

# FcεRI cross-linking-induced actin assembly mediates calcium signalling in RBL-2H3 mast cells

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- 1 To determine the role of actin assembly in the Ca<sup>2+</sup> signalling of mast cells activated by cross-linking of FcεRI, we examined the effects of cytochalasin D, an inhibitor of actin polymerization.
  - 2 In the RBL-2H3 cells, F-actin content was increased by sensitization with anti-dinitrophenol (DNP) IgE. In these cells, cytochalasin D induced oscillatory increases in cytosolic Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>); these increase were inhibited by jasplakinolide, a stabilizer of actin filaments.
  - 3 In the IgE-sensitized RBL-2H3 cells, DNP-human serum albumin (DNP-HSA) augmented actin assembly. DNP-HSA also increased the production of IP<sub>3</sub>, [Ca<sup>2+</sup>]<sub>i</sub> and degranulation. Cytochalasin D enhanced all of these DNP-HSA-induced effects.
  - 4 In a Ca<sup>2+</sup>-free solution, DNP-HSA induced a transient increase in [Ca<sup>2+</sup>]<sub>i</sub>, and this increase was accelerated by cytochalasin D. After cessation of the DNP-HSA-induced Ca<sup>2+</sup> release, the re-addition of Ca<sup>2+</sup> induced a sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> through capacitative Ca<sup>2+</sup> entry (CCE), and this increase was enhanced by cytochalasin D.
  - 5 The effect of cytochalasin D in enhancing the CCE activity was prevented by xestospongin C.
  - 6 In contrast, neither the Ca<sup>2+</sup> release nor the CCE activation that was induced by thapsigargin was affected by cytochalasin D.
  - 7 These results suggest that actin de-polymerization stimulates the FcεRI-mediated signalling to augment the release of Ca<sup>2+</sup> from the endoplasmic reticulum in RBL-2H3 cells.
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**Keywords:** actin; RBL-2H3; calcium; cytochalasin D; IP<sub>3</sub>

**Abbreviations:** CCE, capacitative calcium entry; DNP-HSA, dinitrophenyl-human serum albumin; ER, endoplasmic reticulum; FcεRI, high-affinity receptor for the Fc region of IgE; IgE, immunoglobulin E; IP<sub>3</sub>, inositol 1,4,5-triphosphate; RBL, rat basophilic leukaemia; SOCs, store-operated channels

## Introduction

The release of histamine and other inflammatory mediators from mast cells is the primary event in a variety of acute allergic and inflammatory stages. Rat basophilic leukaemia cell (RBL-2H3) is a tumour mast cell line used frequently as an experimental model for mucosal mast cells. RBL-2H3 cells express receptors with a high-affinity receptor for the Fc region of IgE (FcεRI) on the cell-surface membrane. Antigen-mediated cross-linking of FcεRI is a critical step for triggering the degranulation of mast cells (Jouvin *et al.*, 1995). Furthermore, an increase in the cytosolic calcium level ([Ca<sup>2+</sup>]<sub>i</sub>) is considered to be an essential and ubiquitous mechanism in the process of the degranulation of mediators in mast cells.

The activation of receptors coupled to inositol 1,4,5-trisphosphate (IP<sub>3</sub>) production evokes a biphasic increase in intracellular Ca<sup>2+</sup> in which an initial Ca<sup>2+</sup> release is followed by a sustained Ca<sup>2+</sup> influx (Berridge, 1993; Taylor & Thorn, 2001). In non-excitable cells, including mast cells, IP<sub>3</sub>-induced depletion of intracellular Ca<sup>2+</sup> in the endoplasmic reticulum (ER) activates Ca<sup>2+</sup> entry across the plasma membrane. This process, termed capacitative calcium entry (CCE), is also

known as store-operated calcium entry (Putney, 1986, 1990; Clapham, 1995). CCE is mediated by store-operated channels (SOCs) in the plasma membrane (Zweifach & Lewis, 1996; Parekh *et al.*, 1997; Lewis, 1999).

It is well known that an actin cortex, a network of actin filaments organized and regulated by actin-binding proteins, supports the plasma membrane of the cell. In general, half of the actin in a cell is monomeric (G-actin), and the rest is polymerized into filaments (F-actin) (Barkalow & Hartwig, 1995; Carlier & Pantaloni, 1997). The polymerization-depolymerization transition constitutes an important component of the cellular response to receptor activation. In platelets and smooth muscle cells, for instance, polymerization of actin at the cell periphery prevents CCE from occurring (Patterson *et al.*, 1999; Rosado *et al.*, 2000). In mast cells, on the other hand, inhibition of F-actin polymerization slows the time-dependent decrease of this antigen-stimulated tyrosine phosphorylation of FcεRI β subunit (Holowka *et al.*, 2000). However, the role of actin in the regulation of FcεRI-mediated Ca<sup>2+</sup> signalling has not been clarified.

We report here that, although actin disassembly does not enhance the thapsigargin-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> in RBL-2H3 mast cells, it enhances the antigen-induced increase in

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[Ca<sup>2+</sup>]<sub>i</sub> in the IgE-sensitized cells. Our results indicate for the first time that FcεRI-mediated Ca<sup>2+</sup> release is regulated by the actin cytoskeleton in the RBL-2H3 cells.

## Methods

### Cells

RBL-2H3 cells (ATCC, VA, U.S.A.) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin. Trypsinized cells were plated into culture dishes 1–3 days before usage. In most of the experiments, RBL-2H3 cells were incubated overnight with anti-DNP IgE (0.05 μg ml<sup>-1</sup>) in DMEM before use.

### β-Hexosaminidase degranulation

As an index of degranulation, the release of β-hexosaminidase was measured (Ortega *et al.*, 1989). IgE-sensitized RBL-2H3 cells (2 × 10<sup>5</sup> cells per well) in 24-well plates were washed three times with PIPES buffered solution (in mM: NaCl 140, KCl 5, glucose 5.5, MgCl<sub>2</sub> 0.6, PIPES 10, pH 7.4, and either CaCl<sub>2</sub> 1 or EGTA 0.5; BSA 0.1%). The attached cells were stimulated at 37°C under gentle rotation. The supernatants were collected and transferred to a 96-well plate. Triton X-100 solution (0.5%) was added to the cells to quantify the enzyme activity remaining in the cells. The extracts were transferred to the 96-well plate. To each well of the 96-well plate, 50 μl of substrate solution, 1.3 mg ml<sup>-1</sup> *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide in 0.04 M sodium citrate, pH 4.5, was added. The 96-well plate was incubated at 37°C for 60 min under gentle rotation. A stop solution (150 μl) containing 0.2 M glycine adjusted to pH 10.0 with NaOH was added to each well. The absorbance at 405 nm (optical density: OD) of each well was measured with the microplate reader (Model 3550, BIO-RAD, Tokyo, Japan). The percentage of degranulation was calculated by the following formula:

$$\% \text{Degranulation} = \text{OD}_{\text{supernatant}} / (\text{OD}_{\text{supernatant}} + \text{OD}_{\text{pellet}}) \times 100$$

### F-actin assay

Total F-actin content in RBL-2H3 cells was measured as described by Frigeri & Apgar (1999) with some modification. RBL-2H3 cells (2.5 × 10<sup>5</sup> cells per well) in 24-well cluster plates were fixed with 3.7% formaldehyde in HEPES buffered solution (in mM: NaCl 125.4, glucose 11.5, KCl 5.9, MgCl<sub>2</sub> 1.2, HEPES 10, pH 7.4, either CaCl<sub>2</sub> 1.5 or EGTA 0.5) for 15 min at 37°C after the incubations specified in the Results section below. Cells were washed with HEPES buffered solution, and permeabilized by 0.2% Triton X-100 in HEPES buffered solution for 90 s. Cells were washed twice and stained by FITC-labelled phalloidin (FITC-phalloidin, 6 μg ml<sup>-1</sup>) for 2.5 h in a dark room at room temperature. The cells were washed three times with HEPES buffered solution, and FITC-phalloidin bound to F-actin was extracted by incubating the cells with 75% methanol for 2 h in a dark room at 4°C. The relative fluorescence of the

supernatant, which included the extracts, was measured using a spectrofluorometer (JASCO, Tokyo, Japan) with an excitation wavelength of 495 nm and an emission wavelength of 520 nm. The percentage of F-actin was given by taking the resting value to be 100%.

### Measurement of [Ca<sup>2+</sup>]<sub>i</sub>

RBL-2H3 cells grown on glass cover slips were washed twice with HEPES buffered solution. Cells were loaded with fura-PE3 by exposure to HEPES buffered solution containing 5 μM fura-PE3 acetoxymethyl ester with 0.01% cremophor EL for 40 min in a dark room at 37°C. For fluorescence measurements, cells on glass cover slips were placed in a bath on the stage of an inverted microscope (TE-300, Nikon, Tokyo, Japan) equipped with a 40 fold objective lens. The acquisition and analysis were done with a Ca<sup>2+</sup>-imaging system (PTI-4700, Photon Technology International, NJ, U.S.A.). Images of 510 nm fluorescence were captured using 340 and 380 nm wavelength light. Every 3 s, the images at 340 nm were divided by the images at 380 nm to provide resultant ratio images that are indicators of [Ca<sup>2+</sup>]<sub>i</sub>. Data are expressed as relative values that are derived by taking the resting level of [Ca<sup>2+</sup>]<sub>i</sub> as 0% and the level in the presence of 3 μM ionomycin with 3 mM Ca<sup>2+</sup> as 100%. [Ca<sup>2+</sup>]<sub>i</sub> was measured within regions of interest that consisted of pixel arrays in which all points were averaged together. All experiments were performed at 37°C.

### Quantification of the [Ca<sup>2+</sup>]<sub>i</sub> oscillations

In RBL-2H3 cells, the changes in [Ca<sup>2+</sup>]<sub>i</sub> induced by antigens were oscillatory, asynchronous, irregular and varied tremendously amongst cells. In this type of cell, measuring the area under the antigen-induced Δ [Ca<sup>2+</sup>]<sub>i</sub> per time curve (area under the curve; AUC) was effective for quantifying the antigen-induced response (Narenjkar *et al.*, 1999). The following equation was used for calculating the AUC of each response:

$$\text{AUC} = \sum \Delta X(Y_i + Y_{i+1})/2$$

The area for each section was calculated as a trapezoid whose heights were Y<sub>i</sub> and Y<sub>i+1</sub> and whose base was equal to the interval time between each two samples (ΔX = 3 s). Finally, the calculated areas for all sections were summed.

### Assay of IP<sub>3</sub>

IP<sub>3</sub> was measured as described by Ishii *et al.* (1997), with some modification, using an IP<sub>3</sub> [<sup>3</sup>H] radioreceptor assay kit. IgE-sensitized RBL-2H3 cells (4 × 10<sup>5</sup> cells per well) in 24-well cluster plates were washed three times with HEPES buffered solution. The attached cells were stimulated with DNP-HSA at 37°C in HEPES buffered solution. The reaction was halted using ice-cold 16% trichloroacetic acid. The amount of IP<sub>3</sub> in the extract was detected according to the manufacturer's instructions.

### Chemicals

Drugs and chemicals used were dinitrophenyl (DNP)-human serum albumin (DNP-HSA), mouse monoclonal anti-DNP

(anti-DNP-IgE), cytochalasin D, thapsigargin, U73122, U73343, Triton X-100, *p*-nitrophenyl-*N*-acetyl- $\beta$ -D-glucosamide, DMEM powder, BSA (Sigma chemicals, St. Louis, MO, U.S.A.), jasplakinolide, FITC-phalloidin (Molecular Probes, Eugene, OR, U.S.A.), fura-PE3/AM (TEF LABS, Austin, TX, U.S.A.), cremophor EL (Nacalai Tesque, Tokyo, Japan), EGTA (Dojindo Laboratories, Kumamoto, Japan) and foetal bovine serum (Filtron, Brooklyn, Australia). IP<sub>3</sub> [<sup>3</sup>H] radioreceptor assay kit (DuPont NEN, Boston, MA, U.S.A.). Mycalolide B and xestospongine C were kindly donated by Dr N. Fusetani (The University of Tokyo, Japan) and Dr M. Kobayashi (The University of Osaka), respectively.

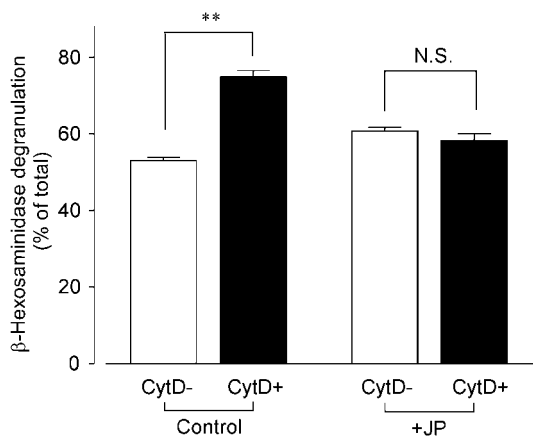
### Statistical analysis

The results of the experiments were expressed as means  $\pm$  s.e.mean. Statistical evaluation of the data was performed using the unpaired Student's *t*-test for comparisons between pairs of groups and by one-way analysis of variance (ANOVA) followed by either Dunnett's test or the Tukey test for comparisons among more than two groups. A value of  $P < 0.05$  was taken as significant.

## Results

### Cytochalasin D increased DNP-HSA-induced degranulation

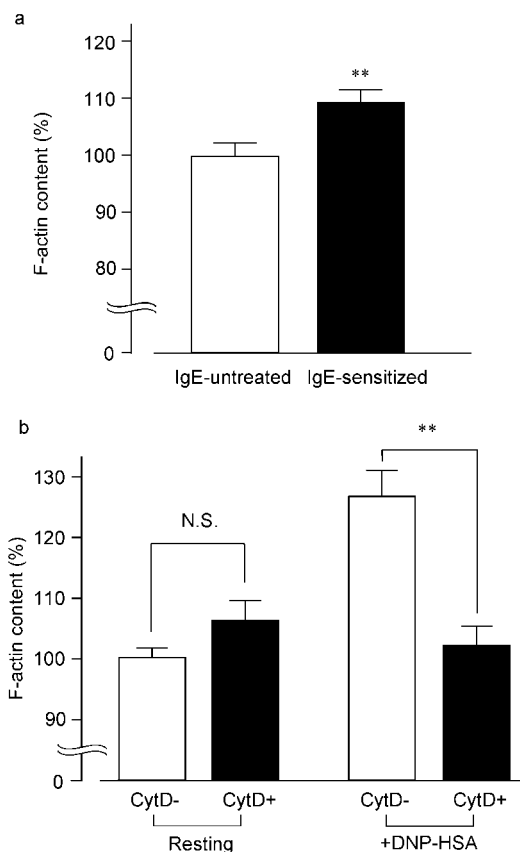
In the IgE-sensitized RBL-2H3 cells, the intracellular  $\beta$ -hexosaminidase was released by DNP-HSA ( $0.01$ – $100$  ng ml<sup>-1</sup>) stimulation for 30 min in PIPES buffered solution in a concentration-dependent manner. Because  $10$  ng ml<sup>-1</sup> DNP-HSA induced the submaximum degranu-



**Figure 1** Effect of cytochalasin D on DNP-HSA-induced  $\beta$ -hexosaminidase degranulation. RBL-2H3 cells were preincubated without (Control) or with jasplakinolide ( $3$   $\mu$ M) for 45 min (+JP) in the presence (CytD+) or absence (CytD-) of cytochalasin D ( $300$  nM) for the last 15 min followed by the addition of DNP-HSA ( $10$  ng ml<sup>-1</sup>) for 30 min to induce degranulation. The percentages of released  $\beta$ -hexosaminidase were calculated against the total amount of  $\beta$ -hexosaminidase. Results are expressed as means  $\pm$  s.e.mean of 10–14 experiments. \*\*Significantly different with  $P < 0.01$ . N.S. Not significantly different.

lation, this concentration of DNP-HSA was employed in the following experiments. Treatment of the cells with  $300$  nM cytochalasin D, a widely utilized membrane-permeate inhibitor of actin polymerization, had no effect on the basal degranulation of  $\beta$ -hexosaminidase ( $5.5 \pm 0.4\%$ ,  $n = 3$ , control;  $7.6 \pm 1.0\%$ ,  $n = 3$ , in the presence of cytochalasin D). However,  $10$  nM– $3$   $\mu$ M cytochalasin D enhanced the degranulation induced by DNP-HSA ( $10$  ng ml<sup>-1</sup>) in a concentration-dependent manner and found that degranulation was enhanced submaximally by cytochalasin D at  $300$  nM ( $n = 4$ ). As shown in Figure 1, pretreatment of the cells for 15 min with cytochalasin D ( $300$  nM) significantly increased DNP-HSA-induced degranulation.

We then examined the effect of jasplakinolide, a cell-permeate stabilizer of actin filaments (Patterson *et al.*, 1999; Rosado *et al.*, 2000), on  $\beta$ -hexosaminidase degranulation. Treatment of the cells with jasplakinolide ( $3$   $\mu$ M) for 75 min had no effect on the degranulation ( $4.5 \pm 0.6\%$ ,  $n = 4$ ) under the resting condition. On the other hand, the effect of



**Figure 2** Effect of cytochalasin D on DNP-HSA-induced F-actin polymerization. (a) RBL-2H3 cells were treated without (IgE-untreated) or with IgE ( $0.05$   $\mu$ g ml<sup>-1</sup>; IgE-sensitized) overnight. The F-actin content is shown as a percentage of the F-actin content in IgE-untreated cells. (b) IgE-sensitized RBL-2H3 cells were preincubated with (CytD+) or without cytochalasin D ( $300$  nM) for 15 min (CytD-), followed by incubation without (Resting) or with DNP-HSA ( $10$  ng ml<sup>-1</sup>) for 5 min (+DNP-HSA). The F-actin content is shown as a percentage of the F-actin content at the resting state. Results are expressed as means  $\pm$  s.e.mean of 8–20 experiments. \*\*Significantly different with  $P < 0.01$ . N.S. Not significantly different.

cytochalasin D to enhance the DNP-HSA-induced degranulation was suppressed by pretreatment with 0.1–3  $\mu$ M jasplakinolide for 45 min in a concentration-dependent manner (data not shown). As shown in Figure 1, 3  $\mu$ M jasplakinolide completely inhibited the effect of cytochalasin D on degranulation.

#### Cytochalasin D inhibited DNP-HSA-induced F-actin polymerization

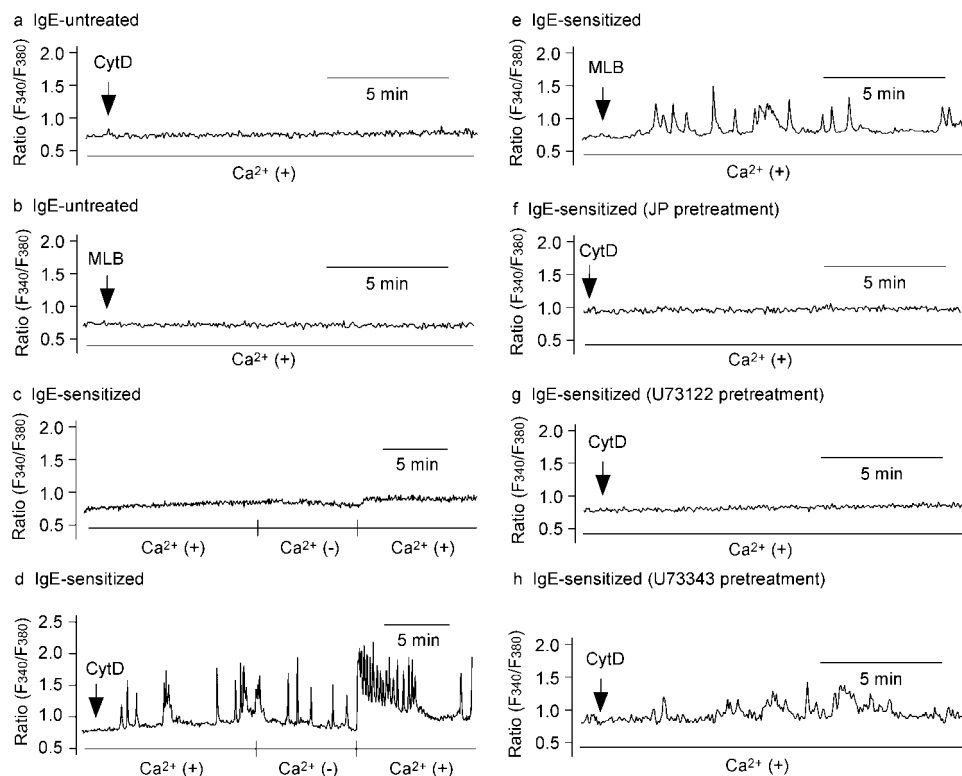
As shown in Figure 2a, IgE-sensitization (overnight) significantly increased F-actin content. Incubation of IgE-sensitized RBL-2H3 cells with cytochalasin D (300 nM) for 20 min did not change the F-actin content under the resting condition (Figure 2b). Exposure of the RBL-2H3 cells to DNP-HSA (10 ng ml<sup>-1</sup>) for 5 min increased F-actin. Preincubation with cytochalasin D for 15 min completely prevented the F-actin polymerization induced by DNP-HSA.

#### Actin disassembly increased $[Ca^{2+}]_i$ in IgE-sensitized cells

In the absence of anti-DNP IgE sensitization, neither cytochalasin D (300 nM) (Figure 3a,  $n=37$ ) nor mycalolide B (100 nM) (Figure 3b,  $n=49$ ), another inhibitor of actin polymerization that severs F-actin (Hori *et al.*, 1993; Saito *et al.*, 1994; Saito & Karaki, 1996), changed the basal  $[Ca^{2+}]_i$  in RBL-2H3 cells.

In RBL-2H3 cells sensitized to anti-DNP IgE (0.05  $\mu$ g ml<sup>-1</sup>) for 12–24 h, the basal level of  $[Ca^{2+}]_i$  was stable regardless of the existence of external  $Ca^{2+}$  (Figure 3c). The addition of cytochalasin D (300 nM) induced frequent  $[Ca^{2+}]_i$  oscillations in 59 out of 99 cells (59.6% of cells).  $Ca^{2+}$  oscillations were observed at least 7 min after removal of external  $Ca^{2+}$ . Re-addition of  $Ca^{2+}$  increased  $[Ca^{2+}]_i$  in the presence of cytochalasin D (Figure 3d), but failed to increase it in the absence of cytochalasin D (Figure 3c).  $[Ca^{2+}]_i$  oscillations were also observed in 41 out of 45 cells (91.1% of cells) when mycalolide B (100 nM) was applied for 15 min (Figure 3e). Pretreatment of the cells with jasplakinolide (3  $\mu$ M) for 30 min abolished the  $[Ca^{2+}]_i$  oscillations induced by cytochalasin D in all IgE-sensitized cells (Figure 3f,  $n=62$ ).

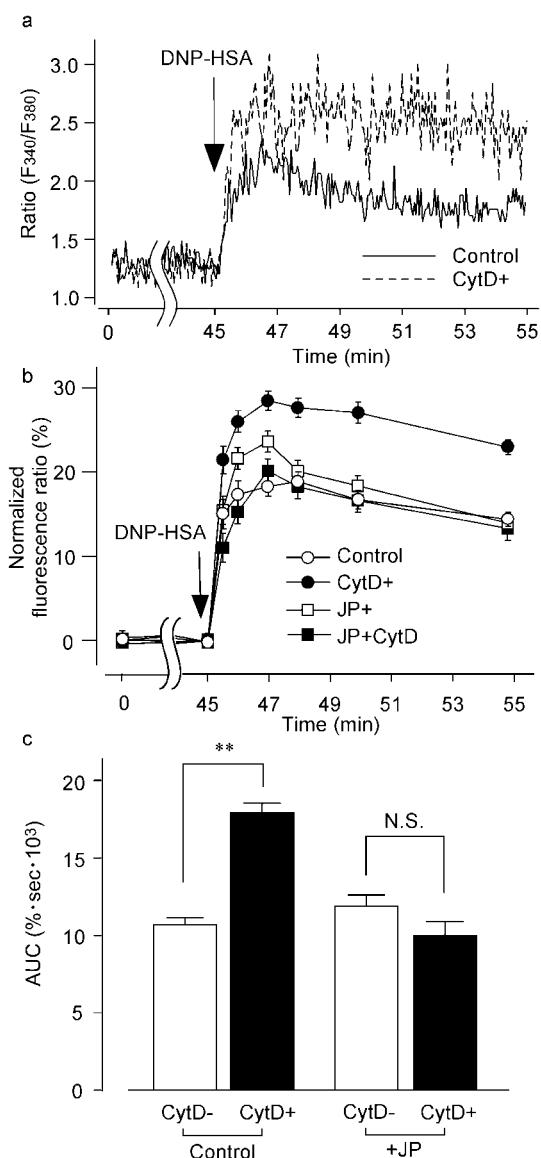
Pretreatment of the cells with U73122 (100 nM), an inhibitor of phospholipase C, for 10 min abolished  $[Ca^{2+}]_i$  oscillations induced by cytochalasin D (300 nM) in all IgE-sensitized cells (Figure 3g,  $n=38$ ). It is well known that this concentration of U73122 is selective for phospholipase C (Seebeck *et al.*, 1998). In the presence of U73343 (100 nM), an analogue of U73122 that acts as a weak inhibitor of phospholipase C (Seebeck *et al.*, 1998), cytochalasin D induced  $[Ca^{2+}]_i$  oscillations in 23 out of 42 cells (54.8% of cells), indicating that U73343 is ineffective (Figure 3h).



**Figure 3** Effect of actin disassembly on  $[Ca^{2+}]_i$  in IgE-sensitized and untreated cells. Cytochalasin D (CytD) (300 nM) (a) or mycalolide B (MLB) (100 nM) (b) was added to the RBL-2H3 cells without a sensitization by anti-DNP IgE. The IgE-sensitized cells were incubated without (c) or with cytochalasin D for 15 min (d).  $Ca^{2+}$  was removed from the medium for 7 min, and re-added for 10 min. (e) The IgE-sensitized cells were incubated with mycalolide B for 15 min. (f) The IgE-sensitized cells were preincubated with jasplakinolide (JP) (3  $\mu$ M) for 30 min and subsequently incubated with cytochalasin D for 15 min. The IgE-sensitized cells were preincubated with U73122 (g) or U73343 (h) for 10 min and subsequently incubated with cytochalasin D for 15 min. Typical recordings are shown ( $n=37-99$ ).

### DNP-HSA-induced increase in $[Ca^{2+}]_i$ and $IP_3$ production was enhanced by actin disassembly

DNP-HSA ( $10 \text{ ng ml}^{-1}$ ) induced an oscillatory increase in  $[Ca^{2+}]_i$  (Figure 4a), which was enhanced by pretreatment with cytochalasin D (300 nM) for 15 min. These results were summarized in Figure 4b and also analysed by measuring the area under the curve (AUC) for 10 min after the DNP-HSA stimulation. As shown in Figure 4c, cytochalasin D significantly increased AUC. To further clarify the role of



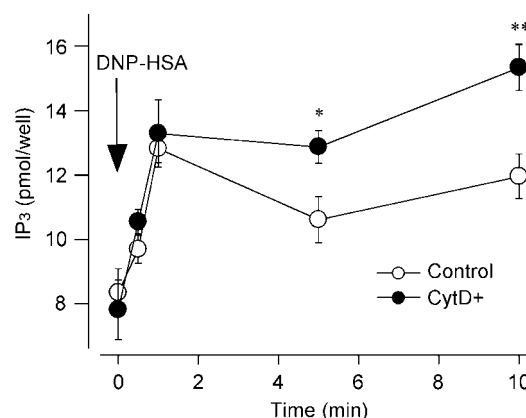
**Figure 4** Effect of actin disassembly on the DNP-HSA-induced increase in  $[Ca^{2+}]_i$ . (a) Typical recording of  $10 \text{ ng ml}^{-1}$  DNP-HSA-induced increase in  $[Ca^{2+}]_i$  in the fura-PE3-loaded RBL-2H3 cells without (Control) or with cytochalasin D (CytD+). Cells were preincubated with cytochalasin D (300 nM) for 15 min. (b) Quantified results of  $[Ca^{2+}]_i$  induced by DNP-HSA ( $10 \text{ ng ml}^{-1}$ ). The RBL-2H3 cells were preincubated without (Control) or with jasplakinolide ( $3 \mu\text{M}$ ) for 45 min (+JP) in the presence (CytD+) or absence (CytD-) of cytochalasin D (300 nM) for the last 15 min. (c) AUC for 10 min after the DNP-HSA stimulation. Results are expressed as means  $\pm$  s.e. mean of 34–49 cells. \*\*Significantly different with  $P < 0.01$ . N.S. Not significantly different.

actin polymerization on the DNP-HSA-induced increase in  $[Ca^{2+}]_i$ , we examined the effect of jasplakinolide. Pretreatment of the cells with jasplakinolide ( $3 \mu\text{M}$ ) for 45 min did not change the resting  $[Ca^{2+}]_i$  ( $n = 49$ ), and had no effect on the DNP-HSA-induced increase in  $[Ca^{2+}]_i$  (Figure 4c). The effect of cytochalasin D in augmenting  $[Ca^{2+}]_i$  was completely suppressed by jasplakinolide.

As shown in Figure 5, DNP-HSA ( $10 \text{ ng ml}^{-1}$ ) induced  $IP_3$  production that lasted for at least 10 min after the DNP-HSA stimulation. In RBL-2H3 cells treated with cytochalasin D (300 nM) for 15 min, the levels of DNP-HSA-induced  $IP_3$  production at 5 and 10 min were significantly increased.

### $Ca^{2+}$ release was activated by cytochalasin D

In the absence of extracellular  $Ca^{2+}$ , DNP-HSA ( $10 \text{ ng ml}^{-1}$ ) induced a phasic increase in  $[Ca^{2+}]_i$  that may be attributable to  $Ca^{2+}$  release from the endoplasmic reticulum (ER) (Gericke *et al.*, 1995). DNP-HSA-induced  $Ca^{2+}$  releases were diverse in frequency, amplitude and shape. Figure 6a shows the two representative patterns of DNP-HSA-induced  $Ca^{2+}$  release. In 24 out of 36 cells (66.7%), mono-phasic  $Ca^{2+}$  release was observed after DNP-HSA stimulation (Figure 6a left), whereas, in the remaining 12 cells (33.3%), several  $Ca^{2+}$  spikes were observed (Figure 6a right). In the cells pretreated with cytochalasin D (300 nM) for 15 min, the time lag between stimulation and  $Ca^{2+}$  release was shorter than that which occurred in the absence of cytochalasin D (Figure 6b). To characterize the effect of cytochalasin D on  $Ca^{2+}$  release due to DNP-HSA, we calculated the time required to initiate the  $[Ca^{2+}]_i$  increase after the addition of DNP-HSA (Figure 6c), and the time-to-peak after the addition of DNP-HSA (Figure 6d). Pretreatment of the cells with cytochalasin D significantly shortened the lag period. The time-to-peak period was also significantly shortened by cytochalasin D. We also calculated the piles of AUC for  $Ca^{2+}$  release induced by DNP-HSA (Figure 6e). The data show that, compared to the response in the control,  $Ca^{2+}$  was released at a significantly faster rate in the cytochalasin D-treated cells.



**Figure 5** Effect of cytochalasin D on DNP-HSA-induced  $IP_3$  production. Quantified results of  $IP_3$  production induced by DNP-HSA. The RBL-2H3 cells were preincubated without (Control) or with 300 nM cytochalasin D (CytD+) for 15 min.  $IP_3$  production was measured at 0, 30 s, 1, 5 and 10 min after the DNP-HSA ( $10 \text{ ng ml}^{-1}$ ) stimulation. Results are expressed as means  $\pm$  s.e. mean of six experiments. Significantly different with \* $P < 0.05$ , \*\* $P < 0.01$ .

As shown in the inset in Figure 6e, the total amount of  $Ca^{2+}$  released from the ER in the cytochalasin D-treated cells did not differ from that released from the ER in the control cells. To evaluate the amount of  $Ca^{2+}$  remaining in ER after DNP-HSA stimulation in  $Ca^{2+}$ -free solution, thapsigargin (100 nM) was applied 5 min after the addition of DNP-HSA. Both in the cytochalasin D-treated cells and in the untreated cells, thapsigargin did not induce  $Ca^{2+}$  release ( $n=13, 16$ ).

#### DNP-HSA-induced $Ca^{2+}$ influx was enhanced by cytochalasin D

In the absence of extracellular  $Ca^{2+}$ , the addition of DNP-HSA (10 ng ml<sup>-1</sup>) evoked a transient increase in  $[Ca^{2+}]_i$  as described above. The subsequent addition of 1.5 mM  $Ca^{2+}$  to the external medium induced a sustained increase in  $[Ca^{2+}]_i$  (Figure 7a). The  $Ca^{2+}$  influx induced by the addition of 1.5 mM  $Ca^{2+}$  was enhanced by cytochalasin D (300 nM). Pretreatment of the cells with mycalolide B (100 nM) for 15 min also enhanced  $Ca^{2+}$  influx. The increase in  $[Ca^{2+}]_i$  due to the addition of  $Ca^{2+}$  as analysed by AUC in Figure 7b indicated that both cytochalasin D and mycalolide B significantly increased the  $Ca^{2+}$  influx.

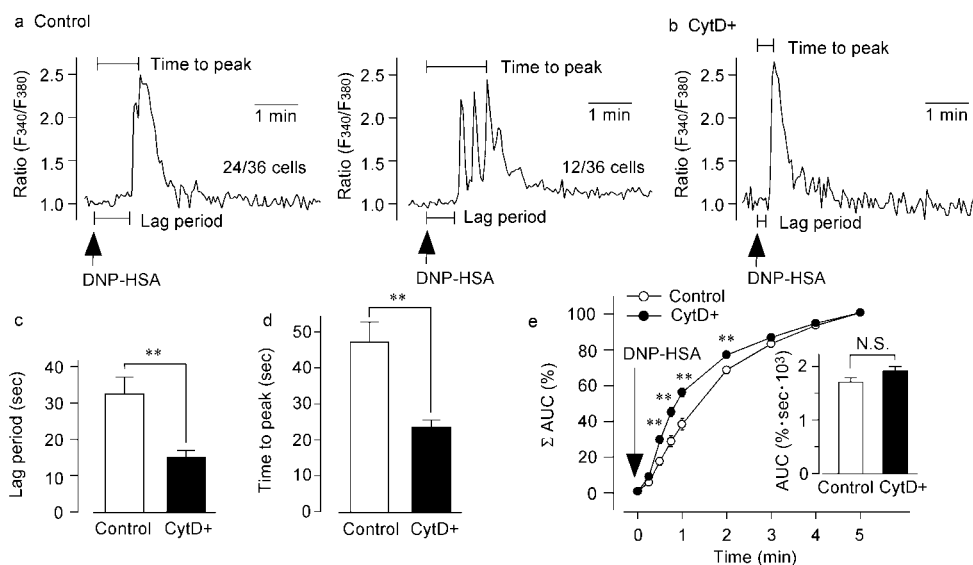
To elucidate the role of IP<sub>3</sub> in the sustained  $[Ca^{2+}]_i$  increase, we examined the effect of xestospongine C, a novel membrane-permeable inhibitor of inositol 1,4,5-triphosphate (IP<sub>3</sub>) receptor (Gafni *et al.*, 1997). After stimulation with DNP-HSA for 5 min in the absence of external  $Ca^{2+}$  to deplete the  $Ca^{2+}$  store, cells were treated with xestospongine C (10  $\mu$ M) for 15 min with or without cytochalasin D (300 nM). The subsequent addition of 1.5 mM  $Ca^{2+}$  to the medium induced a rapid and sustained increase in  $[Ca^{2+}]_i$  in these cells

(Figure 8a). Xestospongine C decreased the  $[Ca^{2+}]_i$  that was induced by the re-addition of  $Ca^{2+}$ . As shown in Figure 8c, the AUC in the presence of xestospongine C was significantly smaller than that in the control. Cytochalasin D also significantly increased  $[Ca^{2+}]_i$  (Figure 8b), and this effect was decreased by xestospongine C to the level in the presence of xestospongine C alone (Figure 8c;  $P>0.05$ ).

We also examined the effect of xestospongine C on thapsigargin-induced increment in  $[Ca^{2+}]_i$ . Xestospongine C (10  $\mu$ M) did not affect the thapsigargin-induced increase in  $[Ca^{2+}]_i$  ( $n=37, 40$ ), suggesting that xestospongine C did not affect directly SOCs activation (Oka *et al.*, 2002).

#### Thapsigargin-induced $Ca^{2+}$ release and influx were not enhanced by cytochalasin D

As shown in Figure 9a, thapsigargin (100 nM) evoked a transient increase in  $[Ca^{2+}]_i$  in the absence of extracellular  $Ca^{2+}$ . After reaching the peak, the thapsigargin-induced transient increase in  $[Ca^{2+}]_i$  gradually decreased to a level below the resting level in 5 min. DNP-HSA (10 ng ml<sup>-1</sup>) that was added 5 min after the thapsigargin stimulation did not have any effect ( $n=15$ ). The addition of 0.1 mM  $Ca^{2+}$  induced a sustained increase in  $[Ca^{2+}]_i$ . Pretreatment of the cells with neither cytochalasin D (300 nM) for 15 min nor jaspalaginolide (3  $\mu$ M) for 45 min changed the increase in  $[Ca^{2+}]_i$  that was induced not only by thapsigargin but also by the addition of 0.1 mM  $Ca^{2+}$  to the medium containing thapsigargin (Figure 9a). Measurements of AUC supported these conclusions as shown in Figure 9b,c. We also calculated the time required to initiate the  $[Ca^{2+}]_i$  increase and the time-to-peak after the addition of thapsigargin in the absence of



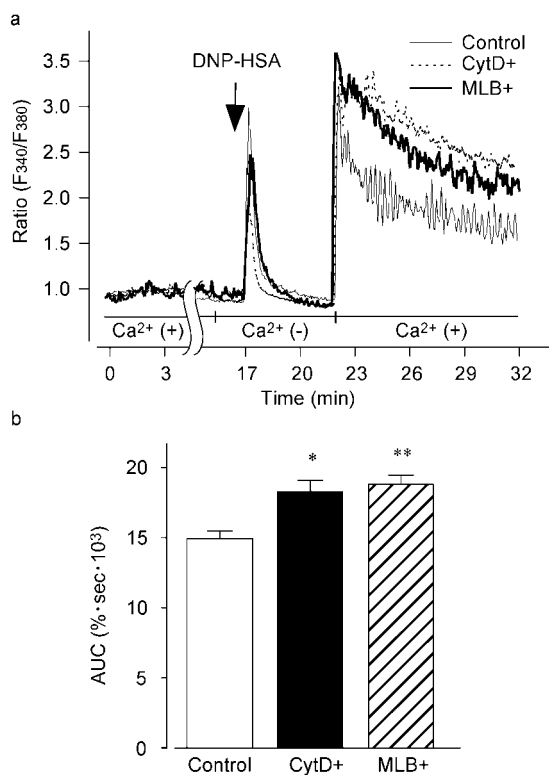
**Figure 6** Effect of cytochalasin D on DNP-HSA-induced  $Ca^{2+}$  release. (a) Typical recordings of 10 ng ml<sup>-1</sup> DNP-HSA-induced  $Ca^{2+}$  release in the absence of extracellular  $Ca^{2+}$  and in the presence of 0.5 mM EGTA. DNP-HSA was applied 2 min after RBL-2H3 cells were exposed to  $Ca^{2+}$ -free solution. (b) Typical recordings of DNP-HSA-induced  $Ca^{2+}$  release in the cells preincubated with cytochalasin D (300 nM) for 15 min. (c) Time between DNP-HSA stimulation and the first  $Ca^{2+}$  release (Lag period) in the cells preincubated without (Control) or with cytochalasin D (300 nM) for 15 min (CytD+). (d) Time between DNP-HSA stimulation and the  $[Ca^{2+}]_i$  peak (Time to peak) in the cells preincubated without (Control) or with cytochalasin D (300 nM) for 15 min (CytD+). (e) Normalized-piles of AUC of the DNP-HSA-induced  $Ca^{2+}$  release in the cells preincubated without (Control) or with cytochalasin D (300 nM) (CytD+). The total AUC for a 5 min DNP-HSA stimulation was taken as 100%. Inset: AUC for 5 min after DNP-HSA stimulation of the cells preincubated without (Control) or with cytochalasin D (CytD+). Results are expressed as means  $\pm$  s.e.mean of 33–36 cells. \*\*Significantly different with  $P<0.01$ . N.S. Not significantly different.

$\text{Ca}^{2+}$ , and found that there were no differences between the cells incubated with or without cytochalasin D (lag period;  $5.8 \pm 0.9$  s,  $n=16$ , control cells;  $5.4 \pm 0.7$  s,  $n=20$ , cytochalasin D-treated cells: time-to-peak;  $30.6 \pm 2.6$  s,  $n=16$ , control cells;  $27.3 \pm 3.3$  s,  $n=20$ , cytochalasin D-treated cells).

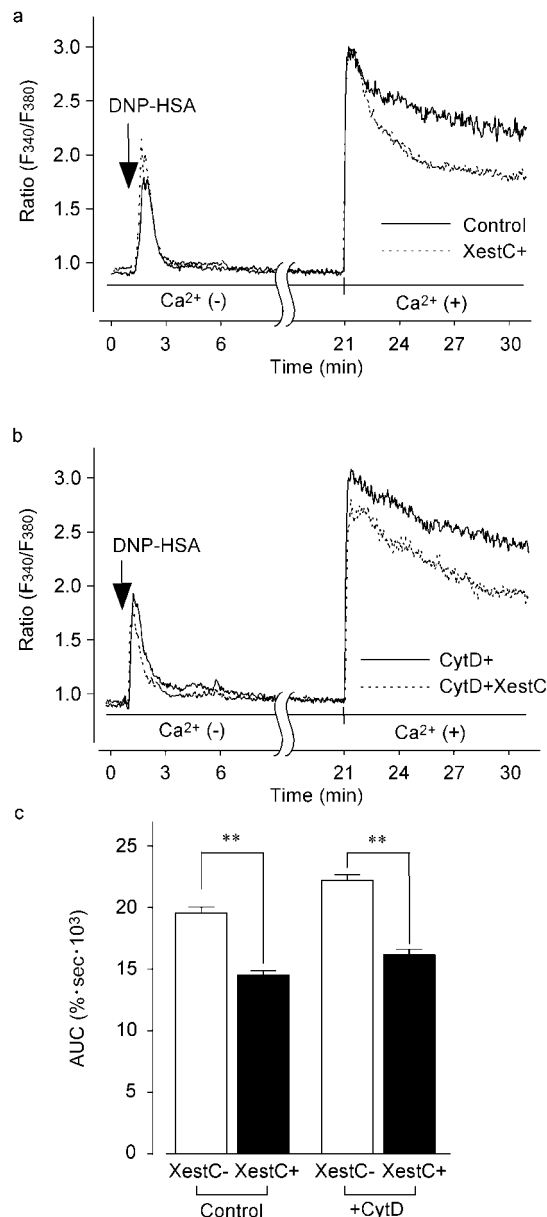
## Discussion

Reorganization of actin assembly has been shown to be involved in the degranulation of mast cells (Tasaka, 1994; Holowka *et al.*, 2000). As demonstrated in Figure 2b, DNP-HSA increased F-actin in RBL-2H3 cells, and cytochalasin D prevented this antigen-induced actin polymerization. However, as was observed in the platelets (Rosado *et al.*, 2000), cytochalasin D did not change the basal level of F-actin. Because cytochalasin D inhibits actin polymerization by binding and capping the barbed end of the actin filaments (Cooper, 1987; 1991), it is suggested that the 'treadmilling' of actin in RBL-2H3 cells at the resting condition is very slow (Fox & Phillips, 1981). As demonstrated in Figure 1, the degranulation of  $\beta$ -hexosaminidase induced by DNA-HSA was augmented by cytochalasin D, and this augmentation was inhibited by jasplakinolide, an actin stabilizer (Patterson

*et al.*, 1999; Rosado *et al.*, 2000). These results suggest that actin assembly plays an important role in the mast cell degranulation. The aim of this study is to clarify the role of actin assembly mediated by the cross-linking of IgE-Fc $\epsilon$ RI in mast cells.

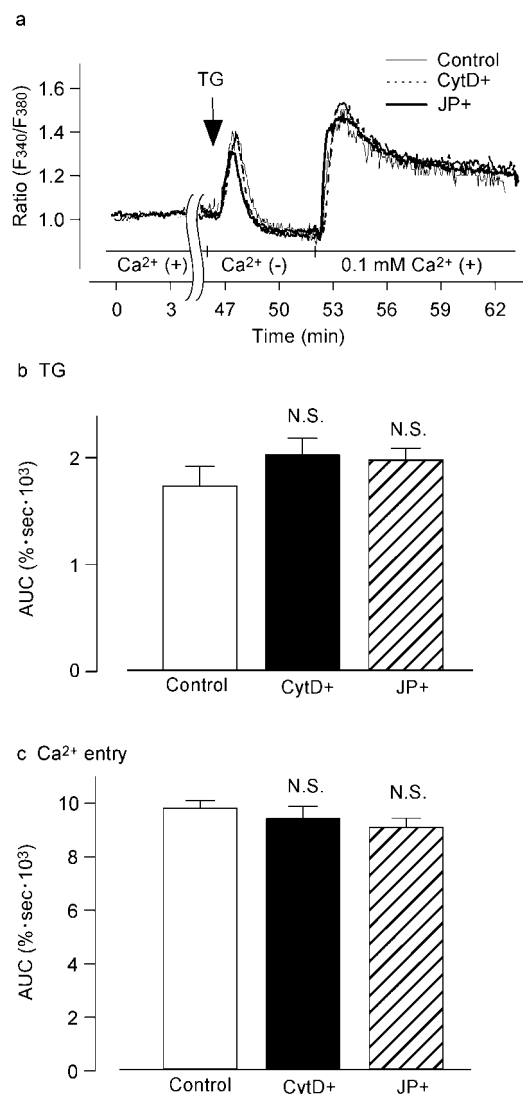


**Figure 7** Augmentation of DNP-HSA-induced  $\text{Ca}^{2+}$  influx by actin disassembly. (a) The fura-PE3-loaded RBL-2H3 cells were treated with 300 nM cytochalasin D (CytD+), 100 nM mycalolide B (MLB+) or with neither agent (Control) in normal HEPES buffered solution for 15 min. After stimulation with DNP-HSA ( $10 \text{ ng ml}^{-1}$ ) for 5 min in the absence of  $\text{Ca}^{2+}$  (with 0.5 mM EGTA), 1.5 mM  $\text{Ca}^{2+}$  was added to the medium without EGTA. Typical recordings are shown in panel a. (b) AUC for 10 min after the addition of 1.5 mM  $\text{Ca}^{2+}$ . Results are expressed as means  $\pm$  s.e. mean of 27–57 cells. Significantly different with \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 8** Augmentation of DNP-HSA-induced  $\text{Ca}^{2+}$  influx by actin disassembly and its inhibition by xestospongin C. (a) The fura-PE3-loaded RBL-2H3 cells were stimulated with DNP-HSA ( $10 \text{ ng ml}^{-1}$ ) in a  $\text{Ca}^{2+}$ -free solution for 5 min. The cells were subsequently incubated without (Control) or with xestospongin C ( $10 \mu\text{M}$ ) for 15 min (XestC+). Then 1.5 mM  $\text{Ca}^{2+}$  was added to external space. Typical recordings are shown in panel a. (b) The fura-PE3-loaded RBL-2H3 cells were stimulated with DNP-HSA ( $10 \text{ ng ml}^{-1}$ ) in the  $\text{Ca}^{2+}$ -free solution for 5 min. The cells were subsequently incubated with cytochalasin D (300 nM), and without (CytD+) or with xestospongin C ( $10 \mu\text{M}$ ) for 15 min (CytD+XestC). Then 1.5 mM  $\text{Ca}^{2+}$  was added to external space. Typical recordings are shown in panel b. (c) AUC for 10 min after the addition of  $\text{Ca}^{2+}$ . Results are expressed as means  $\pm$  s.e. mean of 37–45 cells. Significantly different with \*\* $P < 0.01$ .

As demonstrated in Figure 3a,b, neither cytochalasin D nor mycalolide B, another actin depolymerizing agent (Hori *et al.*, 1993; Saito *et al.*, 1994; Saito & Karaki, 1996), changed the resting level of  $[\text{Ca}^{2+}]_i$  in the absence of IgE sensitization. However, these actin depolymerizers induced oscillatory increments in  $[\text{Ca}^{2+}]_i$  in the IgE-sensitized cells (Figure 3d,e), and these  $[\text{Ca}^{2+}]_i$  oscillations were eliminated by an actin stabilizer, jasplakinolide (Figure 3f), suggesting that IgE sensitization alone changes the responsiveness of cells to actin depolymerizers. Indeed, IgE sensitization increased F-actin



**Figure 9** Effect of actin disassembly on the thapsigargin-induced increase in  $[\text{Ca}^{2+}]_i$ . (a) The fura-PE3-loaded RBL-2H3 cells were treated without (Control) or with 300 nM cytochalasin D for 15 min (CytD+) or 3  $\mu\text{M}$  jasplakinolide for 45 min (JP+). The cells were subsequently stimulated with 100 nM thapsigargin (TG) for 5 min in the absence of  $\text{Ca}^{2+}$  (with 0.5 mM EGTA). Then 0.1 mM  $\text{Ca}^{2+}$  was added to the medium without EGTA. Note that 0.1 mM  $\text{Ca}^{2+}$ , instead of 1.5 mM  $\text{Ca}^{2+}$ , was applied to the medium in this series of experiments because capacitative  $\text{Ca}^{2+}$  entry (CCE) was strongly and maximally activated by thapsigargin. Typical traces are shown in panel a. (b) AUC for 5 min after stimulation with thapsigargin. (c) AUC for 10 min after the addition of 0.1 mM  $\text{Ca}^{2+}$ . Results are expressed as means  $\pm$  s.e. mean of 29–37 cells. N.S. Not significantly different.

content (Figure 2a). The  $\text{Ca}^{2+}$  oscillations induced by cytochalasin D continued for at least 7 min after the removal of extracellular  $\text{Ca}^{2+}$  and subsequent addition of  $\text{Ca}^{2+}$  induced  $\text{Ca}^{2+}$  influx (Figure 3d), which was not induced in the absence of cytochalasin D (Figure 3c), suggesting that cytochalasin D-induced  $\text{Ca}^{2+}$  oscillations may be largely attributable to the  $\text{Ca}^{2+}$  release from the ER. U73122, a phospholipase C (PLC) inhibitor (Seebeck *et al.*, 1998), completely inhibited the  $[\text{Ca}^{2+}]_i$  oscillations induced by cytochalasin D (Figure 3g). Considering that cross-linking of Fc $\epsilon$ RI by multivalent antigen with receptor-bound antigen-specific IgE leads to the activation of non-receptor tyrosine kinases, Lyn and Syk, which is involved in the activation of PLC (Reischl *et al.*, 1999), IgE-Fc $\epsilon$ RI-mediated  $[\text{Ca}^{2+}]_i$  oscillations in the presence of actin depolymerizing agents may be induced through the activation of PLC.

We next examined whether or not these actin depolymerizers affect the  $[\text{Ca}^{2+}]_i$  mobilization, which was induced by antigen-mediated cross-linking of Fc $\epsilon$ RI. As shown in Figure 4, cytochalasin D enhanced the DNP-HSA-induced increase in  $[\text{Ca}^{2+}]_i$  in the IgE-treated cells, and jasplakinolide completely prevented this enhancement. The actin depolymerizer also increased  $\text{IP}_3$  production, which was induced by cross-linking of Fc $\epsilon$ RI (Figure 5). These results suggest that PLC phosphorylation may mediate the release of DNP-HSA-induced  $\text{Ca}^{2+}$  by inhibiting actin polymerization, as discussed above.

It is well known that in non-excitable cells there are two crucial sources of  $\text{Ca}^{2+}$  for signalling:  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  release from internal  $\text{Ca}^{2+}$  stores and the subsequent  $\text{Ca}^{2+}$  influx, CCE, from external space (Putney & Bird, 1993; Parekh & Penner, 1997). Therefore, we investigated the effect of actin disassembly on both  $\text{Ca}^{2+}$  release and CCE.

To investigate the possibility that actin assembly modulates  $\text{Ca}^{2+}$  release, we examined the effect of cytochalasin D on the DNP-HSA-induced  $\text{Ca}^{2+}$  release from ER in  $\text{Ca}^{2+}$ -free solution. As shown in Figure 6, cytochalasin D shortened the time lag for DNP-HSA-induced  $\text{Ca}^{2+}$  release, suggesting that cytochalasin D accelerates  $\text{Ca}^{2+}$  release from ER in the RBL-2H3 cells stimulated by DNP-HSA. Recently, it has been shown that the inhibition of F-actin polymerization enhances antigen-stimulated tyrosine phosphorylation of Fc $\epsilon$ RI, and that RBL-2H3 cells stimulated with antigen in the presence of cytochalasin D exhibited an increase in tyrosine phosphorylation of the  $\beta$  subunit of Fc $\epsilon$ RI (Holowka *et al.*, 2000). These results suggest that the inhibition of F-actin polymerization regulates antigen-stimulated increase in  $[\text{Ca}^{2+}]_i$ , but further investigation is needed to clarify this point.

As demonstrated in Figures 7 and 8, the re-addition of  $\text{Ca}^{2+}$  to the store-depleted cells, which had been stimulated by DNP-HSA in  $\text{Ca}^{2+}$ -free solution, induced a prolonged increase in  $[\text{Ca}^{2+}]_i$  by CCE, and this sustained increase was enhanced by both cytochalasin D and mycalolide B. To investigate whether  $\text{IP}_3$  production is related to the sustained increase in  $[\text{Ca}^{2+}]_i$ , we used a membrane-permeable blocker of  $\text{IP}_3$  receptor, xestospongine C (Gafni *et al.*, 1997). As demonstrated in Figure 8, xestospongine C significantly decreased the DNP-HSA-induced sustained increase in  $[\text{Ca}^{2+}]_i$ . Furthermore, xestospongine C decreased the sustained  $[\text{Ca}^{2+}]_i$  that had been augmented by cytochalasin D to the control level. Several papers have suggested that one obvious role of CCE is to replenish the



intracellular Ca<sup>2+</sup> stores (Takemura & Putney, 1989; Parekh & Penner, 1997), and that, in the sustained phase of oscillatory increase in [Ca<sup>2+</sup>]<sub>i</sub>, IP<sub>3</sub> participates in the release of Ca<sup>2+</sup> (Figures 4 and 5) (Millard *et al.*, 1989; Nash *et al.*, 2001). We propose that these results, taken together, suggest that actin disassembly may enhance the sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> that is induced by DNP-HSA through the activation of IP<sub>3</sub> production.

One crucial remaining question was whether the augmented Ca<sup>2+</sup> release directly participates in the enhancement of the increase in [Ca<sup>2+</sup>]<sub>i</sub> or the potentiation of CCE. As shown in Figure 6e, cytochalasin D accelerated the Ca<sup>2+</sup> release without changing the total amount of released Ca<sup>2+</sup>, suggesting that the sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> is not dependent on the Ca<sup>2+</sup> release but on the Ca<sup>2+</sup> influx from external space, CCE. Sedova *et al.* (2000) showed that, in the vascular endothelium, the activity of CCE is proportional to the level of store filling over the wide range. These results suggest that the release of replenished Ca<sup>2+</sup>, which is accelerated by actin depolymerizers, may result in the augmentation of further Ca<sup>2+</sup> depletion in the ER, and that this profound depletion of stored Ca<sup>2+</sup> may eventually enhance the total activity of CCE.

To evaluate the possibility that actin may affect CCE by directly changing the SOCs activity, we activated SOCs without a concomitant rise in IP<sub>3</sub> by depleting the store using thapsigargin (Ribeiro *et al.*, 1997; Rosado *et al.*, 2000). As demonstrated in Figure 9, neither the thapsigargin-induced Ca<sup>2+</sup> release nor the activation of CCE was affected by cytochalasin D and jasplakinolide, suggesting that the actin does not directly affect SOCs. In NIH3T3 cells, it has been also reported that cytochalasin D did not alter the thapsigargin-activated CCE (Ribeiro *et al.*, 1997).

The degranulation in mast cells is closely related to [Ca<sup>2+</sup>]<sub>i</sub> (Sullivan *et al.*, 1999; Aketani *et al.*, 2001). In the present experiments, cytochalasin D augmented both the degranula-

tion and [Ca<sup>2+</sup>]<sub>i</sub>, which were induced by DNP-HSA. Moreover, the cytochalasin D-induced augmentations were inhibited by jasplakinolide (Figures 1 and 4). These results suggest that the inhibition of actin polymerization might be involved in the augmentation of degranulation through the increment in [Ca<sup>2+</sup>]<sub>i</sub>.

Graier *et al.* (1998) have demonstrated that the increase in [Ca<sup>2+</sup>]<sub>i</sub> due to a submaximal concentration of bradykinin in porcine endothelial cells can be observed when the cells are treated with nocodazol, an inhibitor of microtubules. They speculated that the disruption of the microtubule assembly may affect the subcellular Ca<sup>2+</sup> signalling and thus change the responsiveness of fura-2 to the agonist. In the present study, however, we observed close correlation between the change in fura-PE3 signals and IP<sub>3</sub> production (Figures 4 and 5). In addition, degranulation was also closely associated with the changes in [Ca<sup>2+</sup>]<sub>i</sub> as detected by fura-PE3 (Figure 1). These data suggest that fura-PE3 signals in the RBL-2H3 cells represent the averaged concentration of cytosolic Ca<sup>2+</sup> and that the disruption of actin assembly does not affect the subcellular Ca<sup>2+</sup> signals, unlike the case with porcine endothelial cells, in which the microtubule assembly was disrupted.

In conclusion, in RBL-2H3 cells, the inhibition of antigen-induced actin assembly may regulate the activity of PLC in a manner that increases [Ca<sup>2+</sup>]<sub>i</sub> and degranulation without any direct effect on SOCs activity. It is suggested that the antigen-induced acceleration of actin assembly may serve as a negative feedback control in the mast cell degranulation.

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