

Subthreshold desensitization of human basophils re-capitulates the loss of Syk and FcεRI expression characterized by other methods of desensitization

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Abstract

Background Clinical desensitization of patients to drugs involves progressive exposure to escalating doses of drug over a period of 24 h. In prior studies, this method was re-capitulated *in vitro* to also demonstrate loss of mast cell or basophil responsiveness. However, most signalling studies of human basophils have identified changes in signalling by using other methods of inducing cellular desensitization.

Objective This study examined two well-described endpoints of basophil desensitization, loss of syk or FcεRI expression, under conditions of subthreshold desensitization.

Methods The loss of FcεRI and syk was examined in human basophils.

Results It was shown that both loss of syk and FcεRI/IgE occurred during an escalating series of stimulation (anti-IgE Ab) and that expression loss occurred despite the presence of little histamine release. If basophils were first cultured for 3 days in 10 ng/mL IL-3, the concentration-dependence of histamine release shifted to 100-fold lower concentrations of stimulus. However, loss of syk did not show any change in its EC₅₀ while loss of FcεRI also shifted 100-fold. From the perspective of early signal element activation, the marked shift in the EC₅₀ for histamine release was not accompanied by similar shifts in the EC₅₀s for several signalling elements. The EC₅₀s for phospho-Src, phospho-SHIP1, phospho-Syk, or phospho-Cbl did not change while the EC₅₀s for phospho-Erk and the cytosolic calcium response did shift 100-fold.

Conclusions These studies show that under normal conditions, subthreshold desensitization leads to loss of two critical signalling molecules (FcεRI and syk) but under at least one condition, treatment with IL-3, it is possible to markedly blunt the loss of syk, but not FcεRI, while executing a proper subthreshold titration. These data also suggest that IL-3 modifies only the sensitivity of signalling elements that are downstream of syk activation.

Keywords allergy, basophil, Fc Receptors, human, syk

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Introduction

Clinical desensitization is used in the treatment of some allergic conditions. Most generally, it is only applied to allergies of drugs such as penicillin and is used to allow patients with hypersensitivity to the drug to be treated therapeutically [1, 2]. The protocol includes titration of the drug into the patient, progressively escalating the amount of drug by starting at doses that do not induce overt symptoms of a hypersensitivity reaction. With careful timing and dosing, the patient does not

experience a reaction but is ultimately able to be dosed with therapeutic levels of the drug. The mechanism underlying clinical desensitization is not yet understood.

To explain the operative mechanism underlying clinical desensitization, an obvious place to turn is the mast cell response biochemistry. All forms of receptor stimulation result in the engagement of signal termination events, otherwise external signalling would result in runaway activation of cells. There are a wide variety of mechanisms that mediate termination. A common

mechanism is down-regulation of the receptor from the cell surface but the list of mechanisms is long. During IgE-mediated activation of basophils (or mast cells), the mechanisms depend on the species being studied. Early studies of RBL cells (rat basophilic leukaemia cells) strongly suggested that FcεRI down-regulation was a major component of the termination process [3, 4]. In contrast, studies in human basophils suggested that down-regulation of FcεRI was not even operative [5]. Subsequent studies of human basophils have shown that down-regulation of receptor expression does occur but the timing cannot account for termination of signalling; down-regulation of FcεRI in human basophils requires many hours to days [6].

There have been two operational methods to study desensitization mechanisms in basophils and mast cells. Both operate on a variation of the principle of suboptimal stimulation. The protocol that has generated most of the mechanistic details of cellular desensitization involves stimulation in the absence of extracellular calcium [7, 8]. But the method that re-capitulates the technique of clinical desensitization relies of progressively escalating concentrations of allergen exposure [9–11]. It is found that if the timing of escalation is properly chosen, very little secretion is ever observed although one does eventually reach concentrations that would have induced secretion if not for the escalating method. The question for this study was whether the mechanisms discovered using the first operational protocol, calcium-free stimulation, were re-capitulated with the progressive protocol. This information would be required to properly design a mechanistic study of clinical desensitization.

There have been three mechanisms discovered that may contribute to termination of the secretory signals in human basophils. These include activation of SHIP [12, 13], processing of syk by ubiquitination [14, 15] and degradation and loss of cell surface receptor [6]. Parenthetically, there is a fourth mechanism whose characteristics can be described and which appears to involve resorting of receptor in the membrane, but without details for how it occurs [16]. A study of subthreshold activation requires a way to examine signals too weak to induce secretion and at this point in time, all signalling steps thus far studied titrate like histamine release and are therefore undetectable at subthreshold levels of stimulation. However, two of the identified desensitization mechanisms appear to result from an integration of weak signalling. For example, it is known that syk down-regulation occurs at concentrations of allergen that do not induce secretion [14, 17]. Although the process requires long times (relative to secretion), the integrative characteristic may allow detection during the subthreshold method of inducing desensitization. Likewise, loss of receptor may be detectable over a period of time. However, studies in mice indicate that receptor

down-regulation that occurs during stimulation that is optimal for secretion, it does not occur at all during a subthreshold desensitization protocol [18]. Therefore, as a first step in testing whether the known mechanisms of desensitization apply to the subthreshold method, and therefore may be applicable as an explanation of clinical desensitization, we examined whether or not subthreshold desensitization results in loss of syk and receptor expression on human basophils.

Methods

Materials & buffers

Materials, buffers and antibodies. The following materials were purchased: PIPES, bovine serum albumin (BSA), EGTA, EDTA, D-glucose, NaF, Na₃VO₄, 2-ME (2-mercaptoethanol), NP-40; PP1 (Calbiochem, San Diego, CA, USA); crystallized human serum albumin (HSA) (Miles Laboratories, Elkhart, IN, USA); fetal calf serum (FCS) and RPMI 1640 containing 25 mM HEPES and L-glutamine (BioWhittaker, Walkersville, MD, USA); Percoll (Pharmacia, Piscataway, NJ, USA); Tris(hydroxymethyl)-aminomethane, Tween-20 (Bio-Rad, Hercules, CA, USA); 4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF) (Sigma-Aldrich, St. Louis, MO, USA); Anti-phosphotyrosine mAb (4G10) (Upstate Biotechnology, Lake Placid, NY, USA); Rabbit anti-phospho-ERK Ab, anti-phosphoSHIP, anti-phospho Src (Tyr 416) (New England Biolabs, Beverly, MA, USA, now Cell Signalling); anti-syk mAb, 4D10 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); monoclonal anti-human IgE Ab (6061P) (Hybridoma Labs, Baltimore, MD, USA); anti-HRP-conjugated donkey anti-rabbit Ig Ab, HRP-conjugated Sheep anti-mouse Ig Ab, protein G sepharose beads (Amersham Life Science, Arlington Heights, IL, USA). 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 CaCl₂ and 1 mM MgCl₂. Countercurrent-flow elutriation and labelling with antibodies for flow cytometry was conducted in PAG containing 0.25% BSA in place of 0.003% HSA. ESB is Novex electrophoresis sample buffer containing 5% 2-mercaptoethanol. SDS stripping buffer for Western blots was 65 mM Tris (pH 6.7), 100 mM 2-mercaptoethanol and 2% SDS.

A penicillin (BPO)-specific IgE was partially purified from the sera of penicillin allergic patients as previously described [5]. A gp120-specific monoclonal IgE was the gift of Tanox, Inc, (Houston, TX, USA). This antibody was biotinylated and could be detected by flow cytometry using streptavidin-alexa 647 (Molecular Probes, Eugene, OR, USA), which was calibrated with absolute IgE/basophil by methods described elsewhere [6].

Basophil purification

Residual cells of normal donors undergoing leukapheresis were enriched in basophils using Percoll density gradients and countercurrent-flow elutriation, as previously described [19]. Basophils were further purified by negative selection as described above. Basophil purity exceeded 99%.

Subthreshold desensitization

Prior to culture, basophils were sensitized with either BPO-specific IgE or biotinylated gp120-specific IgE at 5 µg/mL, on ice for 1 h, washed and resuspended for culture. Basophils were incubated in RPMI-1640 media (0.03% HSA, 8 µg/mL gentamycin, with media supplemented to generate a final Ca^{++} concentration of 1 mM). Each experiment included a full range of single concentrations for the 24 h incubation as well as various versions of escalating concentrations of anti-IgE Ab. The primary series for day-0 basophils was 0.006, 0.02, 0.06, 0.15 µg/mL separated by 6 h. The final volume of the cultures in tilted microtubes was 200 µL and at each step, 1 µL of an appropriate concentration of anti-IgE was added to minimize the volume change during sequential additions. To determine if the subthreshold series blunted histamine release, one condition included a final incubation with 0.5 µg/mL anti-IgE for 45 min. In some experiments, partial subthreshold series were included to examine some partial endpoints. Histamine release was measured from supernatants of the cultured cells while the cell pellets were either lysed for measurement of syk expression by Western blotting or the cells were fixed (2% paraformaldehyde) for subsequent analysis by flow cytometry for the presence of either gp120-specific IgE or BPO-specific IgE. Spontaneous histamine release (non-stimulated) in the 24 h cultures averaged $9 \pm 4\%$, a value typical for purified basophils under these conditions.

Detection of cell surface IgE:

Two methods were used to detect loss of cell surface IgE/FcεRI when using anti-IgE antibody to stimulate the cells. The first method has been previously described [6]. Basophils were sensitized with a biotinylated IgE (basophils generally express a low density of unoccupied FcεRI) and after fixing the cells, its presence was detected with streptavidin-alexa647. A second method was developed. Basophils were sensitized with penicillin-specific IgE and its presence detected with a sandwich assay. Prior to fixing the cells, the suspension was chilled to 4°C, 10 µg/mL of BPO(32)-HSA added for 10 min, the cells washed twice in ice-cold buffer and fixed with 2% paraformaldehyde for 20 min prior

to blocking and storage overnight. Before flow cytometry, the fixed cells were incubated with rabbit anti-BPO IgG ± BPO-EACA at 1 mM. The presence of BPO-EACA (monovalent penicillin, penicillin coupled to ε-aminocaproic acid) generates a negative control for the measurements. Using calibrated flow cytometry it was determined that this assay can detect fewer than 50 molecules of penicillin-specific IgE/basophil (online supplement Fig. 1E, panel A, shows that this assay is sensitive to very low densities of BPO-specific IgE). In a desensitization experiment, activation with the pan-crosslinking reagent, anti-IgE antibody (6061P) results in an average of $52 \pm 13\%$ loss of detectable BPO-specific IgE after 24 h, a result commensurate with prior studies (Fig. 2E of the online supplement).

Multiplex western blots

Pelleted cells were lysed in 20 µL of hot ESB, the tube being placed in boiling water for 5 min and the samples stored at -80°C until electrophoresis. Samples were run in a 15 well 8% tris-glycine gel with molecular weight markers in the first and last lanes. The nitrocellulose was cut horizontally at the 64 kD marker level. The > 64 kD and < 64 kD portions were blocked with 4% BSA or 2% milk respectively. The nitrocellulose was blotted on two or three successive days with a choice of antibodies that did not interfere with each other. No stripping step occurred between blotting. The blotting order was phospho-Src followed by phospho-Erk for the portion < 64 kD and phospho-Cbl followed by phospho-SHIP followed by p85α. The order was chosen to take advantage of differences in the strength of the signals detected and the selectivity of the antibodies, with the weaker antibodies analysed first. We have previously established that p85α is a good lane-loading control antibody [6, 13, 20] because its expression is unmodified during a wide variety of experimental designs and because it is easily detected with few cells and is easy to detect as the last step in this multiplex Western blot design (e.g., as an average ($\pm$ SD.) of these experiments, the band intensity of p85 at optimal stimulation was 1.06 ± 0.17 -fold of the non-stimulated band intensity). Multiple film exposures were made to optimize linearity, the films were digitized and the band intensities determined by NIH Image.

For the analysis of syk expression, cell lysates were run on 8% gels and syk detected with 4D10 antibody by using Western blotting.

$[\text{Ca}^{2+}]_i$ and mediator measurements

Basophils were labelled with 4 µM fura-2/AM and cytosolic calcium determined by using digital microscopy as previously described [21].

Results

Pilot studies

Two series of pilot studies were needed to perform these studies. These experiments used a pan-crosslinker, anti-IgE, to induce a response. Pilot studies roughly determined the dose response curve for the anti-IgE in use (6061P). Below 0.01 $\mu\text{g/mL}$ of 6061, histamine release in an overnight culture was generally zero although small levels of release can occasionally be observed from some preparations.

An additional series of pilot experiments refined the method of detecting cell surface IgE/receptor. There have been several methods used in previous studies [5] and most recently, flow cytometric methods appear the most sensitive. But the three flow-cytometric methods previously developed show some discrepancies [6]. Notably, detecting loss of receptor with anti-receptor antibody is confounded by aggregation. The best methods rely on loading a small amount of specific IgE antibody and using loss of this specific IgE as a marker of overall IgE & receptor loss (only when a pan-stimulus like anti-IgE is used). The methods section describes a new method (sensitizing the cells with penicillin-specific IgE) that relies on this approach and this method provides support for a previously described but related method (sensitizing the cells with a biotinylated IgE) [6]. An important test of the reliability of the method is whether or not the src-family kinase inhibitor, PP1, can reverse the loss of receptor to the same extent as it inhibits desensitization. Previous studies demonstrated that 10 μM PP1 would reverse desensitization by ~80% [22]. Likewise, 10 μM PP1 reverses loss of IgE/Fc ϵ RI, using either biotinylated IgE or BPO-specific IgE to detect loss, by $73 \pm 11\%$ and $68 \pm 11\%$ respectively (online supplement Fig. 2E shows these loss studies). Parenthetically, this assay should also have utility in the measurement of BPO-specific IgE densities on basophils of penicillin allergic patients.

Subthreshold stimulation

Detecting loss of either syk or IgE/Fc ϵ RI requires extended times but this requirement is compatible with the long process of subthreshold desensitization. Using the pilot experiments as a guide, basophils were cultured with progressively escalating subthreshold concentrations of anti-IgE spaced over 24 h. Four concentrations, differing by one-half-log units, were used prior to stimulation with an optimal concentration for 45 min to ascertain the extent of desensitization. This is an abbreviated subthreshold approach (half-log increments vs. twofold increments) that captures the important outcome of not observing significant

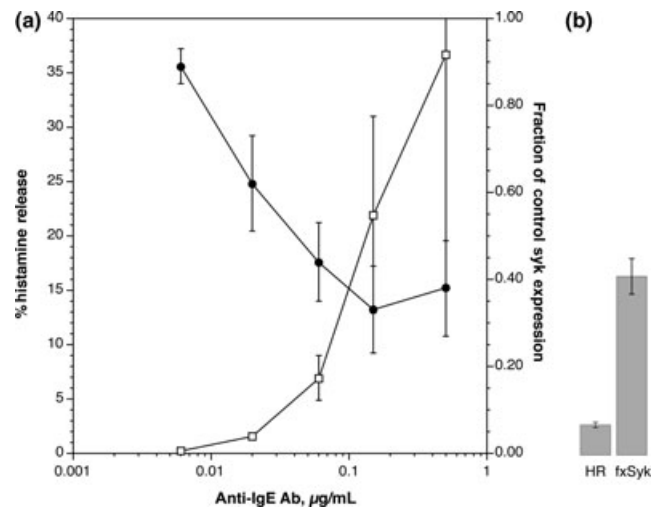


Fig. 1. Subthreshold dosing effects on syk expression ($n = 3$). (a) Concentration-dependence of histamine release (□) and loss of syk expression (●) in 24-h cultures of purified human basophils. The EC₅₀ for histamine release is approximately 0.11 $\mu\text{g/mL}$ and EC₅₀ for loss of syk expression is ~0.02 $\mu\text{g/mL}$. Histamine release was sampled from the supernatants after 24 h and the cells lysed for syk expression and analysed by Western blotting. (b) Loss of syk expression or the accumulated histamine release in basophils cultured with progressively higher concentrations of anti-IgE Ab (0.006, 0.02, 0.06, 0.15 $\mu\text{g/mL}$) over a 24-h period. Histamine release was sampled from the supernatants after 24 h and the cells lysed for syk expression.

histamine release at any point, including after optimal stimulation. Figs 1 and 2 show results for histamine release, syk and IgE/Fc ϵ RI expression respectively. For each experiment, the natural response to the five concentrations of anti-IgE antibody is included in the protocol and it can be seen that the starting concentration, 0.006 $\mu\text{g/mL}$, is a good starting point for the subthreshold protocol, showing no release. It can also be seen that at this concentration, there is some loss of syk expression, $\approx 10\%$, over the ensuing 24 h. As demonstrated previously [14], the EC₅₀ for loss of syk expression ($\approx 0.017 \mu\text{g/mL}$) is about sevenfold lower than the EC₅₀ for histamine release (0.11 $\mu\text{g/mL}$). The histogram to the right of the figure shows the total (cumulative) histamine release in the samples that were treated with the progressively escalating concentrations of anti-IgE antibody, in these experiments, an average of 2% release (6% of the response without the serial dosing). While histamine release was clearly blunted, loss of syk was unaffected, showing a loss similar to that observed at optimal levels of stimulation alone.

Fig. 2 shows the results for loss of IgE/Fc ϵ RI. The differential between the EC₅₀ for loss of receptor vs. histamine release is not as apparent, approximately twofold, although the EC₅₀ for loss of receptor is still less than for histamine release. There continues to be loss of receptor in the subthreshold series although it is, on

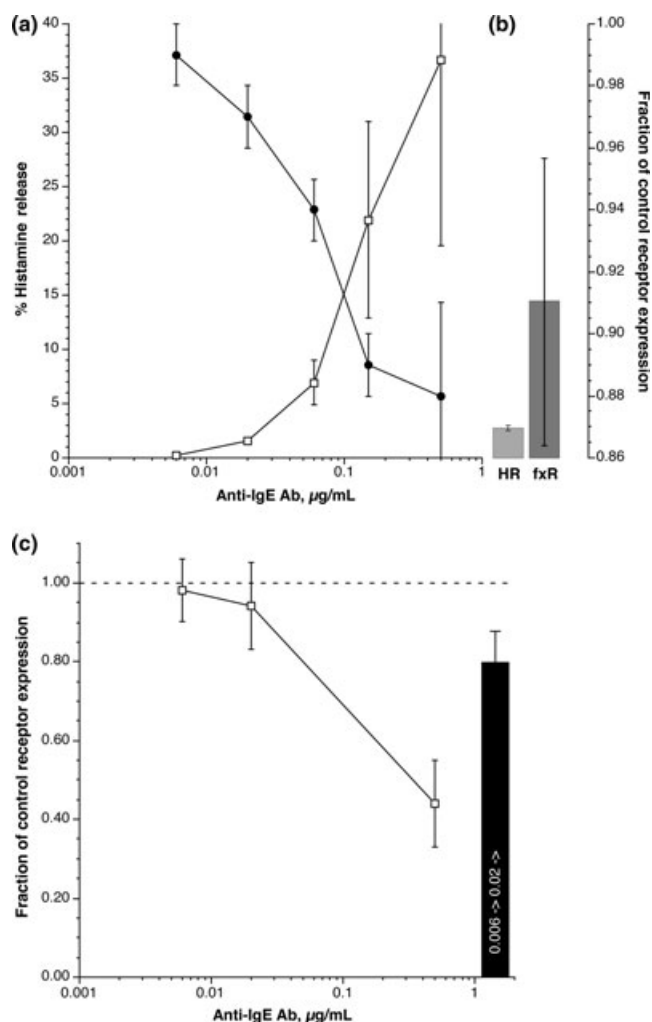


Fig. 2. Subthreshold dosing effects on FcεRI expression. (a) Concentration-dependence of histamine release (□) and loss of FcεRI expression (●) in 24-h cultures of purified human basophils ($n = 3$). The EC₅₀ for histamine release is ~ 0.11 μg/mL and EC₅₀ for loss of syk expression is ~ 0.06 μg/mL. Histamine release was sampled from the supernatants after 24 h and the cells analyzed by flow cytometry for the presence of the probe IgE, biotinylated gp120-specific IgE (see methods). (b) Loss of syk expression or the accumulated histamine release in basophils cultured with progressively higher concentrations of anti-IgE Ab (0.006, 0.02, 0.06, 0.15 μg/mL) over a 24 h period. Histamine release was sampled from the supernatants after 24 h and the cells analyzed by flow cytometry. (c) the design was similar to that used in (a) & (b) but the IgE probe was penicillin-specific IgE. An abbreviated stimulation series was used to determine the sensitivity of FcεRI loss in the early phase of the series where single concentrations had little effect ($n = 3$).

average, not quite the level observed for optimal stimulation. In a separate series, with an abbreviated dosing schedule to examine the two lowest concentrations for loss and using BPO-specific IgE to label the cells, Fig. 2c shows that the sequence of the first two doses generates more loss than either single dose alone.

Effect of IL-3

While exploring the optimal conditions required to perform these culture experiments, it was noticed that treatment with IL-3 for 3 days alters the behaviour of the system in an unexpected way. It has been known for some time that 24-h culture with 10 ng/mL IL-3 significantly enhances IgE-mediated histamine release [23–25] but we noted that after 3 days, the concentrations required for release shift leftward by 100-fold. This is shown in the online supplement Fig. 3e. This remarkable shift in sensitivity led to a re-examination of the subthreshold style experiment but in this case with the starting subthreshold concentration necessarily being 100-fold lower, 0.00006 μg/mL of anti-IgE Ab. Figs 3a and b summarize the experience with this protocol. Quite unexpectedly, the loss of syk expression did not shift with the increased sensitivity for histamine release. Under these conditions, the EC₅₀ for loss of syk was essentially the same as for the studies discussed above, 0.012 μg/mL while the EC₅₀ for histamine release was 0.00018 μg/mL, in other words, a reversal of sensitivity with syk no longer displaying the unique feature of occurring in the absence of histamine release. It can be seen that syk loss still occurs with the subthreshold method (Fig. 3c) but if the dosing were halted as one approached the near maximum for histamine release, the loss of syk would have been only modest. In contrast, loss of receptor expression shifted precisely with the shift in histamine release; the EC₅₀ for receptor loss was 0.00005 μg/mL vs. 0.00018 for histamine release, retaining the twofold to threefold differences observed in the cells not first treated for 3 days with IL-3. In addition, a subthreshold protocol induced loss of receptor expression (Fig. 3c).

The requirements for syk loss appear unaltered by the culture with IL-3 while the requirements for receptor loss mimic the requirements for histamine release. This led to experiments to determine which signalling steps shift with histamine release and which do not. Six signalling elements were examined, phosphorylation of lyn, syk, c-cbl, SHIP and Erk, and the elevation in cytosolic calcium. Fig. 4 shows that in the basophils treated for 3 days with IL-3, the phosphorylation of lyn, syk, c-cbl and SHIP do not track with the relative shift in histamine release (Fig. 4E in the online supplement shows representative Western blots from these experiments). Fig. 5E in the online supplement shows a synopsis of prior published studies for several signalling steps in freshly isolated cells, demonstrating the similarity in concentration-dependence for histamine release and the signalling steps (see discussion). For the purposes of this study, this was done again for the current anti-IgE Ab and once again, the concentration-dependence was concordant with histamine release in the

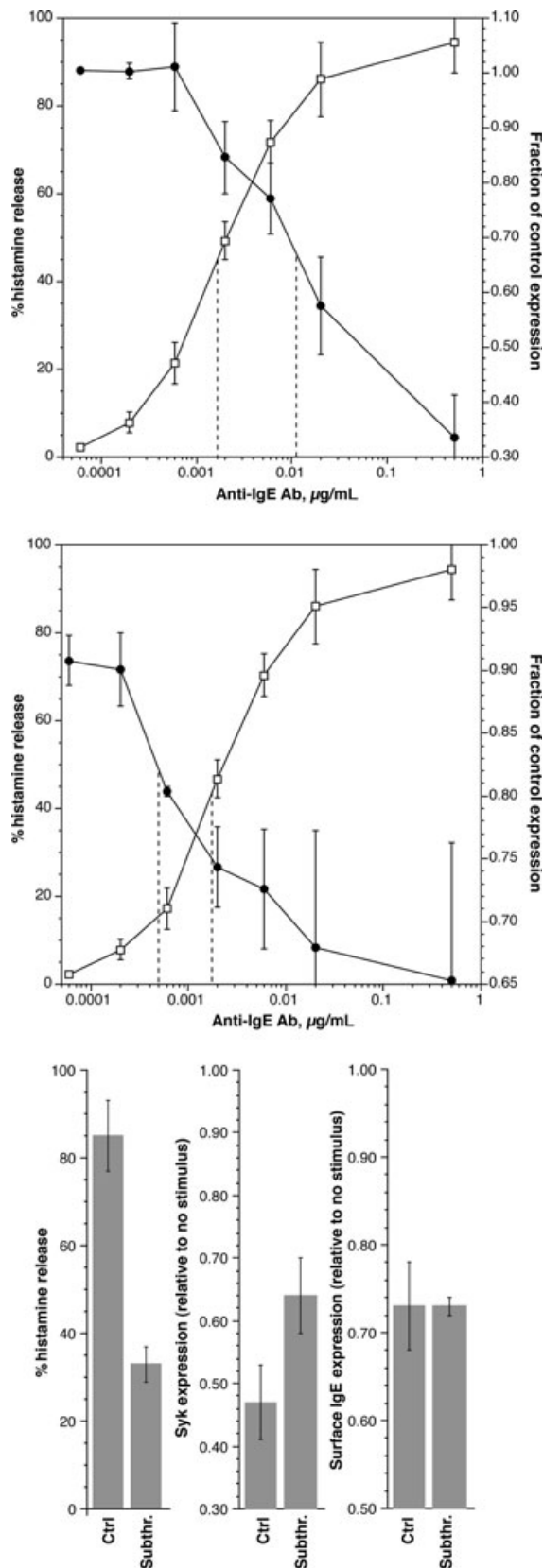


Fig. 3. Subthreshold dosing effects on syk or FcεRI expression on basophils first cultured for 3 days with 10 ng/mL IL-3. After 3 days of culture in IL-3, the cells were recovered, counted and re-established in a 24 h culture (with IL-3) with various levels of stimulation. (a) Concentration-dependence of histamine release (□) and loss of syk expression (●) in 24-h cultures ($n = 3$). Histamine release was sampled from the supernatants after 24 h and the cells analyzed by cell lysis and Western blotting. The EC₅₀ for histamine release is ~ 0.00018 µg/mL and EC₅₀ for loss of syk expression is ~ 0.06 µg/mL. (b) Concentration-dependence of histamine release (□) (which is the same data plotted in (a)) and loss of FcεRI expression (●) in 24-h cultures ($n = 3$). Histamine release was sampled from the supernatants after 24 h and the cells analyzed by flow cytometry for the presence of the probe IgE, biotinylated gp120-specific IgE (see methods). The EC₅₀ for histamine release is ~ 0.00018 µg/mL and EC₅₀ for loss of FcεRI expression is ~ 0.00008 µg/mL. (c) Run in parallel with the results shown in (a) and (b), (c) shows the loss of syk expression, FcεRI expression or the accumulated histamine release in basophils cultured with progressively higher concentrations of anti-IgE Ab (0.00006, 0.0002, 0.0006, 0.002, 0.006 µg/mL with a final histamine release test concentration of 0.02 µg/mL) over a 24 h period. Histamine release was sampled from the supernatants after 24 h and the cells analyzed by flow cytometry or by Western blotting ($n = 3$).

non-cultured basophils (data not shown). In contrast, the phosphorylation of Erk and the cytosolic calcium response moved leftward in a manner commensurate with the shift in histamine release (Fig. 6E in the online supplement shows an example cytosolic calcium response – note the delay in cytosolic calcium at the 0.006 µg/mL concentration, consistent with prior observations on the timelag characteristic [26]). Note that while c-cbl is already shifted leftward of the other signalling species, it does not shift its relative position following culture with IL-3.

In addition, in the 3-day cultured cells, despite the apparent absence of syk phosphorylation at very low concentrations of anti-IgE Ab (<0.005 µg/mL) that continue to induce histamine release, treatment of the cells with a syk inhibitor, NVP-QAB205 (at a concentration considered reasonably selective for syk, 0.3 µM [13, 15]), inhibited histamine release at both high and low concentrations of anti-IgE Ab ($95 \pm 4\%$ inhibition, $n = 3$).

These results suggest that IL-3 induces changes in signalling downstream of the earliest steps in signalling, a result consistent with previous studies of shorter treatments with IL-3 [27], although with much greater effect. Indirectly, this conclusion suggests that the crosslinking that occurs at concentrations of anti-IgE Ab in the range of 0.0001–0.01 µg/mL generate an undetectable signal that is revealed by the changes that occur to the signalling apparatus downstream of syk activation. The results also suggest that perhaps the nature of the crosslink

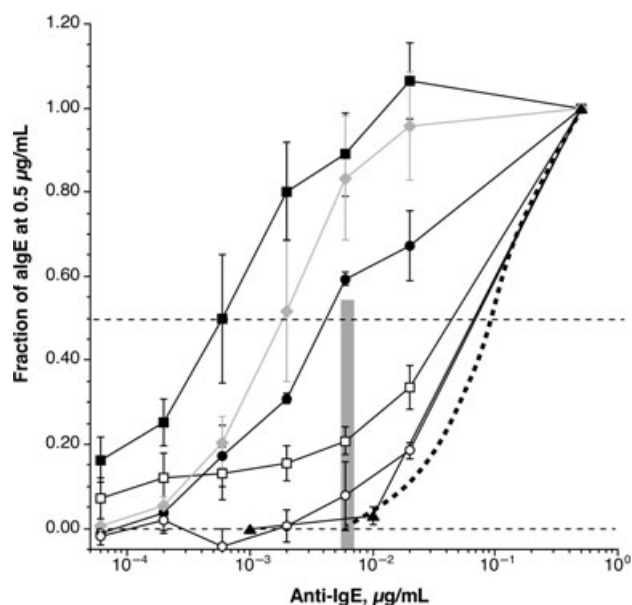


Fig. 4. Concentration-dependence of IgE-mediated signalling in basophils first cultured for 3 days in 10 ng/mL IL-3. Purified basophils were stimulated with the concentrations of anti-IgE Ab shown in the figure and cell pellets lysed after 20 min for a multiplex Western blotting (see methods). (♦) histamine release, (■) Erk phosphorylation, (●) c-cbl phosphorylation, (□) SHIP1 phosphorylation, (○) lyn phosphorylation (using a anti-phospho Src Ab), (▲) syk phosphorylation (using immunoprecipitation of syk followed by Western blotting with anti-phosphotyrosine Ab). Cytosolic calcium was analysed at two concentrations (0.5 µg/mL vs. 0.006 µg/mL) and the time-average of the kinetic curve plotted as a grey histogram. The heavy dotted line is placed in the figure to provide context for the concentration-dependence of day-0 histamine release.

is different. Notably, the anti-IgE antibody used is a monomeric monoclonal which at low concentrations may only generate small aggregates. Several studies in both basophils and RBL cells have suggested that dimeric-only aggregates generate both quantitatively and qualitatively different signals [28, 29]. Previous studies have noted that a bivalent hapten vs. a multivalent haptenated protein generate either predominantly small vs. large aggregates respectively [5]. To determine if small aggregates (primarily dimeric) generate signals are not readily translated to loss of syk expression, basophils were loaded with a series of penicillin-specific IgE densities and stimulated with either BPO2 or BPO (21)-HSA. Although histamine release differed for the two types of stimulation (the EC₅₀ for BPO2 being fourfold shifted from the EC₅₀ for BPO-HSA or tenfold for equivalent histamine release), the loss of syk was similar for either stimulus at a given density of IgE. Fig. 5 demonstrates that for similar loading of penicillin-specific IgE, BPO2 and BPO-HSA induced only slightly different amounts of syk loss. Therefore, the type of aggregate does not appear to be the determinant of whether syk is lost during activation.

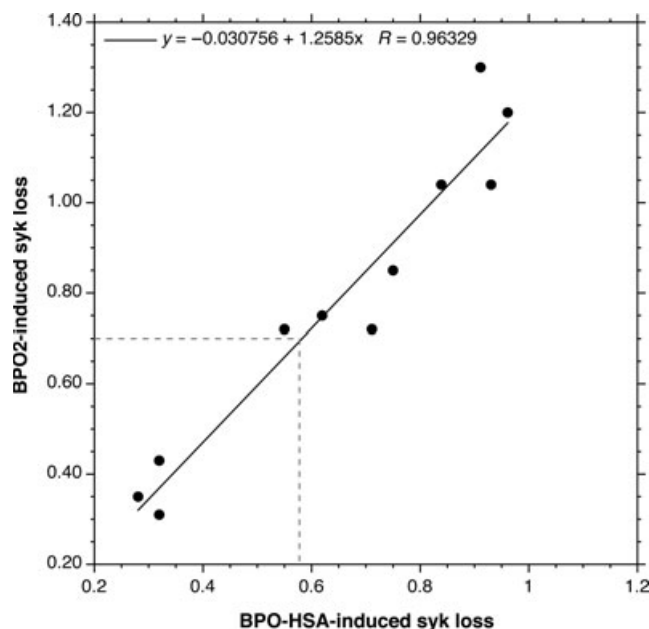


Fig. 5. Relationship between syk loss induced by overnight culture of BPO-sensitized basophils with either BPO(12)-HSA or BPO2 (penicillin linked by 1,8 diamino-octane). The concentration for BPO-HSA was chosen to be optimal for induction of histamine release (0.5 µg/mL) and slightly sub-optimal for BPO2 (5×10^{-9} M) in order to skew aggregate size towards dimers [29]. Previous studies have demonstrated that the EC₅₀ for these two antigens show an approximately fourfold difference in ability to induce histamine release or a tenfold difference for equivalent histamine release (since BPO2 does not induce the same maximum release as BPO-HSA) [5]. Basophils were sensitized with different amounts of BPO-specific IgE and ratio of stimulated/control plotted for BPO-HSA vs. BPO2. A fourfold difference in efficacy would have appeared to have a y-intercept that was significantly negative. The slope of the curve might also not be near one. The slope of the experimental results was essentially one with an intercept of zero.

The ability of the basophil to induce marked secretion when the phosphorylation of syk is not detectable (Fig. 4) is now seen as compatible with earlier observations that antigens like BPO-HSA induce very transient syk phosphorylation (unlike anti-IgE Abs) [12] but nevertheless induce expected levels of IL-4 as a rate much slower than histamine release in even day 0 cells. In Fig. 7E (panel A) of the online supplement is shown an experiment (representative of three similar experiments) that demonstrates the transient nature of syk phosphorylation with antigenic stimulation while simultaneously measuring the secretion of IL-4. After syk phosphorylation has returned to near resting levels, IL-4 secretion is still occurring. We have previously published that the addition of monovalent penicillin (BPO-EACA) at 30 min immediately stops the IL-4 secretion [30] and Fig. 7e (panel b) also shows that it stops IL-4 mRNA generation also, supporting the idea that signalling must be maintained, although syk phosphorylation is not detectable.

Discussion

These studies demonstrate that subthreshold desensitization of human basophils results in two outcomes, loss of syk or cell surface FcεRI, that are also characteristic of desensitization performed in the absence of extracellular calcium or during full activation at optimal concentrations of stimulus. These two outcomes may be considered integrative outcomes because they occur progressively over an extended period of time and in the case of day-0 basophils, occur with little histamine release. Fig. 5e summarizes our previous experience with various signalling steps and their relationship to histamine release. With the exception of c-cbl phosphorylation, all these signalling steps essentially coincide with the concentration-dependence of histamine release. It is probably reasonable to assume that during subthreshold stimulation, when histamine release is not occurring, that there remains some presence of signalling that cannot be readily observed but can mediate the desensitization that is the ultimate result of escalating subthreshold stimulation.

This latter point is highlighted in the experiments using basophils cultured for 3 days with IL-3. The results of these experiments raised a number of interesting questions about the signalling apparatus. First, signalling at, or before, the activation of syk is not observable at low concentrations of anti-IgE Ab that induce nearly maximal release. Both lyn and syk phosphorylation are easily apparent at 0.5 µg/mL anti-IgE Ab, a concentration that is optimal in day-0 cells, but are not apparent at concentrations below 0.01 µg/mL, the new maximum in day-3 cells. In previous studies, phosphorylation of SHIP has appeared dependent on lyn activity rather than syk activity [13] so that the absence of observable phosphorylation at low concentrations of anti-IgE Ab is not surprising. Phosphorylation of c-cbl has already been observed to be shifted leftward of histamine release by several folds [14] and this characteristic is consistent with the previously noted leftward shift (relative to histamine release) of syk loss. But like syk loss, c-cbl phosphorylation does not appear to shift further leftward with IL-3 treatment. However, both Erk phosphorylation and the cytosolic calcium response are shifted with histamine release. Taken together, these observations suggest that IL-3 induces a change in signalling that is downstream of the earliest signalling events, remarkably amplifying signalling at some point downstream of syk itself (with the caveat that phosphorylation is a reasonable surrogate for syk's activity). These characteristics are consistent with the phenotypic changes that are induced by much shorter incubations with IL-3 [27], which suggests that both early and late changes induced by IL-3 follow a similar pattern of not modifying the early steps that occur during FcεRI aggregation.

Second, these results indicate that the process that causes loss of syk expression is not influenced by IL-3. A priori, there is no expectation for this behaviour but given that syk is a step that follows aggregation of the receptor, which is presumably necessary to observe its loss from the cell surface, it is unexpected that cell surface FcεRI loss tracks with the shift in histamine release while syk does not. In addition, we have previously shown that receptor loss is not sensitive to the activities of syk [31] (but is sensitive to the activities of src-family kinases – as shown in the pilot studies and previous studies [22]). This result suggests that receptor internalization is a process that is sensitive to a change induced by IL-3 although it may be a change that is not the same as the change(s) that markedly amplify early signals like syk. Whatever the mechanism, these studies point to the possibility that loss of syk and receptor expression can be disconnected from each other under the proper conditions.

Third, the observation that the EC₅₀ for stimulated syk loss does not follow changes in the EC₅₀ for histamine release also implies that subthreshold desensitization could either be arranged to minimize the loss of syk or that subthreshold desensitization does not work as well. In the experiments done, it was found to be harder to choose concentrations of anti-IgE Ab that still induced a desensitized state without greater histamine release (see Fig. 3c). But this could be a technical issue, related to the need for a more subtle escalation of subthreshold concentrations of anti-IgE Ab.

The nature of the aggregates induced by very low concentrations of a monoclonal anti-IgE Ab are not known but since monoclonal antibodies should be capable of only inducing linear aggregates of IgE/FcεRI – a condition for other bivalent haptens like BPO2 – theoretical considerations would predict that a higher percentage of the aggregates would be dimeric at low concentrations [29, 32]. This possibility led to the hypothesis that perhaps small linear FcεRI aggregates (≤ 3) are inefficient for induction of syk loss but when IL-3-induced amplification steps are engaged, these same small aggregates are sufficient for enhanced downstream signalling. However, when we compared the ability of BPO2 and BPO(12)-HSA to induce loss of syk in basophils sensitized with BPO-specific IgE, there was only a small difference in the loss of syk for similar loading of the cell even though histamine release differed considerably. Therefore, it seems unlikely that aggregate size is a strong determinant of syk loss.

It is also apparent from both the day-3 results and results like those shown in the online supplement, Fig. 7E, that syk activity (assuming phosphorylation is an indicator of activity) does not need to be measurable for there to be a signal that induces mediator secretion. Despite that apparent absence of phosphorylation, the

reaction remains sensitive to a syk inhibitor or dis-aggregation by monovalent hapten [30].

In summary, these studies demonstrate that with respect to two well studied outcomes of basophil desensitization, loss of syk or FcεRI expression, subthreshold methods are efficient at engaging these mechanisms of down-regulation. However, these studies also demonstrated that it may be possible dissociate the loss of syk from mediator release and loss of FcεRI under the right conditions, notably incubation in cytokines that prime the basophil response; conditions that are common in cell cultures of mast cells derived from progenitors.

These studies also demonstrated that long exposures to IL-3 markedly alter signalling steps downstream of the earliest steps in signalling without any measurable effect on the early signals.

Acknowledgements

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Conflict of interest: There are no conflicts to report.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Use of penicillin-specific IgE to detect changes in cell surface IgE levels. The assay was initially developed by removing some endogenous IgE from basophils using a lactic acid protocol previously used in numerous studies [1, 2]. However, it was necessary to document the sensitivity of this assay to detect the potentially low levels of BPO-specific IgE that might result from sensitization of basophils without lactic acid removal. Panel A: purified basophils were sensitized without lactic acid treatment with two concentrations of BPO-specific IgE. The experiments were done with multiple replicates to determine the natural variation in background and sensitized cells. The cells were also analyzed for unoccupied receptor density using a monoclonal anti-FcεRI receptor antibody that only detects unoccupied receptors (15A5, Hoffmann-LaRoche). This antibody has been previously calibrated against absolute receptor densities, thus allowing an absolute 'per-cell' detection limit to be estimated from the variation in the replicates and background. See reference [3] for a more complete description of the calibration procedure. To calibrate the signal that detects BPO-specific IgE, one aliquot of cells was sensitized with a high concentration of BPO-specific IgE and one at a lower concentration. The cells were labeled with either 15A5 (or appropriate isotype control Ab) or by the procedure for detecting BPO-specific IgE. By flow cytometry (and running with pre-established PMT settings and calibrating against a standard bead), the net difference in the BPO-specific signal between the two loading conditions and the net difference in the 15A5 signal was determined (as the BPO-signal increases, the 15A5 signal decreases). Since 15A5 has been calibrated against an absolute receptor measurement, the delta for BPO-specific signal and the

delta for the 15A5 signal sets the equation; $BPO = 15A5 * (\text{calibrated value for } 15A5)$. The figure shows an example of the linearity of the assay and the coefficient of variation that suggests the lower of sensitivity would be ~50 molecules of BPO-specific IgE.

Figure S2. Detection of IgE-mediated down-regulation of cell surface IgE ($\pm$ PP1) using the BPO probe method. Basophils were sensitized (without lactic acid removal) and cultured with 10 μ M PP1 or the equivalent amount of DMSO vehicle (a 1/2000 dilution) overnight with and without stimulation with 0.5 μ g/mL anti-IgE Ab (6061). Flow cytometry of the remaining cell surface BPO-specific IgE (which presumably also indicates the total cell surface IgE density) was performed using the described procedure. Panels A and B: Examples of the flow cytography for this type of experiment; panel A, the gray filled histogram represents labeling with the anti-BPO-IgG in the presence of BPO-EACA (acts as the unlabeled control), the blue-filled histogram is the non-stimulated cells after overnight culture and the open red tracing, the stimulated cells after overnight culture (in this case 60% loss of signal). In panel B, gray is the unlabeled control, blue is the non-stimulated cells after overnight culture in 10 μ M PP1 and the open red tracing the stimulated cells after overnight culture in 10 μ M PP1 (in this case, 10% loss). Panel C; average of four experiments similar to those in panels A&B. Relative expression of BPO-specific IgE is plotted. These results are consistent with previous methods and functional changes [3, 4].

Figure S3. Histamine release induced by anti-IgE Ab (6061) before (●) and after (□) treatment for 3 days with 10 ng/mL IL-3.

Figure S4. Example Western blots for the signaling species examined. For the four signaling species, pErk, pSHIP, p-cbl, and p-Src, a single gel was run and the nitrocellulose cut at the approximately 64 kD line. The blotting order was phospho-Src followed by phospho-Erk for the portion < 64 kD and phospho-Cbl followed

by phospho-SHIP followed by p85a. The order was chosen to take advantage of differences in the strength of the signals detected and the selectivity of the antibodies, with the weaker antibodies analyzed first. We have previously established [5] that p85a is a good lane-loading control antibody because its expression is unmodified during a wide variety of experimental designs and because it is easy to detect as the last step in this multiplex Western blot design. To detect syk phosphorylation, we prefer the use of immunoadsorption (IP) because existing phospho-syk antibodies show backgrounds that are not observed with immunoadsorption and are considerably less selective. Therefore, the bottom portion of figure shows the Western blots for syk phosphorylation run using cells stimulated in parallel with the cells used in top portion. Two time points were examined to confirm the expectation that for anti-IgE antibody, the 20 min time point was sufficient. Previous studies of later signaling such as Erk phosphorylation may be transient, but usually the period between 5–20 min is similar enough to not complicate the interpretation of using a single time point for these measurements. However, it is possible that the concentration-dependence curves could be skewed in favor of lower concentrations (the EC₅₀ appearing leftward) because lower concentrations were only reaching their maximum by 20 min while higher concentrations may have been subsiding from their maximum at 20 min. Given the qualitative nature of the question, with signaling observed at very low concentrations, the timing should not be critical. The phospho-Src measurement includes three bands, p53, p56 and p59 (approximately). The p53 and p56 bands match well with the two lyn splice variants while the p59 band is unassigned. It might be consistent with fyn. In previous studies, we observe that the p53 form of lyn is the band that most clearly increases its state of phosphorylation. The reasons for the differences from p56 are not understood. We have also previously shown in some detail that the band representing c-cbl phosphorylation is broad and shows generally as a smear at approximately 110 kD [6]. Densitometric measurements readily cover the region but it isn't as obvious to the eye when presenting small slices of the blot as shown in the figure.

Figure S5. Synopsis of previously published studies of the relative concentration-dependence of histamine release vs. signaling element activation in basophils

stimulated with an anti-IgE Ab. The figure is a synthesis of various studies in separate publications [2, 7–9]. (○) phospho-Shc, (●) phospho-SHIP1, (■) phospho-c-cbl, (◆) phospho-Erk 1/2, (□) phospho-syk, and (■) histamine release. The gray histogram represents the relative time-average of the cytosolic calcium response kinetics for a 15 min period of observation.

Figure S6. Cytosolic calcium response in purified basophils after 3 days of culture with 10 ng/mL IL-3 and stimulation with either 0.5 µg/mL (○) or 0.006 µg/mL of anti-IgE Ab (6061). The 0.006 µg/mL concentration was chosen to represent stimulation that was well below concentration needed to observe a significant basophil response in untreated basophils but still high enough to generate a strong response in basophils treated for 3 days with IL-3. The time-lag for this low concentration is consistent with previous studies of basophil stimulation [10].

Figure S7. Kinetic differences between syk phosphorylation and secretion of IL-4. Panel A: Basophils were sensitized with BPO-specific IgE and stimulated with an optimal concentration of BPO(12)-HSA for the times shown. Samples of supernatant were analyzed by an IL-4 ELISA (data is shown without normalizing for cell number) or cell pellets lysed and syk phosphorylation analyzed by Western blotting (relative phosphorylation expressed in band densities, arbitrary units). The plot shows one representative experiment of three. Panel B; basophils were sensitized with BPO-specific IgE and stimulated with BPO(12)-HSA. At 45 min, monovalent penicillin (BPO-EACA) was added to dissociate aggregates. Cell lysates were analyzed for IL-4 mRNA by real-time PCR. Previous studies provide the primers used for qPCR and have shown that IL-4 mRNA decays with a half-life of ~1–2 h when actinomycin D is added [11], a value that is consistent with the decay rate observed in this experiment after BPO-EACA was added. Previously published studies also noted that IL-4 secretion stops nearly immediately after addition of BPO-EACA

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Frequency

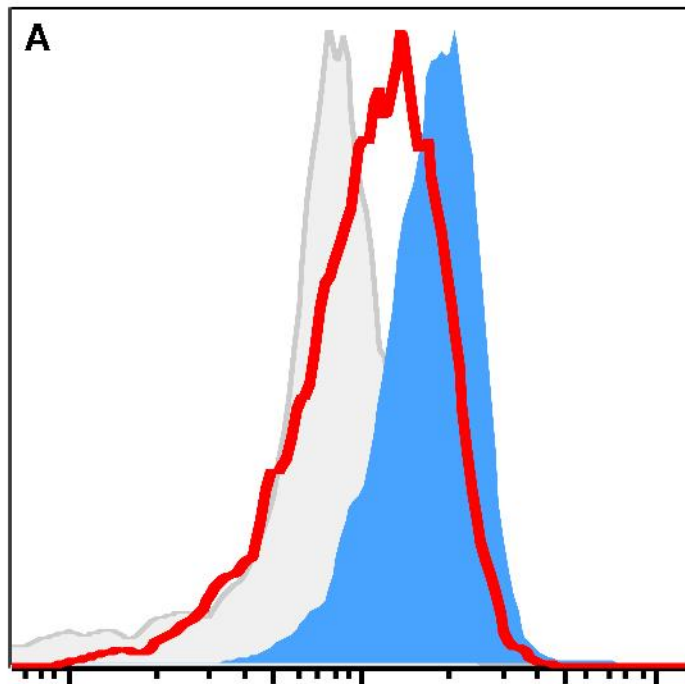
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- 400 IgE/cell, net MFI = 20

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Penicillin-specific IgE (alexa647 fluorescence)

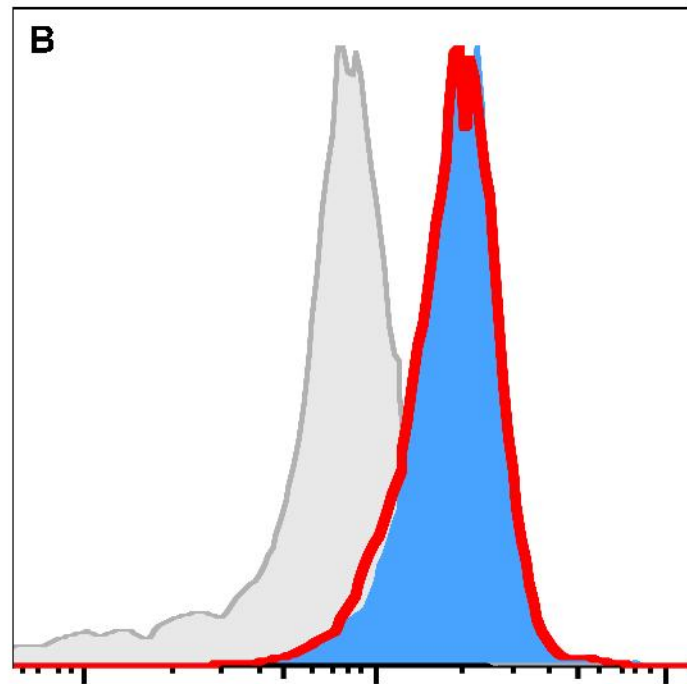
Figure 1E

Online Supplementary Figures

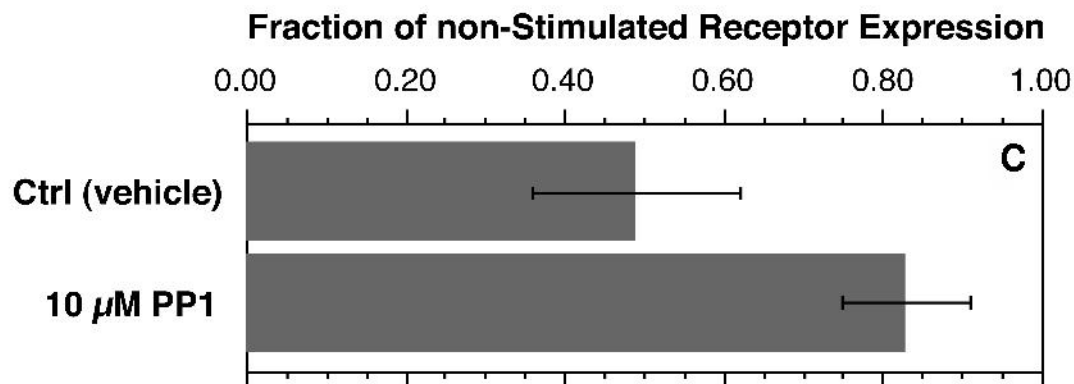
Figure 1E: Use of penicillin-specific IgE to detect changes in cell surface IgE levels. The assay was initially developed by removing some endogenous IgE from basophils using a lactic acid protocol previously used in numerous studies [1, 2]. However, it was necessary to document the sensitivity of this assay to detect the potentially low levels of BPO-specific IgE that might result from sensitization of basophils without lactic acid removal. Panel A: purified basophils were sensitized without lactic acid treatment with two concentrations of BPO-specific IgE. The experiments were done with multiple replicates to determine the natural variation in background and sensitized cells. The cells were also analyzed for unoccupied receptor density using a monoclonal anti-FcεRI receptor antibody that only detects unoccupied receptors (15A5, Hoffmann-LaRoche). This antibody has been previously calibrated against absolute receptor densities, thus allowing an absolute 'per-cell' detection limit to be estimated from the variation in the replicates and background. See reference [3] for a more complete description of the calibration procedure. To calibrate the signal that detects BPO-specific IgE, one aliquot of cells was sensitized with a high concentration of BPO-specific IgE and one at a lower concentration. The cells were labeled with either 15A5 (or appropriate isotype control Ab) or by the procedure for detecting BPO-specific IgE. By flow cytometry (and running with pre-established PMT settings and calibrating against a standard bead), the net difference in the BPO-specific signal between the two loading conditions and the net difference in the 15A5 signal was determined (as the BPO-signal increases, the 15A5 signal decreases). Since 15A5 has been calibrated against an absolute receptor measurement, the delta for BPO-specific signal and the delta for the 15A5 signal sets the equation; $BPO = 15A5 * (\text{calibrated value for } 15A5)$. The figure shows an example of the linearity of the assay and the coefficient of variation that suggests the lower of sensitivity would be approximately 50 molecules of BPO-specific IgE.



Fluorescence (BPO-specific IgE)



Fluorescence (BPO-specific IgE)



Online Supplementary Figures

Figure 2E: Detection of IgE-mediated down-regulation of cell surface IgE ($\pm$ PP1) using the BPO probe method. Basophils were sensitized (without lactic acid removal) and cultured with 10 μ M PP1 or the equivalent amount of DMSO vehicle (a 1/2000 dilution) overnight with and without stimulation with 0.5 μ g/ml anti-IgE Ab (6061). Flow cytometry of the remaining cell surface BPO-specific IgE (which presumably also indicates the total cell surface IgE density) was performed using the described procedure. Panels A and B: Examples of the flow cytography for this type of experiment; panel A, the gray filled histogram represents labeling with the anti-BPO-IgG in the presence of BPO-EACA (acts as the unlabeled control), the blue-filled histogram is the non-stimulated cells after overnight culture and the open red tracing, the stimulated cells after overnight culture (in this case 60% loss of signal). In panel B, gray is the unlabeled control, blue is the non-stimulated cells after overnight culture in 10 μ M PP1 and the open red tracing the stimulated cells after overnight culture in 10 μ M PP1 (in this case, 10% loss). Panel C; average of 4 experiments similar to those in panels A&B. Relative expression of BPO-specific IgE is plotted. These results are consistent with previous methods and functional changes [3, 4].

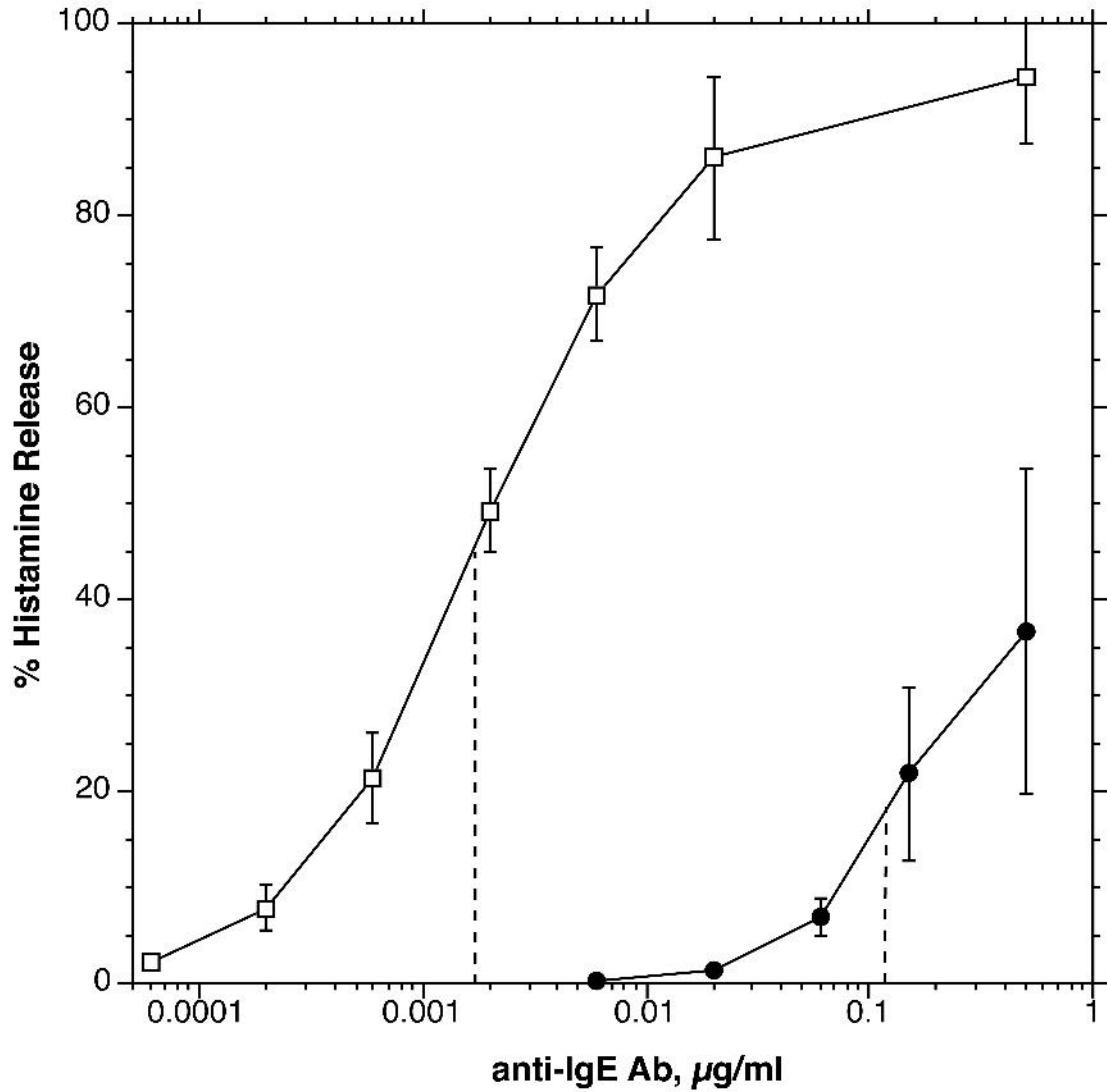


Figure 3E

Online Supplementary Figures

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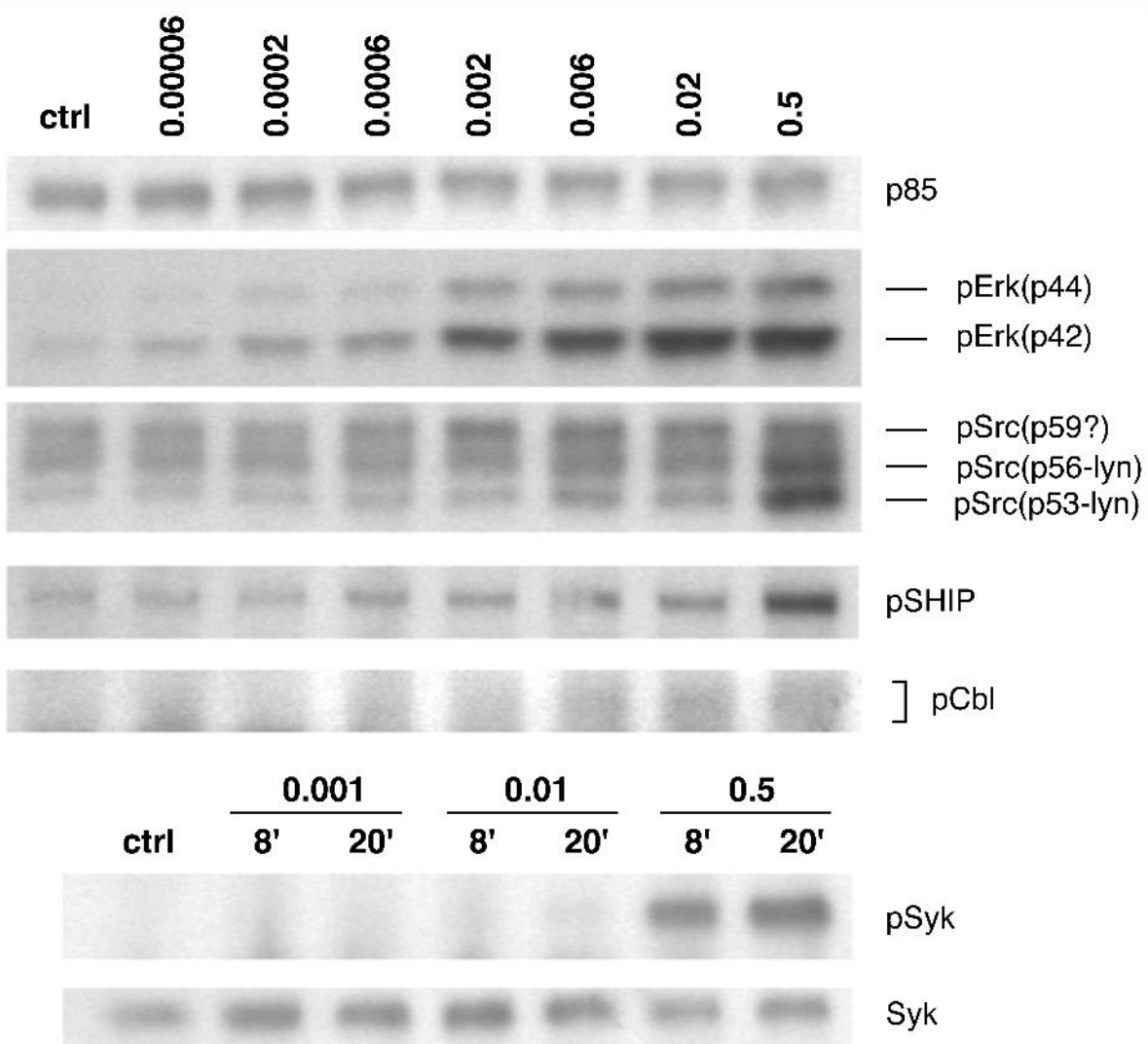


Figure 4E

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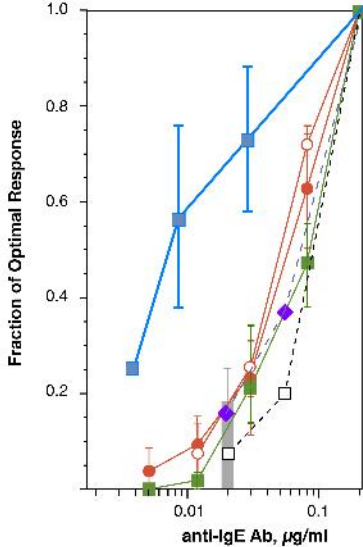


Figure 5E

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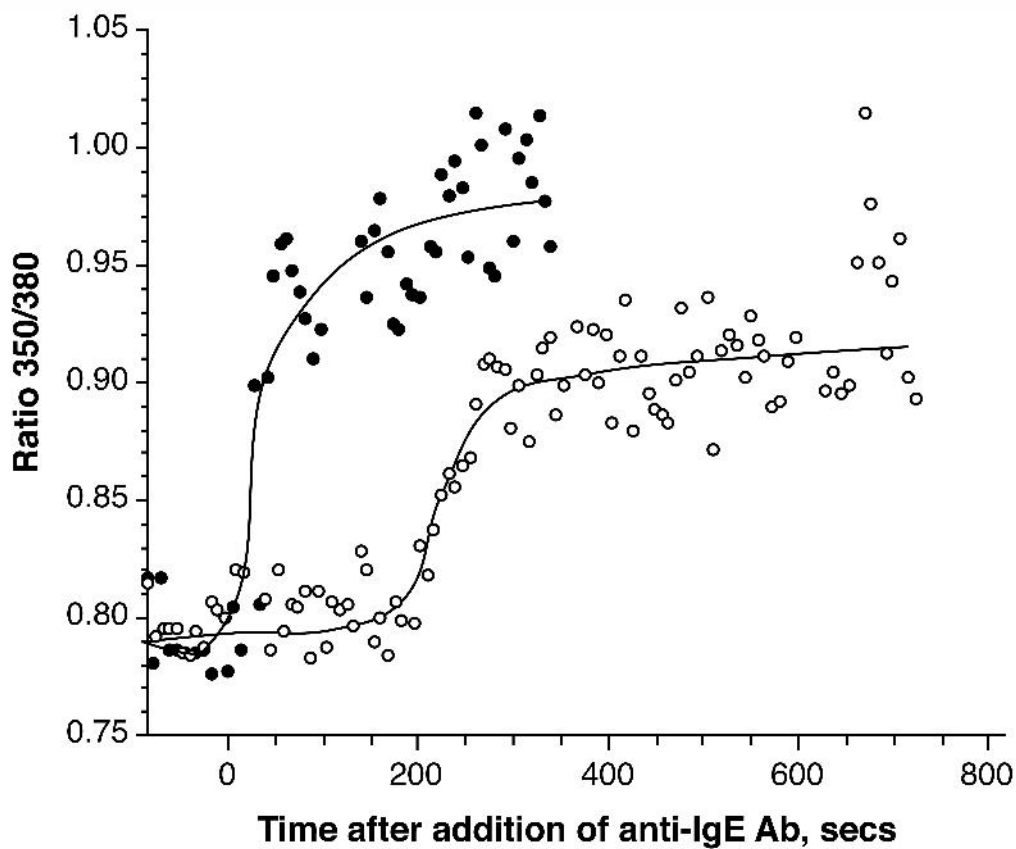


Figure 6E

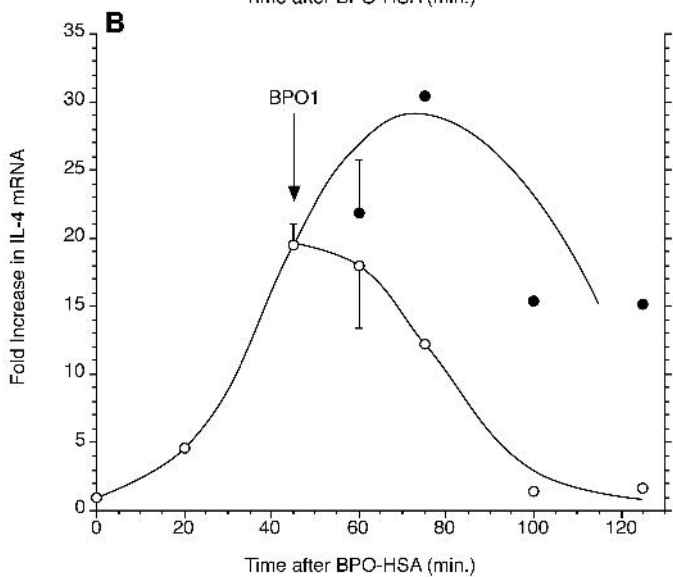
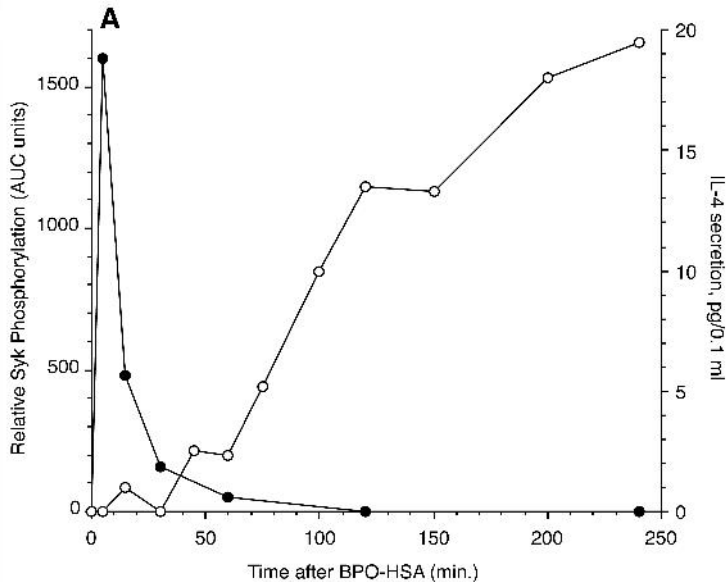


Figure 7E

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Figure 1E: Use of penicillin-specific IgE to detect changes in cell surface IgE levels. The assay was initially developed by removing some endogenous IgE from basophils using a lactic acid protocol previously used in numerous studies [1, 2]. However, it was necessary to document the sensitivity of this assay to detect the potentially low levels of BPO-specific IgE that might result from sensitization of basophils without lactic acid removal. Panel A: purified basophils were sensitized without lactic acid treatment with two concentrations of BPO-specific IgE. The experiments were done with multiple replicates to determine the natural variation in background and sensitized cells. The cells were also analyzed for unoccupied receptor density using a monoclonal anti-FcεRI receptor antibody that only detects unoccupied receptors (15A5, Hoffmann-LaRoche). This antibody has been previously calibrated against absolute receptor densities, thus allowing an absolute 'per-cell' detection limit to be estimated from the variation in the replicates and background. See reference [3] for a more complete description of the calibration procedure. To calibrate the signal that detects BPO-specific IgE, one aliquot of cells was sensitized with a high concentration of BPO-specific IgE and one at a lower concentration. The cells were labeled with either 15A5 (or appropriate isotype control Ab) or by the procedure for detecting BPO-specific IgE. By flow cytometry (and running with pre-established PMT settings and calibrating against a standard bead), the net difference in the BPO-specific signal between the two loading conditions and the net difference in the 15A5 signal was determined (as the BPO-signal increases, the 15A5 signal decreases). Since 15A5 has been calibrated against an absolute receptor measurement, the delta for BPO-specific signal and the delta for the 15A5 signal sets the equation; $BPO = 15A5 * (\text{calibrated value for } 15A5)$. The figure shows an example

of the linearity of the assay and the coefficient of variation that suggests the lower of sensitivity would be approximately 50 molecules of BPO-specific IgE.

Figure 2E: Detection of IgE-mediated down-regulation of cell surface IgE ($\pm$ PP1) using the BPO probe method. Basophils were sensitized (without lactic acid removal) and cultured with 10 μ M PP1 or the equivalent amount of DMSO vehicle (a 1/2000 dilution) overnight with and without stimulation with 0.5 μ g/ml anti-IgE Ab (6061). Flow cytometry of the remaining cell surface BPO-specific IgE (which presumably also indicates the total cell surface IgE density) was performed using the described procedure. Panels A and B: Examples of the flow cytography for this type of experiment; panel A, the gray filled histogram represents labeling with the anti-BPO-IgG in the presence of BPO-EACA (acts as the unlabeled control), the blue-filled histogram is the non-stimulated cells after overnight culture and the open red tracing, the stimulated cells after overnight culture (in this case 60% loss of signal). In panel B, gray is the unlabeled control, blue is the non-stimulated cells after overnight culture in 10 μ M PP1 and the open red tracing the stimulated cells after overnight culture in 10 μ M PP1 (in this case, 10% loss). Panel C; average of 4 experiments similar to those in panels A&B. Relative expression of BPO-specific IgE is plotted. These results are consistent with previous methods and functional changes [3, 4].

Figure 3E: Histamine release induced by anti-IgE Ab (6061) before (●) and after (□) treatment for 3 days with 10 ng/ml IL-3.

Figure 4E: Example Western blots for the signaling species examined. For the 4 signaling species, pErk, pSHIP, p-cbl, and p-Src, a single gel was run and the nitrocellulose cut at the

approximately 64 kD line. The blotting order was phospho-Src followed by phospho-Erk for the portion < 64 kD and phospho-Cbl followed by phospho-SHIP followed by p85a. The order was chosen to take advantage of differences in the strength of the signals detected and the selectivity of the antibodies, with the weaker antibodies analyzed first. We have previously established [5] that p85a is a good lane-loading control antibody because its expression is unmodified during a wide variety of experimental designs and because it is easy to detect as the last step in this multiplex Western blot design. To detect syk phosphorylation, we prefer the use of immunoadsorption (IP) because existing phospho-syk antibodies show backgrounds that are not observed with immunoadsorption and are considerably less selective. Therefore, the bottom portion of figure shows the Western blots for syk phosphorylation run using cells stimulated in parallel with the cells used in top portion. Two time points were examined to confirm the expectation that for anti-IgE antibody, the 20 minute time point was sufficient. Previous studies of later signaling such as Erk phosphorylation may be transient, but usually the period between 5-20 minutes is similar enough to not complicate the interpretation of using a single time point for these measurements. However, it is possible that the concentration-dependence curves could be skewed in favor of lower concentrations (the EC50 appearing leftward) because lower concentrations were only reaching their maximum by 20 minutes while higher concentrations may have been subsiding from their maximum at 20 minutes. Given the qualitative nature of the question, with signaling observed at very low concentrations, the timing should not be critical. The phospho-Src measurement includes three bands, p53, p56 and p59 (approximately). The p53 and p56 bands match well with the two lyn splice variants while the p59 band is unassigned. It might be consistent with fyn. In previous studies, we observe that the p53 form of lyn is the band that most clearly increases its state of phosphorylation. The reasons for the differences

from p56 are not understood. We have also previously shown in some detail that the band representing c-cbl phosphorylation is broad and shows generally as a smear at approximately 110 kD [6]. Densitometric measurements readily cover the region but it isn't as obvious to the eye when presenting small slices of the blot as shown in the figure.

Figure 5E: Synopsis of previously published studies of the relative concentration-dependence of histamine release vs. signaling element activation in basophils stimulated with an anti-IgE Ab. The figure is a synthesis of various studies in separate publications [2, 7-9]. (○) phospho-Shc, (●) phospho-SHIP1, (■) phospho-c-cbl, (◆) phospho-Erk 1/2, (□) phospho-syk, and (■) histamine release. The gray histogram represents the relative time-average of the cytosolic calcium response kinetics for a 15 minute period of observation.

Figure 6E: Cytosolic calcium response in purified basophils after 3 days of culture with 10 ng/ml IL-3 and stimulation with either 0.5 µg/ml (○) or 0.006 µg/ml of anti-IgE Ab (6061). The 0.006 µg/ml concentration was chosen to represent stimulation that was well below concentration needed to observe a significant basophil response in untreated basophils but still high enough to generate a strong response in basophils treated for three days with IL-3. The time-lag for this low concentration is consistent with previous studies of basophil stimulation [10].

Figure 7E: Kinetic differences between syk phosphorylation and secretion of IL-4. Panel A: Basophils were sensitized with BPO-specific IgE and stimulated with an optimal concentration of BPO(12)-HSA for the times shown. Samples of supernatant were analyzed by an IL-4 ELISA (data is shown without normalizing for cell number) or cell pellets lysed and syk phosphorylation

analyzed by Western blotting (relative phosphorylation expressed in band densities, arbitrary units). The plot shows one representative experiment of three. Panel B; basophils were sensitized with BPO-specific IgE and stimulated with BPO(12)-HSA. At 45 minutes, monovalent penicillin (BPO-EACA) was added to dissociate aggregates. Cell lysates were analyzed for IL-4 mRNA by real-time PCR. Previous studies provide the primers used for qPCR and have shown that IL-4 mRNA decays with a half-life of approximately 1-2 hours when actinomycin D is added [11], a value that is consistent with the decay rate observed in this experiment after BPO-EACA was added. Previously published studies also noted that IL-4 secretion stops nearly immediately after addition of BPO-EACA [12].

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