Stimulated Release of Histamine by a Rat Mast Cell Line Is Inhibited during Mitosis

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ABSTRACT Stimulated histamine release was depressed at least tenfold in mitotic 2H3 rat basophilic cells when compared with interphase cells even though both contained comparable amounts of histamine. Antigen stimulation of IgE-sensitized interphase cells initiated an influx of Ca^{2+} that preceded secretion of histamine and a similar Ca^{2+} influx occurred in stimulated mitotic cells. This strongly suggests that during mitosis there is a dramatic inhibition of one or more of the steps on the pathway leading from elevated intracellular Ca^{2+} to the fusion of secretory granules with the plasma membrane.

There appears to be a general cessation of vesicular traffic in cultured animal cells undergoing mitosis. Endocytosis (1–3) and phagocytosis (3) are inhibited and newly synthesized plasma membrane proteins are not transported to the cell surface (4). These processes share a number of basic features. All, for example, involve the budding of vesicles from donor membranes and the fusion of these vesicles with specific acceptor membranes. These similarities suggest that the same mechanism might suffice to inhibit all of these processes during mitosis and the inhibited step in endocytosis and phagocytosis appears to be the budding of the vesicles from the plasma membrane (1, 3). There are, however, reasons that will be discussed later for suggesting that fusion of vesicles with their acceptor membranes is also inhibited. Unfortunately, none of the systems so far used to study vesicular traffic during mitosis is suitable for testing this possibility. The best-studied fusion processes are those in regulated secretory cells where the secretory granules fuse with the plasma membrane after an appropriate stimulus. Assuming this to be a general model for membrane fusion, at least with respect to events during mitosis, we have studied secretion in a rat basophilic leukemic cell line (2H3) which has many of the properties of rat peritoneal mast cells (5). 2H3 cells can be sensitized with IgE (6) and a Ca^{2+} influx follows addition of antigen (7). Histamine is then released over 20–30 min, somewhat slower than the 1–2 min observed with normal rat mast cells (8, 9). They also grow as a monolayer and are amenable to the techniques available for synchronizing animal cells. Mitotic 2H3 cells have been found not to secrete histamine in the studies presented here. The results also extend previous work (4), which suggested a general cessation of vesicular traffic in the mitotic cell.

MATERIALS AND METHODS

2H3 Cell Culture and Sensitization with IgE: The rat basophilic cell line 2H3 (samples of which were donated by Dr. R. P. Siraganian, National Institutes of Health, Bethesda, MD) was established as monolayer and spinner cultures by the method of Barsumian et al. (10). Monolayer cultures were maintained in 850 cm^2 tissue culture roller bottles (Falcon Labware, Oxnard, CA) and cells were grown in Eagle's minimal essential medium with Earle's balanced salt solution supplemented with 15% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco Laboratories, Grand Island, NY) in 5% CO_2/95% air at 37°C.

To sensitize cells for degranulation by aggregated ovalbumin, IgE-specific for ovalbumin (MAS 038c, Sera-lab, Crawley, United Kingdom) was diluted 500-fold into fresh medium which was then used either to replace the medium in roller bottles or for resuspension of spinner cells after centrifugation (200 g, 10 min). Cells were incubated with IgE for >3 h and cell suspensions (10^6 cells/ml) were inverted on a rotary mixer at 1 rpm (7).

Isolation of Mitotic Cells: Monolayer cells grown to ~75% confluence were washed twice by incubating with fresh medium for 20 min before rotating the roller bottles at 200 rpm for 5 min to remove loose cells. Nocodazole (0.04 μg/ml, 0.13 μM, [Sigma Chemical Co.] [11]) was then added for up to 5 h and mitotic cells were removed at 1-h intervals using the shake-off procedure described by Klevecz (12), rotating the roller bottles at 50 rpm for 3 min at 37°C. The mitotic index in cells thus harvested was >90% as determined by staining with Hoechst dye 33258 (3).

For measurements of histamine release cells were washed (200 g, 10 min) and suspended in complete medium; cell suspensions were incubated on a rotary mixer as described above at 10^6 cells/ml and monolayer cultures were established by dispensing 400 μl at 0.5×10^6 cells/ml in complete medium into multi-well (1.6-cm diam) plates (Nunc, GMBH, Federal Republic of Germany). Cells were preincubated as described in the figure legends before stimulation of histamine release.

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Histamine Release: Degranulation was stimulated by the addition of 2.5 or 10 μg/ml aggregated ovalbumin (crystalline ovalbumin (Sera-lab, Crawley, United Kingdom) (7). 50-μl samples of cell suspensions or monolayer medium, taken at the times indicated in Figs. 1, 2, 3, and 5 and in Table II, were centrifuged (18,000 g, 1 min). The cell pellet was resuspended in 200 μl of fresh medium and the cells disrupted by three cycles of freezing in liquid N_2 and thawing at room temperature. The histamine content of 20-μl samples of cell extract or supernatant solution was determined by a single step radioenzymatic assay (13). For the results presented in Figs. 1, 2 and 5, values for histamine release were corrected for spontaneous release (5–10% in 30 min) in the absence of antigen.

Quin 2 Loading and Fluorescence Measurements: Interphase or mitotic cells were loaded with quin 2 (14) after IgE sensitization by centrifugation (200 g, 10 min) and resuspension at 10^6 cells/ml in medium A containing 10 μM quin 2 acetoxymethyl ester (quin 2-AME) added from a stock solution in Me_2SO. After rotary mixing (1 rpm) for 1 h at 37°C the cells were centrifuged and suspended in medium A at 10^6 cells/ml. Intracellular quin 2 fluorescence changes were measured in 2 ml samples of continuously stirred cell suspensions in 1-cm quartz cuvettes at 37°C in a Perkin Elmer 44E spectrofluorimeter (excitation at 339 nm, emission at 492 nm). Complete hydrolysis of quin 2-AME to quin 2 was confirmed by the shift in emission maximum from 435 to 492 nm (15). Estimation of the free, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) from quin 2 fluorescence measurements was made as described previously for thymocytes (15) and 2H3 cells (7). After each experiment cells were lysed by the addition of 10 μl Triton X-100 (10%) to give 100% saturation of quin 2 with Ca^{2+}, followed by 10 μl of 100 mM $MnCl_2$, which quenches the fluorescence by >99%. The $[Ca^{2+}]_i$ was estimated from the calculated percent of Ca^{2+} saturation of quin 2, determined using the formula given in reference 15 and a value of log K_{Ca} for quin 2 of 7.05 at 37°C.

Immunofluorescence Microscopy: 2H3 cells were grown as monolayers on cover slips and treated as required with IgE, nocodazole, and ovalbumin as described above. Cells were fixed and labeled with a rhodamine-conjugated rabbit anti-mouse IgG (16) and then stained with Hoechst dye 33258 (3).

RESULTS

Histamine Is Released from Interphase But Not from Mitotic Cells

Monolayer cultures of 2H3 cells were sensitized with IgE and arrested in mitosis using nocodazole, as described in Materials and Methods. Nocodazole prevents spindle assembly (17) and cells were held at an early stage after entering mitosis, with chromosomes resembling those of prometaphase cells (see Fig. 4). Samples of mitotic cells were shaken off and washed to remove nocodazole, suspended in complete medium, and dispensed into culture plates. Removal of nocodazole allowed the arrested cells to proceed through mitosis and they took about 1 h to reach telophase (Table I). Histamine release was measured following the addition of optimal concentrations of aggregated ovalbumin at progressively later times and it is evident from Fig. 1 that 1 or 2 h after removal of nocodazole the rate and extent of histamine release is closely similar to that from normal interphase cells in suspension (Fig. 3) or in monolayers (7). In the continuous presence of nocodazole, however, the cells were held in the mitotic state and released only basal levels of histamine in the 30 min following ovalbumin addition (Fig. 1). The levels of basal release (<10%) were similar in mitotic and interphase cells and presumably arise from the presence of damaged cells. The data of Table I, taken together with those of Fig. 1, show that the recovery of the degranulation response coincided closely with the completion of mitosis.

TABLE I

Time Taken for 2H3 Cells to Complete Mitosis after Removal of Nocodazole

Time after removal of nocodazole	mitotic cells
h	%
0	92
0.5	71
1	9
2	<1

Cells were arrested in mitosis using nocodazole, shaken off, and then dispensed into culture plates in the absence of nocodazole, as described in Materials and Methods. The proportion of mitotic cells was estimated by staining with Hoechst dye (3).

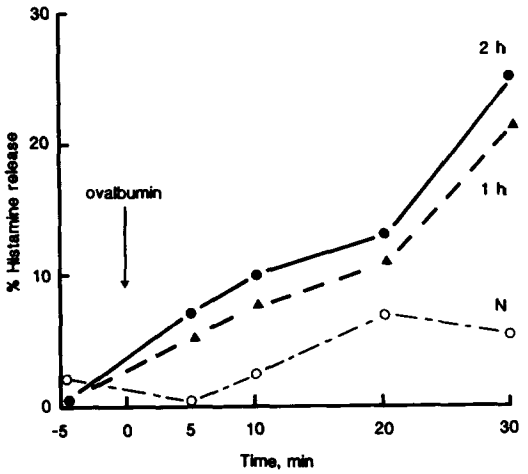

FIGURE 1 Histamine release from mitotic and postmitotic 2H3 cells. Monolayer cultures were sensitized with IgE, accumulated in mitosis using nocodazole, isolated, washed, and dispensed into multi-wells as described in Materials and Methods. Aggregated ovalbumin (2.5 μg/ml) was added at time zero and the histamine release from cells measured: O continuously incubated with 0.04 μg/ml nocodazole (*N*); ▲, 1 h; and ● 2 h after removal of nocodazole. The release is expressed as a percentage of the total histamine content of the cells, after subtraction of the spontaneous release in the absence of antigen (<10%).

The absence of secretion by mitotic 2H3 cells was not a side-effect of the nocodazole used to arrest the cells in mitosis (see also below). 30 min after its removal the cells were still undergoing mitosis (Table I), but there was very little secretion of histamine (Fig. 2). 1 h after its removal (Fig. 2) the histamine released was less than that observed in the experiments presented in Figs. 1 and 5. Variability at this time point was a consistent observation and probably reflects the abrupt transition as the cells complete mitosis and change from a nonsecreting to a secreting state. By 2 h the cells were fully responsive and secreted approximately 10 times as much histamine over a 30-min period as mitotic cells (Fig. 2). The cells continued to grow normally as indicated by the level of histamine release at 22 h (Fig. 2) and by the appearance of the cells when stained with Hoechst dye or observed with Nomarski optics (not shown). The absence of secretion during mitosis could also not be explained by an absence of secretory granules: the total histamine content of mitotic 2H3 cells (0.4 pg/cell, $n = 5$) did not differ significantly from that of normal interphase cells (0.42 pg/cell, $n = 4$).

Interphase Cells Degranulate Normally in the Presence of Nocodazole

Although there was a striking kinetic parallel between the recovery of the degranulation response and the progression into G1 following the removal of nocodazole, it was important to confirm that the drug itself did not inhibit release in

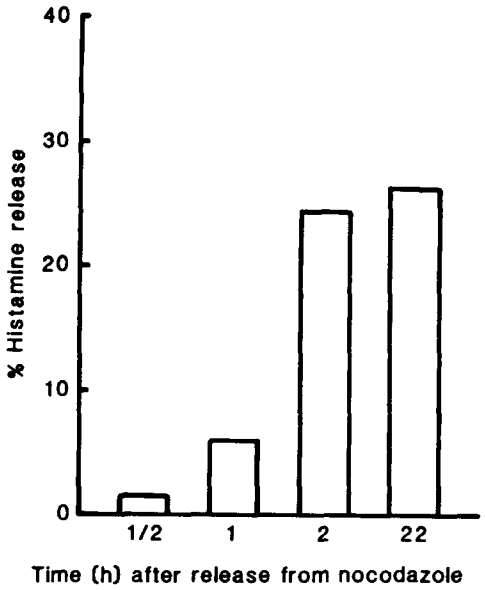

FIGURE 2 Histamine released from sensitized mitotic and postmitotic cells after stimulation with 2.5 μg/ml ovalbumin. The experiments were performed as described in the legend to Fig. 1. Histamine release was measured at different times after the removal of nocodazole. The histograms show the percentage of total histamine released after 30 min and each is the mean of two experiments.

interphase cells. The absence of any effect of 0.04 μg/ml nocodazole on histamine release from interphase 2H3 cells in suspension is confirmed in Fig. 3 for two concentrations of aggregated ovalbumin antigen (2.5 and 10 μg/ml). The release of 25–30% of total histamine within 30 min of stimulation constituted a typical response of this cell line to antigenic challenge (7, 10).

Sensitized interphase cells that remained attached to roller bottles as monolayers following nocodazole treatment and the shake-off procedure also degranulated normally when challenged with aggregated ovalbumin (Table II). The stimulation of 30% histamine release from these cells within 30 min indicated that, provided the cells had not entered mitosis, even prolonged (3 h) exposure to nocodazole did not impair their sensitivity.

Receptor Cross-Linking Elevates $[Ca^{2+}]_i$ in both Mitotic and Interphase Cells

The diffuse, surface immunofluorescent staining seen with rhodamine-conjugated anti-immunoglobulin antibody indicated that mitotic 2H3 cells retain the F_c receptors and IgE binding capacity of interphase cells at all stages of mitosis (Fig. 4), as has been reported by Meyer et al. (18). We have shown previously that the addition of aggregated ovalbumin (2.5 or 10 μg/ml) to suspensions of interphase 2H3 cells sensitized with IgE and loaded with the fluorescent Ca^{2+} indicator quin 2 causes an immediate increase in the fluorescence signal corresponding to an increase in $[Ca]_i$ from 105 to 1,200 nM (7). The same results were obtained with sensitized mitotic 2H3 cells, as shown in Fig. 5*A*. The increase in $[Ca^{2+}]_i$ indicated by the fluorescence change is similar to, though generally slightly smaller than, that in normal unsynchronized cells, rising from a resting level of 58% Ca-quin 2 (159 nM $[Ca^{2+}]_i$) to 85% Ca-quin 2 (650 nM $[Ca^{2+}]_i$) within 3 min of ovalbumin addition. A $[Ca]_i$ increase of this magnitude in normal interphase cells would induce >85% of the maximum histamine release (7). As was also the case for interphase cells, no increase in fluorescence was observed in mitotic cells not sensitized with IgE (data not shown). The presence of quin 2 did not affect the pattern of histamine release. The mitotic cells showing increased $[Ca^{2+}]_i$ did not release histamine whereas a parallel sample washed free of nocodazole and incubated for 1 h before addition of ovalbumin gave normal levels of release (Fig. 5).

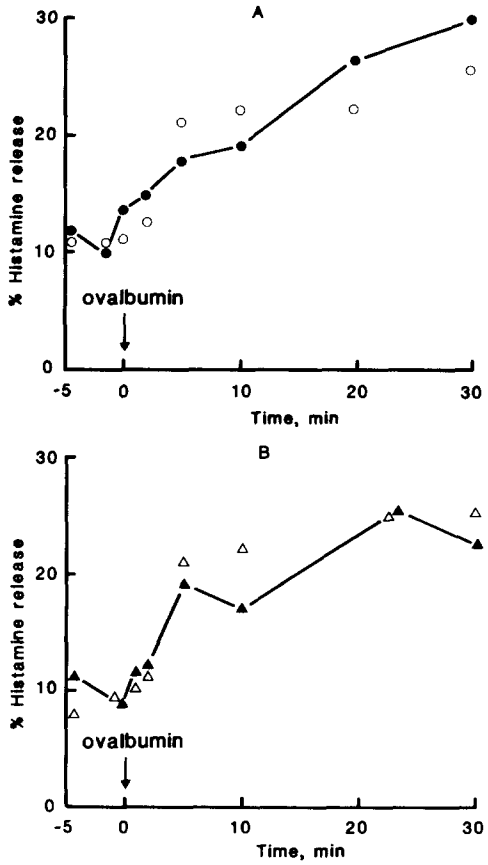

FIGURE 3 Effect of nocodazole on histamine release from interphase 2H3 cells in suspension. 10^6 spinner cells per ml were sensitized with IgE for 3 h with continuous mixing (see Materials and Methods), either without (○, △) or in the presence of (●, ▲) 0.04 μg/ml nocodazole. After washing, the cells were suspended with continuous mixing in medium with or without nocodazole before addition of (*A*) 2.5 (○, ●) or (*B*) 10 (△, ▲) μg/ml aggregated ovalbumin at time zero.

TABLE II

Histamine Release from Interphase Cells Pre-treated for 3 h with Nocodazole

Time after ovalbumin addition	Histamine release
min	%
−1	0
1	0
28	32
31	29
41	46

Roller bottle cultures were sensitized with IgE and incubated with 0.04 μg/ml nocodazole for 3 h before removal of the mitotically arrested cells, as described in Materials and Methods. Fresh medium was added to the roller bottle and histamine release from the remaining, adherent, interphase cells was stimulated by the addition of 10 μg/ml aggregated ovalbumin at time zero.

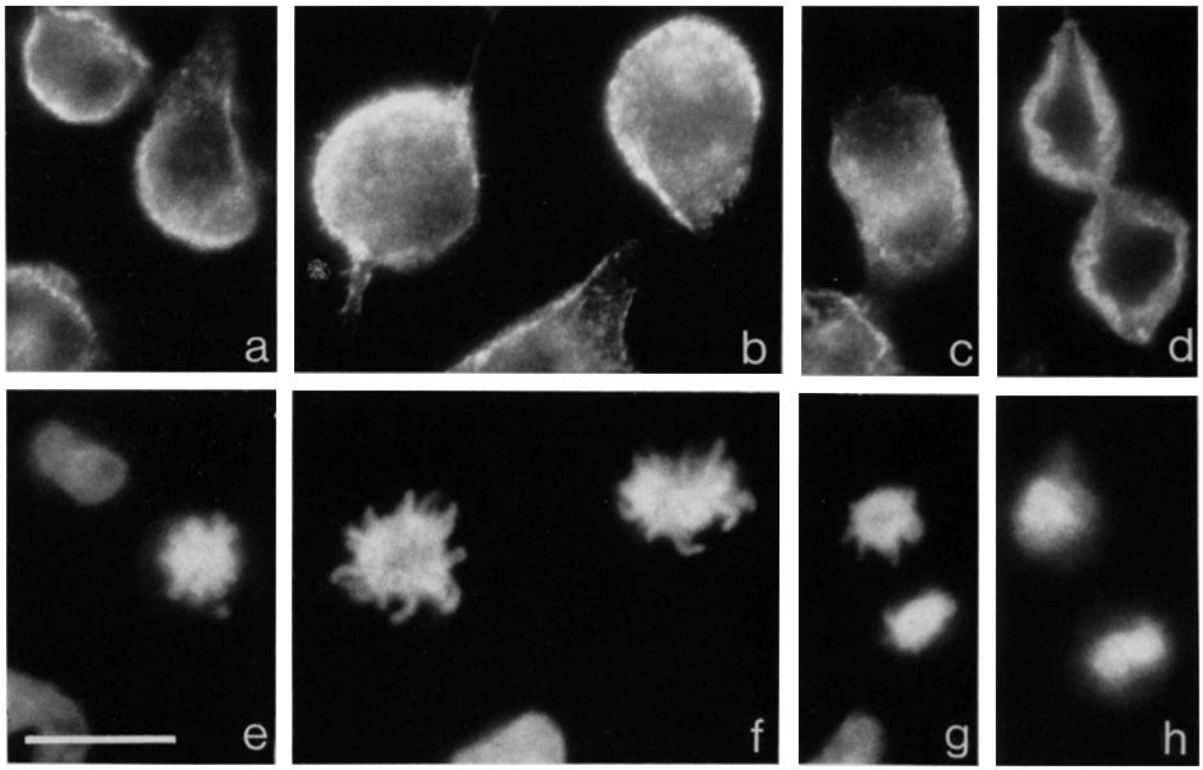

FIGURE 4 IgE in interphase and mitotic 2H3 cells visualized by indirect immunofluorescence. Monolayer 2H3 cells were incubated with IgE for 3 h and in one case (*a* and *e*) with nocodazole for 1 h before fixation. Cells were then labeled with a rhodamine-conjugated anti-Ig antibody (*a–d*) followed by staining with Hoechst dye 33258 (*e–h*). Cells shown are in prometaphase (*e* and *f*), anaphase (*g*), and telophase (*h*). Bar, 20 μm. × 1,000.

DISCUSSION

Mitotic 2H3 cells bind IgE and binding of aggregated antigen is followed by an influx of Ca^{2+} similar to that seen in interphase cells (7). A later step on the pathway must be inhibited, however, because secretion of histamine is at least 10-fold lower than in interphase cells. Metalloendoprotease inhibitors (21) and Zn^{2+} (7, 19, 20) have a similar effect but mitosis is the first physiological condition found to uncouple antigenic stimulus from the secretory response. The inhibition cannot be explained by an absence of histamine because mitotic and interphase cells contain equivalent amounts.

Earlier work on secretion during mitosis gave apparently conflicting results. Some workers (22) found it was depressed, but synthesis of secretory proteins was also depressed so that no conclusions regarding the secretory process itself could be drawn. Other workers (23, 24) observed secretion in synchronized populations of cells undergoing mitosis but the synchrony was not perfect so that cells undergoing mitosis were always contaminated by late G2 and early G1 cells which were presumably responsible for the observed secretion. Another difficulty is that the time taken for a cell to complete mitosis is often shorter than the time needed to transport newly-synthesized proteins to the cell surface (4). These difficulties were avoided in our studies by purifying mitotic cells using a shake-off procedure and arresting them in mitosis using a drug that had no effect on secretion in interphase cells.

The drug nocodazole, used to obtain high yields of mitotic cells, has been shown by Zieve et al. (11) and De Brabander et al. (17) to be superior in its specificity to other inhibitors of microtubule function. They found, for example, that although mitotic block could be induced by colcemid, recovery and progression through mitosis was at least twice as rapid when nocodazole was the arresting agent. On the basis of the present data there are two reasons why it is extremely unlikely that the inhibition of secretion in mitotic cells arises from a direct effect of nocodazole on microtubules involved in degranulation. Firstly, interphase cells degranulate normally in the presence of nocodazole at a concentration that effects mitotic arrest (Fig. 3). This result again contrasts with the effect of antimicrotubule drugs such as colchicine which inhibits histamine release (25) but probably does so by nonspecific means, such as depletion of cellular ATP. Secondly, although Zieve et al. (11) have shown that nocodazole is rapidly removed from cells by washing, 2H3 cells so treated that are still completing mitosis show no significant response to aggregated ovalbumin (Fig. 2). The restoration of normal degranulation proceeds with a time course that closely follows that of the transition of the cells from mitosis to G1 (cf. Fig. 2 and Table I).

It was suggested in the introduction to this paper that reasons existed for thinking that fusion of vesicles with their acceptor membrane might be inhibited during mitosis. These reasons derive from microscopic observations of the Golgi complex. As cells enter mitosis the Golgi complex fragments (26, 27) and the available data suggest that the ordered stacks of Golgi cisternae are converted into tens of thousands of small vesicles (see reference 28). It seems probable that these vesicles mediate the division of the Golgi complex since they appear to randomize throughout the mitotic cell cytoplasm (27, 28) and thus ensure that each daughter cell receives an equal complement of Golgi membrane. Of interest, therefore, is the mechanism that gives rise to these vesicles. During

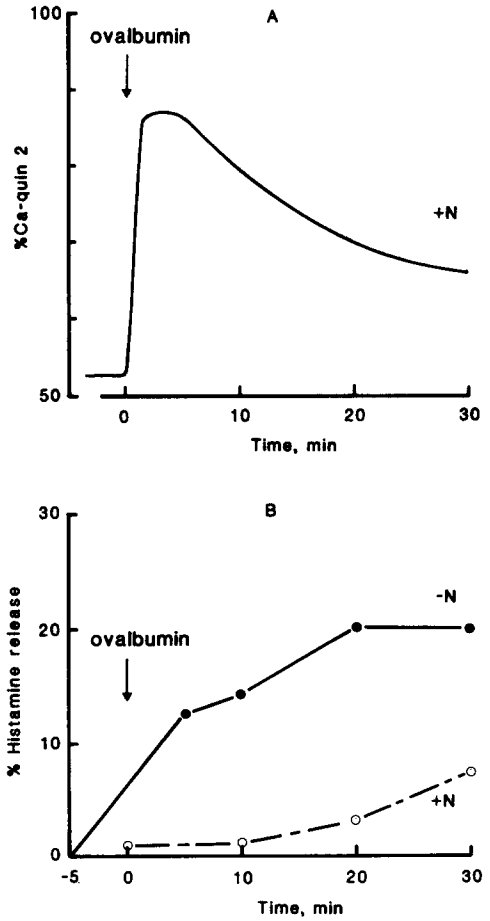

FIGURE 5 The change in intracellular free Ca^{2+} and histamine release from quin 2 loaded cells. Cells were sensitized with IgE and accumulated in mitosis (1 h with nocodazole) before isolation and suspension in medium A containing 10 μM quin 2-AME either with (○) or without (●) nocodazole (*N*). After a further 1 h at 37°C the cells were washed and transferred to continuously stirred suspensions for fluorescence measurements (see Materials and Methods). (*A*) The change in quin 2 fluorescence due to the addition of 10 μg/ml aggregated ovalbumin at time zero to sensitized mitotic cells. (*B*) Histamine release caused by the addition of 10 μg/ml ovalbumin at time zero. ○, mitotic cells incubated with nocodazole throughout; ●, stimulated with ovalbumin ~1 h after removal of nocodazole.

intracellular transport in interphase cells, vesicles are thought to bud from the rims of the Golgi cisternae and fuse with the next cisterna in the stack towards the *trans* side (29). If, at the onset of mitosis, these vesicles could continue to bud but could no longer fuse, each cisterna would be rapidly converted into small vesicles in a manner consistent with the microscopic observations (28). A further result would be the observed cessation of intracellular transport (4). At the end of mitosis, during telophase, vesicle fusion would resume with the Golgi stack reassembling spontaneously. It would only be necessary for the cell to specify the location at which this reassembly should occur. This simple hypothesis goes a long way towards explaining the division of the Golgi complex in animal cells. It does not contradict the suggestion that vesicle budding is inhibited when endocytosis and phagocytosis cease (1, 3): it is only necessary to postulate that fusion of vesicles is inhibited first and that vesicle budding is inhibited a few minutes later, after the Golgi complex has fragmented. Experiments are in progress to test this point. The observations in this paper are consistent with the hypothesis and imply that a common mechanism may control all intracellular membrane fusion events during mitosis.

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