ELECTRON MICROSCOPE EVIDENCE OF CALCIUM-INDUCED EXOCYTOSIS IN MAST CELLS TREATED WITH 48/80 OR THE IONOPHORES A-23187 AND X-537A

M. KAGAYAMA and W. W. DOUGLAS. From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

INTRODUCTION

The recognition of calcium as a general requirement for secretory activity in diverse cells storing their products in membrane-limited granules led to the concept of stimulus-secretion coupling involving operation of a common secretory mechanism, calcium-activated exocytosis (6; see also 7, 8). On this hypothesis, physiological stimuli evoking various secretions act by promoting the appearance of an excess of free calcium ions someplace in the cell, either by increasing the permeability of the plasma membrane to allow entry of extracellular calcium or by mobilizing cellular calcium, and the rise in the calcium ion concentration at some critical site then induces exocytosis. The present experiments were designed to assess, by electron microscopy, some recent observations on mast cells which appear to offer fresh support for this scheme and suggest new approaches.

Mast cells, long known to require calcium for anaphylactic release of histamine (15), were amongst the first suspected of utilizing the calcium-activated exocytosis mechanism (6, 9). Such cells, readily harvested from the peritoneal cavity of rats, offer numerous experimental advantages, and the exocytotic nature of their response to antigen has been clearly established (1). Until recently, however, their usefulness as a model system has been suspect since they continue to respond to the drug 48/80, a classic "degranulating" agent producing typical exocytotic responses (2, 12, 17), when suspended in calcium-free media. This objection has now been countered by evidence indicating that such responses to 48/80 utilize calcium from some cellular store, as both histamine release (10) and the characteristic granule extrusion observed by light microscopy (3) are abolished by EDTA and restored by calcium. In addition, calcium-dependent extrusion of mast cell granules has been observed in response to the ionophores A-23187 and X-537A (3), substances known to facilitate transmembrane fluxes of calcium (16); and mast cells "primed" with these ionophores (or 48/80) in calcium-free media promptly extrude granules when calcium is introduced (3). The critical question examined here is whether these various responses are indeed exocytosis.

MATERIALS AND METHODS

For comparative purposes mast cells were isolated, incubated, and treated with drugs by essentially the same procedures used in the parallel light microscope study (3). Male Sprague-Dawley rats (200–300 g) were decapitated and the peritoneal cavity was injected with 8 ml of a medium 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 medium, and all others used, was equilibrated with air and used at room temperature unless otherwise stated. After massaging the abdomen for 90 s, the peritoneal cavity was opened and the fluid pipetted into a plastic tube. In each experiment, suspensions of peritoneal mast cells from several rats were pooled and aliquots were centrifuged for 5 min at 270 *g*. The cells were washed with and suspended in the same medium with the addition of $CaCl_2$ (1.0 mM) or EDTA (2.0 mM) and incubated for 3 h at 37°C. After further centrifuging, the cells incubated with calcium were resuspended in the same medium, whereas those incubated with EDTA were resuspended in the calcium-free medium but with the concentration of EDTA reduced to 0.1 mM. The procedure yielded aliquots of control cells in calcium-containing medium and aliquots of EDTA-incubated cells suspended in the calcium-free, EDTA-containing medium. The latter, termed EDTA-treated cells, do not extrude granules in response to 48/80 or the ionophores (3). Drugs were added to 2-ml aliquots of cell suspensions in 0.1 ml of the same suspending medium. Compound 48/80 was dissolved directly before use. A-23187 and X-537A were added from stock solutions containing 10 and 25 mg/ml, respectively, of the drugs in dimethyl sulfoxide (DMSO). The final concentration of DMSO did not exceed 0.1% which affects neither histamine release (10) nor granule extrusion (3).

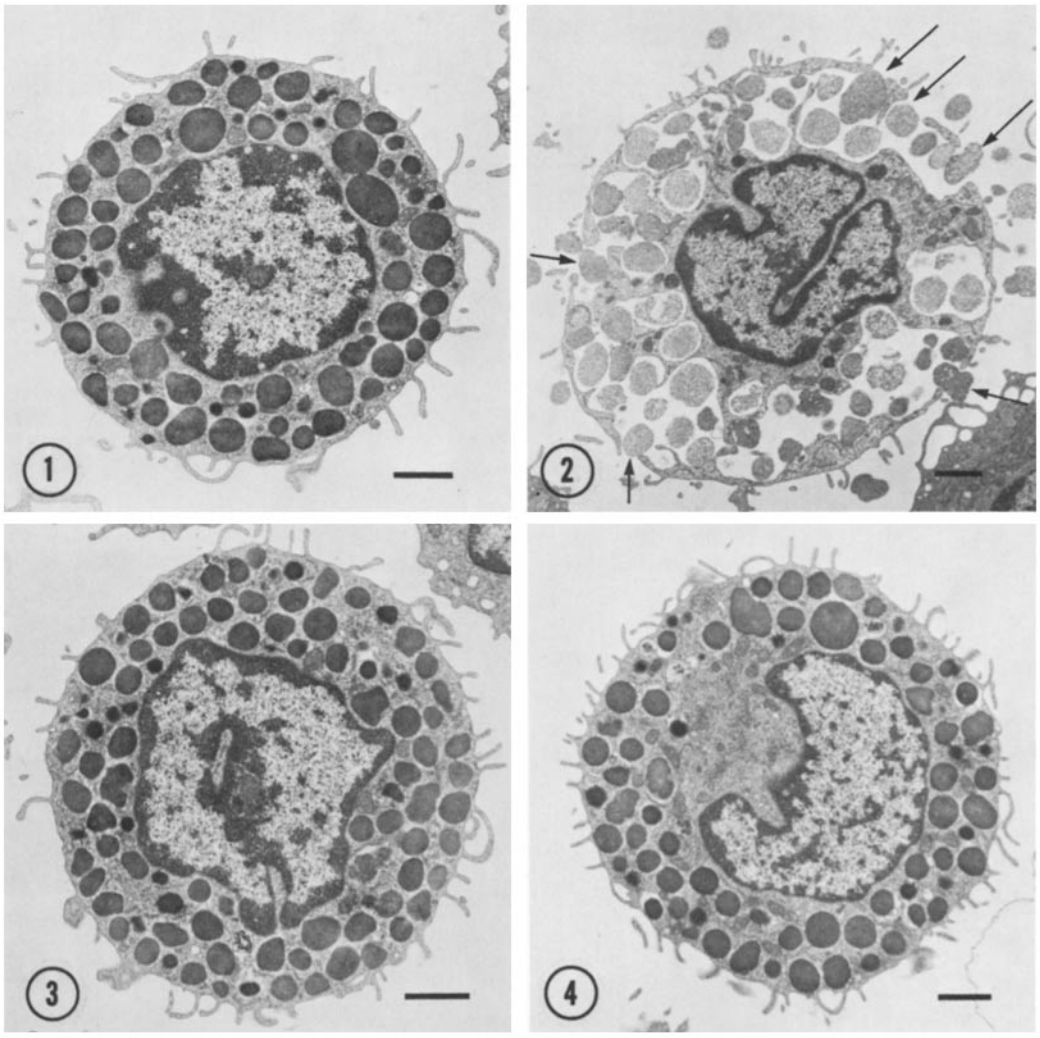
Cells were fixed by mixing suspensions with equal volumes of 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature. After 1 h the cells were centrifuged (770 *g* for 5 min). The pellet was rinsed with 0.1 M cacodylate buffer and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer at 4°C for 1 h, dehydrated, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined in a Zeiss electron microscope. Thick sections were stained with 1% aqueous toluidine blue and observed by light microscopy, as were also small samples of each cell suspension.

RESULTS

Light microscope examination showed that the mast cells used here for electron microscope analysis behaved like those used in the initial light microscope study (3). Thus, most mast cells extruded granules when exposed to 48/80, A-23187, or X-537A; this response was inhibited by pretreatment with EDTA; and EDTA-treated cells primed with any one of the three drugs extruded granules when subsequently exposed to calcium. After incubation with Ca-containing or Ca-free EDTA-containing media, the mast cells had a normal appearance. The fraction showing granule extrusion after the different treatments was estimated on duplicate samples of 200 mast cells. 5 min after adding 48/80, A-23187, or X-537A to cells suspended in Ca-containing medium, granule extrusion was evident in more than 95% of the mast cells exposed to the first two drugs and in more than 80% of those exposed to X-537A. By contrast, fewer than 5% of the EDTA-treated mast cells showed granule extrusion 5 min after any of the three drugs. After introducing calcium to EDTA-treated preparations primed with 48/80 or A-23187, more than 95% of the mast cells showed extruded granules after 5 min, and more than 75% of the cells primed with X-537A had so responded. By contrast, fewer than 5% of the EDTA-treated cells not exposed to the drugs, or treated only with the drug solvent DMSO (0.1%), showed granule extrusion after adding calcium. The electron micrographs selected for illustration are representative of the typical responses.

The ultrastructural appearance of mast cells after isolation, incubation for 3 h, and resuspension in conventional calcium-containing medium conformed to previous descriptions of normal peritoneal mast cells (1, 2, 12, 14, 17). The cell surface generally showed many cytoplasmic protrusions. Secretory granules with distinct limiting membranes filled the cytoplasm and were generally round or oval and dense (Fig. 1). Moreover, exposure to 48/80 caused the familiar spectrum of changes indicative of secretion by repetitive exocytosis (1, 2, 12, 14, 17): secretory granules became less dense, stippled in appearance, and often obviously swollen; and many of them, devoid of limiting membrane, lay outside the cell or in membrane-bounded cisternae frequently seen communicating with the extracellular space (Fig. 2).

EDTA-treated cells were not noticeably different from the controls (compare Fig. 3 with Fig. 1) but failed to respond to 48/80 (compare Fig. 4 with Fig. 2). However, EDTA-treated cells exposed to 48/80 almost all yielded the familiar exocytotic pattern when calcium was introduced (Figs. 5–7). The response occasionally involved only a few granules in a single plane of section (Figs. 5 and 6), but usually most granules were affected (Fig. 7). In the absence of 48/80, calcium had no obvious



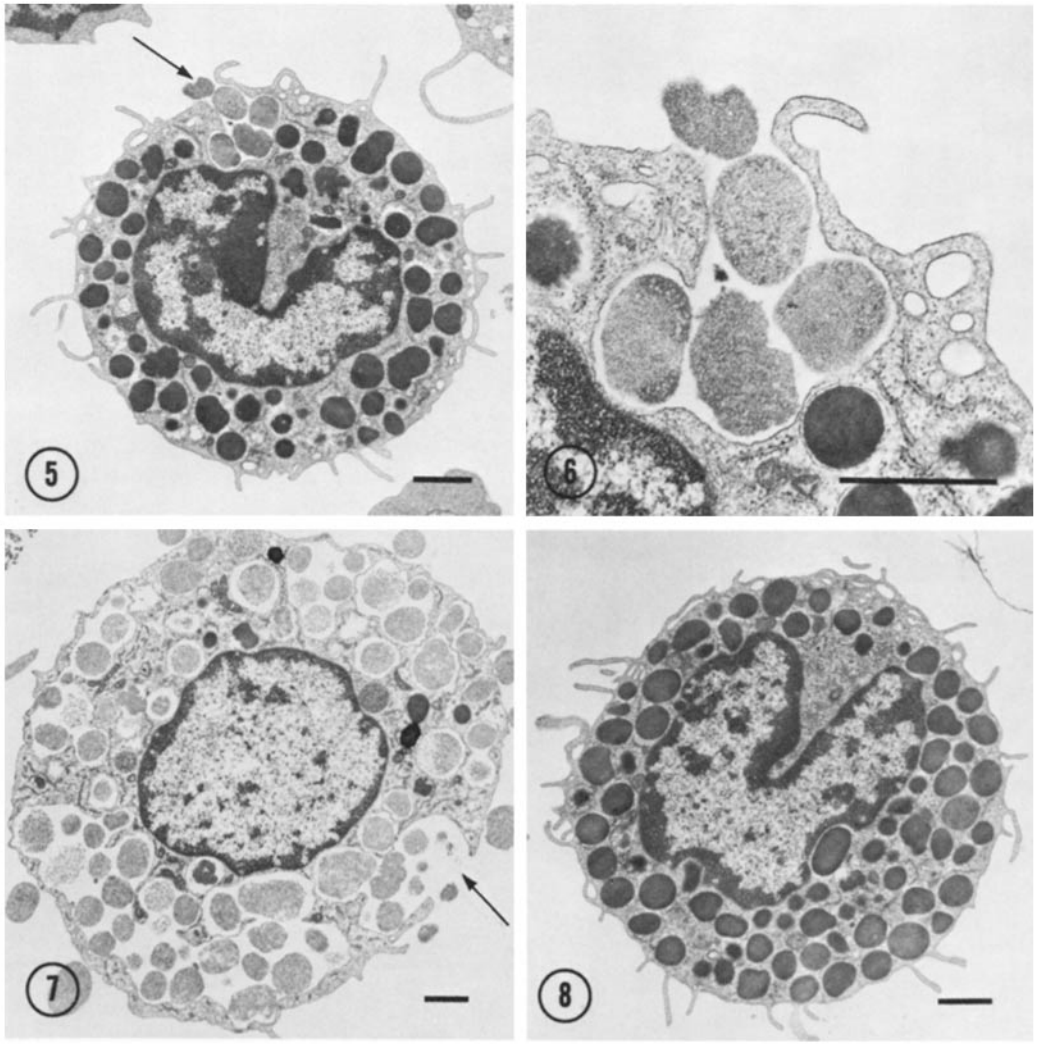
FIGURES 1–4 Prevention of the exocytotic response of peritoneal mast cells to compound 48/80 by pretreatment with EDTA. The arrows here and in subsequent figures indicate openings of exocytotic pits (or cisternae) to the cell surface, and the horizontal bars represent 1 μm.

FIGURE 1 A control mast cell incubated 3 h in conventional Ca-containing medium. The granules are dense and there is no evidence of exocytosis. × 7,700.

FIGURE 2 Exocytotic response of a mast cell in Ca-containing medium to compound 48/80 (2.5 μg/ml for 30 s). Almost all the granules are less dense and many, devoid of limiting membrane, lie outside the cell or in large cisternae sometimes seen to open to the extracellular space (arrows). This is the classic response to 48/80 (2, 12, 17), and is to be compared with that of the EDTA-treated cell shown in Fig. 4. × 6,300.

FIGURE 3 A mast cell incubated with 2 mM EDTA for 3 h and suspended in Ca-free medium containing 0.1 mM EDTA (an "EDTA-treated mast cell"). The cell retains a normal appearance. × 8,300.

FIGURE 4 A mast cell treated with EDTA as in Fig. 3 and further exposed to 48/80 (2.5 μg/ml for 12 min). The cell shows none of the signs of exocytosis exhibited by the cell in conventional Ca-containing medium. (Compare with Fig. 2.) × 6,900.



FIGURES 5–8 Exocytosis caused by introducing calcium to EDTA-treated mast cells exposed to compound 48/80; and the failure of calcium to elicit exocytosis in the absence of 48/80.

FIGURE 5 An EDTA-treated mast cell exposed to 1.0 mM Ca for 2 min after a 10-min exposure to 48/80 (2.5 μg/ml), showing what appears to be an early phase of the response involving only a single group of granules in this section. × 7,300.

FIGURE 6 A higher power view of the affected granules shown in Fig. 5. Four granules are seen lying within a cisterna whose membranous lining is continuous with the cell surface. A fifth granule has apparently been extruded through the opening of this complex exocytotic pit. × 21,000.

FIGURE 7 Another, more advanced (and more typical), example of Ca-induced exocytosis in an EDTA-treated mast cell exposed to compound 48/80. (Same conditions as Fig. 5.) × 6,000.

FIGURE 8 An EDTA-treated mast cell exposed to 1 mM Ca for 10 min in the absence of 48/80. The cell appears normal and shows no sign of exocytosis. × 7,000.

FIGURES 9–13 Effects of the ionophore A-23187.

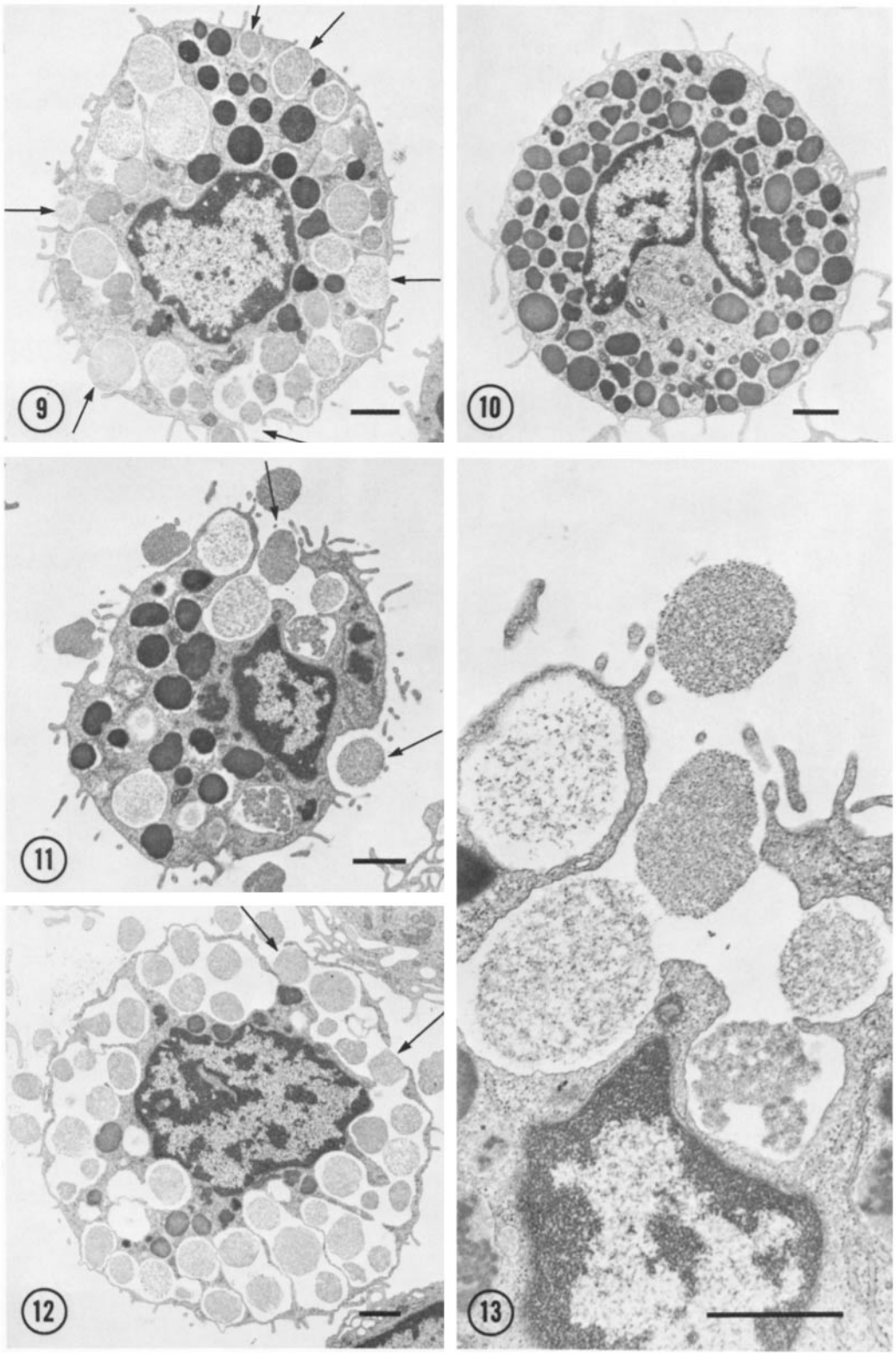
FIGURE 9 Exocytosis in a mast cell suspended in Ca-containing medium and exposed to A-23187 (2.5 μg/ml for 60 s). × 7,300.

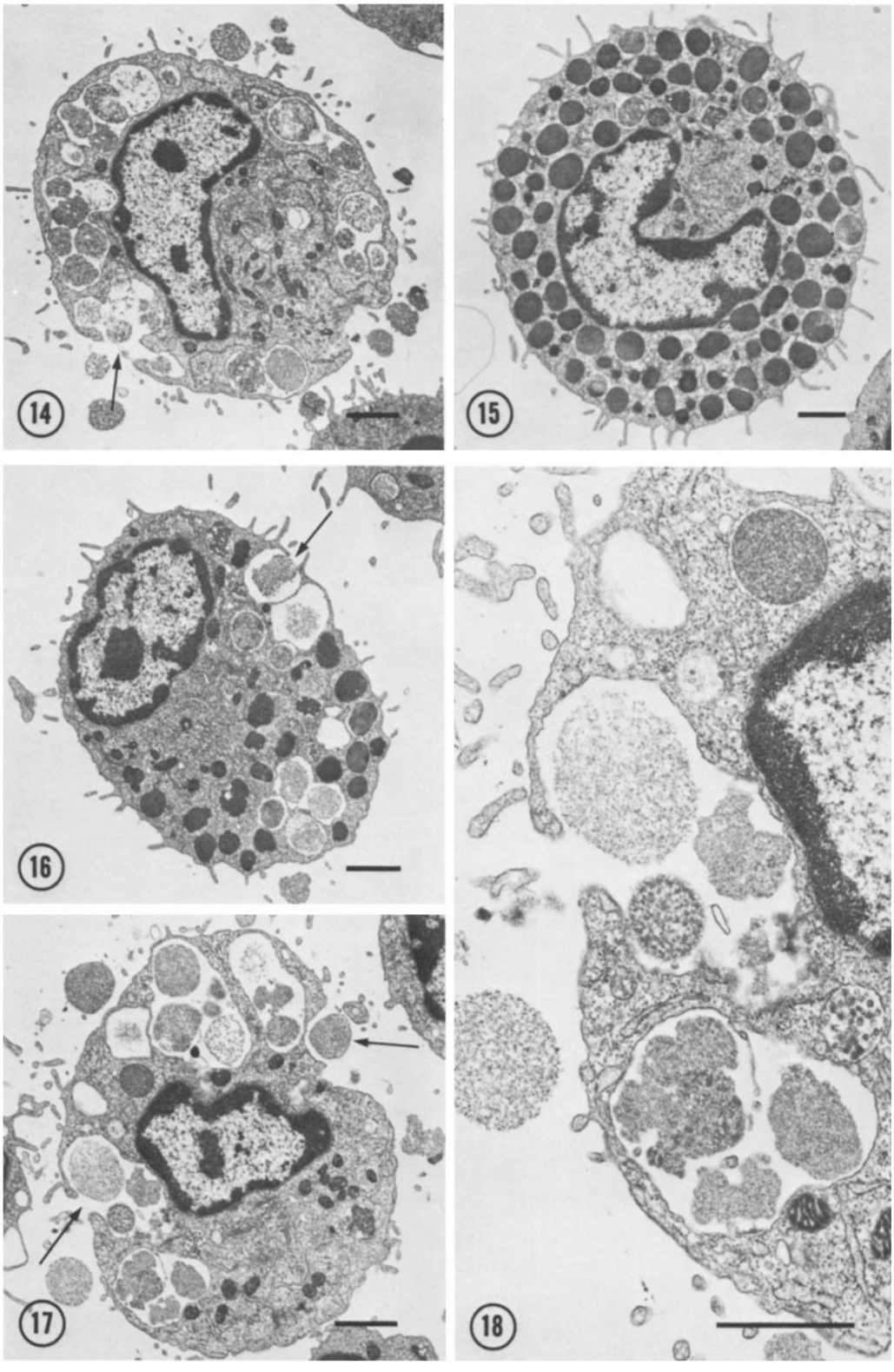
FIGURE 10 Failure of A-23187 (2.5 μg/ml for 10 min) to elicit exocytosis in an EDTA-treated mast cell. × 6,800.

FIGURE 11 Calcium-induced exocytosis in an EDTA-treated mast cell exposed to A-23187 (2.5 μg/ml for 10 min). Ca (1.0 mM) introduced 60 s before fixing. × 8,000.

FIGURE 12 Another, more complete, exocytotic response to Ca. (Conditions as in Fig. 11.) × 6,100.

FIGURE 13 Higher magnification view of the exocytotic pit shown in Fig. 11. × 21,000.


9
10
11
12
13


14
15
16
17
18

effect on the EDTA-treated cells (Fig. 8).

Essentially similar results were obtained with A-23187 and X-537A (Figs. 9–13 and 14–18, respectively). Each drug elicited the typical complex exocytotic response in the presence of calcium (Figs. 9 and 14). And each drug failed to do so in EDTA-treated cells (Figs. 10 and 15) although it primed them to respond with exocytosis on the introduction of calcium (Figs. 11–13 and 16–18).

DISCUSSION

The electron microscope evidence establishes that the granule extrusion response of mast cells to A-23187 and X-537A is, as suspected (3), exocytosis, and shows further that this response conforms to the complex pattern—termed "sequential" (17) or "compound" (7) exocytosis—observed here and in previous studies with 48/80 (2, 12, 17), polymyxin B sulfate (14) and antigen (1). Moreover, the evidence reveals that incubation and suspension in calcium-free, EDTA-containing media, which suppresses granule extrusion in response to all three drugs (3), is without discernible effect on mast cell ultrastructure, and establishes the main point that calcium introduced to mast cells primed with any of the three drugs also elicits the same compound exocytotic secretory response.

The stimulant and "priming" effects of the ionophores may simply reflect their ability to promote influx of calcium across the cell membrane (16); and it is not improbable that the priming effects of 48/80, which is also believed to act on the mast cell membrane (5), are similarly explained. Light microscope analysis has indicated that calcium is the only ion needed for the responses and that changes in membrane potential are probably unimportant (3). The results could thus be considered support for the suggestion that calcium influx mediates antigen-induced histamine release (9) and for the general hypothesis that exocytosis activated by calcium is the central event in stimulus-secretion coupling (6, see also 7, 8).

Since our experiments were completed, and a preliminary account presented at a meeting in the summer of 1973 (8), it has been reported that histamine release from mast cells elicited by A-23187 requires not only calcium but metabolic energy (11). This is consistent with release occurring by the type of secretion demonstrated here and by light microscopy (3): exocytosis generally seems to require calcium and energy (6, 7). On the other hand, in the same study (11), histamine release in response to X-537A required neither energy nor calcium and it was therefore suggested that this drug may release histamine from granules intracellularly. However, the present and other (3) morphological evidence demonstrates that X-537A elicits granule extrusion by calcium-dependent exocytosis. Whether X-537A has more than one action remains to be determined but it is evident that histamine release is not an adequate index of exocytotic activity.

Although we consider our results as support for the "calcium-activated exocytosis" concept of stimulus-secretion coupling (6, see also 7, 8) we wish to emphasize that the ionophores and 48/80 may produce effects, as yet unidentified, that are essential for the expression of the exocytotic response to calcium. For example, lipophilic molecules such as the ionophores (16) may alter properties of cell membranes besides ion permeability. The possibility that stimuli that induce exocytosis in various cells may simply act by elevating the concentration of free calcium ions at some critical intracellular site must be tested more directly. A step in this direction is the recent demonstration that intracellular microinjection of calcium into mast cells elicits granule extrusion (13).

FIGURES 14–18 Effects of the ionophore X-537A.

FIGURE 14 Exocytosis in a mast cell suspended in Ca-containing medium and exposed to X-537A (10 μg/ml for 90 s). × 8,000.

FIGURE 15 Failure of X-537A (10 μg/ml for 10 min) to elicit exocytosis in an EDTA-treated mast cell. × 7,600.

FIGURE 16 Calcium-induced exocytosis in an EDTA-treated mast cell exposed to X-537A (10 μg/ml for 10 min). Ca (1.0 mM) introduced 60 s before fixing. × 8,300.

FIGURE 17 Another, more complete, exocytotic response to Ca. (Conditions as in Fig. 16.) × 9,400.

FIGURE 18 Higher magnification view of the exocytotic pit shown in Fig. 17. × 21,000.

We thank Hoffman-La Roche Inc. and Eli Lilly Co. for gifts of X-537A and A-23187, respectively.

This work was supported by grants NS-08564 and NS-09137 from the United States Public Health Service.

Received for publication 23 November 1973, and in revised form 17 April 1974.

REFERENCES

1. BLOOM, G. D., and N. CHAKRAVARTY. 1970. Time course of anaphylactic histamine release and morphological changes in rat peritoneal mast cells. *Acta Physiol. Scand.* **78**:410–419.
2. BLOOM, G. D., and Ö. HAEGERMARK. 1965. A study on morphological changes and histamine release induced by compound 48/80 in rat peritoneal mast cells. *Exp. Cell Res.* **40**:637–654.
3. COCHRANE, D. E., and W. W. DOUGLAS. 1974. Calcium-induced extrusion of secretory granules (exocytosis) in mast cells exposed to 48/80 or the ionophores, A-23187 and X-537A. *Proc. Natl. Acad. Sci. U. S. A.* **71**:408–412.
4. DIAMANT, B., and P. G. KRÜGER. 1967. Histamine release from isolated rat peritoneal mast cells induced by adenosine-5′-triphosphate. *Acta Physiol. Scand.* **71**:291–302.
5. DIAMANT, B., P. G. KRÜGER, and B. UVNÄS. 1970. Local degranulation of individual rat peritoneal mast cells induced by compound 48/80. *Acta Physiol. Scand.* **79**:1–5.
6. DOUGLAS, W. W. 1968. Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. The First Gaddum Memorial Lecture. *Br. J. Pharmacol.* **34**:451–474.
7. DOUGLAS, W. W. 1974. Involvement of calcium in exocytosis and the exocytosis-vesiculation sequence. Calcium and cell regulation. *Biochem. Soc. Symp.* No. 39. 1–28.
8. DOUGLAS, W. W. 1974. Exocytosis and the exocytosis-vesiculation sequence. *In* Alfred Benzon Symposium VII. O. H. Peterson, editor. Munksgaard, Copenhagen. 801–814.
9. DOUGLAS, W. W., and R. P. RUBIN. 1961. The role of calcium in the secretory response of the adrenal medulla to acetylcholine. *J. Physiol. (Lond.).* **159**:40–57.
10. DOUGLAS, W. W., and Y. UEDA. 1973. Mast cell secretion (histamine release) induced by 48/80: calcium-dependent exocytosis inhibited strongly by cytochalasin only when glycolysis is rate-limiting. *J. Physiol. (Lond.).* **234**:97–98P.
11. FOREMAN, J. C., J. L. MONGAR, and B. D. GOMPERTS. 1973. Calcium ionophores and movement of calcium ions following the physiological stimulus to a secretory process. *Nature (Lond.).* **245**:249–251.
12. HORSFIELD, G. I. 1965. The effect of compound 48/80 on the rat mast cell. *J. Pathol. Bacteriol.* **90**:599–604.
13. KANNO, T., D. E. COCHRANE, and W. W. DOUGLAS. 1973. Exocytosis (secretory granule extrusion) induced by injection of calcium into mast cells. *Can. J. Physiol. Pharmacol.* **51**:1001–1004.
14. LAGUNOFF, D. 1973. Membrane fusion during mast cell secretion. *J. Cell Biol.* **57**:252–259.
15. MONGAR, J. L., and H. O. SCHILD. 1962. Cellular mechanisms in anaphylaxis. *Physiol. Rev.* **46**:51–66.
16. PRESSMAN, B. C. 1973. Properties of ionophores with broad range cation selectivity. *Fed. Proc.* **32**:1698–1703.
17. RÖHLICH, P., P. ANDERSON, and B. UVNÄS. 1971. Electron microscopic observations on compound 48/80-induced degranulation in mast cells. *J. Cell Biol.* **51**:465–483.