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Dual Phase Priming by IL-3 for Leukotriene C₄ Generation in Human Basophils: Difference in Characteristics Between Acute and Late Priming Effects

Katsushi Miura and Donald W. MacGlashan, Jr.

Previous studies have suggested that enhancement of mediator release from human basophils by IL-3 occurs in at least two phases, and the current studies further characterize the signaling changes that accompany these two phases of the basophil in response to IL-3. The test stimulus for these studies was anaphylatoxin split product of C component (C5a), which does not induce leukotriene C₄ release without prior IL-3 treatment. Functionally, IL-3 priming occurs after 5 min, disappears by 2 h, and returns by 18 h. In contrast, the kinetics of cytosolic phospholipase A₂ (cPLA₂) and extracellular signal-regulated kinase (ERK1/2) phosphorylation, induced by IL-3, do not show the second rise by 18 h. The kinetics of cPLA₂ and ERK1/2 phosphorylation following stimulation with C5a are the same for cells that were not treated with IL-3 as for those treated for 18 h, i.e., a lag in phosphorylation of cPLA₂ and ERK1/2 lasting 30 s before its eventual rise. Previous studies showed that a 5-min treatment with IL-3 induced little change in the C5a-induced cytosolic calcium response, while 24 h of treatment resulted in a marked and sustained cytosolic calcium elevation during the C5a-induced response. The first phase of the IL-3 priming effect (5–15 min of treatment) was unaffected by cycloheximide, while the second phase (18 h) was inhibited. These data suggest that early IL-3 priming results from preconditioning cPLA₂, i.e., causing its phosphorylation, while late priming results from a qualitative change in the cytosolic calcium response. The Journal of Immunology, 2000, 164: 3026–3034.

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stimulation of human basophils by cross-linking FceRI (high affinity IgE receptor) or agonists for G protein-coupled receptors (e.g., FMLP) elicits the release of pre-formed mediators (histamine) and de novo synthesized mediators (e.g., leukotriene C₄ (LTC₄) and IL-4) (1, 2), but the profile of mediators depends on both the particular secretagogue and the cytokine environment. For human basophils, a variety of cytokines (e.g., IL-3, IL-5, and GM-CSF) mediates up-regulation of secretion, of which IL-3 is generally the most efficacious (3–5). With the exception of preparations of basophils from certain donors, these cytokines alone are poor stimuli of basophil secretion (3–5). These cytokines are also found at sites of allergic inflammation (6–10), suggesting that they can modify allergic reactions by priming basophil secretion as well as affecting the function of other leukocytes. However, the mechanisms underlying the priming effect of cytokines such as IL-3 in basophils are only partially understood.

For a variety of studies, we have used as a model of the priming effect induced by IL-3 the change in LTC₄ release during stimulation with C5a. C5a is an excellent secretagogue for histamine release, but for basophils from most donors it does not induce LTC₄ release (11–13). A 5-min preincubation of the cells with IL-3 permits C5a to induce marked LTC₄ release (12, 13). To understand how IL-3 brings about this change, we have examined some of the signaling steps involved in generating LTC₄ in human basophils. Current evidence supports the view that cytosolic phospholipase A₂ (cPLA₂) is required for generation of the arachidonic acid (AA) used for LTC₄ synthesis (14, 15). This enzyme is activated by the combined effects of its phosphorylation (for its enzymatic activity) and an elevation in cytosolic free calcium (for its binding to phospholipid pools) (16, 17). Thus, these two signals must be present at the same time for cPLA₂ to hydrolyze phospholipids and produce AA. Previous studies have shown that C5a induces a relatively brief transient rise in the cytosolic calcium response ([Ca²⁺]), lasting about 30–45 s (12). In addition, cPLA₂ is phosphorylated, but measurable changes in its phosphorylation state are not observed for the first 30–45 s in human basophils (14). These data indicate that there is little overlap in the two signals needed for activation of cPLA₂, and this lack of overlap most likely explains the inability of C5a to induce the generation of free AA and consequently LTC₄ (14).

A short treatment of basophils with IL-3 leads to phosphorylation of cPLA₂ (14). Previous studies led us to speculate that IL-3 may induce phosphorylation of ERK1/2, the antecedent kinases most likely responsible for the phosphorylation of cPLA₂ (16, 18). We have also shown that a short treatment of basophils with IL-3 does not alter the characteristics of the [Ca²⁺] that follows stimulation with C5a (12). Consequently, it appears that the ability of IL-3 to allow C5a to initiate LTC₄ release results from its preconditioning of cPLA₂, so that the brief transient [Ca²⁺], that normally follows C5a stimulation is able to overlap with the pre-existing phosphorylated state of cPLA₂ to allow its full activity (14).
It has been known for some time that the priming effect of IL-3 is apparent after overnight (18–24 h) treatment (11). Indeed, secretion-induced LTC4 release is often greater after 24 h of IL-3 treatment than after only 5–15 min of treatment. Unpublished functional studies have indicated that the priming effect is actually biphasic. A similar conclusion could be drawn from studies of the C5a-induced [Ca2+]; as noted above, a short treatment does not alter the character of the response, while previous studies have also demonstrated a marked enhancement of the [Ca2+], characterized as a sustained elevation, occurring after 18–24 h of IL-3 incubation (11, 12). In preliminary results to be presented, we note that priming is not readily apparent after 2 h of priming, i.e., priming occurs after 5 min, disappears by 2 h, and returns by 24 h. The current studies explore the nature of the signaling events that lead to activation of cPLA2, comparing results for cells treated for 5–15 min to those after 18 h of treatment.

Materials and Methods

Materials

The following were purchased: PIPES, BSA, EGTA, EDTA, d-glucose, NaF, Na3P2O7, Na2VO4, 2-ME, Nonidet P-40, cycloheximide, and Tris-HCl (Sigma, St. Louis, MO); crystallized human serum albumin (HSA; Miles Laboratories, Elkhart, IN); PMSF and gentamicin (Life Technologies, Grand Island, NY); RPMI 1640 containing 25 mM HEPES and 1-glutamine (BioWhittaker, Walkersville, MD); Percoll (Pharmacia, Piscataway, NJ); Tris and Tween-20 (Bio-Rad, Hercules, CA); leupeptin, DTT, and PMSF (Roche, Indianapolis, IN); rabbit anti-ERK1 Ab and rabbit anti-ERK2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-phospho-ERK (mitogen-activated protein kinase) Ab, phosphorylated and nonphosphorylated recombinant ERK2 proteins, and biotinylated m.w. markers (New England Biolabs, Beverly, MA); peroxidase-conjugated donkey anti-rabbit Ig Ab (Amersham, Arlington Heights, IL); PDA98059 (Calbiochem, La Jolla, CA); recombinant human IL-3 (BioSource, Camarillo, CA); and fura-2/AM (Molecular Probes, Eugene, OR). Rabbit anti-cPLA2 Ab was provided by Dr. Lisa Marshall (SmithKline Beecham, King of Prussia, PA). Goat anti-human IgG was prepared as previously described (1).

Buffers and media

PIPER-albumin-glucose (PAG) buffer consisted of 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 0.1% glucose, and 0.003% HSA. PAGCM was PAG supplemented with 1 mM CaCl2 and 1 mM MgCl2. PAG-EDTA consisted of PAG supplemented with 4 mM EDTA. Countercurrent elutriation was conducted in PAG containing 0.25% BSA in place of 0.003% HSA.

Basophil purification

Basophils were purified from residual cells of normal donors undergoing leukapheresis using Percoll density gradient and countercurrent-flow elutriation as previously described (19, 20). Basophils and contaminating cells (lymphocytes and monocytes) that typically contaminate enriched basophil preparations expressed essentially equivalent levels of cPLA2 (14), ERK1, and ERK2 (15). In experiments involving the use of Western blot analysis of phosphorylated proteins or measurements of [Ca2+], basophil purities ranged from 80–99% to minimize the contribution from contaminating cells. The purity of basophils was determined by alcin blue staining (21).

Basophils are the major, if not sole, leukocytes to release histamine and to generate LTC4 in response to a combination of IL-3 and C5a, and IL-3 affects the basophil response directly (13). We also confirmed that basophils were the major source of LTC4 release stimulated by IL-3 (for 15 min or 18 h) plus C5a under our experimental conditions (see Results). Basophil preparations that were not as enriched as those used for phosphorylation and calcium studies were used for the experiments of histamine release and LTC4 generation (mean purity, 57 ± 6.3%; ranged from 15–96%).

Phosphorylation of ERKs and cPLA2.

The phosphorylation of ERKs was assessed using two different techniques: 1) phospho-ERK Ab (22), and 2) the electrophoretic mobility shift using anti-ERK1 and anti-ERK2 Abs (23, 24) as described previously (15). The phosphorylation of cPLA2 was assessed using the electrophoretic mobility shift with anti-cPLA2 Ab (14, 18). After basophils were incubated with or without IL-3 in RPMI 1640 containing 2% FCS and gentamicin (25 μg/ml) at 37°C in 5% CO2 incubator for the times indicated, reactions were stopped by adding ice-cold PAG (4-fold volume) and microfuged for 5–10 s. Cell pellets were immediately lysed in lysis buffer (20 mM Tris (pH 7.5), 2 mM EDTA, 2 mM EGTA, 5 mM DTT, 1 mM PMSF, 10 mM benzamidine, 100 μg/ml aprotinin, 200 μg/ml leupeptin, 50 mM NaF, 5 mM Na3P2O7, 1 mM Na2VO4, and 1% Nonidet P-40). Extracts containing equal basophil cell number (1 × 106 cells equivalents/lane) were diluted with an equal volume of 2× loading buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 0.005% bromophenol blue, and 20% glycerol; NOVEX, San Diego, CA) containing 5% 2-ME and subjected to 10% Tris glycerine gel (NOVEX). After electrophoresis (160 V and 1.5 h for detection of ERKs phosphorylation, and 160 V and 3.5 h for cPLA2 phosphorylation), Gels were then transferred to supported nitrocellulose membranes (OPTITRAN, Schleicher & Schuell, Keene, NH) with a Trans Blot (NOVEX). The membranes were immersed overnight in Tris-buffered saline/Tween 20 containing 5% nonfat dry skim milk (Carnation, Los Angeles, CA). Immunoreactive proteins were detected using anti-phospho-ERK Ab, anti-ERK1 Ab, anti-ERK2 Ab, or anti-cPLA2 Ab, which were diluted in Tris-buffered saline/Tween 20 containing 1% skim milk for 4 h. After washing, the membranes were incubated for 1 h with HRP-conjugated anti-rabbit Ab. After washing, membrane-bound anti-rabbit Ig Ab was visualized with enhanced chemiluminesence Western blotting detection reagents (Amersham) and HyperECL luminescence detection film (Amersham).

LTC4 and histamine measurements

Fifty thousand basophils were stimulated in a final volume of 100 μl of RPMI 1640 containing 2% FCS and 25 μg/ml gentamicin at 37°C in 5% CO2 incubator. The reactions were terminated with 900 μl of ice-cold PAG-EDTA, and the cells were then centrifuged in PAG in a microfuge at 14,000 rpm for 10 s. RIA was performed using 100 μl of sucrose solution to determine LTC4 levels as previously described (1, 26). Supernatants (300 μl) were mixed with an equal volume of PAG buffer with 1.6% HClO4. After an overnight incubation at 4°C, the protein precipitate in each tube was pelleted by centrifugation (27), and the supernatants were assayed for histamine content by automated fluorometry (28). The percentage of total histamine release was calculated for the diluted supernatants after subtraction of spontaneous histamine release (29). Each condition tested was performed in duplicate.

Results

Biphatic characteristics of priming by IL-3

For most donor basophil preparations, C5a induces marked histamine release from human basophils with little or no free AA/LTC4 release (11–13). However, the treatment with IL-3 for 15 min or overnight (18–24 h) enhances C5a to induce a significant amount of LTC4 (11–13). The kinetic characteristics of this priming effect were examined. Basophils were treated with IL-3 for 15 min, 2 h, or 18 h before challenge with C5a. Supernatants were harvested after 20 min of challenge, and LTC4 was measured. As shown in Fig. 1A, treatment with IL-3 for 15 min significantly enhanced C5a-induced LTC4 release; this enhancement was reduced to levels similar to those observed for untreated cells by 2 h and was markedly enhanced again by 18 h. This result suggested that there are two distinct priming phases induced by IL-3. The supernatants from these experiments were also examined for histamine release. Similar to LTC4 release, but with far less contrast, treatment with IL-3 for 15 min or 18 h enhanced C5a-induced histamine release,
because C5a is a strong secretagogue for histamine release. Histamine release induced by C5a (no IL-3), IL-3 (15 min) plus C5a, IL-3 (2 h) plus C5a, and IL-3 (18 h) plus C5a was 50 ± 7%, 70 ± 6%, 58 ± 12%, and 93 ± 7%, respectively.

Kurimoto et al. (13) previously demonstrated that basophils are the major, if not sole, leukocytes to generate LTC4 in response to a combination of IL-3 (early effects) and C5a, and IL-3 affects the basophil response directly. To confirm these results under our experimental conditions we also examined LTC4 release from pure (70–96%) and impure (6%) basophil suspensions (from the same donor preparations) in response to combinations of IL-3 and C5a. From cells treated for either 15 min or 18 h, LTC4 release was proportional to the basophil purity (data not shown), indicating that basophils are the major leukocytes to generate LTC4 in response to a combination of IL-3 and C5a.

The appearance of two phases in the kinetics of enhancement suggested that the two phases resulted from two different mechanisms. We sought out other indicators that these two phases of enhancement were mechanistically different. Fig. 1 demonstrates that the concentration dependence of enhancement was different for the two phases. The ED50 for acute priming (15 min) was 0.2 ng/ml (13.3 pM), a potency similar to that required for phosphorylation of cPLA2 and to that for enhanced free AA/LTC4 generation induced by low dose ionomycin (0.1 μg/ml) (14). In contrast, the potency of IL-3 for the late phase (18 h) of enhancement was much higher (ED50 5.2 ng/ml; 133 pM).

The presence of a late priming effect that requires hours rather than minutes suggested that protein synthesis may be required for this phase. Thus, the effect of cycloheximide (protein synthesis inhibitor) on both the acute (15 min) and late (18 h) priming by IL-3 was examined. As shown in Fig. 2, cycloheximide did not alter the effects of IL-3 on the C5a-induced release of LTC4 or histamine when cells were pretreated for only 15 min. In contrast, the enhancement caused by 18 h of pretreatment with IL-3 was inhibited by cycloheximide; complete inhibition occurred at 10 μM with an ID50 of ~0.3 μM. Both the enhancement of LTC4 and histamine release were altered by the inclusion of cycloheximide, although reversal of the IL-3 effect was more complete.
when examining LTC₄ release. Cell viability was examined by the trypan blue exclusion test; cell viability was not affected by cycloheximide (results with no IL-3, with IL-3 for 18 h, and with cycloheximide plus IL-3 for 18 h were 90.3 ± 5.5, 92.8 ± 4.8, and 91.3 ± 4.3%, respectively; n = 3). These results suggested that the effects of cycloheximide might not result solely from an effect on viability. The observation that histamine release was the same in cells treated with IL-3 and cycloheximide as in cells not treated with IL-3 also suggested that degranulation pathways remained functional.

**Phosphorylation of ERKs (ERK1 and ERK2) and of cPLA₂ induced by IL-3 in human basophils**

We have previously demonstrated that human basophils expressed ERK1, ERK2, and cPLA₂ (14, 15), and that ERKs might regulate free AA for LTC₄ generation by phosphorylating cPLA₂ following stimulation with anti-IgE Ab or FMLP in human basophils (15). In addition, phosphorylation of cPLA₂ by IL-3 is associated with increased free AA and LTC₄ generation (14). However, a direct relationship between activation of ERKs and of cPLA₂ in response to IL-3 has not been reported yet. Phosphorylation of ERKs was assessed by two different techniques: a change in the electrophoretic mobility could be detected by anti-ERK1 and -2 Abs and changes in ERK phosphorylation could be detected by immunoblotting with specific anti-phospho-ERKs Ab, as previously described (15). Phosphorylated and nonphosphorylated ERK2 proteins were used as a reference. As shown in Fig. 3A, IL-3 induced phosphorylation of ERKs at 15 min, and the phosphorylation returned toward the basal level at 2 h. It was at basal levels at 18 h. The same results were obtained with the electrophoretic mobility shift assay using anti-ERK1 Ab and anti-ERK2 Ab, respectively. These immunoblottings by anti-ERK1 Ab and anti-ERK2 Ab also demonstrated essentially equal protein loading for each lane, because total protein mass from phosphorylated and nonphosphorylated forms were the same (see Fig. 3A). Coinciding with phosphorylation of ERKs, IL-3 also induced phosphorylation of cPLA₂ at 15 min, and this phosphorylation decreased toward the basal level by 2 h and remained at basal levels at 18 h. These results suggest a kinetic association between activation of ERKs and that of cPLA₂ in response to IL-3 in the context of the acute priming effect. However, the priming effect after 18 h is not explained by these temporal patterns of phosphorylation.

Previous studies noted that the full expression of the acute priming effect of IL-3 required 5 min of pretreatment (13), consistent with the time period that is required for maximum phosphorylation of cPLA₂ induced by IL-3 (14). We therefore examined the IL-3-induced phosphorylation of ERKs within this short time frame. Phosphorylation of ERKs was not detected at 1 min, but significant phosphorylation was observed from 5–15 min (data not shown). A similar kinetic pattern was observed for phosphorylation of cPLA₂ in response to IL-3 (14). These results also suggested the kinetic association of activation of ERKs with that of cPLA₂ and the acute priming effect of IL-3 for LTC₄ release.

It appears that the acute priming effect requires activation of the ERK1/2 pathway. PD98059 is thought to be a specific inhibitor of MEK1/2, the immediately antecedent kinases to the ERKs (15, 30, 31). This compound is often used to determine the involvement of MEK/ERK activation. As shown in Fig. 3B, PD98059 at 100 μM was capable of inhibiting the phosphorylation of both ERK1/2 and cPLA₂ when the cells were stimulated with IL-3, suggesting that MEK/ERK regulates phosphorylation of cPLA₂.

**Kinetics of phosphorylation of ERKs and of cPLA₂ and LTC₄ release induced by C5a after pretreatment with IL-3 (18 h)**

C5a alone induces phosphorylation of cPLA₂ (14) and ERKs (data not shown). However, the normal C5a-mediated [Ca²⁺]ᵢ response is rapid and transient and does not overlap with phosphorylation of cPLA₂ (12, 14). Previous studies have also found that an 18-h pretreatment with IL-3 enhanced the C5a-induced [Ca²⁺]ᵢ response by specifically allowing the expression of a sustained [Ca²⁺]ᵢ (11). It therefore became of interest to examine the characteristics of ERK and cPLA₂ phosphorylation following stimulation with C5a in cells that had been treated with IL-3 for 18 h. As noted above, in the presence of IL-3, phosphorylation of both ERKs and cPLA₂ had returned to resting levels by 18 h. With this as a starting point, the phosphorylation of these proteins was followed for the first 5 min following stimulation with C5a. Modest phosphorylation of ERKs induced by C5a was observed at 30 s with a maximum phosphorylation from 1–5 min (Fig. 4A). Similar
results were obtained with electrophoretic mobility shift assay using anti-ERK1 Ab and anti-ERK2 Ab. These results also confirmed essentially equal protein loading (data not shown). Phosphorylation of cPLA₂ followed phosphorylation of ERKs, with no significant change at 30 s and a maximum change from 1–5 min (Fig. 4A). A similar time course was observed for C5a-stimulated cells that had been incubated for 18 h without IL-3 (data not shown), which is similar to the pattern observed in freshly isolated cells as previously reported (14). In the cells treated with IL-3 for 18 h, LTC₄ release followed phosphorylation of cPLA₂ (Fig. 4B). It should be noted that the kinetics of LTC₄ release in these cells treated with IL-3 for 18 h were much slower (12 ± 0.5% of maximum release at 1 min and half-maximum at ~3.5 min) than the release observed in cells treated for only 15 min (12, 13), which was complete by 30–45 s. The rate of histamine release was similar for cells treated for 15 min (13) and those treated for 18 h (86 ± 5% of maximum at 30 s).

Effect of PD98059 on C5a-induced LTC₄ release after pretreatment of IL-3 for 15 min or 18 h

As shown above, PD98059 inhibits the phosphorylation of ERK1/2 and cPLA₂ in response to IL-3 (Fig. 3B). We have previously shown that it inhibits LTC₄ release induced by FMLP and anti-IgE Ab (15). Thus, the effect of PD98059 on the LTC₄ release enhanced by IL-3 (15 min or 18 h) was examined. FMLP-induced LTC₄ release was also examined as a control, because PD98059 inhibits LTC₄ release (but not histamine release) induced by FMLP (15). As shown in Fig. 5, PD98059 inhibited LTC₄ release induced by IL-3 (15 min or 18 h) plus C5a as well as that by FMLP (no IL-3 pretreatment) at comparable percent inhibition (the percent inhibitions of LTC₄ release induced with IL-3 (15 min) plus C5a, IL-3 (18 h) plus C5a and FMLP by PD98059 were 64.1 ± 6.3, 78.0 ± 2.8, and 64.5 ± 8.5%, respectively). However, histamine release induced by IL-3 (15 min or 18 h) plus C5a was not affected by this compound (percent inhibitions of histamine release induced with IL-3 (15 min) plus C5a and IL-3 (18 h) plus C5a by PD98059 were 11.5 ± 2.7 and 15 ± 2.5%, respectively; n = 3).
This result suggests that MEK-ERK activation is essential for LTC$_4$ release induced by IL-3 (15 min) plus C5a and that induced by IL-3 (18 h) plus C5a.

We considered the possibility that LTC$_4$ release stimulated by C5a in IL-3-pretreated cells might not depend on a pathway similar to FMLP in cells not treated with IL-3. As noted above, PD98059 effectively inhibited the phosphorylation of ERK1/2 and cPLA$_2$ induced by IL-3 (Fig. 3B). Because this compound requires a long preincubation (~1 h) with the cells (15, 22, 32–35) and is not effectively washed from the cells (data not shown), there was no method to isolate the actions of the compound to the C5a-mediated pathway vs its effects on the IL-3-induced pathway. For example, incubation of the cells with PD98059 for 1 h before a 15-min treatment with IL-3 and a subsequent challenge with C5a significantly inhibited LTC4 release (Fig. 5A), but we could not distinguish between its effects on the IL-3 vs C5a-mediated events in these acute priming experiments. Therefore, the effects of this compound were tested on the C5a-stimulated release of LTC$_4$ in cells first treated with IL-3 for 18 h. Cells were incubated for 18 h with IL-3, and 100 μM PD98059 was added 1 h before challenge with C5a. Under these conditions, PD98059 inhibited C5a-induced LTC$_4$ release (79.7 ± 10% inhibition compared with cells not treated with PD98059), with no effect on histamine release (n = 2). These results suggest that the effects of PD98059 on C5a-induced LTC$_4$ release from cells pretreated with IL-3 for 18 h mainly result from inhibition of C5a-induced MEK/ERK activation, but not from that induced by IL-3. Indeed, C5a-induced phosphorylation of ERK and cPLA$_2$ was also inhibited by PD98059 (data not shown).

Effect of EGTA or cycloheximide on [Ca$^{2+}$]$_i$ and LTC$_4$ release induced by C5a after pretreatment of IL-3 (18 h)

For cells incubated with IL-3 for 18 h, the data suggest that the sustained [Ca$^{2+}$]$_i$ is the new element that allows C5a to induce LTC$_4$ release (as will be discussed below). The kinetic characteristics of the C5a-induced [Ca$^{2+}$]$_i$ in cells treated for 18 h with IL-3 are very similar to those of the natural [Ca$^{2+}$]$_i$ in cells stimulated with FMLP (with sustained [Ca$^{2+}$]$_i$) (11). If EGTA is added with FMLP, the sustained phase of [Ca$^{2+}$]$_i$, (that dependent on calcium influx) is ablated, and LTC$_4$ release is effectively inhibited, while histamine release remains largely intact (11). However, these characteristics are different from those of 15-min priming of IL-3 and stimulation by C5a. C5a-induced LTC$_4$ release after pretreatment with IL-3 (15 min) are not affected by EGTA (12). We therefore examined the effect of EGTA on LTC$_4$ release in cells treated with IL-3 for 18 h and stimulated with C5a. EGTA (2 mM) was simultaneously added with C5a, as performed previously (12). The inclusion of EGTA completely inhibited LTC$_4$ release (LTC$_4$ releases without and with EGTA were 79 ± 50 and 4.5 ± 3.3 pmol/10$^6$ basophils, respectively; 95 ± 1% inhibition by EGTA; n = 2). As expected, the C5a-induced sustained phase of [Ca$^{2+}$]$_i$, was also inhibited by EGTA, although the initial peak of [Ca$^{2+}$]$_i$, was not affected (Fig. 6). Histamine release induced by C5a was only marginally affected by EGTA (percent histamine release without and with EGTA were 97 ± 4 and 80 ± 2%, respectively; n = 2).
We then examined the effect of cycloheximide on the sustained C5a-induced $[Ca^{2+}]_i$ after pretreatment with IL-3 for 18 h. Consistent with results for LTC4 release (Fig. 2), the sustained C5a-induced $[Ca^{2+}]_i$ after pretreatment with IL-3 (18 h) was also inhibited by cycloheximide (Fig. 7). Taken together, these results suggest that new protein synthesis might be required for the sustained C5a-induced $[Ca^{2+}]_i$ and for the enhanced LTC4 release after treatment with IL-3 for 15 min (b) or 18 h (c) are shown.

Discussion

Priming of basophils by IL-3 for LTC4 generation appears to occur in two phases (11, 12, 14). With respect to LTC4 generation induced by C5a, the mechanisms underlying the changes can be summarized by the idealized plots shown in Fig. 8. cPLA2 requires synchrony between its phosphorylation (for enzymatic activity) and the increase in $[Ca^{2+}]_i$ (for its binding to phospholipids) for its action to liberate AA (16, 17, 36, 37). In human basophils, cPLA2 is likely to play a role in liberating AA for LTC4 synthesis (as discussed below). In the absence of IL-3, C5a induces both activation of ERK-cPLA2 and an increase in $[Ca^{2+}]_i$ (12, 14). However, the C5a-induced $[Ca^{2+}]_i$ response is transient (<30–45 s) (12) on a time scale that does not overlap with phosphorylation of cPLA2 (no phosphorylation <30 s) (14), as portrayed in Fig. 8A. Because the translocation (or the binding) of cPLA2 to the membrane is dependent on the level in $[Ca^{2+}]_i$ (16, 17, 36, 37), the transient C5a-induced association of cPLA2 to the membranes may be dissociated (due to the transient increase in $[Ca^{2+}]_i$) before phosphorylation of cPLA2. This separation in the times between these two events explains the inability of C5a to induce free AA/LTC4 generation (14). Although both FMLP and C5a are pertussis toxin-sensitive stimuli in human basophils (38), they differ in their characteristic $[Ca^{2+}]_i$ responses (11, 12). In the absence of IL-3 treatment, the FMLP-induced $[Ca^{2+}]_i$ is sustained and overlaps with the increase in cPLA2 phosphorylation (14). We postulate that the overlap in the two events allows LTC4 release to follow stimulation with FMLP, but not that with C5a. For basophils pretreated with IL-3 for 5–15 min, synchrony between the two events is adjusted by the fact that IL-3 induces phosphorylation of cPLA2, so that the brief transient of $[Ca^{2+}]_i$ follows C5a overlaps in time with a phosphorylated cPLA2, allowing free AA/LTC4 generation (14) (Fig. 8Bb). The generation of free AA/LTC4 is rapid and complete within 45 s, which is consistent with the nature of a transient increase in $[Ca^{2+}]_i$ following C5a stimulation (it returns to the basal level within 45 s) (12, 13). In the current studies we have shown that the overlap in the two events was also adjusted by treatment of IL-3 for 18 h, but by a different mechanism. After treatment with IL-3 for 18 h, phosphorylation of cPLA2 had returned to basal levels (as did ERK phosphorylation; Fig. 3A). However, this treatment resulted in C5a inducing a sustained $[Ca^{2+}]_i$, while the time course of cPLA2 phosphorylation induced by C5a (Fig. 4A) was similar to that of cells not treated with IL-3 (14). The overlap in the two events occurred during the sustained rise in $[Ca^{2+}]_i$, much like the response to FMLP in the absence of IL-3. Indeed, eliminating the second phase of the $[Ca^{2+}]_i$, which appears to result from an influx of extracellular calcium, by inclusion of EGTA with C5a (Fig. 6), ablates LTC4 release in a manner similar to that found for FMLP (11). The apparent dependence of LTC4 secretion (and free AA generation, as shown previously (14)) on the synchrony between these two events lends further support to the conclusion that it is the activity of cPLA2 in human basophils that is required for generating the free AA used for LTC4 synthesis.
We have previously shown that the activation of ERKs follows stimulation of basophils with FMLP or anti-IgE Ab and appears to be responsible for free AA/LTC₄ generation by phosphorylating cPLA₂, because PD98059 inhibits ERK and cPLA₂ phosphorylation and selectively inhibits LTC₄ release, but not histamine release or IL-4 production, in human basophils (15). A variety of studies in other cell models also suggest that ERKs are responsible for phosphorylating cPLA₂ (31, 39, 40). The current study shows that treatment by IL-3 or stimulation by C5a results in ERK phosphorylation that is inhibitable by PD98059 (Fig. 3B and data not shown). This compound also inhibited the C5a-induced LTC₄ release in IL-3-primed cells, with both acute (15 min) and late (18 h) priming (Fig. 5), suggesting that the activation of MEK/ERK is essential for LTC₄ generation induced by either acute priming plus C5a or late priming plus C5a. However, we could not clearly isolate and distinguish between two probable inhibitory events by this compound, because this compound does not wash out of the cells (see Results).

Treatment of the cells with cycloheximide also distinguished the two forms of priming. Most notable was that cycloheximide effectively inhibited the priming process that required overnight (18-h) incubation with IL-3. However, this inhibition was restricted to functional changes that were related to the late priming effects of IL-3. C5a-mediated histamine release from cells treated with cycloheximide and IL-3 was similar to release from cells not incubated with IL-3 for 18 h. Likewise, the [Ca²⁺]i, in cells treated with IL-3 in the presence of cycloheximide resembled the normal transient [Ca²⁺]i observed in cells not treated with IL-3 (Fig. 7). In contrast, cycloheximide did not inhibit priming that required only 15 min. The sensitivity to cycloheximide of LTC₄ release and the enhanced [Ca²⁺]i, induced by treatment of IL-3 for 18 h suggest that this priming process requires new protein synthesis.

The fact that both LTC₄ release and the sustained [Ca²⁺]i, are sensitive to cycloheximide (Figs. 2 and 7) and the apparent dependence of enhanced LTC₄ release on the sustained [Ca²⁺]i, (Fig. 6) suggest that one important element of late priming is a change in the regulation of cytosolic calcium levels during stimulation. Overnight (18-h) priming with IL-3 also markedly enhances [Ca²⁺]i, following anti-IgE Ab and FMLP, indicating that the alteration is a globally effective change. It is curious, however, that cells that have not been treated with IL-3 do have the means to sustain a cytosolic calcium response; both FMLP and anti-IgE Ab induce reasonably sustained [Ca²⁺]i, (11, 41). Thus, IL-3 does not specifically alter the [Ca²⁺]i, by making sustained responses possible; it appears to change the effectiveness of whatever process allows a stimulus to induce an influx of extracellular calcium. For C5a, a link to this influx process is normally deficient for reasons not yet understood. IL-3 either up-regulates some component(s) of the influx pathway, such that even C5a is capable of using it, or IL-3 also enables the linkage for C5a to use this normally present pathway. Until more is understood about the regulation of the [Ca²⁺]i, it is not possible to discriminate among various alternatives. It should also be noted that while the purity of the basophils for many of these experiments was very high, and we did not detect a dependence of the priming effects on basophil purity, it remains possible that priming could result from an indirect effect of IL-3 on the remaining contaminating cells in these preparations.

In conclusion, the mechanistic characteristics of late priming by IL-3 differ from those of the acute effect. The acute effect by IL-3, but not the late effect, is associated with ERK-cPLA₂ activation induced by IL-3. However, the late effect is related to an enhancement of the C5a-induced [Ca²⁺]i, response, and the late priming process appears to require protein synthesis.


