Capacity for repeatable leukotriene generation after transient stimulation of mast cells and macrophages

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Leukotriene (LT) synthesis is initiated by the enzyme 5-lipoxygenase (5-LO). Prolonged cell stimulation causes the translocation of 5-LO to the nuclear envelope and the synthesis of LT, with subsequent inactivation and persistent membrane association of 5-LO. In this study, we examined whether persistent membrane association of 5-LO, as well as the inactivation of 5-LO, could be prevented by shortening the length of cell stimulation or by blocking LT synthesis. As expected, stimulation of rat basophilic leukaemia (RBL) cells, a mast cell model, or alveolar macrophages (AMs) with calcium ionophore for 15 min caused 5-LO translocation, LT generation and the inactivation and persistent membrane association of 5-LO. When RBL cells or AMs instead were stimulated for 0.5–5 min, translocation of 5-LO and synthesis of LT still occurred. However, after washing and resting, the 5-LO enzyme returned to its original intracellular distribution. Furthermore these cells showed a retained capacity

for LT synthesis on subsequent re-stimulation. Similar results were obtained when cells were stimulated with either formyl peptide or zymosan, instead of ionophore. In contrast, blockade of LT synthesis during the initial stimulation, with the selective inhibitors zileuton or MK-886, did not inhibit 5-LO translocation, inactivation or persistent membrane association resulting from prolonged cell stimulation. We conclude that, in long-lived immune cells, 5-LO translocation is reversible when cell stimulation is short, but persistent after prolonged stimulation. In addition 5-LO remains active and LT synthetic capacity is retained after transient stimulation, whereas significant inactivation of 5-LO occurs after prolonged stimulation. Finally, results with LT synthesis inhibitors indicate that inactivation and persistent membrane association of 5-LO can result independently of 5-LO activation.

INTRODUCTION

Leukotrienes (LTs) are lipid mediators with important roles in normal immune responses and inflammatory disease states [1,2]. For example, LTB₄ acts both to attract leucocytes to sites of inflammation and to activate them for host defence. The cysteinyl LTs, including LTC₄, LTD₄ and LTE₄, cause oedema and contraction of smooth muscle and thus might have central roles in a variety of diseases including asthma. LTs are synthesized from arachidonic acid (AA) in a process initiated by the enzyme 5-lipoxygenase (5-LO) [3]. On cell stimulation, 5-LO translocates to the nuclear envelope and endoplasmic reticulum [4,5], where it interacts with the 5-lipoxygenase-activating protein (FLAP) to begin LT synthesis. The ensuing LT secretion can be as rapid as the release of preformed molecules like histamine (see, for example, [6]) and is much faster than the release of mediators whose synthesis requires transcription and translation *de novo*, such as peptide mediators. Thus the immediate response to an insult can be rapid granule release with concomitant LT generation.

At mucosal surfaces such as pulmonary alveoli and airways as well as the gut, which are constantly exposed to particles, antigens and microbial pathogens, long-lived immune cells such as mast cells and macrophages can be frequently stimulated. In this setting, the ideal defence system would be able to respond both rapidly and repeatedly. Although the LT pathway can respond rapidly, its capacity for repeated activation is unclear. The critical rate-limiting enzyme, 5-LO, is 'suicide-inactivated',

as it is inactivated by end products [7–9]. If inactivation of 5-LO were an inevitable consequence of cell stimulation, then immune effector cells in the airways would respond rapidly to an initial insult by releasing preformed mediators and LTs, but these cells would then be refractory to re-stimulation.

A different picture of LT action might emerge if cell stimulation did not inevitably cause 5-LO inactivation. If, instead, 5-LO inactivation was a function of the length or type of cell stimulation, then a second burst of LT generation on re-stimulation could occur in certain cases. Supporting this possibility, the translocation of 5-LO has been shown to be reversible after stimulation with antigen, but not with calcium ionophore, in mast cells [10]. This suggests that inactivation of 5-LO might result from only certain types of stimuli in these cells. In another study the pretreatment of neutrophils with zileuton, a specific, reversible 5-LO inhibitor, blocked both AA processing and 5-LO inactivation, but not translocation, on stimulation [11]. Under these experimental circumstances, therefore, inactivation of 5-LO could be avoided by pharmacologically blocking AA processing. However, the factors that determine inactivation, such as length of stimulation, type of stimulus or intracellular pathway of activation, remain uncharacterized.

The purpose of this study was to evaluate the effect of the duration of an initial stimulation on the capacity to generate LTs on subsequent re-stimulation. We focused on the pivotal enzyme 5-LO, and in particular on the relationships between its translocation, its processing of AA and the subsequent loss of LT synthetic capacity. We used the rat basophilic leukaemia (RBL)

Abbreviations used: AA, arachidonic acid; AM, alveolar macrophage; FLAP, 5-lipoxygenase-activating protein; IPF, idiopathic pulmonary fibrosis; 5-LO, 5-lipoxygenase; LT, leukotriene; α MEM, minimal essential medium; RBL, rat basophilic leukaemia.

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cell line (a mast cell model) and alveolar macrophages (AMs), two cell types that reside at mucosal surfaces and have high LT synthetic capacities.

EXPERIMENTAL

Cells

RBL cells (RBL-1, American Type Culture Collection) were cultured in minimal essential medium (α -MEM; Gibco BRL) supplemented with 10% (v/v) heat-inactivated fetal calf serum and penicillin/streptomycin, fed 2 days later and used on the third day as described previously [12]. AMs were obtained from pathogen-free female Wistar rats (Charles River Laboratories) by bronchoalveolar lavage as described [13], washed with PBS and used immediately. Differential counts by microscopic examination indicated that lavaged preparations were predominantly (more than 95%) macrophages. Cell viability was routinely examined by Trypan Blue exclusion and found to be more than 95%.

Cell stimulation

PBS-washed cells were resuspended in α -MEM at 10^6 cells/ml and stimulated by the addition of an equal volume of α -MEM containing the calcium ionophore A23187 (2 μ M). After varying lengths of time, this first stimulation was stopped by the addition of EGTA (2 μ M final concentration). Cells were pelleted and resuspended in Ca^{2+} -free PBS and rested for 30 min at 37 °C. Cells were then resuspended in α -MEM and re-stimulated with α -MEM containing A23187 (1 μ M final concentration) with or without AA (5 μ M final concentration). In some experiments, cells were initially stimulated with the particulate agonist zymosan (1 mg/ml), washed and rested, then re-stimulated with either zymosan or with formyl peptide (*N*-formyl-Met-Leu-Phe, 1 μ M) plus cytochalasin b (5 μ g/ml) for 15 min. In other experiments, cells were pretreated with the FLAP inhibitor MK-886 (1 μ M) or the 5-LO inhibitor zileuton (1 μ M), both of which effectively inhibited LT generation, for 15 min before and during the first stimulation; inhibitors were not included in the rest period or second stimulation.

Indirect immunofluorescent microscopy

Immediately after the first stimulation had been stopped with EGTA, or after washing and resting in PBS, aliquots of cells were fixed in methanol at -20 °C, mounted on slides by cytospin (500 rev./min, 1 min), permeabilized in acetone at -20 °C and then air-dried [5]. Cells were rehydrated and blocked with 1% (w/v) BSA in PBS containing non-immune goat serum. Rabbit polyclonal antibody raised against purified human leucocyte 5-LO (a gift from Dr. J. Evans, Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada) [4] was prepared in 1% (w/v) BSA/PBS (titre 1:150) and applied for 1 h at 37 °C. Mounts were washed with 1% (w/v) BSA/PBS incubated with rhodamine-conjugated goat anti-rabbit antibody (1:200; Sigma) for 1 h, 37 °C, then washed extensively and covered with a coverslip. Similar results were obtained if cells were instead fixed with 2% (w/v) paraformaldehyde for 30 min at 22 °C and permeabilized with 0.1% (v/v) Tween-20 in 3% (w/v) BSA/PBS. Fluorescence was detected with a Nikon Labophot 2 microscope equipped for epifluorescence.

Measurement of LT production by enzyme immunoassay

Enzyme immunoassay (Cayman Chemical), performed in accordance with the supplier's instruction, was used to quantify

immunoreactive LTB_4 and LTC_4 in conditioned medium from unlabelled cells. Average values of duplicate determinations were obtained for each experiment.

Uptake and release of radiolabelled AA

RBL cells were incubated in α -MEM medium with [5,6,8,9- 11,12,14,15 - ^3H]AA (approx. 4×10^5 d.p.m.; specific radioactivity 100 mCi/mmol; Dupont–New England Nuclear) during the 0.5 h rest period, at 37 °C. Incorporation of labelled AA was determined by scraping washed cells into methanol and scintillation counting. In parallel wells, prelabelled cells were washed with PBS and activated in serum-free medium containing 0.1% BSA (to trap released AA and AA metabolites [14]) with 1 μ M A23187 at 37 °C for 15 min. Release of radiolabel was quantified by scintillation counting the conditioned media [15] and expressed as a percentage of the incorporated radioactivity.

Statistical analyses

Statistical significance was evaluated by Student's *t* test, with *P* < 0.05 as indicative of statistical significance.

RESULTS

Transient stimulation and 5-LO translocation

Resting RBL cells stained for 5-LO were characterized by bright heterogeneous staining within the nucleus, with a less intense homogeneous staining across the cytoplasm (Figure 1A), as described previously [5]. Unstimulated RBL cells washed and rested in PBS for 30 min showed a comparable distribution (Figure 1B). Cells stimulated for 0.5 min (Figure 1C) to 5 min (Figure 1E) displayed translocation (i.e. loss) of the cytosolic pool as well as a change in the nuclear pool to diffuse and homogeneous. Both LTB_4 and LTC_4 were also detectable in conditioned media after these time points (results not shown). After washing and resting of the cells, the distribution of 5-LO in these transiently stimulated cells consistently resembled that seen in unstimulated cells (Figures 1D and 1F), with the bright, granular nuclear staining and the diffuse cytosolic fluorescence evident in preparations such as that presented in Figures 1(A) and 1(B). In addition to the elaboration of LTs, the decreased cytosolic volume, consistent with degranulation, supported the view that these cells had been previously activated. Approx. 10% of the cells stimulated initially for 5 min, then washed and rested, had the diffuse nuclear 5-LO distribution of activated cells combined with the conspicuous cytosolic pattern of resting cells (arrow, Figure 1F), indicating a return of the cytosolic 5-LO, but not the nuclear 5-LO, to the resting locale. The return of 5-LO to the cytoplasm was rapid, evident within 0.5 min of removal of the stimulus (results not shown).

Cells initially activated for 10 min or longer were characterized by a pronounced accumulation of 5-LO at the nuclear envelope (Figures 1G and 1I); staining was also evident at the perinuclear endoplasmic reticulum but not within the cytoplasm. Resting for 30 min after an initial 10 min of stimulation did not affect 5-LO distribution (Figure 1H); resting after a 15 min stimulation seemed to enhance the localization of 5-LO at the nuclear envelope and endoplasmic reticulum (Figure 1J). These results indicate that, in RBL cells, translocation of 5-LO can be reversible after a short stimulation (i.e. 5 min or less), whereas translocation can result in persistent membrane association after prolonged cell stimulation (10 min or more).

Similar results were obtained with rat AMs (Figure 2). In resting AMs, 5-LO stained heterogeneously within the nucleus

Figure 1 Localization, by indirect immunofluorescent microscopy, of 5-LO in RBL cells after stimulation with A23187 and after washing and resting for 30 min

RBL cells were stimulated with $1 \mu\text{M}$ A23187 for the indicated times, then washed and rested in Ca^{2+} -free PBS for 30 min. Cells were fixed either after stimulation (left micrographs) or after resting (right micrographs), mounted on slides and stained for 5-LO. The arrow in (F) points to a cell showing diffuse, homogeneous staining of both nucleus and cytosol as well as staining at the nuclear envelope. Scale bar, $10 \mu\text{m}$ for all panels. Images are representative of at least five independent experiments.

and diffusely in the cytoplasm, with the nucleus being much brighter (Figure 2A), as in resting RBL cells. On activation (Figures 2C and 2E), only the nuclear pool changed in distribution, as described previously [5,16]. Initially, nuclear 5-LO changed to a diffuse distribution, evident in seconds (results not shown) and persisting to 5 min (Figure 2C); this progressed to a more pronounced localization at the nuclear envelope at later time points (Figure 2E). LTB_4 release into the medium was evident as early as 0.5 min (results not shown). As in RBL cells,

Figure 2 Localization, by indirect immunofluorescent microscopy, of 5-LO in AMs after stimulation with A23187 and after washing and resting for 30 min

AMs were stimulated with $1 \mu\text{M}$ A23187 for the indicated times (left panels), subsequently washed and rested in Ca^{2+} -free PBS for 30 min (right panels), then fixed and stained for 5-LO. Scale bar, $10 \mu\text{m}$ for all panels. Images are representative of at least three independent experiments.

translocation was reversible after a short stimulation (Figure 2D) but not after prolonged cell stimulation (Figure 2F).

Relationship between 5-LO translocation and inactivation

RBL cells, like mast cells, generate both LTB_4 and LTC_4 . When RBL cells were initially stimulated for only 1 or 2 min and then rested, they retained their capacity to generate both LTB_4 and LTC_4 on re-stimulation (Figure 3A). In case the initial stimulation resulted in depletion of the endogenous pool of AA (the substrate for 5-LO), all experiments were performed with $5 \mu\text{M}$ exogenous AA added with the agonist during the second stimulation. As noted above (Figure 1), when RBL cells were initially stimulated for 10 or 15 min then washed and rested, 5-LO remained associated with the nuclear envelope. The ability of these cells to make either LTB_4 or LTC_4 on re-stimulation was significantly impaired, even though cells were supplied with exogenous AA (Figure 3A). This loss of LT synthetic capacity was not accompanied by parallel changes in capacity to either take up or release AA. Both the uptake of exogenous radiolabelled AA and the subsequent release on re-stimulation decreased with increasing length of the initial stimulation (Figure 3B); however, these changes could not account for the substantially greater loss of LT synthetic capacity. These changes, combined with a capacity to exclude Trypan Blue, indicated that the loss of LT synthetic capacity did not result from a loss of cell viability.

As observed in RBL cells, AMs showed both the retained LT synthetic capacity after transient stimulation and the diminished LT synthetic capacity after a prolonged initial stimulation (Figure

Figure 3 Effect of length of initial cell stimulation on subsequent AA processing and LT synthesis on re-stimulation in RBL cells

(A) LT synthesis. Cells were stimulated for the indicated lengths of time, washed and rested for 30 min, then re-stimulated with 1 μ M A23187 with AA (5 μ M) for 15 min at 37 °C. Results are means $\pm$ S.E.M. for one experiment and are representative of four independent experiments. *Statistically different from no first stimulation. (B) AA processing. Cells were stimulated for the indicated lengths of time, then washed and rested in PBS containing [³H]AA for 30 min at 37 °C. Cells were then washed to remove unincorporated label and either extracted for scintillation counting to determine the uptake of AA, or stimulated with A23187 in the presence of 0.1% BSA, with radiolabel in the media measured to evaluate the release of AA and AA metabolites. Results are means $\pm$ S.E.M. for one experiment and are representative of two independent experiments.

4). This was evident when AMs were treated with ionophore at both the first and the second stimulations (Figure 4A). It was also observed when AMs were stimulated first with zymosan and secondly with either zymosan or formyl peptide, or first with formyl peptide and secondly with either formyl peptide or zymosan (Figure 4B). The loss of LT synthetic capacity after prolonged stimulation with the biological agonists was statistically significant but typically less pronounced than that produced by ionophore.

Like ionophore, zymosan and formyl peptide each caused translocation of the intranuclear pool of 5-LO, but not the cytosolic pool, to the nuclear envelope of AMs (Figures 5A and 5B). Translocation of the intranuclear pool was evident in more than 50% of the cells, whereas the remaining cells showed no apparent translocation of 5-LO. This can be compared with the response to ionophore, which caused translocation of the intranuclear pool in virtually all cells. Washing and resting AMs after treatment with formyl peptide for 5 min resulted in a return of the intranuclear 5-LO in essentially all cells (Figure 5C). Washing and resting AMs after treatment with formyl peptide for 15 min resulted in persistent nuclear localization of 5-LO being evident in approximately one-half of all cells (Figure 5D); cells treated

Figure 4 Effect of transient stimulation on LTB₄ synthetic capacity in AMs

(A) Stimulation with ionophore. Cells were initially stimulated with 1 μ M A23187 for the times indicated, washed and rested for 30 min, then re-stimulated with 1 μ M A23187 with AA (5 μ M) for 15 min. (B) Receptor-mediated stimulation. Cells were initially stimulated with either zymosan (1 mg/ml; $\square$, $\blacksquare$) or formyl peptide (1 μ M; $\circ$, $\bullet$) with cytochalasin b (5 μ g/ml) for the times indicated, washed and rested for 30 min, then re-stimulated with AA (5 μ M) and either zymosan ($\circ$, $\square$) or formyl peptide with cytochalasin b ($\bullet$, $\blacksquare$) for 15 min. Results are presented as percentages of product generated on re-stimulation of cells given no first stimulation (2896 ± 296 and 4781 ± 385 pg/ml for formyl peptide and zymosan respectively). Results are means $\pm$ S.E.M. for one experiment and are representative of two independent experiments. *Statistically different from no first stimulation.

with zymosan for this period could not be evaluated because of the fluorescence from persistently bound zymosan particles.

Relationship between 5-LO action and inactivation

The hypothesis that prolonged 5-LO processing of AA causes inactivation of 5-LO was tested directly with zileuton, a specific, reversible inhibitor of 5-LO, in the protocol outlined above. When RBL cells were initially stimulated in the presence of zileuton (1 μ M), few or no detectable LTs were generated, regardless of the length of stimulation (results not shown). The capacity to make LTB₄ on re-stimulation, in the presence of exogenous AA but without zileuton, was not affected by the presence or absence of zileuton during the initial stimulation (Table 1), consistent with zileuton's action being reversible. Both the enhancement of LTB₄ production in transiently stimulated cells (seen best at 0.5 min) and the loss of LTB₄ synthetic capacity after prolonged stimulation were evident when 5-LO action was originally blocked by zileuton. As reported previously [11], cells stimulated for 5 min in the presence of zileuton retained some 5-LO activity, although we found that it was diminished relative to controls. These results indicate that 5-LO action is not necessary for 5-LO inactivation.

Figure 5 Translocation of nuclear 5-LO in AMs after receptor-mediated cell stimulation

Cells were stimulated with (A) formyl peptide ($1 \mu\text{M}$) for 5 min, (B) zymosan (1 mg/ml), (C) formyl peptide for 5 min, washed and rested for 30 min, or (D) formyl peptide for 15 min, washed and rested for 30 min. Cells treated with formyl peptide were also given cytochalasin b ($5 \mu\text{g/ml}$). After treatments, cells were fixed, permeabilized and stained for 5-LO. Images are representative of two independent experiments.

Table 1 Effect of inhibition of 5-LO action on loss of LT synthetic capacity

RBL cells were initially stimulated with $1 \mu\text{M}$ A23187 for the times indicated, with or without zileuton ($1 \mu\text{M}$), washed and rested in PBS without zileuton, then restimulated for 15 min with $1 \mu\text{M}$ A23187 with $10 \mu\text{M}$ AA in medium without zileuton. LTB₄ secreted into the medium was measured by enzyme immunoassay. Results are means $\pm$ S.E.M. for one experiment and are representative of three independent experiments. *Statistically different from no first stimulation.

Length of first stimulation (min)	[LTB ₄] (pg/ml)	
	Control	Zileuton
0	1563 $\pm$ 124	1563 $\pm$ 124
0.5	3260 $\pm$ 348*	4382 $\pm$ 421*
2.0	2339 $\pm$ 203	1366 $\pm$ 118
5.0	1141 $\pm$ 120	487 $\pm$ 133*
15.0	166 $\pm$ 35*	237 $\pm$ 75*

Relationship between 5-LO action and persistent membrane association

Although 5-LO became inactivated even when inhibited by zileuton, it was not clear whether translocation had occurred on cell stimulation or whether inactivation could be linked with nuclear envelope localization. To address this, RBL cells were pretreated with or without either zileuton or MK-886 (an irreversible inhibitor of FLAP). Analysis of media verified that A23187 alone induced LT synthesis and that the inhibitors blocked LT synthesis by more than 95% (results not shown). Neither zileuton nor MK-886 blocked translocation of 5-LO to

Figure 6 Effect of inhibition of 5-LO action on translocation of 5-LO and redistribution after removal of the stimulus

RBL cells were pretreated with or without zileuton ($1 \mu\text{M}$) or MK-886 ($1 \mu\text{M}$) for 15 min, then stimulated (with or without inhibitor) with $1 \mu\text{M}$ A23187 for 15 min. Cells were either fixed and stained for 5-LO (left panels) or washed and rested for 30 min and then fixed and stained (right panels). The arrow in (C) points to a binucleate cell, which shows a translocation of 5-LO qualitatively identical with that seen in the mononucleate cell in the same frame. Scale bar, $10 \mu\text{m}$ for all panels. Images are representative of three independent experiments.

the nuclear envelope when RBL cells were stimulated with $1 \mu\text{M}$ A23187 for 15 min (Figures 6A, 6C and 6E). This result is consistent with the reported failure of MK-886 to block ionophore-induced 5-LO translocation in AMs, as evaluated by immunoelectron microscopy [16]. After washing and resting, 5-LO remained membrane-associated in all conditions tested (Figures 6B, 6D and 6F). These results indicate that, like inactivation, both translocation and persistent membrane association of 5-LO are not affected by 5-LO inhibitors.

DISCUSSION

The results of this study extend our understanding of the regulation of 5-LO action in LT synthesis. Specifically, the results indicate that (1) translocation of 5-LO to the nuclear envelope is readily reversible when cell stimulation is transient, but persistent when the stimulation is prolonged; (2) after transient stimulation of cells, 5-LO remains active and LT synthetic capacity on re-stimulation is maintained or actually enhanced, whereas significant inactivation of 5-LO, as judged by loss of LT synthetic capacity, occurs only after prolonged cell stimulation; and (3) inhibition of 5-LO's processing of AA, particularly by the specific reversible inhibitor zileuton, does not interfere with translocation, inactivation or persistent membrane association of 5-LO.

Perhaps the most physiologically important of these findings relates to the effect of short-term transient stimulation on LT synthetic capacity. Long-lived cells such as mast cells and macrophages can be stimulated repeatedly. If initial stimulation resulted in 5-LO inactivation, then this first line of defence would

be compromised until new 5-LO enzyme could be supplied. Instead, the opposite occurs. In this study both RBL cells and AMs retained full LT synthetic capacity after a short stimulation. This retained capacity for LT synthesis was evident after treatment with ionophore or either of the biological agonists zymosan or formyl peptide. The return of 5-LO from the nuclear envelope to its resting distribution was correlated with the retained LT synthetic capacity and gave the impression that the leucocytes were 'resetting' for LT generation.

A very different situation was observed after prolonged stimulation, which resulted in a diminished ability to elaborate LTs on re-stimulation. This effect was less pronounced in AMs than in RBL cells, and less so in response to biological agonists than to ionophore. It is perhaps noteworthy that, in AMs, the cytosolic pool of 5-LO does not translocate to the nuclear envelope (Figures 2 and 5) [5,16], whereas both nuclear and cytosolic 5-LO translocates in RBL cells (Figure 1) [5]. If membrane association is necessary for the inactivation of 5-LO, perhaps it is the non-translocating cytosolic pool of 5-LO in AMs that avoids inactivation and remains to generate LTB_4 even after prolonged cell stimulation. Further, the biological agonists do not cause the translocation of 5-LO in all AMs, whereas ionophore does. Cells that fail to show translocation during the first stimulation with the biological agonists might remain able to respond to the subsequent stimulation.

In this study, persistent membrane association of 5-LO resulted from prolonged cell stimulation. Persistent membrane association of 5-LO has also been reported in certain chronic disease states and might reflect an immediate past history of prolonged cell stimulation. An example of this is idiopathic pulmonary fibrosis (IPF). We recently reported that homogenates of lung samples from patients with IPF have higher LT levels than do homogenates of lung samples from control patients [17]. In that study, 5-LO was found by immunohistochemistry to be persistently localized to the nuclear envelope of numerous AMs in lung sections from IPF patients. The abnormally high proportion of cells showing membrane-associated 5-LO indicates a condition characterized by prolonged cell activation. Similar findings have also been reported for irreversible reperfusion injury in ischaemic brain [18]. The use of immunohistochemistry to identify cell types in which 5-LO has been persistently activated therefore has promise in the investigation of pathophysiological conditions. The current results add a new dimension to our ability to interpret these results: leucocytes demonstrating a 'resting' distribution of 5-LO might truly be resting, or they might have been recently stimulated for a short period. Leucocytes that seem to be 'activated' because 5-LO is associated with the nuclear envelope might indeed be activated, or they might have previously been persistently activated and are no longer capable of generating LTs on re-stimulation.

Zileuton is representative of a group of pharmacological compounds that block 5-LO enzymic activity. We had hypothesized that if enzymic action were necessary for subsequent enzyme inactivation, then zileuton might ultimately block inactivation. In fact, zileuton has been previously reported to allow the persistent membrane association of 5-LO with retained enzymic activity in neutrophils stimulated with calcium ionophore for 5 min [11]. We found similar results with zileuton-treated RBL cells when a 5 min stimulation time was employed. However, with longer stimulations, zileuton treatment did not block the inactivation of 5-LO. 5-LO inactivation, then, can occur in the presence of zileuton and this might enhance the efficacy of the drug.

The mechanism of inactivation of 5-LO remains unclear; 5-LO is recognized as being inactivated by its own action [7–9].

Our results indicate that 5-LO activity can be completely lost even if zileuton is used to prevent 5-LO from processing AA. Only persistent association with the nuclear envelope of activated cells was correlated with 5-LO inactivation. However, it is not clear whether persistent membrane association leads to inactivation or vice versa. On the one hand, AMs, which do not show membrane association of the cytosolic 5-LO pool, also do not show complete 5-LO inactivation, suggesting that membrane association might mediate inactivation. Jakschik and co-workers [10] theorized that the inactivation of 5-LO resulted from excessive intracellular Ca^{2+} during ionophore treatment, given that high levels of Ca^{2+} can inactivate 5-LO *in vitro* [19]. Membrane association of 5-LO is known to be driven by Ca^{2+} [20]. Also, 5-LO can be inactivated by oxidation [20,21]. Conceivably, Ca^{2+} might force the persistent association of 5-LO with the nuclear envelope, with the inactivation of 5-LO resulting from oxidants that accumulate, independently of 5-LO action, in the nuclear envelope during prolonged cell stimulation. Alternatively, persistent membrane association might result from 5-LO inactivation, particularly if inactivation alters the 5-LO molecule to make it more hydrophobic. Membrane association might then represent a step subsequent to inactivation of 5-LO, on the way to its degradation.

In AMs, qualitatively similar results were obtained when either ionophore or physiological agonists were used. This suggests that our three central conclusions listed above can be generalized across agonists. This contrasts with the findings of Jakschik and co-workers [10], who found that ionophore, but not antigen, caused irreversible translocation of 5-LO in mast cells. These differences can be reconciled by an understanding that (1) many, but not all, cells respond during stimulation with physiological agonists, whereas essentially all cells respond to ionophore (Figures 1, 2 and 5), (2) the cell's immediate response (e.g. magnitude and length of a Ca^{2+} transient) might depend on both the dose and length of exposure to the agonist, and (3) the ultimate result (e.g. persistent 5-LO translocation or loss of LT synthetic capacity) depends on the length of the initial stimulation (Figures 1–4) and most probably also on the dose of the agonist. Thus ionophore should be able to mimic physiological agonists closely in their effect on 5-LO, particularly if the length of stimulation and the dose of ionophore used evoke comparable Ca^{2+} changes to those produced by the physiological agonists.

Aberrant LT synthesis, either overproduction or underproduction, might contribute to numerous diseases. Previously, LT generation was thought to be regulated largely by the amount of either 5-LO or FLAP protein present in the source cell. More recently, the discovery that 5-LO can occur in both the cytosol and the nucleus of resting cells [5,12,16,22] presented the possibility that compartmentation of 5-LO can regulate LT synthetic capacity. This study underlines the importance of other processes, including translocation, inactivation and persistent membrane association, in 5-LO action. Conceivably, an error in any of these processes could also lead to aberrant LT synthesis and contribute to different disease processes.

In summary, the present investigation shows that the loss of LT synthetic capacity can be a function of the length of stimulation. This work suggests that, depending on the length or type of initial stimulation, LT synthetic capacity can be abolished, maintained or even enhanced. The capacity for rapid, repeatable responses distinguishes the LT response pathway from other response pathways utilizing preformed molecules (which lack repeatability) or peptide mediators (which lack rapidity).

This work was supported by National Heart, Lung and Blood Institute Grant R01 HL47391, National Institutes of Health Training Grant T32 HL07749 and Specialized

Center of Research Grant P50 HL46487 to M.P.G., as well as an American Lung Association Award to T.G.B., who is a Parker B. Francis Fellow in Pulmonary Research, an Edward Livingston Trudeau Scholar of the American Lung Association, and Amgen's Research Grant Awardee.

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Received 7 August 1997; accepted 17 September 1997