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THE RELATIONSHIP BETWEEN HISTAMINE SECRETION AND ^{45}CALCIUM UPTAKE BY MAST CELLS

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SUMMARY

1. Unstimulated mast cells from the peritoneal cavity of the rat take up ^{45}Ca: the initial phase of rapid uptake being complete after 1 min incubation of the cells with the isotope. Stimulation of the mast cells with an antigen-antibody reaction, dextran or concanavalin A induces an increase in the uptake of ^{45}Ca which is accompanied by a release of granular material: this increase in ^{45}Ca uptake is also complete in 1 min. The majority of the stimulated ^{45}Ca uptake cannot be explained in terms of binding of Ca to released granular material, or to an enlargement in either the extracellular compartment or the cell surface area.

2. The magnitude of the increase in ^{45}Ca uptake caused by stimulating the mast cells increases when the degree of histamine secretion increases.

3. The increased ^{45}Ca uptake induced by stimulation of the mast cells and the degree of histamine secretion are both dependent on extracellular H ion concentration. Changes of pH cause similar changes in ^{45}Ca uptake and secretion with maxima at pH 7·5.

4. Two thirds of the ^{45}Ca uptake induced by an antigen-antibody reaction or by the Ca ionophore A 23187 is unaffected by inhibiting glycolysis and oxidative phosphorylation. Histamine secretion on the other hand is practically abolished by this metabolic inhibition. Thus, ^{45}Ca uptake proceeds in the absence of the discharge of granules.

5. Dibutyryl cyclic AMP or theophylline inhibit both the increase in ^{45}Ca uptake and the histamine secretion caused by stimulating mast cells with an antigen-antibody reaction. Cyclic AMP, cyclic GMP and dibutyryl cyclic GMP have no effect on uptake or secretion.

6. The Ca ionophore, A 23187, induces uptake of ^{45}Ca and histamine secretion, neither effect being inhibited by either dibutyryl cyclic AMP or theophylline.

7. Phosphatidyl serine increases both ^{45}Ca uptake and the histamine release induced by an antigen-antibody reaction, dextran or concanavalin A.

INTRODUCTION

Many cells secrete preformed granules stored within the cytoplasm, and in most cases, Ca couples the stimulus on the cell membrane with the actual release of the granules (Douglas, 1968).

The release of histamine induced by an antigen-antibody reaction on the mast cell membrane is accompanied by the selective release of the granule contents whilst other intracellular material such as potassium, lactic dehydrogenase and ATP is retained within the cell (Johnson & Moran, 1969). Histamine secretion is dependent on the presence of extracellular Ca (Mongar & Schild, 1958), and it appears that an influx of Ca from the extracellular environment into the mast cell is a sufficient stimulus to initiate histamine secretion. Such an influx of Ca may be produced experimentally with the Ca ionophore, A 23187 (Foreman, Mongar & Gomperts, 1973). Similarly, transmitter release has been induced by the injection of Ca into squid nerve terminals (Miledi, 1975) and into mast cells (Kanno, Cochrane & Douglas, 1973). However, it should be pointed out that not all attempts to inject calcium into mast cells have resulted in secretory activity (Tasaka, Sugiyama, Komoto & Yamasaki, 1970*a*).

Stimulus-induced changes of mast cell membrane permeability to Ca have been studied by measuring ^{45}Ca uptake. Previous work has demonstrated that stimulation of mast cells with ATP (Dahlqvist, 1974*a*, *b*) or compound 48/80 (Spataro & Bosmann, 1976) results in an uptake of ^{45}Ca by the cells. A preliminary account of some of our results has already been published (Foreman, Hallett & Mongar, 1975).

METHODS

Preparation of mast cells

Male Lister Hooded rats from a closed, random-bred colony were used for the experiments: they weighed 150–300 g. The rats were given an injection of 0·25 ml., intramuscularly into each hind limb, of a solution containing dried egg white (Sigma), 50 mg/ml. and 8×10^8 dead *Bordetella pertussis* organisms per ml. (Burroughs Wellcome), 15–30 days before the experiment. The injection of antigen (ovalbumin) with *B. pertussis* organisms sensitizes the rats by the formation and fixation to mast cells of IgE antibody (Mota, 1964). On the day of the experiment, sensitized rats were decapitated after anaesthetizing them with nitrous oxide. Three ml. saline (NaCl, 154 m-mole/l.) was injected into the peritoneal cavity of each rat and the abdomen was massaged for 1 min to cause circulation of the saline in the peritoneum. The peritoneal cavity was then opened by a mid-line incision and the fluid withdrawn. The fluid from several rats was pooled for an experiment and contained about 3 % mast cells. Separation of mast cells from the other cells present was achieved by density gradient centrifugation over human serum albumin.

Human serum albumin (Kabi) was dissolved in distilled water and then the concentrated stock solution was diluted with appropriate salt solutions to give final concentrations of albumin of 0·20 and 0·26 g/ml. which had an osmolality of 300

m-mole/kg, a Ca concentration of 1 m-mole/l. and a pH of 7·0. The gradient comprised a lower layer of 1 ml. albumin containing 0·26 g/ml. and an upper layer of 1 ml albumin containing 0·20 g/ml. on to which about 5 ml. peritoneal washings were placed. All interfaces were gently stirred before centrifugation in swing-out buckets at 90 *g* for 10 min. The mast cells formed a pellet at the base of the tube, the other cells remaining at the interface of the two concentrations of albumin. More than 80% of the cells in the pellet were mast cells. The cells were washed free from albumin by resuspension in 10 ml. saline and recentrifugation at 90 *g* for 10 min, before being divided into aliquot parts for the experiment.

^{45}Ca uptake measurement

The medium in which the cells were suspended was Tyrode solution, with the following composition: NaCl, 137 m-mole/l.; KCl, 2·7 m-mole/l.; NaH_2PO_4, 0·4 m-mole/l.; $NaHCO_3$ 12 m-mole/l.; glucose, 5·6 m-mole/l.; $MgCl_2$, 1·0 m-mole/l.; $CaCl_2$, 1·0 m-mole/l. Unless otherwise indicated, the pH of this medium was 7·6. Versilube F 50 silicone oil (General Electric Company, U.S.A.), 100 μl. was placed in the bottom of a conical microcentrifuge tube (capacity 400 μl.) and 50 μl. Tyrode solution containing ^{45}Ca, 0·2 mc/ml., was layered on top of the silicone oil. The specific activity as purchased was 900 mc/m-mole and was 4·5 mc/m-mole when used in the experiment. The tubes were brought to a steady-state temperature of 37° C and then at zero time, 50 μl. cell suspension at 37° C were added to the tube. Incubation at 37° C was allowed to proceed for 5 min, unless otherwise indicated in the results. At the end of the incubation, the cells were separated from the radioactive incubation medium by centrifuging them through the silicone oil to form a pellet at the bottom of the tube. Centrifugation was carried out at 15,000 *g* for 30 sec in a Beckman 152 Microfuge. The number of cells added to each tube was about 5×10^5: a cell count being performed in a modified Fuchs–Rosenthal chamber on an aliquot of the suspension which had been fixed in ethanol and stained with toluidine blue (Moore & Watson, 1953). Cell diameter was measured on a stained preparation using a calibrated eyepiece in the microscope. Addition of agents to stimulate or modify the secretory activity of the cells was made to the Tyrode solution containing the ^{45}Ca before the addition of the cells. The cell pellet formed during the centrifugation was recovered by freezing the tubes in liquid nitrogen and slicing off the bottom of the tube containing the pellet. The pellet was thawed, vigorously shaken with 1ml. of an aqueous solution of Triton X-100, 100 mg/ml. at room temperature, and left overnight to dissolve. ^{45}Ca was assayed by adding Instagel scintillant to the Triton X-100 solution and counting in the preset ^{14}C channel of a liquid scintillation spectrometer (Packard Tricarb 3380). The counting efficiency was 87% and did not change significantly from sample to sample.

Experiments were performed to show that the centrifugation transferred all of the mast cells from the incubation medium to the bottom of the silicone layer. The specific gravity of Versilube F 50 is 1.045. Equal aliquot parts of cells were placed either over silicone oil as described above or into empty centrifuge tubes, and were incubated for 5 min at 37° C. All the aliquot portions were then centrifuged at 15,000 *g* for 30 sec. The pellets from tubes with or without silicone oil were boiled in Tyrode solution for 5 min to release cellular histamine which was then assayed. The histamine in the supernatant from the tubes containing no silicone oil was also assayed. Histamine in the pellets beneath the silicone oil was expressed as a percentage of the total histamine content for each tube, and this is compared in Table 1 with the spontaneous release of histamine into the supernatant before centrifugation. On average, 94% of the histamine in an aliquot of cells was transferred to the pellet

beneath the silicone oil as compared with an average spontaneous secretion into the supernatant of 7·8%.

Centrifugation of tubes containing ^{45}Ca in incubation medium layered over silicone oil but no cells, produced no radioactivity above background in the silicone oil layer.

TABLE 1. Transfer of histamine from incubation medium (Tyrode solution) into the pellet below the silicone oil (Versilube F-50). Each value is a mean of triplicate determinations

Experiment	Histamine in pellet cellular histamine (% of total)	Spontaneous histamine release in a separate aliquot of cells (% of total)
1	94·4	9·6
2	88·8	9·1
3	91·5	11·5
4	96·0	5·2
5	93·0	4·4
6	95·0	6·0
7	94·0	10·1
8	96·0	5·1
9	94·0	8·0
10	93·0	9·0
Mean	93·6	7·8

As the cells are centrifuged through the silicone oil, they carry with them a certain amount of the radioactive incubating medium which has been called the 'entrained space'. The volume of this extracellular space was estimated with two radioactive markers which penetrate the cells poorly or not at all. The markers were (hydroxy [^{14}C]methyl) inulin and Na [^{35}S] sulphate and the method used to determine the spaces occupied by these substances was exactly the same as that employed to determine ^{45}Ca uptake except that the medium in which the cells were incubated contained either [^{35}S]sulphate 63 mc/ml. (specific activity 6·3 mc/m-mole) or (hydroxy[^{14}C]methyl) inulin 10·3 mc/ml. (specific activity 182 μc/m-mole).

Determination of histamine secretion

The method of determining histamine secretion from samples of the cells has already been described (Foreman & Mongar, 1972). Histamine was assayed either by bio-assay on the guinea-pig ileum using the method of Boura, Mongar & Schild (1954) or by fluorimetric assay as described by Shore, Burkhalter & Cohn (1952) but without the extraction steps (Loeffler, Lovenberg & Sjordsma, 1971).

All isotopes were obtained from the Radiochemical Centre, Amersham. Phosphatidyl serine was obtained from Lipid Products and the method of preparing it for these experiments has already been described (Foreman & Mongar, 1973). The Ca ionophore A 23187 was a gift from Dr R. L. Hamill, Lilly Research Labs. It was dissolved in ethanol to produce a stock solution containing 6 m-mole/l. and this was diluted with Tyrode solution to give the desired concentration. The final concentration of ethanol (less than 20 m-mole/l.) did not not affect the response of the cells to antigen stimulation. In the experiments in which pH was varied the $NaHCO_3$ in Tyrode was replaced by HEPES (4-(2 hydroxyethyl)-1-piperazine ethane sulphonic

acids), 20 m-mole/l. (Burroughs Wellcome), and the pH adjusted with NaOH, 1 mole/l. or HCl, 1 mole/l.

The sources of other chemicals were as follows: $N^6O^{2'}$ dibutyryl cyclic 3'5'-adenosine monophosphate (dibutyryl cyclicAMP) – Sigma; antimycin A – Calbiochem; concanavalin A – Sigma; Dextran 110 60 mg/ml. aqueous solution – Fisons; theophylline and NaCN – B.D.H. Inorganic chemicals for Tyrode solution were Analar quality.

RESULTS

Extracellular space measurement

It has been shown in the Methods section that the cells are all transferred from the aqueous layer by centrifugation, but some extracellular fluid accompanies them. (Hydroxy[^{14}C]methyl) inulin and [^{35}S]sulphate spaces

TABLE 2. Volumes of extracellular fluid entrained with 10^6 mast cells during the separation of cells from incubating medium (see Methods). Each value is the mean from five experiments together with S.E. of mean.

Space marker		Volume of space (nl.)	
Time of incubation with marker (min)	Stimulus	[^{35}S]Sulphate	Hydroxy [^{14}C]methyl inulin
1	None	16·0 ± 2·0	39·5 ± 2·5
20	None	16·5 ± 0·5	31·0 ± 4·0
40	None	15·0 ± 3·0	29·5 ± 1·5
60	None	22·0 ± 5·0	35·5 ± 0·5
5	None	24·1 ± 3·1	27·8 ± 11·1
5	Antigen*	28·2 ± 7·2	34·9 ± 11·3

* Histamine secretion induced by antigen stimulation was 40·2 ± 12·6 % for the inulin space determination and 47·0 ± 13·4 % for the sulphate space determination. The spaces were determined on different populations of cells.

were determined to assess the magnitude of this extracellular space entrained with the cells, and investigate the effects on this space of stimulating the mast cells. Table 2 shows that the mean size of the entrained spaces was 20 and 30 nl. for sulphate and inulin spaces respectively. The values refer to the entrained space of 10^6 mast cells which have a volume of about 1 μl. calculated from mean cell diamter of 12 μm (Fig. 1).

Thus, the entrained space is equivalent to only 2–3 % of the cell volume. Table 2 also shows that size of the entrained space was independent of the time for which cells were incubated with marker isotope, indicating that the marker was not being taken up by the cells.

Stimulation of the mast cells by the antigen-antibody reaction did not result in any change in the entrained space (Table 2). It can be shown that the amount of Ca contained in the entrained space, given an extracellular

concentration of 1 m-mole/l. is only about 20–30 p-mole (equivalent to about 200 c.p.m.) which is small compared with the stimulated or resting ^{45}Ca uptakes described below (about 2000–6000 c.p.m.). In other words, if the ^{45}Ca uptake by the cells were to be explained in terms of an increase in entrained space then for antigen-induced uptake associated with a histamine secretion of 25%, the entrained space would have to increase by about fifteen fold.

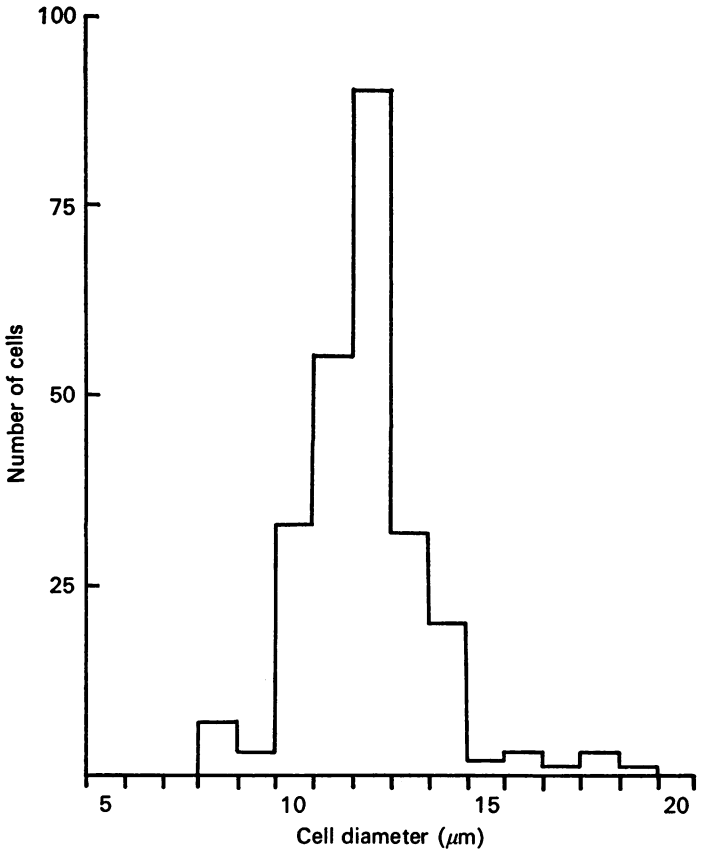

Fig. 1. Distribution of rat mast cell diameters. 250 mast cells were counted after they had been separated from other cells by density-gradient centrifugation as described in the text. The mean diameter is 12 μm.

^{45}Ca uptake in resting and stimulated cells

Unstimulated mast cells rapidly take up ^{45}Ca: the initial phase of rapid uptake being complete within 1 min of adding cells to the incubating medium (Fig. 2*A*). Between 1 and 30 min of incubation, there is a subsequent slow and comparatively small increase in the non-stimulated ^{45}Ca uptake. In

three experiments the uptake at 1 min was 3900 ± 400 c.p.m./10^6 cells (mean ± S.E.) which increased to 4800 ± 500 c.p.m./10^6 cells at 30 min. Assuming that this uptake represents a transmembrane flux of calcium, it can be calculated from the counting efficiency and specific activity that there was a Ca uptake of 100 p-mole/10^6 cells.

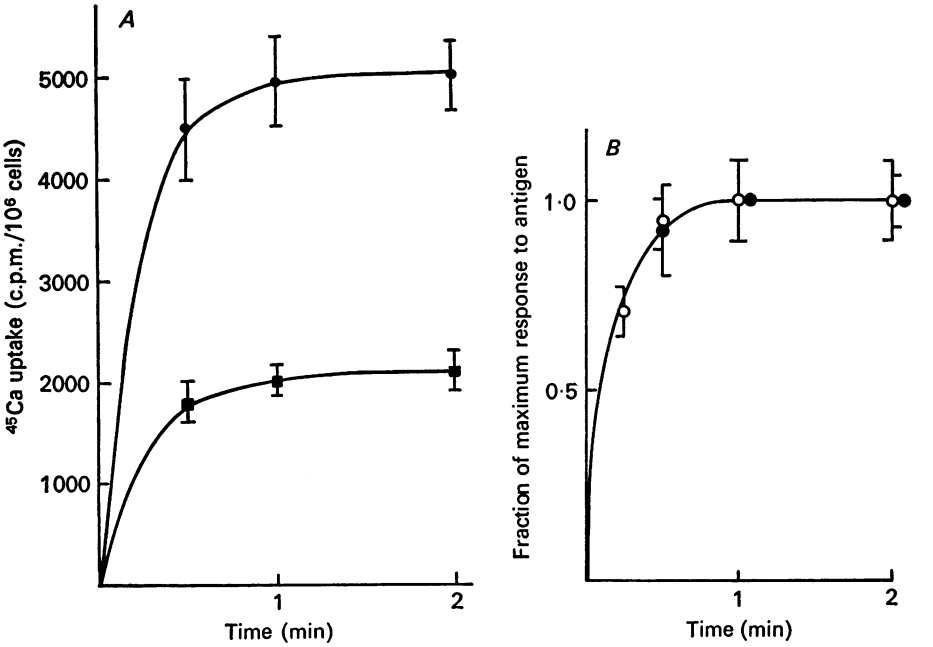

Fig. 2. Rates of ^{45}Ca uptake and histamine secretion. *A*, the rate of ^{45}Ca uptake in unstimulated cells (■—■) and cells stimulated by the antigen-antibody reaction (●—●). Each point is the mean from four experiments in which the mean histamine secretion was 4 ± 2 % for unstimulated cells and 32 ± 6 % for antigen-stimulated cells. Vertical bars indicate the S.E. of mean. *B*, the rates of ^{45}Ca uptake (●—●) and histamine secretion (○—○) in antigen-stimulated cells. Both measurements were made on the same cell populations and have been corrected by subtracting the values for unstimulated cells. Response is expressed as a fraction of maximum; the maximum histamine secretion was 28 ± 4 % and the maximum ^{45}Ca uptake was 3000 ± 275 c.p.m/10^6 cells. Each point is the mean of four experiments and vertical bars indicate the S.E. of the mean.

Stimulation of rat mast cells by means of an antigen-antibody reaction increases the ^{45}Ca uptake compared with unstimulated cells (Fig. 2*A*) and this increase in uptake is also complete within 1 min. Fig. 2*B* shows the rate of increase of ^{45}Ca uptake caused by antigen stimulation compared with the rate of histamine secretion after stimulation. It can be seen that

15 sec after stimulation the majority of the secretory activity has taken place and at 30 sec after stimulation the ^{45}Ca uptake is 90 % complete.

The magnitude of the increase in ^{45}Ca uptake induced by antigen stimulation was not constant when different populations of mast cells were compared. It is known that different populations of mast cells do not release similar amounts of histamine when challenged with a maximal antigen stimulus, and this variation is assumed to be due to the differing

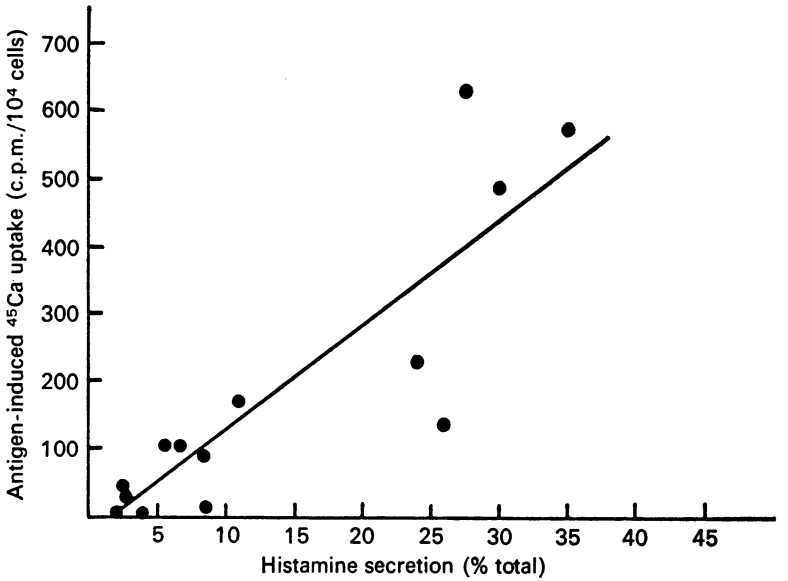

Fig. 3. Correlation of the magnitude of ^{45}Ca uptake and histamine secretion induced by antigen, measured after incubation for 5 min. Each point represents the mean of five replicate determinations on a single population of mast cells. The coefficient of rank correlation is 0·85 ($P < 0·01$).

extent to which IgE antibody is fixed to the cell membrane during the sensitizing process. Using cells from rats which showed varying degrees of sensitization, maximum histamine secretion was compared with ^{45}Ca uptake under the same conditions. Fig. 3 shows the correlation between the histamine secretion and ^{45}Ca uptake. Each point represents a different pool of cells and the coefficient of rank correlation for the two variables was found to be 0·85 ($P < 0·01$). The magnitude of the ^{45}Ca uptake induced by antigen, therefore, appears to be related to the magnitude of the secretory response. The results shown in Fig. 4 are in agreement with this conclusion since the concentration-effect relationship for antigen and histamine secretion is coincident with that for antigen and ^{45}Ca uptake. In this case, both measurements were made on the same cell population over the whole antigen concentration range 10 ng/ml. to 1 mg/ml.

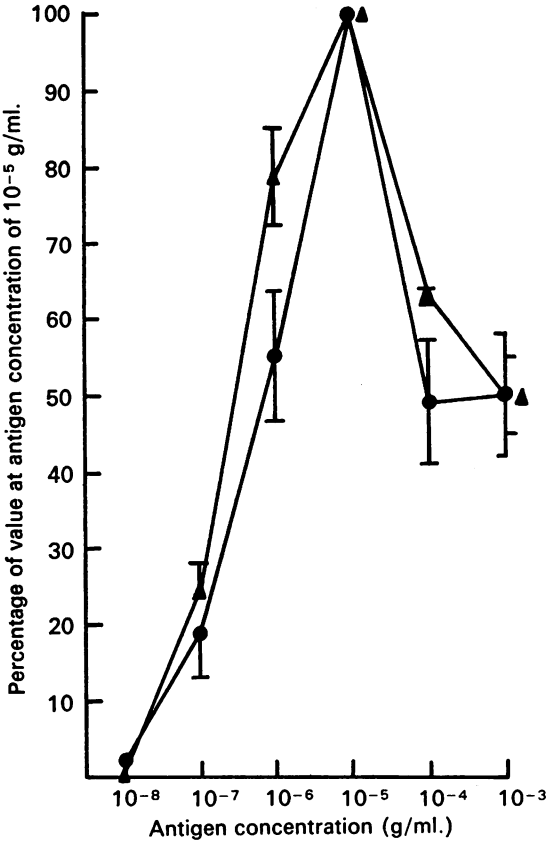

Fig. 4. Concentration–effect curves for antigen stimulated histamine secretion (▲—▲) and ^{45}Ca uptake (●—●) measured after incubation for 5 min. The response is expressed as a percentage of the value measured at an antigen concentration of 10 μg/ml., which was 43 ± 3 % for histamine secretion and 4025 ± 820 c.p.m./10^6 cells for ^{45}Ca uptake. Each point represents the mean of four experiments with vertical bars indicating the S.E. of mean.

Effect of adding ^{45}Ca to cells after antigen stimulation

When mast cells are stimulated with antigen in the absence of extracellular calcium no secretion of histamine takes place. If Ca is then added the response obtained depends on the time interval between antigen-stimulation and addition of Ca. The response to Ca under these conditions decays with time; the half-time of decay being about 1 min (Foreman & Garland, 1974; Diamant, Grosman, Stahl Skov & Thomle, 1974). It has been suggested that this decay in the response to Ca after antigen-stimulation is due to reduction of the increased membrane permeability to Ca which the stimulus initially brings about. The experiments shown in Fig. 5

were designed to shed further light on this suggestion. Mast cells were stimulated with antigen in the presence of unlabelled Ca and then at various intervals after stimulation, ^{45}Ca was added. The ^{45}Ca uptake, measured when 1 min was allowed to elapse between antigen-stimulation and tracer addition, was only about half the ^{45}Ca uptake measured when

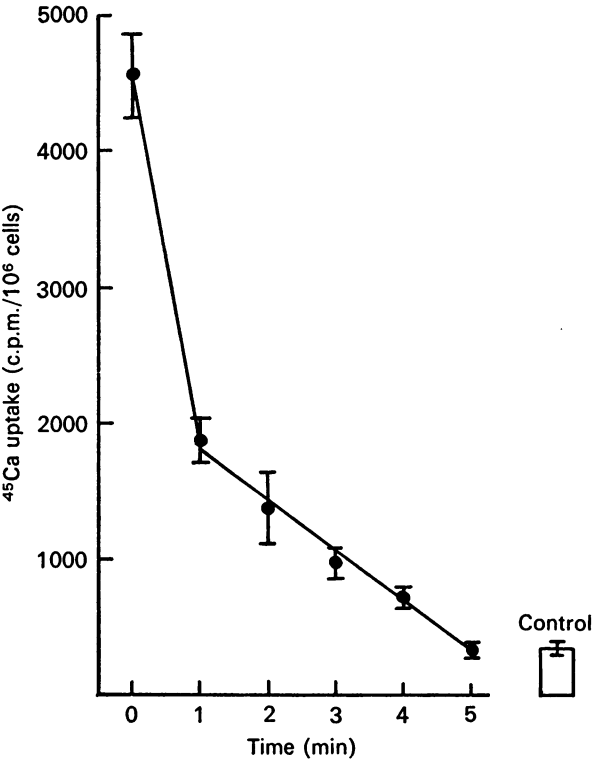

Fig. 5. Time course of the change in ^{45}Ca uptake after antigen stimulation. Cells from the same pool were either challenged with antigen in the presence of ^{45}Ca (total Ca concentration 1 m-mole/l.): $t = 0$, or cells were challenged with antigen in the presence of non-labelled calcium, 1 m-mole/l. and then ^{45}Ca was added at t min after antigen. Incubation was allowed to proceed for 5 min after the addition of ^{45}Ca. The control bar represents ^{45}Ca uptake in the absence of antigen stimulation. Each point is the mean of three experiments and vertical bars indicate the S.E. of mean.

the cells were stimulated with antigen in the presence of ^{45}Ca. As the time between stimulation and addition of tracer was extended over the period between 1 and 5 min, there was a steady decline in the ^{45}Ca uptakes measured (Fig. 5). At 5 min after antigen stimulation, no significant ^{45}Ca uptake could be recorded.

Effect of extracellular Ca concentration on ^{45}Ca uptake

The specific activity of the ^{45}Ca was kept constant while the extracellular Ca concentration was varied over the range 0·5–8 m-mole/l. Fig. 6 shows that over the concentration range used both the stimulated and the unstimulated ^{45}Ca uptakes were linearly related to the extracellular Ca concentration, with no sign of saturation.

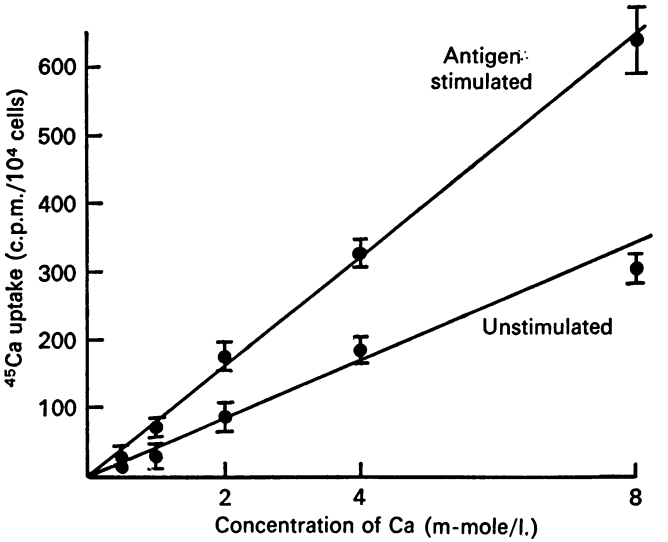

Fig. 6. The relationship between ^{45}Ca uptake and total external Ca concentration for unstimulated cells and antigen-stimulated cells. The specific activity of the ^{45}Ca was constant. Incubation was carried out for 5 min at 37° C and each point represents the mean of three experiments, with vertical bars to indicate the S.E. of mean.

Effect of pH on ^{45}Ca uptake

Antigen-induced histamine release from mast cells in guinea-pig lung has been shown to be dependent on pH (Mongar & Schild, 1958). Both antigen-evoked histamine secretion and ^{45}Ca uptake in rat mast cells are dependent on the extracellular H ion concentration in the range pH 6·5–8·5 (Fig. 7). The maximum histamine secretion and ^{45}Ca uptake induced by antigen occurs at pH 7·5 and there is a close parallel between histamine secretion and ^{45}Ca uptake over the whole pH range tested.

The effects of metabolic inhibitors on ^{45}Ca uptake

The secretion of histamine from mast cells appears to depend upon an intact system for the generation of ATP within the cells (Chakravarty, 1968; Diamant, Norn, Felding, Olsen, Ziebell & Nissen, 1974; Johansen &

Chakravarty, 1972). In the absence of glucose, antimycin A, 1 μmole/l. or NaCN, 5 m-mole/l., almost completely inhibit antigen-induced histamine secretion. The increase in ^{45}Ca uptake which accompanies antigen-evoked histamine secretion is, however, reduced by only about one third in the presence of these inhibitors (Fig. 8). When histamine secretion is induced by the Ca ionophore, A 23187, 6 μmole/l., an increased ^{45}Ca uptake is observed and this too is inhibited by only about one third in the presence of antimycin A or NaCN, whereas the histamine secretion is almost completely inhibited.

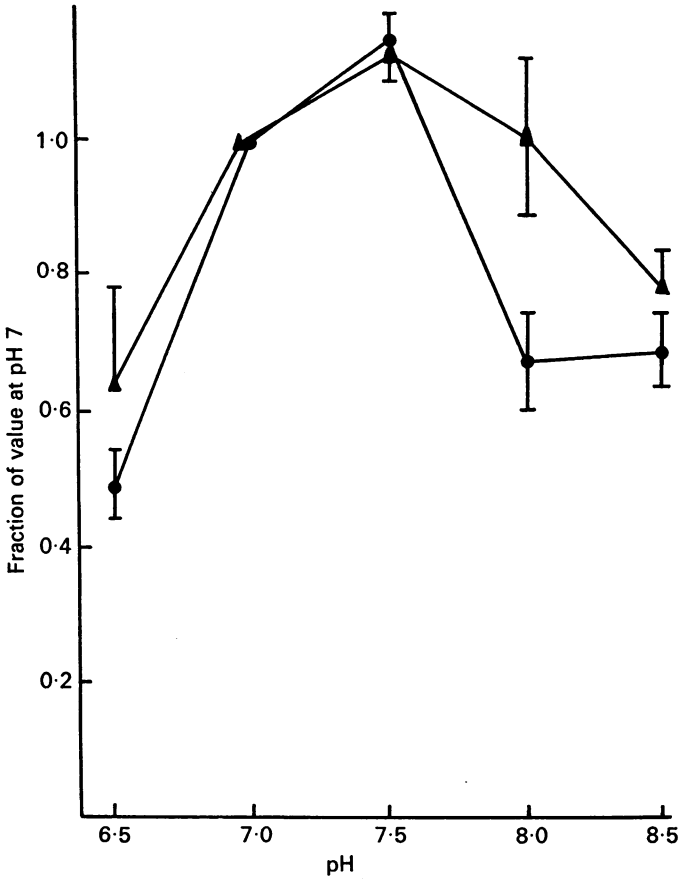

Fig. 7. The effect of pH on antigen-stimulated ^{45}Ca uptake and histamine secretion. The histamine secretion (●—●) is expressed as a fraction of the value at pH 7·0 which was 32 ± 7 % and ^{45}Ca uptake induced by antigen (▲—▲) is expressed as a fraction of the value at pH 7·0 which was 2600 ± 240 c.m.p./10^6 cells. The vertical bars are the S.E. of mean.

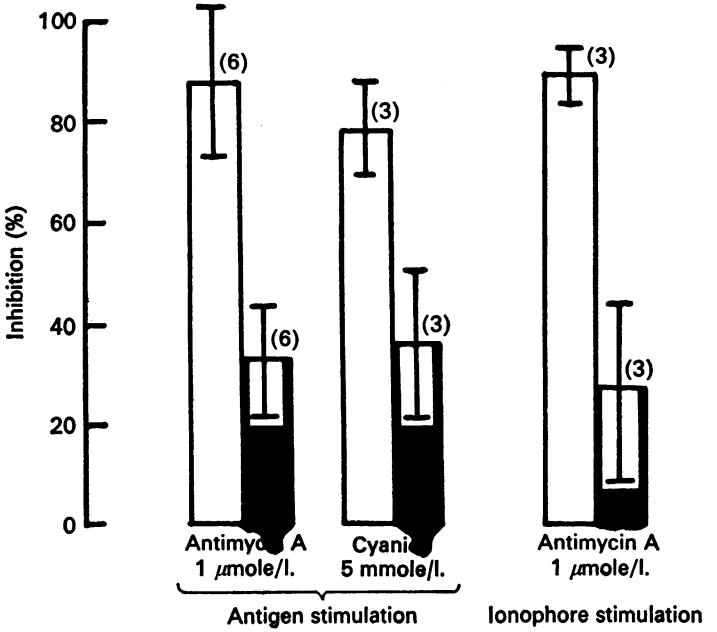

Fig. 8. The effect of metabolic inhibitors on ^{45}Ca uptake and histamine secretion. The response is expressed as % inhibition of ^{45}Ca uptake (filled columns) or of histamine secretion (open columns) induced by either A 23187 6 μmole/l. or by antigen 10 μg/ml. The figures in parentheses indicate the number of experiments contributing to the column and the vertical bars represent the S.E. of mean. The uninhibited values for ^{45}Ca uptake were 2600 ± 210 c.p.m./10^6 cells for antigen and 3160 ± c.p.m./10^6 cells for A 23187. The uninhibited values for histamine secretion were: 33 ± 2% for antigen and 50 ± 4% for A 23187.

The effects of cyclic nucleotides on ^{45}Ca uptake

Histamine secretion from mast cells can be inhibited by dibutyryl cyclic AMP (Assem, 1970; Baxter, 1972; Foreman, Mongar, Gomperts & Garland, 1975) in the concentration range 0·1–10 m-mole/l. Fig. 9 shows that in addition to inhibiting histamine secretion, dibutyryl cyclic AMP also inhibits the antigen-induced ^{45}Ca uptake in a dose-related manner in the concentration range 0·1–10 m-mole/l. The unstimulated ^{45}Ca uptake by mast cells is not affected by dibutyryl cyclic AMP in these concentrations. Furthermore, the other nucleotides tested including cyclic AMP, cyclic GMP and dibutyryl cyclic GMP, did not affect antigen-evoked ^{45}Ca uptake at concentrations up to 10 m-mole/l.

Phosphodiesterase inhibitors are known to increase mast cell levels of cyclic AMP (Johnson, Moran & Meyer 1974; Kaliner & Austen 1974). Fig. 9 shows that the phosphodiesterase inhibitor, theophylline, inhibits

antigen induced ^{45}Ca uptake at a concentration of 1 m-mole/l.: the same concentration as that required to inhibit histamine secretion.

Foreman *et al.* (1975) showed that histamine secretion induced by the calcium ionophore, A 23187, was not inhibited by dibutyryl cyclic AMP in contrast to histamine release induced by antigen, which was inhibited by this substance. Fig. 9 shows that ^{45}Ca uptake induced by the calcium ionophore is also not inhibited by either dibutyryl cyclic AMP, 10 m-mole/l or theophylline, 1 m-mole/l.

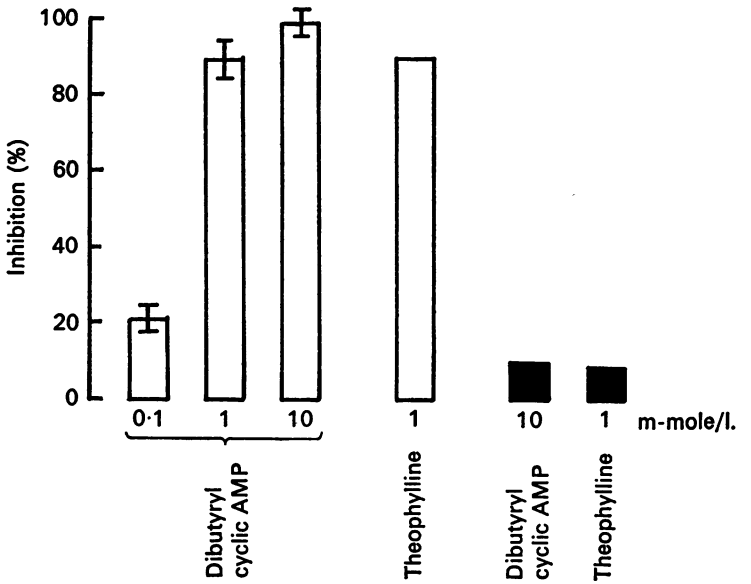

Fig. 9. Inhibition by dibutyryl cyclic AMP of antigen-evoked ^{45}Ca uptake (open columns) and of A 23187-induced ^{45}Ca uptake (filled columns). Each bar is the mean of five experiments and the vertical bar represent the S.E. of mean. The uninhibited values from which percentage inhibitions were calculated were 3100 ± 300 c.p.m./10^6 cells for antigen-induced ^{45}Ca uptake, 5500 ± 350 c.p.m./10^6 cells for A 23187-induced ^{45}Ca uptake, 43 ± 11 % for antigen-induced histamine secretion and 62 ± 2 % for A 23187-induced histamine secretion. The concentration of A 23187 was 6 μmole/l.

The effect of phosphatidyl serine

Phosphatidyl serine has been shown to increase the amount of histamine secreted from rat peritoneal mast cells in response to antigen-antibody stimulation (Goth, Adams & Knoohuizen, 1971; Mongar & Svec, 1972; Foreman & Mongar, 1973). The effect is quite specific for phosphatidyl serine but several different stimuli induce secretion which is potentiated in the presence of this phospholipid. It has already been suggested on the

basis of indirect evidence (Foreman & Garland, 1974) that phosphatidyl serine acts by enhancing the mast cell membrane permeability to calcium. Fig. 10 shows the effect of phosphatidyl serine, 10 μg/ml., on ^{45}Ca uptake induced by three different stimuli of histamine secretion. The potentiation of ^{45}Ca uptake produced by phosphatidyl serine is about twofold, which is similar to the potentiation of histamine secretion produced by this optimum concentration of phosphatidyl serine. It must be pointed out that the unstimulated ^{45}Ca uptake is not affected by the addition of phosphatidyl serine.

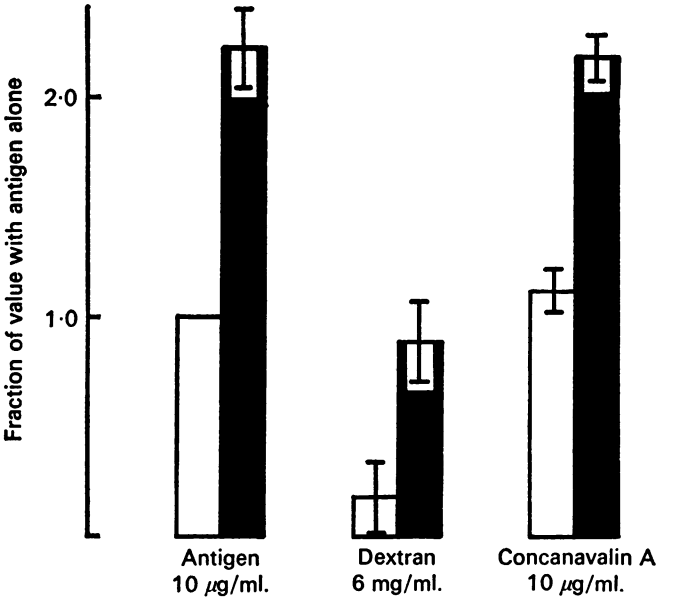

Fig. 10. The action of phosphatidyl serine 10 μg/ml. (filled columns) on ^{45}Ca uptakes induced by antigen, dextran and concanavalin A. Open columns represent the ^{45}Ca uptake in the absence of phosphatidyl serine. The response is expressed as a fraction of the value recorded for antigen-induced ^{45}Ca uptake in the absence of phosphatidyl serine which was 2600 ± 510 c.p.m./10^6 cells. Each column is the mean from three experiments and the vertical bar represents the S.E. of mean.

DISCUSSION

The technique used to measure ^{45}Ca uptake in mast cells was based on a method described by Harris & Berent (1969), who worked with mitochondria. The technique is based on the rapid separation of the mast cells from the ^{45}Ca-containing incubation medium, after varying periods of incubation, by high velocity centrifugation of the cells into a water immiscible fluid: silicone oil. Results are presented to show that the centrifugation procedure transfers, on average, 94% of the histamine in a cell

sample into the silicone oil and this represents the transfer of intact cells. The remainder of the histamine which is not transferred can be accounted for in terms of spontaneous release from cells into the incubating medium above the silicone. No aqueous material other than that associated with the cells is transferred into the silicone layer.

TABLE 3. Comparison of the membrane permeability to Ca of some different types of cell

Tissue	Resting permeability (f-mole/cm^2.sec)	Stimulated permeability (f-mole/cm^2.sec)	Reference
Squid giant axon	76	600	Hodgkin & Keynes (1957)
Guinea-pig atria	20	460	Winegrad & Shanes(1962)
Frog heart	9	110	Niedergerke (1963)
Mast cell	8	—	This paper
antigen stimulation		380	
calcium ionophore (0·6 μmole/l.)		190	

The results show that non-stimulated cells rapidly take up ^{45}Ca from the incubation medium; the rapid uptake being complete after a 1 min incubation. A slow component of ^{45}Ca uptake is observed in non-stimulated cells between 1 and 30 min after incubation. The rapid ^{45}Ca uptake in non-stimulated cells probably represents exchange between the label and Ca bound to the surface of the mast cell. The binding sites equilibrate rapidly with the labelled Ca, and the exchange does not appear to saturate as the external Ca concentration is raised from 0·5 to 8 m-mole/l., with the specific activity of the ^{45}Ca being kept constant. The non-stimulated uptake is not affected by cyclic AMP at concentrations up to 10 m-mole/l., by phosphatidyl serine, 10 μg/ml., or by inhibitors of oxidative and glycolytic metabolism.

The slower component of ^{45}Ca uptake in non-stimulated mast cells may represent entry of Ca into the cell across the resting membrane. It was calculated above that the Ca uptake by the non-stimulated cells was 100 p-mole/10^6 cells, assuming that all the ^{45}Ca uptake represents the influx of Ca. Using this figure and a value of 10 cm^2/10^6 cells for the cell surface area (Helander & Bloom, 1974) it can be calculated that the resting permeability of the membrane to Ca is about 6 f-mole/cm^2.sec^{-1}, which is similar to values calculated for other tissues (Table 3).

Stimulation of mast cells by an antigen-antibody reaction, concanavalin

A or dextran, induces an increase in both ^{45}Ca uptake and histamine secretion. The Ca ionophore A 23187 also caused an uptake of ^{45}Ca and histamine secretion. We could obtain no evidence which suggested that the stimulated increase in ^{45}Ca uptake was due to an increase of the entrained extracellular space: antigen stimulation did not affect either the sulphate or the inulin space. It will, however, be noted that the sulphate space is only about two thirds as large as the inulin space and it is thought that this may be due to charge repulsion between the cell surface and the SO_4^{2-} ion.

The results show that there is a close relationship between the magnitude of the secretion of histamine and the amount of ^{45}Ca uptake. The stimulus (antigen) concentration–effect curves for ^{45}Ca uptake and histamine secretion are coincident and the time courses of histamine secretion and ^{45}Ca uptake are the same. Some important information about this association between the two measurements is gained from the experiments with metabolic inhibitors. Total inhibition of histamine secretion by the addition of antimycin A in the absence of glucose is not accompanied by abolition of the ^{45}Ca uptake. ^{45}Ca uptake is only reduced by about a third when histamine secretion is completely inhibited by substances which stop ATP production. Thus, when granular material is not set free from the cells, ^{45}Ca uptake still proceeds, but is reduced. It is likely that this reduction of ^{45}Ca uptake in the presence of metabolic inhibitors is due to the loss of Ca binding capacity presented by the released granular material which is known to have a high affinity for calcium (Bergendorff & Uvnas, 1973). Nevertheless, the conclusion is that about two thirds of the ^{45}Ca uptake induced by antigen stimulation, or for that matter by ionophore, is independent of the process of granule extrusion. It follows that the majority of antigen-induced ^{45}Ca uptake results from an increase in membrane permeability to calcium, and if, as in other cells, the free intracellular Ca concentration is very low relative to the extracellular concentration (Baker, 1972), Ca would be expected to enter the cells along a concentration gradient.

It can be seen from the results that the increase in ^{45}Ca uptake induced by antigen is about 2000 c.p.m. for 28% histamine secretion (Fig. 2), after subtracting one third of the counts actually measured: this being the component which is sensitive to metabolic inhibitors. Assuming that this ^{45}Ca uptake represents the influx of Ca into the cell, the permeability of the membrane after antigen (or ionophore) stimulation can be calculated in a manner similar to that used for calculating the permeability of the resting membrane. A Ca uptake of 230 p-mole/10^6 cells for 28% histamine secretion which occurs in 1 min (Fig. 2*B*), gives membrane permeabilities of 380 and 190 f-mole/cm^2.sec for antigen and ionophore treated cells respectively which is similar to values calculated for other tissues (Table 3).

It is unlikely that all of the ^{45}Ca uptake measured represents net accumulation of Ca by the cell, since an exchange across the membrane between intra- and extracellular calcium could account for some ^{45}Ca uptake.

The data presented in Fig. 5 show the decline of membrane permeability to Ca following antigen stimulation. Although antigen is present throughout the period of the experiment, the permeability increase rapidly subsides to 36% of the initial level within 1 min, and further to the resting level within the next 4 min. This rapid shutting off of the membrane permeability may account for the time course of ^{45}Ca uptake after antigen stimulation shown in Fig. 2*A*. After 1 min most of the ^{45}Ca uptake has occurred, and the increased membrane permeability has subsided before an equilibrium can be reached. The time course of the subsidence of membrane permeability is of interest in the context of changes in secretory response which occur during this time. When cells are stimulated in the absence of Ca, virtually no secretion occurs (less than 10%), but when Ca is added back secretion occurs, the magnitude of which depends on the time interval between stimulation and the addition of Ca (Foreman & Garland, 1974). Such a decay in the response to calcium seen after antigen stimulation is not observed when release of histamine is induced by the ionophore, A 23187 (Foreman & Garland, 1974) and so it is considered that the decay phenomenon occurs at the level of Ca entry into the cell caused by antigen. The time course of the declining ^{45}Ca uptake (Fig. 5) is similar to the decay of the secretory response after antigen stimulation and it is suggested, therefore, that after antigen stimulation has induced an increase in membrane permeability to calcium, the permeability decreases; complete return to resting permeability being achieved 5 min after stimulation. Similar decay of secretory response and membrane Ca permeability has been observed in other secretory systems (Nordmann, 1976: Borowitz, Leslie & Baugh, 1975).

The extracellular pH is known to influence the degree of histamine secretion from mast cells (Mongar & Schild, 1958; Chakavarty, 1960; Uvnas & Thon, 1961). Ca uptake and antigen-stimulated secretion from rat mast cells both show dependence on pH with an optimum secretion at pH 7·5 (Fig. 7) which suggests that the influence of hydrogen ions on histamine secretion may be the result of variation in permeability of the membrane to calcium occuring at different pH.

It has already been pointed out that inhibitors of glycolytic and oxidative metabolism allow dissociation of ^{45}Ca uptake from secretion. In contrast, raised intracellular levels of cyclic AMP produced either with theophylline, a phosphodiesterase inhibitor, or with dibutyryl cyclic AMP, prevent antigen-induced ^{45}Ca uptake and histamine secretion. It is important to note that cyclic AMP does not affect the action of the ionophore

either in producing an uptake of ^{45}Ca or a secretion of histamine (Foreman *et al.* 1976; Garland & Mongar, 1976). The action of cyclic AMP is, therefore, selective against ^{45}Ca uptake and histamine secretion induced by antigen. Clearly, if cyclic AMP inhibited a stage in the secretory process occuring after Ca entry, histamine secretion induced by ionophore as well as that induced by antigen would be expected to be inhibited by cyclic AMP. Cyclic AMP, therefore, appears to inhibit histamine secretion by preventing the antigen-induced increase in membrane permeability to Ca, and it is suggested that cyclic AMP might be the physiological agent which reduces membrane permeability to Ca after the stimulus. It is known that mast cell cyclic AMP levels fall during the period when ^{45}Ca uptake and histamine secretion are occuring, and then the levels return towards resting level with a time course similar to that of the decay in membrane permeability to Ca and histamine secretion described above (Kaliner & Austen, 1974).

Phosphatidyl serine increases the ^{45}Ca uptake induced by the antigen-antibody reaction, dextran or concanavalin A and the magnitude of the increase produced by the phospholipid, about twofold, is of about the same magnitude as the increase in histamine secretion which it produces (Mongar & Svec, 1972; Foreman & Mongar, 1973). Phosphatidyl serine had no effect on the ^{45}Ca uptake in resting cells and so we conclude that the phospholipid acts by potentiating the antigen-induced increase of membrane permeability to Ca. In brain and muscle tissue there is evidence that phospholipid affects an ATPase involved with ion transport (Fenster & Copenhaver, 1966; Martonosi, 1968; Wheeler & Whittam, 1970). There is some evidence that mast cells possess a Ca-activated ATPase (Cooper & Stanworth, 1976) but the role of this enzyme and the action of phosphatidyl serine on it are not known.

The conclusions from the discussion of the results presented in this paper are that the antigen-antibody reaction and some other mast cell membrane stimuli, induce an increase in the permeability of the membrane towards Ca, and this change is associated with histamine secretion probably resulting from an entry of Ca into the cell. The change appears to be transient so that the cell does not remain permanently permeable to Ca. Phosphatidyl serine may be involved in the actual transport of Ca across the cell membrane. Cyclic AMP appears to inhibit secretion by reducing the membrane permeability to Ca and may be involved in limiting Ca entry after stimulation of the cell.

Our results do not provide any direct evidence that a net increase in intracellular Ca is a requisite occurrence for histamine secretion, but our previous experiments with the Ca ionophore make this an extremely likely possibility (Foreman, Mongar & Gomperts, 1973).

Further work using X-ray microprobe analysis and efflux measurements are helping to resolve the question about net Ca uptake and the distribution of Ca across the membrane, while electrophysiology may help to resolve the kinetics of calcium movement. Histamine secretion is known to be independent of monovalent cations and of the depolarization of the mast cell membrane (Mongar & Schild, 1958). However, depolarization does seem to occur during secretion when the normal resting potential of about -12 mV is reduced to zero (Boyde, Johnson Littlejohns & Mongar, 1970; Tasaka *et al.* 1970*b*; Gushchin, Orlov & Tsiyu, 1973). In fact, using the estimate of Ca uptake derived above, and assuming a membrane capacitance of 1 μF/cm^2, a flux of 23 p-mole/cm^2 of a divalent cation would be expected to transfer sufficient charge to change the cell membrane potential by 4400 mV. It, therefore, seems likely that the influx of Ca ions is accompanied by an efflux of another cation, probably K.

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