

Research report

The influence of actin microfilaments on signaling by the receptor with high-affinity for IgE

Chikako Torigoe^{a,c}, Jinming Song^b, B. George Barisas^b, Henry Metzger^{a,*}^a Section on Chemical Immunology, Arthritis and Rheumatism Branch, NIAMS, Bethesda, MD 20892-1820, USA^b Department of Chemistry, Colorado State University, Fort Collins, CO 80523, USA^c Department of Pathology, Division of Cell Pathology, Cancer Research Facility, University of New Mexico School of Medicine, 2325 Camino de Salud, Suite 201, Albuquerque, NM 87131-5301, USA

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Abstract

Aggregation of the receptors with high-affinity for IgE (FcεRI) stimulates a variety of cellular responses, but excessive aggregation inhibits such responses. Actin filaments have been implicated in the inhibitory phenomenon because disrupting the filaments enhances the cellular reactions stimulated by the aggregated receptors. To clarify further the molecular mechanism and physiological importance of the actin-mediated inhibition, we assessed the effect of inhibitors of actin polymerization on the initial signaling events of mast cells alternatively stimulated by nitrophenyl ligands that dissociate slowly (high-affinity) or rapidly (low-affinity) from receptor-bound anti-dinitrophenyl IgE. The inhibitors amplified the phosphorylation of FcεRI and of Syk induced by addition of either ligand but at physiological temperatures, the augmentation of the response to the low-affinity ligand was especially exaggerated. The effect of actin is on the earliest events, and although the molecular mechanism(s) by which the filaments regulate the intensity of proximal signaling remains unclear, several possibilities have been excluded. That the inhibitors only minimally augment the responses stimulated by preformed dimers of IgE, and in general show smaller effects with more limited aggregation, suggests that the actin-mediated “down-regulation” may be more prominent in laboratory experiments than under physiological circumstances.

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1. Introduction

Aggregation of the receptor with high-affinity for IgE (FcεRI) stimulates a variety of cellular responses but it has long been known that the dose response to the aggregating stimulus (bivalent antibody or antigen) shows an optimum dose effect. Studies in the early 1970s revealed that at very high doses, inhibition of degranulation was, as expected, due to inhibition of aggregation (as observed in a precipitin reaction in antigen excess). Surprisingly, however, at somewhat lower doses, inhibition was not due to diminished aggregation but to excessive aggregation (Becker et al., 1973) (see also Magro and Alexander, 1974; DeLisi and Siraganian, 1979). The studies by Becker et al. examined the effect of the dose of antigen on the degree of degranulation and surface re-distribution of receptor-bound fluorescent IgE. Later studies, showing that at least some

of the re-distribution of FcεRI at high doses of antigen was inhibitable by cytochalasin (Carson and Metzger, 1974) suggested that actin microfilaments might be involved in the functional inhibition, and direct evidence for such a connection began accumulating. Although the initial published results failed to resolve whether the effect of disrupting f-actin was principally on early or late events (Urata and Siraganian, 1985; Narasimhan et al., 1990; Narasimhan et al., 1990), more recent data clearly implicate an effect on both.

Here we consider only the effect on early responses (Seagrave and Oliver, 1990; Pierini et al., 1997; Frigeri and Apgar, 1999; Holowka et al., 2000). The reason for focusing on the effect on the early steps is related to the increasing interest in explicating cell signaling phenomenon sufficiently to be able to make quantitative predictions. The most advanced of such efforts to model the signaling by FcεRI do not incorporate actin filaments as one of components in the early steps (Faeder et al., 2003) and so we wished to determine how this effect could be incorporated

* Corresponding author. Tel.: +1-301-435-6126; fax: +1-301-402-0012.
E-mail address: metzgerh@exchange.nih.gov (H. Metzger).

into such modeling schemes. No such detailed models for the downstream events are as yet available.

We describe experiments that explore further the relationship between cytoskeletal rearrangements focusing particularly on the influence of the cytoskeletal filaments on the early signaling induced by alternative antigens that dissociate rapidly or slowly from the receptor-bound IgE. Prior work using these ligands revealed that “kinetic proofreading” governs at least some of the cellular responses initiated by FcεRI (Torigoe et al., 1998). The kinetic proofreading constraint proposes that an activated receptor’s ability to propagate distal steps is related to the length of time the unit ligand–receptor interaction is maintained (McKeithan, 1995). In interactions such as those involving FcεRI the critical time relates to the interaction of antigen with the receptor-bound IgE because the latter’s binding is essentially irreversible on the time scale of the experimental protocols. In general, the rate at which small ligands bind to antibody combining sites varies little, and it is the rate of dissociation that varies the most. Hence, the kinetic proofreading constraint in the context of this system means that the number of distal steps activated by FcεRI is sensitive to the affinity of the ligands that aggregate the receptors via their bound IgE. We were interested in how the actin microfilaments might influence the proofreading phenomenon, and whether this could help to clarify the mechanism by which the cytoskeleton regulates the intensity and kinetics of signal transduction.

2. Materials and methods

2.1. Reagents

Reagents for enhanced chemiluminescence were obtained from Amersham Pharmacia Biotech (Piscataway, NJ); polyacrylamide gels were from Invitrogen (Carlsbad, CA). Mouse monoclonal IgG2b anti-phosphotyrosine, 4G10 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). PY-*Plus* (the mixture of monoclonal anti-phosphotyrosine antibodies) was obtained from Zymed Laboratories Inc. (San Francisco, CA). Goat anti-mouse IgE was affinity purified and rendered monospecific on appropriate columns of IgE. Cytochalasin D was obtained from Sigma (St. Louis, MO); latrunculin A from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA).

2.2. IgE

Mouse monoclonal anti-dinitrophenyl (DNP) IgE (Liu et al., 1980) was affinity purified from ascites fluid by using a column of Sepharose conjugated with trinitrophenyl-lysine (TNP-Lys). Bispecific IgE was purified as described (Subramanian, 1995). Briefly, cell culture supernatant of a quadroma—the fusion product of an anti-DNP IgE-producing and an anti-dansyl (DNS) IgE-producing

hybridoma—was concentrated and affinity purified through DNS- and TNP-Lys columns sequentially. Chemically-crosslinked rat IgE (IR 162 (Bazin et al., 1974)) was prepared as described (Segal et al., 1977) and dimers were purified by gel-filtration using high performance liquid chromatography.

2.3. Nitrophenylated antigens

DNP- and 2-nitrophenyl (2NP)-conjugated Fab fragments of bovine IgG were prepared as described previously (Torigoe et al., 1998). Briefly, the εNH₂-caproate derivative of either hapten was directly conjugated to the Fab fragment and purified by gel-filtration and dialysis.

2.4. Cells, cell culture, and the treatment with the inhibitors of actin polymerization

RBL cells of the 2H3 line were maintained as described previously (Barsumian et al., 1981). When indicated, cells were pre-treated with the inhibitors of actin polymerization for 10 min at 37 °C. At the time of these studies the cells expressed approximately 4×10^5 receptors per cell, and 1×10^6 molecules of Lyn kinase (long and short forms in roughly equal amounts). Cells of clone A11-Chinese hamster ovary (CHO) fibroblasts that had been transfected with the α, β, and γ subunits of FcεRI as well as with the short form of Lyn kinase have been described (Vonakis et al., 1997).

2.5. Cell lysates and immunoprecipitation

Cells were harvested with trypsin-EDTA and washed with buffer A [150 mM NaCl, 5 mM KCl, 5.4 mM glucose, 1.8 mM CaCl₂, 1 mM MgCl₂, 25 mM Pipes (pH 7.2) containing 0.1% BSA] and re-suspended in buffer A at 6.2×10^6 cells/ml. Two volumes of cell suspension was mixed with one volume of ice-cold 3× solubilization buffer giving final concentration of 0.1% TX-100, 50 mM Tris, 50 mM NaCl, 5 mM EDTA, 1 mM Na₃VO₄, 5 mM Na₄P₂O₇, 50 mM NaF, 2 mM iodoacetate, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml each of aprotinin, leupeptin, and pepstatin A (pH 7.6), and incubated for 30 min at 4 °C. After a 10 min centrifugation, FcεRI were immunoprecipitated from the supernatant with polyclonal anti-IgE antibodies using protein A Sepharose beads (Amersham Pharmacia Biotech). Anti-Syk serum (a kind gift from Dr. U. Blank) was used to immunoprecipitate Syk.

2.6. Phosphotyrosine assay

Immunoprecipitated proteins were extracted with hot Na-dodecylsulfate sample buffer and electrophoresed on polyacrylamide gels. Phosphotyrosine was detected by Western blotting using antibody 4G10 or PY-*Plus* under conditions that favored accurate quantitation as described previously (Torigoe et al., 1997).

2.7. Hexosaminidase assay

The activity of hexosaminidase was measured as described (Alber et al., 1992), using *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide as a substrate.

2.8. Chemical cross-linking

The membrane-permeable cross-linking reagent, dimethyl-3-3'-dithiobispropionimidate (DTBP) (Pierce, Rockford, IL), was used at 10 mM for trapping receptor-associated Lyn and at 4 mM for the assay of the activity of receptor-associated Lyn. The cross-linking reaction was performed at room temperature for 30 min and quenched with 40 mM glycine as previously described (Mao et al., 1995).

2.9. Kinase assay

After chemical cross-linking with DTBP as described above, receptor-associated Lyn was co-immunoprecipitated with protein A beads conjugated with anti-IgE antibody. As a control, goat anti-mouse IgG (Fc γ specific) (Jackson ImmunoResearch, West Grove, PA) was used. Precipitated proteins were washed three times with RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS) and three times with kinase assay buffer (0.5 mM Na₃VO₄, 2 mM CHAPS in A-buffer). A peptide corresponding to residues 6–20 of p34cdc2, which is a specific substrate for Src-family kinases (Cheng et al., 1991) (final concentration, 2 mM), 5 mM MnCl₂, 0.1 mM ATP, and 10 μ Ci of [γ -³²P]ATP were added to the washed beads. The reaction was conducted for 3 h at room temperature with occasional vortexing and the activity of Lyn was measured as described (Vonakis et al., 2001) using SpinZyme (Pierce).

2.10. Time-resolved phosphorescence anisotropy (TPA) instrumentation and analysis

TPA experiments were performed using methods previously described, as adapted for 2H3 cells (Song et al., 2002). Samples of $1\text{--}5 \times 10^6$ cells were washed, deoxygenated, and examined in a 5 mm $\times$ 5 mm Suprasil quartz cuvette mounted in a thermostated housing. Excitation was generally provided by the frequency-doubled 532 nm output of a Spectra-Physics DCR-11 Nd:YAG laser operated at 10 Hz with a vertically-polarized TEM₀₀ output of 250 μ J at the sample cuvette (Peng and Barisas, 1997). However, in selected experiments, a LightWave 203G diode-pumped Nd:YAG laser operating at 500 Hz was employed. Sample phosphorescence was isolated by a holographic notch filter (Kaiser Optical Systems Inc., Ann Arbor, MI), a 1 M Na₂Cr₂O₇ solution chemical filter, a KV 550 color filter (Schott Glass Technologies Inc., Duryea, PA) to block scattered light, a 3 mm thick RG 665 filter to eliminate delayed

fluorescence and a rotating polarizer to select phosphorescence emitted with vertical $I_{||}(t)$ and horizontal $I_{\perp}(t)$ polarizations. The phosphorescence signal was detected by a thermoelectrically-cooled EMI 9816A photomultiplier tube protected from light scattered during laser pulses by a fast gating circuit (Herman et al., 1992). The photomultiplier output was amplified by a Tektronix 476 oscilloscope and a 35 MHz bandwidth buffer amplifier. In experiments using the Spectra-Physics laser, 4096 traces in each polarizer orientation were averaged by a Nicolet 12/70 computer equipped with a 20 MHz, 8-bit ADC while, in high-speed experiments with the LightWave laser, an EG&G 9826 low re-arm time signal averager was employed. Data were downloaded into a PC-compatible computer for analysis. Phosphorescence intensities $I_{||}(t)$ and $I_{\perp}(t)$ were analyzed (Austin et al., 1979) to yield a phosphorescence intensity function $s(t) = I_{||}(t) + 2I_{\perp}(t)$ and a phosphorescence anisotropy function $r(t) = [I_{||}(t) - I_{\perp}(t)]/s(t)$. The phosphorescence intensity function was fitted to a sum of six fixed exponential decays of 3, 10, 30, 100, 300, and 1000 μ s, respectively. To facilitate comparison of phosphorescence decay between samples, mean decay half-times were calculated from the lifetimes and amplitudes of each step. The lifetime results were used to weight points in a non-linear least squares fit of the anisotropy data. Anisotropy data were, thus, satisfactorily analyzed according to a single average exponential decay model $r(t) = r_{\infty} + (r_0 - r_{\infty}) \exp(-t/\varphi)$ yielding the initial anisotropy value r_0 , the limiting anisotropy value r_{∞} , and the rotational correlation time (RCT) φ as well as the statistical uncertainties in these quantities (Bevington, 1969).

2.11. Quantitation of polymerized actin

The relative amounts of polymeric actin were measured as previously described (Draberova et al., 2003). Briefly, cells were incubated in PBS containing 100 μ g lysophosphatidylcholine, 3.6% formaldehyde, and 0.75 μ g/ml FITC-phalloidin for 10 min at 37 °C. The cells were then centrifuged, resuspended in PBS, and the mean fluorescence intensity was determined on a cell sorter (FACSCalibur (BD Biosciences, San Jose, CA).

3. Results

3.1. Effect of cytochalasin D on phosphorylation of Fc ϵ RI and Syk and on degranulation

The earliest observable molecular modification that results from the aggregation of Fc ϵ RI is the phosphorylation of the receptors and it had previously been determined that substantially higher concentrations of more rapidly dissociating ligands, that is, ligands of lower affinity such as 2NP, were required to stimulate equivalent levels of modified receptors than was the case with the ligands of higher affinity such as DNP (Torigoe et al., 1998). It was, therefore,

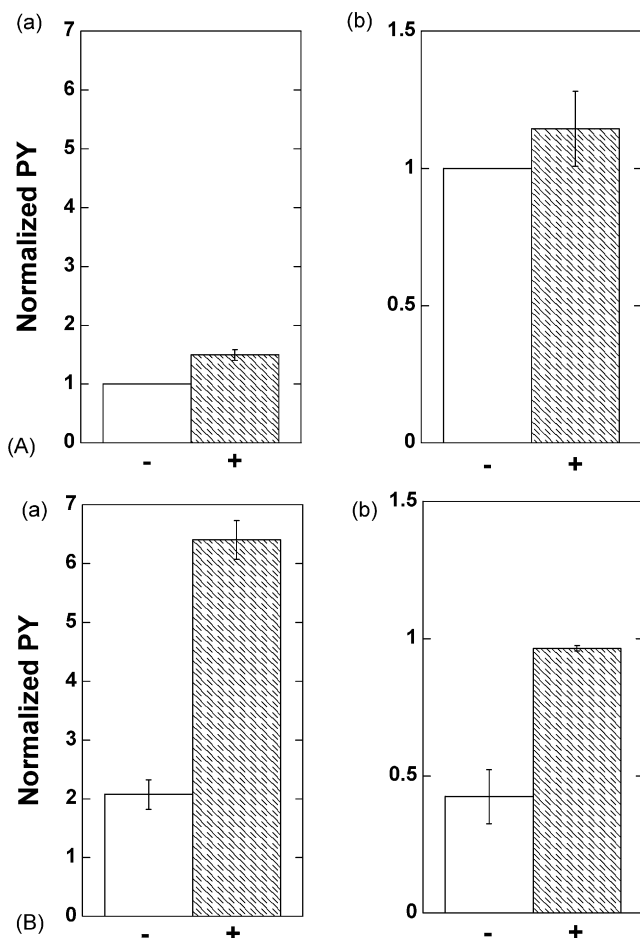


Fig. 1. Effect of cytochalasin D on receptor-mediated phosphorylation. Cells were stimulated with 3 ng/ml (DNP-cap)_{3.5}-Fab (Panel A) or 250 ng/ml (2NP-cap)_{3.9}-Fab (Panel B) for 3 min at 37 °C. Cells were pre-treated (+) or not (–) with 2 μ M cytochalasin D for 10 min at 37 °C. (a) Phosphotyrosine on FcεRI. (b) Phosphotyrosine on Syk. All the data are relative to the level of phosphorylation induced by the DNP antigen in the absence of cytochalasin D. The results shown are the average ($\pm$ S.E.M.) of the ratios observed in two experiments.

of interest to see if modifying actin filaments would affect this earliest stage of the signaling cascade. If so, one might anticipate that the *relative* phosphorylation by the low- and high-affinity ligands would be altered. Similarly, the relative efficiency of signaling of further downstream events by the low-affinity ligand should likewise be enhanced.

Fig. 1 summarizes two experiments in which we examined the phosphorylation of FcεRI and Syk in cells stimulated alternatively with the high-affinity, slowly dissociating DNP antigen or the low-affinity, rapidly dissociating 2NP antigen, in the presence and absence of cytochalasin D. The data were normalized to the difference between the resting cells and the cells stimulated for 3 min with 3 ng/ml (DNP-cap)_{3.5}-Fab.

Panel (A) shows the effect of cytochalasin D on the phosphorylation of FcεRI (a) and Syk (b) on cells reacted with the DNP antigen. In the cells treated with 2 μ M cytochalasin D for 10 min at 37 °C prior to the addition of antigen, phos-

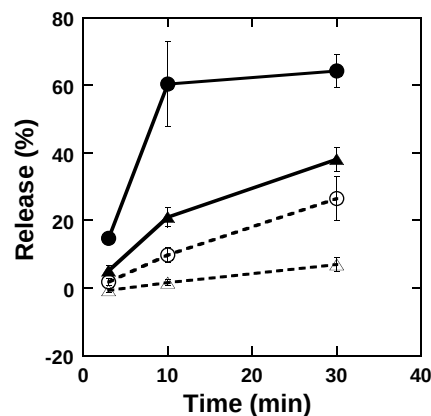


Fig. 2. Effect of cytochalasin D on FcεRI-mediated release of hexosaminidase. Filled symbols: cells treated with cytochalasin. Open symbols: cells not treated with cytochalasin. Circles: cells stimulated with DNP antigen. Triangles: cells stimulated with 2NP antigen. Each data point represents the average ($\pm$ S.D.) of three independent samples. The results in this experiment were representative of two other similar experiments.

phorylation of FcεRI increased by almost 50%; the increase in phosphorylation of Syk was not statistically significant.

Panel (B) shows the effect of cytochalasin D on phosphorylation of FcεRI (a) and Syk (b) on cells reacted with 250 ng of the 2NP antigen ((2NP-cap)_{3.9}-Fab). As shown previously (Torigoe et al., 1998), higher doses of the more rapidly dissociating 2NP are required to phosphorylate the FcεRI to a level comparable to that induced by the DNP analogue. As can be seen in the figure, cytochalasin D had a greater stimulatory effect on the response to this antigen, augmenting phosphorylation of the receptor > three-fold. However, even at doses of the 2NP sufficient to stimulate phosphorylation of FcεRI two-fold greater than that induced by the DNP, Syk, the proximal downstream component stimulated by aggregation of FcεRI, is phosphorylated much less. Cytochalasin stimulated the phosphorylation of Syk > two-fold. In our earlier studies, more distal events were progressively less efficiently stimulated by 2NP, and the latter induced minimal degranulation (Torigoe et al., 1998). However, significant degranulation is induced by the 2NP antigen after the treatment of cells with cytochalasin D (Fig. 2). Likewise, the cytochalasin enhanced the degranulation induced by the low dose of the DNP antigen.

The experimental protocols described subsequently were designed to probe at which site(s) in the earliest events, the concentration of actin filaments had an effect. First, however, we wished to confirm that the effect of cytochalasin D was attributable to its action on actin filaments and not on some other unanticipated process.

3.2. Effect of different concentrations of cytochalasin D and latrunculin A

That the amplification induced by cytochalasin D was based on its effect on actin filaments was validated by the

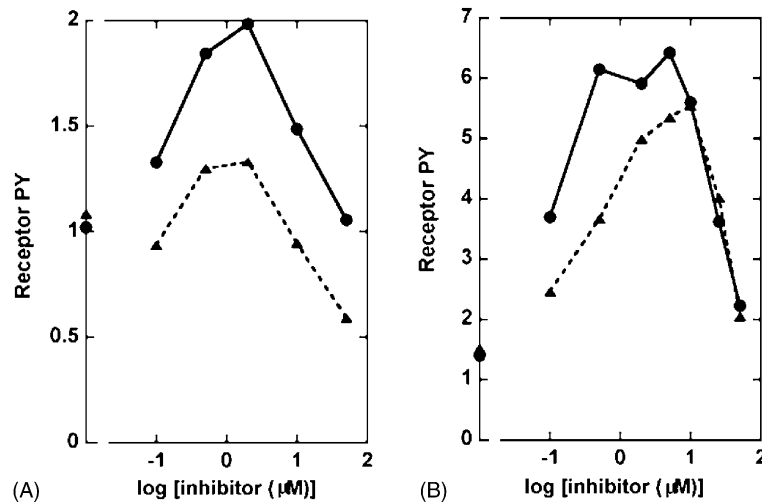


Fig. 3. Effects of dose of inhibitors of actin polymerization on phosphorylation of tyrosines on FcεRI. Cells were stimulated for 3 min at 37°C with (A) 3 ng/ml (DNP-cap)_{3.5}-Fab or (B) 250 ng/ml (2NP-cap)_{3.9}-Fab after pre-treatment for 10 min with cytochalasin D (●) or latrunculin A (▲). The data points on the left ordinate show the level of phosphorylation in the absence of inhibitors. A repeat experiment gave comparable results.

results with a second reagent that interferes with polymerization of actin. Whereas, cytochalasin D inhibits polymerization of actin by capping the barbed end of actin filaments, latrunculin A binds to monomeric actin (Coue et al., 1987). As shown in Fig. 3A, at ≥ 0.1 μM, both inhibitors amplified the phosphorylation of FcεRI induced by DNP antigen, although at 20-fold higher doses the amplification disappeared. A similar though exaggerated dose response was observed with cells stimulated by the 2NP antigen (Fig. 3B).

3.3. Effect of temperature

As noted previously (Torigoe et al., 1998), the concentration of phosphorylated receptors induced by the antigen with low-affinity is dramatically reduced at higher temperatures, relative to the response to the antigen with high-affinity even though no large effect of temperature was observed with respect to the affinity of either antigen for the IgE. This result suggests that some process other than dissociation of the ligand that reverses the phosphorylation, is enhanced at the higher temperatures. We wished to test whether the temperature dependence would provide some hints about the mechanism by which decreasing the actin filaments enhanced the signaling response. The effect of temperature on the response to the 2NP antigen can be seen by the data shown as the open circles in Fig. 4B. The effect is much less pronounced for the DNP antigen (Fig. 4A). The corresponding enhancements by cytochalasin are shown by the filled circles. There is a modest enhancement induced by cytochalasin D in cells stimulated with the DNP antigen at physiological temperatures. But the effect of temperature on the augmentation produced by cytochalasin D is much greater on the cells stimulated with the low-affinity ligand.

3.4. Effect of size of aggregates

Prior studies with pre-aggregated IgE indicated that the rapid dephosphorylation of the receptors normally seen particularly with higher levels of antigen, was much less prominent when small oligomers of IgE were used to stimulate the cells (Kent et al., 1994). To see if this was peculiar to chemically-crosslinked IgE or, more likely, related to the limited size of the receptor aggregates, cells were incubated with a bispecific IgE that was monovalent for anti-DNP (Subramanian, 1995). Such cells were compared to cells incubated with mono-specific (bivalent) anti-IgE after incubation with the paucivalent DNP antigen (Fig. 5). It is apparent that the response of the cells stimulated by dimers formed by the (reversible) binding of ligand is the same as those of cells stimulated by covalent dimers (Kent et al., 1994), that is, with smaller-sized aggregates of receptors the response is considerably prolonged.

3.5. Relative phosphorylation of FcεRI β and γ chains

As reviewed in the Section 4, a variety of experimental results suggest that the β and γ chains are phosphorylated sequentially. Previously we had not reproducibly seen that the low-affinity ligand was less efficient in stimulating the full sequence, but more thorough analysis indicates that this is in fact the case: in the absence of additives, and relative to the DNP antigen, the 2NP antigen induces a lower ratio of phosphorylated γ:β (Table 1). Treatment with cytochalasin D or latrunculin A increases that ratio induced by 2NP antigen so it becomes closer to the ratio induced by DNP antigen (Fig. 6). At very high doses of the inhibitors, the ratio declines. A similar increase in the ratio was observed by lowering the temperature (data not shown).

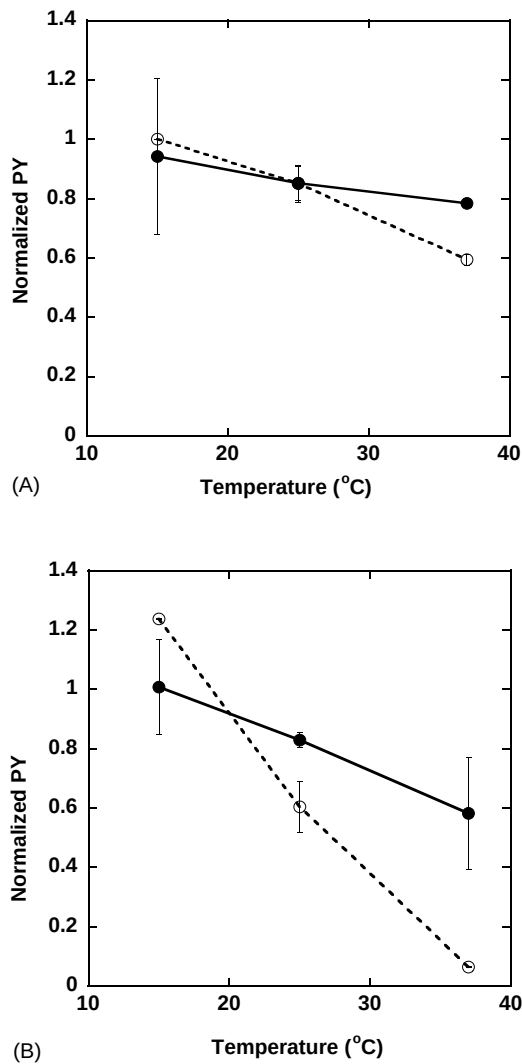


Fig. 4. Temperature dependence of the effect of cytochalasin D on phosphorylation of FcεRI. Filled symbols: cells pre-incubated with 2 μM cytochalasin D. Open symbols: cells not reacted with cytochalasin D. Cells were stimulated with 50 ng/ml DNP antigen (A) or 250 ng/ml 2NP antigen (B) for 3 min. All the data have been normalized to the level of phosphorylation induced by the DNP antigen at 15 °C in the absence of cytochalasin D. The data shown are the average (±S.E.M.) of two experiments.

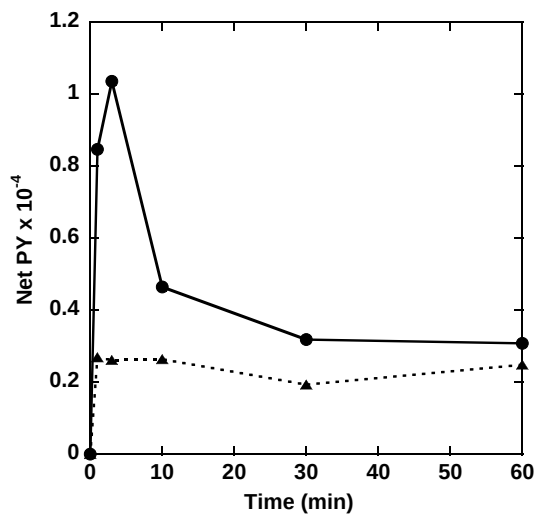


Fig. 5. Effect of size of aggregates on their functional longevity. Cells (6.2×10^6 cells/ml) were incubated either with saturating amounts of either normal bivalent anti-DNP IgE (solid line and circles) or with bispecific (effectively monovalent) anti-DNP, anti-DNS IgE (dashed line and triangles) and then challenged with 50 ng/ml of (DNP-cap)_{4,6}-Fab at 37 °C. A second experiment gave virtually identical results.

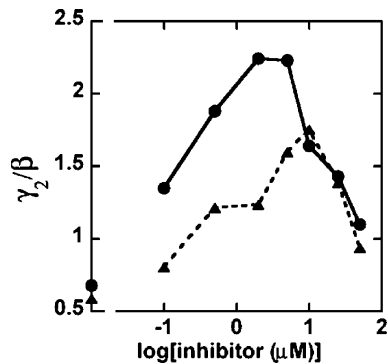


Fig. 6. The effect of the inhibitors of actin polymerization on the ratio (phosphorylated γ chains)/(phosphorylated β chains). Cells were stimulated with 250 ng/ml (2NP-cap)_{3,9}-Fab for 3 min at 37 °C. Cytochalasin D (●), latrunculin A (▲). The data points on the left ordinate show the relative levels of phosphorylation in the absence of inhibitors.

Table 1
Effect of stimulant on the relative phosphorylation of the subunits of FcεRI

Stimulation	Cd ^a	Total PY on receptors (normalized) ^b	γ ₂ /β ^c	Syk PY (normalized) ^d
3 ng/ml (DNP-cap) _{3,5} -Fab	—	“1”	1.7 ± 0.1	“1”
3 ng/ml (DNP-cap) _{3,5} -Fab	+	1.2 ± 0.1	2.0 ± 0.1	1.1 ± 0.1
250 ng/ml (2NP-cap) _{3,5} -Fab	—	1.6 ± 0.2	1.1 ± 0.2	0.45 ± 0.1
250 ng/ml (2NP-cap) _{3,5} -Fab	+	3.8 ± 0.6	2.4 ± 0.2	1.0 ± 0.005

^a CD: cytochalasin.
^b Total PY on receptors: three experiments.
^c γ₂/β: DNP, three experiments; 2NP, 11 experiments.
^d Syk: two experiments.

3.6. Interaction of receptor with Lyn

Although as noted in Section 1, phosphorylation of the receptor is the first observable modification, a variety of experimental results have led to a model involving a several molecular interactions that precede the covalent changes (Faeder et al., 2003). In order to investigate whether the increased phosphorylation of receptors in the presence of latrunculin A results from the enhanced interaction of receptors with Lyn, the amount of Lyn associated with receptor was assessed by co-immunoprecipitation after chemical cross-linking using permeabilized cells (Yamashita et al., 1994). However, in permeabilized cells, the increase of receptor phosphorylation by latrunculin A was not observed (data not shown). Possibly, permeabilization leads to the loss of monomeric actin molecules so that formation of polymerized actin is reduced much as it is inhibited by addition of latrunculin. We modified our procedure and used a permeant cross-linking reagent with intact cells. The results revealed no difference in the level of Lyn kinase associated with the Fc ϵ RI in the presence and absence of latrunculin (Fig. 7A). No increase

in receptor-associated kinase was observed after aggregation with the 2NP ligand possibly because cross-linking takes a finite time whereas the small, aggregation-induced increase in receptor-associated kinase (Yamashita et al., 1994) is transient, particularly when the rapidly dissociating ligand is used.

3.7. Activity of Lyn

It was possible that the actin-disrupting agents were directly or indirectly affecting the activity of Lyn (Section 4). Using conditions similar to those described in the prior paragraph but using the minimal concentration of cross-linking reagent necessary to stabilize the full complement of receptor-associated Lyn, we immunoprecipitated the chemically-crosslinked but unaggregated receptors via their bound IgE and subjected them to an *in vitro* kinase assay. The results are shown in Fig. 7B. The amount of receptor-associated Lyn was unaffected by the actin-disrupting reagents (above) so to compare validly the receptor–Lyn complexes from the treated and untreated cells, little correction of the gross counts was necessary. It is apparent that there was no significant difference. No increase in the activity of the receptor-associated kinase was observed after aggregation with the 2NP ligand (data not shown).

3.8. Interaction of receptor with phosphatases

3.8.1. Dephosphorylation by disaggregation of receptors

In order to determine whether disruption of actin filaments altered the susceptibility of the phosphorylated Fc ϵ RI to phosphatases, or the activities of the phosphatase(s) that dephosphorylate the receptors upon disaggregation, we first compared the rate of dephosphorylation in the presence and absence of actin inhibitors of receptors disaggregated by addition of hapten. As shown in Fig. 8A, we found no evidence that cytochalasin D inhibited the dephosphorylation of the disaggregated receptors.

3.8.2. Dephosphorylation by competition

We next tested to see if the rate of dephosphorylation induced simply by dissociation of Lyn from the receptors was affected by the inhibitors of actin. In the RBL cells, the amount of Lyn available to Fc ϵ RI appears to be limited, and the phosphorylation of aggregated Fc ϵ RI reflects a dynamic balance between the action of kinase and phosphatases (Kent et al., 1994; Mao and Metzger, 1997). We demonstrated this previously with a protocol that establishes a competition between distinguishable aggregates of receptors for the limited Lyn (Torigoe et al., 1997). In those experiments, cells were first incubated with monomeric anti-DNP mouse IgE to partially fill the Fc ϵ RI, and then with dimeric rat IgE. The receptors that bound the dimeric IgE became phosphorylated whereas those associated with the monomeric anti-DNP IgE (and, hence, unaggregated) remained unphosphorylated. Upon addition of a multivalent

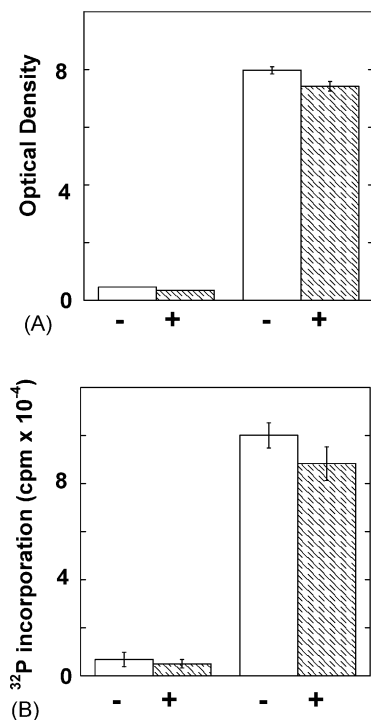


Fig. 7. Effect of latrunculin on the interaction of unaggregated Fc ϵ RI with Lyn kinase (A) and the specific activity of Lyn kinase (B). Cells were reacted (+) or not (–) with latrunculin at 2 μ M for 10 min at 37 $^{\circ}$ C and then reacted with a chemical cross-linking reagent for 30 min at room temperature in the continued presence of latrunculin. The cells were then solubilized and the amount of Lyn was estimated from Western blots. The activity of Lyn kinase bound to the immunoprecipitated Fc ϵ RI was assayed *in vitro* against a synthetic peptide. Left two bars: control immunoprecipitates; right two bars: anti-IgE immunoprecipitates. (A) Average results from a single experiment consisting of duplicate samples. The error bars show the range. A second experiment gave comparable results. (B) Average results ($\pm$ S.D.) from six independent samples.

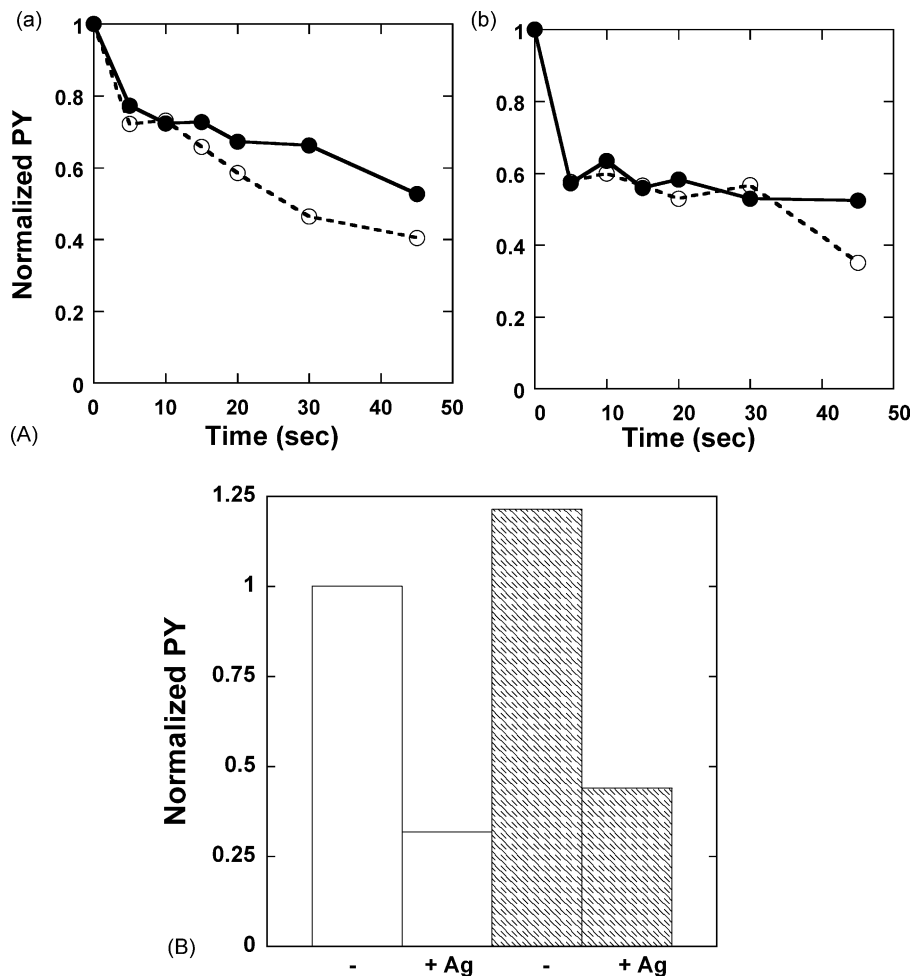


Fig. 8. Effect of cytochalasin on rate of dephosphorylation. (A) Dephosphorylation of disaggregated receptors. Cells were stimulated for 3 min at 37 °C after which hapten was added; (a) 50 ng/ml (DNP-Fab)_{3.5}-Fab + 3 μ M DNP-caproic acid and (b) 250 ng/ml (2NP-cap)_{3.9}-Fab + 3 μ M 2NP-caproic acid. Open circles: no cytochalasin D; filled circles: 2 μ M cytochalasin D. Data are from a single experiment; a second experiment gave similar results. (B) Dephosphorylation of aggregated Fc ϵ RI. Cells were reacted with sufficient monomeric anti-DNP IgE to saturate ~50% of the receptors. After washing the cells were reacted with 0.3 μ g/ml dimeric rat IgE. At 9 min an aliquot was removed and added to ice-cold solubilization buffer. At 10 min 0.1 μ g/ml DNP₇-BSA was added to the remainder and 2 min later these cells were likewise solubilized. Phosphotyrosine on the receptors bound to dimeric IgE was analyzed in each sample. (–) cells analyzed at 9 min, (+ Ag) cells analyzed at 12 min. Open bars: no incubation with latrunculin. Hatched bars: cells pre-treated with 2 μ M latrunculin A.

DNP antigen, vigorous phosphorylation of the receptors bound to the newly aggregated anti-DNP IgE was observed, whereas, the receptors associated with the dimeric IgE became rapidly dephosphorylated. We proposed that upon addition of the DNP antigen, progressive phosphorylation of the excess larger aggregates sequestered the Lyn, depriving the smaller aggregates of their associated kinase. This led to the dephosphorylation of the small aggregates because the action of the phosphatases was now unopposed. We repeated this experiment in the presence and absence of latrunculin A (Fig. 8B). As shown in the figure, generation of the larger aggregates decreased the phosphorylation of the receptors aggregated by dimeric IgE by two-thirds. This decrease was the same in the absence (open bars) or presence (hatched bars) of latrunculin even though the larger aggregates from the treated cells showed the expected enhanced

phosphorylation (not shown). Together these results show that the enhanced phosphorylation of Fc ϵ RI induced by the disruption of actin filaments is not explained either by an enhanced association of Lyn with the Fc ϵ RI or a decreased rate of dephosphorylation of aggregated receptors.

3.9. Role of Csk-binding protein (Cbp/PAG)

There are experimental data supporting a role for the transmembrane adaptor protein, Cbp/PAG, as an indirect molecular governor of Src-family kinase activity (Kawabuchi et al., 2000; Brdicka et al., 2000). When phosphorylated, this protein, exclusively localized to so-called lipid rafts, recruits the cytosolic kinase Csk via the latter's SH2 domain. The membrane associated Csk can then phosphorylate the inhibitory tyrosine on Src-family kinases. Evidence has also

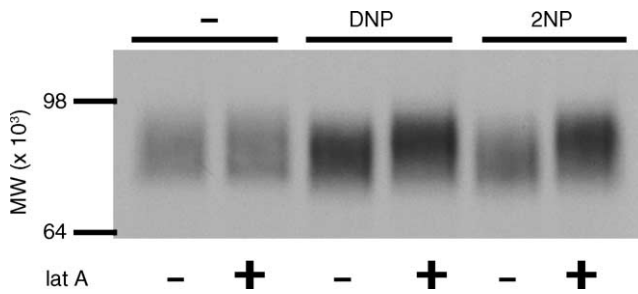


Fig. 9. Effect of latrunculin A on the phosphorylation of Cbp. The figure is a phosphotyrosine blot of Cbp. RBL-2H3 cells were stimulated with 10 ng/ml (DNP-cap)_{4,6}-Fab or 300 ng/ml (2NP-cap)_{5,3}-Fab for 3 min at 37 °C with (+) or without (–) 2 μ M latrunculin A (lat A). Two additional experiments gave similar results.

been presented that this mechanism occurs in RBL-2H3 cells and acts as a feed-back loop with respect to the phosphorylation of Fc ϵ RI (Ohtake et al., 2002). We investigated whether treatment of the cells with actin-disrupting agents affected this mechanism. Cells were pre-treated with 2 μ M latrunculin A for 10 min at 37 °C and stimulated with 10 ng/ml (DNP-cap)_{4,6}-Fab or 300 ng/ml (2NP-cap)_{5,3}-Fab for 3 min at 37 °C conditions that lead to approximately equivalent levels of phosphorylation of Fc ϵ RI. Cbp was immunoprecipitated and phosphotyrosine was analyzed. As shown in Fig. 9, in our experiments, Cbp showed significant constitutive phosphorylation and this phosphorylation was only modestly increased by addition of a cross-linking ligand. The 2NP ligand induced only slightly weaker phosphorylation of Cbp compared to that induced by the DNP ligand. If anything, the level of phosphorylation of Cbp induced by both ligands was increased by the treatment of cells with latrunculin A, and interestingly, the Cbp migrated at an apparently higher molecular weight than that in untreated cells.

3.10. Rotational diffusion measurements

We examined the rotational diffusion of Fc ϵ RI bound IgE to see if we could gain further insight into the interaction

between actin filaments and Fc ϵ RI. Such studies were previously used to examine the membrane dynamics of Fc ϵ RI before and after aggregation as well as to monitor interactions between the receptor and the “mast cell function-associated antigen” ((Song et al., 2002) and references therein). In such time-resolved anisotropy experiments, samples are pulsed with a laser and one determines the initial anisotropy, r_0 , of the erythrosine-conjugated IgE, its final value, r_∞ , and from the rate of decay of the anisotropy, the rotational correlation time. The results on the RBL cells are presented on the upper portion of Table 2.

In the present study, the RCT of the unaggregated receptors on RBL cells was determined to be 21 μ s at 37 °C (line 1) and 99 μ s at 4 °C (line 7) in reasonable agreement with the values of 27 and 81 μ s, determined in a previous study (Song et al., 2002). The data after addition of the paucivalent nitrophenyl-conjugated Fab fragments (cf. lines 2 and 3 with line 1; lines 5 and 6 with line 4) the differences are not statistically significant. With a multivalent BSA-conjugate (line 7) a more substantial slowing of rotation is observed. Treatment with cytochalasin D failed to decrease the RCT (increase the rate of rotational diffusion) of either the unaggregated or aggregated receptors significantly.

3.11. Studies on CHO transfectants

In order to investigate early signaling events in isolation, we had previously transfected CHO cells with Fc ϵ RI and Lyn (Vonakis et al., 1997). In those transfectants, we observed an unusually high level of spontaneous receptor phosphorylation (Torigoe and Metzger, 2001). To gain additional insight into the possible mechanism by which actin filaments influence the early signaling events, the effect of cytochalasin D on both spontaneous and antigen-induced receptor phosphorylation of one of those clones (A11) was examined. Fig. 10 compares the responses of RBL and A11 cells to stimulation with the 2NP antigen in the presence and absence of cytochalasin D. In RBL cells (Panel a), cytochalasin D amplified the response more than three-fold, whereas in the cells of clone A11 it induced no enhancement. In a

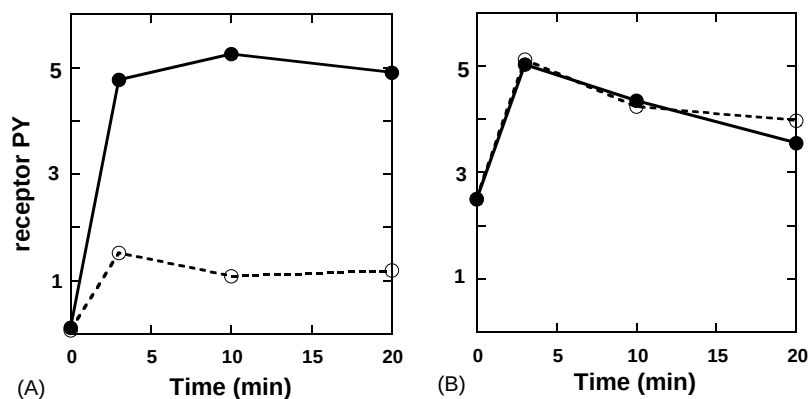


Fig. 10. The effect of cytochalasin D on the phosphorylation of Fc ϵ RI in CHO transfectants. (A) RBL cells and (B) CHO cells transfected with both Fc ϵ RI and Lyn kinase were stimulated with 250 ng/ml (2NP-cap)_{3,9}-Fab at 37 °C for 3 min, in the presence (●) or absence (○) of 2 μ M cytochalasin D.

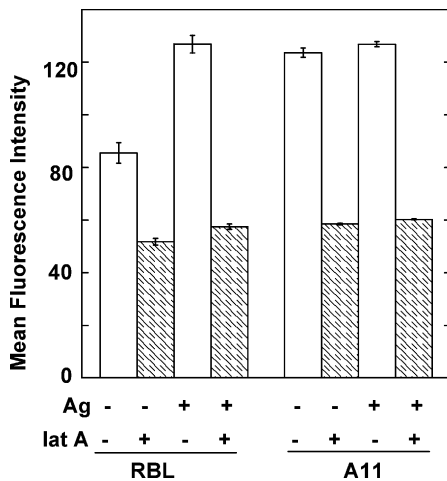


Fig. 11. The effect of latrunculin A on actin polymerization. Cells were stimulated with 1 μ g/ml DNP₃₀-human serum albumin (Ag) for 3 min at 37°C in the presence or absence of 2 μ M latrunculin A (lat A). Cells were stained with FITC-phalloidin, washed, and analyzed by FACS. The range of the results on duplicate samples that were analyzed for each condition is shown. Two additional experiments gave comparable results.

repeat experiment in which the responses were measured at 3 min only, similar results to those observed with cytochalasin were seen with latrunculin A (data not shown).

In the earlier study we observed an altered electrophoretic mobility of the α chain of Fc ϵ RI likely due to changed glycosylation of the subunit's exodomain and proposed that this might have led to spontaneous association of the Fc ϵ RI either with other surface proteins or with each other. The elevated values of r_0 , r_∞ , and RCT shown in the lower half of Table 2 are consistent with that proposal.

A possible explanation for the failure of actin-disrupting agents to enhance the early signaling events in the transfectants might relate to the failure of the aggregated receptors to stimulate formation of f-actin in those cells. Fig. 11 compares the amount of phalloidin bound by resting and stimulated RBL-2H3 cells and the A11 transfectants in the presence and absence of latrunculin. In the RBL cells, binding of phalloidin increases when Fc ϵ RI on the cells are vigorously aggregated. Incubation with latrunculin suppresses both the resting level of bound phalloidin and eliminates the receptor-induced enhancement. In the transfectants the resting level is equivalent to that observed in the RBL cells but no enhancement is observed when the receptors are aggregated. Addition of the actin-disrupting agent reduces the binding of phalloidin to the level observed in the latrunculin treated resting RBL cells.

4. Discussion

Considerable experimental evidence favors a specific molecular model for how a multivalent antigen (or a surrogate stimulus) reacting with Fc ϵ RI-bound IgE on the surface of mast cells stimulates a variety of responses. In

Table 2
Rotational diffusion of Fc ϵ RI on RBL and transfected CHO cells in the presence and absence of actin-disrupting agents

Cell	Ag	Temperature (°C)	$r_0 \times 1000$			$r_\infty \times 1000$ inhibitor			RCT (μ s)		
				CytD	Lat.		CytD	Lat.		CytD	Lat.
1	RBL	37	30 $\pm$ 1.8(5)	32 $\pm$ 0.9(5)	26 $\pm$ 1.1(3)	14 $\pm$ 2.2(5)	16 $\pm$ 3.1(5)	11 $\pm$ 5.2(3)	21 $\pm$ 1.8(5)	19 $\pm$ 2.0(4)	39 $\pm$ 9.2(3)
2	RBL	37	41 $\pm$ 1.2(3)	43 $\pm$ 1.2(3)	14 $\pm$ 1.7(3)	18 $\pm$ 2.3(3)	-	19 $\pm$ 4.0(3)	21 $\pm$ 2.3(3)	-	-
3	RBL	37	46 $\pm$ 1.2(3)	43 $\pm$ 1.2(3)	-	16 $\pm$ 1.2(3)	16 $\pm$ 1.2(3)	-	31 $\pm$ 1.7(3)	21 $\pm$ 5.8(3)	-
4	RBL	25	41 $\pm$ 1.2(3)	49 $\pm$ 2.9(3)	40 $\pm$ 0.6(3)	26 $\pm$ 3.5(3)	20 $\pm$ 2.9(3)	15 $\pm$ 4.0(3)	25 $\pm$ 8.1(3)	30 $\pm$ 2.9(3)	48 $\pm$ 3.5(3)
5	RBL	25	49 $\pm$ 5.8(3)	57 $\pm$ 2.9(3)	-	16 $\pm$ 1.7(3)	25 $\pm$ 2.3(3)	-	35 $\pm$ 7.5(3)	33 $\pm$ 6.9(3)	-
6	RBL	25	43 $\pm$ 5.2(3)	58 $\pm$ 1.7(3)	-	13 $\pm$ 1.7(3)	27 $\pm$ 3.5(3)	-	45 $\pm$ 12(3)	30 $\pm$ 7.5(3)	-
7	RBL	4	43 $\pm$ 0 (2)	43 $\pm$ 4.9(2)	-	32 $\pm$ 0 (2)	32 $\pm$ 0.7(2)	-	81 $\pm$ 7.1(2)	61 $\pm$ 2.1(2)	-
8	A11*	37	52 $\pm$ 2.0(4)	-	-	79 $\pm$ 3.5(4)	-	-	165 $\pm$ 14(4)	-	-
9	All	37	55 $\pm$ 1 (3)	-	-	79 $\pm$ 3.5(4)	-	-	252 $\pm$ 64(4)	-	-
10	All	25	56 $\pm$ 3.8(7)	-	-	81 $\pm$ 7.6(7)	-	-	289 $\pm$ 80(7)	-	-
11	All	25	60 $\pm$ 5.2(3)	-	-	83 $\pm$ 2.9(3)	-	-	244 $\pm$ 15(3)	-	-
12	All	15	53 $\pm$ 2.9(6)	-	-	70 $\pm$ 4.1(6)	-	-	248 $\pm$ 55(6)	-	-
13	All	15	72 $\pm$ 7.5(4)	-	-	92 $\pm$ 7.0(4)	-	-	366 $\pm$ 124(4)	-	-
14	All	4	58 $\pm$ 0.7(2)	-	-	70 $\pm$ 2	-	-	201 $\pm$ 71(2)	-	-

brief, this model posits that in resting cells, a fraction of the receptors are weakly associated with Lyn kinase. Although incapable of phosphorylating the monomeric receptor with which it is associated, the kinase can “transphosphorylate” tyrosines in the immunoreceptor tyrosine activation motifs (Reth, 1989; Cambier, 1995) on receptors that have become approximated through the antigen-mediated aggregation of the IgE. Once phosphorylated, such receptors associate more stably with additional molecules of Lyn. The phosphorylated receptors—specifically their phosphorylated γ chains—recruit Syk kinase whose subsequent phosphorylation is required for many if not all of the downstream responses. A dynamic balance between the kinase and still-unidentified phosphatases that rapidly dephosphorylate the receptors, favors the kinase only as long as the receptors remain aggregated. At least in the cells of the mucosal mast cell line RBL-2H3, the stimulation of phosphorylation of the Fc ϵ RI appears not to involve a dramatic increase in the specific activity of Lyn kinase (Yamashita et al., 1994) nor changes in the activity of phosphatases or susceptibility of the phosphorylated receptors to them (Mao and Metzger, 1997). These early post-aggregation events, need not (Yamashita et al., 2000; Vonakis et al., 2001; Kovarova et al., 2001), but normally likely do occur in the context of lipid rafts (Field et al., 1995).

The model accounts for the stimulated phosphorylation of the receptors, but until recently no corresponding molecular explanation for the “spontaneous” dephosphorylation of the aggregated receptors has been proposed. Evidence for one inhibitory mechanism has been presented by two groups (Kawabuchi et al., 2000; Brdicka et al., 2000) and extended to RBL cells by another (Ohtake et al., 2002). The former groups discovered a 46 kDa transmembrane protein localized in glycosphingolipid-enriched membrane microdomains (GEMs) and dubbed Cbp (for “Csk-binding protein”) or PAG (for “phosphoprotein associated with GEMs”), which was phosphorylated by Src-family protein tyrosine kinases. Phosphorylation of Cbp recruited C-terminal Src-family kinase (Csk) to the membrane where it could phosphorylate and thereby inactivate the critical Src-family kinase. Ohtake et al. observed enhanced phosphotyrosine on Cbp and recruitment of Csk to the membrane after aggregation of Fc ϵ RI. Over-expression of Cbp inhibited cellular responses to Fc ϵ RI. The authors concluded that, whereas, in T lymphocytes the action of Cbp may be largely to repress Src-family kinases in resting cells (Brdicka et al., 2000), its action in RBL cells was to suppress excessive responses. If the actin filament disrupting agents were working by this mechanism, then one would expect activation-induced phosphorylation to have been reduced in the presence of the inhibitors. But in our experiments, the modest stimulus-enhanced phosphorylation of Cbp was if anything *augmented* by inhibiting the formation of microfilaments, making it an unlikely that the diminished inactivation of Lyn by Csk accounts for the enhancing effect of disrupting actin microfilaments.

As mentioned in Section 1, excessive aggregation inhibits aggregation-dependent signaling (Becker et al., 1973; Magro and Alexander, 1974; DeLisi and Siraganian, 1979) and this phenomenon is significantly dependent on actin-containing microfilaments. Some insight was gained from experiments that investigated the shuttling of Lyn kinase between Fc ϵ RI in RBL cells (Torigoe et al., 1997). We observed that extensively aggregated receptors became phosphorylated at the expense of minimally aggregated (dimerized) receptors, but that with time, the latter became re-phosphorylated at the expense of the larger aggregates. We interpreted these findings as indicating that the Lyn kinase that had been sequestered by the extensively aggregated receptors, had dissociated from the latter, and became re-associated with the dimeric ones. What remained unclear, however, was whether the dephosphorylation of the larger aggregates resulted from, or was responsible for, the dissociation of the Lyn kinase. Frigeri and Apgar (1999) hypothesized that, “...microfilaments are uncoupling Lyn from the crosslinked receptors” possibly by altering the access of aggregated receptors to the detergent-resistant domains, and others (Wilson et al., 2000; Holowka et al., 2000) likewise emphasize the role of microfilaments in segregating raft components from the aggregated Fc ϵ RI (Table 2).

The results of the present studies have provided further insight into the enhancing action of the actin-disrupting agents in the following ways.

- (1) We have obtained further evidence that at least some of the effects of f-actin relate to the very earliest events. This was most convincingly shown by the changes in the relative phosphorylation of the β and γ subunits when the receptors were aggregated by the rapidly rather than the slowly dissociating ligand. Although, our earlier studies using either DNP (Pribluda et al., 1997) or 2NP (Torigoe et al., 1998) antigens had failed to demonstrate a clear-cut sequential phosphorylation of the subunits, a more detailed examination (Table 1) clearly provides evidence for such a phenomenon with the rapidly dissociating ligand. These results are consistent with evidence for an ordered phosphorylation of the β chain (Pribluda et al., 1997; Jouvin et al., 1994; Lin et al., 1996; Ortega et al., 1999) and for a β -dependent phosphorylation of the γ subunit (Jouvin et al., 1994; Lin et al., 1996). That the ratio of phosphorylated γ_2 : β was normalized by the actin filament disrupting agents underscores that the inhibitory effect of actin filaments occurs at the earliest point in the biochemical cascade.
- (2) Our experiments have ruled out several possible mechanisms any one of which could have led to an augmented phosphorylation of receptors. Treatment of the cells with cytochalasin or latrunculin prior to aggregating their Fc ϵ RI failed to reveal any increase in the amount of constitutively associated Lyn kinase (Fig. 6A) nor in its specific activity (Fig. 6B). Nor did it stabilize the interaction between the dimerized receptors and

Lyn (Fig. 7B). It has been suggested that by segregating aggregated receptors from rafts one removes them from an environment protected from phosphatases (Holowka et al., 2000). But other data suggest that proteins in rafts are not protected from such attack (Peirce and Metzger, 2000). That the actin filament disrupting reagents did not diminish the rapid dephosphorylation of aggregated FcεRI (Fig. 7B) also seems inconsistent with a mechanism based on exclusion from phosphatases.

- (3) The results of experiments in which the level of actin was measured directly in RBL cells and the transfected CHO cells suggest that it is only the actin newly formed by stimulation that constrains the level of phosphorylation of the receptors.
- (4) Our new data also support the basic notion that large aggregates become segregated from Lyn kinase (Torigoe et al., 1997; Frigeri and Apgar, 1999; Wilson et al., 2000; Holowka et al., 2000). We would add, however, that such segregation may more frequently occur in laboratory experiments where one frequently uses more aggressive stimulation than may occur physiologically to promote easily detectable cellular responses. The experiments showing that small stable (Kent et al., 1994; Torigoe et al., 1997), or reversible (Fig. 5) aggregates can continue to generate signals for prolonged times, may be closer to reality. However, the specific molecular interactions that promote the actin filament mediated segregation of larger aggregates from Lyn kinase remain to be determined.

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