# Changes in the State of Actin during the Exocytotic Reaction of Permeabilized Rat Mast Cells

**Anna Koffer, Peter E. R. Tatham, and Bastien D. Gomperts**

Department of Physiology, University College London, London, WC1E 6JJ United Kingdom

*Abstract.* The major part of mast cell actin is Triton-soluble and behaves as a monomer in the DNase I inhibition assay. Thus, actin exists predominantly in monomeric or short filament form, though filamentous actin is clearly apparent in the cortical region after rhodamine–phalloidin (RP) staining. The minimum actin content is estimated to be ~2.5 $\mu$g/$10^6$ cells (cytosolic concentration ~110 $\mu$M.

After permeabilization of mast cells by the bacterial cytolysin streptolysin-O, ~60% of the Triton-soluble actin leaks out within 10 min. However, the staining of the cortical region by RP remains undiminished, and the cells are still capable of exocytosis when stimulated by GTP-$\gamma$-S together with $Ca^{2+}$. In the presence of cytochalasin E the requirement for $Ca^{2+}$ is decreased, indicating that disassembly of the cytoskeleton may be a prerequisite for exocytosis. This disassembly is likely to be controlled by $Ca^{2+}$-dependent actin regulatory proteins; their presence is indicated by a $Ca^{2+}$-dependent inhibition of polymerization of extraneous pyrene-G-actin by a Triton extract of mast cells. The effect of cytochalasin E on secretion is similar to that of phorbol myristate acetate, an activator of protein kinase C; both agents enhance the apparent affinity for $Ca^{2+}$ and cause variable extents of $Ca^{2+}$-independent secretion.

Exposing the permeabilized cells to increasing concentrations of $Ca^{2+}$ caused a progressive decrease in F-actin levels as measured by flow cytometry of RP-stained cells. In this respect, both cytochalasin E and phorbol ester mimicked the effects of calcium. GTP-$\gamma$-S was not required for the $Ca^{2+}$-dependent cortical disassembly. Thus, since conditions have not yet been identified where secretion can occur in its absence, cortical disassembly may be essential (though it is not sufficient) for exocytosis to occur.

PERMEABILIZED rat mast cells provide an excellent system for investigating the mechanism of exocytosis. In response to provision of $Ca^{2+}$ and GTP (or its analogues), they release up to 100% of their contained histamine and hexosaminidase, both of which can be rapidly and sensitively measured (16). The exocytotic process, which may be studied by providing appropriate effectors (bypassing the receptor–agonist interaction), involves fusion of the granular and plasma membranes followed by the release of granule contents. Since all mammalian cells contain an actin-rich network in the cortical region (6), it has been suggested that disassembly of the cortical cytoskeleton might be a prerequisite for exocytosis (3, 7, 26, 42). On the other hand, the motile elements of the cytoskeleton are also thought to be involved in the transport of vesicles to the plasma membrane. Several studies have demonstrated increased actin filament assembly after cell stimulation (10, 39, 41). Controversial conclusions have also been presented on the basis of experiments involving the use of microfilament-specific agents such as phalloidin and cytochalasin (2, 13, 16, 19, 29, 30, 34, 42, 44). Thus, the exact role (if any) of the cytoskeleton in the exocytotic reaction remains an open question.

In the following work, the presence of actin in rat mast cells has been established by gel electrophoresis, peptide mapping, and DNase I inhibition assay. Actin has been shown to be the most abundant Triton-soluble protein component. A major part of the actin in these cells appears to be either in the monomeric or short filament form and can be released from the cells after their permeabilization by a bacterial toxin, streptolysin-O (SL-O)[1]. However, a stable cortical actin filament network is present and can be visualized by rhodamine–phalloidin (RP) staining. Treatment of the permeabilized cells with cytochalasin E enhances the apparent affinity of the exocytotic reaction for $Ca^{2+}$. This effect is similar to that achieved by the protein kinase C activator, PMA (see reference 32). Both these agents, as well as $Ca^{2+}$, cause a decrease in the content of filamentous actin in the cortical region of the permeabilized mast cells. In contrast, the cortical F-actin of intact mast cells has been found resistant to both cytochalasin E and PMA treatment and the two agents did not significantly change the dose response of intact cells to compound 48/80. In all aspects examined here, secretion from permeabilized rat mast cells and cortical F-actin disassembly were correlated, implying an important,

1. *Abbreviations used in this paper*: GTP-$\gamma$-S, guanosine-5′-0-(3-thiotriphosphate); pCa, $-\log_{10}$ [$Ca^{2+}$]; RP, rhodamine-phalloidin; SL-O, streptolysin-O.

though not sufficient, role for cortical disassembly in exocytosis. We have also demonstrated the presence in rat mast cells of calcium-dependent actin regulatory factors.

## Materials and Methods

### Cells and Cell Fractionation

Mast cells were purified to >95% purity by centrifuging peritoneal washings from adult Sprague Dawley rats through a 2-ml cushion of Percoll (final density 1.12 g $ml^{-1}$). The cells were washed twice by resuspension and centrifugation and finally suspended in a buffered salt solution (pH 6.8), which comprised 137 mM NaCl, 2.7 mM KCl, 20 mM Pipes, 5.6 mM glucose and 1 mg $ml^{-1}$ BSA (45).

When preparing the cell fractions, the purified cells were washed again and resuspended in a buffered sucrose solution (pH 7.4), consisting of 0.3 M sucrose, 10 mM Pipes, 10 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.2 mM DTT, 0.2 mM ATP, 0.2 mM PMSF and 5 $\mu g$ $ml^{-1}$ of each of the following proteinase inhibitors: aprotinin, leupeptin, and *p*-tosylarginine methylester. Triton-soluble extracts were prepared by high ($10^5$ *g*, 1 h) or low ($10^4$ *g*, 15 min) speed centrifugation of cells after lysis in 0.5% Triton X-100 ($\sim 1.5 \times 10^7$ cells $ml^{-1}$).

### SDS-PAGE

To prepare samples for gel electrophoresis, the cells were pretreated with 2 mM diisopropylphosphofluoridate for 5 min at room temperature to inhibit serine proteinases (1). They were washed and then either permeabilized by treatment with streptolysin-O (SL-O) (0.4 IU $ml^{-1}$) in the buffered salt solution (but the albumin was now omitted and 0.025 mM EGTA included) or lysed with 0.5% Triton X-100 in buffered sucrose as described above and centrifuged as indicated. The supernatants were dissolved in Laemmli sample buffer (giving a final concentration of 1% SDS) and heated for 2–5 min in a boiling water bath. The electrophoresis was carried out on 10% vertical slab gels (25). The gels were silver-stained by the method of Morrissey (28) omitting the glutaraldehyde treatment.

### Actin Concentration

The concentrations of actin in whole cell lysates or in Triton-soluble extracts were estimated by the DNase I inhibition assay, following the method of Blikstad et al. (5). The cells were lysed in 0.5% Triton X-100 in buffered sucrose and promptly assayed. Alternatively, the content of actin was assessed by densitometry of silver-stained electrophoretic gels. Skeletal muscle actin, prepared by the method of Spudich and Watt (43), was used as a standard in both cases.

### Peptide Maps

The band containing the peptide co-migrating with the actin standard was cut out from the gels and treated with *Staphylococcus aureus V8* protease (10 $\mu l$ of 1 $\mu g$ $ml^{-1}$), as described by Cleveland et al. (11). The digested proteins were analyzed by electrophoresis on 15% polyacrylamide SDS gels.

### RP Staining

***Intact Cells.*** Aliquots of cell suspension in the buffered salt solution, were transferred to a microtitre plate (96 wells, Falcon Labware, Oxnard, CA), $\sim 10^5$ cells per well, and fixed overnight with 3% paraformaldehyde in PBS supplemented with 3 mM EGTA and 2 mM $MgCl_2$. The cells were then treated (a 2-min spin at 500 *g* followed each treatment) as follows: residual aldehyde was quenched by treatment with 50 mM $NH_4Cl$ for 10 min. On occasions when the fixation period was <12 h, the cells were permeabilized with 0.5% Triton X-100 in the buffered salt solution containing 3 mM EGTA and 2 mM $MgCl_2$. After prolonged fixation this step was omitted since the treatment with paraformaldehyde permeabilizes the cells sufficiently. The prolonged fixation was also found to improve the morphology and intensity of staining of intact cells. The cells were finally washed and stained with 1 $\mu M$ (a saturating concentration) RP. After five washes, the cells were photographed (using a Leitz Fluovert microscope) or analyzed by flow cytometry.

***Permeabilized Cells.*** Using the 96-well microtitre plate, the cells were permeabilized using SL-O (0.4 IU $ml^{-1}$) for 15 min in the buffered salt solution supplemented with 3 mM EGTA or $Ca^{2+}$. EGTA buffers and 2 mM $MgCl_2$. They were then fixed for 15 min with 3% paraformaldehyde in PBS, 3 mM EGTA, 2 mM $MgCl_2$, stained for 15 min with 1 $\mu M$ RP, rinsed and photographed, or analyzed by flow cytometry (15 min fixation was sufficient for permeabilized cells).

### Flow Cytometry

Measurements of individual cell fluorescence were made using a cytometer (EPICS CS; Coulter Electronics Inc., Hialeah, FL). The laser operated at 514.5 nm (200 mW) and fluorescence was collected using a 514.5-nm blocking filter and a 550-nm long pass filter. Forward light scatter and 90° light scatter signals were also collected. The fluorescence and scatter histograms (3,000–5,000 cells) were generally unimodal and any weak fluorescence emission resulted from noise and particles other than mast cells. The parameter reported here is a statistic derived from the fluorescence histograms that provides a measure of the relative cell F-actin content. It was obtained by calculating the product of the proportion of cells in the main fluorescence population and their mean fluorescence intensity.

### Measurement of Secretion

To stimulate secretion from permeabilized cells, 30 $\mu l$ of suspension ($\sim 2 \times 10^5$ cells $ml^{-1}$) were transferred to 90 $\mu l$ of buffer containing SL-O (0.4 IU $ml^{-1}$ final), CaEGTA buffer (3 mM EGTA and 2 mM $Mg^{2+}$ final, to regulate $-\log_{10}$ [$Ca^{2+}$] [hereafter, pCa] as indicated) and guanosine-5′-0-(3-thiotriphosphate) (GTP-γ-S) as indicated. Alternatively, for stimulation of intact cells, the buffered salt solution was supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, and the cells were triggered by addition of compound 48/80. After incubation at 30°C for 15 min, the reactions were terminated by addition of 0.5 ml of ice-cold NaCl (0.15 M) buffered at pH 7 with 10 mM potassium phosphate, and the cells sedimented by centrifugation. Samples of supernatant were removed for measurement of β-*N*-acetyl-D-glucosaminidase (hexosaminidase) or histamine as previously described (16). The values were related to the total cellular content as measured in the supernatants from cells lysed by treatment with Triton X-100 (0.2%).

### Measurement of Actin Polymerization

G-actin labeled with *N*-(1-pyrenyl)iodoacetamide was prepared as described by Kouyama and Mihashi (23). This was added (final concentration 2.3 $\mu M$)

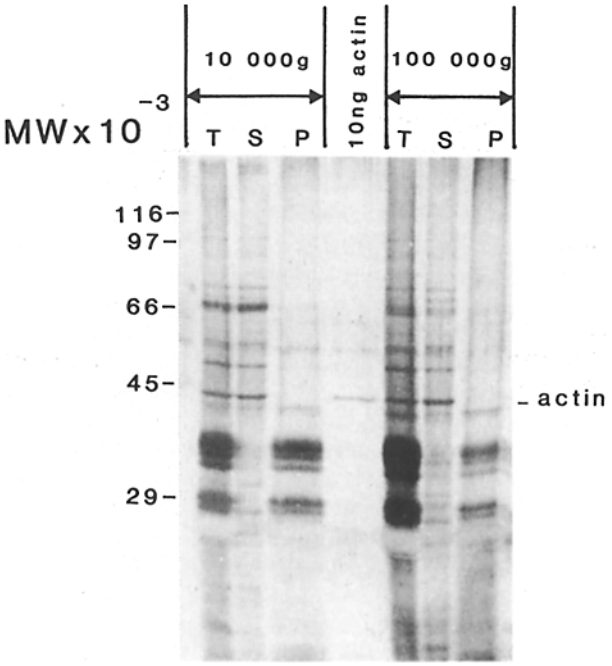


*Figure 1.* Polyacrylamide/SDS gel electrophoresis of total cell lysates (*T*) and Triton X-100 soluble (*S*) and insoluble (*P*) fractions obtained by high (100,000 *g*) or low (10,000 *g*) speed centrifugation. Each lane corresponds to 30,000 cells.

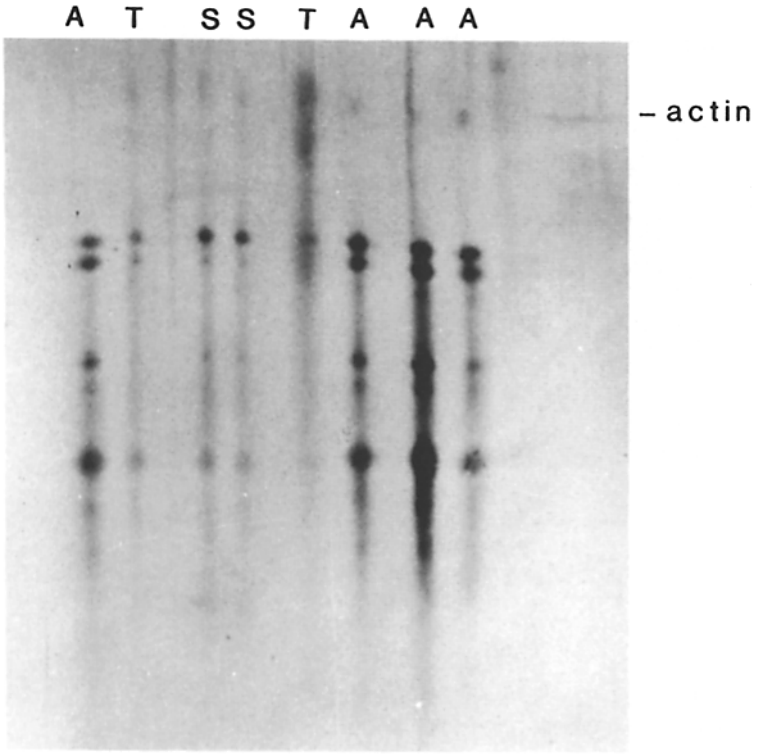


*Figure 2.* One-dimensional peptide maps obtained by digestion with *S. aureus V8* protease of the protein (43 kD) in excised electrophoretic zones. (*A*) skeletal muscle actin. (*T* and *S*) A protein co-migrating with actin in total cell lysate and in Triton-soluble extract, respectively.

at time zero to a cuvette containing the high speed supernatant from ~7.5 × $10^5$ cells lysed with Triton X-100, in a total volume of 400 μl. For this purpose, we used the sucrose-containing buffer to which was added 1 mM ATP. Where indicated, 1 mM $CaCl_2$ was added to give a free [$Ca^{2+}$] of ~5 μM. Fluorimetric measurements (excitation 365 nm, emission 408 nm) were carried out as described previously (20).

### *Materials*

Special reagents were obtained from the following sources: RP and phalloidin, cytochalasins D and E, and PMA from Sigma Chemical Co. Ltd. (Poole, Dorset, UK); GTP-γ-S, in the form of a 100-mM solution, was from Boehringer Mannheim UK (Lewes, Sussex, UK); SL-O from Wellcome Diagnostics (Dartford, Kent, UK).

## *Results*

### *State and Content of Actin in Rat Mast Cells*

Rat mast cell lysates were analyzed by differential centrifugation to determine the cellular content of filamentous actin: cytoskeletal F-actin is insoluble in Triton X-100 and can be sedimented by low speed centrifugation, while single long filaments sediment only at high speed. Figure 1 shows an electrophoretic analysis of cell fractions obtained from cells lysed by 0.5% Triton X-100. It is evident that very little actin sedimented at low or high speed (lanes *P*). The bulk of the actin remained buoyant even when the total concentration in the lysate exceeded its critical concentration (~0.5 μM). Thus, the actin content of the Triton soluble extract (lanes *S*) constitutes the major proportion of the total cellular actin; (compare lanes *T* and *S*). Examination of the Triton extracts (lanes *S*) by densitometry of silver-stained gels and by comparison with the actin standard showed the soluble actin content of mast cells to be 2.5 μg/$10^6$ cells (2.4 and 2.6 μg/$10^6$ cells, two experiments). Actin constituted ~10% of the total Triton-soluble protein.

Peptide mapping of the protein co-migrating with the actin standard (Fig. 2) showed that the dominant 43-kD protein present in mast cells is indeed actin and the densitometric estimates are therefore appropriate.

The content of monomeric actin in total Triton lysates and in low speed supernatants was also analyzed by the DNase I inhibition assay (5). The results, which are summarized in Table I, are close to those obtained by densitometry of SDS-PAGE gels. These again indicate that most of the cellular actin is either in a monomeric form (most probably complexed) or in the form of short filaments that do not pellet and can be readily dissociated by the DNase I. However, the presence of filaments in the cortical region of the cells is clearly demonstrated by RP staining (Fig. 3 *A*), revealing an actin containing network.

No significant increase in monomeric actin content could be detected by the DNase method after stimulating the cells with the compound 48/80 (17 μg $ml^{-1}$, 5 and 90 s after adding 48/80), after treatment with cytochalasin E (up to 50 μM, 10 min at 37°C), or when ~5 μM free $Ca^{2+}$ was pres-

*Table I. DNase I Inhibition Assay of Actin Content in Mast Cells*

| | μg/$10^6$ cells |
|---|---|
| Total cell lysate | 2.8 ± 1.2 ($n$ = 6) |
| Triton extract | 2.0 ± 0.5 ($n$ = 5) |

The determinations were carried out as described in Materials and Methods. The cells were lysed in 0.5% Triton X-100 and either assayed immediately (*Total cell lysate*) or after a 15-min centrifugation at 10,000 *g* (*Triton extract*).

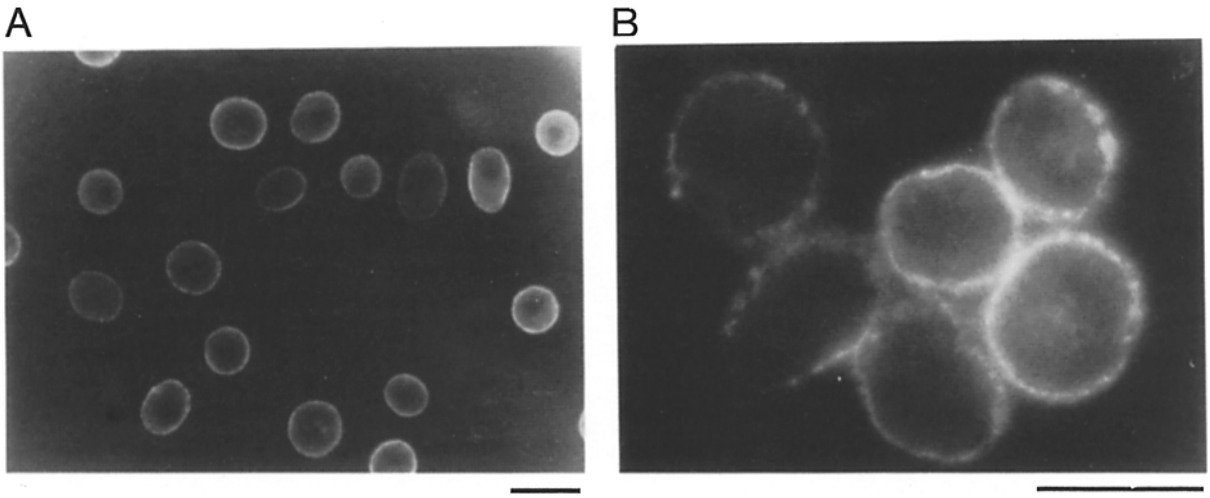


*Figure 3.* Presence of the cortical filamentous actin network in (*A*) intact and (*B*) SL-O permeabilized mast cells. The cells were stained with 1 μM RP as described in Materials and Methods. Note that SL-O permeabilized cells showed the same pattern of staining whether RP was added concomitantly with SL-O, 15 min later (as in Fig. 3 *B*) or 1 h later. The fluorescence intensity was also undiminished. Bar, 20 μm.

ent during the cell lysis. This is not surprising since most of the actin in unstimulated cells is already capable of inhibiting DNase I.

### *Leakage of Actin from SL-O Permeabilized Cells*

The proteins that leak from SL-O permeabilized cells were examined by gel electrophoresis (Fig. 4 *A*). Actin is clearly the most abundant protein released; the heavy band of 66 kD is BSA, present in the commercial preparation of SL-O. Densitometry of a similar gel (Fig. 4 *B*) showed that ~60% of the total Triton-soluble actin was released from the cells during 10–15 min after permeabilization. However, the SL-O permeabilized cells could still be stained by RP in the cortical region, and the fluorescence intensity did not decrease as the time of permeabilization was extended up to 1 h. This indicates the presence of a very stable cortical network (Fig. 3 *B*).

### *Effect of Cytochalasin, PMA, and Phalloidin on Secretion from Permeabilized Mast Cells*

The following experiments were designed to test the effect of compounds, understood to perturb the cortical network, on the secretory reaction of permeabilized mast cells. Cells were pretreated with 100 μM cytochalasin E (10 min at 30°C; 25 μM final) and then stimulated to secrete by permeabilization in the presence of GTP-γ-S (15 μM) and a range of $Ca^{2+}$ concentrations. Two effects were observed. (*a*) Cytochalasin treatment by itself caused a calcium-independent release of histamine, which was apparent even in the absence of GTP-γ-S (Fig. 5 *A*, and Fig. 6). No significant release was observed from intact cytochalasin-treated cells (Fig. 6). (*b*) In the presence of GTP-γ-S (Fig. 5 *B*), cytochalasin-treated cells showed a decrease in the calcium requirement for secretion; i.e., the apparent affinity for $Ca^{2+}$

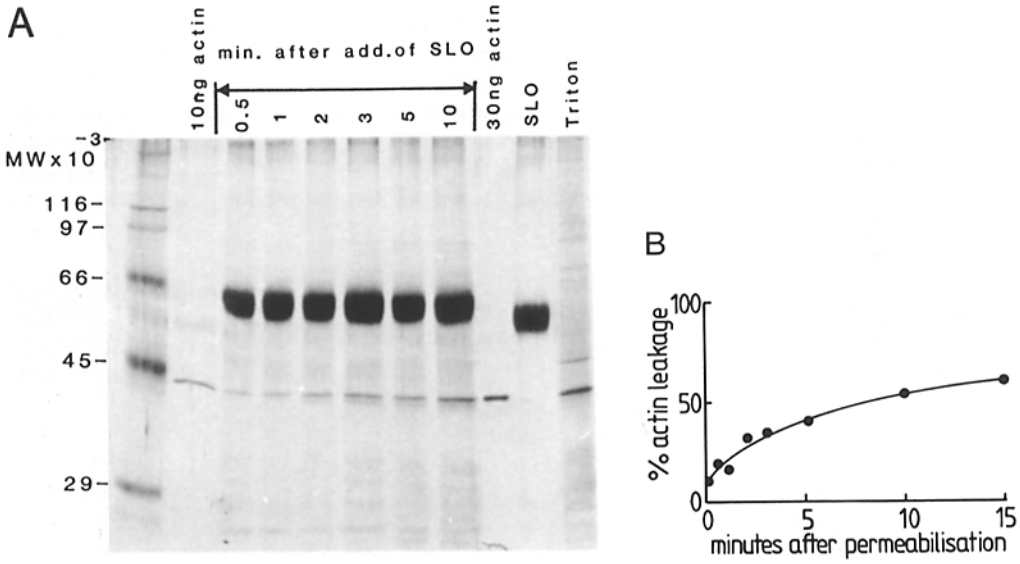


*Figure 4.* Leakage of actin from mast cells permeabilized by SL-O. (*A*) The cells were permeabilized by SL-O (0.1 IU $ml^{-1}$) or lysed by 0.5% Triton X-100 and pelleted after the indicated intervals. Each lane corresponds to 17,000 cells. Positions of molecular weight markers, actin standards, and BSA from the corresponding amount of the SL-O sample (*SLO*) are also indicated. (*B*) Actin leakage expressed as a percentage of the total actin content in the Triton soluble extract (2.6 μg/$10^6$ cells) estimated by densitometry of silver-stained gels.

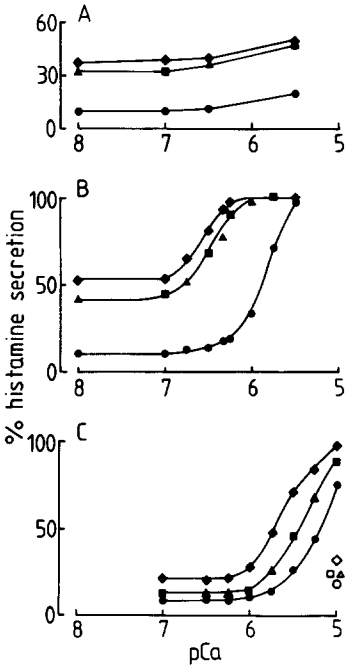


*Figure 5.* Effect of cytochalasin E and PMA on histamine secretion from permeabilized mast cells. (*A* and *B*) Cells were incubated 10 min at 30°C with (●) no additions except 1% DMSO for controls; (▲) 100 μM cytochalasin E; (■) 200 nM PMA; (◆) or both drugs. PMA was present only during the last 2 min of the incubation. After the above treatment, the cells were added to SL-O (0.4 IU $ml^{-1}$); calcium buffers (pCa as indicated); and either (*A*) none or (*B*) 15 μM GTP-γ-S. Final concentrations of cytochalasin E and PMA were 25 μM and 50 nM, respectively. Secretion was determined after a 15-min incubation at 30°C. (*C*) Cells were incubated 2 min at 30°C with metabolic inhibitors (10 μM antimycin and 6 mM deoxyglucose) followed by a further 10-min incubation with cytochalasin E and/or PMA as described above (i.e., 12 min total with the inhibitors), and finally added to SL-O, calcium buffers, and 15 μM GTP-γ-S. Open symbols indicate histamine release at pCa5 in the absence of GTP-γ-S.

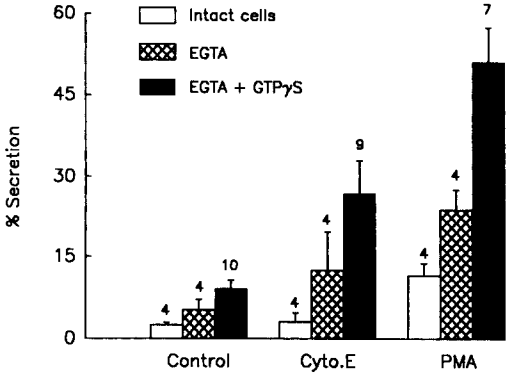


*Figure 6.* Calcium-independent secretion from intact and permeabilized rat mast cells: effect of cytochalasin and PMA. The conditions were as described for Fig. 5 (*A* and *B*). Intact cells were in 2 mM $MgCl_2$ and 40 μM EGTA in the buffered salt solution. The bars represent SEM. The number of experiments is shown above each bar.

was increased. In both these respects, the effects of cytochalasin can be said to mimic those obtained after treatment of cells with PMA, an activator of protein kinase C (200 nM, 2 min at 30°C before the permeabilization). The effects of the two agents were not additive. Intact cells treated with PMA released ~10% of their histamine, which was significantly less than the release from permeabilized cells (Fig. 6).

To demonstrate that the effects of PMA are mediated through a kinase reaction, similar experiments were carried out using metabolically inhibited cells. Fig. 5 *C* shows results obtained with cells that had been pretreated (12 min at 30°C) with metabolic inhibitors (6 mM deoxyglucose and 10 μM antimycin A); under these conditions the intracellular levels of ATP are reduced from ~1 mM to 10–30 μM, as measured by bioluminescence assays (P. E. R. Tatham and A. Koffer, unpublished results). As previously shown (14), the metabolically depleted cells remain competent to secrete but require higher concentrations of $Ca^{2+}$. A low level of calcium-independent secretion from the depleted cells was apparent only when they were treated with both cytochalasin and PMA. Individually, PMA as well as cytochalasin E caused much smaller shifts to lower $Ca^{2+}$ requirements than those obtained with uninhibited cells, and in this case their effects were approximately additive.

The extent of both of the above effects varied between individual experiments. Following treatment with cytochalasin or PMA, $Ca^{2+}$-independent release of histamine (in the presence of GTP-γ-S) varied between 15 and 60% (Fig. 6). Usually (but not always), the extent of the $Ca^{2+}$-independent component of secretion was greater for PMA than for cytochalasin (Fig. 6). The magnitude of the increase in apparent affinity for $Ca^{2+}$ also varied. In these experiments, the effects of the cytochalasins D and E were indistinguishable from each other. Both were active at concentrations in the range 1–100 μM.

To test the effect of phalloidin, which is membrane impermeable, it was necessary to add it to the cells at the time of permeabilization together with $Ca^{2+}$ and GTP-γ-S. It induced no apparent changes in the secretory response at concentrations up to 5 μM (not shown).

### *Effect of Calcium, Cytochalasin E and PMA on the Staining of Mast Cells by RP*

Flow cytometric analyses of RP fluorescence were carried out to examine how the level of F-actin in permeabilized cells might be affected by $Ca^{2+}$. Increasing calcium concentration caused a marked decrease in the content of filamentous actin (Fig. 7 *A*). After treatment of cells with metabolic inhibitors, a higher concentration of $Ca^{2+}$ was required to reduce the content of F-actin. This correlates well with the decrease in the apparent affinity for $Ca^{2+}$ in the exocytotic reaction of metabolically inhibited cells (Fig. 5 *C* and reference 16).

Since phalloidin had no effect on secretion from permeabilized cells (see above), it was important to establish whether phalloidin actually prevents cortical disassembly. This question was addressed by changing the order in which the reagents were provided. That is, in parallel with the usual procedure of permeabilization, fixation and staining, cells were permeabilized in the presence of RP (with or without $Ca^{2+}$) and then fixed. Alternatively, cells were permeabilized in the presence of RP and EGTA, and 6 min later treated with $Ca^{2+}$ and finally fixed. In all cases the decrease in RP stain-

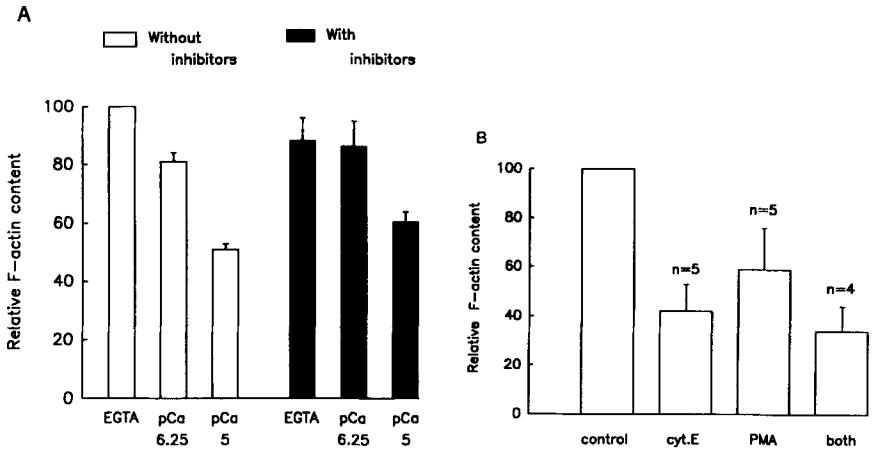


*Figure 7.* Effect of calcium (*A*) and cytochalasin E and PMA (*B*) on RP staining of mast cells. (*A*) Cells were permeabilized for 15 min in the presence of SL-O (0.4 IU $ml^{-1}$), 2 mM $MgCl_2$, and 3 mM calcium. EGTA buffers as indicated, fixed with 3% paraformaldehyde and stained with 1 $\mu$M RP. The fluorescence was assayed by flow cytometry. Metabolically inhibited cells were treated with 10 $\mu$M antimycin and 6 mM deoxyglucose for 12 min at 30°C before the permeabilization. The bars represent SEM, $n = 3$. (*B*) Cells were treated for 10 min with either 1% DMSO (controls) or 100 $\mu$M cytochalasin E, or for 2 min with 200 nM PMA. When used together, PMA was added for the last 2 min of the cytochalasin treatment. Cells were then permeabilized for 15 min in the presence of SL-O (0.4 IU $ml^{-1}$), 2 mM $MgCl_2$, and 3 mM EGTA, and then fixed and stained as above. The bars represent SEM; *n*, number of experiments.

ing in response to calcium was similar to that shown in Fig. 7 *A*. Thus, phalloidin does not prevent cortical disassembly.

To investigate the effects of cytochalasin E and PMA, the cells were pretreated with either one or both agents (10 and 2 min, respectively), permeabilized by SL-O in the presence of EGTA (15 min) and processed as above. The results (Fig. 7 *B*) show that both compounds cause a significant decrease in the content of RP-stained filamentous actin and therefore provoke disassembly of the cortical network. In one experiment, various concentrations and times of exposure to cytochalasin E were tested. The decrease in the relative F-actin content was the same for 5, 15, or 30-min treatment with 5, 20, or 100 $\mu$M cytochalasin E.

The decrease caused by PMA depended on the metabolic competence of the cells. If the cells were metabolically inhibited before the PMA treatment, the decrease in the F-actin level was negligible (<10%, not shown). Interestingly, similar observations were made concerning the effects of cytochalasin E. Thus, metabolically inhibited cells became incapable of F-actin disassembly in response to cytochalasin E, and this resistance increased with the increasing time of incubation with the metabolic inhibitors (not shown). Both results correlate well with the greatly reduced effects of these two agents on secretion from metabolically inhibited cells (Fig. 5 *C*).

### *Effect of Cytochalasin and PMA on RP Staining, and Secretion from Intact Mast Cells*

Intact cells were also examined. These (control, PMA-treated, and cytochalasin E-treated) were fixed overnight in 3% paraformaldehyde, and then stained with RP. The decrease in fluorescence intensity was never >10% whether the cytochalasin E and PMA had been applied separately or together (not shown).

The responsiveness of control and cytochalasin- or PMA-treated intact cells to compound 48/80, a well-known secretagogue for mast cells (35), was tested. Neither agent had any significant effect on the extent of secretion due to 48/80 at concentrations up to 10 $\mu$g $ml^{-1}$ (not shown), although treatment with PMA by itself caused low levels of release (Fig. 6).

### *Presence of Calcium-dependent Actin Regulatory Factor(s)*

To test for the presence of actin regulatory proteins, we have used the pyrene-labeled derivative of actin, polymerization of which is accompanied by a large increase in fluorescence intensity (23). The effect of the Triton-soluble extract from rat mast cells on pyrene–actin polymerization is shown in Fig. 8. In the presence of 5 $\mu$M free $Ca^{2+}$, strong inhibition of actin polymerization was observed, while no effect was evident in the presence of EGTA.

## *Discussion*

### *State and Content of Actin in Rat Mast Cells*

Actin is present in rat mast cells at a concentration of at least 110 $\mu$M. This is within the range of published estimates for other types of cell; (BHK cells contain ~70 $\mu$M [22] and platelets up to 1 mM [38]). The estimation of the actin concentration in mast cells was made as follows: the mean cell diameter is 13.5 $\mu$m (24), which, assuming spherical geometry, gives a mean cell volume of $1.3 \times 10^{-12}$ liter. Since the cytosol comprises only ~42% of the total cell volume (15), actin (43 kD) at 2.5 $\mu g/10^6$ cells is present at ~110-$\mu$M concentration in the cytosol. This represents a minimum estimate since the contribution of F-actin (sedimentable and/or not detectable by the DNase I assay method) is not included.

A cortical network of filamentous actin is clearly evident, as revealed by RP staining (Fig. 3), so it was surprising to find that most of the actin could be recovered in the Triton-soluble fraction. The amount of Triton-insoluble actin is negligible (Fig. 1), and the permeabilization data (Fig. 4) indicate that some two-thirds of the cellular actin leak from SL-O permeabilized cells within 10–15 min and are therefore

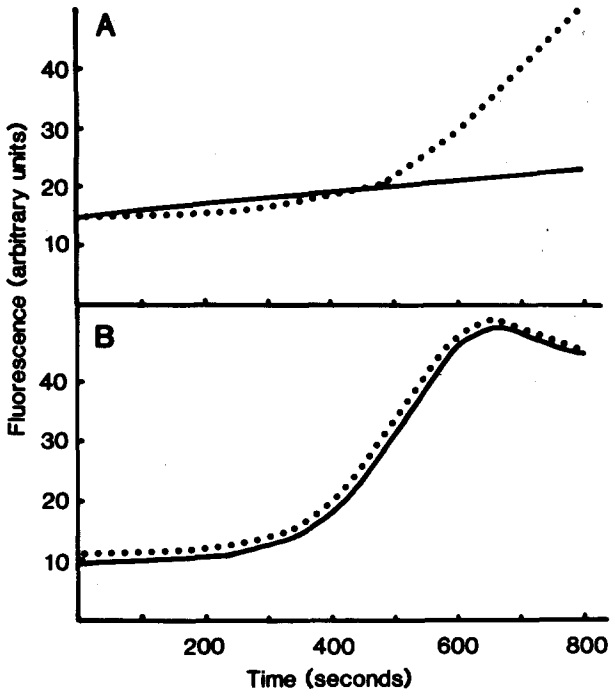


*Figure 8.* Effect of Triton-soluble extract from rat mast cells on polymerization of pyrene-G-actin in the presence (*A*) and absence (*B*) of calcium. Final concentration of pyrene actin was 2.3 μM, the endogenous actin from the cells contributed ~+5% (extract from ~7.5 × $10^5$ cells/400 μl). The ionic conditions were those of the buffered sucrose solution with 1 mM ATP and in (*A*) 1 mM $CaCl_2$, producing 5 μM free $Ca^{2+}$. (*Dotted line*) Control; (*full line*) with the added extract.

present as a monomeric pool (most probably complexed with monomer-sequestering proteins) and/or as a population of oligomers. The remainder probably constitutes actin that is associated with intracellular structures, although much of it must be soluble in Triton. The amount of Triton-soluble actin assessed by densitometry of SDS gels is comparable to the quantity estimated by the DNase I method, suggesting that most mast cell actin is capable of inhibiting the enzyme (Table I). Complexes of G-actin with profilin or actin-depolymerizing factor can inhibit DNAase I to the same extent as G-actin alone (5, 37), as do short actin filaments that readily depolymerize in the presence of DNase I (22, 36). Thus, both complexed G-actin and short oligomers can register as monomers in the DNase I inhibition assay.

RP staining of intact cells (Fig. 3 *A*) revealed the presence of F-actin–enriched cortices but no other filamentous structures were visible. The staining pattern of intact cells was similar to that of permeabilized cells that had lost ~60% of their actin (Fig. 3 *B*). This suggests that the cortical F-actin constitutes most, if not all, of the filamentous actin of (resting) mast cells and, for the reasons stated above, that it is made up predominantly of short filaments. Evidence for the presence of short microfilaments in rat mast cells has been obtained previously by electron microscopy (D. Lawson, personal communication).

Leakage of actin from SL-O permeabilized cells does not in any way diminish their secretory responsiveness; on the contrary, we have previously demonstrated that secretion from mast cells, permeabilized by treatment with $ATP^{4-}$ to permit selective dialysis of only low molecular mass solutes (while retaining most of the cytosol proteins), requires much higher concentrations of $Ca^{2+}$ and GTP-γ-S than those necessary to activate more extensively permeabilized, SL-O–treated cells (21). It remains to be established whether it is the actin that leaks out of SL-O permeabilized cells that causes the inhibition imposed by the cytosol.

### *Calcium Causes Disassembly of the Cortical Cytoskeleton in Permeabilized Cells*

As shown in Fig. 7 *A*, there is a progressive decrease in the fluorescence intensity of F-actin bound RP as the concentration of $Ca^{2+}$ is increased. This decrease is independent of GTP-γ-S, and, since a guanine nucleotide is an essential effector for exocytosis in these cells under the specified conditions (14, 17), this observation indicates that disassembly of the cortical network by itself is not a sufficient stimulus for secretion. Another process, mediated via a GTP-binding protein (GE) must be involved, although low levels of release were observed in the absence of GTP-γ-S under conditions that led to cortical disassembly (see below).

### *Phosphorylation by Protein Kinase C May Regulate Cortical Actin Disassembly*

After treatment with metabolic inhibitors to reduce the intracellular levels of ATP and phosphoproteins, higher concentrations of $Ca^{2+}$ are required to induce cortical disassembly (assessed by RP staining, Fig. 7 *A*). Furthermore, treatment with PMA also reduces RP staining of cortical F-actin to about the same extent as cytochalasin E (Fig. 7 *B*). This raises the question of whether protein kinase C could regulate cortical actin disassembly through phosphorylation of an actin-binding protein. Reduction in the content of cytoskeletal actin after treatment with PMA has previously been reported in adrenal chromaffin cells (8). These effects parallel those observed when secretory responses are measured. Thus, metabolically inhibited cells require higher concentrations of $Ca^{2+}$ for secretion (Fig. 5 *C*; reference 17); on the other hand, PMA increases the apparent affinity for $Ca^{2+}$ (Fig. 5 *B*; reference 18).

### *Effect of Cytochalasin, PMA, and Phalloidin on Secretion from Permeabilized Rat Mast Cells*

Phalloidin and cytochalasin, which cause stabilization and destabilization, respectively, of the microfilamentous network have frequently been used to investigate the involvement of actin in cellular processes (12). Their effects on secretion from mast cells and a related, rat basophilic leukemia cell line have been examined in many studies but with controversial conclusions (13, 16, 27, 29, 30, 34).

Our results support the idea that the cortical cytoskeleton acts as a barrier, preventing the fusion of granule and plasma membranes (3, 7, 26, 42). Thus cytochalasin, which causes a decrease in the level of filamentous actin in SL-O permeabilized cells (Fig. 7 *B*), increases the apparent affinity for $Ca^{2+}$ in the exocytotic reaction and evokes a variable extent of $Ca^{2+}$-independent secretion (Figs. 5 and 6). These effects are discernible only at lower concentrations of $Ca^{2+}$, but this is not surprising as the extent of disassembly at pCa5 already matches that achieved by cytochalasin (Fig. 7).

We have also demonstrated that PMA, an activator of pro-

tein kinase C, has similar effects on secretion and disassembly as those of cytochalasin. The effects of both these agents on secretion are greatly reduced after metabolic inhibition of the cells (Fig. 5 *C*); this correlates with loss of their ability to induce F-actin disassembly in metabolically inhibited cells. That the effect of PMA is dependent on the metabolic competence of the cells is not surprising. The observation concerning cytochalasin is somewhat unexpected but not unique: metabolic inhibition was found to prevent the effect of cytochalasin D on cytoskeletal networks of cultured kidney cells (40).

Cortical actin disassembly, whether caused by calcium, cytochalasin or PMA is always accompanied by a small but significant level of secretion even in the absence of GTP-γ-S (Figs. 5 *A* and 6). There is also a limited amount of GTP-γ-S independent secretion at high $Ca^{2+}$ concentrations. Thus, histamine release at pCa5 in the absence of GTP-γ-S was 14% ± 2 (SEM, $n = 5$) while at pCa8 it was 5% ± 2 ($n = 4$). It is possible that disassembly of cortices facilitates release from those vesicles immediately adjacent to the plasma membrane while an additional signal is required for further release.

Phalloidin, a filamentous actin stabilizing agent, added to the permeabilized cells at concentrations up to 5 μM together with $Ca^{2+}$ and GTP-γ-S, had no detectable effects on exocytosis. However, our results show that phalloidin is incapable of preventing cortical network disassembly due to increased calcium concentration. Phalloidin has also been shown to be unable to protect F-actin from the effects of gelsolin (46). This, together with our observations, is an important consideration when using phalloidin as a stabilizer of actin filaments.

### *Effect of Cytochalasin E and PMA on Secretion from Intact Cells*

In contrast to the permeabilized cell preparation, the F-actin content of intact cells was not significantly reduced after treatment with cytochalasin or PMA, and, in parallel, neither were any significant changes in secretion observed. It is possible that the presence of the cytosol protects the cortical filaments from disassembly induced by these agents. Resistance of cortical filaments of various intact cells to cytochalasin has been reported (7, 12). In intact cells, the cortical F-actin is in equilibrium with the pool of cytosolic actin, and thus the conditions are quite different from those of the permeabilized cells, which lose their monomeric actin pool rapidly (Fig. 4). In the absence of this pool, it is not possible to study any oscillations of actin assembly that may occur and that have been detected in some intact cell systems (4, 33). Scanning electron microscopy of mast cell plasma membranes has revealed that a rearrangement of the cortical cytoskeleton occurs after activation of intact cells with compound 48/80 (31).

### *Presence of Calcium-dependent Actin Regulatory Factor(s)*

The elevation of intracellular $Ca^{2+}$ that occurs in many types of cells after stimulation (9) may be expected to cause numerous changes in the organization of the microfilamentous network, since many of the actin regulatory proteins are themselves calcium binding proteins (37). Here, we have demonstrated that such factors are indeed present in the Triton-soluble fraction of rat mast cells, since polymerization of extraneous actin was strongly inhibited by the addition of the Triton extract in the presence of $Ca^{2+}$ (Fig. 8).

### *Cortical Actin Disassembly Correlates with Secretion from Permeabilized Mast Cells*

In conclusion, in all aspects examined here, secretion from permeabilized mast cells and disassembly of cortical F-actin are related. The disassembly is controlled directly by the levels of $Ca^{2+}$ and by a protein kinase C-mediated phosphorylation. However, unlike exocytosis (14), disassembly does not seem to be under direct control of a GTP-binding protein. While conditions in which secretion occurs in the absence of disassembly have not yet been identified, it remains likely that cortical disassembly is essential (though not sufficient) for exocytosis. To confirm this requirement, it would be necessary to show that no secretion can occur when disassembly is prevented, but we have been unable to achieve this with phalloidin, the conventional stabilizer of filamentous actin.

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