

Inducing an anergic state in mast cells and basophils without secretion

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Background: IgE-mediated secretion from mast cells or basophils depends on the activity of both spleen tyrosine kinase (syk) and phosphatidylinositol 3' kinase (PI3K), but several specific downregulatory pathways (eg, loss of syk expression) do not.

Objective: We tested whether stimulation with antigen in the presence of a syk inhibitor (NVP-QAB205) would ablate secretion while simultaneously allowing anergy.

Methods: The anergic or desensitized state in human basophils, cultured-derived mast cells, and *in situ* stimulated airway mast cells (in organ baths) was assessed after stimulation with antigen in the presence of syk inhibitor.

Results: Antigen caused $35 \pm 7\%$ and $62 \pm 10\%$ histamine release from basophils and mast cells, respectively, and it caused an $87 \pm 5\%$ histamine/leukotriene D_4 -dependent contraction of human isolated bronchi. All of these responses were blocked $>95\%$ by the syk inhibitor. Rechallenging the preparations with antigen, after first washing out the syk inhibitor and antigen, revealed that near complete anergy (92% to 100%) occurred in each case. A similar result was found when using a PI3K inhibitor, LY294002, in studies of basophils.

Conclusion: Although the syk inhibitor nearly abolished the antigen-induced secretion from mast cells and basophils, it had little effect on the pathways involved in anergy. These results suggest that syk and PI3K are not involved in downregulation leading to anergy. (*J Allergy Clin Immunol* 2008;121:1500-6.)

Key words: Desensitization, spleen tyrosine kinase, phosphatidylinositol 3' kinase

All receptor-mediated events must have termination mechanisms to prevent a runaway response by the cell. Some of these mechanisms may be enhanced versions of the homeostatic mechanisms that maintain low receptor activity until ligand is present, or the mechanisms may be specialized to explicitly downregulate an activated receptor. There is also interest today in collateral mechanisms that serve to keep the threshold for a response high. Examples of these 3 categories in the IgE/FcεRI-

Abbreviations used

HSA: Human serum albumin

LT: Leukotriene

PI3K: Phosphatidylinositol 3' kinase

SHIP: Src homology domain 2-containing inositol phosphatase

syk: Spleen tyrosine kinase

dependent signaling cascade might include the local feedback loop associated with regulation of src-family kinase,^{1,2} the loss of spleen tyrosine kinase (syk) by ubiquitylation,³⁻⁶ and the association of FcεRI (and its immunotyrosine activation motif-bearing subunits) with immunotyrosine inhibition motif-bearing receptors like FcγRIIb.⁷⁻⁹ Each of these mechanisms has its place in controlling an IgE-mediated reaction. In recent years, a number of other mechanisms have been elucidated, but they all serve to terminate an ongoing IgE-mediated signal.

It has been demonstrated by several different methods that syk activity is both critical and nonredundant to initiating most mast cell/basophil responses to IgE-mediated activation.¹⁰⁻¹² However, in the process of sorting out the various mechanisms involved in signal termination of the IgE-mediated reaction, our recent studies revealed an interesting pattern. Third-generation syk inhibitors made these observations possible because they have enough selectivity¹³ to provide some confidence in interpreting the various studies where they have been used. First, these inhibitors have a limited effect on the phosphorylation of Src homology domain 2-containing inositol phosphatase (SHIP).¹³ SHIP is a phosphatase that probably has a complex role in signaling, but its primary enzymatic activity includes the dephosphorylation of phosphatidylinositol triphosphate (PIP3), a lipid that is critical for recruiting some positive signaling elements to the plasma membrane. There is reasonable evidence from studies of bone marrow-derived murine mast cells that SHIP is a counterregulatory element—that is, a mechanism to terminate the ongoing response.¹⁴ There is less direct evidence in human basophils that this is true, but current data support the idea.^{13,15} Therefore, studies showing that syk inhibitors do not potently inhibit SHIP phosphorylation, a marker of SHIP participation in the signaling complex, suggest that at least 1 downregulatory step is not directly dependent on syk activity. Second, we also noted that downregulation of syk after long-term aggregation of FcεRI, presumably by ubiquitylation,⁵ did not depend on the enzymatic activity of syk.¹⁶ These 2 processes may have some role in signal termination, but in one case, the precise role of SHIP is not yet known, and in the second case, the downregulation of syk requires a period that is too slow to account for the fast signal termination that limits histamine and leukotriene release. It is not clear whether termination of histamine release is independent of syk activity in the same

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way as the more lengthy process of downregulation associated with a process like syk ubiquitylation. In other words, a full listing of the mechanisms of downregulation is not in hand, so it may be the case that only some of the downregulatory mechanisms would be independent of syk activity. If so, a syk inhibitor might have a limited effect on downregulation of basophil functions. The only way to test for generality of the role of syk in downregulation is to examine the complete secretory response for signs that downregulation occurs in the absence of syk activity. The following studies were initiated to determine whether it is possible to engage downregulation fully of the basophil or mast cell—that is, fully desensitize the cell's ability to respond to antigenic challenge, while disallowing secretion. It was demonstrated in the early 1970s that it was possible to accomplish this outcome by excluding extracellular calcium during a period of exposure to antigen.¹⁷ This form of desensitization has been studied many different ways during the intervening years. A second method has been to perform the experiment under normal secretory conditions but to titrate in the antigen slowly, beginning with concentrations that induce only very weak secretion.^{18–20} The following study approaches the question by focusing on which signal activation elements—specifically asking about syk kinase—are necessary for any form of signal termination/desensitization.

METHODS

Materials and buffers

The [Online Repository](http://www.jacionline.org) at www.jacionline.org lists the common reagents and buffers used in these studies, and they are also listed in previous publications.¹³ PIPES-albumin-glucose and PAGCM were the primary buffers (piperazine-N,N'-bis[2-ethanesulfonic acid], albumin, glucose, $\pm$ calcium magnesium). NVP-QAB205 was a gift from GlaxoSmithKline (Stonhedge, United Kingdom), and its characteristics have been published.²¹

Basophil and mast cell preparation

Basophils, impure and purified, were prepared as previously described.¹³ Human mast cells were obtained by culturing CD34-positive cells according to methods previously described.^{22,23} Briefly, CD34⁺ cells were cultured in the presence of recombinant IL-3 and stem cell factor for a period greater than 8 weeks.

Cell handling

The [Online Repository](http://www.jacionline.org) contains detailed, but standard, methods on IgE dissociation and sensitization, basophil culture, flow cytometry, and immunoprecipitation.^{13,24}

Airway tissue challenges

The [Online Repository](http://www.jacionline.org) contains the previously published methods²⁵ for handling human lung specimens. The contractions caused by ragweed antigen were obtained in a cumulative fashion and expressed as a percentage of that obtained by a maximally effective concentration of carbachol (10 μ mol/L) added at the end of the experiment.

The syk inhibitor was added to the tissue baths 30 minutes before the beginning of the antigen-E concentration-response curve. In some cases, NVP-QAB205 was washed from the tissues by replacing the buffer with fresh solution every minute times 5 minutes, then every 15 minutes times 60 minutes. NVP-QAB205, washed out in this manner, had no effect on the magnitude of the carbachol-induced contractions as quantified as grams of tension ($P > .1$) while inhibiting contractions by 98% when present with antigen.

RESULTS

Basophils and syk inhibition

Previous studies have shown that a third generation inhibitor of syk kinase inhibited activation steps and mediator release with an inhibitory concentration 50 of 0.04 μ mol/L,¹³ but did not inhibit the loss of syk during an overnight incubation with anti-IgE Ab.¹⁶ The inhibitor did result in some inhibition of syk loss at 400 times the expected IC₅₀, but this was considered a nonselective effect. In recent studies, we demonstrated that there is a slow loss of Fc ϵ RI from the cell surface that requires approximately 2 to 4 hours to observe the initial loss and 2 days to reach maximal loss from the cell surface.²⁴ Degradation was slow and usually incomplete, reaching a maximum of about 50% after 24 hours. This loss is also a form of downregulation, albeit one that requires a longer time frame than the self-limiting kinetics of histamine release. The ability of the syk inhibitor, NVP-QAB205, to inhibit either loss from the cell surface or degradation of internalized receptor was examined. [Fig 1](#) demonstrates that the inhibition of syk activity had no influence on either loss from the cell surface or degradation of receptor, at least not at concentrations consistent with the drug's activity toward syk. Previous studies have not shown whether the slow loss of antigen-specific IgE and its receptor from the cell surface is accompanied by loss of receptors bound to IgE that have a different antigen specificity. Basophils sensitized with dinitrophenyl-specific and gp120-specific IgE (the latter of which had been labeled with biotin) were incubated for 18 hours with dinitrophenyl(7)-human serum albumin (HSA), and then cell surface expression levels of the gp120-specific IgE were assessed by flow cytometry using streptavidin-alexa647. Basophils treated with dinitrophenyl-HSA bound $84 \pm 3\%$ ($n = 4$; $P = .008$; value $\neq 100\%$) of the streptavidin bound to untreated cells. Although a subtle effect, this is another form of nonspecific desensitization, like the loss of syk, that would down-regulate the response to noncross-reacting antigens.

We next asked whether desensitization in basophils, as a general functional phenomenon, was inhibited by syk inhibition. Previous studies have shown that inhibitors of the src-family kinases, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP) 1 or PP2, inhibit desensitization, whereas third-generation protein kinase C (PKC) inhibitors do not.²⁶ The current experiments were performed by using 2 methods. The first method uses the well described technique of inducing cellular desensitization. In this technique, the cells are stimulated in the absence of extracellular calcium, which therefore prevents mediator release. A variety of unpublished studies in this laboratory have shown that many signaling steps continue to occur but without a sustained rise in intracellular calcium (while the release of internal stores of calcium in the first 30–60 seconds continues to occur²⁷). After a period, calcium is returned to the buffer, and the cells are monitored for mediator release. In human basophils, a period of 60 minutes is usually sufficient nearly to ablate any subsequent response when calcium is returned to the buffer, but 45 minutes was chosen to produce partial desensitization because, *a priori*, it wasn't known whether the drug would have no effect, accelerate, or inhibit the desensitization process. If a drug is introduced into the first phase of the experiment (the calcium-free phase) and is then effectively washed away, it is possible to test whether the drug inhibits desensitization. This type of experiment was performed by using NVP-QAB205 in the first phase of the experiment. There are several controls in this experimental design.

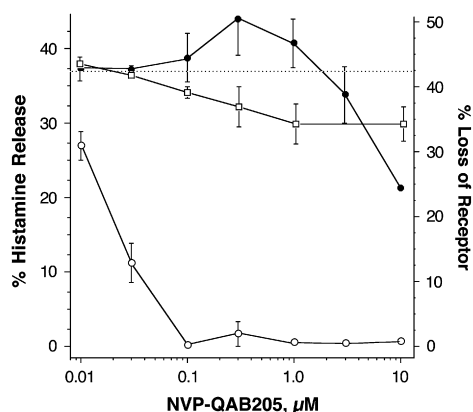


FIG 1. Lack of inhibition of receptor loss in the presence of a syk inhibitor. Purified basophils were stimulated for 18 hours with optimal anti-IgE Ab (0.2 μ g/mL) in the presence of either vehicle control (dimethyl sulfoxide) or NVP-QAB205 at the concentrations shown. The loss of 22E7-positive Fc ϵ RI in whole cell lysates (●) or from the cell surface (□) is compared with histamine release (○) in the same samples. The dotted line represents the stimulated response in the presence of the dimethyl sulfoxide control vehicle: histamine release, $37 \pm 3\%$; loss of total p60 receptor in cell lysates, $47 \pm 2\%$; loss of cell surface receptor, $42 \pm 3\%$. See Methods in the Online Repository for details of the 3 techniques used to assess the remaining surface receptor.

First, one set of conditions tests for the effectiveness of washing out the drug by incubating nonstimulated cells with NVP-QAB205 for the same desensitization period, 45 minutes, washing the cells once, and stimulating with several concentrations of anti-IgE Ab or antigen to assess any remaining inhibition throughout a range of stimulation conditions (lower levels of stimulation tend to be more sensitive to the presence of inhibitors). A second control condition demonstrates that the drug is acting as expected on normal secretion. Fig 2 shows the various results of this design. It is first notable that the washing was effective in removing enough drug that no inhibition was observed at any of the 3 concentrations of stimulation (Fig 2, A). Second, at 0.3 μ mol/L, which has been previously shown to inhibit completely many signaling steps as well as secretion, histamine release was inhibited 100% (Fig 2, B, column 4). On average, for these 4 experiments, histamine release after the desensitization was $22 \pm 5\%$ of the nondesensitized response. In the presence of NVP-QAB205, desensitization was $29 \pm 6\%$, not statistically different from desensitization without the drug present. A similar result was observed for stimulation with an antigen (data not shown). In addition, this article's Fig E1 in the Online Repository at www.jacionline.org shows that desensitization of syk phosphorylation was also not inhibited in the presence of NVP-QAB205—that is, syk activity is not necessary for the processes that lead to loss of syk phosphorylation.

In the second method, the design was similar except that full secretion was allowed during the first phase. In this design, the question being asked was whether cells optimally stimulated in the presence of NVP-QAB205 would fail to secrete but continue to desensitize. This was tested by washing the cells with a single addition of ice-cold buffer and centrifugation and restimulating with anti-IgE Ab or antigen. The set of controls was similar to those described but in addition, the amount of release during the first phase was monitored, which also allowed an assessment of the drug's effectiveness in inhibiting histamine release. For the cells that received stimulus but no drug, the rechallenge necessarily

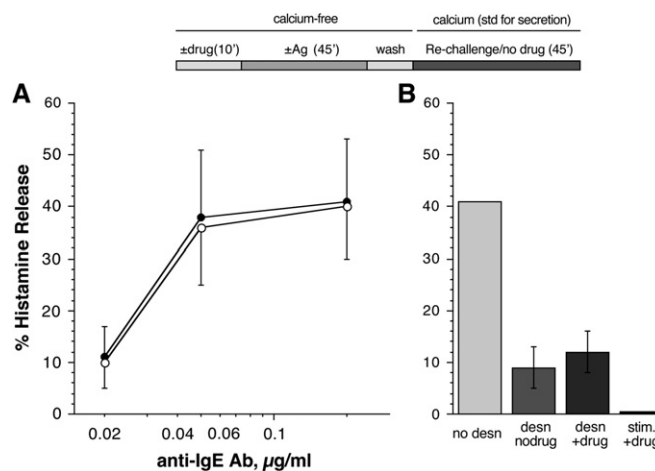


FIG 2. Effect of NVP-QAB205 on desensitization induced by anti-IgE Ab in calcium-free buffer. The schematic above the figure shows the timeline for the incubations. **A**, In the washout portion of the experiment (see text; $n = 4$), cells were incubated in the presence or absence of drug for 45 minutes, washed once, resuspended in PAGCM for challenge with antigen (●) no drug present during the 45 minutes, (○) NVP-QAB205 at 0.3 μ mol/L for 45 minutes before washing. **B**, In the remaining portion of the experiment ($n = 4$), cells were not desensitized (no desn), cells were desensitized without drug present (desn nodrug), cells were desensitized with drug present (desn + drug), or cells were incubated without drug or anti-IgE, washed, and stimulated with anti-IgE in the presence of 0.3 μ mol/L NVP-QAB205 (stim. + drug). std, Standard; Ag, antigen.

resulted in the total histamine content to be reduced proportionally to the amount of release that occurred during the first phase. However, for cells stimulated in the first phase in the presence of drug, the total histamine content of the cells should have been, and was, the same as that of nonstimulated cells. Fig 3, A, illustrates the sequence of steps in this protocol. Fig 3, B, shows that washing effectively eliminated the drug before rechallenge in the second phase. Fig 3, C, shows that cells that had been stimulated in the presence of NVP-QAB205 and then washed could not respond to further stimulation. Although 0.3 μ mol/L NVP-QAB205 is considered adequate for inhibiting syk activity, a concentration of 1 μ mol/L was also examined to rule out the possibility that desensitization mechanisms were more difficult to inhibit while still being dependent on syk. The design was similar, as were the results (data not shown). A second class of mediators, leukotriene (LT) C₄, was examined in 3 separate experiments using a similar experimental design. Fig 3, D, shows that there was no statistical difference in the LTC₄ release from cells in the second phase of the protocol (column bars 2 and 3) whether or not NVP-QAB205 was included in the first phase. Not shown is that the histamine release was also similar to the LTC₄ results for these 3 experiments, and there was not a statistically significant inhibition in the washout test portion of the experiment.

Previous studies have demonstrated 2 forms of desensitization: that specific for the antigen being used to desensitize the cell, and a form of nonspecific desensitization that results in a diminished response to all other noncross-reacting antigen-induced release.²⁸ The nonspecificity is limited to IgE-mediated stimuli; non-IgE-mediated stimuli are unaffected.²⁹ The degree of nonspecific desensitization is a function of the relative ratio of the strength of desensitizing stimulus and the rechallenge, noncross-reacting stimulus, and it is uncommon to observe complete nonspecific desensitization even when specific desensitization is complete.³⁰

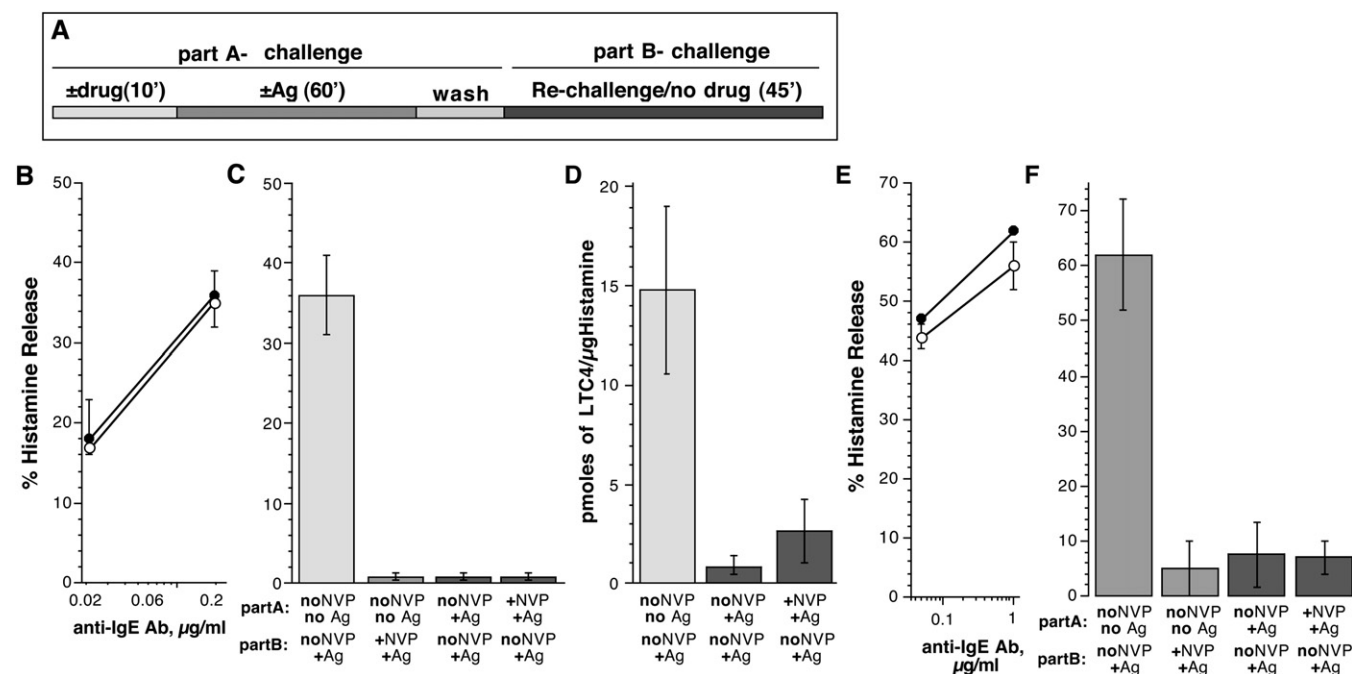


FIG 3. Effect of NVP-QAB205 on desensitization induced by anti-IgE Ab under full secretory conditions (ie, all incubations in PAGCM). The schematic above the figure shows the timeline for the incubations. **A**, Schematic of the time line for the protocol. **B-D**, Experiments using human basophils. **E** and **F**, Human cultured mast cells. **B**, In the washout portion of the experiment (see text; $n = 5$), cells were incubated in the presence or absence of drug for 45 minutes (in PAGCM), washed once, resuspended in PAGCM for challenge with anti-IgE (●) no drug present during the 45 minutes, (○) NVP-QAB205 at 0.3 µmol/L (in PAGCM) for 45 minutes before washing. **C**, In the remaining portion of the experiment ($n = 5$), abscissa shows conditions of the 2 incubations; Ag refers to the stimulus, anti-IgE Ab (0.2 µg/mL). The NVP-QAB205 (denoted NVP in the figure, ie, no NVP or +NVP) concentration used in these experiments was 0.3 µmol/L. **D**, Similar experimental design as in **A** and **B**, but LTC₄ was the measured mediator. **E** and **F**, Effect of NVP-QAB205 on desensitization induced by anti-IgE Ab under full secretory conditions (ie, all incubations in PAGCM) in cultured-derived human mast cells. Before the experiments, the cells had been sensitized with 10 µg/mL SE44 IgE antibody for 30 minutes at 4°C and washed. **E**, In the washout portion of the experiment (see text; $n = 2$). Cells were incubated in the presence or absence of drug for 40 minutes (in PAGCM), washed once, resuspended in PAGCM for challenge with anti-IgE (1 µg/mL of 6061 anti-IgE Ab) (●) no drug present during the 45 minutes, (○) NVP-QAB205 at 0.3 µmol/L (in PAGCM) for 40 minutes before washing. **F**, In the remaining portion of the experiment ($n = 2$), abscissa shows conditions of the 2 incubations; Ag refers to the stimulus, anti-IgE Ab, 2 µg/mL. The NVP-QAB205 concentration used in these experiments was 0.3 µmol/L.

The next series of experiments tested whether NVP-QAB205 also had no effect on the progression of nonspecific desensitization. A desensitization protocol was used that was similar to that used for the experiments shown in Fig 2. However, to examine nonspecific desensitization, the cells were sensitized with both gp120-specific and dinitrophenyl-specific IgE. To prepare the cells for this sensitization, the cells were treated with lactic acid for 3 seconds to remove a fraction of the endogenous IgE.³¹ The concentrations of sensitizing IgE were chosen to result in approximately equivalent release to the 2 antigens. Cells were desensitized with dinitrophenyl(7)-HSA ± NVP-QAB205 with appropriate controls. The cells were rechallenged with gp120-ovalbumin to test for the extent of nonspecific desensitization. Controls in these experiments also included rechallenging with dinitrophenyl-HSA to ensure complete specific desensitization. All rechallenging conditions, as well as the wash, included 1 mmol/L dinitrophenyl-lysine to both dissociate any bound dinitrophenyl-HSA and prevent any remaining dinitrophenyl-HSA signaling. Fig 4 shows that although not complete, nonspecific desensitization occurred as expected, an approximately 62% loss of response to gp120-ovalbumin after desensitizing with dinitrophenyl-HSA.

NVP-QAB205 in the first phase of the experiment did not change the extent of nonspecific desensitization. Therefore, neither specific nor nonspecific desensitization requires the activity of syk.

Basophils and phosphatidyl inositol 3' kinase inhibition

The experiments with the syk inhibitor suggested that desensitization/signal termination does not require syk activity and might imply that inhibition of elements downstream of syk would also not inhibit desensitization. The phosphatidyl inositol 3' kinase (PI3K) inhibitors wortmannin and LY294002 are very effective inhibitors of all forms of mediator secretion,³² as complete an inhibition as observed with NVP-QAB205. It seems likely that PI3K activity lies downstream of syk because studies in other cell types suggest phosphorylation of the regulatory subunit of PI3K, p85, requires syk activity, indirectly or directly.³³⁻³⁶ We tested whether this was true for human basophils and found that NVP-QAB205 at 0.3 µmol/L effectively inhibited p85 phosphorylation (an example Western blot is shown in this article's Fig E2, A, in the Online Repository at www.jacionline.org; the IC50

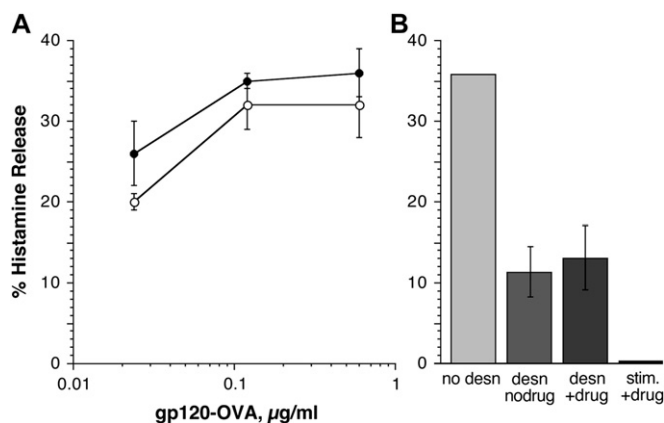


FIG 4. Effect of NVP-QAB205 on nonspecific desensitization. The experimental design was similar to that used for Fig 2. However, the basophils were first very briefly treated (3 seconds) with lactic acid (see Methods in the Online Repository) to remove a fraction of the endogenously bound IgE and then resensitized with a mixture of antidinitrophenyl mIgE and anti-gp120-human IgE. In the desensitization phase of the experiment, the cells were challenged with 3 µg/mL dinitrophenyl(7)-HSA. In the second phase of the experiment, the cells were stimulated with 0.6 µg/mL gp120-ovalbumin (OVA). Histamine release was assessed. **A**, In the washout portion of the experiment (see text; $n = 4$), cells were incubated in the presence or absence of drug for 45 minutes, washed once, resuspended in PAGCM for challenge with antigen (●) no drug present during the 45 minutes, (○) NVP-QAB205 at 0.3 µmol/L for 45 minutes before washing. **B**, In the remaining portion of the experiment ($n = 4$), cells were not desensitized (*no desn*), cells were desensitized without drug present (*desn nodrug*), cells were desensitized with drug present (*desn + drug*), or cells incubated without drug or gp120-OVA, washed, and stimulated with gp120-OVA in the presence of 0.3 µmol/L NVP-QAB205 (*stim. + drug*).

<0.1 µmol/L is consistent with the IC₅₀ for other signaling elements). To test whether another critical signaling element downstream of syk could also allow desensitization/energy without secretion, the PI3K inhibitor LY294002 was examined by using an experimental design similar to that shown in Fig 2. Pilot studies have shown that this drug is effectively washed out of the cells with a single wash, and this can be seen in Fig E2. Like NVP-QAB205, LY294002 did not reverse desensitization/signal termination while completely inhibiting histamine release. Indeed, it appears that desensitization was slightly enhanced. LY294002 was also used in a full secretion protocol, like that shown in Fig 3, and like the syk inhibitor, secretion was fully inhibited while signal termination/desensitization allowed the cell to be unresponsive when drug was washed away (LY294002 data not shown).

Mast cells and syk inhibition

The next question was whether these results could be repeated with human mast cells. Using culture-derived, 9-week-old human mast cells, NVP-QAB205 also inhibited secretion without altering desensitization/signal termination. Fig 3, E and F, shows these results. The design of the experiment was similar to that shown in Fig 3, A, for studies using human basophils.

To test whether the same behavior was observed in bronchial tissue mast cells, human isolated bronchial preparations were passively sensitized (see Methods) and tested by using a similar sequence of stimulation as discussed. The antigen-induced tissue mast cell activation was assessed as bronchial smooth muscle contraction.

Antigen E consistently activated the tissue mast cells, resulting in a contraction that averaged nearly 70% of the carbachol maximum (Fig 5, A and B). The contractions were completely prevented by blocking histamine H1 and cysLT1 receptors with pyrilamine (1 µmol/L) and ICI 198615 (1 µmol/L; $n = 3$ data not shown). Stimulating with antigen for 30 minutes followed by repeated washing for 60 minutes effectively desensitized the cells to further stimulation (Fig 5, A, and bar 3 in B).

Incubating paired bronchi with 1 µmol/L NVP-QAB205 virtually abolished the antigen-induced response (Fig 5, B, bar 4). In control experiments, we found that NVP-QAB205 had no effect on the ability of exogenously applied histamine to contract the tissues. In 3 experiments, the effective concentration 50 (M) values for histamine-induced contractions averaged 1 µmol/L and 1.3 µmol/L in the absence and presence of NVP-QAB205, respectively. The maximum histamine-induced contractions averaged 87% and 82% of the carbachol maximum.

For obvious reasons, it is more difficult to remove a drug from isolated tissues than from cell suspensions. In preliminary experiments, we incubated the bronchi with 1 µmol/L NVP-QAB205, then evaluated whether the effect of the drug could be reversed by washing the tissues with drug-free solution for 65 minutes as described in Methods. In bronchi obtained from 5 lungs, we noted that the antigen-induced contraction averaged $66 \pm 12\%$ in control tissues and $56 \pm 13\%$ ($P > .1$ compared with control) in paired bronchi that were treated with NVP-QAB205, then washed with drug-free buffer. These data suggest that we were able effectively to wash the drug and its effect from the bronchial tissue. In the 3 specific experiments in which desensitization was studied, the effect of NVP-QAB205 against the antigen-induced contractions was removed by repeated washing; however, the antigen contractions in these tissues averaged only about 50% of the control tissues (this difference is likely accountable by the variability in the antigen-induced contraction among the paired tissues (Fig 5, A, and bar 2 in B). In any event, consistent with the data obtained with basophils and cultured mast cells; NVP-QAB205, at a concentration that virtually abolished the tissue mast cell activation, did not inhibit the desensitization process (Fig 5, A, and bar 5 in B).

DISCUSSION

These studies demonstrate that the process of desensitization, whatever the mechanism and whether operationally demonstrated in the presence or absence of extracellular calcium, is not sensitive to inhibition of syk or PI3Ks. One could argue that the syk inhibitor used may not be strictly selective for syk kinase, but because no effect was observed, collateral inhibitory effects are also not relevant. The concentration chosen for the syk inhibitor has been shown in many previous experiments to be a balance between complete inhibition of mediator release (and a variety of signaling elements known to be involved in IgE-mediated release¹³) and higher concentrations that might begin to show nonselective effects. A similar argument could be made about the use of LY294002, although in these experiments, the presence of some enhanced desensitization might be a result of either the selected inhibition of PI3K or some collateral/nonselective effects of LY294002.

From previous studies and the current studies, we now know that there are at least 3 mechanisms of downregulation that do not appear sensitive to the activity of syk: the downregulation of

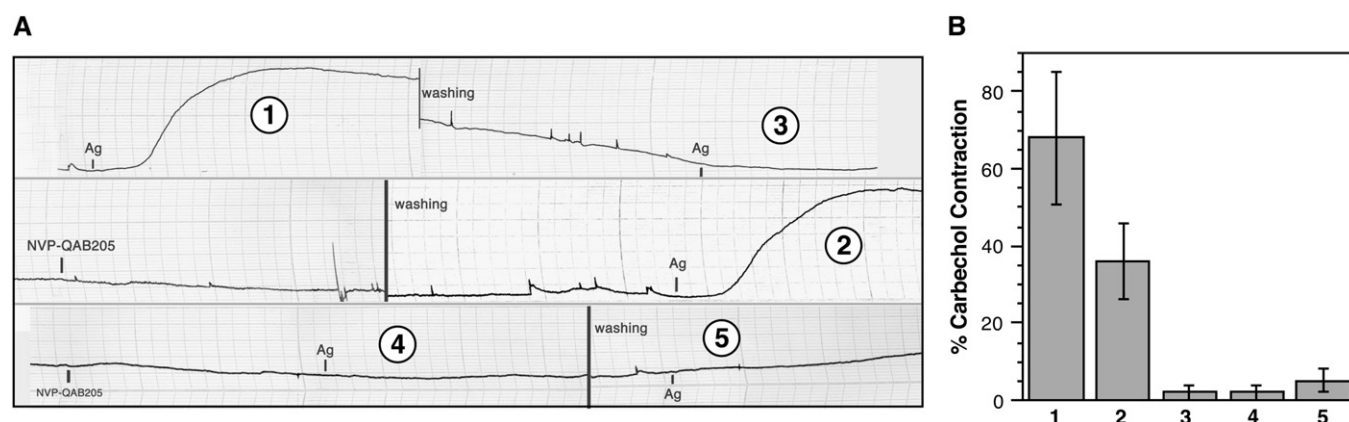


FIG 5. Effect of NVP-QAB205 on desensitization induced by ragweed antigen under full secretory conditions in human bronchial airway tissue. **A**, Smooth muscle contraction tracings for the various conditions: 1, tissue stimulated with antigen (Ag); 2, tissue exposed to NVP-QAB205 for 30 minutes, washed for 60 minutes (in the tracing, the designation of washing refers to the 60-minute washing that is cut from the tracing), and stimulated with antigen; 3, tissue exposed to antigen for 30 minutes, washed for 60 minutes, and reexposed to antigen; 4, tissue exposed to drug, then stimulated with antigen; and 5, tissue exposed to drug for 5 minutes, then stimulated with antigen for 30 minutes, washed for 60 minutes, and re-exposed to antigen. The NVP-QAB205 concentration used in these experiments was 1.0 $\mu\text{mol/L}$. The antigen concentration, 1 $\mu\text{g/mL}$, was optimal for these tissues, which were sensitized overnight with ragweed-specific IgE containing serum. **B**, Summary of the 3 experiments, each condition performed in duplicate, with the data expressed as a percent of maximum contraction induced by 1 mmol/L carbachol. The numbers on the abscissa refer to the conditions in A and described here.

syk,¹⁶ the activation of SHIP¹³ and from the current study, and the downregulation of surface Fc ϵ RI and its degradation late in the reaction. The current study also adds to the list that the functional consequences of desensitization/self-termination do not appear to require syk activity. Therefore, no matter what additional mechanisms are discovered to mediate desensitization/self-termination, the functional results suggest that syk is also not required for these unknown downregulatory steps. Signaling downstream of syk activity would also be expected to not be involved in desensitization, and the results using the PI3K inhibitor LY294002 appear to support this view. In human basophils, only PI3K delta (of the class 1A family) is expressed,³² so the LY294002 effect directly reflects the lack of PI3K delta involvement in desensitization.

Although the secretion of IL-4 or other cytokines has not been examined, the observation that the results are similar for LTC₄ secretion and the fact that IL-4 release also desensitizes (albeit somewhat more slowly²⁷) suggest that all secreted mediators would be desensitized. In addition, nonspecific desensitization also was insensitive to inhibition of syk. We have speculated that nonspecific desensitization results from a combination of the effect of syk downregulation and the activation of SHIP.^{13,37} Both of these events are insensitive to syk inhibition, which is consistent with the absence of an effect of the syk inhibitor on the functional aspects of nonspecific desensitization. It should be noted that desensitization through the IgE receptor has no influence on activation of basophils by ligands that operate through heterotrimeric GTPases (formyl-met-leu-phe [fMLP], C5a, chemokines, and so forth).^{11,29,38,39} Therefore, it is not anticipated that the desensitized state induced by antigen would inhibit activation by these ligands. It is less clear whether ligands that use receptors that also share signaling steps with the IgE receptor would be inhibited. There is now evidence that syk downregulation occurs for several receptors that use syk for activation, and one could

speculate that at least this aspect of IgE-mediated anergy would cross-desensitize these receptors (leukocyte immunoglobulin-like receptor, subfamily A and human recombinant histamine-releasing factor).⁴⁰

We found that the results obtained from studies of basophils also applied to mast cells, whether they were obtained from long-term culture or tested indirectly in airway tissue. To date, the IgE-mediated signaling characteristics of human mast cells have not differed markedly from the signaling characteristics of human peripheral blood basophils, although the mast cell reaction occurs somewhat faster. These studies suggest that signal termination in mast cells also does not require the activity of syk.

Beyond the mechanistic implications of these results, the idea that one can prevent mast cell/basophil activation without inhibiting desensitization may have practical implications. It is not possible to induce an anergic state *in vivo* by stimulation in the absence of extracellular calcium. However, the current studies suggest that anergy could be induced by a short pretreatment with antigen in the presence of a syk inhibitor (or inhibitor of downstream signaling like PI3K). It is not yet known how long anergic/desensitized states persist in mast cells and basophils. *In vitro* studies suggest that they persist for at least 3 days.^{13,41,42} *In vivo* studies have only been anecdotal. If the mechanism of clinical desensitization with penicillin is in fact a reflection of a desensitized mast cell, then the desensitized state is reported to persist for days to weeks.⁴³ Therefore, although it is not precisely known how long such a desensitized state would persist, an induction of desensitization in the presence of a syk inhibitor would allow refractory periods to be induced without concern for anaphylaxis. This would open up a class of drugs for use acutely that would be considered too dangerous to use chronically.

These speculations aside, the results demonstrate that an early tyrosine kinase considered critical for the activation steps leading to IgE-induced mediator release has no role in the

self-termination of mediator release. This applies to basophils and mast cells and applies to both forms of desensitization, specific and nonspecific, that have been described for IgE-mediated release. It seems likely that signaling elements that rely on syk activity, but are also considered critical for mediator release, such as PI3K, are also not necessary for desensitization processes.

We thank Val Alexander for her technical assistance and Dr Stephan von Guten for providing the cultured-derived mast cells for these experiments.

Clinical implications: These results suggest that it would be possible to induce mast cell/basophil anergy *in vivo* without a significant induction of mast cell/basophil secretion.

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METHODS

Materials

The following were purchased: PIPES, BSA, ethyleneglycol-bis-(β -amino-ethylether)-N,N,N',N'-tetraacetic acid, EDTA, D-glucose, NaF, $\text{Na}_4\text{P}_2\text{O}_7$, Na_3VO_4 , 2-mercaptoethanol, NP-40, fMLP (Sigma, St Louis, Mo); crystallized human serum albumin (HSA; Miles Laboratories, Elkhart, Ind); FCS and RPMI-1640 containing 25 mmol/L HEPES and L-glutamine (BioWhittaker, Walkersville, Md); Percoll (Pharmacia, Piscataway, NJ); TRIS (hydroxy-methyl)-aminomethane, Tween-20 (Bio-Rad, Hercules, Calif); leupeptin, dithiothreitol, phenylmethylsulfonyl fluoride (Boehringer Mannheim, Indianapolis, Ind); biotinylated molecular weight markers (New England Biolabs, Beverly, Mass, now Cell Signaling); rabbit anti-Fc ϵ RI α Ab (Upstate Biotechnology, Inc, Lake Placid, NY); mouse anti-Fc ϵ RI α mAbs, 22E7 and 15A5 (gifts from Hoffman-LaRoche, Nutley, NJ); horseradish peroxidase-conjugated donkey antirabbit Ig Ab, horseradish peroxidase-conjugated sheep antimouse Ig Ab, and protein G Sepharose beads (Amersham Life Science, Arlington Heights, Ill). Goat antihuman IgE Ab was prepared as previously described.^{E1} gp120-Ov-albumin was provided by Tanox, Inc (Houston, Tex), and dinitrophenyl(7)-HSA was prepared as described previously. LY294002 (Calbiochem, La Jolla, Calif) and NVP-QAB205 was a gift from GlaxoSmithKline (Stoehedge, United Kingdom), and its characteristics have been published.^{E2}

Two IgE antibodies were used in these studies. A gp120-specific monoclonal IgE was the gift of Tanox, Inc. The second IgE, antidinitrophenyl-mouse IgE, H1-dinitrophenyl- ϵ 26, was a gift of T. Ishizaka (when at Johns Hopkins University, Baltimore, Md).

Buffers

PIPES-albumin-glucose (PAG) buffer consisted of 25 mmol/L PIPES, 110 mmol/L NaCl, 5 mmol/L KCl, 0.1% glucose, and 0.003% HSA. PAGCM was PAG supplemented with 1 mmol/L CaCl_2 and 1 mmol/L MgCl_2 . PAG-EDTA consisted of PAG supplemented with 4 mmol/L EDTA. Countercurrent elutriation was conducted in PAG containing 0.25% BSA in place of 0.003% HSA. Lactic acid buffer was used for removing endogenous cell bound IgE; 0.01 mol/L lactic acid, 0.14 mol/L NaCl, 0.005 mol/L KCl, pH 3.9.^{E1,E3} The electrophoresis sample buffer is Novex electrophoresis sample buffer containing 5% 2-mercaptoethanol. Nitrocellulose stripping buffer was 65 mmol/L TrisHCl (pH 6.7), 100 mmol/L 2-mercaptoethanol, and 2% SDS.

Basophil purification

For most of these experiments, basophils were enriched from whole blood by a single-step Percoll gradient as previously described. For some of these experiments, residual cells of normal donors undergoing leukapheresis were enriched in basophils by using a combination of Percoll density gradients and countercurrent-flow elutriation, as previously described.^{E4} The cells were further purified by negative selection by using a magnetic cell sorting basophil isolation kit (Miltenyi Biotec, Auburn, Calif). A cocktail of antibodies for negative selection from Stem Cell Technologies (basophil purification kit; Vancouver, British Columbia, Canada) and columns from Miltenyi Biotec were used. The purity of basophils was determined by alcian blue staining,^{E5} and basophils purified from leukapheresis packs generally exceeded 99% purity.

IgE dissociation and sensitization

For some of the experiments, basophils were treated with lactic acid to dissociate endogenous IgE from its receptor. To minimize harm to the cells, the exposure time to lactic acid buffer was 3 seconds. Dinitrophenyl-mIgE and gp120-specific human IgE have been shown to not induce measurable signaling.^{E6} However, to minimize concerns that some low-level but undetectable signaling might occur during the sensitization, sensitization was performed at 4°C for 1 hour (5 $\mu\text{g}/\text{mL}$ IgE), and the cells were washed once with ice-cold PAG and resuspended in cold media before warming and culture. Viability after lactic acid-induced dissociation was $92 \pm 4\%$.

Basophil cultures

Purified basophils were cultured at 2 million/mL in RPMI-1640 medium supplemented with 10 $\mu\text{g}/\text{mL}$ gentamycin and 300 $\mu\text{g}/\text{mL}$ HSA. In

experiments in which anti-IgE antibody was used to downregulate receptor expression, the media included enough additional calcium to equal 1 mmol/L and excluded IL-3.

Flow cytometry and detection of aggregated cell surface receptor

Before stimulation with anti-IgE Ab, the cells were sensitized with a biotinylated anti-gp120-specific IgE. Receptor on the surface was monitored by 3 methods,^{E7} using flow-cytometric detection for each. In the first approach, cell surface receptor was detected with antireceptor antibody (22E7). In the second, presence of the gp120-specific IgE antibody on the cell surface, acting as surrogate detector of all IgE, was detected by flow cytometry with an anti-idiotypic antibody. For the third method, the biotinylated anti-gp120-specific IgE on the cell surface was detected by labeling with a streptavidin-alexa647 conjugate. Three methods were used because of a concern that epitopes on either the receptor or its bound IgE might be masked in a maturing surface receptor aggregate. The average of the 3 methods was used in the final data presentation.

Immunoprecipitation

To detect phosphorylation of the p85 subunit of PI3K, basophils were lysed in a 10-mmol/L Tris buffer, pH 7.8, containing 1% NP-40, 10% glycerol, 100 $\mu\text{g}/\text{mL}$ aprotinin, 10 mmol/L benzamidine, 1 mmol/L 4-(2-aminoethyl)benzene sulfonyl fluoride, 100 $\mu\text{g}/\text{mL}$ leupeptin, 50 mmol/L NaF, and 1 mmol/L Na_3VO_4 (Sigma), and the lysis was centrifuged at 16,000g for 4 minutes to remove nuclei. The antiphosphotyrosine antibody PY20 (Santa Cruz Biotechnology, Santa Cruz, Calif), was used to immunoadsorb phosphotyrosine proteins, and the adsorbed proteins were examined by Western blotting with p85 antibody (Santa Cruz Biotechnology).

Culture-derived human mast cells

Human mast cells were obtained by culturing CD34-positive cells according to methods previously described.^{E8,E9} Briefly, CD34⁺ cells were cultured in the presence of recombinant IL-3 and stem cell factor for a period greater than 8 weeks.

Airway tissue challenges

Human lung specimens were obtained from anonymous donors through the nonprofit International Institute for the Advancement of Medicine. Intralobar bronchi (2-5 mm diameter) were dissected from human donor lungs. The tissues were incubated overnight in minimal essential media growth medium containing a 1:10 dilution of serum pooled from highly ragweed-allergic donors. These tissues were used on day 2 for the study of ragweed antigen extract (antigen E)-induced contractions.

On day 2, the bronchi were suspended in 10 mL water-jacketed tissue baths containing Krebs bicarbonate buffer solution containing the following in mmol/L: NaCl, 118; KCl, 5.4; NaH_2PO_4 , 1.0; MgSO_4 , 1.2; CaCl_2 , 1.9; NaHCO_3 , 25.0; dextrose, 11.1; indomethacin, 3; and gassed with 95% O_2 , 5% CO_2 , pH 7.4, 37°C. The buffer was exchanged with fresh buffer every 15 minutes for 2 hours. The tissues were maintained at a resting tension of 1 g.

The antigen-induced bronchial contractions were studied exactly as described previously.^{E10} The contractions caused by ragweed antigen were obtained in a cumulative fashion and expressed as a percentage of that obtained by a maximally effective concentration of carbachol (10 $\mu\text{mol}/\text{L}$) added at the end of the experiment.

In 3 separate experiments, we evaluated the effect of 1 $\mu\text{mol}/\text{L}$ pyrilamine and 1 $\mu\text{mol}/\text{L}$ ICI 198615 (provided by Zeneca Pharmaceuticals, Sodertalje, Sweden) to confirm that the responses obtained were entirely a result of the release of histamine and cysLT. As expected, and consistent with previous findings,^{E10} the histamine H1 and cysLT1 receptor antagonists completely prevented the antigen-induced contractions (data not shown).

The syk inhibitor was added to the tissue baths 30 minutes before the beginning of the antigen E concentration-response curve. In some cases, NVP-QAB205 was washed from the tissues by replacing the buffer with fresh solution every minute times 5 minutes, then every 15 minutes times 60

minutes. NVP-QAB205, washed out in this manner, had no effect on the magnitude of the carbachol-induced contractions as quantified as grams of tension ($P > .1$) while inhibiting contractions by 98% when present.

Statistics

In general, data are shown as the means $\pm$ SEMs, both in text and figures. In some instances, the figure shows means $\pm$ SEMs, although the analysis is performed with paired statistics.

RESULTS

Downregulation of syk phosphorylation

We have previously shown that when basophils are stimulated with antigens, the phosphorylation of syk is transient. This occurs whether cells are stimulated in the presence or absence of extracellular calcium. Therefore, we asked whether this transience is inhibited by treatment of the cells with NVP-QAB205 during stimulation with antigen. The experiment needed 2 phases because we have also shown that NVP-QAB205 is an effective inhibitor of syk phosphorylation (therefore, syk activity is needed for phosphorylation of syk). In the absence of extracellular calcium, cells were treated using 4 conditions, shown in Fig E1, and after 45 minutes, diluted with ice-cold buffer, centrifuged, resuspended in warm buffer, and rechallenged in the presence of extracellular calcium. In this design, bulk antigen is diluted in the procedure, but what is bound to the cell surface likely remains bound (as we have shown previously^{E11}). Therefore, during the restimulation, the tubes labeled *blanks* are likely to produce a signal that is very similar in syk phosphorylation to the tubes that received additional antigen (labeled *DH*), and this was observed. Parenthetically, the kinetics of syk phosphorylation after desensitization was the same as that of nondesensitized cells (data not shown). There was some loss of syk expression (29%) in the 1 hour of desensitization, as we have previously observed,^{E12} so the band intensities of phospho-syk were normalized for the relative presence of syk. Fig E1 shows that the presence of NVP-QAB205 did not reverse the loss of syk phosphorylation that occurred in the absence of the drug. Therefore, the process that results in downregulation of syk phosphorylation does not depend on syk activity.

Basophils and PI3K inhibition

The PI3K inhibitor LY294002 was used in an experimental design similar to that shown in Fig 2. It was first necessary to

demonstrate that phosphorylation of PI3K through its p85 subunit was sensitive to the activity of syk. We tested whether this was true for human basophils and found that NVP-QAB205 at 0.3 $\mu\text{mol/L}$ effectively inhibited p85 phosphorylation (an example, Western blot is shown in Fig E2, A; the $\text{IC}_{50} < 0.1 \mu\text{mol/L}$ is consistent with the IC_{50} for other signaling elements). Fig E2, B and C, tests whether desensitization occurred in the presence of LY294002. Pilot studies have shown that this drug is effectively washed out of the cells with a single wash, and this can be seen in Fig E2, B. Like NVP-QAB205, LY294002 did not reverse desensitization/signal termination while completely inhibiting histamine release. Indeed, it appears that desensitization was slightly enhanced.

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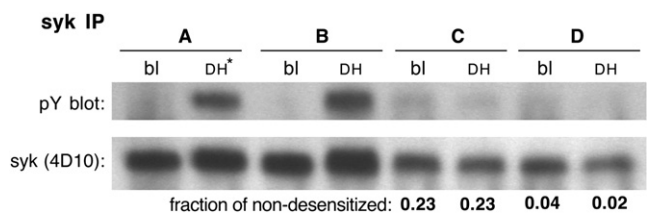


FIG E1. Test of the effects of NVP-QAB205 on the downregulation of syk phosphorylation. Purified basophils were treated briefly with lactic acid stripping buffer to remove a fraction of the endogenous IgE and resensitized with mouse dinitrophenyl-specific IgE (a nonstimulatory mouse monoclonal IgE^{E6} that was the gift of Dr F. T. Liu through T. Ishizaka). After washing, the cells were incubated under 4 conditions. In phase 1, the absence of extracellular calcium, **A** = no antigen, no drug; **B** = no antigen, NVP-QAB205 at 0.3 μ mol/L; **C** = antigen, no drug; and **D** = antigen, NVP-QAB205 at 0.3 μ mol/L. The antigen was dinitrophenyl(7)-HSA at an optimal concentration of 3 μ g/mL. After 45 minutes, ice-cold PAG was added. The cells were centrifuged for 4 minutes at 350g, resuspended in 37°C PAGCM, and quickly added to the stimulation tubes. For each condition, the cells were split and added to either PAGCM or PAGCM containing 3 μ g/mL dinitrophenyl(7)-HSA. After 5 minutes, the cells were lysed, syk immunoprecipitated, the immunoadsorbed syk analyzed by PAGE electrophoresis, and nitrocellulose blotted first with antiphosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY) and after stripping, blotted with anti-syk Ab (4D10; Santa Cruz Biotechnology).^{E6} The figure shows the Western blot with 4G10 in the upper half and the reblot with anti-syk Ab, 4D10. The band intensities were normalized for the amount of syk present, and these data were expressed as a fraction of the nondesensitized control responses (conditions **A** and **B**), which are values shown under the Western blot. *bl*, Blank—that is, no antigen added during rechallenge; *DH*, dinitrophenyl(7)-HSA. *IP*, Immunoprecipitation; pY, phosphotyrosine.

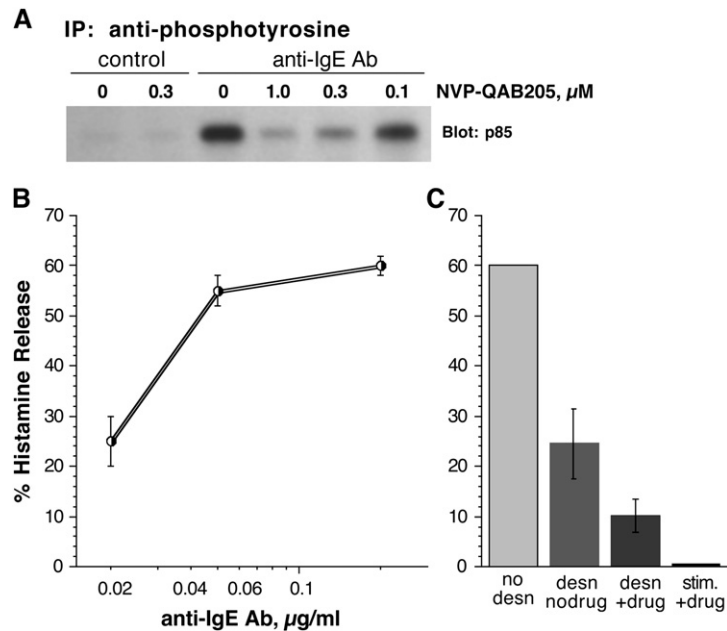


FIG E2. Test of the effects of NVP-QAB205 on p85 phosphorylation and the effect of LY294002 on desensitization induced by anti-IgE Ab in calcium-free buffer. **A**, The syk inhibitor, NVP-QAB205 inhibits phosphorylation of the p85 subunit of PI3K in basophils. Cell lysates of basophils stimulated $\pm$ NVP-QAB205 at the concentrations shown $\pm$ anti-IgE Ab at 0.2 μ g/mL for 8 minutes were immunoadsorbed with antiphosphotyrosine Ab (PY20) and the Western blots developed with anti-p85 Ab. The drug concentration is shown above the blot, and the 0 refers to the presence of dimethyl sulfoxide at a concentration equivalent to that of the 0.3- μ mol/L concentration of NVP-QAB205. **B**, The washout portion of the experiment (see text; $n = 4$), cells incubated in the presence or absence of drug for 45 minutes, washed once, resuspended in PAGCM for challenge with antigen ($\bullet$) no drug present during the 45 minutes, ($\circ$) LY294002 at 5 μ mol/L for 45 minutes before washing. **C**, In the remaining portion of the experiment ($n = 4$), cells were not desensitized (*no desn*), cells were desensitized without drug present (*desn nodrug*), cells were desensitized with drug present (*desn + drug*), or cells were incubated without drug or anti-IgE, washed, and stimulated with anti-IgE in the presence of 5 μ mol/L LY294002 (*stim. + drug*). IP, Immunoprecipitation.