

# Polymerization of actin does not regulate desensitization in human basophils

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**Abstract:** Previous studies have suggested that maintenance of IgE-mediated signaling results from regulation of the activity of signaling complexes by actin polymerization. This process is also hypothesized to be related to desensitization of basophils and mast cells. Recent studies demonstrated that any signaling process dependent on syk or PI-3K activity cannot be a mechanism of desensitization, and in this context, syk and PI-3K inhibitors were found to inhibit actin polymerization. Inhibitors of actin polymerization were tested for their effect on desensitization of human peripheral blood basophils. Latrunculin A, in particular, removed all resting and stimulated f-actin but did not inhibit desensitization. Cytochalasin D and latrunculin A also did not reverse the loss of syk phosphorylation that accompanies desensitization. These results demonstrate that desensitization mechanisms are not dependent on actin polymerization. In this context, it was also shown that progressive immobilization of FcεRI during aggregation was sensitive to syk or actin polymerization inhibition. Therefore, desensitization is also not dependent on receptor immobilization. These studies demonstrate that desensitization is not the result of two signaling pathways once considered relevant to down-regulation of IgE-mediated signaling. *J. Leukoc. Biol.* 85: 627–637; 2009.

**Key Words:** signal termination · FcεRI · histamine release

## INTRODUCTION

The self-termination of receptor-mediated stimulation is a necessary component of any cellular response. There are various terms for this process; desensitization or tachyphylaxis is in common use. The signaling initiated by aggregation of the high-affinity IgE receptor (FcεRI) on mast cells and basophils has been shown to result in several forms of self-termination. For example, previous studies have identified a process that occurs early in the signaling cascade that results in transient activation of syk: the ZAP-70 family kinase, which is critical for coupling receptor aggregation to many downstream signaling steps [1]. In rodent mast cells, some of the transience may be related to internalization of the receptor, which occurs in minutes in these cells [2]. However, in human cells, there is no measurable loss of receptor from the cell surface for several

hours, so the transient activation of syk that follows stimulation with antigens appears to be related to another proximal self-termination process [3, 4]. Studies in rodent cells also first demonstrated that polymerization of actin is associated with signal termination; inhibition of actin polymerization with cytochalasin D results in a sustained, early signal [5–7]. Consistent with the prolongation of the early signaling, inhibitors such as cytochalasin D markedly enhanced mediator release [8–10]. In a similar manner, stimulation in human basophils was also found to be sensitive to the presence of actin polymerization inhibitors [11, 12]. We showed previously that stimulation of human basophils with antigen leads to transient activation of syk and naturally, events downstream of syk, such as the activation of p21ras and the MAPK pathway [1, 13]. However, if an inhibitor of actin polymerization, such as latrunculin A, is included in the reaction, syk phosphorylation becomes sustained, and the MAPK pathway remains transient [13]. Thus, these studies partially recapitulated the studies done on the mucosal mast cell-like cell, the rat basophilic leukemia (RBL), and mouse bone marrow-derived mast cells. A larger body of evidence derived from studies of RBL cells has led to the conclusion that the polymerization of actin drives early signal termination. Parenthetically, if actin polymerization is one early process leading to signal termination, our studies in basophils also suggested that there was at least one other self-termination process that we have speculated to be related to the activities of SHIP [1]. A similar conclusion has been drawn from studies in rodent cells [14, 15].

The mechanism of the early process of self-termination or desensitization remains unclear. The studies in RBL cells have suggested that actin polymerization induces a rearrangement of membrane or membrane-signaling components so that the necessary intermolecular associations in lipid rafts are diminished [5, 16, 17]. However, the nature of what is happening and how actin polymerization controls this re-sorting of proteins are not understood. In our studies of the human basophil, there are some characteristics to actin polymerization and the effects of actin polymerization inhibitors that raise some questions about the role of actin polymerization in mediating desensitization. For example, stimulation of basophils with phorbol esters (PMA), fMLP, or anti-IgE leads to actin polymerization and

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histamine release [18]. Notably, the magnitude of actin polymerization correlates with the magnitude of degranulation, i.e., lies on the same regression line, regardless of which stimulus is used. The mechanisms by which each of these three types of stimulation induces release are very divergent. So too are the down-regulatory mechanisms. Indeed, it is not even clear whether a nonreceptor-dependent, stimulus like PMA induces desensitization in the canonical sense. These results suggest that stimulated actin polymerization is playing a role in the granule fusion process. Consistent with this view are early studies in RBL cells that first suggested that cytochalasin B altered terminal steps in exocytosis [10]. As other types of mediator secretion are subject to the effects of down-regulation, these results raise a question about whether actin polymerization could also be modulating a desensitization process. In our studies of the effect of latrunculin A on mediator release, we found that although this polymerization inhibitor markedly enhanced secretion, the kinetics of secretion was essentially unchanged [13]. This observation suggested that although prolongation of early signaling occurred with inhibitor, it was not reflected in the kinetics of secretion.

In studies of human basophil desensitization, a longstanding approach to dissecting the desensitization process has been to induce desensitization prior to secretion by stimulating the cells with antigen in the absence of extracellular calcium ( $Ca_e$ ) [19, 20]. Under these conditions, the cells appear to initiate terminating signals. However, without a prolonged increase in cytosolic Ca as a result of the absence of the normal influx of  $Ca_e$  [21, 22], all secretory processes are inhibited. Several studies have suggested that the absence of an increase in cytosolic Ca also alters the timing and perhaps the nature of the engagement of these self-terminating processes, but the methodological approach still retains use for asking questions about desensitization [21]. Recent studies have also shown that the desensitization and signal termination under full secretory conditions were not dependent on the activities of syk. This result suggested that if any process could be shown to be dependent on syk activity, then the process could not be relevant to desensitization. By examining the role of PI-3K, whose phosphorylation was shown to be dependent on syk, this logic was tested, and it was found that desensitization was also not dependent on PI-3K activity. In the current study, this logic was also applied to the question of a role for actin polymerization in self-termination/desensitization. The conclusions of these results were validated in experiments where specific actin polymerization inhibitors were examined for an effect on desensitization.

## MATERIALS AND METHODS

### Materials

The following were purchased: PIPES, BSA, EGTA, EDTA, D-glucose, NaF,  $Na_4P_2O_7$ ,  $Na_3VO_4$ , 2-ME, Nonidet P-40 (NP-40), fMLP, PMA, and 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF; Sigma Chemical Co., St. Louis, MO, USA); crystallized human serum albumin (HSA; Miles Laboratories, Elkhart, IN, USA); Percoll (Pharmacia, Piscataway, NJ, USA); Tris (hydroxymethyl)-aminomethane and Tween-20 (Bio-Rad, Hercules, CA, USA); leupeptin and DTT (Boehringer Mannheim, Indianapolis, IN, USA); and anti-syk

mAb, 4D10, HRP-conjugated donkey anti-rabbit Ig antibody, HRP-conjugated sheep anti-mouse Ig (mIg) antibody, and protein G sepharose beads (Amersham Life Science, Arlington Heights, IL, USA). Goat anti-human IgE antibody was prepared as described previously [23]. The following were purchased: mIgM anti-human IgE, HP6061P (Hybridoma Reagent Laboratory, Baltimore, MD, USA); antiphosphotyrosine mAb (4G10; Upstate Biotechnology, Lake Placid, NY, USA); rabbit antiphospho-ERK antibody, antiphospho-Akt (Thr308-specific), anti-Akt, antiphospho-p21-activated kinase 1/2 (PAK1/2; S144/141), antiphospho-Shc (Y239/240), antiphospho-pyk2 (Y402), anti-Nck, and biotinylated molecular weight markers (New England Biolabs, Beverly, MA, USA; now Cell Signaling, Danvers, MA, USA); anti-syk mAb, 4D10, antiphosphotyrosine mAb (PY20), anti-actin (C-11), anti-phospho-VAV (Y174), and anti-clp/PIPS-albumin-glucose (PAG; Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-Src homology-2-containing tyrosine phosphatase 1 (SHP1; PTP1C; Transduction Laboratories, Lexington, KY, USA); and anti-HRP-conjugated donkey anti-rabbit Ig antibody, HRP-conjugated sheep anti-mIg antibody, and protein G sepharose beads (Amersham Life Science). Goat anti-human IgE antibody was prepared as described previously [23]. Others include: PPI, latrunculin A, and LY294002 (Calbiochem, San Diego, CA, USA); NVP-QAB205 (gift of GlaxoSmithKline, UK) [24]; cytochalasin B or D and DNP-lysine (Sigma Chemical Co.); and Oregon Green phalloidin (Molecular Probes, Eugene, OR, USA). The clone to generate anti-DNP mIgE (H1 DNP- $\epsilon$ -26) was the gift of Teruko Ishizaka. Anti-DNP mIgE was purified partially from ascites fluid as described previously [25]. DNP(7)- and benzylpenicilloyl determinant (11) [BPO(11)]-HSA [26] were synthesized as described previously. gp120-OVA was provided by Tanox, Inc. (Houston, TX, USA), and penicillin (BPO)-specific IgE was purified from patient sera as described previously [26]. DNP-GFP was synthesized by the method used for DNP-HSA using a purified, cloned GFP developed in-house.

### Buffers

PAG buffer consisted of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 0.1% glucose, and 0.003% HSA. PAGCM was PAG supplemented with 1 mM  $CaCl_2$  and 1 mM  $MgCl_2$ . PAG-EDTA consisted of PAG supplemented with 4 mM EDTA. Countercurrent elutriation was conducted in PAG containing 0.25% BSA in place of 0.003% HSA. ESB is Novex (San Diego, CA, USA) electrophoresis sample buffer containing 5% 2-ME. Complete lysis buffer (CLB) is 20 mM Tris-HCl, pH 7.5, 100  $\mu$ g/ml aprotinin, 10 mM benzamide, 1 mM AEBSF or 1 mM PMSF, 100  $\mu$ g/ml leupeptin, 50 mM NaF, 1 mM  $Na_3VO_4$ , 1% NP-40, and 10% glycerol. Incomplete lysis buffer is CLB without the protease inhibitors, NP-40, glycerol, or vanadate. Nitrocellulose stripping buffer was 65 mM Tris-HCl (pH 6.7), 100 mM 2-ME, and 2% SDS. Lactic acid buffer was for removing endogenous cell-bound IgE, 0.01 M lactic acid, 0.14 M NaCl, and 0.005 M KCl, pH 3.9 [23, 27].

### Histamine release

Histamine was measured in supernatants by automated fluorimetry [28].

### Basophil purification

For many of these experiments, residual cells of normal donors undergoing leukapheresis were enriched in basophils using a combination of Percoll density gradients and countercurrent-flow elutriation, as described previously [29]. The cells were purified further by negative selection using MACS basophil isolation kit (Miltenyi Biotec, Auburn, CA, USA). We use a cocktail of antibodies for negative selection from Stem Cell Technologies (Vancouver, BC; basophil purification kit) and columns from Miltenyi Biotec. The purity of basophils was determined by alcian blue staining [30], and basophils purified from leukapheresis packs generally exceeded 99% purity. For the remaining experiments, basophils were enriched on a single-step Percoll gradient from blood obtained by venipuncture (purities range from 1% to 3%).

### IgE dissociation and sensitization

For many of the experiments, basophils were treated with lactic acid (above) to dissociate endogenous IgE from its receptor. To minimize harm to the cells, the exposure times to lactic acid buffer ranged from 6 to 20 s. To minimize concerns that some low-level but undetectable signaling might occur during the sensitization, sensitization was performed at 4°C for 1 h (5  $\mu$ g/ml IgE); the cells were washed once with ice-cold PAG and resuspended in cold media

before warming prior to culture. Viability after lactic acid-induced dissociation was  $92 \pm 4\%$ , as determined by erythrocin B exclusion. Spontaneous histamine release in lactic acid-treated cells is not different than nontreated cells, typically  $<5\%$  for nonpurified cells and  $5\text{--}10\%$  for purified cells.

## Phosphorylation of signaling elements

Cell lysates were analyzed by Western blotting with antiphospho-specific antibodies. After stimulating basophils ( $0.5\text{--}1 \times 10^6$  cells/sample) in PAGCM buffer, reactions were stopped by brief, high-speed centrifugation for  $5\text{--}10$  s, supernatant collected, and the cell pellet lysed with hot ESB (Novex) and separated on  $8\%$  or  $10\%$  Tris glycine gels (Novex). As determined previously, one of the better lane-loading controls is to re-blot with anti-p85 $\alpha$  antibody, as p85 levels do not change expression levels during stimulation [31]. ECL films were converted to digital images with a Kodak DC290 camera, and the bands were analyzed with National Institutes of Health (NIH) Image.

## Immunoprecipitation

Immunoprecipitation was done for some studies for which good antiphospho-antibodies were not available. After stimulating basophils ( $1.5\text{--}6 \times 10^6$ ) in PAGCM buffer at  $37^\circ\text{C}$ , the reaction was stopped by adding ice-cold PAG and centrifuged in a microfuge for  $15$  s. The supernatants were recovered for mediator detection, and the pellets were lysed immediately in CLB by vortexing and incubating on ice for  $10$  min. The lysates were then centrifuged for  $3$  min at  $16,000 g$  and precleared with protein G sepharose beads for  $30$  min at  $4^\circ\text{C}$ . Then, the clarified lysates were incubated with antibodies (syk, SHP1, PY20) prebound to protein G sepharose beads ( $1\text{--}5 \mu\text{g}$  antibody/ $20 \mu\text{l}$  beads) for  $1$  h at  $4^\circ\text{C}$ . The beads were washed three times, and the immunoprecipitated proteins were eluted by boiling for  $5$  min in ESB. After electrophoresis and transfer, the membranes were blotted with 4G10 antibody. The membranes were then stripped with SDS buffer and re-blotted with relevant antibody to determine loading (proteins obtained by PY20 had no loading control step).

## f-Actin measurement

Intracellular f-actin levels were measured using Oregon Green phalloidin with a few modifications from the method described previously [29]. Basophils ( $0.1\text{--}0.2 \times 10^6$ ) were stimulated at  $37^\circ\text{C}$ , and the reaction was stopped with ice-cold fixation buffer ( $3.2\%$  paraformaldehyde and  $0.25\%$  lysophosphatidylcholine in PBS). After overnight incubation at  $4^\circ\text{C}$ , basophils were washed once and incubated with  $0.2 \mu\text{M}$  Oregon Green phalloidin (in PBS containing  $1\%$  BSA) for  $20$  min at  $20^\circ\text{C}$  in the dark. The fluorescent dye was washed away, and fluorescence was measured by flow cytometry.

## Desensitization protocol $\pm$ actin inhibitors

There are two sequential incubations: a desensitization phase, where cells are challenged in a Ca-free buffer (PAG+ $50 \mu\text{M}$  EDTA), followed by a single centrifugation and a release phase, where cells are challenged in a Ca-containing buffer (PAGCM). There are five parallel conditions for the desensitization phase of the experiment: cells + carrier solvent, no antigen; cells + drug, no antigen; cells + drug, no antigen; cells + carrier solvent + antigen; and cells + drug + antigen. The second and third conditions are similar in the desensitization phase but in the release phase, the third condition is stimulation without drug after washing the cells, and the second condition is stimulation with drug with no washing. The second condition determines that the drug was operating as expected during release. The third condition controls for drug-carryover effects. Ideally, the response of cells from the third condition (after the washing step between the desensitization and release phases of the protocol) should be similar to the first condition. The fourth and fifth conditions determine the extent of desensitization in the absence and presence of drug, respectively. After the five parallel conditions during the desensitization phase, the cells are washed once, and for the release phase, cells from each condition are resuspended in Ca-containing buffer and divided equally into three tubes, to which buffer  $\pm$  stimulus or perchloric acid were added immediately to determine total histamine content, spontaneous release, or stimulated release. For these experiments, the cells were stimulated with a concentration of anti-IgE antibody shown to be optimal for histamine release,  $0.2 \mu\text{g}/\text{ml}$ .

## Antigen binding measured by flow cytometry

Basophils were treated with a mild acid-stripping buffer (see Materials and Methods) to dissociate a portion of the surface IgE and then sensitized at  $4^\circ\text{C}$

with DNP-specific mIgE for  $30$  min. A portion of the cells was not sensitized, and these acted as a negative control for the experiments. Binding was detected in a flow cytometer with samples incubated in an adjacent water bath at  $37^\circ\text{C}$ . The cell density of the purified basophils was high enough that the cytometer could count  $1000\text{--}3000$  cells in  $15$  s (out of the water bath). Pilot studies established the concentration of DNP-GFP, which was optimal for signaling and histamine release, and this was the concentration used in these experiments ( $1.5 \mu\text{g}/\text{ml}$ ) [32].

In pilot experiments, this method showed that the binding goes through an optimum that occurs at  $15$  min. By  $60$  min, only  $50\%$  of the signal observed at  $15$  min is present. This is not a result of internalization of the receptor, as determined by testing the presence of cell-surface IgE with an anti-mIgE antibody ( $60\text{-min}$  binding was  $1.04 \pm 0.03$  of  $0\text{-min}$  binding), a result consistent with several previous studies. We noted that when cells were incubated with PP1 or NVP-QAB205, which would ablate secretion completely, the  $5\text{-min}$  time-point was more similar to the  $60\text{-min}$  time-point. The pilot studies showed that there was some nonspecific uptake of DNP-GFP and that it was probably degraded at longer times. Therefore, the fluorescence signal that represents actual bound antigen is the net signal found after  $60$  min of incubation.

To measure elution kinetics, antigen was bound for  $60$  min,  $0.4$  mM DNP-lysine added, and cells monitored by flow cytometry for a period of  $30$  min. Each time-point in the elution curve represents a separate tube to avoid contamination of samples when running samples through the cytometer.

## Statistics

In general, data are shown as the mean  $\pm$  SEM in text and figures. In some instances, the figure shows mean  $\pm$  SEM, although the analysis is done with paired statistics (e.g., see Fig. 3).

## RESULTS

### Actin polymerization is dependent on syk or PI-3K activity

In studies published recently, we found that no aspect of signal termination/desensitization was dependent on the activity of syk; i.e., desensitization is complete in the presence of complete inhibition of syk activity [33]. In addition, desensitization was not dependent on the activity of PI-3K. These results suggested a strategy for asking whether a particular signaling process might be relevant to desensitization mechanisms. If the activation of a signaling element is found to be dependent on syk activity, then logically, it cannot be important for the form of desensitization that we are studying. If it were not sensitive to a syk inhibitor, it should be sensitive to a src family kinase (SFK) inhibitor, as we have shown that SFK inhibitors inhibit desensitization [34]. We therefore tested whether actin polymerization was dependent on syk activity and PI-3K activity. The third generation syk inhibitor, NVP-QAB205, was used, as we have tested its behavior extensively. **Figure 1** shows that syk inhibition ablates actin polymerization completely with an  $\text{IC}_{50}$  that is consistent with its inhibition of all other syk-dependent endpoints (NVP-QAB205 did not change resting f-actin levels; data not shown). The SFK inhibitor, PP1, also inhibits with an  $\text{IC}_{50}$ , consistent with prior studies that used this drug. The PI-3K inhibitor, LY294002, also inhibited actin polymerization (as did wortmanin, wortmannin; data not shown).

### Inhibition of actin polymerization by LY294002 is not complete

Inhibition of stimulated actin polymerization by LY294002 was not complete at high concentrations. The  $\text{IC}_{50}$  for LY294002 to

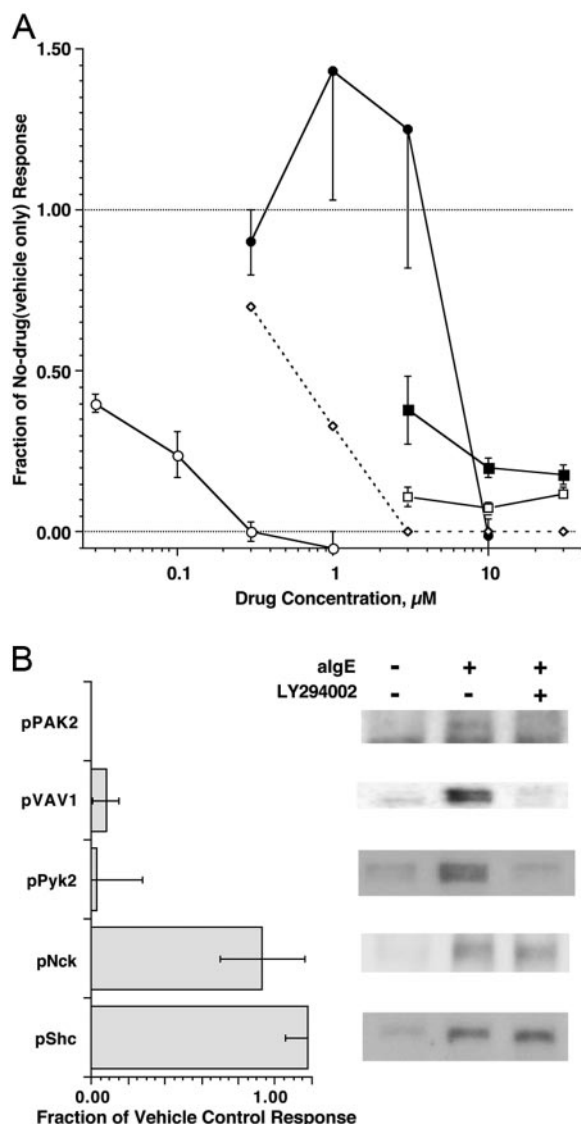


**Fig. 1.** Inhibition of actin polymerization and the actin polymerization signaling pathway with early signaling inhibitors. (A) Inhibition of the anti-IgE antibody-stimulated f-actin increase with (○) NVP-QAB205 with anti-IgE antibody as a stimulus (0.2  $\mu\text{g/ml}$ ;  $n=2$ ), (●) PP1 with anti-IgE antibody as a stimulus (0.2  $\mu\text{g/ml}$ ;  $n=2$ ), (□) LY294002 with BPO-HSA (0.5  $\mu\text{g/ml}$ ) as a stimulus ( $n=5$ ), (■) LY294002 with anti-IgE antibody (0.2  $\mu\text{g/ml}$ ) as a stimulus ( $n=5$ ). Inhibition of Akt phosphorylation (◇, dotted line) with LY294002 and anti-IgE antibody as the stimulus (derived from ref. [32]). The dotted, horizontal lines show the resting and stimulated levels of response in the presence of the vehicle control. (B) Effect of LY294002 on stimulated phosphorylation of several components thought to be in the pathway that mediates actin polymerization. Basophils were stimulated with anti-IgE antibody (0.2  $\mu\text{g/ml}$ ) for 5 min in the presence or absence of 10  $\mu\text{M}$  LY294002 or vehicle control. For phospho-VAV1 (pVAV1), pPAK2, pPyk2, and pShc, the cells were lysed in hot electrophoresis sample buffer, and for pNck, the cells were lysed in CLB, and lysates immunoadsorbed with antiphosphotyrosine antibody (PY20) and the Western blot developed with anti-Nck antibody. The left histogram shows the average results for three experiments each, and the right side shows an example of Western blots. (The band for PAK2 is very faint and difficult to image for the figure but is readily digitized for quantitative analysis.)

inhibit phosphorylation of Akt is  $\sim 0.6 \mu\text{M}$  (dotted line in Fig. 1A). Nevertheless, it was not possible to inhibit stimulated actin polymerization more than  $\sim 85\%$  (Fig. 1A, contrast this

result with the efficacy of PP1 or NVP-QAB205). This result occurred whether anti-IgE or antigen (in these studies, BPO-HSA was used on cells sensitized with BPO-specific IgE) was used as the stimulus. This result suggested that stimulated actin polymerization was composed of a LY294002-sensitive pool and a LY294002-resistant pool. We examined some of the signaling elements thought to mediate the polymerization of actin. In human basophils, we found that phosphorylation of PAK2 (we found no evidence for the expression of PAK1 or PAK3), pyk2, and VAV1 (basophils did not express VAV2) were inhibited completely by 10  $\mu\text{M}$  LY294002, and phosphorylation of Shc and Nck were not inhibited at all (Fig. 1B).

## Functional desensitization $\pm$ actin inhibitors

The syk dependence of actin polymerization suggested that actin polymerization would not play a role in self-termination/desensitization. To test whether this conclusion is valid, desensitization was examined in the presence of actin polymerization inhibitors. Stimulating basophils in the absence of  $\text{Ca}_e$  prevents secretion but allows a down-regulatory process to continue. Adding  $\text{Ca}$  to buffer after a period of time, in effect, queries the state of the functional desensitization. After 45–60 min, the basophil no longer responds to stimulation. To test whether actin polymerization is required for desensitization, an actin polymerization inhibitor was included in the desensitization phase of the experiment (see Materials and Methods). The ideal behavior for this type of study is to find a drug that is washed readily from the cells prior to re-introduction of antigen and  $\text{Ca}_e$ . Basophil desensitization  $\pm$  cytochalasin B or latrunculin A was examined with this protocol. **Figure 2** shows that desensitization was insensitive to the presence of these drugs. The most-clear results occurred with cytochalasin B, as washing between the desensitization and release phase of the experiment was effective in removing the enhancing effect of the drug (Fig. 2B). It was more difficult to remove the enhancing effects of latrunculin A (Fig. 2C). However, even with this residual, enhancing effect, the cells continued to be unresponsive after desensitization. If latrunculin A were included in both phases (no wash step), the cells displayed similar desensitization kinetics (the half-life for desensitization remains similar), although there is much greater histamine release at the 0-min time-point when latrunculin A is present (Fig. 2D). An experiment similar in design to that shown in Figure 2D examined leukotriene C4 (LTC4) release as an endpoint. The results were similar: At 20 and 60 min of desensitization time, LTC4 release was  $0.13 \pm 0.01$  and  $0.00 \pm 0.00$  of the no-desensitization control, respectively, without latrunculin A and  $0.07 \pm 0.01$  and  $0.00 \pm 0.00$ , respectively, with 200 nM latrunculin A ( $n=2$ ; LTC4 release in nondesensitized cells was sixfold greater in the presence of latrunculin A).

Some previous studies have used a different method to generate a desensitized state in basophils and mast cells, so-called subthreshold desensitization [35–37]. The progressive escalation of concentrations of stimulus over an extended period of time in the presence of  $\text{Ca}_e$ , beginning at a concentration that does not induce histamine release directly, will ultimately produce a cell that is in a desensitization state. Although this methodological approach does not routinely produce a completely desensitized cell, it does offer a way to test

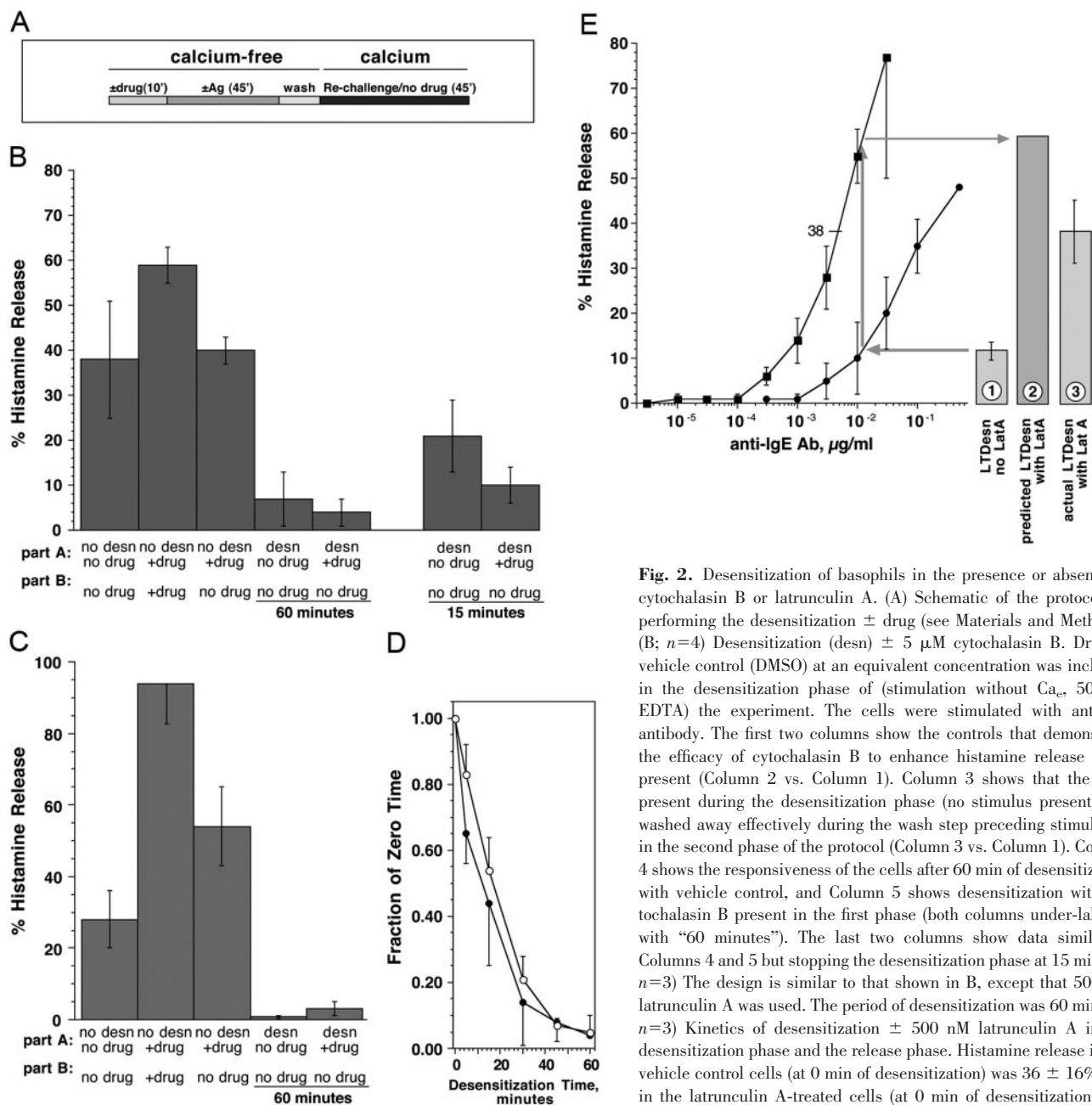


**Fig. 2.** Desensitization of basophils in the presence or absence of cytochalasin B or latrunculin A. (A) Schematic of the protocol for performing the desensitization  $\pm$  drug (see Materials and Methods). (B;  $n=4$ ) Desensitization (desn)  $\pm$  5  $\mu$ M cytochalasin B. Drug or vehicle control (DMSO) at an equivalent concentration was included in the desensitization phase of (stimulation without  $\text{Ca}_e$ , 50  $\mu$ M EDTA) the experiment. The cells were stimulated with anti-IgE antibody. The first two columns show the controls that demonstrate the efficacy of cytochalasin B to enhance histamine release when present (Column 2 vs. Column 1). Column 3 shows that the drug present during the desensitization phase (no stimulus present) was washed away effectively during the wash step preceding stimulation in the second phase of the protocol (Column 3 vs. Column 1). Column 4 shows the responsiveness of the cells after 60 min of desensitization with vehicle control, and Column 5 shows desensitization with cytochalasin B present in the first phase (both columns under-labeled with "60 minutes"). The last two columns show data similar to Columns 4 and 5 but stopping the desensitization phase at 15 min. (C;  $n=3$ ) The design is similar to that shown in B, except that 500 nM latrunculin A was used. The period of desensitization was 60 min. (D;  $n=3$ ) Kinetics of desensitization  $\pm$  500 nM latrunculin A in the desensitization phase and the release phase. Histamine release in the vehicle control cells (at 0 min of desensitization) was  $36 \pm 16\%$  and in the latrunculin A-treated cells (at 0 min of desensitization) was  $73 \pm 11\%$ . (E;  $n=3$ ) Subthreshold desensitization  $\pm$  200 nM latrunculin A (LTDesn). The left plot shows histamine release  $\pm$  latrunculin A for 260 min with single concentrations of anti-IgE antibody; without ( $\bullet$ ) and with ( $\blacksquare$ ) latrunculin A. The histograms on the right show histamine release for basophils stimulated with escalating concentrations of anti-IgE antibody  $\pm$  latrunculin A (LatA). The concentrations and timing of stimulation were in the absence of latrunculin A (histogram marked 1) 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1  $\mu$ g/ml for 40, 40, 40, 30, 30, 20 min, followed by 0.5  $\mu$ g/ml for 30 min. In the presence of 200 nM latrunculin A (histogram marked 3), the concentrations and timing were 0.00003, 0.0001, 0.003, 0.001, 0.03, 0.01, 0.03  $\mu$ g/ml for 30, 40, 40, 40, 40, 20, 20 min, followed by 0.1  $\mu$ g/ml for 30 min. The histogram marked "2" is the predicted amount of release for latrunculin A-treated cells (follow arrows) following escalating concentrations of anti-IgE antibody.

the desensitization process during full secretory conditions. Stimulation with anti-IgE started at a concentrations threefold below the concentrations required to observe minimal histamine release. As latrunculin A markedly enhances histamine release, the starting anti-IgE antibody concentration was 0.00003  $\mu$ g/ml for cells stimulated in the presence of 200 nM latrunculin A and 0.0003  $\mu$ g/ml in the absence of latrunculin A. The full time-frame for the escalating concentrations was 4.5 h. Figure 2E, left plot, first shows the anti-IgE dose-response curves  $\pm$  200 nM latrunculin A (single concentration

of anti-IgE throughout the 4.5-h incubation). The right-most histograms show the amount of release following the escalating concentrations of anti-IgE antibody. In the absence of latrunculin A, the escalating concentrations of anti-IgE resulted in only  $13 \pm 2\%$  histamine release; i.e., desensitization was  $\sim 75\%$  (histogram labeled 1). As latrunculin A is a good enhancing agent, a cell releasing 13% would be expected to release 60% in the presence of 200 nM latrunculin A (following the arrows in Fig. 2E). Actual release,  $38 \pm 7\%$ , for the escalating concentrations of anti-IgE antibody in the presence

of latrunculin A was less than the predicted 60%, suggesting that desensitization continued unabated in the presence of latrunculin A.

## Early signaling $\pm$ $\text{Ca}_e$

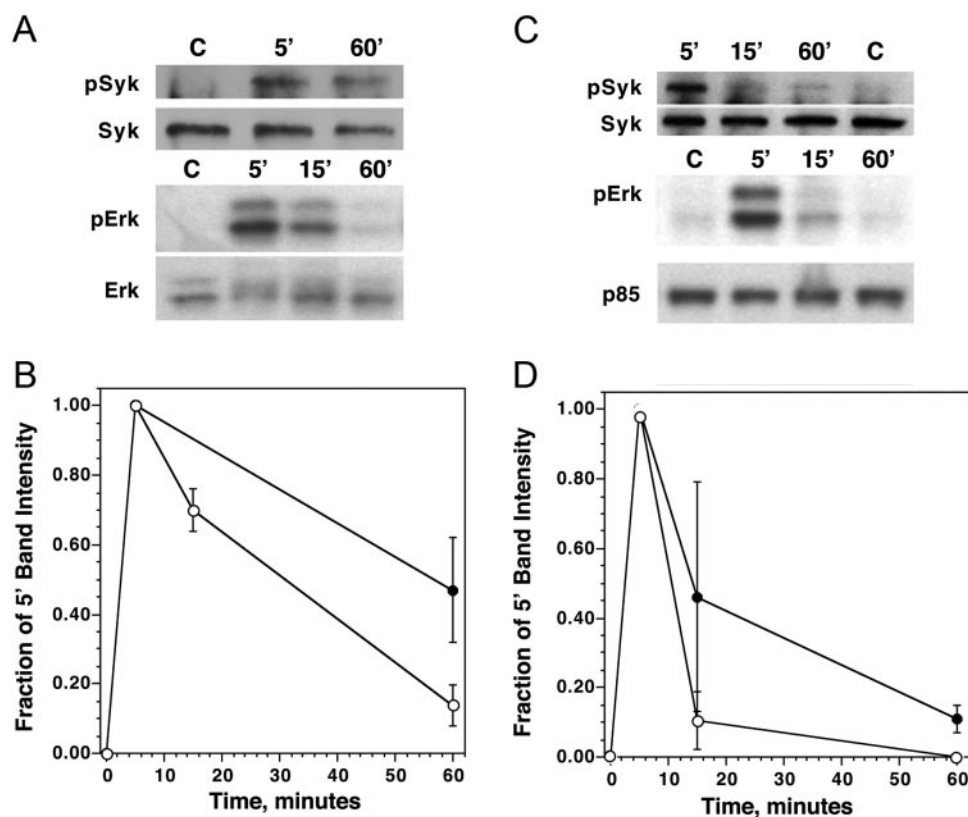
The studies above suggested that at a functional level, inhibition of actin polymerization had no influence on desensitization. We were next interested in how this observation translated to the early steps in activation, given that our previous studies demonstrated that latrunculin A prolonged syk phosphorylation. However, to date, signaling has been studied primarily in the presence of  $\text{Ca}_e$  only. At the signaling level, there are two characteristics of the type of self-termination that occurs in human basophils. The first relates to differences in the signaling that follows stimulation with antigen versus stimulation with goat polyclonal anti-IgE (and several other anti-IgE antibodies). Stimulation with antigen results in transient syk phosphorylation (and several other early signaling steps), and stimulation with anti-IgE leads to a sustained, early signal [1]. The second characteristic is the transience of the MAPK pathway, regardless of whether the cells are stimulated with antigen or anti-IgE antibody. These characteristics have been defined during full secretion but not during a desensitization-style protocol (stimulation without  $\text{Ca}_e$ ). **Figure 3** shows that stimulation with antigen (BPO-HSA) in the absence of  $\text{Ca}_e$  results in a transient increase of syk that is similar to that observed in the presence of  $\text{Ca}_e$  [1]. The only notable difference is that the magnitude of the increase is  $\sim 50\%$  of the increase observed when Ca is present (data in the figure are plotted as the fraction of the

5-min observation to highlight the characteristic of transience). Parenthetically, after 60 min, when Ca is returned to the buffer, there is little further increase in level of syk phosphorylation observed at the 60-min time-point (data not shown). In other words, although the signal remains weakly present in these desensitized cells, they are unresponsive to the addition of  $\text{Ca}_e$  at the level of the earliest signaling steps, just as they do not release histamine. If the stimulus is polyclonal anti-IgE antibody, syk phosphorylation is relatively persistent in the absence of  $\text{Ca}_e$ , and Erk phosphorylation is transient (Fig. 3). This behavior is also similar to stimulation with  $\text{Ca}_e$ . Syk phosphorylation is somewhat more transient in the absence of  $\text{Ca}_e$ , because as shown previously, syk loss by ubiquitylation is somewhat faster under these conditions [31]. The figure shows syk phosphorylation kinetics without normalization for syk loading; if normalized for remaining syk, there is little decrease in the syk signal following anti-IgE antibody. In other words, the syk that remains is phosphorylated with the same efficiency at 5 and 60 min, which was not the case following stimulation with antigen. The data indicate that at the level of early signaling, the behavior of the cells without Ca recapitulates their behavior in the presence of Ca, at least for these two characteristics of the response.

## Early signaling was not altered with polymerization inhibitors

Previous studies showed that the addition of latrunculin A converts the transient syk phosphorylation that follows stimulation with antigen to a response that is sustained [13]. This

**Fig. 3.** Kinetics of syk or Erk phosphorylation following stimulation in the absence of  $\text{Ca}_e$  (50  $\mu\text{M}$  EDTA). To perform these experiments, the cells were treated briefly with lactic acid to remove a fraction of the endogenous IgE, resensitized with penicillin (BPO)-specific IgE on ice (see Materials and Methods), washed, and stimulated with BPO(11)-HSA at 0.5  $\mu\text{g}/\text{ml}$  in PAG (+50  $\mu\text{M}$  EGTA) or goat anti-IgE antibody. After the times shown, cells were lysed with electrophoresis sample buffer or CLB and processed for Western blotting (see Materials and Methods). The Western blot bands were digitized, and data are expressed as the fraction of the response observed at 5 min. (A) Example blots for syk and Erk phosphorylation following stimulation with an optimal concentration of anti-IgE antibody, 0.2  $\mu\text{g}/\text{ml}$  (C, control). Also shown are the re-blot for lane-loading control. (B) Average kinetics (○) pErk response in cells stimulated with anti-IgE antibody ( $n=3$ ); (●) syk phosphorylation in cells stimulated with anti-IgE antibody ( $n=7$ ). (C) Example blots for syk and Erk phosphorylation following stimulation with an optimal concentration of BPO(11)-HSA, 0.5  $\mu\text{g}/\text{ml}$ . Also shown are the re-blot for lane loading; p85 was used as a lane-loading control in the pErk example [38]. (D) Average kinetics (○) pErk response in cells stimulated with BPO(11)-HSA ( $n=2$ ); (●) syk phosphorylation in cells stimulated with BPO(11)-HSA ( $n=3$ ). Not shown is that the 5-min peak response for syk phosphorylation in the absence of  $\text{Ca}_e$  was  $\sim 50\%$  of the peak in the presence of  $\text{Ca}_e$  and  $\sim 35\%$  for Erk phosphorylation.



was considered consistent with the hypothesis that actin polymerization mediated the down-regulation of the earliest steps in signaling. The transience of syk phosphorylation  $\pm$   $\text{Ca}_e$  was then examined in the presence or absence of latrunculin A. **Figure 4** shows that during stimulation in the presence of  $\text{Ca}_e$  and latrunculin A, syk phosphorylation switched from transient to persistent, as observed in our previous studies. In contrast, syk phosphorylation remained transient in the presence of

latrunculin A when the cells were stimulated without  $\text{Ca}_e$ . This was a surprising result, and it raised the question of whether the characteristics of actin polymerization changed when basophils were stimulated without  $\text{Ca}_e$  (relative to stimulation +  $\text{Ca}_e$ ). **Figure 5, A and B**, shows that stimulation –  $\text{Ca}_e$  resulted in the same peak response of actin polymerization as stimulation +  $\text{Ca}_e$  and that latrunculin A continued to completely suppress the stimulated increase as well as the resting level of f-actin. Taken together with the results shown in Figure 4B, the logical conclusion is that polymerization of actin is not required to observe the transient phosphorylation of syk.

Although cytochalasins are used to alter the polymerization of actin, the mechanism for altering actin polymerization differs from that of latrunculin A. Many of the studies showing the influence of actin polymerization on down-regulation of early signals in nonhuman mast cells have used cytochalasin B or D. We repeated the syk phosphorylation experiments using cytochalasin D. Figure 4, C and D, shows that cytochalasin D did not alter the transience of syk phosphorylation  $\pm$   $\text{Ca}_e$ . Figure 5D shows that the presence of polymerized actin in basophils has a complex relationship with the presence of cytochalasin D. The reported  $\text{IC}_{50}$  for cytochalasin D in cells should be in the 0.5- $\mu\text{M}$  range, with a maximum effect occurring at 2–5  $\mu\text{M}$ . However, in human basophils, the addition of cytochalasin D without stimulation results in increased f-actin. The  $\text{IC}_{50}$  for the stimulated increase is  $\sim 0.5 \mu\text{M}$  (inset to Fig. 5D), with complete inhibition of the stimulated increase above 1  $\mu\text{M}$ . However, there is no point where the resting f-actin level is below untreated cells. Indeed, there is an increase in resting f-actin at higher concentrations. As found for latrunculin A, Figure 5E shows that the presence or absence of  $\text{Ca}_e$  does not change the ability of cytochalasin D to alter actin polymerization.

### Actin polymerization augments receptor- and nonreceptor-mediated histamine release

Together, these results suggest that the enhancement of secretion in the presence of actin polymerization inhibitors is not a consequence of an inhibition of desensitization processes. This enhancement might be a result of a more generalized process, which alters all forms of stimulation. Basophils were stimulated with fMLP or PMA  $\pm$  latrunculin A. fMLP is a secretagogue that operates through a G-protein. PMA directly activates a variety of protein kinase C family members in basophils [39] and is therefore a nonreceptor-dependent process that is poorly understood. We previously examined the effect of latrunculin on a single, near-optimal concentration of PMA [13] but in the current study, examined lower concentrations as well. For fMLP and PMA, 500 nM latrunculin A enhanced histamine release (data not shown), but for PMA, it was only apparent at lower concentrations (at 3, 10, and 30 ng/ml, histamine release was  $8 \pm 4$ ,  $10 \pm 8$ , and  $25 \pm 10\%$  without latrunculin A, respectively, and  $28 \pm 2$ ,  $29 \pm 2$ , and  $27 \pm 7\%$  with 500 nM latrunculin A, respectively).

The studies above tested whether actin polymerization was dependent on syk activity and found that it was. We concluded that actin polymerization was not a necessary process to mediate desensitization, and the demonstration that actin polymerization was not needed for desensitization using actin po-

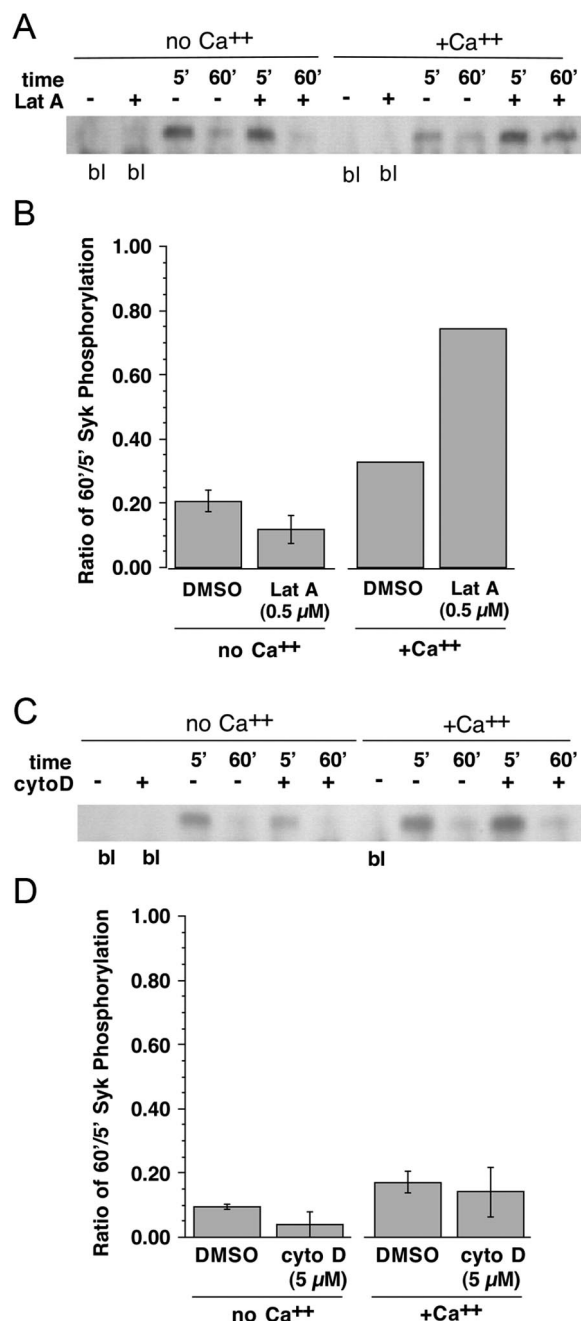
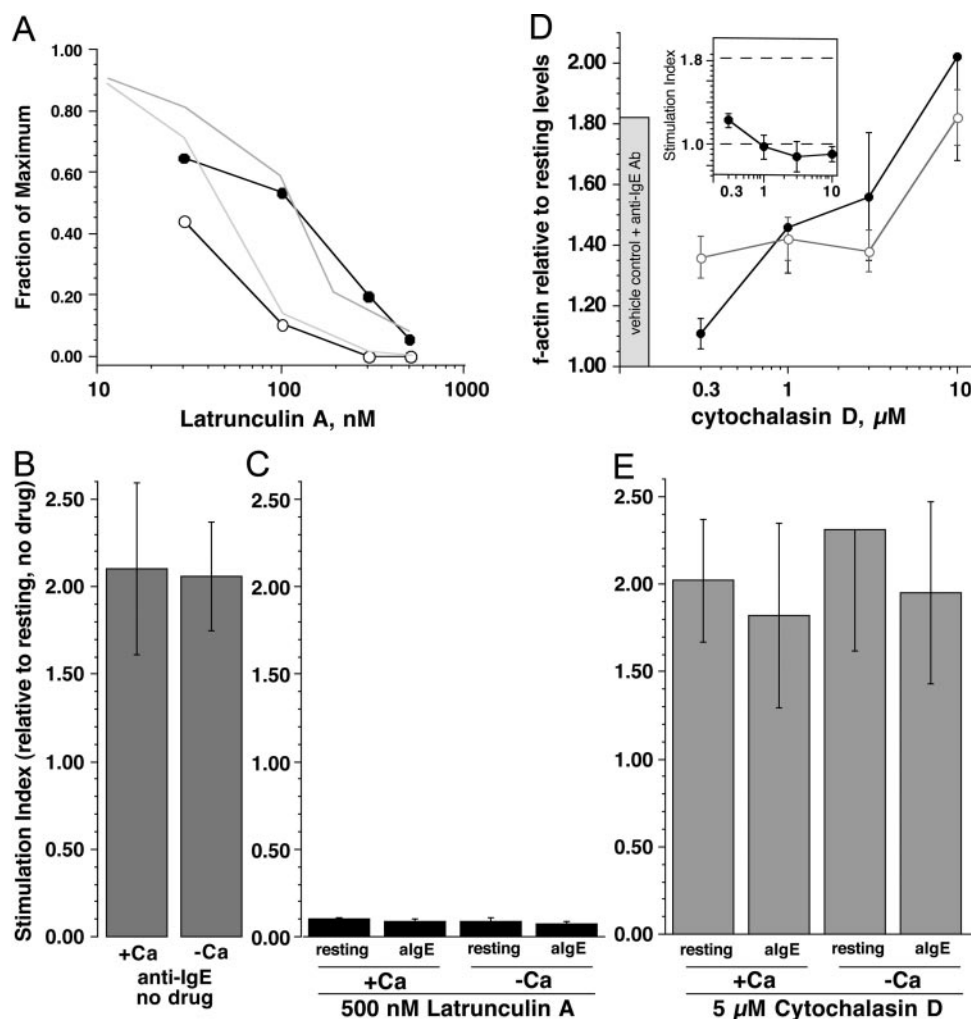


**Fig. 4.** Syk phosphorylation in the presence and absence of  $\text{Ca}_e$  and the presence or absence of latrunculin A or cytochalasin D. (A) Western blot (4G10, antiphosphotyrosine antibody) for one example experiment (the syk re-blot is not shown, but loading was similar for all lanes). The latrunculin A concentration was 0.5  $\mu\text{M}$  (vs. vehicle control, DMSO). bl, no stimulus. (B) Average results for two experiments like that shown in A. (C) An example experiment similar in design to that shown in A; the drug was 5  $\mu\text{M}$  cytochalasin D (cytoD). (D) Average of two experiments as shown in C.

**Fig. 5.** Polymerization of actin under resting and stimulated conditions  $\pm$   $\text{Ca}_e$ . (A) Concentration dependence of actin polymerization with latrunculin A at the shown concentrations ( $n=1$ ). (●) Stimulated increase in f-actin, (○) resting level of f-actin. The light gray lines show data from a previous study, which was done in the presence of  $\text{Ca}_e$  [13] ( $n=3$ ). (B;  $n=3$ ) Stimulation index of actin polymerization  $\pm$   $\text{Ca}_e$ ; cells were stimulated with 0.5  $\mu\text{g/ml}$  anti-IgE antibody (6061) for 8 min prior to fixation and labeling. (C;  $n=3$ ) Presence of f-actin in basophils, resting versus stimulated (anti-IgE antibody),  $\pm$   $\text{Ca}_e$  in the presence of 500 nM latrunculin A. Data are plotted relative to the resting f-actin in cells not treated with drug. (D;  $n=3$ ) f-Actin in resting (●) or stimulated basophils (anti-IgE antibody; ○) in the presence or absence of cytochalasin D at the concentrations shown. Data are calculated relative to resting cells in the presence of vehicle control (DMSO). The inset shows the stimulation index (anti-IgE vs. resting) for each concentration of cytochalasin D. The upper dotted line at  $\sim 1.8$  is the stimulation index with vehicle control. (E;  $n=3$ ) Stimulation index (relative to resting cells with vehicle control)  $\pm$   $\text{Ca}_e$  in the presence of 5  $\mu\text{M}$  cytochalasin D.



lymerization inhibitors validated the logic of the approach. We then extended the syk-dependence/independence strategy to an early event that has been speculated to require actin polymerization and to possibly mediate desensitization.

### Receptor immobilization is dependent on actin polymerization

Several studies have found that elution of IgE-bound multivalent antigen, using a monovalent form of the antigen to dissociate antigen from an activated basophil or mast cell, occurs quickly in the early binding reaction but slowly late in the reaction [26, 40, 41]. Theoretical studies have suggested that this is not a result of the multipoint attachment of antigen to many surface IgE molecules but is more likely the result of a change in the mobility of the IgE in the membrane [42]. Such a change in mobility has been proposed to be a reflection of a desensitization process [5]. We therefore examined whether inhibition of syk would modify this change in elution rate induced by monovalent antigen (hapten).

To study antigen binding to basophils, we synthesized DNP(18)-GFP, sensitized human basophils with DNP-specific IgE, and detected antigen binding by flow cytometry (see Materials and Methods). **Figure 6A** shows the elution kinetics of the bound antigen in the presence of 0.1 mM DNP-lysine at 5 min versus 60 min, recapitulating previous studies with the

finding that antigen dissociates slowly late in the reaction. Figure 6, B–D, shows that incubation in the cold or incubation with latrunculin A, NVP-QAB205, or PP1 partially ablated the slowing of elution at 60 min. These results lead to the conclusion that this form of receptor immobilization is dependent on syk activity and actin polymerization but is therefore not playing a role in desensitization/self-termination.

### DISCUSSION

Given the longstanding suggestion that actin polymerization acts as a negative regulator of early signaling and mediator release, it was surprising that inhibitors of actin polymerization, indeed dissolution of f-actin under resting conditions, had no influence on basophil desensitization. Several observations were unexpected.

First, latrunculin A induced a sustained phosphorylation of syk when stimulation was done in the presence of  $\text{Ca}_e$  but not in its absence. This was not the result of a loss in the ability of receptor aggregation to induce actin polymerization; stimulation  $\pm$   $\text{Ca}_e$  resulted in equivalent increases in f-actin. In fact, latrunculin A was also just as effective at eliminating all of the f-actin that could be detected with phalloidin in resting cells in the absence of  $\text{Ca}_e$ . It is not clear from the available data why

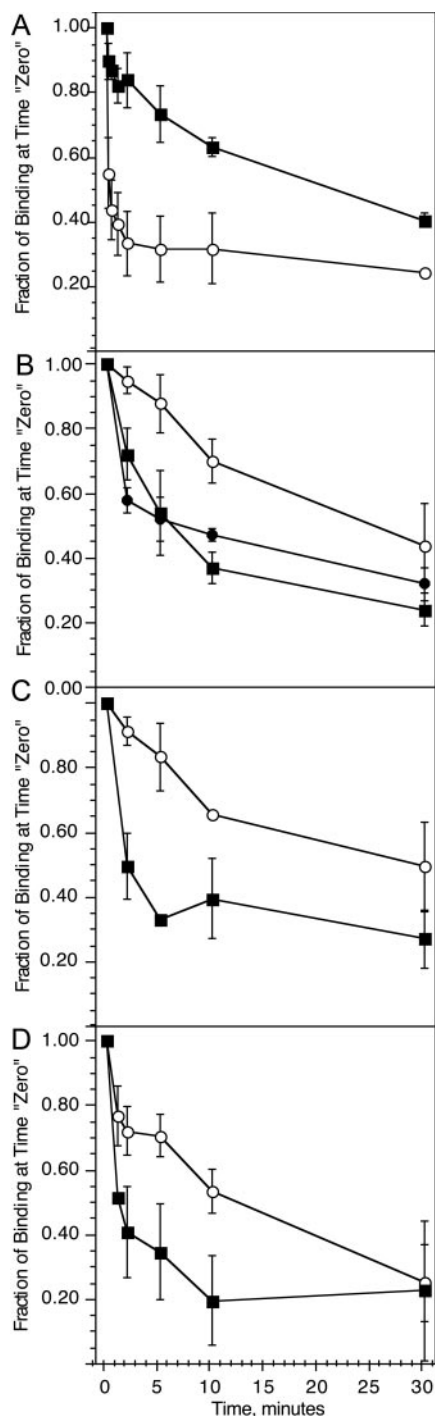


**Fig. 6.** Kinetics of DNP-GFP elution from basophils under various conditions. Basophils were sensitized with and without DNP-specific IgE. The nonsensitized cells were used as controls for all of the time-points and conditions shown. Fluorescence was monitored by flow cytometry. Data are expressed as a fraction of the maximum or starting net signal (sensitized-nonsensitized fluorescence). (A;  $n=5$ ) Kinetics of antigen elution after 5 min of antigen binding ( $\circ$ ) or 60 min ( $\blacksquare$ ) of antigen binding; 0.4 mM DNP-lysine was added and the amount of fluorescence monitored for the times shown. (B;  $n=3$ ) Kinetics of elution in the presence of vehicle control (DMSO;  $\circ$ ), 0.3  $\mu$ M NVP-QAB205 ( $\bullet$ ), or 10  $\mu$ M PPI ( $\blacksquare$ ). The drugs were present throughout the first 60 min of binding as well as the 30-min elution. (C;  $n=2$ ) Kinetics of elution at 37°C ( $\circ$ ) or 4°C ( $\blacksquare$ ). The temperature was 4°C or 37°C throughout the first 60 min of binding as well as the 30 min elution. (D;  $n=3$ ) Kinetics of elution in the presence of vehicle control (DMSO;  $\circ$ ) or 0.5  $\mu$ M latrunculin A ( $\blacksquare$ ; drugs present throughout the binding and elution).

the absence of  $\text{Ca}_e$  decouples the actin response from the processes controlling the transience of syk phosphorylation. Previous studies have also noted that in the presence of  $\text{Ca}_e$ ,  $\text{Fc}\epsilon\text{RI}\gamma$  phosphorylation, lyn phosphorylation (activation loop tyrosine), and SHIP1 phosphorylation (which is dependent on SFK activity) become more sustained in the presence of latrunculin A [32]. Therefore, during full secretion, the effects of latrunculin A influence the persistence of signaling elements that precede syk phosphorylation. It is difficult to visualize a model that ties together these various observations. However, regardless of the explanation, the studies in  $\text{Ca}$ -free buffers suggested that actin polymerization was not always associated with early signal termination.

We then found that incubation with cytochalasin D did not result in a change in the transience of syk phosphorylation, regardless of the presence or absence of  $\text{Ca}_e$ . Interpretation of this result is complicated by the unexpected increase in f-actin in resting cells. It was clear that at the concentrations used, stimulation did not increase f-actin, but “resting” f-actin remained present if not enhanced. However, this result contrasts with observations about the effect on cytochalasin D in RBL cells, where cytochalasin D has little effect on resting f-actin and prolongs early signaling (syk phosphorylation and phosphorylation of the  $\beta$  subunit) [7]. The results with human basophils could be interpreted as resting f-actin having a role in the signal termination. However, complete dissolution of the resting f-actin with latrunculin A did not reverse the transience of syk phosphorylation ( $-\text{Ca}_e$ ).

Not only was an early termination event sometimes independent of actin polymerization, functional desensitization occurred in the presence of latrunculin A or cytochalasin D. This result is similar to that found when an effective syk inhibitor was shown not to alter functional desensitization (or the transience of syk phosphorylation) [33]. In addition, the lack of an effect of latrunculin A on desensitization was true whether the desensitization was performed in the absence or presence of  $\text{Ca}_e$ . The method of subthreshold desensitization is complicated by the difficulty in achieving complete desensitization in a short enough time-frame that other long-term, down-regulatory events, such as loss of syk or receptor expression, are not also operating. In our approach, the time was limited to 4 h, and this resulted in less than complete desensitization. Nevertheless, these studies also showed that subthreshold desensitization still occurred in the presence of latrunculin A. Was the amount of desensitization that occurred the same as in the absence of latrunculin A? This was not as easy to assess, but knowing the relationship between release in the presence and absence of latrunculin A, one could predict the amount of release expected in the presence of latrunculin A when desensitization was not complete. The amount of release following escalating concentrations of stimulus in the presence of latrunculin A was less than predicted; i.e., not only did desensitization continue to occur, but also, desensitization may have been greater than expected.

As noted above, when we first found that actin polymerization was dependent on syk activity, we could have predicted that actin polymerization was not involved in desensitization/signal termination. Finding that actin polymerization inhibitors did not reverse desensitization helps to validate the strategy of

using the dependence of a pathway on syk as a guide to whether the pathway is involved in desensitization. It should be acknowledged that a small amount of residual actin polymerization might be undetectable but critical to desensitization, so these studies cannot entirely exclude a role for actin polymerization.

We also found that inhibition of PI-3K markedly inhibited actin polymerization. As previous studies also concluded that inhibition of PI-3K does not affect desensitization, the dependence of actin polymerization on PI-3K, such as its dependence on syk, supported the conclusion that actin polymerization is not relevant to desensitization. However, the LY294002 studies were somewhat more complicated to interpret than the results with syk inhibition. Full inhibition of actin polymerization was not possible with LY294002 or wortmannin. In previous studies, inhibition of several signaling elements (and secretion) by LY294002 is complete [43]. In the current study, this was also true of pyk2, PAK2, and VAV phosphorylation. These latter three elements have been associated with one of the signaling cascades leading to actin polymerization [44–47]. In contrast, phosphorylation of Nck, an adaptor protein involved in the activation of Wiskott-Aldrich syndrome protein and the actin-related protein 2/3 complex [48–50], was not inhibited by LY294002. These results suggest that there are two pathways to actin polymerization: one dependent on Nck and one dependent on the VAV/pyk2/PAK2 pathway. These pathways control separate pools of actin or converge on a single pool in such a way that some polymerization can occur even with one pathway inactive. These issues will need to be resolved in a future study.

If actin polymerization does not play a role in desensitization, why do inhibitors of actin polymerization markedly enhance mediator release? It is interesting to note that in human basophils, the magnitude of actin polymerization (as a stimulation index) is correlated tightly with the amount of histamine release, regardless of the stimulus [18]. In particular, stimulation of human basophils with the phorbol ester, PMA, leads to substantial histamine release, and the data points for PMA-induced actin polymerization versus histamine release lie on the same line as those generated with anti-IgE antibody or fMLP as the stimulus. This observation suggests that actin polymerization is serving a role during degranulation that is shared amongst the stimuli. In a similar context, latrunculin A enhances IgE-, fMLP-, and PMA-mediated histamine release (although the effect on PMA-induced release is restricted to suboptimal concentrations). Although actin polymerization could regulate each of these stimuli by different mechanisms, each of which depended on actin polymerization, perhaps the enhancing effect results from a shared secretory event that is important to granule fusion rather than only modifying the sorting of FcεRI in and out of lipid domains. This conclusion is more consistent with early studies of cytochalasin B effects in RBL cells [10]. However, having noted this point, inhibition of actin polymerization also enhances secretion of LTC<sub>4</sub> and does not reverse desensitization so that any unified explanation for the role of actin polymerization would ultimately need to take into account how it influences secretion of a newly synthesized mediator such as LTC<sub>4</sub>.

The actin polymerization studies strengthen the motivation to use a syk inhibitor to explore the role of other postulated mechanisms of desensitization. In this context, the ability of syk inhibitors to alter the kinetics of antigen elution late in the reaction was examined. It was predicted that this phenomenon would be the result of activation and more specifically, the result of actin polymerization [42]. The theoretical background is that simple binding to even multiple IgE molecules could only reduce the elution rate by two- to sixfold, not the 25- to 100-fold observed experimentally. However, if the IgE molecule lost mobility [51] by some mechanism, this would slow dissociation, as IgE dissociating from a multivalent antigen:IgE aggregate would not readily escape the region of the aggregate, and rebinding would occur more readily. One established mechanism for slowing the mobility of the receptor is to entrap it in a cytoskeletal “cage.” This led to the prediction that actin polymerization might accomplish this effect. Our studies demonstrate that the elution rate can be converted to one that is much faster, although not quite as fast as found when the antigen is eluted early in the reaction by incubation in NVP-QAB205, PP1, cold, or latrunculin A. These are all consistent with a requirement for activation and more specifically, actin polymerization. However, this result leads to the conclusion that this mechanism of receptor immobilization does not result in or is not associated with desensitization.

These studies lead to the conclusion that actin polymerization is not an important element underlying desensitization of human basophils and neither is immobilization of the receptor. They also further validate the strategy of using a syk inhibitor to explore the possible mechanisms of desensitization. These results may have application to other immunoreceptor-dependent, self-termination processes. In particular, actin polymerization may have some regulatory role, but final signal termination does not depend on its activities.

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