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Calcium-Induced Extrusion of Secretory Granules (Exocytosis) in Mast Cells Exposed to 48/80 or the Ionophores A-23187 and X-537A

(histamine secretion/monovalent and divalent cations/light microscopy/stimulus-secretion coupling)

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ABSTRACT **Isolated peritoneal mast cells from rats were observed, by phase contrast microscopy, to extrude secretory granules when exposed to 48/80 or the ionophores A-23187 and X-537A, which are known to facilitate transmembrane fluxes of calcium. These effects were abolished when the cells were treated with EDTA and suspended in a Ca-free environment. Ca-deprived cells exposed to any one of the three drugs promptly extruded granules when calcium, but not magnesium, was added to the incubation medium. Such Ca-evoked or Ca-dependent responses persisted when Na was omitted from the incubation medium and replaced with sucrose, choline, or K. The responses thus seem independent of possible shifts in the alkali metal ions. The results are considered support for the view that Ca influx mediates stimulus-secretion coupling and does so by initiating exocytosis.**

When various observations on the medullary chromaffin cell led Douglas and Rubin (1) to propose the calcium-influx hypothesis of stimulus-secretion coupling for the medullary chromaffin cell, they suggested that a similar mechanism might operate in the mast cell, whose secretory response in anaphylaxis was known at the time to be Ca-dependent (2). Later, when exocytosis was established beyond all doubt as the mode of medullary secretion, and when the coexistence of exocytosis and calcium-dependence was demonstrated in other cells, the behavior of the mast cell was suggested to be but one example of a seemingly general secretory mechanism, namely calcium-activated, or dependent, exocytosis (2). On this hypothesis, various secretagogues (substances eliciting secretion) act by promoting Ca-influx or by mobilizing cellular Ca, and it is the appearance of an excess of free Ca ions someplace in the cell that then initiates this secretory response. More recently the list of cells appearing to fit this pattern has been greatly extended (3).

The mast cell offers an interesting model system for studying stimulus-secretion coupling, since the large size of its secretory granules allows light microscopic observation of granule extrusion in the living cell (e.g., 4) and there is extensive electron microscopic evidence confirming that such extrusion occurs by exocytosis (4–6). Doubts about the validity of the isolated mast cell as a model system for studying exocytosis based upon evidence that responses to 48/80 (the classical mast cell secretagogue or histamine-releasing substance) can be obtained in Ca-free media (7) have recently been dispelled by the evidence that the response can utilize tightly bound Ca. Thus, histamine release promoted by 48/80 was abolished by incubation with EDTA and reappeared after reintroduction of Ca (7).

Our main purpose in the present experiments is to present evidence that the simple introduction of calcium induces massive granule extrusion from mast cells maintained in a Ca-free environment and primed by 48/80 or by the ionophores X-537A and A-23187, which are known to facilitate fluxes of Ca ions through various membranes (8). The results encourage the view that Ca-activated exocytosis is a key event in stimulus-secretion coupling and heighten interest in the usefulness of the mast cell for study of the intimate nature of calcium's involvement.

METHODS AND MATERIALS

Preparation of Mast Cells. Male Sprague–Dawley rats weighing 200–300 g were decapitated. *Control mast cells* were prepared as follows: about 8 ml of Locke solution at room temperature was injected into the peritoneal cavity, the abdomen was gently massaged for 90 sec, and the peritoneal washing, containing mast and other cells, was aspirated and centrifuged at 400 × *g* for 5 min. The supernatant was discarded and the cells resuspended in the same medium. Aliquots (0.5 ml) were pipetted into Sykes–Moore culture chambers, allowed to settle for about 30 min, and observed through an inverted microscope under phase contrast (×400). Representative fields were selected, their stage coordinates recorded, and photomicrographs taken. Thereafter, one of the three drugs (48/80, A-23187, and X-537A), made up in 0.1 ml of the same medium, was added, effects on the whole population of cells were noted, and the representative fields were rephotographed. *EDTA-treated cells* were prepared and studied along the same general lines except that they were incubated with EDTA by the procedure previously shown to prevent histamine release in response to 48/80 (7). Ca-free Locke was used to wash the peritoneal cavity and the cells, harvested by centrifuging the peritoneal washing, were resuspended in Ca-free Locke containing 2 mM EDTA, incubated for 3 hr at 37° with gentle agitation, centrifuged, and washed once more with this medium, and finally suspended in Ca-free Locke containing 0.1 mM EDTA and pipetted into Sykes–Moore chambers. After the cells had settled and representative fields had been photographed, 0.1 ml of the same medium containing one of the three drugs was introduced and 15 min later the fields were rephotographed. Then Ca or Mg (again in 0.1 ml of the appropriate Locke) was added, and 1–3 min later the procedure of inspecting the population and photographing the representative fields was repeated.

In some experiments the cells pipetted into the culture chamber were suspended in one of three Na-free media. In such instances the same medium was also used for the preceding wash and served also as the vehicle for subsequent addition of drugs or ions.

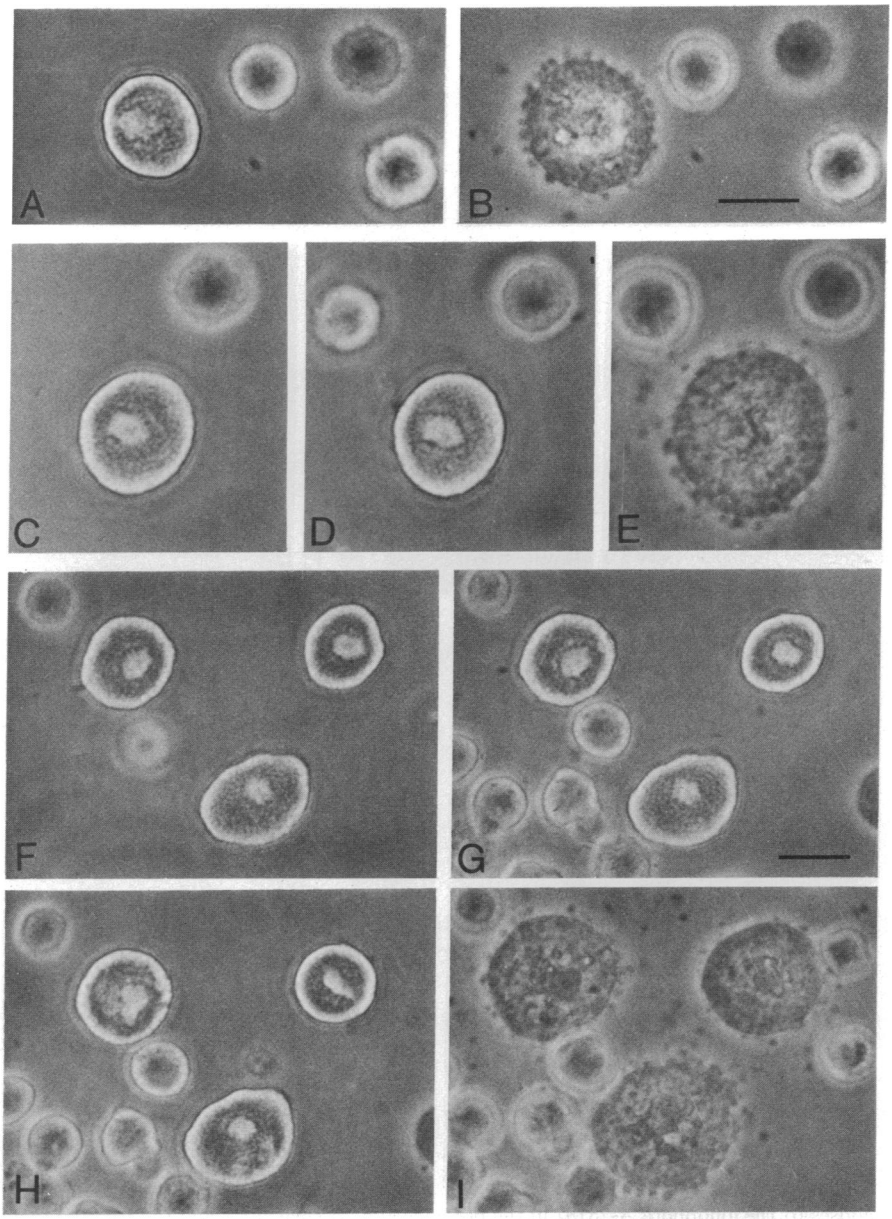

FIG. 1. Responses of mast cells to 48/80 in the presence and absence of Ca, and the stimulant effect of Ca on cells primed with 48/80. This and the subsequent figures show peritoneal cells viewed through an inverted microscope by phase contrast. The large, highly refractile, cells are the mast cells. The horizontal *bars* represent 10 μm. (*A*) A mast cell in conventional (Ca-containing) Locke. (*B*) The same cell 2 min after addition of 48/80 (2 μg/ml of final concentration), showing the well-known degranulating response. Many extruded granules lie round the cell surface or a short distance away. (*C*) An EDTA-treated mast cell (bathed in *0.1 mM EDTA-Locke* after incubation in *2.0 mM EDTA-Locke*): the cell appears normal. (*D*) The same cell 15 min after addition of 48/80 (2 μg/ml) shows absence of degranulating response (compare with *B*). (*E*) The same cell 2 min after further addition of Ca (4 mM), showing Ca-induced granule extrusion. Series *F–I*, A group of 3 EDTA-treated mast cells showing again a normal appearance (*F*) and lack of degranulation 15 min after addition of 2 μg/ml of 48/80 (*G*) and, in addition, the failure of Mg (4 mM, 10 min before) to induce degranulation (*H*) or to prevent degranulation on the further addition of 4 mM Ca (*I*, taken 3 min after addition of Ca).

Solutions. Locke contained 150 mM NaCl, 5 mM KCl, and 2 mM $CaCl_2$. In *Ca-free Locke*, $CaCl_2$ was omitted; this medium, with appropriate amounts of ethylenediaminetetraacetic acid, yielded *2 mM EDTA* or *0.1 mM EDTA-Locke*. *Sucrose-Locke without Ca*, *K-Locke without Ca*, and *choline-Locke without Ca* were essentially Ca-free Locke with all NaCl replaced by sucrose (300 mM), KCl (150 mM), or choline chloride (150 mM), respectively. Versions of these various solutions were made up to contain 20 mM Ca or 20 mM Mg; tonicity was then maintained by appropriate reduction of NaCl, sucrose, KCl, or choline chloride. When added to Ca-free incubation media, these solutions yielded a final concentration of the alkaline earth of about 3.0 mM. All solutions were equilibrated with air, used at room temperature unless otherwise stated, and contained bovine-serum albumin (1.0 mg/ml), glucose (1.0 mg/ml), and heparin (0.1 mg/ml). All but one, *Locke*, were buffered with 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer with pH adjusted to 7.0 by addition of NaOH (or KOH for the Na-free solutions). *Locke* was buffered to the same pH with 3 mM NaH_2PO_4–Na_2HPO_4.

Drugs. Compound 48/80 (Burroughs Wellcome Co.) was dissolved in the appropriate experimental solution im-

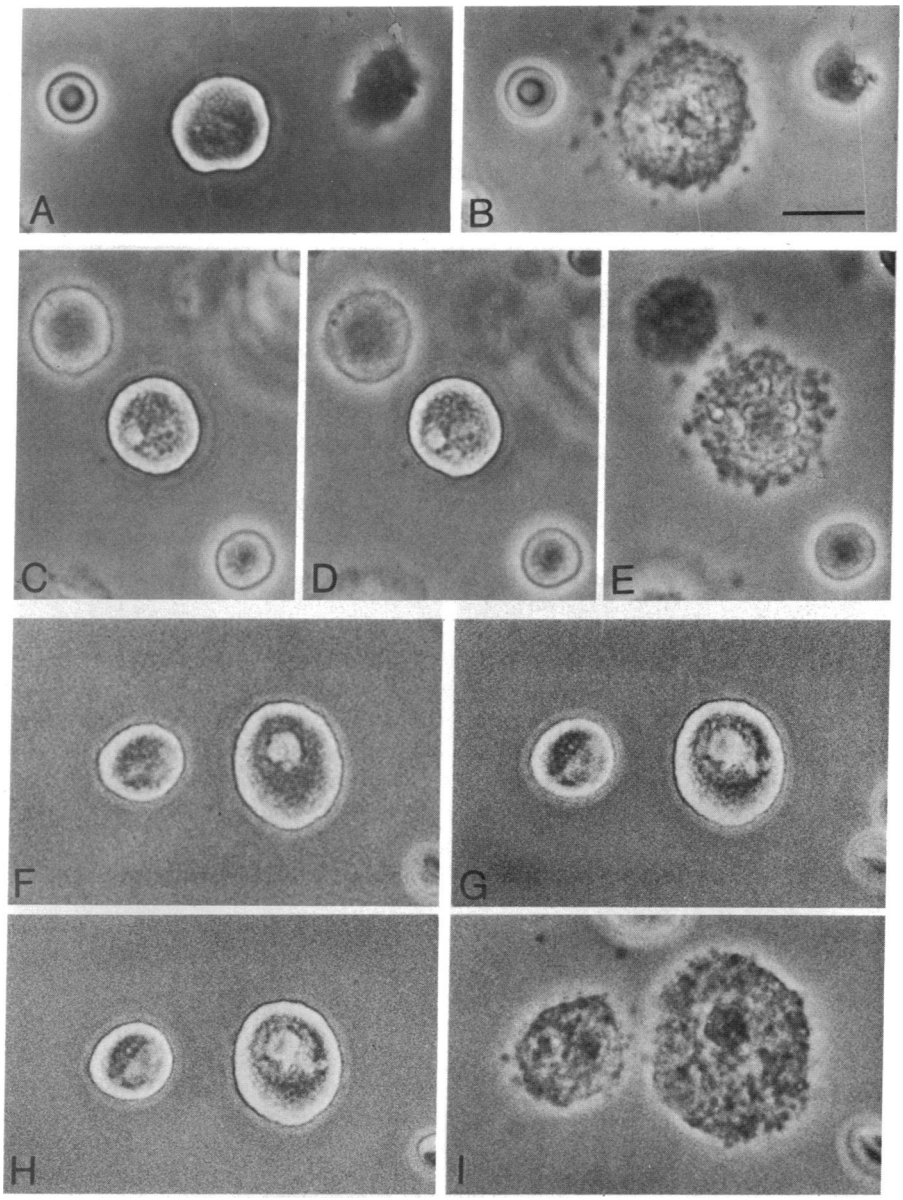

FIG. 2. Mast cell responses to the ionophore A-23187 in the presence and absence of Ca and the stimulant effect of Ca on cells primed with A-23187. The pattern of experiment and illustration is similar to that of Fig. 1. (*A*) A mast cell in Locke. (*B*) Same cell 2 min after addition of A-23187 (3 μg/ml); granules are extruded as with 48/80 (compare with Fig. 1*B*). (*C*) An EDTA-treated mast cell. (*D*) The same cell 15 min after addition of A-23187 (3 μg/ml). (*E*) The same cell 2 min after further addition of Ca (4 mM). (*F–I*) Two EDTA-treated mast cells before (*F*) and 15 min after (*G*) addition of A-23187 (3 μg/ml). (*H*) Ten minutes after further addition of Mg (4 mM). (*I*) two minutes after further addition of Ca (4 mM).

mediately before use. The ionophores, X-537A (Hoffman–La Roche) and A-23187 (Eli Lilly), were prepared as stock solutions in dimethylsulfoxide (Me_2SO) at concentrations of 25 mg/ml and 10 mg/ml, respectively, and stored in the refrigerator until used. Dilution with the appropriate experimental solution to the desired final concentration (25 μg/ml) was done on the day of the experiment. The final concentration of Me_2SO was never greater than 0.1%. In control experiments this concentration of Me_2SO was itself without any apparent effect on the morphology of isolated mast cells or their response to drugs, as was to be expected from experiments on histamine release from the same cells; thus, this concentration of Me_2SO neither elicits histamine release nor prevents 48/80-evoked release (7).

RESULTS

Rat peritoneal mast cells isolated and examined in conventional Ca-containing Locke solution were generally spherical or nearly so, smooth-surfaced, filled with fine granules surrounding a prominent nucleus, and highly refractile (Fig. 1*A*). Within a few seconds of addition of 48/80 (final concentration 2 μg/ml) cells began to 'bubble', swell, and extrude granules forcefully in the manner now familiar from many reports and demonstrated by electron microscopy to result from exocytosis (4–6). This concentration of 48/80 has been shown to release more than 90% of the total histamine from isolated peritoneal mast cells of the same population of rats (ref. 7, and unpublished observations of Douglas and Ueda), and

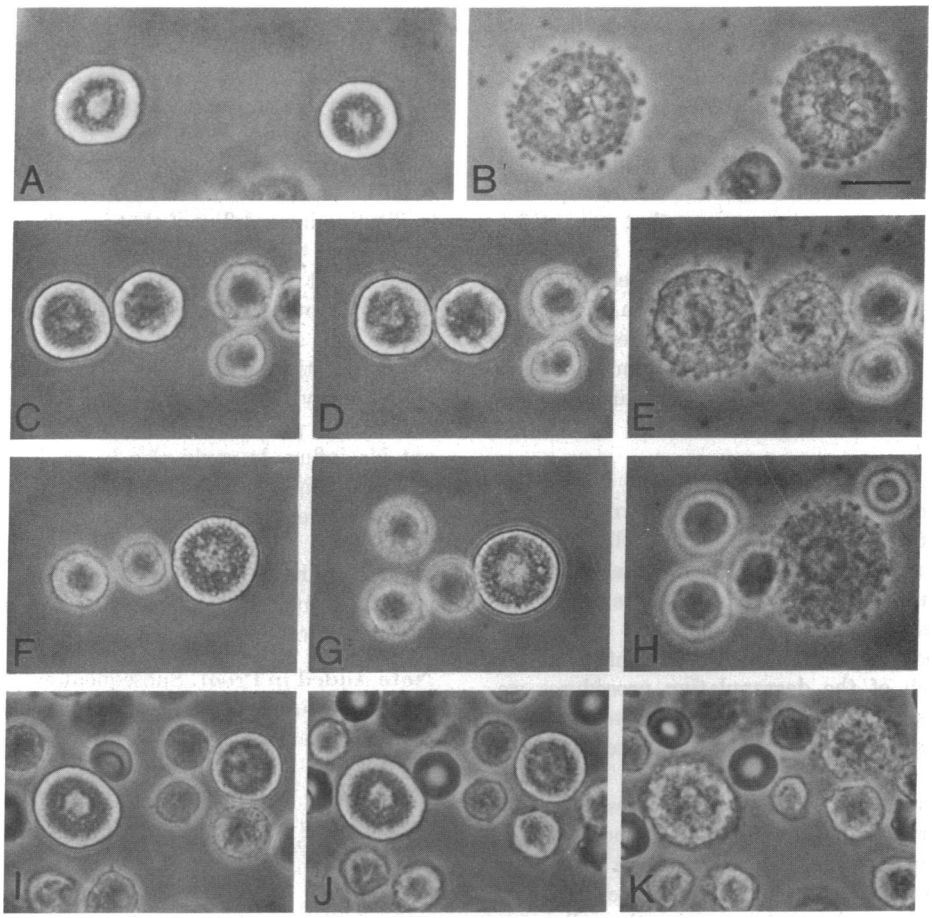

FIG. 3. Mast cell responses to A-23187 in Na-free media and to X-537A. (*A*) Two mast cells in Na-free *sucrose-Locke* containing 2 mM Ca. (*B*) The same cells 2 min after addition of A-23187 (3 μg/ml). (*C*) Two EDTA-treated mast cells in Na-free *choline-Locke without Ca*. (*D*) The same cell 10 min after addition of A-23187 (3 μg/ml). (*E*) After further addition of Ca (4 mM). (*F*) An EDTA-treated mast cell in a medium containing 150 mM KCl (*K-Locke without Ca*). (*G*) The same cell 10 min after addition of A-23187 (3 μg/ml). (*H*) Two minutes after further addition of Ca (4 mM). (*I*) Two EDTA-treated mast cells in *0.1 mM EDTA-Locke*. (*J*) The same cells 5 min after X-537A (3 μg/ml) was added. (*K*) The same cells, four minutes after Ca (4 mM) was added.

very few mast cells failed to show the massive degranulation illustrated in Fig. 1*B*. By contrast, cells isolated in Ca-free Locke, incubated with 2 mM EDTA-Locke and suspended in 0.1 mM EDTA-Locke, although of normal appearance (Fig. 1*C*) showed no such response—or indeed, any other change—when 48/80 was added as shown in Fig. 1*D*, obtained some 15 min after addition of 48/80. Even exposure to 48/80 for an hour or more was without effect. This striking absence of the classical norphological response to 48/80 (4–6) is in harmony with the previously demonstrated failure of the drug to release histamine under the same circumstances (7). However, such EDTA-treated cells exposed to 48/80 extruded granules within a few seconds of addition of calcium to the incubation medium; this secretory response (Fig. 1*E*)—indistinguishable from the effect of adding 48/80 to the cells in normal media—rapidly ran to completion to involve almost all cells in the population. This effect was dependent on the presence of 48/80; the simple addition of calcium to EDTA-treated cells did not evoke it. Nor does Ca introduced in such circumstances elicit histamine release (7). In contrast to calcium, magnesium did not induce granule extrusion in the EDTA-treated mast cell primed with 48/80, nor did it prevent the response of such cells to calcium (Fig. 1*F–I*).

Essentially similar results were obtained with the ionophores, A-23187 and X-537A. The former has been shown to release histamine from mast cells and to require calcium for this effect (9), and we have shown the same is true of the latter. Using a standard procedure applied to measure histamine release in response to 48/80 (7), we have found that 3 μg/ml of X-537A released more than 80% of the histamine present in isolated peritoneal mast cells from these same rats, but failed to evoke this response after cells had been incubated with 2 mM EDTA-Locke and suspended in 0.1 mM EDTA-Locke. On light microscopic observation, we found that both ionophores cause granule extrusion from peritoneal mast cells bathed in conventional (Ca-containing) Locke, but did not elicit this response from EDTA-treated cells incubated in 0.1 mM EDTA-Locke, although the subsequent addition of Ca (but not Mg) elicited degranulation. This pattern of behavior is essentially the same as that illustrated for 48/80 in Fig. 1 and need be illustrated fully only for one compound, A-23187 (Fig. 2*A–I*). For compound X-537A we illustrate the main point: absence of effect in Ca-free environment and bubbling response when calcium is introduced (Fig. 3 *I–K*). A comparison of the two ionophores may prove interesting; we have noticed that the response to X-537A is more sluggish than that to A-23187.

While calcium is clearly an essential element in the response to the three drugs, the question arises whether other ions participate. This seems especially relevant to the action of

the ionophores, A-23187 and X-537A, both of which, the latter especially, have a capacity to influence movement of monovalent cations (8). Whether secretory responses to these agents depend to any important extent on disturbances in the monovalent cationic environment—for example, entry of Na or efflux of K, or resulting changes in membrane potential such as depolarization—are questions that cannot be fully answered at present, but such experiments as we have performed to date have offered no grounds for believing any of these factors are important. As illustrated in Fig. 3, A-23187 elicited granule extrusion under three different conditions where all sodium in the bathing medium had been replaced by osmotically equivalent amounts of sucrose, choline, or potassium.

DISCUSSION

On the basis of the published light and electron microscopic studies on 48/80-induced secretion in mast cells (4–6) it seems likely that the similar granule extrusion observed in the various conditions used in present experiments is indeed exocytosis. A parallel electron microscopic study (M. Kagayama and W. W. Douglas, unpublished) has confirmed this and shown that each of the degranulating procedures we have used yields mast cells with abundant exocytotic figures resembling those obtained from normal cells exposed to 48/80 (4–6).

Therefore, the principal significance of the present results is that they offer striking new and visible testimony on the living cell of the capacity of calcium to induce exocytosis under various conditions, at least two of which may be fairly supposed to allow calcium to traverse the cell membrane. Thus, the description of A-23187 and X-537A as 'ionophores' derives from their very ability to carry calcium and other ions across lipid barriers, including biological as well as artificial membranes. As for other ionophores, it has been suggested that they do this by enveloping (complexing with) an ion at the membrane interphase, diffusing across the membrane as a cation complex, releasing the ion at the opposite interphase, and diffusing back uncomplexed to the original interphase to complete the catalytic cycle. Both A-23187 and X-537A acting in this way as mobile carriers for cations effectively transfer Ca across biological as well as artificial membranes and are believed, for example, to owe to this property their ability to induce or facilitate Ca-dependent contractions of muscle (8). Indeed, one of these compounds, A-23187 has been shown to promote Ca uptake into suspensions of mixed peritoneal cells, which include mast cells, and this effect has been offered in explanation of the stimulant effect of A-23187 on histamine release (9). However, ionophores are rather new pharmacological agents and on historical precedent it would not be surprising if they are demonstrated in the future to exert biological effects quite unrelated to their action on movement of inorganic cations. In point of fact, it is already known that some ionophores form complexes with organic amines, including ethanolamine, epinephrine, and norepinephrine (8). It might be argued that such an action could explain the transfer of histamine from storage sites in the cells to the bathing medium noted here with X-537A and in the earlier work with A-23187 (9). However, our observation that the ionophores not only release histamine but cause degranulation renders such conjecture unnecessary. The mechanism seems to involve the classical secretory response of the mast cell, exocytotic extrusion of whole granules. While it seems simplest at present to suppose that the ionophores allow calcium to activate exocytosis by facilitating inward flux of that ion, there is less precedent for concluding that conditioning with 48/80 is similarly explained, but this is one possibility. The response to 48/80 is complex but is certainly believed to involve actions of the drug on the cell membrane (10).

The question whether calcium ions alone are sufficient to induce exocytosis is still open but the degranulating response to A-23187 proceeded nicely in Na-free media, thus ruling out Na influx. It could also be readily obtained from cells suspended in 150 mM KCl, which should prohibit K efflux. Any movements of these alkali metal ions do not seem then to be required adjuvant factors, but whether 48/80 and the ionophores have some other ill-defined conditioning effect remains to be seen, and the inference that calcium ions are effective intracellularly obviously needs more direct support.

Note Added in Proof. Subsequent experiments have demonstrated extrusion of secretory granules after intracellular injection of calcium ions into mast cells not exposed to any drug (11).

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