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Acute IL-3 Priming Up-Regulates the Stimulus-Induced Raf-1-Mek-Erk Cascade Independently of IL-3-Induced Activation of Erk[1]

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IL-3 is a potent priming cytokine for human basophils, inducing an increase of mediator release after stimulation. The mechanism of IL-3 priming of the basophil response to FcεRI aggregating stimuli remains unknown. We explored the regulation of several elements of IgE-mediated signaling by a short priming with IL-3. Early signaling events such as phosphorylation of Syk, Shc, linker for activation of T cells, and the calcium signal were not statistically affected by acute IL-3 priming. Downstream in the signaling cascade, a point of up-regulation was found at the level of Raf-1-Mek-Erk. Although the phosphorylation of Raf-1 was not changed by IL-3 priming, IL-3-primed anti-IgE-stimulated basophils showed a strong synergism for Mek and Erk phosphorylation when compared with either IL-3 or anti-IgE alone; pre-exposure to IL-3 induced a final 13-fold average increase over anti-IgE-induced Erk phosphorylation (6-fold above the sum of anti-IgE and IL-3 alone). The kinetics, dose response, and pharmacologic characteristics of the IL-3 priming of stimulus-induced Erk phosphorylation support the involvement of a yet unknown mechanism that is independent of IL-3-induced Erk and PI3K activation. This type of preactivation can be mimicked by incubation with the Ser-Thr kinase inhibitors, Ro-81–3220, or bisindoylmaleimide II. *The Journal of Immunology,* **2005, 175: 3006–3014.**

Interleukin-3, together with IL-5 and GM-CSF, constitute a family of cytokines characterized by sharing the same βc subunit of their respective receptors. The IL-3/IL-5/GM-CSF family has an essential role in the proliferation, differentiation, and survival of several hemopoietic lineages, among them stem multipotential cells, eosinophils, neutrophils, lymphocytes, and basophils (1). Besides these well-known effects, these cytokines also have the ability to enhance the functional responses of inflammatory cells, such as basophils, eosinophils, and neutrophils, to several stimuli (2–5). This ability, also shared by other cytokines, has been termed priming and despite several attempts to study its mechanism (3, 6, 7), not much is known about how priming occurs. The recruitment of basophils to sites of allergic inflammation (8–11) suggests that their priming by members of the IL-3/IL-5/GM-CSF family of cytokines may play a role in the pathophysiology of allergy because these cytokines have been detected in tissues undergoing allergic inflammation (12–15).

In human basophils, priming with IL-3 induces an increase of mediator release (histamine, leukotriene C_4 (LTC_4),[3] and IL-4) to several stimuli, among them immunologic stimuli such as Ag, C5a, and FMLP (4, 5, 7, 16). To date, only the IL-3 priming of C5a-induced LTC_4 has been studied mechanistically. C5a is a good stimulus for histamine release, however it does not induce appreciable LTC_4 release in the absence of IL-3. A preincubation with IL-3 primes the basophils to release LTC_4 upon C5a stimulation. The available data support a model in which the temporal overlap, or the absence thereof, between the rise in cytosolic calcium and the phosphorylation of cytosolic phospholipase A_2 ($cPLA_2$) determines the amount of C5a-induced LTC_4 release. This outcome is presumably due to the dual requirements of elevated cytosolic calcium and phosphorylation for the translocation and activation of $cPLA_2$. Because C5a alone induces a calcium response that returns to resting levels before $cPLA_2$ becomes phosphorylated, there is no overlap between the calcium signal and $cPLA_2$ phosphorylation, so LTC_4 is not generated. IL-3 induces $cPLA_2$ phosphorylation, so the typical transient calcium response following C5a necessarily overlaps the state of phosphorylated $cPLA_2$ established by IL-3 (7). Therefore acute IL-3 priming appears to result from preactivation of Erk before the stimulus is added. However the mechanism by which IL-3 primes the IgE-mediated response is not known, and because the calcium signal and Erk phosphorylation elicited by IgE-mediated stimulation occur in a very similar time frame, the previous mechanistic explanation did not seem applicable.

Previous studies showed that IL-3 activates several signaling pathways in basophils including STAT5, JAK2, Shc, Grb2, *ras*, Erk, and Src homology protein 2 (17), in which some pathways are common with IgE-mediated signaling. However the modulation of IgE-mediated signaling by IL-3 has not been addressed yet. Like the priming of C5a responses (7), the priming of IgE-mediated response in basophils seems to be biphasic. The acute response is apparent at times as short as 2 min after exposure to IL-3, and a late response (5) can occur in the 18–24 h time frame, with at least some of its effects being dependent on protein synthesis. In this study we will focus on the effects of acute IL-3 priming on IgE-mediated signaling. We hypothesized that some of the signaling pathways activated by aggregation of FcεRI will be enhanced by acute IL-3 priming, and because the priming effect is evident for the release of both histamine and LTC_4 mediators, we predicted that the signaling target of IL-3 priming would be located in the common early signaling steps. The results will show that acute

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[3] Abbreviations used in this paper: LTC_4, leukotriene C_4; LAT, linker for activation of T cell; PKC, protein kinase C; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $cPLA_2$, cytosolic phospholipase A_2.

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IL-3 priming does not induce a general up-regulation of all signaling elements studied; on the contrary it exerts its effect at localized points of the signaling cascades. One of these points is Erk, whose preactivation is not necessary for priming.

Materials and Methods

Materials

The following were purchased: PIPES, BSA, EGTA, EDTA, D-glucose, NaF, Na_3VO_4, 2-ME, Nonidet P-40, FMLP, and Tris (Sigma-Aldrich); LY294002, Ro-81-3220, and bisindoylmaleimide II (Calbiochem); crystallized HSA (Miles Laboratories); FCS and RPMI 1640 containing 25 mM HEPES and L-glutamine (BioWhittaker); Percoll (Amersham Biosciences); Tris and Tween 20 (Bio-Rad); fura 2-AM (Molecular Probes); PMSF (Boehringer Mannheim); anti-phosphotyrosine mAb (4G10; Upstate Biotechnology); anti-phosphorylated linker for activation of T cells (anti-phospho-LAT, Tyr^{171}), anti-phospho-Shc (Tyr^{239}/Tyr^{240}), anti-phospho-Akt (Thr^{308}), anti-phospho-Raf-1 (Ser^{338}), anti-phospho-Mek1/2 (Ser^{217}/Ser^{221}), anti-phospho-Erk p42/44 (Thr^{202}/Tyr^{204}), anti-Erk, anti-Akt, and biotinylated m.w. markers (Cell Signaling Technology); anti-Syk mAb (Santa Cruz Biotechnology); and HRP-conjugated donkey anti-rabbit Ig Ab, HRP-conjugated sheep anti-mouse Ig Ab, and protein G-Sepharose beads (Amersham Biosciences). Goat anti-human IgE Ab was prepared as described previously (18).

Buffers

PIPES-albumin-glucose (PAG) buffer consisted of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 0.1% glucose, and 0.003% HSA. PAG buffer was supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PAGCM). Countercurrent elutriation was conducted in PAG buffer containing 0.25% BSA in place of 0.003% HSA. Electrophoresis sample buffer (ESB) is Novex buffer containing 5% 2-ME. Complete lysis buffer (CLB) is 20 mM Tris-HCl, pH 7.5, 100 μg/ml aprotinin, 10 mM benzamidine, 1 mM PMSF, 50 mM NaF, 1 mM Na_3VO_4, 1% Nonidet P-40, and 10% glycerol. Incomplete lysis buffer is CLB without the protease inhibitors Nonidet P-40, glycerol, or vanadate. Stripping buffers were 7 M guanidine hydrochloride or 65 mM Tris-HCl (pH 6.7), 100 mM 2-ME, and 2% SDS depending on the sensitivity of the subsequent blotting.

Basophil purification

For these experiments, residual cells of normal donors undergoing leukapheresis were enriched in basophils using Percoll density gradients and countercurrent flow elutriation, as previously described (19). The procedure included an overnight incubation before negative selection; to slow loss of cells, 30 pg/ml IL-3 were added to the medium. Previous studies of acute and long-term effects of IL-3 on function determined that 30 pg/ml overnight did not alter the general behavior of the cells to further IL-3 stimulation (7). Basophils were further purified by negative selection with a basophil purification kit (StemCell Technologies) and columns from Miltenyi Biotec. The purity of basophils was determined by Alcian blue staining (20) and generally exceeded 99% when basophils were purified from these leukapheresis packs.

LTC_4 measurements

A radioimmunoassay was performed using 50 μl of supernatant to determine LTC_4 levels as previously described (21). LTC_4 was measured in the supernatant of the same cells that were used to detect protein phosphorylation (1.5–3 $\times$ 10^6 pure basophils/singlet condition).

Phosphorylation of Syk

Syk phosphorylation was detected with the anti-phosphotyrosine Ab clone 4G10 after immunoprecipitation of Syk from basophil lysates. After stimulating basophils (1.5–3 $\times$ 10^6) in PAGCM buffer at 37°C, the reaction was stopped by adding ice-cold PAG buffer and centrifuged in a microfuge for 15 s. The supernatants were recovered for LTC_4 detection, and the pellets were immediately lysed in CLB by vortexing and incubating on ice for 10 min. The lysates were then centrifuged for 3 min at 16,000 $\times$ *g* and precleared with protein G-Sepharose beads for 30 min at 4°C. Then the clarified lysates were incubated with anti-Syk Ab prebound to protein G-Sepharose beads (1 μg of Ab per 20-μl beads) for 1 h at 4°C. The beads were washed three times, and the immunoprecipitated proteins were eluted by boiling for 5 min in ESB. After electrophoresis and transfer, the membranes were blotted with 4G10 Ab. The membranes were then stripped with SDS buffer and reblotted with anti-Syk Ab to determine loading. Data from the anti-phosphotyrosine blots were normalized for loading differences using the band intensities from the anti-Syk reblot.

Phosphorylation of LAT, Shc, Akt, Raf-1, MEK, and Erk1/2

The phosphorylation of these proteins was assessed using the following phospho-specific Abs: anti-phospho-LAT (Tyr^{171}), anti-phospho-Shc (Tyr^{239}/Tyr^{240}),anti-phospho-Akt(Thr^{308}),anti-phospho-Raf-1(Ser^{338}),anti-phospho-MEK1/2 (Ser^{217}/Ser^{221}), and anti-phospho-Erk p42/44 (Thr^{202}/Tyr^{204}). Basophils (0.5–1 $\times$ 10^6) were stimulated in PAGCM buffer, and reactions were stopped by adding ice-cold PAG buffer. The cell pellets were lysed in ESB and boiled for 5 min. The lysates were separated by electrophoresis on 10% Tris-glycine gels (NOVEX) and transferred to a nitrocellulose membrane as described in the product literature. The membranes were incubated overnight at 4°C in TBST containing 5% nonfat dried skim milk (Carnation) or 4% BSA to block nonspecific binding. Phosphorylated proteins were detected by a 90-min incubation with the Abs diluted in TBST containing 3% skim milk or 5% BSA. After washing, the membranes were incubated with HRP-conjugated anti-rabbit Ab for 1 h. ECL detection was performed per manufacturer's instructions (electrochemiluminescence; Amersham Biosciences). The membranes were reblotted with anti-Akt Ab to assess equal loading after stripping with SDS buffer for 1 h at 50°C. ECL films were converted to digital images with a Kodak DC290 camera and the bands analyzed with NIH Image. Erk1/2 data presented are the result of adding the two bands p42/44. In vitro Erk activity was determined using an Erk kinase kit (Cell Signaling Technology), following the manufacturer's guidelines.

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurements

Basophils were labeled with 1 μM fura 2-AM for 20 min at 37°C in RPMI 1640 containing 2% FCS (0.3–0.5 $\times$ 10^6 cells in 200 μl). After washing once with 200 μl of PAG buffer, the cells were resuspended in PAG buffer for loading in the microscope observation chamber (22, 23). Changes in cytosolic-free calcium concentration $[Ca^{2+}]_i$ were determined by digital video microscopy (22, 24). Briefly, 15 μl of cells were loaded onto the siliconized coverslip of the microscope and, after settling, overlaid with 1 ml of PAGCM buffer. After warming to 37°C, monitoring of the cells was begun. After four frames (each frame is a single measurement of a field of 30–100 cells) of resting, $[Ca^{2+}]_i$ levels were acquired, and the cells were challenged with 1 ml of stimulus in buffer. Data were then obtained for 50–150 frames at intervals of 1–10 s to determine the $[Ca^{2+}]_i$ response. The average of all points was plotted because the profile of the response did not change in basophils preincubated with IL-3 for 15 min vs anti-IgE alone.

Data presentation

ECL films were converted to digital images with a Kodak DC290 camera, and the bands were analyzed with NIH Image software. Because both IL-3 and anti-IgE induced the phosphorylation of Shc, Akt, Raf-1, MEK, and Erk, the data are expressed as a ratio to the sum of both conditions alone. In this way if the phosphorylation by IL-3 and anti-IgE together exceeds the value of 1 (see Figs. 1, *A–H*, and 6*B*), it means that in the presence of both cytokine and stimuli the phosphorylation of that protein is higher than the addition of the phosphorylation levels induced by IL-3 and anti-IgE separated. Erk1/2 data presented are the result of adding the two bands p42/44. Most of the experiments were done with different preparations of basophils (see Fig. 1). In a few instances, multiple signaling elements were examined in the same preparations, e.g., phosphorylation of MEK and Erk and Akt.

Results

Effects of IL-3 on known signaling to Erk activation

Because acute priming with IL-3 increases IgE-dependent mediator release in general, we began by examining early IgE-mediated signaling, the steps for which are likely to be common for all secreted mediators. The phosphorylation of Syk after FcεRI aggregation is one of the earliest events on the IgE-mediated signaling pathway. Syk phosphorylation was measured in basophils preincubated for 15 min with IL-3 and then stimulated with anti-IgE. IL-3 did not change the kinetics of Syk phosphorylation induced by aggregation of the FcεRI with anti-IgE, nor did IL-3 induce Syk phosphorylation by itself (Fig. 1*A*, *right histogram*). IL-3 priming for 15 min did not induce a statistically significant increase of anti-IgE-induced Syk phosphorylation at 5 min after stimulation (Fig. 1*A*, *left histogram*) ($n = 11$, $p = 0.0506$). For most of these

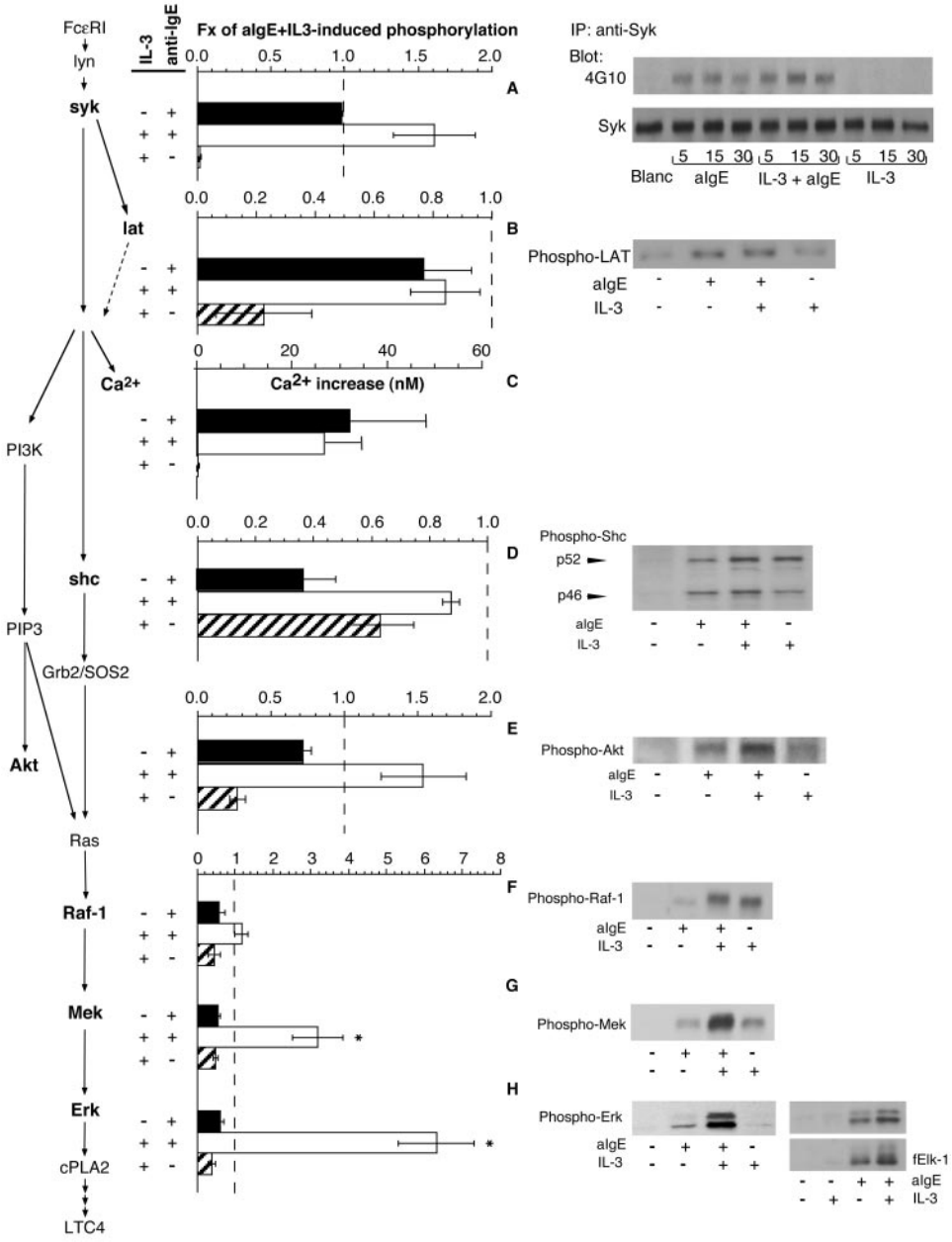

FIGURE 1. Effect of acute IL-3 priming on signaling. The schematic (*left*) outlines the steps that have been studied in human basophils and examined for changes under the influence of IL-3 exposure. The experimental design for each step was similar: basophils were preincubated with 10 ng/ml IL-3 for 15 min and then stimulated with 0.2 μg/ml anti-IgE for 5 min. Representative results for each element or expanded versions (*right*) of the basic experimental design are shown. Using the average of multiple experiments (*left histogram*), generally performed with distinct preparations of cells, basophils stimulated with anti-IgE + IL-3 (□) are compared with anti-IgE alone (■) and IL-3 alone (▨). Except *C*, data are expressed as a ratio of the response compared with the summation of anti-IgE alone plus IL-3 alone. *A*, Kinetics of anti-IgE-induced Syk phosphorylation (*right*) in the presence or absence of IL-3 with $n = 11$ (left histogram) are shown. *B*, LAT phosphorylation ($n = 4$). *C*, Net elevation in cytosolic-free calcium ($n = 3$). *D*, Shc ($n = 3$). *E*, Akt phosphorylation ($n = 4$). *F*, Raf-1 ($n = 5$). MEK ($n = 9$) (*G*) and Erk ($n = 9$) (*H*) phosphorylation are shown. *H*, Two Western blots (*far right*) show the in vitro kinase assay results for Erk activity using a fusion Elk-1 protein as substrate compared with the phosphorylation state of Erk from the same experiment.

measurements, the cells were lysed at 5 min, at the peak, or near-peak of the response for these signaling elements, and the point of 50% of maximum histamine release usually occurred at 8 min at this concentration of anti-IgE Ab. Note that for the purpose of presentation, the data are expressed as a fraction of the summed signals induced by anti-IgE and IL-3 alone. For those signaling events in which IL-3 alone induces phosphorylation, the value of 1.0 represents a strictly additive response. Synergy would be defined as values with statistically significant increases >1.0.

Several other early signaling steps not known to follow stimulation with IL-3 were also examined. Fig. 1, *B* and *C*, show results for LAT phosphorylation and increases in $[Ca^{2+}]_i$. The phosphorylation of the adaptor protein LAT, as detected by a phospho-specific Ab, was the same in anti-IgE-stimulated basophils regardless the presence or absence of IL-3 (Fig. 1*B*). The increase of $[Ca^{2+}]_i$ is also a signaling step required for the IgE-mediated release of histamine, LTC_4, and IL-4. Acute IL-3 priming did not change the intensity or the profile of the anti-IgE-induced increase of $[Ca^{2+}]_i$ (Fig. 1*C* and data not shown). IL-3 alone did not induce an elevation in cytosolic calcium.

Because LTC_4 release is markedly enhanced from IL-3 primed basophils (5, 25) and the activation of the *ras*-Erk pathway is required for LTC_4 release (26), we next studied the effect of IL-3 on the pathways that lead to Erk activation. Two signaling routes have been described to be involved in the activation of the *ras*-Erk pathway and LTC_4 release by IgE-mediated stimulation: Shc/Grb2/SOS2 and PI3K (27, 28). Shc was phosphorylated after incubation with IL-3 or anti-IgE (Fig. 1*D*). In basophils incubated for 15 min with IL-3 and then stimulated with anti-IgE, p52 Shc phosphorylation equaled the sum of the phosphorylation induced by IL-3 alone plus that of anti-IgE alone. p46 Shc followed the same pattern of phosphorylation. The activation of Shc was also measured as association of Shc with Grb2, and in this case no synergism in the activation of Shc was detected either (data not shown).

We tested the priming effect of IL-3 on the activation of PI3K and phosphatidylinositol 3,4,5-trisphosphate generation and used

Akt phosphorylation as a surrogate measure of phosphatidylinositol 3,4,5-trisphosphate levels. Stimulation with anti-IgE and IL-3 induced Akt phosphorylation. The levels of Akt phosphorylation reached in the presence of both IL-3 and anti-IgE together were not significantly different from the addition of phosphorylation induced by IL-3 alone and anti-IgE alone (Fig. 1*E*).

The activation of the *ras*-Erk pathway was detected by phosphorylation of activating sites of Raf-1, Mek, and Erk. Phosphorylation of Raf-1 in Ser^{338} was induced by both IL-3 and anti-IgE, and the addition of Raf-1 phosphorylation in both conditions alone was similar to its phosphorylation in the presence of IL-3 and anti-IgE together (Fig. 1*F*; note the change in scale on the ordinate). MEK phosphorylation, detected with a specific anti-phospho-MEK Ab (Ser^{217}/Ser^{221}), was synergistic in IL-3 preincubated anti-IgE-stimulated basophils (Fig. 1*G*). The average increase was 3.1 ± 0.7-fold ($n = 9$, $p = 0.01$) over the addition of both conditions alone and 5.4 ± 1.4-fold over anti-IgE alone. Erk activation was detected with a phospho-specific Ab raised against the activating sites Thr^{202}/Tyr^{204} (a test of Erk kinase was also performed and is discussed below). IL-3 priming induced a remarkable increase of Erk phosphorylation after stimulation with anti-IgE (Fig. 1*H*). As expected, IL-3 alone and anti-IgE alone also induced Erk phosphorylation; however, the level of Erk phosphorylation in the presence of IL-3 and anti-IgE together was 6.3 ± 1.0-fold ($n = 9$, $p = 0.002$) higher than the summed phosphorylation levels of basophils incubated with IL-3 alone or anti-IgE alone. The increase over anti-IgE-induced Erk phosphorylation was 13.0 ± 3.4-fold (ranging from 2.1- to 40-fold). A single peak time point was analyzed in the described experiments but acute IL-3 priming did not significantly change the overall character of the kinetics of Erk phosphorylation (Fig. 2*A*) or LTC_4 release (Fig. 2*B*) induced by optimal stimulation with anti-IgE (the peak time for maximum LTC_4 release and for the peak of Erk phosphorylation was somewhat sooner in IL-3-primed cells, but Erk phosphorylation remained transient).

Characteristics of acute IL-3 priming of Erk phosphorylation

The kinetics of the IL-3-priming effect on phosphorylation of Erk was explored next. For this purpose, the basophils were primed for different times with IL-3, and then stimulated for 5 min with anti-IgE. For comparison, a kinetic of Erk phosphorylation induced by IL-3 alone was included in the same experiment. The kinetics of IL-3-induced Erk phosphorylation peaked at 5 min and returned to basal levels by 60 min (Fig. 3*B*, *bottom*). Fig. 3*A*, *lanes 4–6*, shows that when IL-3-induced Erk phosphorylation was back to resting levels, IL-3 still induced a strong increase of Erk phosphorylation triggered by anti-IgE (Fig. 3*A*, *lanes 10–12*). The up-regulating effect of IL-3 on anti-IgE-induced Erk phosphorylation was sustained for over 1 h (Fig. 3*B*, *top*). The IL-3-priming effect (Fig. 3*B*, *top*) was represented as the fold increase of Erk phosphorylation in IL-3-primed/anti-IgE-stimulated basophils over the anti-IgE-matched control after subtracting IL-3 alone-induced Erk phosphorylation at the same time point. The matched control was the phosphorylation of Erk 5 min after stimulation with anti-IgE in basophils incubated in medium alone for the same time lengths as the IL-3 preincubations. When 10 ng/ml IL-3 and 0.2 μg/ml anti-IgE are added simultaneously, the synergism on Erk phosphorylation was already apparent and the signal is as strong as at later time points (Fig. 3*A*, *lane 7*, and *B*, *top*, first circle). To check whether measuring Erk phosphorylation was a useful surrogate of Erk activity, phospho-Erk was immunoprecipitated and its activity assessed by the phosphorylation of a GST-Elk-1 fusion protein as a substrate. For this experiment, basophils were first incubated with 10 ng/ml IL-3 for 60 min before the addition of anti-IgE Ab for 5 min. As shown in Fig. 1*H*, *far right*, the increase in phospho-Erk was mirrored by an increase in Erk activity.

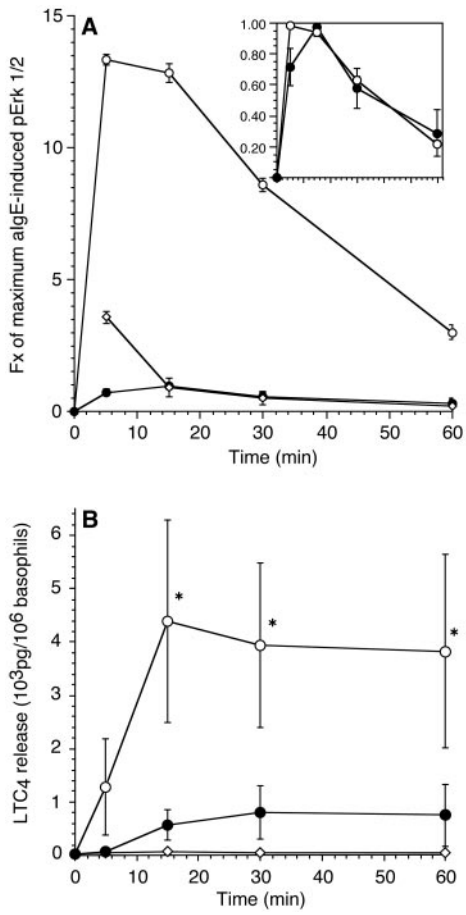

FIGURE 2. Kinetics of anti-IgE-induced Erk phosphorylation and LTC_4 release in human basophils primed with IL-3. Basophils were preincubated for 15 min in the presence of 10 ng/ml IL-3 and stimulated with 0.2 μg/ml anti-IgE for 5, 15, 30, and 60 min. Anti-IgE alone (●), IL-3 plus anti-IgE (○), and IL-3 alone (◇) are represented. *A*, Erk phosphorylation. *B*, LTC_4 release ($n = 3$). Within each experiment for Erk phosphorylation, the data were first expressed as a fraction of maximum response for that particular condition (anti-IgE alone, anti-IgE + IL-3, IL-3 alone), the three experiments were averaged, and this result is shown in *A* (*inset*). The actual difference in the magnitude of the response for each condition is shown (*A*).

The IL-3-priming effect on anti-IgE-induced Erk phosphorylation is present at concentrations of IL-3 that do not induce any Erk phosphorylation when added alone (Fig. 3*C*). In this experiment, the priming of anti-IgE-induced Erk phosphorylation was analyzed as described in Fig. 1, after a 15 min preincubation with IL-3 and an additional 5 min incubation time with anti-IgE. For these experiments, Erk phosphorylation following stimulation with IL-3 alone was measured at both a 5- and 20-min time point. The concentration-response curve for IL-3 priming shows an EC_{50} of ~0.1 ng/ml (IL-3 priming of anti-IgE-induced Erk phosphorylation, Fig. 3*D*, ○), remarkably similar to the EC_{50} of IL-3 for acute priming of LTC_4 release (7). However the EC_{50} for IL-3 alone-induced Erk phosphorylation was 10 times higher (Fig. 3*D*).

Mechanisms of IL-3 priming

There are many issues raised by these results. Most notably, the kinetic data presented in Fig. 3 lead to a model of up-regulation quite different from previous studies of priming of the C5a response. Specifically, concurrent activation of Erk (as measured by

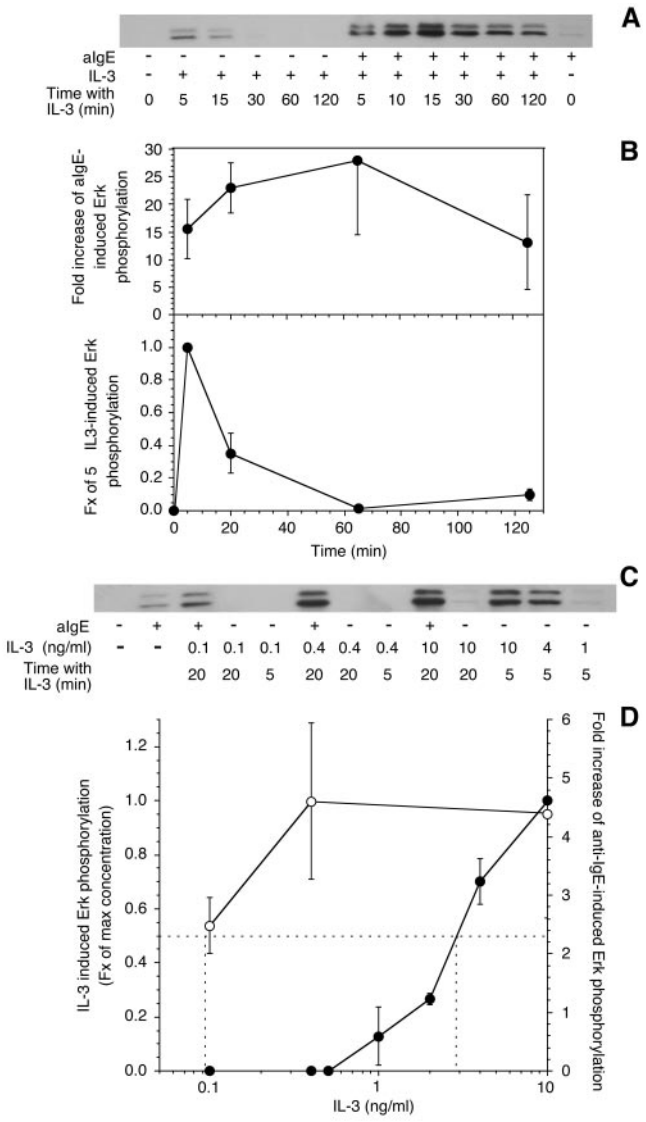

FIGURE 3. Kinetics and EC_{50} of IL-3 priming of anti-IgE-induced Erk phosphorylation in human basophils. *A*, Comparative kinetics of IL-3-induced Erk phosphorylation and IL-3/anti-IgE synergism on Erk phosphorylation. Basophils were preincubated for different times in the presence of 10 ng/ml IL-3 and then stimulated or not with 0.2 μg/ml anti-IgE for 5 min (anti-IgE was added for the last 5 min of time with IL-3, which means that at the 5-min time point IL-3 and anti-IgE were added together). *B*, Averaged kinetics of IL-3-induced Erk activation and priming. Basophils were preincubated for 0 (IL-3 and anti-IgE added together), 15, 60, and 120 min in the presence of 10 ng/ml IL-3 and then stimulated or not with 0.2 μg/ml anti-IgE for 5 min. Kinetics of Erk phosphorylation induced by IL-3 alone (*bottom*) are represented as a fraction of Erk phosphorylation at 5 min. The kinetics of priming of anti-IgE-induced Erk phosphorylation (*top*) obtained in the same experiments were represented as the fold increase of Erk phosphorylation in the presence of IL-3 and anti-IgE over anti-IgE alone-induced Erk phosphorylation after subtracting Erk phosphorylation induced by IL-3 alone at each time point. The ratio was calculated to their anti-IgE-matched control, meaning that Erk phosphorylation at 5 min of stimulation with anti-IgE was measured after incubation for 0, 15, 60, and 120 min in medium alone for each experiment ($n = 3$). *C*, Priming of anti-IgE-induced Erk phosphorylation at low doses of IL-3. Basophils were stimulated for 5 min with anti-IgE after priming for 15 min with 0.1, 0.4, and 10 ng/ml IL-3. Beside the 20-min time point, IL-3 alone-induced Erk phosphorylation was also analyzed at 5 min for 0.1, 0.4, 1, 4, and 10 ng/ml IL-3. *D*, IL-3 concentration-response curve for priming of anti-IgE-induced (○) Erk phosphorylation ($n = 3$) and IL-3 alone-induced (●) Erk phosphorylation ($n = 3$). The concentration-response for Erk phosphorylation was analyzed at 5 min, with the one for priming at 20 min (15 min of preincubation with IL-3 plus 5 min of stimulation with anti-IgE), which are the kinetic peaks for each of these IL-3 effects shown in *A*. One experiment shown in *C* included both dose-response curves for priming of anti-IgE-induced Erk phosphorylation and for IL-3-induced Erk phosphorylation. The other two experiments were not matched.

its phosphorylation state) is no longer a prerequisite for the priming effect. We have examined several aspects of this conclusion.

First, we examined the possibility that IL-3 priming altered the dependence on signaling steps thought to be upstream of Erk phosphorylation. Basophils, with or without priming with IL-3 (10 ng/ml) for 15 min, were stimulated with anti-IgE Ab in the presence or absence of 10 μM MEK inhibitor, U0126 (U0126 was added for 10 min, then the cells were primed for 15 min, 0.2 μg/ml anti-IgE Ab added and the reaction stopped at 5 min post anti-IgE). U0126 inhibited Erk phosphorylation in both primed and unprimed cells stimulated with anti-IgE Ab; therefore, there is not a newly established Erk phosphorylation pathway by IL-3 priming.

It seemed likely that this mechanism of IL-3 priming could be generalized. We tested the effect of longer priming by IL-3 on Erk activation when cells were stimulated with either FMLP or C5a. Fig. 4, *A* and *B*, shows that once again, when the IL-3-induced phosphorylation of Erk has returned to resting levels (at 45 min), there remains a marked priming of Erk phosphorylation following stimulation with either FMLP of C5a. Notably, this priming effect increases both the rate and extent of Erk phosphorylation induced by C5a (Fig. 4*C*), whereas the otherwise normal delay in Erk phosphorylation (following C5a in the absence of IL-3) makes it asynchronous with the calcium response (7).

Generalizing the priming effect to FMLP (or C5a) allowed a subtle question to be addressed; we could ask whether the activity of PI3K is required for the priming effect. The PI3K inhibitor, LY294002, is known to completely inhibit IgE-mediated stimulation of all elements of the *ras*-Erk pathway. In contrast, LY294002 has no effect on IL-3-induced (17) or FMLP-induced (our unpublished observation) Erk phosphorylation. We examined the effect of LY294002 on IL-3 priming of anti-IgE- and FMLP-induced Erk phosphorylation. As expected, LY294002 had no effect Erk phosphorylation induced by IL-3 alone, and reduced the Erk phosphorylation that occurred with the combination of anti-IgE Ab and IL-3 back to levels observed with IL-3 alone (data not shown). However, because of the linkage established between Erk activation and PI3K activity when stimulating with anti-IgE, it was not possible to address whether the priming mechanism required PI3K activity. By using FMLP as the stimulus, we could ask whether this PI3K activity induced by IL-3 was indirectly responsible for the priming/synergy observed for Erk phosphorylation. Fig. 4*D* shows that IL-3 priming of Erk phosphorylation induced by FMLP was insensitive to the presence of LY294002, indicating that PI3K activation does not appear to be involved in the occult pathway that results in synergy of MEK or Erk phosphorylation. Note, unlike the absence of effects on Erk phosphorylation (Fig. 4*D*) induced by IL-3, LY294002 did completely inhibit Akt phosphorylation induced by IL-3 alone (data not shown).

The Raf-1-Mek-Erk cascade has been modeled as an ultrasensitive signaling cascade. Under these conditions, very small increases of Raf-1 activity would generate a larger increase of Erk activation. Considering this behavior of the cascade, it is possible that a small increase in Raf-1 activity by IL-3 is responsible for the strong priming of Erk phosphorylation. Although the studies described show that Raf-1 phosphorylation at Ser^{338}, which happens after its recruitment to *ras* and contributes to Raf-1 kinase activity,

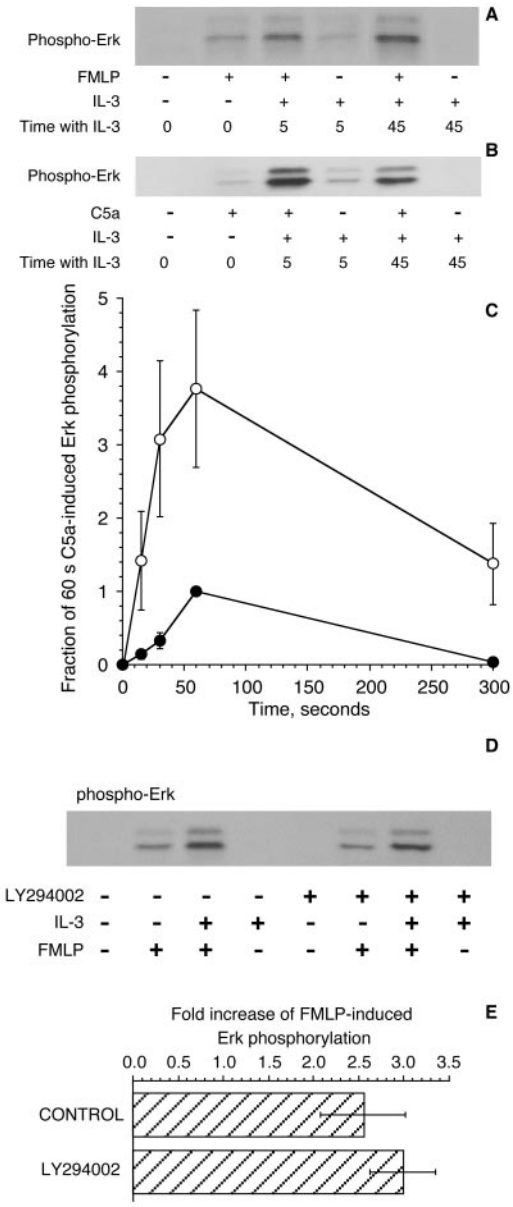

FIGURE 4. Priming of Erk phosphorylation following stimulation with non-IgE-dependent stimuli. *A*, Incubation in buffer with (+) or without (−) IL-3 at 10 ng/ml for 15 min before stimulation with 200 nM FMLP for 1 min. *B*, Incubation in buffer with or without IL-3 at 10 ng/ml for 15 or 45 min before stimulation with 50 ng/ml C5a for 1 min. *C*, Incubation in buffer with or without IL-3 at 10 ng/ml for 15 min before stimulation with 50 ng/ml C5a for multiple times. *D*, LY294002 effect on IL-3 priming of FMLP-induced Erk phosphorylation. Basophils were preincubated for 30 min with 10 μM LY294002 and then 10 ng/ml IL-3 or 1 μM FMLP were added for 5 and 1 min, respectively (loading controls not shown for *A*, *B*, and *D*). *E*, Average of two experiments like that in *D*. The data are represented as the fold increase of Erk phosphorylation in the presence of IL-3 and FMLP together over FMLP alone-induced Erk phosphorylation after subtracting Erk phosphorylation induced by IL-3 alone ($n = 2$).

was not modulated by IL-3 priming, the modulation of the kinase activity of this enzyme is more complex than a single-site phosphorylation. We attempted to develop a Raf-1 enzymatic assay suitable for basophil studies. The conditions for the in vitro kinase assay of Raf-1 isolated by immunoprecipitation were tuned using the leukemia cell line MO7e, in which we detected a stimulation index of ~4-fold over resting levels. However the same experimental conditions did not show any increase in the activity of Raf-1 after the addition of IL-3 or of a stimulus to a basophil suspension. Although there appeared to be a small increase of Raf-1 activity when IL-3 and stimulus were present together (using 6 million cells/condition), the lack of a statistically significant stimulation index in both conditions alone does not allow a straightforward interpretation of the data. Because there is some background kinase activity in this assay in the absence of cells, limitations in the number of basophils available for this type of assay made it difficult to readily exceed this background.

A different approach was followed to test the hypothesis of an increase of Raf-1 activity being responsible for the IL-3 priming increase of stimulus-induced Erk phosphorylation. We used the phosphorylation of Raf-1 at Ser^{338} as an indicator of the participation of Raf-1 in the reaction, and plotted its stimulation index against Erk phosphorylation. A straightforward model of ultrasensitivity predicts that all the points of Erk phosphorylation will follow a single steep sigmoid curve when plotted against the activity of Raf-1 (in this case, measured as phospho-Raf-1) regardless of the extent of Raf activity. That is, if IL-3 primes Erk phosphorylation by increasing Raf-1 activity, with no other mechanism being involved, then the points of Erk phosphorylation in the presence of IL-3 should fit in the same sigmoid curve. We generated different levels of activation of these enzymes by stimulating the basophils with several concentrations of C5a in the presence and absence of IL-3. For each condition Erk phosphorylation and Raf-1 phosphorylation were measured by sequential Western blots with specific Abs. In Fig. 5, the stimulation index of phosphorylated Raf-1 was plotted against Erk phosphorylation normalized to one of the concentrations of C5a (50 ng/ml) (after subtracting the minimal IL-3 effect alone). The curve profile does not correspond with IL-3 having an effect only on Raf-1 activity, because Erk phosphorylation seemed to reach a saturation point when stimulated only with C5a, and a different, higher saturation point when primed with IL-3 before C5a stimulation.

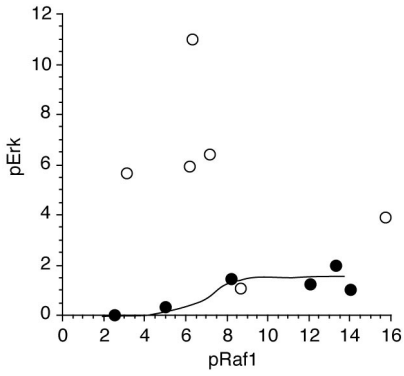

FIGURE 5. Relationship between phosphorylation of Raf-1 and Erk1/2 with or without IL-3. Basophils were incubated with IL-3 for 15 min followed by stimulation with three concentrations of C5a (10, 20, and 50 ng/ml). The points represent data from two separate experiments. For each condition Erk phosphorylation and Raf-1 phosphorylation were measured by sequential Western blots with specific Abs. Erk phosphorylation seemed to reach a saturation point when stimulated only with C5a (●) and a different, higher saturation point when primed with IL-3 before C5a stimulation (○). The stimulation index of phosphorylated Raf-1 (pRaf-1) was plotted against Erk phosphorylation where the phosphorylated Erk (pErk) band intensity was normalized to the 50 ng/ml concentration of C5a after subtracting from both Raf-1 and Erk band intensities the phosphorylation induced by IL-3 alone under the same conditions. For demonstration purposes, the curve represents one possible fit of the results for the relationship in the absence of IL-3 with the expectation that all points would lie on such a curve.

Protein kinase C (PKC) has also been reported to modulate the activation of the Raf-1-Erk cascade. On the one hand, PKC has been described to have a positive role on the activation of Erk in multiple cell models (29–31), among them basophils in which the PKC activator PMA induces Erk phosphorylation (28). In contrast, in some cell models PKC seems to be involved in Erk deactivation (32). The PKC inhibitor Ro-31-8220 induced an increase of MEK and Erk phosphorylation after anti-IgE stimulation both in IL-3-primed and nonprimed basophils, without a significant effect on the phosphorylation induced by IL-3 alone (Fig. 6, *A* and *B*). In the presence of Ro-31-8220 the difference between anti-IgE-induced MEK and Erk phosphorylation in IL-3-primed and nonprimed basophils is reduced compared with conditions in the absence of the inhibitor (Fig. 6*C*). Similar results were obtained with another related PKC inhibitor bisindolylmaleimide II (data not shown). The Ro-31-8220 EC_{50} for the increase of Erk phosphorylation was 1 μM (Fig. 6*D*), considerably higher than the 0.1 μM EC_{50} for most of PMA-induced changes in basophils (33, 34). Consistent with the enhancement of MEK and Erk by Ro-31-8820, this inhibitor also markedly enhanced LTC_4 release. For example, in the experiment shown in Fig. 6*A*, addition of 1 μM Ro-31-8220 increased anti-IgE^+ IL-3 LTC_4 release by 21-fold.

Discussion

In this study we show that one point of modulation of IgE-mediated signaling by acute IL-3 priming is localized at the level of the Raf-1-Mek-Erk cascade. Fig. 1 includes a schematic representation of the signaling cascade that leads to LTC_4 release after IgE-mediated stimulation in basophils. The scale change that is needed to display the MEK and Erk data highlights the synergy that accompanies the changes in signaling at this point in the cascade. Because the IL-3-priming effect on IgE-mediated Erk phosphorylation was more sustained and has a 10-fold lower EC_{50} than the IL-3-induced Erk phosphorylation (Fig. 3), both events can be considered to some extent independent. For example, after 60 min of priming, there is essentially no remaining Erk activation due to IL-3 alone and yet the enhanced response to anti-IgE remains, in absolute terms, nearly as marked. In this context, the IgE-mediated activation of MEK and Erk in IL-3 primed basophils was respectively 5.4 ± 1.4 and 13.0 ± 3.4 times higher than in nonprimed basophils. For the initial elements of the signaling cascade this ratio stays close to 1.0, which means that the IgE-mediated activation of these elements is the same in primed and nonprimed basophils.

The marked synergy between IL-3-induced and anti-IgE-induced Erk phosphorylation raised the possibility that synergy in a pathway element always occurred when two stimuli used the same pathway element. However, this is not the case for several other shared elements, notably Shc, Akt, and Raf-1 phosphorylation. Therefore, the marked effect at the level of MEK and especially Erk phosphorylation is a selective effect on these elements.

This up-regulation of the Raf-1-Mek-Erk cascade that yields a final 13-fold increase of the levels of Erk phosphorylation induced by an IgE-mediated stimulus is consistent with the remarkable increase of IgE-mediated LTC_4 release induced by IL-3 priming. However it does not explain the more modest increase of histamine or IL-4 release because LTC_4 is the only mediator for which release is dependent on Erk phosphorylation (26). The early signaling elements that are common to the release of all mediators do not seem to be affected by acute IL-3 priming. Syk phosphorylation was not increased ($p > 0.05$) in IL-3 primed/anti-IgE-stimulated basophils vs anti-IgE alone. However, it could be argued that the experiment was not statistically powered to detect small levels of increase given the high amount of biological variation observed among different donors. Although we do not exclude the possibility of Syk being weakly modulated by IL-3 under conditions of optimal stimulation (1.5-fold compared with 13-fold for Erk), the relevance of this effect for downstream signaling is also questionable because the phosphorylation of LAT and Shc, both considered to be direct targets of Syk (27, 35), was not affected by IL-3 priming. Additionally the anti-IgE-induced calcium signal, another event dependent on Syk activation in basophils (36), was also unaffected by a short priming with IL-3. It is important to point out

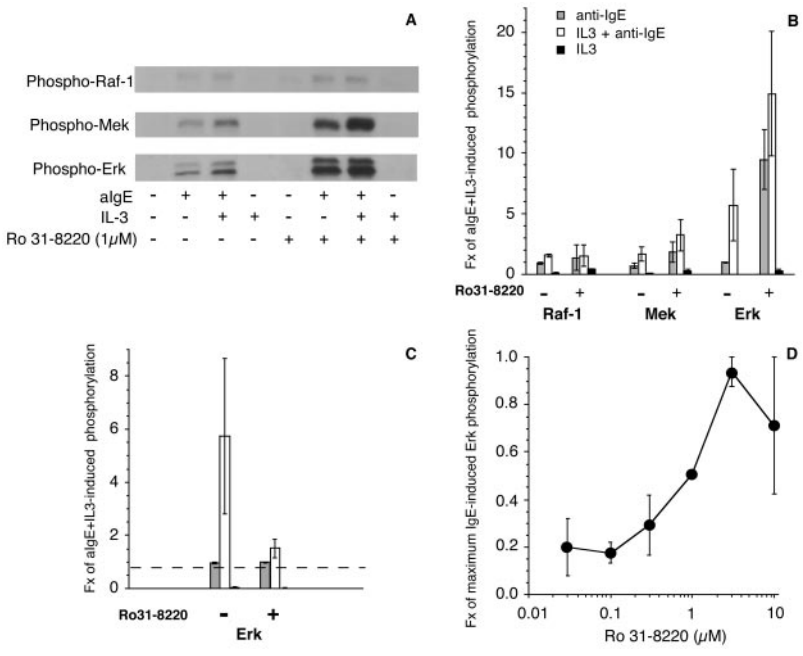

FIGURE 6. Effect of preincubation with Ro-31-8220 on Raf-1, MEK, and Erk phosphorylation following stimulation with anti-IgE Ab in IL-3-primed and nonprimed basophils. *A*, Example of Western blot, basophils were incubated with 1 μM Ro-31-8220 for 10 min before the addition of 10 ng/ml IL-3 for 15 min. Following IL-3, 0.2 μg/ml anti-IgE Ab was added for an additional 5 min. *B*, Average of four experiments performed as shown in *A*. Data are expressed as done for the histograms in Fig. 1; the ratio of the response for a given condition relative to the summation of the response for IL-3 alone + anti-IgE alone (in the absence of Ro-31-8220). *C*, Recasting of the data in *B* for Erk phosphorylation is shown to highlight the extent of IL-3 enhancement in the presence or absence of 1 μM Ro-31-8220. The bars for stimulation in the absence of Ro-31-8220 are calculated as in *B*; however, for stimulation in the presence of Ro-31-8220, the phosphorylation induced by IL-3 and anti-IgE together in the presence of Ro-31-8220 was divided by IL-3 alone + anti-IgE alone in the presence of Ro-31-8220. *D*, Concentration dependence for the enhancing effect of Ro-31-8220 on anti-IgE-induced Erk phosphorylation ($n = 3$). A 10-min preincubation with different concentrations of Ro-31-8220 was followed by stimulation with 0.2 μg/ml anti-IgE. To facilitate the averaging, the data from each experiment were first normalized to the effect observed at 1 μM.

that for all these experiments, the basophils were stimulated with an optimal dose of anti-IgE (a dose that induces maximum histamine and LTC_4 release). The effects of IL-3 priming on IgE-mediated signaling at suboptimal doses of stimulation have not been explored. It remains possible that at suboptimal stimulation, an effect of IL-3 priming on IgE-mediated stimulation of Syk becomes more apparent.

The up-regulation observed in the presence of IL-3 and anti-IgE affects the phosphorylation of MEK and Erk at activating sites, and this translated to greater in vitro Erk activity (Fig. 1*H*). However it did not affect the phosphorylation of Raf-1 at Ser^{338}. Because the phosphorylation of Ser^{338} is an event that follows Raf-1 binding to *ras*, it seems that IL-3 does not have an effect on the recruitment of Raf-1 to the reaction complex. The synergism on MEK phosphorylation points to an up-regulation of the enzymatic activity of Raf-1 or the recruitment of MEK and Erk. Another possibility is that IL-3 down-regulates MEK and/or Erk dephosphorylation. The increase of the synergism from ~3-fold in the case of MEK phosphorylation to 6-fold in the case of Erk also raises the possibility for a second point of up-regulation between MEK and Erk. However, it may not be necessary to implicate more than one regulatory point because the MAPKKK-MAPKK-MAPK cascade has been demonstrated to behave as an ultrasensitive sequence, i.e., small changes in MAPKKK (Raf-1) signal result in disproportionate changes in MAPKK (MEK) and MAPK (Erk) activation (only at certain regions of MAPKKK activation). With the correct starting input, there may be more change in Erk than MEK. However, thus far, this behavior has only been observed using extracts of *Xenopus oocytes*, not in situ mammalian cells (37). Although we were unable to scale an in vitro Raf-1 kinase assay suitable for studies of basophils, the results shown in Fig. 4 suggest that the data for stimulation with or without IL-3 do not fall on the same curve, as one might expect if a full explanation of the results needed only the behavior of an ultrasensitive sequence. However, these studies only examined one of the possible phosphorylation sites of Raf-1 and the activity of Raf-1 may be discordant with this particular phosphorylation site.

The identity of the mechanism that up-regulates MEK and Erk in the presence of IL-3 and anti-IgE remains unknown. However, whatever this mechanism is, it does not seem to be exclusively regulating IgE-mediated stimulation because the characteristics of this enhancement are similar in IL-3 primed basophils after stimulation with FMLP and C5a. As pointed out previously, some characteristics of the IL-3-priming effect on anti-IgE-induced Erk phosphorylation suggest that there is not a parallel signaling pathway leading to Erk phosphorylation, but rather an IL-3-generated signal that modulates the IgE-mediated signaling to LTC_4 release at the level of the Raf-1-Erk cascade. The inhibition of the anti-IgE-mediated Erk phosphorylation by MEK inhibitors in the presence and absence of IL-3 eliminates the possibility of IL-3 inducing a MEK-independent activation of Erk, which has been described in other cell types including RBL cells (32, 38–40). Another piece of evidence that supports this conclusion is that PI3K inhibition with LY294002, which eliminates IgE-mediated Erk phosphorylation but leaves unaffected the IL-3 induced Erk phosphorylation (17), inhibits the phosphorylation of Erk in IL-3 primed/anti-IgE-stimulated basophils to the level of IL-3 alone-induced Erk phosphorylation. These results would be also consistent with LY294002 inhibitable PI3K activated by IL-3 being involved in the priming of Erk phosphorylation. However the lack of inhibition of priming of FMPL-induced Erk phosphorylation, a LY294002-independent event, is a strong argument against this possibility, assuming a common mechanism for all stimuli.

In addition to these pharmacologic data, the kinetics of the IL-3-priming effect on anti-IgE-induced Erk phosphorylation, that is still present 2 h after the addition of IL-3, when the IL-3-induced Erk phosphorylation is back to resting levels, suggests that the signaling pathway that IL-3 uses to activate Erk is different from the mechanism that it used to prime the IgE-mediated Erk phosphorylation. The EC_{50} values of IL-3 for both Erk activation and Erk priming also support this interpretation, because the priming of Erk activation occurs at concentrations of IL-3 that do not induce any detectable Erk phosphorylation. Another implication of these data is that, contrary to what we had previously hypothesized (7), the preactivation of Erk by IL-3 is not a requirement for IL-3 priming of LTC_4 release. The EC_{50} of IL-3 for priming of stimulus-induced Erk phosphorylation is coincident with the EC_{50} for acute priming of LTC_4 release (7). In contrast, the EC_{50} for IL-3-induced Erk phosphorylation is on the order of 10-fold higher, which is more coincident with the EC_{50} for late priming of LTC_4 release (5, 7). Overall, the evidence we present in this study strongly supports the up-regulation of stimulus-induced Erk phosphorylation by IL-3 being responsible for the acute priming of LTC_4 release. Whether the concurrent activation of Erk after short incubations with IL-3 further augment secretagogue-induced secretion remains to be seen. There are a variety of possible pathways that may influence MEK or Erk phosphorylation (e.g., changes in dephosphorylation) but it appears that, for the moment, this is an occult pathway that is activated very rapidly, affects Erk phosphorylation induced by different stimuli, is very sensitive to the presence of IL-3, and persists for relatively long periods. Interestingly, the phosphorylation of STAT5 induced by IL-3 follows similar sustained kinetics (17) and recently we have seen that it has also a similar concentration dependency (data not shown). Because the IL-3 priming affected Erk phosphorylation induced by three different stimuli, it logically follows that this occult priming pathway would influence Erk phosphorylation induced by IL-3 alone. Until the mechanism for priming is identified, this possibility cannot be readily explored.

There are indications in the literature that PKC-dependent pathways can modulate the Raf-1-MEK-Erk cascade. In this study we find that inclusion of relatively selective PKC inhibitors, Ro-81-3320 or bisindoylmaleimide II, mimic the effects of adding IL-3, including the enhancement of LTC_4 release. These inhibitors induce a remarkable increase of anti-IgE-induced Mek and Erk phosphorylation without a significant effect on Raf-1 phosphorylation or on IL-3-induced phosphorylation of these three proteins (previous studies demonstrated no enhancement of the IgE-mediated elevation in cytosolic calcium; see Ref. 33). This observation would be consistent with a down-regulatory role of a PKC isoform on the secretagogue-mediated phosphorylation of Mek and Erk that could be inhibited by IL-3 priming, and this down-regulatory pathway not targeting IL-3-induced Erk activation. The ability of Ro-81-3320 or bisindoylmaleimide II to negate the enhancing effect of IL-3 also suggests that Ro-31-8220 and IL-3 are using the same mechanism to increase Erk phosphorylation. However, the reduction of the difference could be due to Erk phosphorylation reaching a maximum in basophils incubated with Ro-31-8220/IL-3/anti-IgE. Conditions that induce a strong phosphorylation of Erk such as the combination of ionomycin/PMA or Ro-31-8220/IL-3/FMLP at maximum levels of stimulation equaled but did not exceed the levels of Erk phosphorylation induced by Ro-31-8220/IL-3/anti-IgE (data not shown). The discrepancy between the EC_{50} of Ro-81-3320 for the increase of Erk phosphorylation (1 μM) and the EC_{50} for most of the PMA-induced changes in basophils (0.1 μM) (33, 34), together with the lack of complete specificity of

these compounds for PKC (41), suggest that a Ser/Thr kinase different from PKC could be responsible for this regulation. Regardless the molecular identity of the Ser/Thr kinase implicated, it is possible that it shares some common signaling with the IL-3 priming pathway.

In summary, acute priming with IL-3 up-regulates the stimulus-induced signaling that lead to LTC_4 release at the level of the Raf-1-Mek-Erk cascade. The final result of this modulation at the end of the cascade is an average increase of ~13-fold of IgE-mediated Erk activation, consistent with the 15-fold average increase of IgE-induced LTC_4 release. The up-regulation of Erk activation by IL-3 is common to FcεRI-mediated and G protein-coupled receptor-mediated stimulation. We present evidence, based on the kinetics and IL-3 EC_{50} of priming effects, which suggests that acute IL-3 priming of secretagogue-induced Erk activation and LTC_4 release does not require a pre-established state of Erk activity, but it rather follows from an IL-3-dependent regulation of MEK and Erk phosphorylation by a pathway still to be determined.

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Disclosures

The authors have no financial conflict of interest.

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