table 1, in an air phase 93-95% of isolated mast cells were degranulated by compound 48/80 (0.5 $\mu\text{g}/\text{ml}$), α -chymotrypsin (100 $\mu\text{g}/\text{ml}$) and anti-serum (10%), independently of the presence or absence of glucose (5.6 mM) in the incubation medium. In nitrogen gas phase, when glucose was absent, this action of compound 48/80 and α -chymotrypsin was, however, completely inhibited to the same degree of spontaneous degranulation, while 84% of mast cells were degranulated by anti-serum. This inhibition was markedly counteracted by the presence of glucose (5.6 mM), i.e. degranulated mast cells reached 77-80% of the total. Anti-serum was previously dialyzed against distilled water for 48 hours, but glucose was unable to be reduced below one-third of the initial level. Therefore, about 0.2 mM of glucose will present in the reactant medium. The concentration of glucose required for counteracting the anoxic inhibition of degranulation was very low. Thus, 50% of mast cells were still degranulated by compound 48/80 (0.5 $\mu\text{g}/\text{ml}$) in the presence of only 0.14 mM glucose (approximately 1/40 of serum glucose level) and 0.28 mM was sufficient for the maximum counteraction (Fig. 10). This may possibly account for the absence of inhibitory action of oxygen lack on degranulation by anti-serum.

Time-course curves of glucose-dependent anaerobic degranulation caused by compound 48/80 and by α -chymotrypsin were quite similar type respectively to those obtained under aerobic condition, indicating that ultimate mechanism of disruption may not be different under aerobic and anaerobic conditions (Fig. 4).

These evidences obtained with isolated mast cells clearly demonstrate that anoxic degranulation of mast cells and possibly

FIG. 10. The effect of glucose on degranulation of isolated mast cells induced by compound 48/80 (0.5 $\mu\text{g}/\text{ml}$) under nitrogen gas phase. Spontaneous degranulation deducted from all values.

release of histamine from them as well rely entirely on exogenous glucose, apart from any influence of the tissue or other cells.

Uvnäs and Antonsson (12) observed in their experiments using mesentery mast cells that anoxic inhibition of the effect of α -chymotrypsin was counteracted by the presence of Mg^{2+} . However, I could not confirm such an effect of Mg^{2+} (1 mM) on any of isolated or mesentery mast cells.

b. Effects of phlorizin, 2-deoxyglucose and ouabain on glucose-dependent degranulation

Phlorizin is considered to inhibit the transport of glucose probably by binding a glucose carrier in the cell membrane (35), and 2-deoxyglucose to block glycolytic pathway by accumulating its phosphorylated product inside the cells (36). As is well known, ouabain inhibits active ion transport in some biological membranes. Csáky *et al.* (37) reported that this compound completely inhibited the transport of 3-methylglucose across the intestine possibly linking with active ion transport mechanism. More recently it has been demonstrated that phlorizin inhibits the histamine release from sensitized guinea-pig lung tissue induced by antigen and from non-sensitized rat lung tissue by compound 48/80, when elicited under nitrogen in the presence of glucose (10). A similar inhibitory effect has been reported of 2-deoxyglucose with *in vitro* anaphylactic histamine release from guinea-pig lung under the same conditions (7). Lastly, Yamasaki *et al.* (38) have found that ouabain is a more potent inhibitor which acts at the concentrations as low as 10^{-8} M– 10^{-5} M, of the 'glucose effect' counteracting the blocking effect of nitrogen anoxia on the anaphylactic release of histamine from guinea-pig lung tissue. In view of these findings, it is interesting to see the effects of phlorizin, 2-deoxyglucose and ouabain on degranulation of isolated mast cells elicited under nitrogen.

FIG. 11. Effect of phlorizin (1 mM) and 2-deoxyglucose (5.6 mM) on degranulation of isolated mast cells induced by compound 48/80 (0.5 μ g/ml), α -chymotrypsin (100 μ g/ml) and anti-serum (10%) under air (O₂) in the glucose-free medium and under nitrogen (N₂) in the presence of glucose (0.7 and 5.6 mM). Dots indicate individual values in separate experiments. Spontaneous degranulation deducted from all values.

Mast cells were incubated for 15 minutes under air phase in glucose-free medium containing phlorizin (1 mM) or 2-deoxyglucose (5.6 mM). 0.1 ml of this suspension was added to 1.7–1.8 ml of the already sufficiently nitrogenated and incubated medium in a Carrel's flask, containing one of these compounds at the same concentration and 0.7 mM or 5.6 mM of glucose. To this mixture containing mast cells and glucose and an inhibitor, 0.1–0.2 ml of a degranulating agent was introduced. After 15 or 30 minute additional incubation the reaction was stopped. These experiments were accompanied by control experiments carried out in the same way under air instead of nitrogen.

As indicated in Fig. 11 phlorizin and 2-deoxyglucose had no effect on degranulating action of compound 48/80, α -chymotrypsin or anti-serum under aerobic condition, but markedly inhibited glucose-dependent anaerobic degranulation due to any of these agents. The inhibition was generally more remarkable when the concentration of glucose was lower (0.7 mM) than higher (5.6 mM).

The influence of ouabain was investigated in the range of concentrations of 10^{-8} M to 10^{-5} M. Although its inhibitory effect on this glucose-dependent degranulation was observed in some cases, the reproducibility of the results was minimal. Therefore, it seems premature to conclude that ouabain has an inhibitory effect on this reaction as far as isolated mast cells are concerned.

DISCUSSION

Mast cells isolated by the present method may be considered to be biologically intact, since they contain the same quantity of histamine as mast cells in mixed peritoneal cells and have a high sensitivity to the degranulating agents. Mast cells obtained by a similar procedure using a BSA from commercial source, which gives a considerably high acidity when dissolved in the physiological buffer, prove to be deprived of the sensitivity, indicating that the selection of an appropriate albumin preparation is essential for the success of isolation. Isolated mast cells by the present method retained an intact vulnerability to the degranulating agents for a long time even at room temperature when the suspending fluid contained a small amount of BSA, while in the absence of BSA a rapid fall of the vulnerability occurred. This observation on protective action of albumin confirms the experiences by Uvnäs and Thon (20, 28) on mast cells isolated using 'Ficoll'.

The special interest of the present studies may be the success of anaerobic investigations on isolated mast cells. Uvnäs and Thon (28) and Moran *et al.* (15) were unable to demonstrate the anoxic inhibition of compound 48/80-induced histamine release from isolated mast cells or mixed peritoneal cells. It is conceivable that the net oxygen consumption of mast cells *per se* may be so small compared with that of coexisting tissue pieces such as mesentery pieces that a more strict condition will be required for testing the effect of anoxia on isolated mast cells without coexistence of tissue. In the present experiments, a small piece of yellow phosphorus was used to exclude a minute quantity of oxygen dissolved in the suspending medium. By this means an anaerobic

condition sufficient to block the degranulation of mast cells was attained. If yellow phosphorus may not be entirely harmless to mast cells, it is evident that it does not affect mast cells in any way as to alter the biological properties, since under such anaerobic condition they show a high sensitivity to the degranulating agents in the presence of glucose. Moreover, mast cells did not show any morphological change only in the presence of yellow phosphorus, and pH of the suspending medium remained unchanged. The applicability of Thunberg tube evacuated and filled with nitrogen was also tested. However, this was abandoned, since such a drastic change of the pressure variably reduced the sensitivity of mast cells.

The observations under aerobic condition indicate that degranulation caused by either of compound 48/80, α -chymotrypsin or anti-serum is dependent on pH and temperature. The response curves varied slightly with individual agents although the optimum pH and temperature were approximately the same with each other agent. Uvnäs *et al.* (39) reported some variance of pH or temperature-response curves of mast cell degranulation due to the degree of purification of *Ascaris* extract, a histamine liberator.

Ca^{2+} were essential for degranulating action of these three agents on mesentery mast cells *in situ*. On isolated mast cells, Ca^{2+} were required for the action of α -chymotrypsin and anti-serum, but as reported by Uvnäs and Thon (28), degranulation by compound 48/80 occurred freely even in the absence of Ca^{2+} . The reason why only degranulation by compound 48/80 of isolated mast cells is independent of Ca^{2+} is not clear at the stage of the present investigation. Since after the treatment at 56°C for 30 minutes anti-serum has lost the degranulating activity, Ca^{2+} may also be required for the action of complement. The reaction of mast cells caused by compound 48/80 is very rapid regardless of the concentration of this agent while the action of the other agents has a lag phase, and there are some differences among the morphological changes of mast cells caused by these agents. These facts suggest some variance with individual agents in the mode of action on mast cells, even though there is no difference in energy requiring metabolic aspects in the reaction as mentioned below.

The degree of degranulation under aerobic condition by any of these three agents did not vary with the presence or absence of glucose in the medium. Under anaerobic condition brought by the present method, compound 48/80 or α -chymotrypsin was, however, not able to degranulate isolated mast cells at all, provided that glucose was not contained in the medium. The presence of a small quantity of glucose (above 0.14 mM) in the medium counteracted this inhibition. Degranulation by anti-serum was not blocked by anoxia in the glucose-free medium. This may be accounted for by incomplete exclusion of glucose from the serum.

2-Deoxyglucose and phlorizin brought into contact with mast cells for 15 minutes before the addition of glucose largely inhibited the restoring effect of glucose on the anoxic block of degranulation by any of these degranulating agents. 2-Deoxyglucose is phosphorylated by hexokinase (40) but blocks a special transport system for glucose or glycolytic pathway by accumulating its phosphorylated product, 2-deoxyglucose-6-

phosphate inside the cell (36, 41). Wick *et al.* (36) concluded that the primary block in the breakdown of glucose in tissues may be due to the competitive inhibition by 2-deoxyglucose-6-phosphate of the conversion of glucose to ketose. Phlorizin is considered to block biological transport of glucose (competitively or non-competitively) possibly by binding with high affinity a monosaccharide carrier in the cell membrane (35). Therefore, the utilization of exogenous glucose in mast cells may be inhibited by either compounds. It is unlikely that the inhibition by these compounds of the restoring effect of glucose on degranulation is the result of affected biological properties of mast cells, since in an air phase, these compounds had no influence on degranulation of mast cells.

The action on degranulation of KCN, a respiratory inhibitor, and DNP, an uncoupler of oxidative phosphorylation, may be considered similar to that of anoxia. In fact, these metabolic inhibitors almost completely inhibited degranulation of mast cells in response to compound 48/80 and the other two agents, and this inhibition was fully counteracted by the presence of glucose (5.6 mM). In this case, the action of anti-serum was also inhibited by DNP and KCN, while it was not blocked by anoxia. This discrepancy comes into question. It is possible that these inhibitors may also interfere with the breakdown of glucose, since they attack metabolic processes at various points. There are some evidences supporting this assumption with respect to the effect of DNP (8, 47). Iodoacetate strongly inhibited degranulation in the presence as well as in the absence of glucose. Such inhibitory effects of this substance (13, 14, 33, 34, 42, 43) and other SH-inhibitors (8, 13-15, 28, 31-34) have been reported on the reaction of mast cells *in situ* or isolated. The facts that the inhibition of degranulation caused by anoxia, KCN and DNP is counteracted by glucose and that this effect of glucose is inhibited by 2-deoxyglucose and phlorizin indicate that adenosine triphosphate (ATP) produced by glycolysis is required for this reaction, and that this ATP generation is a proper function of mast cells though they do not contain glucose needed for this process. This is in contrast with the presence of endogenous substrates for aerobic oxidation sufficient to yield the energy required for degranulation.

Succinate did not potentiate degranulation of isolated mast cells (unpublished observation). This substrate seems to be an exogenous energy source for histamine release only in guinea-pig tissue (1, 44-46). It is possible, therefore, that endogenous storage of substrates for aerobic degranulation or histamine release are more abundant in mast cells of the rat than in those of the guinea pig. However, another possibility that permeability to this substrate of the cell membrane or of the mitochondrial membrane of mast cells varies with different species can not be neglected.

In all events, the fact that exogenous glucose provides an alternative energy supply for the reaction of mast cells in some species, suggests a possibility that glycolysis may take part as one of the controlling factors in some allergic manifestations since biological transport of glucose is largely subjected to the hormonal control.

SUMMARY

1. Degranulating actions of compound 48/80, α -chymotrypsin and anti-serum have been studied on mast cells isolated from rat peritoneal fluid under aerobic and anaerobic conditions. Anaerobic condition has been achieved by bubbling of nitrogen gas, before introduction of mast cells, through the incubation medium in Carrel's flasks containing a small piece of yellow phosphorus. The presence of a small amount of bovine serum albumin in the medium has been confirmed to be effective for preserving the responsiveness of mast cells.

2. There are some differences between these degranulating agents in the morphology of the affected mast cells and lag phase of the reaction.

3. Degranulating actions of these agents are all dependent on temperature and pH. Ca^{2+} are essential for the actions of α -chymotrypsin and anti-serum on isolated mast cells but not for compound 48/80, while for the actions on mesentery mast cells all the agents require Ca^{2+} . Ca^{2+} are also involved in complement activation in the action of anti-serum.

4. Under aerobic condition the actions of these three degranulating agents are not influenced by the presence or absence of glucose in the medium. In anaerobiosis the actions of compound 48/80 and α -chymotrypsin are completely inhibited when the medium does not contain glucose, although this is not for the action of anti-serum owing to glucose contained in itself. KCN and 2,4-dinitrophenol completely inhibit the actions of these three degranulating agents in the glucose-free medium in air phase. All these inhibitions are largely counteracted by the presence of a small amount of glucose in the medium, and this 'glucose effect' is markedly blocked by phlorizin and 2-deoxyglucose, while the effect of ouabain is uncertain as far as isolated mast cells are concerned. Iodoacetate inhibits the degranulation irrespective of presence or absence of glucose. Mg^{2+} have no effect on the anoxic inhibition of degranulation.

5. These results indicate that the degranulations of mast cells in response to these three agents all rely on energy generation, which is a function localized in mast cells, and that in the presence of exogenous glucose glycolysis takes part in supplying energy required for anoxic degranulation, alternating the aerobic oxidation of endogenous substrates.

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