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ON THE CALCIUM RECEPTOR ACTIVATING EXOCYTOSIS: INHIBITORY EFFECTS OF CALMODULIN-INTERACTING DRUGS ON RAT MAST CELLS

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SUMMARY

1. A series of neuroleptic drugs (five phenothiazines, imipramine, and pimozide) and the smooth muscle relaxant W-7, which all inhibit calcium·calmodulin-activated processes, inhibited rat mast cell secretion elicited by antigen, by 48/80, and by the calcium ionophore A23187.

2. Neither the phenothiazines nor W-7 reduced ^{45}Ca uptake in response to A23187. The drugs thus exert an inhibitory action distal to the rise in intracellular Ca ions that activates exocytosis.

3. Chlorpromazine sulphoxide, which shares several membrane-perturbing actions of the phenothiazines but is a weak inhibitor of calmodulin, did not inhibit secretion. Moreover, the inhibitory effects of the phenothiazines were not overcome by a 5- or 10-fold increase in the concentration of calcium, which should counter unspecific membrane effects.

4. The inhibitory effects of the various neuroleptic drugs appeared to be related to their ability to inhibit calmodulin because the individual potencies of these compounds on secretion evoked by 48/80 or A23187 correlated significantly with their reported potencies in inhibiting calmodulin-activated processes. (The greater potency and different rank order of these compounds on secretion evoked by antigen suggests an additional inhibitory action, perhaps involving Ca entry.)

5. These results, which parallel those obtained with drugs of this sort in smooth muscle where calmodulin seemingly functions as the Ca receptor activating contraction, strengthen the view that calmodulin, or some calmodulin-like protein, is the Ca receptor activating exocytosis.

INTRODUCTION

The general calcium hypothesis of stimulus–secretion coupling (Douglas, 1968), which holds that Ca ions have a widespread mediator function in secretory cells to translate the actions of diverse stimuli into a common response, exocytosis, is now supported by an extensive literature. But how Ca ions induce exocytosis is unknown (see Douglas, 1974; 1978; Rubin, 1974; Meldolesi, Borgese, De Camilli & Ceccarelli, 1978; Hopkins & Duncan, 1979).

From the outset, the mediator function of Ca in secretion has seemed to parallel its function in contraction and this early prompted the suggestion that the mechanism of action of Ca in secretion might best be understood within the broader framework of 'stimulus–response coupling' (Douglas, 1966). Fresh incentive to adopting a broad approach to the problem is provided by the more recent evidence that Ca ions also function in a 'messenger' capacity to control numerous cellular responses besides contraction and secretion; and, moreover, apparently act in each instance through Ca-binding regulator proteins (Kretsinger, 1977) among them calmodulin, an intracellular 'Ca receptor' (Means & Dedman, 1980) apparently ubiquitous in eukaryotic cells (Cheung, 1980; Watterson & Vincenzi, 1980). In view of the particularly close parallels between stimulus–secretion coupling and excitation–contraction coupling in smooth muscle (see Carafoli, Clementi & Drabikowski, 1975; Weiss, 1977) it seems especially noteworthy that in smooth muscle it is calmodulin that apparently acts as the Ca receptor and that it functions there to effect Ca-dependent protein phosphorylation (see Adelstein & Eisenberg, 1980; Hartshorne & Siemankowski, 1981). Certain depressant effects on some exocytotic responses of phenothiazine drugs which, among their other actions, inhibit calmodulin are at least consistent with the involvement of this protein in stimulus-secretion coupling (see Discussion); and Ca-dependent protein phosphorylation accompanies stimulation in several secretory cells (Schulman, 1981).

All this has prompted us to apply lessons from smooth muscle contraction to exocytosis. In smooth muscle, evidence that calmodulin serves as the Ca receptor has been provided by pharmacological studies showing that phenothiazines and certain other drugs have inhibitory effects on contraction that are: (1) exerted at some site distal to the Ca signal yet proximal to the effector mechanism that yields the cellular response; and (2) correlated with the *in vitro* calmodulin-inhibiting activity of the drugs (Hidaka, Yamaki, Totsuka & Asano, 1979; Cassidy, Hoar & Kerrick, 1980). In the present experiments, in which exocytosis was studied in rat mast cells, we have obtained comparable results. The observations encourage the view that calmodulin, or some pharmacologically similar entity, serves as the Ca receptor to initiate exocytosis.

METHODS

Mast cells

Cells from the peritoneal and thoracic cavities of male Sprague-Dawley rats (350–500 g) (Charles River, Wilmington, MA or Camm Research, Wayne, NJ) were harvested, pooled, and resuspended in Locke solution which contained (mM): NaCl, 150; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; Na_2HPO_4/NaH_2PO_4, 3, (pH 6·8); glucose, 5·6, and 1 mg/ml. bovine serum albumin (BSA). When the Ca concentration was to be altered, serosal cells were harvested and resuspended in Locke buffered with HEPES (5 mM; pH 6·8). For most experiments, mixed serosal cells (7–10% mast cells) were used without purification.

Incubation protocol

To plastic tubes, chilled on ice, were added 0·4 ml. of the serosal cell suspension ($2–5 \times 10^5$ mast cells/ml.) followed by 0·1 ml. of the drug to be tested or its vehicle as a control. Preincubation (for 10 min) was initiated by transferring the tubes to a shaking water bath at 37 °C. Histamine secretion was induced by the addition of secretagogue in 0·1 ml. of the appropriate Locke solution. Incubation at 37 °C was for 5 min (for 48/80 and A23187) or 20 min (for ovalbumin). When antigen (75 μg/ml.

ovalbumin, Sigma, grade VI) was used as secretagogue the cells were first passively sensitized with mouse anti-ovalbumin IgE as previously described (Nemeth & Douglas, 1980) except that the final total protein concentration of this preparation was 200 μg/ml. Phosphatidylserine (Sigma, 10·6 μg/ml. final) was included with ovalbumin. In some experiments (Fig. 5) the serosal cells were added to pre-warmed (37 °C) tubes containing either the test substance or vehicle control before incubation was performed as above.

Incubations were terminated by adding 0·9 ml. ice-cold Locke solution (lacking Ca and albumin) and placing the tubes on ice. Cells were separated by centrifugation and the histamine contents of the deproteinized pellets and supernatant fractions were determined fluorimetrically (Nemeth & Douglas, 1978; 1980). None of the drugs tested interfered with this assay.

Mast cell purification and measurement of ^{45}Ca uptake

Mast cells were purified by a procedure similar to that described by Yurt, Leid, Austen & Silbert, (1977). Serosal cells were harvested and resuspended in gelatin Locke (1 mg gelatin replacing 1 mg BSA). Cells from one rat (in 1 ml. Locke) were layered over 2 ml. 24 % (w/v) metrizamide dissolved in gelatin–Locke. The gradients were centrifuged at 264 *g* for 5 min and the non-mast cells that were retained at the buffer–metrizamide interface were aspirated. The mast cell pellet was washed twice with Locke (containing 1 mg/ml. BSA), resuspended in a small volume of Locke (2·5–5·5 $\times$ 10^6 mast cells/ml.) and immediately placed on ice. The purity of such mast cell suspensions was 97·1 $\pm$ 0·1 % (n = 27); cellular viability (as indexed by Trypan Blue exclusion) was never less than 98 %.

To assess the uptake of ^{45}Ca, we followed a procedure similar to that described by Foreman, Hallett & Mongar (1977). To 400 μl. microfuge tubes were added 150 μl. Versilube F50 silicone oil (General Electric, Waterford, NY). Over this was layered 50 μl. Locke solution with or without A23187 and containing 10·2 μc/ml. ^{45}Ca, s.a. = 10·2 $\times$ 10^3 mc/mole (ICN Pharmaceuticals, Inc., Irvine, CA). Purified mast cells were pre-incubated at 37 °C for 10 min with the test substance or vehicle (control) and then 50 μl. (containing 1–3 $\times$ 10^5 cells) was transferred to the microfuge tubes (pre-warmed to 37 °C) and incubated for 5 min. Cells were pelleted by centrifuging for 30 sec at 15000 *g* in a Beckman 152 Microfuge. The bottom of the microfuge tube was sliced off and dissolved in 0·5 ml. 10 mg/ml. Triton X-100. Duplicate 100 μl. samples were mixed with 5 ml. Biofluor (New England Nuclear) and counted in the pre-set ^{3}H + ^{14}C channel of a liquid scintillation spectrometer.

To measure histamine release in the same samples assessed for ^{45}Ca uptake, we used the following procedure: after centrifugation, 90 μl. of the supernatant was transferred to 400 μl. 10 mg/ml. Triton 100. 100 μl. of diluted supernatant and dissolved pellet samples were then mixed with 400 μl. 2·5 % perchloric acid. The samples were rapidly frozen, thawed and centrifuged to remove protein. 200 μl. of each of the resulting supernatants were subsequently processed for the fluorimetric determination of histamine. This procedure minimized interference by Triton in the fluorimetric assay and gave values of histamine release that were similar to those obtained in test samples treated identically except that they were lysed by boiling in 2 % perchloric acid.

Preparation of drugs

In most experiments, the drug to be tested was prepared in the dark in Locke solution immediately before use. Stock solutions of drugs stored in the dark at 4 °C for 2–3 days gave results similar to those obtained with freshly prepared drugs. Trifluoperazine HCl, chlorpromazine sulphoxide HCl (Smith, Kline and French), chlorpromazine HCl (Sigma), and imipramine HCl (Ciba-Geigy) were dissolved directly in Locke solution and the pH was adjusted to 6·8. Thioridazine HCl (Sandoz) and *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide (W-7; a gift from Dr Hiroyoshi Hidaka, Dept. of Pharmaclogy, Mie University, Japan) were dissolved in Locke by acidification with 1 N-HCl to pH 2·3 and stirring in the dark at room temperature; the final pH was adjusted to 6·8. Chlorprothixene (Hoffman-LaRoche) and promethazine (Wyeth) were prepared in the same way as thioridazine and W-7 except that the final pH was adjusted to 6·4. Pimozide (Janssen R. & D., Inc.) was prepared as a 10 mM solution in 10 % (v/v) acetic acid. A portion of this stock solution was diluted to 0.25 mM with Locke and the pH adjusted to 5·8. The final pH in the incubation tube containing pimozide or its vehicle (control) was 6·65.

Stock solutions of 48/80 (Burroughs Wellcome Co.), A23187 (Calbiochem) and antimycin A (Sigma, Type III) were prepared and used as previously described (Nemeth & Douglas, 1978).

RESULTS

Inhibition of exocytosis by neuroleptic drugs

We selected for study seven neuroleptic drugs known to inhibit contraction of smooth muscle cells to various stimuli apparently by inhibiting calmodulin (see Kerrick, Hoar, Cassidy, Bolles & Malencik, 1981). The seven agents, mostly pheno-

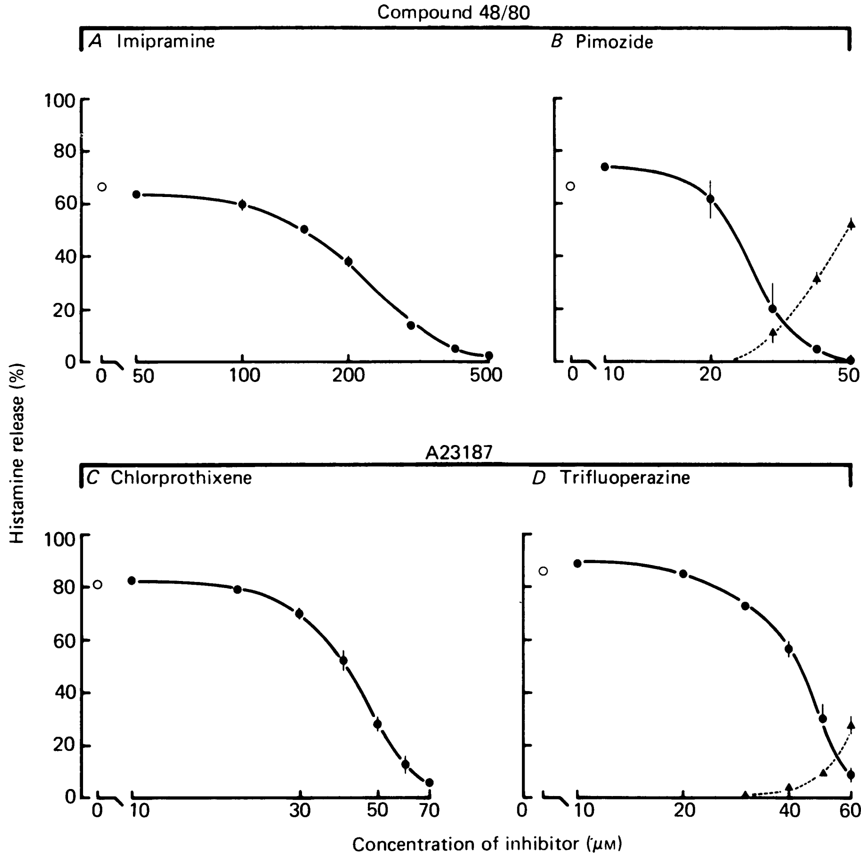

Fig. 1. Inhibition of 48/80- and A23187-induced histamine secretion by neuroleptic drugs. Mixed serosal cells were pre-incubated 10 min, 37 °C, in the absence or presence of neuroleptic drugs and incubated a further 5 min with or without addition of 48/80 (0·2 μg/ml.) or A23187 (0·2 μg/ml.). The continuous lines represent histamine secretion (as % total: see Methods) after correction for basal release which is indicated by the dashed lines only where it exceeded twice the control basal release ($2 \cdot 4 \pm 0 \cdot 1$ %, $n = 33$). Each point is the mean of four to six separate experiments. In this and all subsequent figures the S.E. of mean, where it exceeds the dimensions of the symbol, is indicated by a vertical line.

thiazine derivatives, were studied for their effects on secretion evoked by three different secretagogues: antigen, which (in sensitized cells) promotes the receptor-dependent influx of extracellular Ca; 48/80, which acts on different receptors to mobilize intracellular Ca; and the Ca ionophore A23187 which circumvents early, receptor-dependent, events and elicits exocytosis in mast cells by delivering Ca directly to the cell interior.

All the neuroleptic drugs tested inhibited exocytosis induced by antigen, by 48/80, or by the ionophore A23187. Fig. 1, which is representative of these inhibitory patterns, shows that nearly maximal secretory responses elicited by 48/80 or A23187 could be completely suppressed by these agents. Although pimozide and trifluoperazine tended to increase basal histamine release in concentrations lower than those that completely inhibited evoked secretion (Fig. 1*B* and *D*), this did not occur with imipramine or chlorprothixene (Fig. 1*A* and *C*) or with promethazine. And neither

TABLE 1. Concentrations of neuroleptic drugs required to reduce the secretory responses to A23187 or to 48/80 by 50% (IC_{50})

Neuroleptic drug	IC_{50} (μM) A23187	48/80
Pimozide	29·1 ± 1·9 (5)	27·1 ± 3·8 (4)
Thioridazine	37·8 ± 1·9 (6)	41·6 ± 3·4 (4)
Chlorprothixene	45·1 ± 1·8 (4)	65·5 ± 4·8 (3)
Trifluoperazine	46·0 ± 3·8 (6)	47·3 ± 2·4 (6)
Chlorpromazine	94·4 ± 3·1 (5)	83·9 ± 7·3 (5)
Imipramine	264·0 ± 6·3 (5)	218·0 ± 7·3 (5)
Promethazine	295 (2)	310 (2)

Mixed serosal cells were incubated with drugs as in Fig. 1. Histamine secretion induced by A23187 (0·2 μg/ml.) and 48/80 (0·2 μg/ml.) was 83·2 ± 1·2 (n = 39) and 68·5 ± 0·9% (n = 29), respectively. IC_{50}s were calculated by plotting the values from each experiment on arithmetic-probability co-ordinates. Each value is the mean ± S.E. of mean of the number of experiments in parentheses.

thioridazine nor chlorpromazine increased basal release until evoked secretion was inhibited by more than 90%. Increases in basal release have been noted in mast cells (Jansson, 1970; Frisk-Holmberg, 1971) and in other secretory cells (Williams, Poulsen & Lee, 1977; Elferink, 1979; White & Raynor, 1980) with high concentrations of phenothiazines and this effect apparently results from membrane perturbations leading to cell lysis. It is unlikely, however, that these unspecific membrane actions are causally related to the inhibitory effects of the drugs on mast cell secretion evident at lower concentrations (see below).

The concentration of each of the neuroleptic drugs required to reduce the secretory responses to 48/80 and to A23187 by 50% (the IC_{50}) was determined graphically and the values are listed in Table 1. The IC_{50} for any particular drug was approximately the same whether secretion was evoked by 48/80 or by A23187. The potencies (IC_{50}s) of these neuroleptic drugs as inhibitors of evoked secretion correlated closely with their reported potencies in inhibiting activation of phosphodiesterase by Ca·calmodulin (Fig. 2*A*) which, in turn, reflects their affinity for binding to calmodulin (Weiss, Prozialeck, Cimino, Barnette & Wallace, 1980). On the other hand, there was no significant linear correlation between the tendency of these drugs to increase basal histamine release and their potency in inhibiting evoked secretion (Fig. 2*B*).

When antigen was used to elicit secretion, a different rank order of potency of the neuroleptic drugs was observed. Moreover, these drugs inhibited antigen-induced secretion to a greater extent than they inhibited secretion evoked by 48/80 or by A23187. Concentrations of the drugs that had reduced secretion in response to 48/80

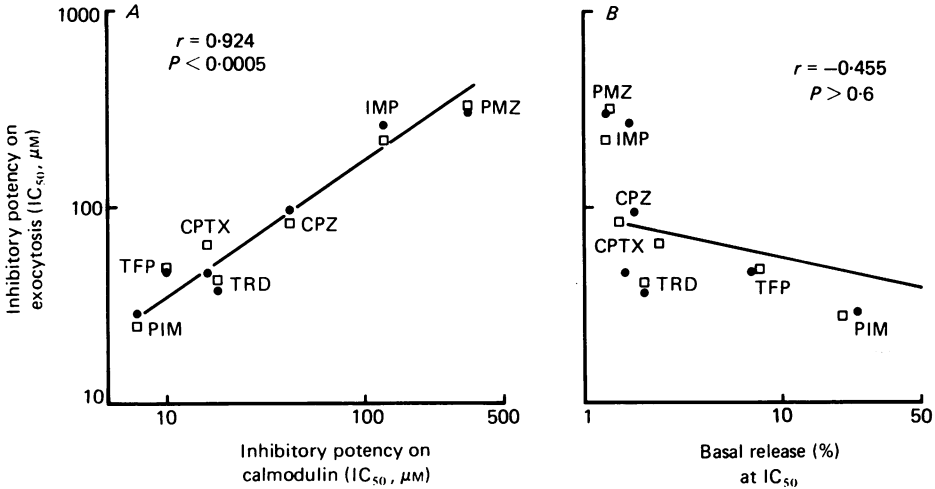

Fig. 2. Inhibitory effects of neuroleptic drugs on exocytosis evoked by A23187 or 48/80 correlate with their inhibitory effects on calmodulin (*A*) but not with their stimulant effects on basal release (*B*). The IC_{50}s of the neuroleptic drugs for inhibiting secretion evoked by 48/80 (□) or A23187 (●) were taken from Table 1 and plotted against their respective IC_{50}s for inhibiting Ca-calmodulin activation of phosphodiesterase (brain; as reported by Levin & Weiss, 1979) or against the basal histamine release produced by the drugs at the IC_{50}s for inhibiting evoked secretion. PIM, pimozide; TRD, thioridazine; CPTX, chlorprothixene; TFP, trifluoperazine; CPZ, chlorpromazine; IMP, imipramine; PMZ, promethazine.

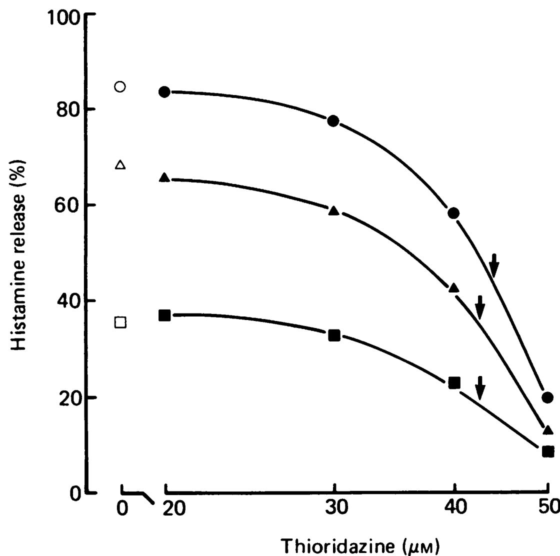

Fig. 3. Inhibitory effect of thioridazine on histamine secretion induced by various concentrations of 48/80. Mixed serosal cells were pre-incubated with (filled symbols) or without (open symbols) thioridazine before the addition of 0·15 (■, □), 0·5 (▲, △) or 1·0 (●, ○) μg/ml. 48/80 as in Fig. 1. All values have been corrected for basal histamine release which was 2·7 % in the absence and 6·1 % in the presence of 50 μM-thioridazine. The IC_{50} for each curve is indicated by an arrow. Each point is the mean of two separate experiments.

or A23187 by about 50% yielded, in four separate experiments with antigen, the following inhibitory pattern: promethazine (300 μM), 94·4 ± 0·3; chlorpromazine (100 μM), 91·5 ± 0·8; thioridazine (40 μM), 91·0 ± 1·7; pimozide (30 μM), 87·1 ± 6·7; chlorprothixene (50 μM), 81·2 ± 7·9; imipramine (230 μM), 76·2 ± 5·7; trifluoperazine (50 μM), 71·2 ± 7·4% inhibition (cf. Table 1).

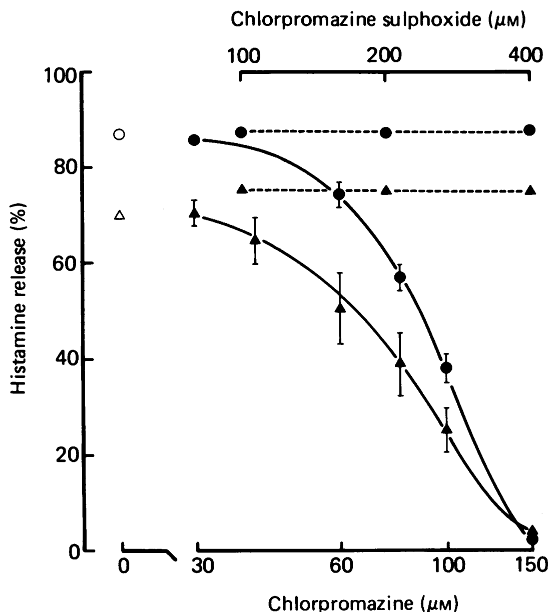

Fig. 4. Contrasting effects of chlorpromazine (continuous line) and chlorpromazine sulphoxide (dashed line) on histamine secretion evoked by 48/80 or by A23187. Mixed serosal cells were pre-incubated in the absence or presence of chlorpromazine or chloropromazine sulphoxide and incubated with or without 48/80 (0·2 μg/ml; △, ▲) or A23187 (0·2 μg/ml.; ○, ●) as in Fig. 1. All values have been corrected for basal histamine release, which was 1·1 ± 0·1% ($n = 5$) in the absence and 6·2 ($n = 2$) ± 3·2% ($n = 3$) in the presence of 150 μM-chlorpromazine. Basal release in the presence of 400 μM-chlorpromazine sulphoxide was 3·1 ± 0·02% ($n = 4$). Each point is the mean of three to five experiments.

To examine whether the greater susceptibility of antigen-evoked secretion to inhibition by the neuroleptic drugs might reflect the lesser intensity of the secretory response to antigen: (50·6 ± 4·1% ($n = 4$) histamine secretion compared with 70–85% in response to 48/80 or A23187) we tested the effects of a representative phenothiazine, thioridazine, on a range of secretory responses evoked by various concentrations of 48/80. However, the percent inhibition achieved by a given concentration of thioridazine against both small and large secretory responses remained fairly constant and the IC_{50} was unrelated to the magnitude of the secretory response. This indicates that the inhibitory effect of thioridazine is non-competitive. In the two experiments illustrated in Fig. 3, the IC_{50}s for thioridazine against concentrations of 48/80 that elicited 35·8, 68·2 and 84·8% secretion were 43·5, 43·0 and 44·0 μM respectively. The greater inhibitory potency of the neuroleptic drugs towards antigen-evoked secretion thus reflects a characteristic of the response to this secretagogue other than its magnitude.

The ability of the neuroleptic drugs to inhibit secretion evoked by three secretagogues with different mechansims of action that deliver Ca by quite different means to induce exocytosis suggested that the inhibitory action is exerted at or beyond the Ca-receptive site. This might be a specific action on a receptor such as calmodulin. Alternatively, unspecific membrane perturbating actions of these lipophilic drugs (see Seeman, 1972) might inhibit exocytosis itself since this phenomenon involves fusion

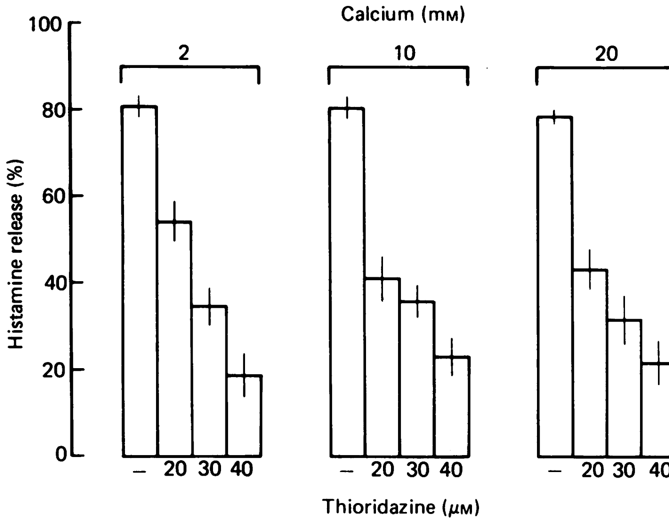

Fig. 5. Effect of increasing the extracellular concentration of Ca on the inhibitory effects of thioridazine on histamine secretion in response to A23187. Mixed serosal cells were added to tubes pre-equilibrated to 37 °C containing the indicated concentration of thioridazine or its vehicle (control) and pre-incubated for 10 min. Incubation (for 5 min) was initiated by the addition of A23187 dissolved in Ca-Locke to obtain a final ionophore concentration of 0·4 μg/ml. and Ca concentrations of either 2, 10 or 20 mM. All values have been corrected for basal release which was $2{\cdot}7 \pm 0{\cdot}3$ ($n = 6$) in the absence and $2{\cdot}8 \pm 0{\cdot}2$ ($n = 6$), $3{\cdot}2 \pm 0{\cdot}3$ ($n = 4$), and $3{\cdot}1 \pm 0{\cdot}5$% ($n = 4$) in the presence of 20, 30 and 40 μM-thioridazine, respectively. Basal release at 10 and 20 μM-Ca was not significantly different from these values. Each value is the mean of four or six separate experiments.

and rearrangement of lipoid membranes. We therefore examined the effect of chlorpromazine sulphoxide which is equipotent with chlorpromazine in its unspecific effects (e.g. inhibition of $Na^{+}K^{+}$-ATPase and alteration of membrane fluidity: Keeffe, Blankenship & Scharschmidt, 1980), yet is 60-fold less potent than chlorpromazine in inhibiting activation of phosphodiesterase by Ca·calmodulin (Weiss *et al.* 1980). Chlorpromazine sulphoxide was without effect on histamine secretion induced by 48/80 or by A23187, even when used in much higher concentrations than chlorpromazine (Fig. 4). Another possibility was that the neuroleptic drugs might inhibit exocytosis by displacing membrane-bound Ca (Lüllmann, Plösch & Ziegler, 1980). Indeed, such an action has been postulated to account for phenothiazine-induced inhibition of membrane fusion reactions including exocytosis (Poste & Allison, 1973). If this were so then it would be expected that the inhibition should be overcome by increasing the concentration of Ca which competes with and displaces phenothiazines from membrane-binding sites (Breton, Viret & Leterrier, 1977). However, increasing

the Ca concentration 5- or 10-fold did not reduce the inhibitory effect of the phenothiazines on A23187-induced secretion. This is depicted for thioridazine in Fig. 5. The results with chlorpromazine and trifluoperazine were similar: the percent inhibitions obtained in 2, 10 and 20 mM Ca were, for chlorpromazine (30 μM), $30{\cdot}7 \pm 4{\cdot}5$ ($n = 6$), $29{\cdot}6 \pm 6{\cdot}6$ ($n = 5$) and $29{\cdot}4 \pm 4{\cdot}9$ ($n = 5$) and, for trifluoperazine (20 μM), $25{\cdot}4 \pm 6{\cdot}6$, $20{\cdot}5 \pm 3{\cdot}6$ and $21{\cdot}2 \pm 1{\cdot}7$ ($n = 3$ in each instance).

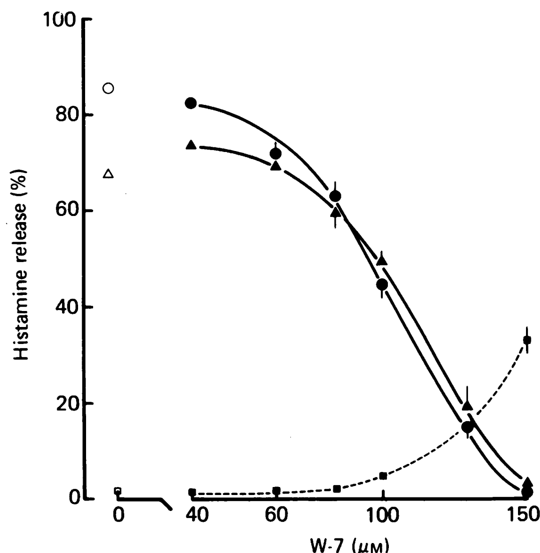

Fig. 6. Inhibitory effect of W-7 on histamine secretion induced by 48/80 or A23187. Mixed serosal cells were pre-incubated in the presence or absence of W-7 and incubated with or without 48/80 (0·2 μg/ml.; ▲, △) or A23187 (0·2 μg/ml.; ●, ○) for 5 min. The continuous lines represent histamine secretion after correction for the respective basal release (dashed line). Each value is the mean of six to eight (A23187) or three (48/80) experiments.

In these experiments to examine the effects of Ca we noted that the three phenothiazines tested exerted a greater inhibitory effect on evoked secretion than in our previous experiments. However, the dose–response curve for each drug was shifted to the left by approximately the same extent and the rank order of potency was not changed. On the other hand, the concentration of each drug producing lytic effects (as indexed by increased basal release) was not altered. This provides further evidence for a dissociation between the inhibitory effects of the phenothiazones and their nonspecific membrane effects. Thus, 90 μM-chlorpromazine and 40 μM-trifluoperazine inhibited A23187–evoked secretion by $87{\cdot}3 \pm 5{\cdot}4$ ($n = 4$) and 95·7 (mean of two experiments) percent respectively, whereas the corresponding basal release in the presence of these drugs was $3{\cdot}4 \pm 0{\cdot}1$ ($n = 4$) and 4·6 (mean) percent. Control experiments indicated that these results were not explained by the somewhat different preincubation protocol used, but resulted from the use of rats from a different supplier. Direct comparison of serosal cell populations indicated that cells prepared from the Camm Research rats used in this series of experiments were more susceptible to the inhibitory effect of phenothiazines than cells prepared from the Charles River rats, which were used in all other experiments.

Inhibition of exocytosis by W-7

In the aggregate, the various results with the neuroleptic drugs seemed to favour some specific action involving the Ca stage of stimulus–secretion coupling and pointed to the possible involvement of calmodulin. Because of this, we next examined the

effect, on mast cell secretion, of the novel smooth muscle relaxing drug W-7 whose various pharmacological effects have been attributed to a single action – inhibition of calmodulin-dependent processes (Hidaka *et al.* 1979; Kobayashi, Tawata & Hidaka, 1979; Hidaka, Yamaki, Naka, Tanaka, Hayashi & Kobayashi, 1980; Nishikawa, Tanaka & Hidaka, 1980). In addition, study of this drug offered us a further opportunity to explore parallels between secretion and contraction. As shown

TABLE 2. Effect of neuroleptic drugs and of W-7 on histamine secretion and ^{45}Ca uptake induced by A23187

	Normal cells	Energy-deprived cells	
Inhibitor	Histamine secretion (%)	Histamine secretion (%)	^{45}Ca uptake (c.p.m. × $10^{-3}/10^{6}$ cells)
None (control)	87·0 ± 0·8 (9)	3·3 ± 0·9 (11)	2·95 ± 0·40 (11)
Chlorpromazine (125 μM)	40·4 ± 4·1 (3)	1·3 ± 0·7 (3)	3·46 ± 0·54 (3)
Thioridazine (50 μM)	30·7 ± 4·4 (5)	0·7 ± 0·4 (4)	3·38 ± 0·50 (4)
Chlorprothixene (100 μM)	47·3 ± 2·4 (3)	1·5 ± 0·8 (3)	3·26 ± 0·64 (3)
W-7 (150 μM)	46·0 ± 5·5 (3)	−0·1 ± 0·3 (3)	3·00 ± 0·16 (3)

Purified mast cells were pre-incubated in the absence or presence of the inhibitor at the indicated concentration before incubation with A23187 (4 μg/ml.). Purified mast cells were somewhat less responsive to both agonist and antagonist: such reduced responsiveness is commonly encountered upon purification (see, for example, Coutts, Nehring & Jariwala, 1980). Histamine secretion and ^{45}Ca uptake were determined as described in Methods. Energy-deprived cells were harvested in the usual glucose-containing Locke and then washed and resuspended in glucose-free Locke to which actimycin A (0·5 μM final) was added during the last 5 min of pre-incubation. The vehicle for antimycin A (ethanol, 0·35 % final) was without effect on either basal or A23187-induced histamine secretion or ^{45}Ca uptake. All values have been corrected for basal histamine release or basal ^{45}Ca uptake. In energy-deprived cells, the basal ^{45}Ca uptake was (c.p.m. × $10^{-3}/10^{6}$ cells): 1·23 ± 0·18 ($n = 11$) in the absence and 3·15 ± 0·79 ($n = 3$), 1·53 ± 0·31 ($n = 4$), 1·78 ± 0·24 ($n = 3$) and 2·32 ± 0·24 ($n = 3$) in the presence of chlorpromazine, thioridazine, chlorprothixene and W-7, respectively.

in Fig. 6, W-7 inhibited secretory responses to both the receptor-active agent 48/80 and the Ca ionophore A23187. Like pimozide and trifluoperazine, W-7 increased basal release at higher concentrations, but caused significant inhibition, particularly of A23187–evoked secretion, at concentrations without effect on basal release. As with the neuroleptic drugs, the IC_{50}s for W-7 against responses evoked by 48/80 and A23187 were similar: 121·6 ± 5·6 μM ($n = 3$) and 104·8 ± 4·3 μM ($n = 8$), respectively. This is close to the IC_{50} of W-7 (129 μM) for inhibition of smooth muscle contraction.

Effect of neuroleptic drugs and W-7 on ^{45}Ca uptake

Experiments were performed to determine whether the inhibitory effects of the various drugs on A23187-induced secretion were due to some action that lessened the ability of the ionophore to promote influx of Ca. Uptake of ^{45}Ca in response to A23187 in the presence or absence of an inhibitory agent was examined in purified mast cells. To obtain a valid measurement of A23187-induced ^{45}Ca influx it was important to suppress the secretory response. Otherwise, much of the ^{45}Ca which becomes associated with the cellular pellet is the consequence (rather than the cause) of secretion. We therefore adopted the procedure of Foreman *et al.* (1977) in which

the mast cells are rendered incapable of secreting by being deprived of metabolic energy through omission of glucose and addition of antimycin A (see also Diamant, 1975). Under such conditions, changes in ^{45}Ca uptake induced by A23187 reflect the ability of A23187 to promote ^{45}Ca influx (Foreman *et al.* 1977). In such metabolically impaired cells, we found that the increased uptake of ^{45}Ca resulting from exposure to A23187 was not reduced by any of the drugs tested: W-7 was quite without effect and the neuroleptic drugs slightly potentiated A23187-induced ^{45}Ca uptake (Table 2).

DISCUSSION

The rat mast cell provides one of the best documented models of Ca-mediated exocytosis (Lagunoff & Chi, 1980; Foreman, 1981). Besides responding to secretagogues such as antigen and 48/80 which act through receptors in the cell membrane to promote Ca entry (Foreman *et al.* 1977) or to mobilize cellular Ca (Douglas & Ueda, 1973; Garland & Payne, 1979) respectively, it readily responds to Ca itself when treated with the ionophore A23187. The assumption that responses to A23187, as we have used it in the mast cell, reflect calcium activated exocytosis seems well founded. Although A23187 is not accurately characterized by the common designation 'Ca ionophore' since it can transport across biological membranes various divalent metal cations in exchange for protons, its action on the mast cell seems dependent on its ability to promote influx of Ca. Thus histamine secretion in A23187-treated mast cells falls progressively as the concentration of extracellular Ca is lowered (Foreman, Mongar & Gomperts, 1973; Johansen, 1978), and is completely abolished in conditions of rigorous Ca-deprivation (Cochrane & Douglas, 1974). Moreover, the response is Ca-specific: Mg, for example, which is likewise transported by A23187, will not elicit secretion even when used in much higher concentrations (Cochrane & Douglas, 1974). Furthermore, the Ca-dependent A23187-induced secretory response of the mast cell conforms to the classic exocytotic pattern not only in its dependence on metabolic energy (Foreman *et al.* 1973; Johansen, 1980) and temperature (Johansen, 1978), but also in its appearance in light (Cochrane & Douglas, 1974) and electron microscopy (Kagayama & Douglas, 1974). There is no need to postulate any additional effect of A23187 since Ca ions are generally accepted as providing an adequate stimulus to exocytosis and, moreover, various other means of introducing Ca into the mast cell likewise elicit an exocytotic response (Kanno, Cochrane & Douglas, 1973; Douglas & Kagayama, 1977; Theoharides & Douglas, 1978). A23187 thus provides a suitable means of circumventing the early, receptor-dependent, steps of stimulus–secretion coupling and of isolating for study those events which detect and transform the Ca signal into the functional response. This has allowed us to study the effects of neuroleptic drugs and of W-7 on the Ca-activated steps of exocytosis and to compare these with their more clearly defined actions on Ca-activated contraction in smooth muscle.

Since neither the neuroleptic drugs nor W-7 lessened the ability of A23187 to transport ^{45}Ca into the cell it follows that the inhibitory effects of these drugs on A23187-induced exocytosis result from some action exerted distal to the Ca signal. This action must also contribute to the inhibitory effects of the various drugs on responses to antigen and to 48/80 each of whose effects are mediated by Ca ions. It

is sufficient to explain the inhibition of responses to 48/80, which was acheived with similar concentrations of each of the drugs; but an additional component seems to contribute to the inhibition of responses to antigen since the drugs were active in lower concentration and in a somewhat different rank order of potency. Because antigen, unlike 48/80, acts mainly by opening membrane Ca channels, this may be the process that is additionally affected; neuroleptic drugs inhibit stimulus-induced Ca influx in various other secretory cells (see Williams *et al.* 1977; Singh, 1980; Fleckman, Erlichman, Schubart & Fleischer, 1981; Valverde, Sener, Lebrun, Herchuelz & Malaisse, 1981) and smooth muscles (see Chaturvedi, Landon & Rama Sastry, 1978).

Those inhibitory effects of the neuroleptic drugs and of W-7 that are exerted distal to the Ca signal indicate an action either on the Ca-receptor or on the processes it activates to effect exocytosis. In smooth muscle an action on the contractile apparatus distal to the Ca receptor is easily excluded (Cassidy *et al.* 1980; Kerrick *et al.* 1981). But it is more difficult to exclude an action on the effector apparatus of exocytosis. Unlike contraction, exocytosis involves complex membrane interactions – and cell membranes are a classic site of action of neuroleptic drugs which not only block ion channels and receptors for various autacoids but additionally have 'unspecific' effects on membranes that include 'stabilization', 'destabilization' and displacement of membrane-bound Ca (Seeman, 1972; Lüllman *et al.* 1980). Indeed, such unspecific membrane effects have been commonly invoked to explain effects of these drugs on exocytosis and on membrane fusion–fission processes in general (see, for example, Poste & Allison, 1973; Williams *et al.* 1977; Elferink, 1979; Singh, 1980). Nevertheless, our results indicate some other, more specific, action. Thus the inhibitory potencies of the various drugs did not correlate with their potencies in producing unspecific membrane effects as reflected by cytolysis. Moreover, chlorpromazine sulphoxide, which shares the unspecific membrane actions of this class of drugs but lacks calmodulin-inhibiting action, did not inhibit exocytosis. And high Ca, which tends to counter unspecific membrane effects, did not reverse the inhibition of exocytosis. It would thus seem that for exocytosis, as for smooth muscle contraction, the drugs we have tested can inhibit by some action in the stimulus–response coupling sequence that is proximal to the effector mechanism yet distal to the Ca signal. In smooth muscle, the correspondingly-sited action is inhibition of the Ca receptor (calmodulin) and our observations in the mast cell encourage a similar interpretation. Thus we found that the various neuroleptic drugs inhibited Ca-induced exocytosis in the same rank order of potency that they inhibit Ca-induced contraction in smooth muscle (Hidaka *et al.* 1979; Cassidy *et al.* 1980) and Ca-activated calmodulin activity *in vitro* (Weiss *et al.* 1980). Moreover, our findings that thioridazine inhibits exocytosis by a non-competitive action and that high Ca fails to overcome the inhibition of exocytosis parallel observations on contraction in smooth muscle (Hidaka, Asano, Iwadare, Matsumoto, Totsuka & Aoki, 1978; Crosby & Diamond, 1980) and calmodulin *in vitro* (Weiss *et al.* 1980). It may be noted further that the various drugs we tested bind rather selectively to calmodulin (Weiss *et al.* 1980): by way of illustration, they bind only weakly to the Ca-receptive protein of *striated muscle*, troponin C (Hidaka *et al.* 1980; Weiss *et al.* 1980), and this is reflected in the refractoriness of contraction in striated muscle to inhibition by the phenothiazines (Andersson, 1972; Kerrick *et al.* 1981). On these various grounds, the target of

neuroleptic drugs and W-7 in the mast cell seems likely to be some Ca-binding protein that closely resembles calmodulin if it is not calmodulin itself: and the results can be taken as indicating the involvement of this protein as the Ca receptor activating exocytosis in the mast cell. The function of this Ca-receptive protein may, as in smooth muscle, involve protein phosphorylation: the mast cell is one of those in which Ca-dependent protein phosphorylation has been observed (Sieghart, Theoharides, Alper, Douglas & Greengard, 1978).

Whether a calmodulin-like entity functions generally in such a capacity in cells secreting by exocytosis is uncertain. Phenothiazines have long been known to have complex effects, including inhibition, on various secretory cell types (see Poste & Allison, 1973). But inhibition, in the absence of evidence that it correlates with inhibtion of calmodulin and not with actions proximal or distal to the Ca receptor, provides little support for involvement of calmodulin. In only one study, on the pancreatic β cell, has a sufficient number of drugs been examined and a correlation been observed between the drug's potency to inhibit calmodulin and its potency to inhibit secretion (Gagliardino, Harrison, Christie, Gagliardino & Ashcroft, 1980). But in this study no evidence was provided to show that the drugs did not inhibit at some stage before the generation of the Ca signal. An inhibitory effect distal to the Ca signal has, however, been demonstrated in other studies (Baker & Knight, 1980; Baker & Whitaker, 1980; Naccache, Molski, Alobaidi, Becker, Showell & Sha'afi, 1980: Schubart, Erlichman & Fleischer, 1980); but in these only one or two drugs were used so that evidence of a correspondence between inhibition of secretion and calmodulin inhibiting activity is lacking. Furthermore, in all of these studies it is uncertain whether the inhibitory effects of the drugs reflect an action on calmodulin or unspecific actions on cell membranes of the sort that have alternatively been advanced to explain inhibitory effects of these drugs on exocytosis (see, for example, Poste & Allison, 1973; Williams *et al.* 1977; Elferink, 1979; Singh, 1980). Indeed, such unspecific membrane effects are evident in many of the studies by increased basal release (Sugden, Christie & Ashcroft, 1979; Baker & Knight, 1980; Krausz, Wollheim, Siegel & Sharp, 1980; Naccache *et al.* 1980; White & Raynor, 1980). The only experiments that have addressed this point and that indicate an additional and more specific action of the neuroleptic drugs are those on the mast cell. The evidence is thus fragmentary. Nevertheless, the pieces are complementary and together comprise an argument for a general involvement of some calmodulin-like protein in stimulus-secretion coupling, perhaps functioning as the Ca-receptor that activates exocytosis.

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