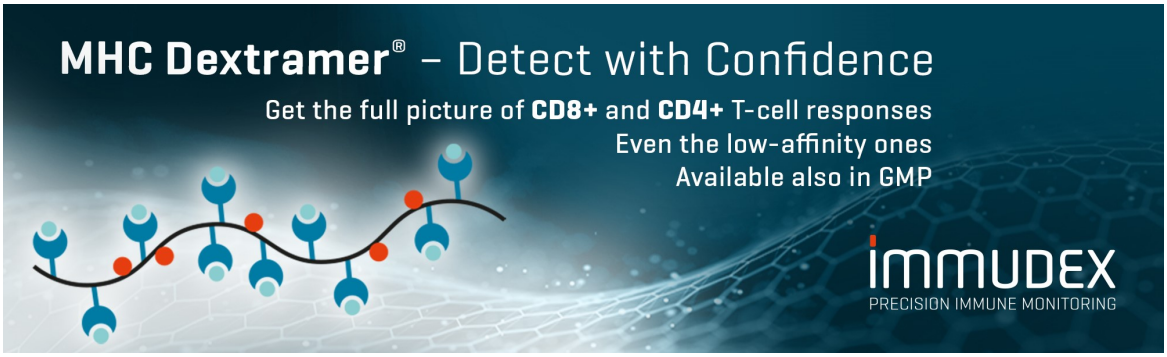

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Two Regions of Down-Regulation in the IgE-Mediated Signaling Pathway in Human Basophils[1]

Donald MacGlashan, Jr.[2]

Previous studies demonstrated that after stimulation of human basophils with a polyclonal anti-IgE Ab, early signaling elements showed sustained phosphorylation, whereas later elements were transient, suggesting that a region of down-regulation involved inhibition of phosphatidylinositol (PI) 3 kinase or its products. However, the current studies show that under some conditions, syk phosphorylation is transient. Generally, stimulation with a variety of Ags makes this early form of down-regulation more apparent. An exploration of the conditions needed to induce early down-regulation indicates that both the nature of aggregation and the cell surface density of IgE play roles. It was also found that the previously described late form of down-regulation (PI3 kinase product transience) can occur in cells displaying early down-regulation (transient syk phosphorylation), but this phenomenon is revealed by testing for subsequent down-regulation of the response to non-cross-reacting stimuli, altering their ability to induce phosphorylation of Akt or extracellular signal-regulated kinase. In contrast, phosphorylation of syk kinase, in response to a non-cross-reacting stimulus, was relatively unaffected by prior stimulation. The magnitude of cross-desensitization of the Akt or extracellular signal-regulated kinase response was a function of the strength of the first stimulus. Mediator release showed a similar cross-desensitization effect. Therefore, stimulation induces two forms of down-regulation, one operating before or at the level of syk phosphorylation, possibly characterizing the process formerly known as specific desensitization, and one that operates in the region of PI3 kinase, accounting for the process formerly known as nonspecific desensitization, which is dependent on the strength of stimulus. *The Journal of Immunology,* **2003, 170: 4914–4925.**

Activation of cells is always accompanied by processes that limit the ongoing response. There are a variety of mechanisms responsible for these down-regulatory events that range from removal of receptors to alterations in signaling elements within the cell. Down-regulation of IgE-mediated secretion from basophils and mast cells was initially characterized 30 years ago and was termed desensitization (1). Although the process of desensitization was typically characterized by first stimulating the cells under suboptimal conditions, e.g., in the absence of extracellular Ca^{2+} to inhibit secretion, the process is active during ongoing secretion (2). Within the general population there is an excellent inverse correlation between the rate that a donor's basophils can be shown to desensitize and the maximum IgE-mediated histamine release that can be obtained (3), suggesting that this process is an important determinant of mediator release from these cells.

The mechanism of desensitization in human cells is not yet known. Whereas a great deal is known about IgE-mediated signaling in rat basophilic leukemia (RBL)[3] cells, the process of desensitization, as it has been characterized in human basophils or mast cells, is not so clear cut in the RBL cell. For example, the operational method of first stimulating the cells in the absence of extracellular calcium to down-regulate the response does not induce significant desensitization in the RBL cell (4, 5). In addition, aggregating FcϵRI in RBL cells results in its rapid internalization (6, 7), whereas in human basophils, internalization, if it occurs at all, does not occur on a time frame coincident with desensitization (8). Because this is a common mechanism of down-regulation for receptor-driven signaling, its absence in human basophils suggests that the mechanism of desensitization needs to be studied in human basophils or mast cells. Nevertheless, numerous studies in RBL cells and other mast cell models have shown that signaling is self-limiting in these cells as well and that some of the mechanisms may still apply to the signaling cascades in human basophils or mast cells. For example, there appears to be a down-regulatory mechanism that involves the cytoskeleton and its regulation of lipid raft constituents (9, 10). Agents that inhibit cytoskeletal formation markedly enhance secretion in RBL cells and have the same functional effect in basophils (11). Recent studies in murine mast cells suggest that Src homology 2-containing 5′-inositol phosphatase (SHIP) is a critical down-regulator of ongoing secretion whose absence leads to hypersecretion (12), and a similar finding may apply to human basophils (13, 14).

Recently, we demonstrated that basophils stimulated with a polyclonal anti-IgE Ab showed a persistence in signaling elements that precede activation of phosphatidylinositol (PI) 3 kinase (syk, shc, growth factor receptor-bound protein-2/son-of-sevenless, p85 subunit of PI3 kinase), but showed a transient activation of $p21^{ras}$ pathway elements ($p21^{ras}$, mitogen-activated protein kinase kinase, extracellular signal-regulated kinase 1/2 (ERK1/2)) as well as transient phosphorylation of Akt (14). Together with studies on the role PI3 kinase plays in basophil secretion, these results suggested that regulation of the ERK pathway was exerted somewhere between the activity of PI3 kinase and its direct or indirect activation of $p21^{ras}$. In the context of mediator secretion from basophils, these results were consistent with the observations that

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[3] Abbreviations used in this paper: RBL, rat basophilic leukemia; SHIP, Src homology 2-containing 5′-inositol phosphatase; PI, phosphatidylinositol; ERK, extracellular signal-regulated kinase; LTC_4, 5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; HSA, human serum albumin; BPO, anti-penicilloyl; EACA, ϵ-aminocaproic acid; h, human; PIP3, phosphatidylinositol 3,4,5-phosphate.

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5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (LTC_4) secretion is rapid, whereas IL-4 secretion requires several hours. In other words, stimulation with anti-IgE Ab results in a sustained phosphorylation of syk kinase and this is consistent with the long duration of IL-4 secretion, whereas anti-IgE also only induces a transient activation of ERK phosphorylation (with ERK activity being required for cytosolic phospholipase A_2 phosphorylation and, therefore, LTC_4 release), which is consistent with the short duration of LTC_4 secretion (15).

While examining the persistence of early signaling, we asked whether the sustained phosphorylation of syk kinase or one of its downstream effectors (16), shc, would be sensitive to cessation of the earliest steps in signaling. Disaggregation of receptors would have been the best approach, but this was not readily available for studies with the particular polyclonal anti-IgE Ab used, so the ability of 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4,-d]pyrimidine, a lyn kinase inhibitor, to stop signaling after 60 min of sustained signal was tested. Although these experiments demonstrated that shc phosphorylation required the sustained activation of upstream signals, presumably provided by lyn kinase, we sought to re-examine the question using a multivalent Ag to initiate and maintain signaling. Disaggregation was to be accomplished with the addition of monovalent hapten. However, these studies demonstrated unexpected behavior, and the results are presented. Furthermore, because the results with Ag-driven signaling revealed down-regulation that occurred earlier than observed after stimulation with anti-IgE Ab, we asked whether Ag-induced signaling could be demonstrated to also induce the type of down-regulation observed with anti-IgE Ab.

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, and FMLP (Sigma-Aldrich, St. Louis, MO); crystallized human serum albumin (HSA; Miles Laboratories, Elkhart, IN); FCS and RPMI 1640 containing 25 mM HEPES and L-glutamine (BioWhittaker, Walkersville, MD); Percoll (Pharmacia, Piscataway, NJ); Tris (hydroxymethyl)-aminomethane, and Tween 20 (Bio-Rad, Hercules, CA); leupeptin, DTT, and PMSF (Boehringer Mannheim, Indianapolis, IN); anti-phosphotyrosine mAb (4G10) and rabbit anti-son-of-sevenless 1 Ab (Upstate Biotechnology, Lake Placid, NY); rabbit anti-phospho-ERK Ab, recombinant ERK-2 ($p42^{MAPK}$) protein, anti-phospho Akt (Thr^{308}-specific), anti-Akt, and biotinylated protein markers (Cell Signaling, Beverly, MA); anti-syk mAb (Santa Cruz Biotechnology, Santa Cruz, CA); and HRP-conjugated donkey anti-rabbit Ig Ab, HRP-conjugated sheep anti-mouse Ig Ab, and protein G Sepharose beads (Amersham Life Science, Arlington Heights, IL). Goat anti-human IgE Ab was prepared as previously described (17). Anti-penicilloyl (BPO) IgE was purified from serum of penicillin-allergic patients as previously described (18) and was typically used at 5 μg/ml in RPMI 1640 buffer containing 0.1 mM BPO-ϵ-aminocaproic acid (BPO-EACA). BPO-HSA and BPO-EACA were synthesized for previous studies (18). Anti-gp120 peptide-specific IgE, its Ag (gp120-OVA), anti-idiotype-specific monoclonal mouse Ab (AB19-4) with specificity for the anti-gp120-IgE Ab (19), and monoclonal anti-IgE Ab were gifts from Tanox (Houston, TX). PS myeloma and the clone to generate anti-DNP mouse IgE (H1 DNP-ϵ-26) were gifts from T. Ishizaka. Anti-DNP mouse IgE was partially purified from ascites fluid as previously described (20). DNP (7)-HSA was prepared as previously described (20). Ragweed-specific IgE was obtained from the serum of a high-titer ragweed-allergic patient, and purified ragweed AgE was a gift from Dr. T. P. King. RBL-SX38 cells (RBL cells transfected with human FcϵRI$\alpha\beta\gamma$) were developed and provided by Dr. J.-P. Kinet (Harvard University, Cambridge, MA).

Buffers

PIPES-albumin-glucose (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) electrophoresis sample buffer containing 5% 2-ME. Complete lysis buffer (CLB) is 20 mM Tris-HCl (pH 7.5), 100 μg/ml aprotinin, 10 mM benzamidine, 1 mM PMSF, 100 μg/ml leupeptin, 50 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P-40, and 10% glycerol. Incomplete lysis buffer (ILB) is CLB without the protease inhibitors, Nonidet P-40, glycerol, or vanadate. Stripping buffers were either 7 M guanidine hydrochloride or 65 mM Tris-HCl (pH 6.7), 100 mM 2-ME, and 2% SDS. The sensitivity of the subsequent blotting to the choice of stripping agent determined which of these two were used.

Basophil purification

For most 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 previously described (21). The cells were further purified by negative selection using MACS basophil isolation kit (Miltenyi Biotec, Auburn, CA). More recently, we have used a mixture of Abs from Stem Cell Technologies (Vancouver, BC, Canada) and columns from Miltenyi Biotec for negative selection (basophil purification kit). The purity of basophils was determined by alcian blue staining (22), and basophils purified from leukapheresis packs generally exceeded 99% purity.

Phosphorylation of ERKs and Akt

The phosphorylation of ERKs and Akt was assessed using phospho-ERK Ab or phospho-Akt Ab, respectively. After stimulating basophils ($0.5–1 \times 10^6$ cells per sample) in PAGCM buffer, reactions were stopped by adding ice-cold PAG and were microfuged for 5–10 s. After collecting the supernatant (for the measurement of histamine and LTC_4), cell pellets were immediately lysed in ESB (electrophoresis buffer; NOVEX) and separated on 10% Tris glycine gels (NOVEX). Electrophoresis and transfer were performed as described in the product literature. The membranes were immersed in TBST containing 2% nonfat dried skim milk (Carnation, Los Angeles, CA) or 4% BSA overnight to block nonspecific binding. Immunoreactive proteins were detected using the Abs, which were diluted in TBST containing 2% skim milk or 4% BSA for 90 min. After washing, the membranes were incubated with HRP-conjugated anti-rabbit Ab for 1 h. After washing, ECL detection (Amersham, Piscataway, NJ) was performed as described in the product literature. The same membranes were sequentially blotted with both anti-phospho-ERK Ab and anti-pAkt mAb and reblotted after stripping in SDS buffer with anti-Akt Ab to assess equal loading.

Immunoprecipitation

After stimulating basophils ($1.5–5 \times 10^6$ cells per sample) in PAGCM buffer at 37°C, the reactions were stopped by centrifugation at $14,000 \times g$ for 5–10 s. The cell pellets were immediately lysed in CLB buffer and, after a 10-min incubation on ice, they were centrifuged for 3 min at $16,000 \times g$ to remove nuclei or undissolved components. Lysates were precleared with protein G Sepharosebeads for 30 min at 4°C to remove any nonspecific binding to the beads. The clarified lysates were then incubated with specific Ab prebound to protein G Sepharosebeads (usually 1 μg per 20 μl of beads) at 4°C. After a 1-h incubation, the beads were washed three times with CLB buffer. The immunoprecipitated proteins were eluted by boiling in ESB. For detection of phosphotyrosines, the membranes were blotted with 4G10. The membranes were stripped with SDS buffer and reprobed with anti-syk Ab to determine loading. ECL films were converted to digital images with a Kodak DC290 camera (Kodak, Rochester, NY), and the bands were analyzed with NIH Image (National Institutes of Health, Bethesda, MD). Data from the anti-phosphotyrosine blots were normalized for loading differences using the band intensities from the secondary blot with anti-syk. By and large, there was a $\pm$16% variation in lane loading.

LTC_4 and histamine measurements

An RIA was performed using 100 μl of supernatant to determine LTC_4 levels as previously described (23). Histamine was measured by automated fluorometry (24). The percentage of total histamine release was calculated after subtraction of spontaneous histamine release. Each condition tested was performed in duplicate in histamine release experiments using impure cells.

Sequential stimulation experiments

Basophils, either purified or obtained from single-step Percoll gradients (1–2% purity), were sensitized with DNP-specific mouse IgE. The concentration of mouse IgE was usually 5 μg/ml, but in some experiments lower concentrations were used. For many of the experiments using purified basophils to measure ERK or Akt phosphorylation, the cells were

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treated with lactic acid briefly (6–15 s) before sensitization to generate many more unoccupied receptors and to increase loading with the anti-DNP-specific mouse IgE (25, 26). There is great variation in the ability of this procedure to dissociate IgE, so there was a wide range in the ability to subsequently load the cells with DNP-specific IgE. In general, the cells were stimulated in PAGCM buffer with optimal concentrations of the relevant stimuli. For DNP(7)-HSA, the peak response for both histamine and LTC_4 release is 3 μg/ml, and for polyclonal goat anti-IgE Ab it is 0.2 μg/ml. For sequential stimulation, the cells were generally first stimulated with 3 μg/ml DNP-HSA, followed by the addition of 0.2 μg/ml anti-IgE Ab. In some experiments with impure cells, the order was reversed.

Flow cytometry

Cell surface expression of FcϵRIα-chain was detected using a mouse IgG1 anti-human FcϵRIα-chain mAb (22E7; generously provided by J. Kochan (Hoffman-LaRoche, Nutley, NJ)) and was compared with labeling with an identical concentration of irrelevant mouse IgG1 (Coulter, Hialeah, FL). The 22E7 Ab has been shown to recognize an epitope that is unaffected by FcϵRIα occupancy. Unoccupied FceRI was detected using another Roche Ab, 15A5. Cell surface IgE that was specific for a gp120 peptide was detected using an anti-idiotypic specific mAb (AB19-4; Tanox, Houston, TX). Aliquots of cells were labeled in PBS containing 0.2% HSA with 1 mg/ml human IgG to minimize nonspecific binding to FcγR. Each of the mAbs were used at concentrations predetermined to be optimal for labeling. Binding of mAbs was detected using saturating concentrations of R-PE-conjugated polyclonal goat anti-mouse IgG (Tago Scientific, Burlingame, CA). Data are expressed as the mean fluorescence in labeled cells minus the mean fluorescence of IgG1 controls.

Results

Preliminary characterization of the Ag-induced response

As noted in the introduction, these studies began with a simple experiment to examine the consequences of disaggregation on the early signaling steps. For the initial studies, we sensitized basophils with penicillin-specific IgE, challenged the cells with BPO(11)-HSA and, after various periods of time, added BPO-EACA to dissociate cross-links. Because syk phosphorylation is presumably one of the earliest signaling steps and is sustained after stimulation with polyclonal anti-IgE Ab, the phosphorylation state of syk was monitored in these experiments. As can be seen in Fig. 1, BPO-HSA, used at a concentration first shown to be optimal for histamine and LTC_4 release, did not induce a sustained phosphorylation of syk. It was possible to observe the effects of BPO-EACA addition, but syk phosphorylation was only marginally above resting levels by 60 min. Because this result was in marked contrast to the relatively sustained signaling after anti-IgE Ab, the conditions for the appearance of a transient early signal were explored.

Concentration of the stimulus

For previous studies, concentrations of Ag or anti-IgE that were previously determined to be optimal for inducing histamine or LTC_4 release were used for stimulation. Because the binding rate of the stimulus partially determines the dynamics of the reaction, syk phosphorylation kinetics were examined across a broader range of concentrations encompassing the suboptimal and supraoptimal regions of the concentration dependence curve for mediator release. Both polyclonal anti-IgE Ab and BPO-HSA were studied. The histamine release dose-response curve for polyclonal anti-IgE Ab is quite narrow, so we examined 0.02, 0.2, and 3 μg/ml, reflecting one suboptimal, one optimal, and one markedly supraoptimal concentration, respectively. For BPO-HSA, cells were challenged with 0.02, 0.1, 0.5, and 5 μg/ml: two suboptimal, one optimal, and one supraoptimal concentration, respectively. For the data shown in Figs. 1 and 2, it is convenient to focus on the ratio of band intensities for syk phosphorylation at 60 min vs 5 min as a measure of the transience of the reaction. Fig. 1*D* demonstrates that even the lowest concentration of BPO-HSA has a 60:5 min ratio of only 0.48 $\pm$ 0.02. For the most part, syk phosphory-

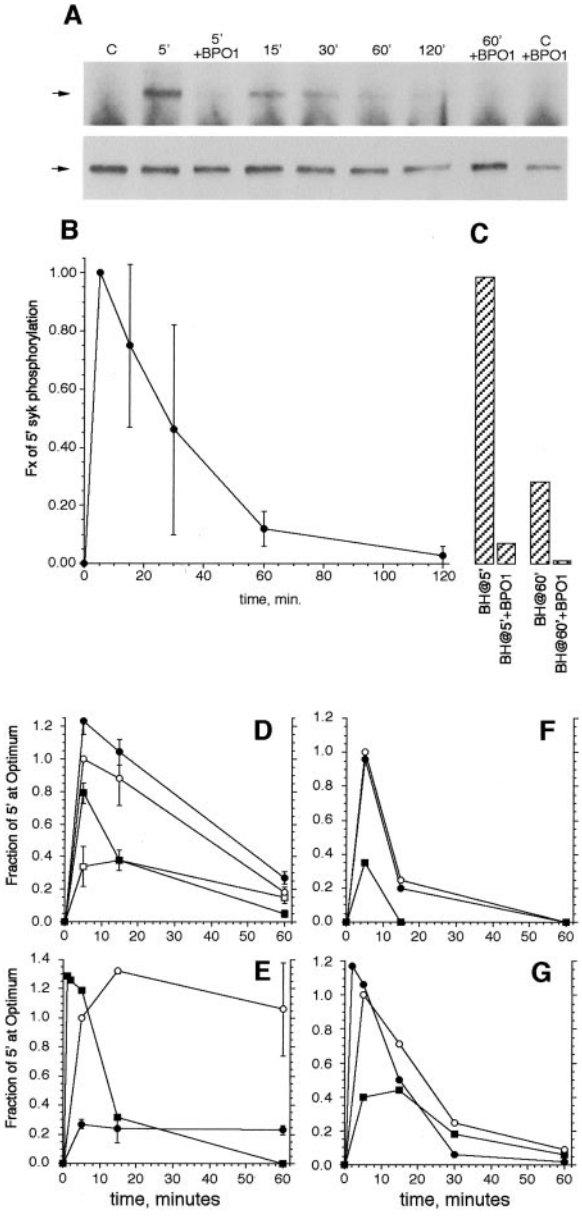

FIGURE 1. Kinetics of syk phosphorylation in BPO-specific IgE-sensitized basophils challenged with 0.5 μg/ml BPO-HSA. *A*, Western blot for one experiment; the top segment represents the 4G10 blot, and the bottom segment represents the syk reblot. The times listed represent the time after the addition of BPO-HSA. *Lanes 3* and *8* show results for the addition of BPO-HSA followed by the addition of 0.1 mM BPO-EACA for 5 min before harvesting. For the three experiments shown in *B*, averaging was done by expressing data at other time points as a fraction of the 5-min time point. *C*, Effect of adding 0.1 mM BPO-EACA to the reaction at the 5- and 60-min time points. Five minutes after the addition of BPO-EACA, the cells were harvested ($n = 2$). *D–G*, Kinetics of syk and ERK phosphorylation after challenge with several concentrations of either BPO-HSA or anti-IgE Ab. *D*, Results for cells sensitized (without IgE dissociation) with BPO-specific IgE and stimulated with 5.0 (●), 0.5 (○), 0.1 (■), or 0.02 (□) μg/ml BPO-HSA ($n = 2$; 0.5 μg/ml is the optimum concentration for histamine and LTC_4 release, although the peak of the concentration dependence is broad). *E*, Results for cells stimulated with three concentrations of polyclonal anti-IgE Ab: 3.0 (■), 0.2 (○), and 0.02 (●) μg/ml ($n = 2$; 0.2 μg/ml is the concentration optimal for histamine and LTC_4 release). *F*, Similar analysis with phospho-ERK as the endpoint after stimulation with BPO-HSA ($n = 1$): 0.5 (○), 0.1 (●), and 0.02 (■) μg/ml. *G*, Phospho-ERK kinetics after anti-IgE Ab at 0.2 (○), 0.05 (●), and 0.02 (■) μg/ml. In all cases the data are expressed as a fraction of the response at 5 min for the optimal concentration of stimulus, 0.5 μg/ml for BPO-HSA and 0.2 μg/ml for anti-IgE.

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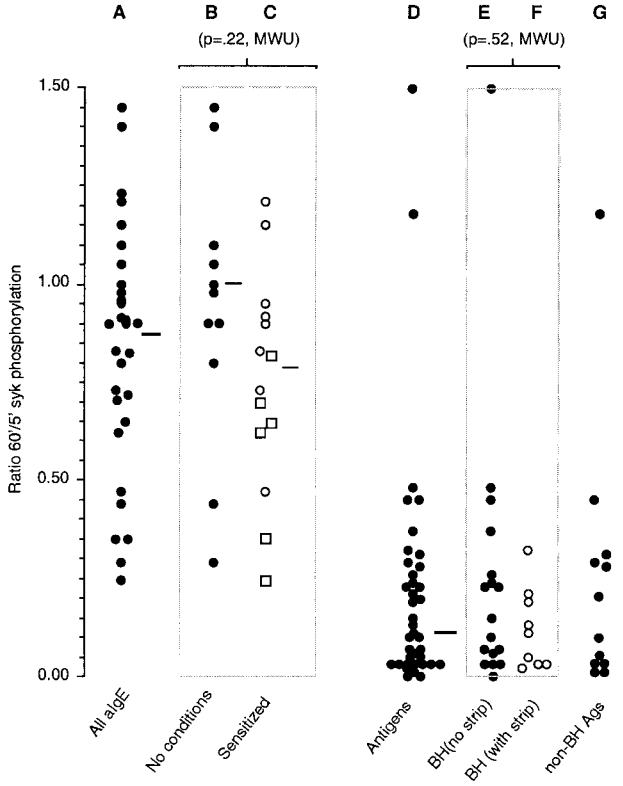

FIGURE 2. Ratios of 60:5 min syk phosphorylation after stimulation with either anti-IgE Ab or Ags. As noted in the text, a number of conditions are represented by the data shown. *A*, All of the data for stimulation with 0.2 μg/ml goat polyclonal anti-IgE Ab. *B* and *C*, Data separated from *A* according to whether the cells had first been sensitized. In this comparison, the medians are not statistically different (Mann-Whitney *U* test). *D*, Aggregate experience with a variety of Ags. In this column, no distinctions are made regarding the nature of Ag or IgE or the conditions of sensitization. The difference in the medians for Ags vs anti-IgE Ab is statistically significant ($p < 0.001$; Mann-Whitney *U* test (MWU)). *E* and *F*, Analysis of the difference between basophils sensitized with or without prior IgE dissociation (using lactic acid buffer). The medians are not statistically different in this comparison. *G*, Non-BPO-HSA Ags tested. The median for this distribution is not statistically different from the median for BPO-HSA Ag.

lation remained relatively transient for all concentrations tested. At concentrations of BPO-HSA that had molar equivalency with the anti-IgE Ab, BPO-HSA continued to show a low 60:5 min ratio, whereas anti-IgE Ab showed sustained syk phosphorylation. The pattern for polyclonal anti-IgE was somewhat more complex. Interestingly, and consistent with prior studies of the supraoptimal side of the polyclonal anti-IgE concentration dependence curve, the 3 μg/ml concentration of anti-IgE did induce a transient phosphorylation of syk (but it induced only 16% of the release observed at its optimal concentration of 0.2 μg/ml).

Transience is not restricted to BPO-HSA

We asked whether transient syk phosphorylation was a unique response to the Ag BPO-HSA and therefore explored several other means of stimulating the basophils. We had available two other purified IgE Abs, specific for either DNP or a peptide of the HIV gp120 protein. The Ags in both cases are multivalent for a simple epitope, a DNP(7)-HSA and a multivalent conjugate of the relevant short peptide of gp120, respectively. We also examined ragweed-specific IgE, where the Ag would have multiple epitopes and the IgE would be a diverse multiclonal preparation with multiepitope specificities. Fig. 2 summarizes our experience. A wide variety of sensitization conditions were used for these experiments, and this is only partially noted in the figure. Specific examples will be discussed below. Fig. 2 also summarizes our current experience with polyclonal anti-IgE Ab. In most cases, the cells were challenged and syk phosphorylation was examined at 5, 15, and 60 min. For the analysis in Fig. 2, the ratio of the 60-min syk phosphorylation state to the 5-min state was calculated. It is evident from the figure that there is a significant difference between the ratios for polyclonal anti-IgE and any antigenic challenge. Specifically, all four Ags are not distinguishable. Ragweed AgE was not different from the single-epitope multivalent Ags, and it didn't matter whether the IgE used for sensitization was monoclonal or polyclonal (e.g., gp120-specific IgE vs BPO-specific IgE). A mix of sensitization conditions was examined. For example, sensitizing the cells on ice vs 37°C was compared, and a similar transience of syk phosphorylation for antigenic challenge was found. This point will be addressed further below but most of the studies that required sensitization were done by sensitizing the cells at near 0°C temperatures. Stripping vs not stripping (prior treatment with lactic acid vs no treatment) to enhance loading of the IgE was also examined. In other words, syk phosphorylation was transient with other Ags even if the IgE was first dissociated to allow high loading densities (more on this below). The transience of a BPO2 (a bivalent BPO-diamino-octane) and BPO(11)-HSA response was compared, and the BPO2 response was found to be as transient as the BPO-HSA response. A comparison of DNP(7)-HSA and DNP(35)-HSA, using cells that had been "stripped" and loaded with DNP-specific IgE, showed a transient response with either DNP-HSA preparation. The transience of syk phosphorylation after challenge with an anti-Id Ab specific for the binding site of gp120-specific IgE (on cells stripped and sensitized with gp120-specific IgE) was examined and it also induced a transient syk response (60:5 min ratio = 0.02). In some of these experiments, both Ag and polyclonal anti-IgE Ab were run in parallel to demonstrate that the general observations shown in Fig. 2 held true within a given basophil preparation. However, the data in Fig. 2 also demonstrate that there is considerable overlap in the distributions for polyclonal anti-IgE Ab and the variety of Ags tested.

We noticed that in many of the direct comparisons of Ag with anti-IgE Ab, there was greater mediator release to the antigenic stimulus than to anti-IgE Ab. For example, mediator release was analyzed in six experiments in which basophils were sensitized with BPO-specific IgE (without "stripping"), and the syk phosphorylation response to BPO-HSA and anti-IgE Ab was compared. On average, the peak phosphorylation of syk after BPO-HSA was 31 $\pm$ 6% of the response to anti-IgE Ab. In contrast, LTC_4 release was 17.0 $\pm$ 7.8 and 9.6 $\pm$ 5.8 ng/10^6 basophils, respectively, and histamine release was 47 $\pm$ 12% vs 28 $\pm$ 6%, respectively. If, for each of six experiments, the mediator release data are normalized for the peak syk phosphorylation response and the ratio of the normalized release for Ag vs anti-IgE is averaged, Ag appears to induce ~10-fold greater LTC_4 release and ~5-fold greater histamine release than does anti-IgE Ab. Comparisons between other Ags (gp120-OVA or AgE) and anti-IgE Ab resulted in similar differences, with Ag being 2.5- to 8-fold better than anti-IgE Ab. Even when the syk phosphorylation response to Ag was greater than to anti-IgE (as occurred in the "stripping" experiments), some difference remained, ranging from 1- to 3.5-fold better mediator release after Ag (per equivalent level of syk phosphorylation). Because of the brevity of the BPO-HSA-induced syk phosphorylation, we examined the kinetics of syk phosphorylation and IL-4 secretion in RPMI 1640 (supplemented with 1 mM calcium) and found that the syk kinetics were similar to those shown in Fig. 1 and that IL-4 kinetics were similar to those previously reported

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(21). Specifically, there was no new IL-4 protein before 60 min, whereas at 60 min syk phosphorylation was 9% of its peak at 5 min. IL-4 release was complete by 160–200 min and syk phosphorylation was not different from control at times ≥120 min (data not shown). Stimulation with anti-IgE Ab generated IL-4 secretion that was similar in onset and duration to that induced by stimulation with BPO-HSA (data not shown).

The density of Ag-specific IgE

For a population median, the basophil expresses ~100,000 FcεRI per cell with only 5,000 of these receptors unoccupied (26). Therefore, sensitization with an Ag-specific IgE (BPO-specific IgE in the experiments above) would typically allow only 5% of the total IgE to be responsive to the specific Ag. In contrast, polyclonal anti-IgE Ab should have access to all cell surface IgE. This leads to a marked difference in the strength of the possible signal. Although there are indications in the literature that a stronger signal sometimes results in more transient responses, it was possible that a higher density of Ag-specific IgE would result in a more sustained syk phosphorylation. To test this hypothesis, basophils needed to be stripped of some of their endogenous IgE. The lactic acid protocol developed by Pruzansky et al. (25) has been used in a variety of previous studies (26), and although it can be harsh on the cells (particularly purified basophils), it is the only practical way of establishing more unoccupied receptors for sensitization. To minimize the effects of the mild acidity on basophil function, the stripping step was restricted to <15 s (typically 6–10 s). With purified basophils, there is a 25–50% loss of cells even with this short treatment, so there were practical limits to our ability to remove IgE. In some experiments, we monitored the loss of endogenous cell surface IgE and appearance of unoccupied receptors by flow cytometry, using anti-IgE Ab, anti-receptor Ab (anti-FcεRIα, 22E7), or anti-unoccupied receptor Ab (anti-FcεRIα, 15A5). Typically, the short lactic acid procedure removes ~50% of the endogenous IgE, with only modest loss (5–25%) of the total receptor density, and this was found to be the case in the following experiments. Purified basophils were treated with lactic acid stripping buffer and split into two conditions, sensitization with 5 μg/ml BPO-specific IgE for 25 min at 37°C or sensitization with a mixture of 0.625 μg/ml BPO-specific IgE and 4.375 μg/ml gp120-specific IgE (or PS myeloma in some cases). Cells from both sensitization conditions were washed and challenged with 0.5 μg/ml BPO-HSA, and syk phosphorylation was measured at 5 and 60 min. The ratio of 60-min syk phosphorylation to 5-min syk phosphorylation was similar for both conditions ($n = 4$): 0.17 ± 0.02 for low-level sensitization and 0.13 ± 0.05 for high-level sensitization, values consistent with the results in Fig. 1. The 60:5 min ratio for stimulation with polyclonal anti-IgE Ab at 0.2 μg/ml in these experiments was 0.66 ± 0.02. It should be noted that in these experiments, the peak of syk phosphorylation after BPO-HSA was 25% of the anti-IgE response in cells sensitized with a low concentration of BPO-specific IgE and 250% of the anti-IgE response in cells sensitized with a high concentration of BPO-specific IgE. These experiments indicated that cell surface density did not alter the ability of BPO-HSA to induce transient syk phosphorylation. In contrast, alterations in IgE density did have some effect on the response to polyclonal anti-IgE Ab. Although it was not possible to dissociate all cell surface IgE with lactic acid, a partial test of stripping on the anti-IgE response was possible. Purified basophils were treated for 20 s with lactic acid, and one-half of the "stripped" cells were resensitized with 5 μg/ml gp120-specific IgE (as an IgE source only) for 1 h on ice. A portion of the cells were set aside before lactic acid treatment and these were also examined for syk phosphorylation kinetics. Another portion of the cells from each condition were analyzed by flow cytometry for the presence of cell surface IgE. This procedure reduced surface IgE to approximately one-fifth the starting density (0.19 ± 0.03), and under these conditions the 60:5 min ratio was reduced from 0.87 ± 0.07 to 0.56 ± 0.14 ($n = 3$). After resensitization, the 60:5 min syk phosphorylation ratio was 1.16 ± 0.08 and the IgE density was 0.52 ± 0.13 of the pre-strip density.

Table I. *syk phosphorylation kinetics for monoclonal and polyclonal anti-IgE ± hIgG*[a]

Condition (min)	Polyclonal Anti-IgE Ab	Monoclonal Anti-IgE Ab
5	1.00	1.00
15	1.01 ± 0.03	1.35 ± 0.17
60	0.84 ± 0.10	0.50 ± 0.04
+HSA (syk 60:5 min ratio)	0.78 ± 0.33	0.36 ± 0.05
+hIgG (syk 60:5 min ratio)	0.98 ± 0.21	0.88 ± 0.21
LTC_4 release (IgG:HSA ratio)	1.29 ± 0.40	3.65 ± 1.10

[a] Comparison of syk phosphorylation kinetics for polyclonal and monoclonal anti-IgE Ab and the effects of nonspecific human IgG. The top half of the table shows the kinetics of syk phosphorylation after stimulation of basophils with 0.2 μg/ml goat polyclonal anti-IgE antibody or 10 μg/ml monoclonal anti-IgE antibody ($n = 3$). Consistent with the method of data reduction used elsewhere, the band densities from 4G10 Western blots were calculated as the fraction of the 5-min time point. For these experiments, the ratio of peak syk phosphorylation for monoclonal anti-IgE Ab/polyclonal anti-IgE Ab was 1.40 ± 0.60. The lower half of the table shows the effects of 25 μM IISA or human IgG (4 mg/ml) on either the duration of syk phosphorylation (the ratio of the band intensities from the 4G10 blot for 60 min vs 5 min is shown) or the amount of LTC_4 release (expressed as the ratio of release in the presence of IgG vs HSA).

Monoclonal anti-IgE Ab was also examined because it may induce a form of aggregation different from polyclonal anti-IgE Ab. Direct comparison of syk phosphorylation at three time points was made for each of three basophil preparations; the data are also shown in Table I. Syk phosphorylation after stimulation with monoclonal anti-IgE Ab was significantly more transient than with polyclonal anti-IgE Ab, although not quite as transient as the bulk of our results with Ags. We considered the possibility that monoclonal anti-IgE Ab might co-cross-link FcγRIIB (known to reside on human basophils) and FcεRI (27, 28). Under these conditions, anti-IgE Ab might deliver two signals, although it would be expected that the signal due to FcγRIIB would be inhibitory (and presumably inhibitory at a site distal to syk). Basophils were stimulated with monoclonal anti-IgE Ab in the presence or absence of 4 mg/ml of human IgG to block binding of the goat IgG (anti-IgE) to FcγRIIB.[4] Syk phosphorylation was the endpoint examined first. A statistically significant increase in the 60:5 min ratio (Table I) was found (0.36 with HSA and 0.88 with human IgG (hIgG)). However, there was no increase in the duration of ERK phosphorylation (data not shown). Nevertheless, LTC_4 release was markedly enhanced with the inclusion of hIgG (Table I). In contrast, cells stimulated with Ag in the presence hIgG did not show enhanced LTC_4 release (data not shown).

The influence of hIgG on the response to polyclonal anti-IgE was then also examined. The 60:5 min ratio for stimulation in the

[4] In the absence of a well-characterized polyclonal anti-IgE Ab in an $F(ab')_2$ form, we have chosen to use human IgG to block potential binding of the goat IgG to any available FcγR receptors. The concentration of solution phase hIgG chosen for these experiments was based on a calculation of the potential local concentration of goat IgG when already bound to surface IgE. The median density of IgE is 100,000 for human basophils, assuming one goat IgG per IgE and a radius of gyration for the unbound Fc portion of the goat IgG of 20 nm beyond the membrane surface, producing a shell volume of 2.5×10^{-14} liters, which any bound goat IgG occupies, or a concentration of bound goat IgG of 1 mg/ml. To compete with this potential concentration of IgG, we preincubated the basophils for 10 min with 4 mg/ml nonspecific human IgG and challenged in the presence of 4 mg/ml human IgG. For a control, an equimolar concentration of HSA was used.

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Table II. *Signaling with monomeric IgE*[a]

Expt.	IgE Source	Stripped	syk Phosphorylation	ERK Phosphorylation	Histamine or LTC_4 Secretion	Ag Response
1	PS myeloma	−	0.05	0.00	−	
	PS myeloma	+	0.25	0.20	+	
2	PS myeloma	−	0.03	<0.005	−	
	gp120IgE	−	0.00	0.00	−	0.37
3	gp120IgE	−	0.00	ND	−	ND
4	gp120IgE	+	ND	<0.005	−	ND
	DNPIgE	+	ND	<0.005	−	ND
5	gp120IgE	−	ND	<0.005	−	0.5
6	gp120IgE	−	0.05	0.00	−	ND
	gp120IgE	−	ND	0.00	−	ND
	DNPIgE	−	ND	0.00	−	ND
	PS myeloma	−	ND	0.00	−	
7	gp120IgE	+	0.00	ND	−	0.2
	DNPIgE	+	0.01	ND	−	ND
	gp120IgE	−	0.01	ND	−	0.2
	DNPIgE	−	0.00	ND	−	ND

[a] syk and ERK phosphorylation after stimulation with several putatively monovalent IgE preparations. Three different preparations of IgE were examined: purified PS myeloma, gp120 peptide-specific monoclonal hIgE, and DNP-specific mIgE. This is indicated in the second column. In some cases the cells were first treated with lactic acid buffer to dissociate endogenous IgE (this is indicated in the third column (+, lactic acid dissociation was performed; −, lactic acid dissociation was not performed). The fourth column indicates the intensity of the syk phosphorylation (4G10 band intensity) for the response to monomeric IgE, relative to the maximum anti-IgE-induced syk phosphorylation done in the same experiment. In general, the cells were stimulated with anti-IgE Ab (0.2 μg/ml), monomeric IgE (5 μg/ml), or Ag (if sensitized), and the cells were harvested at several times after stimulation, usually 5–15 minutes. The fifth column indicates the peak ERK phosphorylation in response to monomeric IgE relative to the maximum response to anti-IgE Ab. Whether the cells secreted histamine or LTC_4 is indicated in the sixth column (+, there was measurable release; −, there was no measurable release). The last column refers to the intensity of the response (ERK or syk phosphorylation) to Ag challenge relative to the response to anti-IgE Ab. To make this measurement, a portion of the cells were sensitized with the IgE being used to directly stimulate the cells and subsequently were challenged with the relevant Ag. In these instances, the IgE was gp120 peptide-specific IgE and the Ag gp120 peptide-OVA. ND, Not done.

presence of HSA (added at a concentration equimolar to the IgG used) was 0.78 ± 0.33, whereas the 60:5 min ratio in the presence of hIgG was 0.98 ± 0.21 (paired analysis revealed no statistically significant difference; $n = 3$; Table I). Stimulation with hIgG alone had no effect (data not shown). LTC_4 release for these and two additional studies using less enriched basophils showed no statistical difference for the two conditions (Table I). A similar study examining the phosphorylation of ERK also showed no difference between stimulation in the presence of HSA or hIgG (data not shown).

The role of monomeric IgE signaling

Recent studies using mouse bone marrow-derived mast cells have indicated that certain monomeric IgE Abs can induce traditional signaling (phosphorylation of the receptor, syk, shc, on down through ERK1/2) (29). We considered the possibility that the act of sensitizing the basophils induced some signaling during the sensitization phase of the experiment. In effect, down-regulation would have already begun before the addition of Ag, resulting in the apparently faster decrease of syk toward resting levels. We have looked at this issue several ways. First, as noted above, the data in Fig. 2 are a composite of several sensitization conditions. Notably, early in this study we switched from sensitizing basophils at 37°C to a longer sensitization near 0°C (in an ice-water bath). Previous studies have shown that no desensitization occurs at these temperatures during Ag challenge (1), so it seemed unlikely that significant signaling of any kind could occur during sensitization. In a direct comparison of warm vs cold sensitization, we could not detect a difference in the transience of syk phosphorylation. As noted above, despite routinely using this approach, a variety of different sensitizations continued to result in syk phosphorylation being transient. In Fig. 2, we also segregated the syk phosphorylation ratio after anti-IgE Ab in cells in which there was no prior sensitization vs cells that had been sensitized with an Ag-specific IgE. Although there is a slight difference in the medians for the two conditions, it is not statistically significant. In a couple of experiments we directly compared the anti-IgE Ab response with and without sensitization and found no significant difference.

Finally, we directly examined signaling after exposure of the cells to various putative monomeric IgE Abs. Three purified Abs were tested: PS myeloma, a mouse DNP-specific IgE, and the anti-gp120 peptide-specific IgE (the latter two having been used in the experiments above). These IgE preparations were not processed or tested for the presence of aggregates. Cells were challenged with 5 μg/ml of the Abs (the concentrations typically used for sensitization and also similar to the concentrations examined in the published studies of mouse mast cells). For comparison, the cells were also challenged with anti-IgE Ab. In some cases, a portion of the cells were fully sensitized with gp120-specific IgE and challenged with Ag to determine the magnitude of the response mediated by the specific IgE with an aggregating stimulus. Two endpoints were examined: syk phosphorylation and/or ERK phosphorylation (we show below that the ERK and syk responses titrate similarly, with ERK being a slightly more sensitive indicator of activation). For a total of seven experiments, using cells both stripped and not stripped, there was only one preparation for which there was a modest syk response in stripped cells. In the same preparation (the cells that hadn't been stripped) there was little or no response. It is relevant to note that in this one experiment, both LTC_4 and histamine release followed the stimulation with PS myeloma. In all other experiments, release was negative. Table II summarizes our experience. These results will be discussed in detail below, but we conclude that these IgE Abs do not usually induce measurable signaling and, combined with the use of cold sensitization, it would seem unlikely that the syk transience observed after Ag challenge could be solely the result of a low level of activation occurring during sensitization.

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Cross-desensitization after antigenic stimulation

Our previous studies on down-regulatory mechanisms after stimulation with polyclonal anti-IgE Ab suggested that control was exerted at the level of PI3 kinase or its direct product, phosphatidylinositol 3,4,5-phosphate (PIP3).[5] In contrast, the current studies using Ag suggest that control is exerted before or at the phosphorylation of syk. We asked whether after Ag stimulation, control was also exerted in the region identified with anti-IgE Ab, only it was not apparent because of the effects on syk phosphorylation. Stimulation with Ag would induce transient phosphorylation of syk but might also induce down-regulation in the PI3 kinase region that might be detected by restimulating with a second non-cross-reactive stimulus. Testing this idea required sequential stimulation with two non-cross-reacting Ags. To facilitate these studies, we chose to load cells with one Ag-specific IgE and to sequentially stimulate the cells with the specific Ag followed by polyclonal anti-IgE Ab. This scheme required that there was no cross-reactivity between the Ag-specific IgE used for sensitization and polyclonal anti-IgE Ab, which would stimulate the endogenous cell surface IgE. DNP-specific mouse IgE was used to sensitize human basophils because this mouse Ab is known to bind to the human FcϵRI. To show that polyclonal anti-IgE Ab did not aggregate the mouse IgE, RBL-SX38 cells were sensitized with either DNP-specific mouse IgE (5 μg/ml for 30 min) or human IgE (gp120-specific IgE, 5 μg/ml for 30 min). The sensitized cells were stimulated with either polyclonal anti-IgE or DNP(7)-HSA at several concentrations ($n = 3$). Cells sensitized with mouse IgE released 0 $\pm$ 0% when stimulated with 0.2 μg/ml of polyclonal anti-hIgE and 28 $\pm$ 3% when stimulated with 3 μg/ml DNP-HSA (the optimum for this Ag). When cells were sensitized with human IgE, polyclonal anti-hIgE Ab induced 31 $\pm$ 3% histamine release, whereas DNP-HSA induced 0 $\pm$ 0% release. In addition, loading the RBL cell with one-tenth of the mouse IgE resulted in equally good secretion when stimulated with DNP-HSA. This suggests that the cells were capable of secreting with much lower levels of aggregates, implying that the inability of anti-IgE Ab to stimulate any release means that if any aggregates were formed with this stimulus, the levels must have been quite low.

Effects on ERK and Akt phosphorylation

Our previous studies demonstrated a transition from sustained signaling to transitory signaling during stimulation with polyclonal anti-IgE Ab, which occurred between syk phosphorylation and ERK phosphorylation, with later studies refining this to sustained p85α (PI3 kinase) phosphorylation[5] and transitory Akt phosphorylation. Therefore, we examined three reactions, using phosphorylation of ERK as a readout of the $p21^{ras}$ pathway activation, phosphorylation of Akt as an indicator of PIP3 levels, and syk phosphorylation as an early signal. Fig. 3 shows three Western blots of ERK phosphorylation under conditions of sequential stimulation. DNP-HSA was the first stimulus and anti-IgE Ab was the second. Note that the magnitude of ERK phosphorylation after stimulation with DNP-HSA varied widely in these three examples (variation in all of the experiments of this type resulted from natural variations in unoccupied receptor density or because the cells were treated with lactic acid, which results in varied densities of unoccupied receptor). As previous studies have shown, ERK phosphorylation is transient, and in these experiments the cells were incubated for 80 min before the addition of anti-IgE Ab to ensure that ERK phosphorylation had returned to resting levels. The panels show that partial to complete loss of the ERK response to anti-IgE Ab occurs after stimulation with DNP-HSA. Fig. 4*A* shows a relationship between the strength of the first stimulus and the amount of cross-down-regulation (from here on this will be called nonspecific desensitization). In this plot, the ratio of peak ERK phosphorylation for DNP-HSA and anti-IgE Ab was used as a measure of the relative strengths of the two stimuli (Fig. 3 also shows that the relative level of nonspecific desensitization is similar whether one examines the peak of ERK response (5 min) or later (15 min)). In a single experiment, the cells were sensitized with a low or high concentration of DNP-specific IgE, and the nonspecific desensitization was assessed for the two conditions. As expected, there was more nonspecific desensitization in the cells loaded with a higher density of DNP-specific IgE. In three experiments, the order of the stimuli was reversed, with anti-IgE Ab used for the first 80 min of incubation followed by DNP-HSA. As expected for a strong stimulus, prior treatment with anti-IgE Ab resulted in nearly complete nonspecific desensitization of the DNP-HSA response (data not shown).

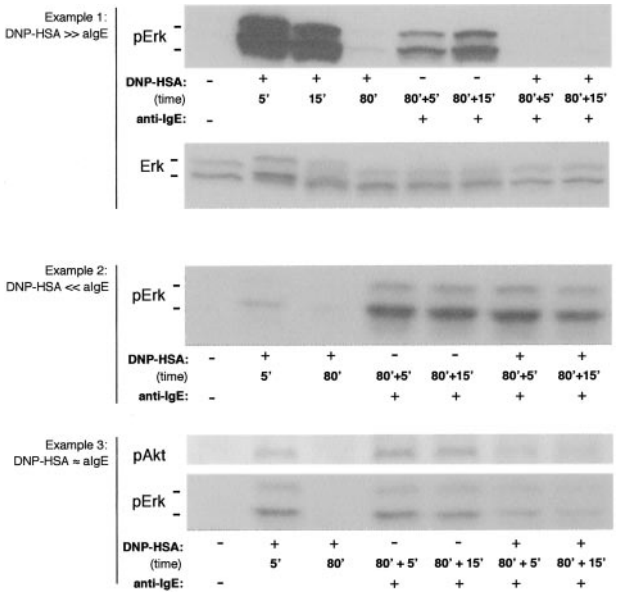

FIGURE 3. Western blots for phospho-ERK and phospho-Akt from basophils sequentially stimulated with DNP-HSA and anti-IgE Ab. Basophils sensitized with DNP-specific mouse IgE were stimulated with DNP-HSA at 3 μg/ml for 80 min, anti-IgE was added at 0.2 μg/ml, and cells were lysed with ESB at 5 or 15 min. Western blots were developed for phospho-ERK or phospho-Akt. Equivalent lane loading was determined by stripping the blots and developing for either ERK or Akt (Akt data not shown).

Phosphorylation of Akt is transient on a time scale similar to ERK phosphorylation. Indeed, thus far, the characteristics of ERK phosphorylation are well mimicked by Akt phosphorylation. This holds true for nonspecific desensitization of the anti-IgE response after prior stimulation with DNP-HSA, as also seen in Fig. 3 (*bottom panel*). A correlation between stimulus strength and nonspecific desensitization of Akt phosphorylation also occurs (Fig. 4*B*). We sensitized basophils with a mixture of two IgE Abs, specific for DNP or gp120 peptide (data not shown). As found for the sequential stimulation with DNP-HSA and anti-IgE Ab, prior stimulation with DNP-HSA caused nonspecific desensitization of the Akt and ERK phosphorylation that followed restimulation with gp120-OVA.

[5] Recent unpublished studies have shown that immunoprecipitation of human basophil lysates with anti-phosphotyrosine Ab results in a wortmannin-sensitive PI kinase activity that is increased approximately fourfold after stimulation with anti-IgE Ab and that is sustained for 60 min (D. W. MacGlashan, Jr., manuscript in preparation). These results further support the idea that PI3 kinase activity is sustained during stimulation with polyclonal anti-IgE Ab and that the transient presence of PIP3 results from the activity of a phosphatase.

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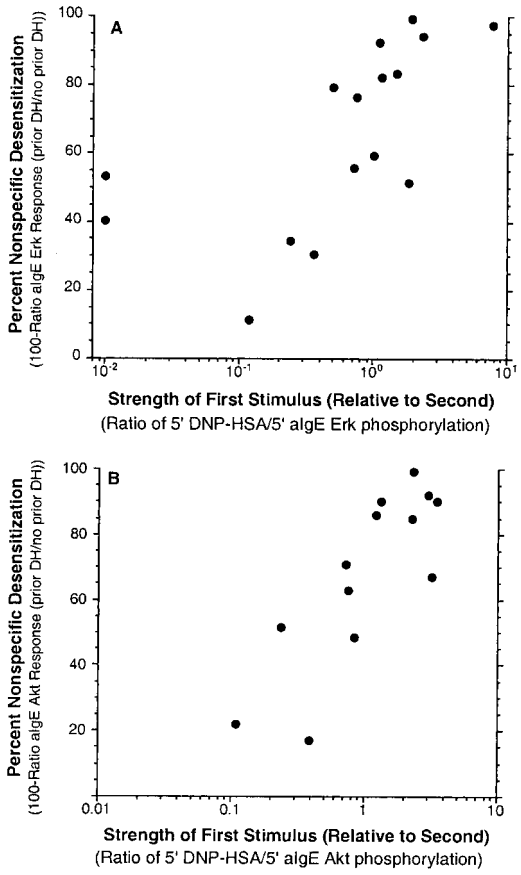

FIGURE 4. Relationship between the strength of the first stimulus (DNP-HSA) response to the amount of nonspecific desensitization observed upon further stimulation with the second stimulus (anti-IgE Ab). Using the protocol shown in Fig. 4 (blots were scanned and band intensities measured), the peak response to DNP-HSA (5-min time point) was divided by the peak response to anti-IgE Ab (without prior stimulation with DNP-HSA) to obtain the value plotted on the abscissa. The ordinate was the 1-ratio of the anti-IgE Ab response after prior stimulation with DNP-HSA divided by the response to anti-IgE Ab without prior stimulation with DNP-HSA expressed as a percentage (percent nonspecific desensitization). *A*, Data for the phospho-ERK response. *B*, Data for the phospho-Akt response. Each point represents a separate experiment with a different preparation of basophils. Variation in the relative strengths of the responses to DNP-HSA and anti-IgE Ab was the natural consequence of differences in ability to sensitize the basophils with DNP-specific IgE. In many cases, the cells had been treated with lactic acid to generate unoccupied receptors, but the natural variation in the cells' response to this treatment also resulted in differences in the relative strengths of the two stimuli for different preparations.

Effects on syk phosphorylation

As noted, previous studies found a locus of down-regulation that occurs between the activation of PI3 kinase and $p21^{ras}$. Therefore, steps preceding the activation of PI3 kinase would not be expected to experience the effect of cross-down-regulation observed with phosphorylation of Akt and ERK, if the mechanism for Ag-induced nonspecific desensitization being observed was similar. The phosphorylation of syk kinase was examined using a simplified protocol in which syk, Akt, and ERK phosphorylation were examined in the same cells. The interpretation of the results is made easier because the phosphorylation of syk after stimulation with DNP-HSA, as we have just shown, is transitory. An 80-min first incubation is generally sufficient to allow syk phosphorylation induced by DNP-HSA to return to near resting levels. Fig. 5, *A* and *B*, shows two examples of this experiment: one in which the first stimulus generates a weaker signal than the second and one in which the first stimulus generates a stronger response than the second. In these experiments, there continued to be nonspecific desensitization of ERK phosphorylation, whereas syk phosphorylation was relatively unaffected. Fig. 5*C* summarizes the results of four experiments done this way.

It was possible that ERK phosphorylation was a significantly less sensitive measure of human basophil stimulation, a condition that might lead to the results in Fig. 5 even if nonspecific desensitization did not occur between syk and ERK. To exclude this possibility, full kinetic curves of ERK and syk phosphorylation were examined at several concentrations of Ag. The area under the curves for syk phosphorylation at each Ag concentration were plotted against the area under the curves for ERK phosphorylation. On the basis of three experiments, the *y*-intercept for this plot was somewhat positive (ERK being a slightly more sensitive indicator of stimulation), although not statistically significantly different from 0, indicating a roughly similar sensitivity of syk and ERK phosphorylation as measures of basophil stimulation (data not shown).

Functional effects

The ability of one Ag to influence the response of the basophil to other non-cross-reacting Ags has been previously characterized as nonspecific desensitization. However, the experimental protocol used in previous studies differed from the one used in the current study in that incubation with the first stimulus was conducted under nonsecreting conditions (the absence of extracellular calcium) (30–32). We examined whether functional nonspecific desensitization could be observed even under secreting conditions. Fig. 6 shows the results of experiments performed with the sequential stimulation protocol used in the studies above. In the data shown, the cells were sensitized with DNP-specific IgE and the first stimulus was DNP-HSA. Note that the secretion after DNP-HSA is essentially complete by 15 min ($t_{1/2}$ of ~8 min, as observed in many previous experiments). At the 45-min time point in these experiments, anti-IgE Ab was added. Here the kinetics were similar to DNP-HSA, complete by 15 min with a $t_{1/2}$ of ~8 min. The histogram bar to the right of the kinetics plot shows the release to anti-IgE Ab in the absence of prior stimulation with DNP-HSA. This release is greater than the release to anti-IgE in the sequential challenge. Fig. 6*B* shows the average results for 10 experiments done in this manner. For each experiment, three values were extracted from the kinetic data: 1) the average of DNP-HSA-induced histamine release at 30, 45, and 60 min; 2) the average of the two release values at the 60- and 75-min time points after the addition of anti-IgE Ab; and 3) the release after stimulation with anti-IgE Ab alone (as measured at 30 and 45 min poststimulus, after a 45-min incubation in PAGCM alone). There are two ways the extent of nonspecific desensitization can be calculated: 1) relative only to the total anti-IgE-induced release (without prior DNP-HSA stimulation) or 2) relative to the increment in release above DNP-HSA alone (above the dotted line shown in Fig. 6, *B* and *C*). The latter method is preferable because 100% nonspecific desensitization would mean that no additional release occurred after anti-IgE Ab was added to cells previously stimulated with DNP-HSA. Fig. 6, *B* and *C*, shows that the sequential challenge resulted in 43 ± 8% nonspecific desensitization when histamine release was the endpoint and 58 ± 6% when LTC_4 release was the endpoint (both of which are statistically significantly different from 0). Not shown in the figure are the results of several other manipulations done in these experiments. For example, the cells were desensitized for 60 min using the traditional approach of excluding calcium in the first

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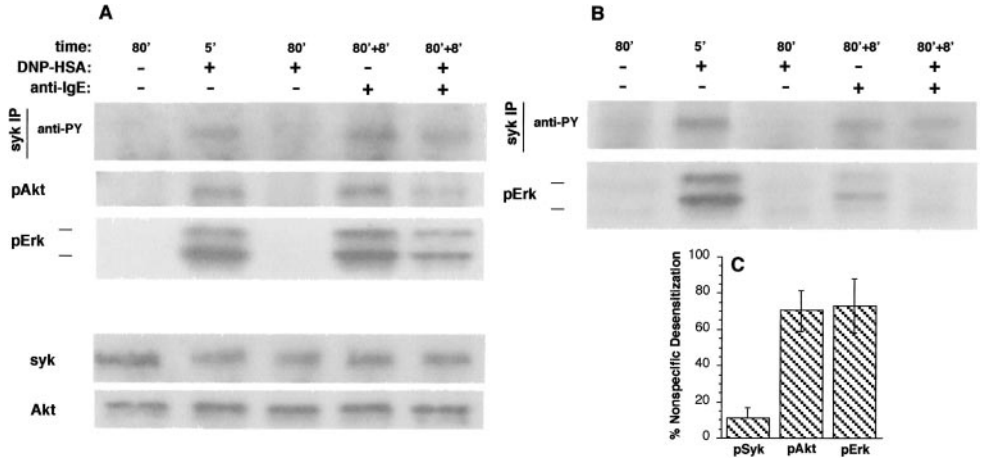

FIGURE 5. Nosnpecific desensitization of the syk phosphorylation response vs ERK or Akt phosphorylation. Using an abbreviated version of the protocol described in Fig. 4, the cell lysates were processed to measure syk, ERK, or Akt phosphorylation. The percent nonspecific desensitization is calculated for each of the endpoints. Shown are two of four experiments, one in which the DNP-HSA response was less than the anti-IgE response (*A*) and one in which the DNP-HSA response was greater than the anti-IgE response (*B*). *C*, Average nonspecific desensitization for four experiments.

phase of the protocol. Under these conditions, specific desensitization was 96 ± 2% complete, as expected, and nonspecific desensitization, with histamine release as the endpoint, was 25%. In addition, the order of the stimuli was reversed. In this instance, anti-IgE Ab was more often the stronger stimulus (as can be seen in Fig. 6), and the subsequent addition of DNP-HSA did not induce further release. Indeed, the general rule of thumb was that the only way to observe partial nonspecific desensitization, using the sequential approach in calcium-containing buffer, was when the first stimulus was weaker than the second. This rule did not apply to measurements of signaling, as observed above.

Previous studies of desensitization observed the transition from specific to nonspecific desensitization as an increase in nonspecific desensitization with greater densities of Ag-specific IgE, akin to the data shown in Fig. 4. This was true in the secretion studies. The cells were sensitized with different concentrations of DNP-specific IgE, and the amount of nonspecific desensitization of the anti-IgE response was determined as above. At 1.25, 2.5, and 5 μg/ml DNP-specific IgE for sensitization, there was 36 ± 8%, 58 ± 10%, and 80 ± 2% nonspecific desensitization, respectively.

Discussion

These results demonstrate that not only is there down-regulation during IgE-mediated activation that lies in the region of PI3 kinase and/or its products, but there is an earlier down-regulatory event lying proximal to syk phosphorylation or at the level of syk activation (because syk kinase phosphorylates some of its tyrosines). Furthermore, both forms of down-regulation can occur after stimulation. The earlier down-regulatory event was most readily observed after various types of antigenic stimulation, but there was a continuum of behavior with respect to the appearance of early down-regulation that depended on the nature and/or strength of the IgE-mediated stimulus. There was significant overlap in the ability of anti-IgE Abs vs Ags to expose this early form of down-regulation.

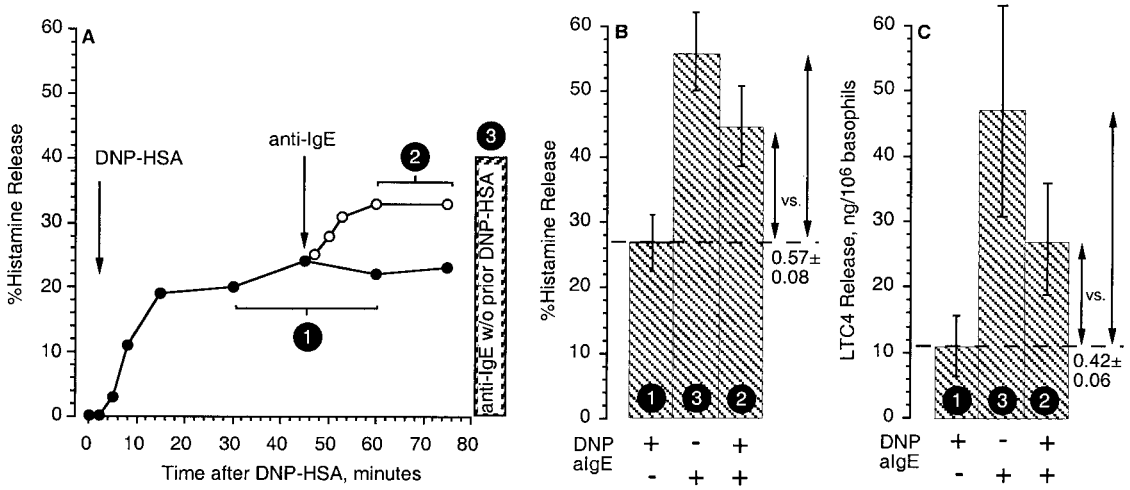

FIGURE 6. Nonspecific desensitization of histamine and LTC_4 release in basophils sequentially stimulated with DNP-HSA and anti-IgE Ab. Basophils prepared by single-step Percoll gradients were sensitized with DNP-specific IgE and stimulated with DNP-HSA at 3 μg/ml. The reactions were stopped by the addition of EDTA and centrifugation at various times (●). Some cells were further challenged at the 45-min time point with anti-IgE Ab at 0.2 μg/ml (○), and the reactions were stopped at various times with addition of EDTA and centrifugation. Harvested supernatants were assayed for histamine and LTC_4. *A*, One representative experiment; the numbers 1–3 designate values obtained for the average data shown in the histograms in *B* (histamine release) and *C* (LTC_4 release). Measurement 3 is derived from cells incubated for 45 min in buffer alone and then stimulated with anti-IgE Ab with supernatants harvested at the 75-min time point.

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Because the early form of down-regulation was most readily observed after sensitization of the cells in order to stimulate with a specific Ag, perhaps the most critical technical consideration was the issue of monomeric IgE signaling. As noted previously, a recent study using cultured mouse mast cells indicated that monomeric IgE could induce a large number of the signaling steps formerly associated with aggregating stimuli (29). Notably, although these signaling elements are active, these apparently activated mast cells do not degranulate or secrete arachidonic acid metabolites. More recent studies by this and a second group suggest that this phenomenon may be restricted to certain sources of IgE (33, 34), but the generality of the phenomenon is not yet clearly established in the literature. In recent unpublished studies, it appears that even for instances in which monomeric IgE induces only survival of mast cells without other overt signaling, this effect may reflect the presence of aggregates or aggregates formed after the IgE is bound to FcϵRI. A current operating hypothesis is that certain IgE Abs may be capable of self-aggregating once engaged in FcϵRI. This self-aggregation appears to require an empty ligand binding site because monovalent ligand can markedly blunt the signals induced by the presence of the putatively monomeric IgE (29). Although the current evidence suggests that this effect may be restricted to certain IgE Abs, it remains a concern for a clear interpretation of our results.

However, on the basis of our results, we do not think that monomeric IgE is signaling in these cells or that the process of sensitization per se can explain the results with Ag. First, cold sensitization doesn't alter the outcome and all current evidence indicates that no signaling can proceed at temperatures $<4°C$. Therefore, until the cells were resuspended in cold PAGCM and then ramped up to 37°C, there should have been little or no signaling taking place (at best, any signaling would occur for only 5–10 additional min before the addition of Ag). Direct stimulation with our preparations of IgE Ab, for the most part, did not result in measurable signals. This was not exclusively the case, however. Our first measurement using PS myeloma as a source of IgE resulted in modest syk phosphorylation when the cells were stripped with lactic acid. Because we have not attempted to isolate strictly monomeric IgE, it is possible that a fraction of the IgE is aggregated in our preparations of PS myeloma. Consistent with this possibility is that mediator release occurred. This is unlike the situation with monomeric IgE stimulation of murine mast cells in which signaling occurred in the absence of mediator release (29). On the basis of the PS myeloma results, we turned to experiments using the IgE Abs used for the Ag studies. Here there were no consistent increases in syk or ERK phosphorylation, although very low-level signals were occasionally noted. Although we could attempt to prepare strictly monomeric IgE from these preparations, the various results taken together (cold load, multiple Ags, extremely low-level signaling) suggested that it was not necessary to pursue this course of action because it seems unlikely that monomeric IgE signaling during sensitization could easily explain the results with Ag.

An examination of a variety of conditions for stimulation suggests that both the nature of aggregation and the density of IgE play roles in determining whether the early process of desensitization occurs. The most obvious difference between most of the Ag experiments and stimulation with anti-IgE Ab lies in the differences in the cell surface density of the target for the stimulus. As noted above, the goat polyclonal anti-IgE Ab presumably has access to all of the cell surface IgE, whereas Ag is restricted to a subset of the IgE. From previous studies we know that the median receptor density on basophils is ~100,000 and that the typical basophil has ~5,000 unoccupied receptors (26). Without lactic acid dissociation of the cell-bound IgE, a typical sensitization results in relatively low densities of Ag-specific IgE relative to the total IgE density. In the early 1980s, we and others proposed that the two forms of desensitization, specific and nonspecific, resulted from different densities of Ag-specific IgE (30–32). Specific desensitization results in down-regulation of the basophil response only to the Ag used to stimulate the cell, whereas nonspecific desensitization results in down-regulation of the cell's response to all non-cross-reacting Ags. Low levels of Ag-specific IgE lead to desensitization that is predominantly specific in character, whereas high densities lead to both complete specific and nonspecific desensitization. In other words, specific desensitization always occurs, but nonspecific desensitization occurs only when the signal is strong enough. One viewpoint on specific desensitization is that although it may always occur, its rate is saturable so that the activation of some signaling elements does not appear to decay quickly at the point in the signaling cascade where specific desensitization acts when the stimulus is strong (35). Instead, the process of nonspecific desensitization becomes dominant and controls the extent of mediator release. Because earlier studies used mediator release as an endpoint for determining the extent of desensitization, either specific or nonspecific, whether some early signaling step would in fact decay more slowly was speculation. However, with the measurements made in this and other recent studies, the relative rates at which Ag and anti-IgE Ab induce down-regulation before syk phosphorylation initially appeared consistent with a saturable process of specific desensitization. Furthermore, the correlation between strength of the first stimulus and the extent of nonspecific desensitization at the level of ERK and Akt phosphorylation is consistent with nonspecific desensitization becoming a dominant aspect of desensitization with strong stimulation. With these observations in mind, specific desensitization would be equated with the early pre-syk down-regulation and nonspecific desensitization equated with down-regulation of signaling somewhere between PI3 kinase and $p21^{ras}$ activation.

However, the current studies indicate that IgE density/signal strength does not fully explain the observations shown in Fig. 1 and indirectly suggest that lack of transience does not result from a saturable rate of early down-regulation. By any measure of signaling, antigenic stimulation of the basophils loaded with high densities of Ag-specific IgE (after treatment with lactic acid to remove some endogenous IgE) resulted in a stronger peak signal than polyclonal anti-IgE tested on the same preparations. syk, Akt, or ERK phosphorylation or histamine and leukotriene release were all significantly greater with the antigenic challenge in these experiments. Despite this significant difference in signal strength (by whatever aggregation mechanism), Ag-induced syk phosphorylation remained transient. In contrast, reduction of surface IgE with lactic acid treatment did allow anti-IgE Ab to induce a more transient phosphorylation of syk kinase. These results suggest a mixed conclusion regarding the conditions that result in transient syk phosphorylation. Both IgE density and the character of the aggregation likely play roles in the induction of this type of down-regulation.

The sustained signaling due to anti-IgE Ab resembles the characteristics of stable aggregate formation using small oligomers of IgE to stimulate RBL cells (5), which also generate sustained syk phosphorylation, whereas the response to Ag is similar to that observed during antigenic stimulation of RBL cells (10), where syk phosphorylation is also transient. An explanation for this difference in RBL cells has not yet been advanced, although many studies have begun to show that the qualitative nature of signaling differs considerably depending on the dynamics and nature of aggregation and therefore on the nature of the stimulus.

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In RBL cells, one early down-regulatory mechanism is thought to require the participation of the cytoskeleton and its influence on the structure of lipid rafts (9, 10). Because some of the same agents, e.g., cytochalasin D, that alter secretion in RBL cells by acting on the cytoskeleton also alter the response in a similar way in human basophils, it is possible that a similar mechanism of down-regulation controls the early signaling events in human basophils. This is being studied.

The current studies also demonstrate that even antigenic stimulation induces a down-regulatory process that acts in the region of PI3 kinase and/or its direct products, such as PIP3. As noted, it has also been known that desensitization with one Ag, under certain conditions, can result in down-regulation of the basophil's response to other non-cross-reacting Ags. The current studies suggest that the point of down-regulation responsible for nonspecific desensitization lies in the same region suggested by the published studies using anti-IgE Ab to study signaling. It should also be noted that stimulation with anti-IgE Ab also induced nonspecific desensitization of Ag-induced secretion, consistent with the fact that this stimulus induces down-regulation in the region of PI3 kinase/PIP3 and consistent with anti-IgE Ab being a strong stimulus.

These conclusions are dependent on the non-cross-reactivity of the anti-IgE Ab and mouse IgE. Although there is no evidence that anti-human IgE would cross-react with mouse IgE, we examined this in an assay that should be extremely sensitive to the presence of some cross-reactivity. In sensitizing RBL-SX38 cells with mouse IgE (anti-DNP), both the endogenous rat receptors as well as the transfected human FcϵRI would bind the mouse IgE. This would lead to a very high density of Ag-specific IgE per cell and, given the sensitivity of the RBL cell to stimulation, this should provide a good detector of any potential low-level aggregation by the anti-hIgE Ab. We could not detect histamine release in this case. To demonstrate that the anti-hIgE Ab was capable of generating an adequate signal from normal cross-linking in these same cells, the cells were sensitized with hIgE. This combination of hIgE and anti-hIgE Ab generated secretion equivalent to that of DNP-HSA (previously published studies establish the similar sensitivity of the rat and human receptors to stimulation in these cells (36)).

The current studies cannot distinguish between a mechanism of nonspecific desensitization that results from active inhibition of a signaling step vs a shared element allocation problem (the dog-in-the-manger scenario).[6] Indeed, if nonspecific desensitization of the response to the second Ag is simply the result of tying up a signaling element (e.g., all PI3 kinase is involved in the signaling compartment generated by the first Ag), then the apparent down-regulation in this region of the pathway after stimulation with the second Ag does not reflect a desensitization process at all. Clarification of these issues will require a better understanding of the precise mechanism of down-regulation that occurs under these various circumstances. However, given the large body of evidence supporting a role for SHIP in regulating signaling caused by increased synthesis of PIP3 in a variety of cell types, it is reasonable to speculate that nonspecific desensitization in human basophils involves the recruitment of SHIP (or possibly PTEN) into the reaction complex.

The ability to sustain syk phosphorylation is not a characteristic only of polyclonal anti-IgE Ab because stimulation with monoclonal anti-IgE Ab in the presence of nonspecific IgG to block binding to FcγRIIB induces a sustained phosphorylation of syk. One interpretation is that the mouse Ab Fc region is capable of recruiting FcγRIIB into the reaction complex to inhibit signaling. However, it was unexpected that changes occurred in syk phosphorylation because the current model of coaggregation with FcγRIIB should result in SHIP recruitment, which would operate downstream of syk kinase. In this context we have shown that inhibiting PI3 kinase has no effect on syk phosphorylation, so reducing a PI3 kinase product, PIP3, with recruited SHIP would also not be expected to feedback inhibit syk phosphorylation. Not only did the introduction of IgG lead to changes in syk phosphorylation, it didn't lead to sustained ERK phosphorylation. The observation that it did markedly enhance LTC_4 release suggests that there may be effects on the cytosolic calcium response that could account for the increase in LTC_4 release. However, these results highlight an additional aspect of the down-regulatory process: no condition thus far, including those that resulted in sustained early signaling, resulted in sustained later signaling. This issue is also the subject of a future study.

The presence of a transient activation of syk phosphorylation during antigenic stimulation is surprising when considering the time course of IL-4 and other chemokine/cytokine secretion. We have previously shown that stimulation results in IL-4 secretion that does not even begin for 45 min and may require another 1–2 h for completion (21). Some cytokine secretion, e.g., IL-13 or macrophage-inflammatory protein 1α, takes 12–36 h for completion (37–39). Furthermore, addition of monovalent hapten, BPO-EACA, at an early time (30−45 min) halts IL-4 secretion, suggesting a requirement for the maintenance of signaling (15). Putting the previous kinetics of secretion data together with the data shown in Fig. 1 leads to the suggestion that syk phosphorylation is near resting levels when the rate of IL-4 secretion is maximal (and in unpublished studies, levels of mRNA for IL-4 are at their maximum). We measured syk phosphorylation and IL-4 secretion in the same cell preparation and obtained results similar to those previously published. These results raise the possibility that strong syk activity may be required for initiating cytokine secretion but that only a very low level of syk activity is required to maintain the signaling needed to finalize cytokine secretion. Indeed, adding monovalent hapten to the reaction to stop IL-4 secretion may have little to do with inhibiting further syk activity, but instead may indicate the need for some other signaling cascade not currently measured. This result is somewhat akin to our previous results suggesting that PI3 kinase was required to initiate cytokine secretion, but that it was not required for the final steps of secretion (14).

In summary, we found that early signaling is transient when the stimulus is any Ag, regardless of the cell surface density of the Ag-specific IgE, and this observation indicates that there is an important region of control exerted before or at the level of syk phosphorylation that was not as apparent in the studies in which polyclonal anti-IgE Ab was used. Although stimulation with Ag appears strongly regulated at the level of syk kinase, we clearly demonstrated that there are effects on non-cross-reacting Ag-induced signaling that lie in the region of PI3 kinase. This suggests that Ag also induces down-regulation in the same region identified in the studies using anti-IgE Ab as a stimulus. The appearance of two regulatory regions, one that acts early in signaling and one that acts late, is consistent with prior speculations on the nature of signaling that follows specific and nonspecific desensitization, and the current studies suggest that nonspecific desensitization results from down-regulation in the region of PI3 kinase.

[6] The dog-in-the-manger scenario was posited by H. Metzger and colleagues (40, 41) for the behavior of lyn kinase in RBL cells stimulated with two Ags. Strictly speaking, in the scenario described by the Metzger studies, the first Ag was a poor stimulus and, although it did not induce secretion, it could tie up the available lyn kinase. In our case, the first stimulus is strong but would have the same impact on a second stimulus. Furthermore, because syk phosphorylation in response to the second Ag is largely intact, the involved element is further downstream than lyn kinase.

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