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CALCIUM AND STIMULUS-SECRETION COUPLING IN THE MAST CELL: STIMULANT AND INHIBITORY EFFECTS OF CALCIUM-RICH MEDIA ON EXOCYTOSIS

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SUMMARY

1. Isolated rat peritoneal mast cells incubated in Ca-free media for 2 h, with or without EDTA, and observed by phase-contact microscopy, became 'bubbled' in appearance when subsequently exposed to media rich in calcium (16–110 mM).

2. Electron microscopy showed the response to be 'compound' exocytosis of the sort elicited by conventional mast cell secretagogues such as antigen (in sensitized cells) and 48/80.

3. The response to Ca was inhibited by withdrawing glucose and adding dinitrophenol and was thus energy-dependent.

4. Mg in similarly high concentration had no such stimulant effect on Ca-deprived cells, and excess Ca stimulated only after Ca deprivation.

5. It is suggested that Ca deprivation may increase the permeability of the plasma membrane of the mast cell thereby allowing some Ca, when subsequently introduced in high concentration, to penetrate and activate exocytosis; and the results are considered further support for the postulated mediator function of Ca in stimulus-secretion coupling.

6. Two inhibitory effects of calcium in high concentration were detected: (*a*) suppression of migration or expulsion of granules from the exocytotic pits within the cellular domain; and (*b*) diminished sensitivity to 48/80.

INTRODUCTION

Some years ago it was argued that a common pattern could be discerned in stimulus-secretion coupling in which various secretagogues exert their effects on diverse cells by acting on the cell membrane to increase permeability, promote Ca influx, and activate a common secretory process, exocytosis; and among the systems suggested to operate in this way was

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the sensitized mast cell responding to specific antigen (Douglas, 1968, 1971). With accumulating evidence that the mast cell does conform to this common pattern there has been increasing interest in its use as a model secretory system (see, for example, Uvnäs, 1971, 1974; Foreman & Mongar, 1972, 1975; Foreman, Garland & Mongar, 1976; Douglas, 1974*a*, *b*, 1975*a*). In making use of Ca influx to effect exocytotic responses to antigen, mast cells behave like chromaffin and several other endocrine cells responding to their physiological secretagogues (Douglas, 1974*a*, 1975*b*); but with other stimuli they seemingly use calcium mobilized from cellular sources (Douglas & Ueda, 1973; Douglas, 1975*a*, 1976; Cochrane & Douglas, 1976) much as certain exocrine cells appear to do (Argent, Case & Scratcherd, 1973; Case & Clausen, 1973; Matthews, Petersen & Williams, 1973). The coexistence, in the same cell, of two basic variations of the stimulus-secretion coupling mechanism involving extracellular and cellular Ca respectively, lends particular interest to the mast cell model.

We are concerned here with examining a central assumption of the Ca hypothesis, namely that an excess of Ca ions somewhere in the cell suffices to initiate the exocytotic response (see Douglas, 1968, 1974*a*). For mast cells, support for this postulated mediator or 'messenger' function of Ca has come from two different approaches. In the first, mast cells treated with the lipophilic antibiotic A 23187, known to act as an ionophore for divalent cations (Reed & Lardy, 1973; Pressman, 1973), were discovered to release histamine (Foreman, Mongar & Gomperts, 1973; see also Diamant & Patkar, 1975) and to extrude secretory granules by exocytosis (Cochrane & Douglas, 1974; Kagayama & Douglas, 1974) in a Ca-dependent manner. In the second, granule extrusion was noted when Ca was injected into mast cells through a micropipette (Kanno, Cochrane & Douglas, 1973). Valuable as they are, both sets of results fall short of establishing the sufficiency of calcium for eliciting exocytosis, for both sorts of experiment involve manipulations whose possible adjuvant effect is unknown – on the one hand the incorporation of foreign lipophilic material in the cell and on the other the passage of substantial ion-ejecting current across the cell membrane. Here we report a third, and quite different, condition that indicates the ability of Ca to elicit exocytosis in the mast cell in the absence of anaphylactic reaction or other secretory stimulus. This involves the reintroduction of Ca in high concentration after a period of Ca deprivation. In the course of our work we have also noted some rather striking inhibitory effects of Ca in excess.

METHODS

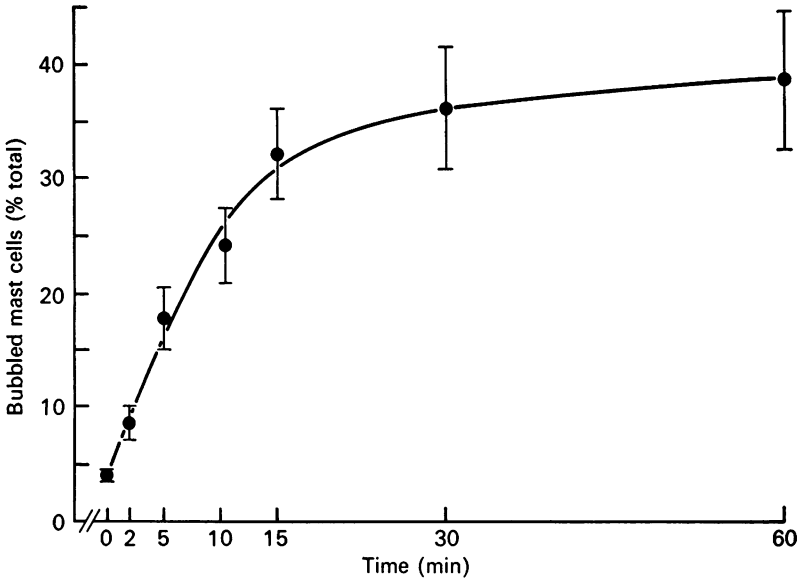
Animals. Male Sprague–Dawley rats (about 250 g) were decapitated and bled. Peritoneal washings containing mast cells were obtained as follows. A large fold of abdominal skin was snipped off with scissors and, with a needle and syringe, the peritoneal cavity was injected, through the mid line, with 8 ml. *Ca-free Locke solution* containing (mM): NaCl, 150; KCl, 5; Na_2HPO_4-NaH_2PO_4 buffer (pH 7·0), 3; glucose 5·6; with the addition of bovine serum albumin 1 mg/ml. and heparin 10 u./ml. This and all the other solutions used, except where stated otherwise, were equilibrated with air and used at room temperature (about 22° C). The abdomen was gently massaged for 90 s after which it was opened in the mid line and the fluid was aspirated with a plastic pipette and transferred to a plastic tube. The cells were spun down by centrifuging at room temperature at 270 *g* for 5 min. The supernatant was decanted and the cells were washed with Ca-free Locke solution. Portions were recentrifuged and the cells were resuspended in Ca-free Locke or *Locke* (the same with the addition of 2 mM-$CaCl_2$). The cell suspensions were then incubated, with gentle shaking, for 2 h at 37° C. Portions (1–2 ml.) were pipetted into Sykes–Moore culture chambers and the cells were allowed to settle on the optical glass bottom and observed by phase contrast (× 400) with an inverted microscope. The medium was then gently drawn off and replaced with *HEPES-Locke* or *Ca-free HEPES-Locke* (for cells previously incubated in Locke or Ca-free Locke respectively). These media were identical to the solutions described previously save that, to prevent precipitation of Ca on exposure to high Ca media, the phosphate buffer was replaced by 5 mM-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) with pH adjusted to 7·0 by addition of NaOH. These two treatments yielded mast cells that were either normal or Ca-deprived. These were then exposed to experimental test media, commonly containing excess Ca, either 4, 8, 16, 32, 64 or 110 mM-$CaCl_2$ (this last is referred to as '110 *mM-Ca-Locke*') with appropriate reductions in the concentration of NaCl to maintain the solutions isosmotic with Locke. In '110 *mM-Mg-Locke*', $MgCl_2$ was used in place of $CaCl_2$. To count the mast cells that had responded at a particular time to any of these treatments, the cells were fixed with glutaraldehyde (2% in 0·1 M cacodylate buffer). The appearances of cells before and after fixation were identical. Mast cells were judged to be 'bubbled' when their outlines, normally smooth, showed many irregular protrusions usually with loss of refractility. For any one count, twenty fields containing a total of about 300–600 mast cells were rated.

For electron microscopy the cells were maintained in suspension in plastic tubes with gentle shaking, and changes in media were effected by centrifuging and resuspending the cells as described above. The cells were fixed with glutaraldehyde, post-fixed with osmium tetroxide and embedded in Epon. Thin sections, stained with uranyl acetate and lead citrate, were examined with a Zeiss 9A electron microscope. Details of the method have been provided (Kagayama & Douglas, 1974).

RESULTS

Effect of high Ca. Most mast cells preincubated for 2 h in Ca-containing media (Locke followed by HEPES-Locke) retained a normal appearance when exposed to 110 mM-Ca-Locke (compare Pl. 1*A* and *B*): in three experiments only 5·8 ± 0·9 % (mean ± S.E.) of the mast cells were 'bubbled' after 30 min in the high-Ca medium and this value was comparable with that found in populations of normal mast cells maintained for the same

total time (150 min) in the conventional medium (Locke) containing 2 mM-Ca (4·2 ± 0·7 %, $n = 3$). By contrast, many mast cells preincubated for 2 h in Ca-free media (Ca-free Locke followed by Ca-free HEPES-Locke) bubbled when exposed to this high-Ca medium (39·5 ± 5·1 %, $n = 4$) and assumed an irregular outline and wrinkled 'prune-like' appearance (Pl. 1*D*). This occurred despite the fact that the cells appeared normal before excess Ca was admitted (Pl. 1*C*) and showed no increase in the incidence



Text-fig. 1. Time course of the bubbling response evoked by exposing mast cells to 110 mM-Ca Locke after 2 h incubation in Ca-free Locke ($n = 3$).

of bubbling if further incubated for 30 min with Ca-free HEPES-Locke (3·9 ± 0·6 %, $n = 4$). Some mast cells bubbled within a minute or so of exposure to the high-Ca medium whereas others responded more slowly. Within about 30 min, however, the phenomenon had run its course and few mast cells bubbled thereafter (Text-fig. 1). Mast cells preincubated in Ca-free media for periods much shorter than 2 h did not bubble as often to 110 mM-Ca; and only 6·5 ± 1·3 % ($n = 2$) became bubbled when Ca-free preincubation was limited to 15 min.

Magnesium. Like Ca, magnesium in excess (110 mM) did not change the appearance of mast cells preincubated in Ca-containing media. But unlike calcium, Mg failed to induce bubbling in Ca-deprived mast cells (compare Pl. 1*E* and *F*): in three experiments only 4·8 ± 0·9 % were bubbled after 30 min exposure to 110 mM-Mg–Locke.

The addition of Mg (1 mM) to the Ca-free preincubation media had little,

if any, effect on the subsequent bubbling response to 110 mM-Ca (compare Pl. 1*G* and *H*): in three experiments 42·2 ± 6·5 % of the mast cells were bubbled after 30 min exposure to 110 mM-Ca.

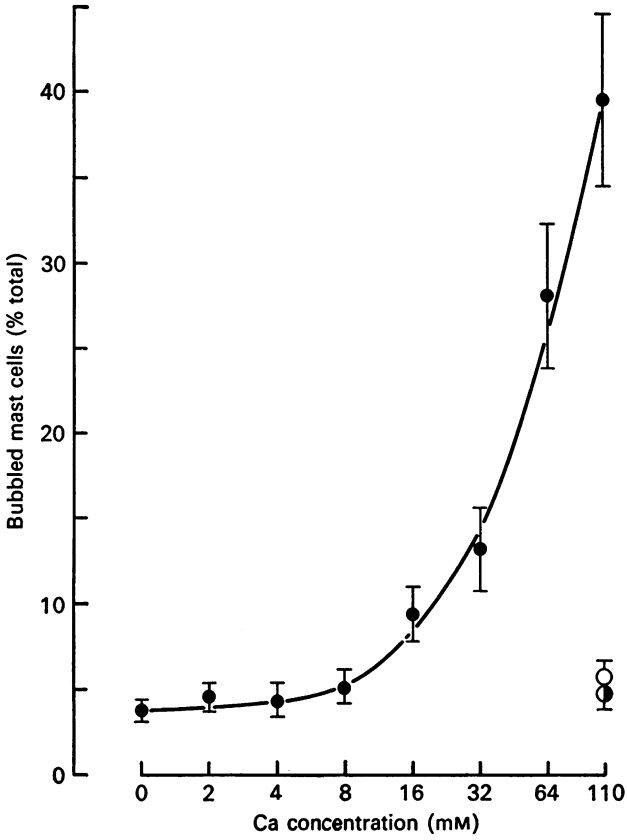
Electron microscopy. Mast cells exposed to 110 mM-Ca without prior Ca deprivation typically showed little, or no, evidence of secretory activity (compare Pl. 2*A* and *B*); and the same was true of mast cells exposed to Ca-free media for prolonged periods (Pl. 2*C*), as previously noted (Kagayama & Douglas, 1974). By contrast, many Ca-deprived exposed to 110 mM-Ca showed the signs of 'compound' exocytosis (Douglas, 1974*a*) familiar from studies of responses to conventional secretagogues such as antigen (Anderson, Slorach & Uvnäs, 1973), 48/80 (Röhlich, Anderson & Uvnäs, 1971), and polymyxin B (Lagunoff, 1972): granules devoid of membranes and of reduced electron density were present in exocytotic pits or cisterns with openings to the cell surface frequently visible in the plane of section. In many instances all, or nearly all, of the granules in the plane of section were involved (Pl. 2*D*). In others, the reaction was less extensive (Pl. 2*E*). Cells exposed to 110 mM-Mg after Ca deprivation did not show these changes (Pl. 2*F*).

Treatment with EDTA. Stimulant actions on mast cell secretion of 48/80 and of Na lack involve mobilization of Ca from some cellular store (Douglas & Ueda, 1973; Cochrane & Douglas, 1976). Excess Ca, however, does not seem to act in this manner: addition of 2 mM-EDTA during the 'conditioning' period of Ca deprivation, which inhibits reversibly responses to 48/80 (Douglas & Ueda, 1973; Cochrane & Douglas, 1974; Kagayama & Douglas, 1974) or Na lack (Cochrane & Douglas, 1976), did not appreciably alter the response to 110 mM-Ca: the number of bubbled cells after 30 min was comparable (44·3 ± 6·2 %, $n = 3$). Mg (110 mM) was again without effect: less than 6 % of the mast cells were bubbled at 30 min in each of two experiments.

Effect of Ca concentration. The response was greatest with 110 mM-Ca, the highest concentration tested, and fell off steeply with Ca concentrations below this with the threshold lying at about 16 mM (Text-fig. 2).

Effects of inhibiting energy production. Exocytotic secretory responses of rat peritoneal mast cells to anaphylaxis and 48/80 are energy-dependent (see Diamant, 1975; Peterson, 1974). The responses to 110 mM-Ca were likewise suppressed by inhibiting energy production. In three experiments, glucose was omitted during the final hour of the Ca-free preincubation and 2,4-dinitrophenol (2×10^{-4} M) was added during the last 10 min. The medium was then replaced with 110 mM-Ca Locke free of glucose with the same concentration of dinitrophenol. After 20 min only 7·1 ± 0·8 % of the mast cells were bubbled compared with 35·5 ± 3·9 % in paired control samples.

Inhibitory effects of excess Ca. Although the bubbling response to excess Ca ceased after about 30 min (Text-fig. 1), changes occurred when excess Ca was removed. Commonly many granules appeared around bubbled cells where few were evident before (compare bottom cell in Pl. 3*B*, *C*). Since this did not occur when cells were washed with high Ca medium



Text-fig. 2. Effect of Ca concentration on the incidence of the bubbling response evoked by introducing Ca to mast cells incubated 2 h in Ca-free Locke (●) (n = 4 except for the values at 4 and 8 mM, where n = 2 and 3 respectively). The half-filled circle (◑) indicates the effect of introducing 110 mM-Mg (n = 3) under the same conditions. The open circle (○) indicates the response to 110 mM-Ca *without* prior Ca-deprivation (n = 3).

(Pl. 3*E–G*), reduction of the Ca concentration evidently facilitated escape of granules. The same impression was given by a second phenomenon (illustrated by the behaviour of the uppermost mast cell in Pl. 3*B*, *C*) in which a few cells that had failed to bubble in the high-Ca medium bubbled

when the Ca concentration was reduced. Whatever the explanation of this 'off response', the point to be made is that expulsion of granules was then much more obvious than when bubbling occurred in high Ca. These observations point up a singular feature of the bubbling response to high Ca: that it rarely involves the frank expulsion of exocytosed secretory granules beyond the cell boundary. This behaviour, which results in the 'prune-like' appearance of cells stimulated with excess Ca (Pl. 1*D*, *H*; Pl. 3*B*, *F*, *G*), contrasts with that obtained with more conventional secretagogues such as specific antigen (in sensitized cells) or 48/80 where, in more normal ionic environments, many granules escape from the exocytotic pits and cluster round the cell giving what Uvnäs (1964) termed a 'mulberry-like appearance' (Pl. 4*B*, *E*; see also Bloom, 1974; Horsfield, 1965). The contrasting behaviour is also discernible in Pl. 3, where, in *D*, 48/80 was given to test the secretory competence of the two mast cells that failed to respond to Ca excess.

Concentrations of 48/80 (2·5 μg/ml.) that caused a massive degranulating response from mast cells in a normal ionic environment were without effect on mast cells in 110 mM-Ca, but a response could be elicited with higher doses of 48/80 and, significantly, it then involved expulsion of very few granules and yielded prune-like cells (Pl. 4).

DISCUSSION

The discovery that Ca elicits secretion from Ca-deprived chromaffin cells was one of the main clues prompting the Ca-influx hypothesis of stimulus-secretion coupling (Douglas & Rubin, 1961, 1963; Douglas, 1968). Ca deprivation was known to increase membrane permeability and secretion was therefore attributed to Ca ions penetrating the abnormally leaky plasma membranes. We interpret similarly the present results wherein Ca elicited granule extrusion from Ca-deprived mast cells but failed to do so when Ca deprivation was avoided. Mast cells that failed to secrete when Ca was reintroduced might simply be those whose membrane permeability was relatively little affected by Ca deprivation: their subsequent responsiveness to 48/80 (Pl. 3*D*) demonstrates secretory competence. The slowing of bubbling in the mast cell population with time might reflect the tendency of Ca to restore the plasma membrane to its normal, relatively impermeable, state.

A feature that sets the mast cell apart from the chromaffin cell is the comparatively rigorous treatment required to elicit secretion with Ca: in the chromaffin cell, reintroduction of 2 mM-Ca after 15 min Ca deprivation suffices. Perhaps the mast cell loses its complement of permeability regulating Ca less readily than does the chromaffin cell so that Ca

subsequently penetrates in effective amount only when its inwardly directed concentration gradient is greatly increased. Alternatively, the contrasting behaviour may reflect the presence, in the plasma membrane of chromaffin cells, of potential-dependent Ca channels that open as transmembrane potential falls (Douglas & Rubin, 1961; Douglas & Poisner, 1961; Douglas, Kanno & Sampson, 1967; Douglas, 1968, 1974*a*, 1975*b*; Baker & Rink, 1976) and the absence of such channels in mast cells (see Douglas, 1974*a*; Cochrane & Douglas, 1976). Chromaffin cells depolarize rapidly when deprived of Ca (Douglas *et al.* 1967) and this should open potential-dependent Ca channels. In mast cells, however, there should be no such effect, and increased tendency for Ca to penetrate after Ca-deprivation must result from some other, more general, increase in membrane permeability. Regarded in this light, the mast cell offers a simpler model than the chromaffin cell since it obviates the complications introduced by the potential-sensitive apparatus and the molecular rearrangements of the plasma membrane associated with depolarization. It follows therefore that the stimulant effect of Ca on the mast cell, albeit requiring more rigorous conditions for its demonstration than in the chromaffin cell, provides a rather better argument than Ca, by itself, is an adequate stimulus to secretion, and that concurrent perturbation of plasmalemmal structure is unnecessary (cf. Douglas, 1968).

Since secretion induced by excess Ca resembled that seen with more conventional secretagogues such as specific antigen, 48/80 or polymyxin B, both in ultrastructural characteristics and energy requirement, the results clearly support the view that Ca ions mediate stimulus-secretion coupling in the mast cell and sustain the broader thesis that energy-dependent Ca-activated exocytosis is the central feature of stimulus-secretion coupling in cells exporting preformed, membrane-packaged, secretory product (Douglas, 1968, 1974*a*).

The effect of excess Ca that hinders expulsion of granules from exocytotic pits may result from the well-known ability of Ca to cause organelles to adhere by annulling mutually repulsive negative charges and forming cationic bridges. More interesting, is the alternative possibility that the full expression of the secretory response involves an expulsive component, conceivably of a contractile nature, that is inhibited by excess Ca. Mast cells, like many others (see Isenberg, Rathke, Hülsmann, Franke & Wohlfarth-Botterman, 1974), possess supposedly contractile elements, such as microfilaments (Röhlich, 1975) whose role in secretion is uncertain (Orr, Hall & Allison, 1972; Douglas & Ueda, 1973).

The action of excess Ca that renders mast cells insensitive to 48/80 in normally effective concentration is seemingly exerted at some early step

in stimulus-secretion coupling for responsiveness to the ionophore A 23187 is little affected (D. E. Cochrane & W. W. Douglas, unpublished results).

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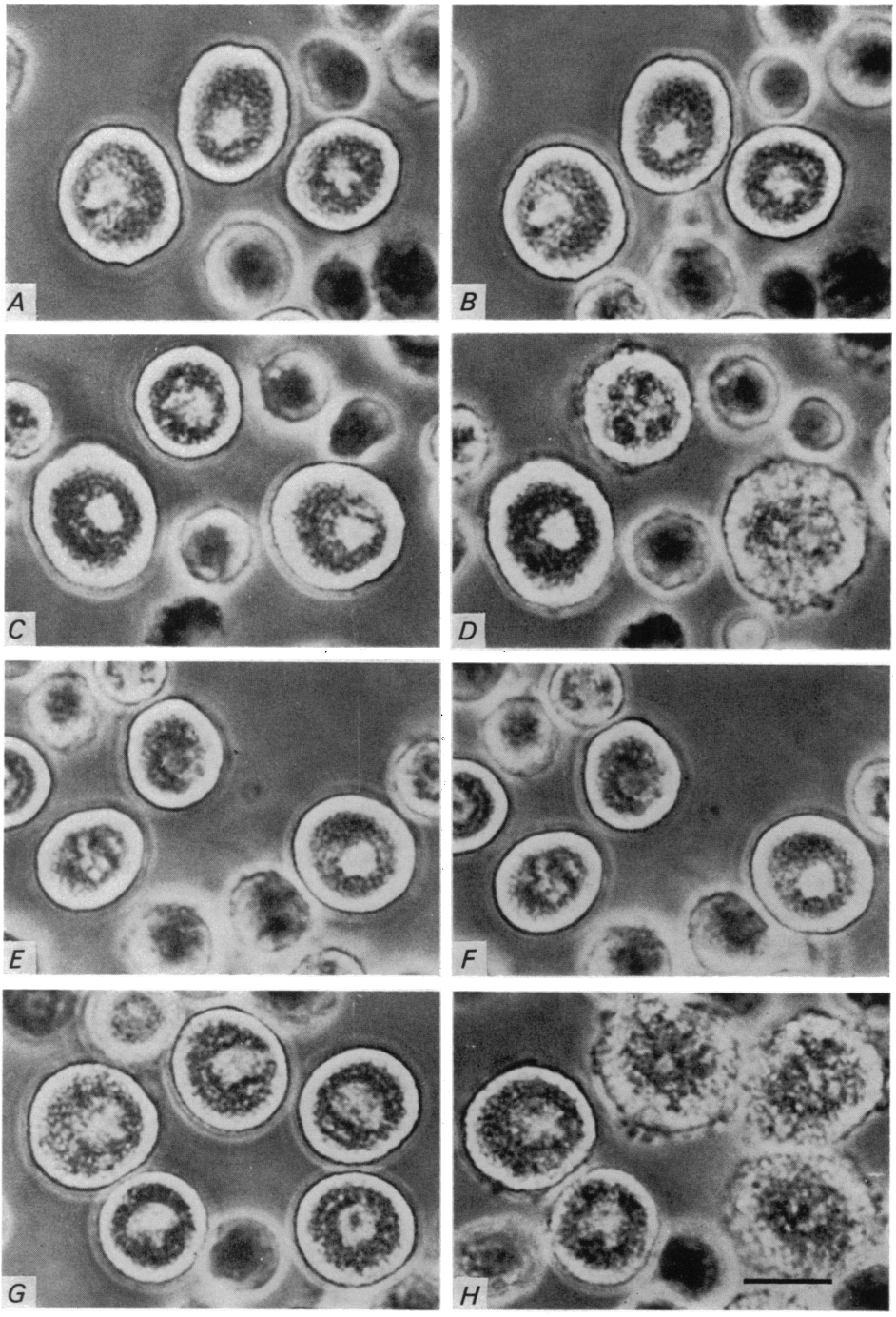

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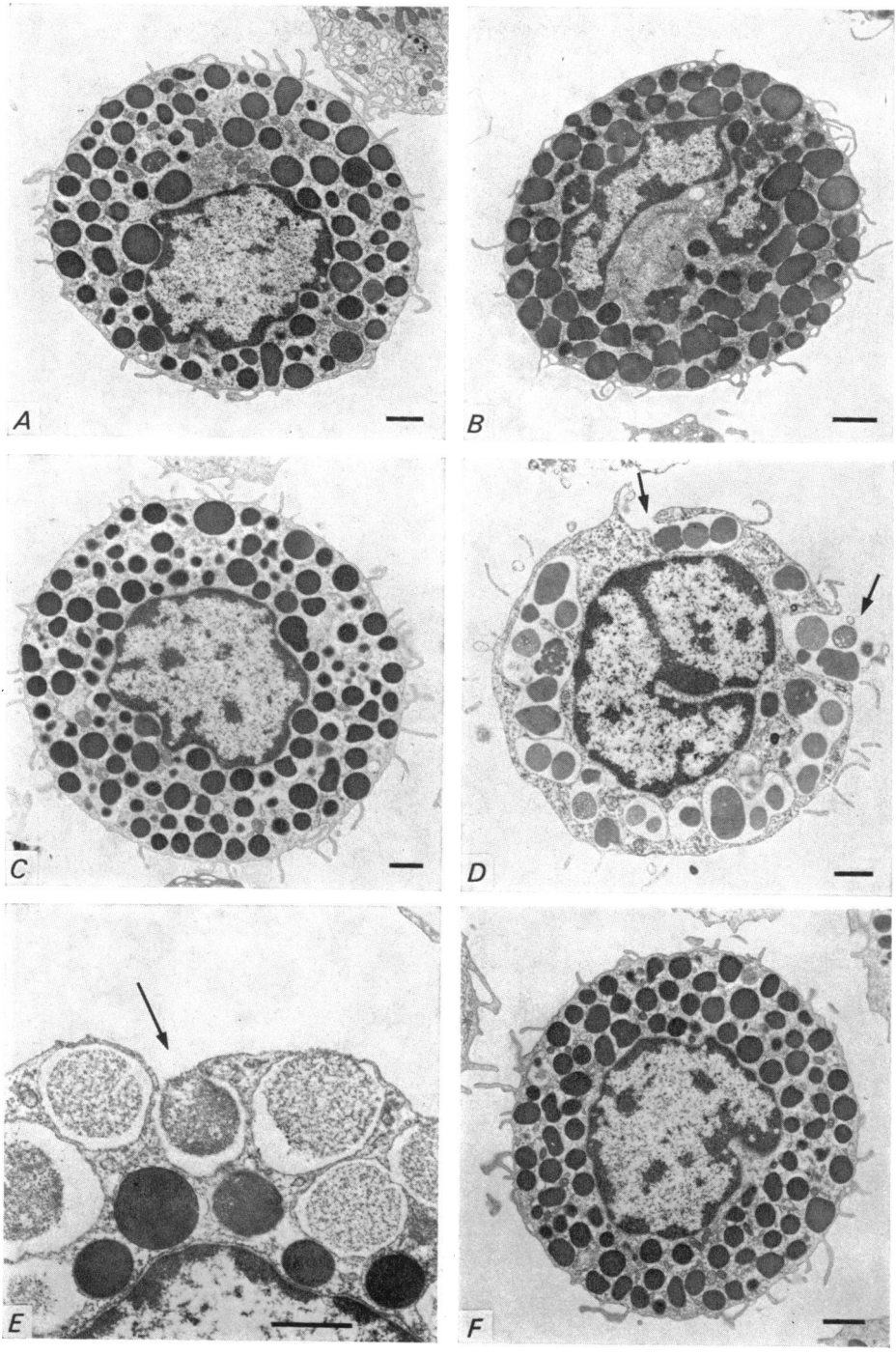
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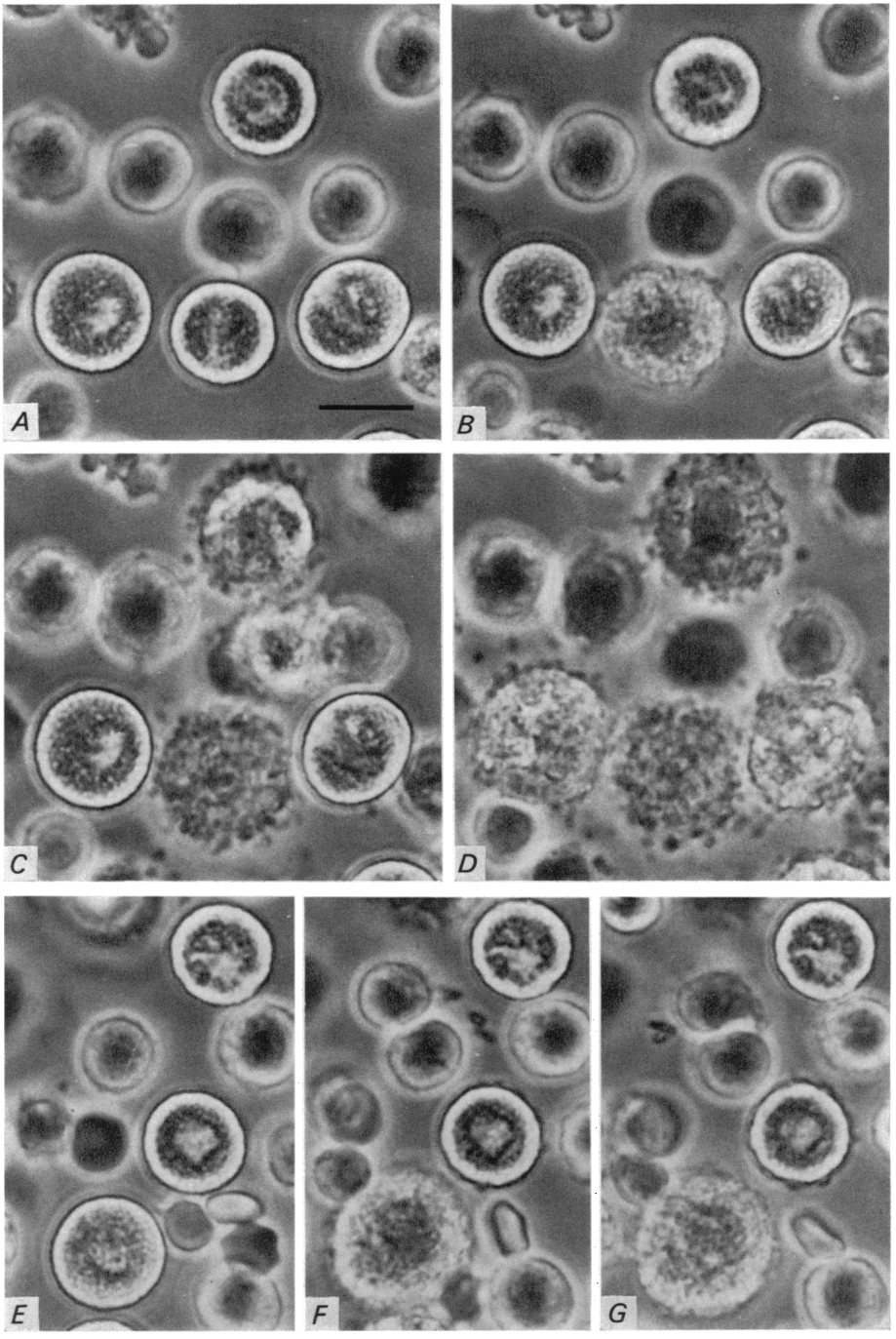
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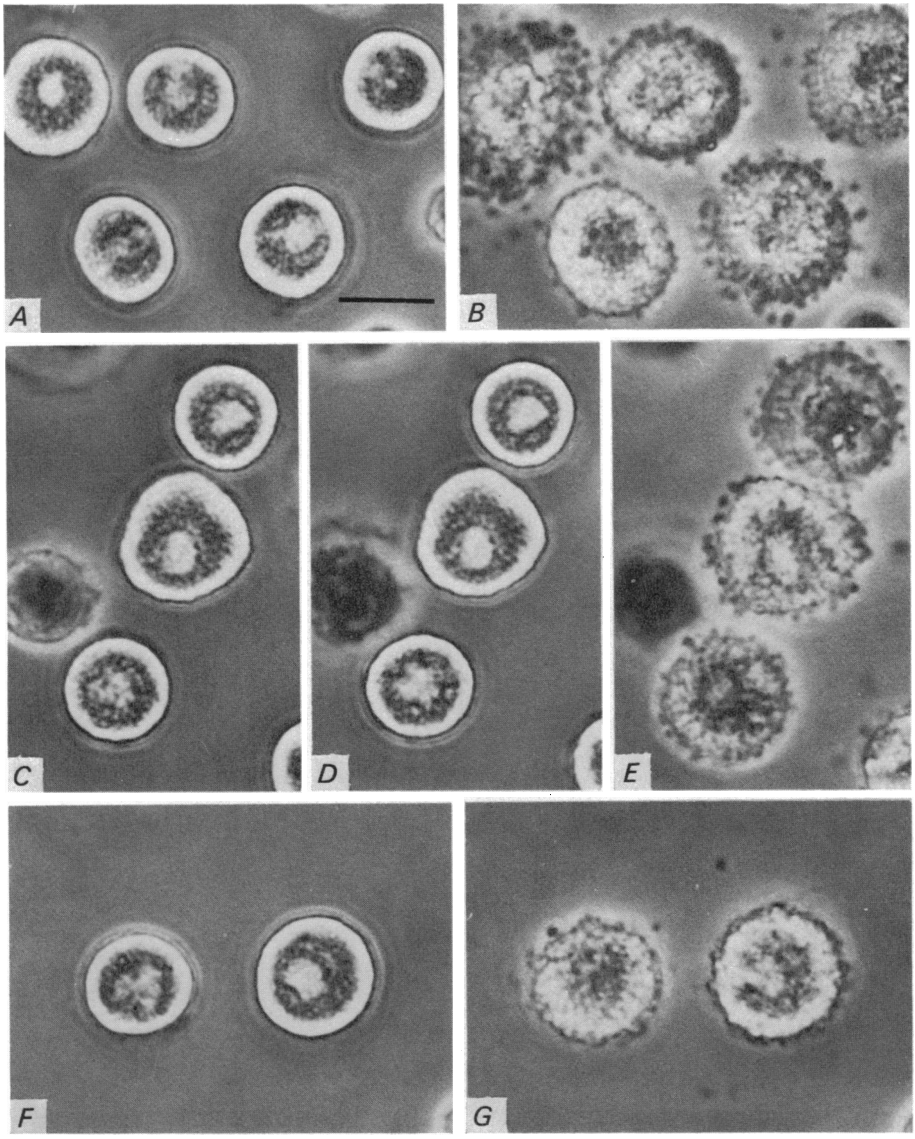
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EXPLANATION OF PLATES

PLATE 1

Light microscopic appearances of mast cells treated with Locke, Ca-free Locke, excess Ca and excess Mg illustrating the 'bubbling' response to 110 mM-Ca and its dependence on calcium deprivation. Here, and in Pls. 3 and 4, the cells are viewed with phase contrast optics (× 40 objective) and the horizontal bar represents 10 μm.

A, three mast cells in HEPES Locke following 2 h preincubation in Locke (controls).

B, the same cells 30 min after introducing 110 mM-Ca Locke. There is no response.

C, three Ca-deprived mast cells in Ca-free HEPES Locke following 2 h preincubation in Ca-free Locke.

D, the same cells 30 min after introducing 110 mM-Ca Locke. The upper and right hand mast cells now have a 'bubbled' appearance with irregular surface and loss of refractility.

E, three Ca-deprived mast cells (same treatment as in *C*).

F, the same cells 30 min after introducing 110 mM-Mg Locke. There is no response.

G, five Ca-deprived mast cells (same treatment as *C*) save that 1 mM-Mg was present throughout.

H, the same cells 30 min after introducing 110 mM-Ca Locke. The three cells to the right are 'bubbled'.

PLATE 2

Representative electron micrographs of mast cells treated with Locke, Ca-free Locke, excess Ca, and excess Mg. Exocytotic nature of the response to excess Ca. The horizontal bars represent 1 μm.

A, mast cell washed with HEPES-Locke following 2 h preincubation in Locke (control).

B, mast cell treated as in *A* and then exposed to 110 mM-Ca Locke for 30 min. No exocytosis is apparent.

C, Ca-deprived mast cell washed with Ca-free HEPES Locke following 2 h preincubation in Ca-free Locke.

D, Ca-deprived mast cell treated as in *C* and then exposed to 110 mM-Ca Locke for 30 min. Note that many granules, less dense and devoid of membranes, now lie in large membrane-bounded cisterns, some of which are seen opening to the extracellular space in the plane of section (arrows). The appearances are those of

'compound' or 'sequential' exocytosis familiar from studies with other mast cell secretagogues (see text).

E, portion of another mast cell treated as in *D* save that exposure to 110 mM-Ca Locke was for 10 min. The response to excess Ca is less extensive. Some granules are normally dense. Others, however, appear swollen, less dense, and separated from their membranes. In one instance (arrow) the membrane-bounded space containing such a granule can be seen to open to the extracellular environment.

F, a Ca-deprived mast cell prepared as in *C* and then exposed to 110 mM-Mg Locke for 30 min. The cell has a normal appearance.

PLATE 3

Responses of Ca-deprived mast cells to 110 mM-Ca followed by 2 mM-Ca illustrating changes ('off responses') associated with the reduction of extracellular Ca concentration to a conventional value and contrasting the 'prune-like' appearance of cells bubbled in high Ca with the 'mulberry-like' appearance of those bubbled in 2 mM-Ca Locke. Phase contrast. Horizontal bar represents 10 μm.

A, four mast cells after 2 h incubation in Ca-free Locke.

B, the same cells 30 min after introducing 110 mM-Ca. One (lower centre) shows the 'prune-like' appearance characteristic of the bubbling response to high Ca.

C, 'off responses' in the same cells 5 min after withdrawing the high Ca medium and replacing it with HEPES Locke (2 mM-Ca). Note that the previously 'prune-like' cell (lower centre) now displays frankly extruded granules, and that the uppermost cell has now reacted and also shows many extruded granules. These two cells now have the familiar 'mulberry' appearance classically associated with the 'degranulating' response.

D, the same cells 5 min later after adding 48/80 (2·5 μg/ml.) showing that the previously refractory cells (right and left) are capable of responding with granule extrusion.

E–G, a sequence illustrating the relative stability of the responses to high Ca when washing does not involve a reduction in the Ca concentration.

E, three mast cells after 2 h incubation in Ca-free Locke.

F, the same cells after 30 min exposure to 110 mM-Ca Locke. One of the three cells has bubbled.

G, the same cells 30 min later following washing and reincubation with 110 mM-Ca.

PLATE 4

Inhibitory effects of 110 mM-Ca on the responses of mast cells to 48/80. Phase contrast. Horizontal bar represents 10 μm.

A, five mast cells incubated in Locke (2 mM-Ca).

B, the same cells 5 min after adding 48/80 (2·5 μg/ml.) showing the familiar 'mulberry-like' response involving frank extrusion of granules (control).

C, three mast cells in 110 mM-Ca Locke (without prior Ca-deprivation) 5 min after adding 48/80 (2·5 μg/ml.). The cells show no response.

D, the same cells 10 min after adding more 48/80 (total 10 μg/ml.). There is still no response.

E, the same cells 5 min after replacing the medium with Locke (2 mM-Ca) containing 2·5 μg/ml. 48/80. Typical bubbling and granule extrusion.

F, two mast cells in 110 mM-Ca Locke (without prior Ca deprivation). The cells appear normal.

G, the same cells 15 min after exposure to a high concentration (25 μg/ml.) of 48/80. The cells have responded but have a 'prune-like' appearance with few extruded granules. This differs from the 'mulberry-like' response to 48/80 in 2 mM-Ca (of *B*) but resembles the 'bubbling' induced by excess Ca after Ca deprivation which is illustrated in Pls. 1 and 3.