

Targeting Ca^{2+} release-activated Ca^{2+} channel channels and leukotriene receptors provides a novel combination strategy for treating nasal polyposis

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Background: Nasal polyposis is a chronic inflammatory disease of the upper respiratory tract that affects around 2% of the population and almost 67% of patients with aspirin-intolerant asthma. Polyps are rich in mast cells and eosinophils, resulting in high levels of the proinflammatory cysteinyl leukotrienes.

Objectives: To better understand the role of the proinflammatory leukotrienes in nasal polyposis, we asked the following questions: (1) How do nasal polyps produce leukotriene C₄ (LTC₄)? (2) Can LTC₄ feed back in a paracrine way to maintain mast cell activation? (3) Could a combination therapy targeting the elements of this feed-forward loop provide a novel therapy for allergic disease?

Methods: We have used immunohistochemistry, enzyme immunoassay, and cytoplasmic calcium ion (Ca^{2+}) imaging to address these questions on cultured and acutely isolated human mast cells from patients with polyposis.

Results: Ca^{2+} entry through store-operated Ca^{2+} release-activated Ca^{2+} (CRAC) channels in polyps produced LTC₄ in a manner dependent on protein kinase C. LTC₄ thus generated activated mast cells through cysteinyl leukotriene type I receptors. Hence Ca^{2+} influx into mast cells stimulates LTC₄ production, which then acts as a paracrine signal to activate further Ca^{2+} influx. A combination of a low concentration of both a CRAC channel blocker and a leukotriene receptor antagonist was as effective at suppressing mast cell activation as a high concentration of either antagonist alone.

Conclusion: A drug combination directed against CRAC channels and leukotriene receptor antagonist suppresses the feed-forward loop that leads to aberrant mast cell activation. Hence our results identify a new potential strategy for combating polyposis and mast cell-dependent allergies. (J Allergy Clin Immunol 2009;124:1014-21.)

Key words: Calcium channel, calcium signal, leukotrienes, cysteinyl leukotriene type I receptor, 5-lipoxygenase, nasal polyposis

Abbreviations used

Ca^{2+} :	Calcium ion
CRAC:	Ca^{2+} release-activated Ca^{2+}
IC ₅₀ :	Inhibitory concentration of 50%
La^{3+} :	Lanthanum ion
LTC ₄ :	Leukotriene C ₄
LTD ₄ :	Leukotriene D ₄
LTE ₄ :	Leukotriene E ₄

Nasal polyps arise from a chronic inflammation of the upper airways and occur in around 2% of the population. Polyposis develops mainly in adults, and symptoms include itchy eyes, sneezing, rhinorrhea, nasal itch, sleep disorders, headaches, and, more severely, the loss of sense of smell.^{1,2} Development of polyps is associated with perennial nonallergic rhinitis, asthma, aspirin intolerance, allergic fungal rhinosinusitis, and cystic fibrosis. Polyposis is particularly prominent in patients with aspirin-induced asthma, with almost two thirds of aspirin-intolerant patients having nasal polyps.³

The molecular basis of aspirin-induced nasal polyposis is not clear. Polyps are rich in mast cells and eosinophils. Mast cell-dependent signals, including histamine and cysteinyl leukotrienes, such as leukotriene C₄ (LTC₄), are present in high concentrations, as is IgE.³ There is also a significant increase in cell-surface expression of cysteinyl leukotriene type I receptors in mast cells⁴ and in expression of 5-lipoxygenase in eosinophils.⁵

LTC₄ production in mast cells is stimulated by an increase in cytoplasmic calcium ion (Ca^{2+}) concentrations^{6,7} mediated through plasmalemmal store-operated Ca^{2+} channels.⁸ In mast cells, like other immune cells, the Ca^{2+} release-activated Ca^{2+} (CRAC) channel mediates store-operated entry.⁹ Orai1 comprises at least part of the CRAC channel pore,¹⁰⁻¹² and mice lacking either Orai1 or the CRAC channel activator stromal interaction molecule 1 (STIM1) exhibit defective mast cell secretion and impaired IgE-mediated anaphylactic responses *in vivo*.^{13,14}

Ca^{2+} entry through CRAC channels in mast cells is tightly coupled to the synthesis and secretion of leukotrienes,¹⁵ and these leukotrienes then act on cysteinyl leukotriene type I receptors to activate CRAC channels in neighboring mast cells. This positive feedback cascade leads to a wave of excitation that spreads through the mast cell population.¹⁶ Because polyps contain very few blood vessels, paracrine signals, such as LTC₄, will build up and could thus result in sustained mast cell activation.

Our finding of a positive feedback cycle suggests a novel tact for treating mast cell-driven disorders like allergic rhinitis and nasal polyposis, namely combined targeting of CRAC channels and leukotriene receptors should be extremely effective in damping down sustained mast cell activation and thus the

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severity of the response. The positive feedback between CRAC channels and leukotrienes implies that modest inhibition of both CRAC channels and leukotriene type I receptors should be effective in attenuating mast cell activity. Such a combination therapy would greatly increase the therapeutic window of the drugs by reducing side effects that are manifest at higher drug concentrations.

Here we have tested this idea and find that the combination of low concentrations of a CRAC channel blocker and a leukotriene receptor antagonist is as effective as a high dose of a single inhibitor. Our results identify a new approach for treating nasal polyposis and related disorders.

METHODS

Cell culture, isolation of human mast cells, Ca^{2+} imaging, generation of mast cell supernatant, LTC_4 measurements, and immunohistochemistry were all carried out as previously reported¹⁶ and are described in detail in the Methods section of this article's Online Repository at www.jacionline.org.

RESULTS

Mast cells from patients with nasal polyposis secrete and respond to cysteinyl leukotrienes

Mast cells isolated from patients with nasal polyposis stained for 5-lipoxygenase (Fig 1). This enzyme is cytosolic at rest but migrates to the nucleus on stimulation,¹⁷ where it is activated by the nuclear protein 5-lipoxygenase-activating protein. In non-stimulated human mast cells, 5-lipoxygenase was distributed throughout the cytoplasm but largely excluded from the nucleus (Fig 1, A). However, on stimulation with thapsigargin (2 $\mu\text{mol/L}$ for 4 minutes), which inhibits the sarcoendoplasmic reticulum Ca^{2+} adenosine triphosphatase pump, leading to store depletion and subsequent opening of the plasma membrane store-operated CRAC channels, 5-lipoxygenase migrated to the nucleus (Fig 1, B). This CRAC channel-driven translocation is functionally important because it led to a significant increase in cysteinyl leukotriene generation (Fig 1, C). Because the LTC_4 enzyme immunoassay detection kit we used cannot wholly discriminate between LTC_4 , leukotriene D_4 (LTD_4), and leukotriene E_4 (LTE_4), we refer to the secreted material using the general term cysteinyl leukotriene, although experiments in Fig 2, D, suggest that the major leukotriene is LTC_4 . Stimulation with thapsigargin in the absence of external Ca^{2+} did not generate any cysteinyl leukotrienes (Fig 1, C), revealing that it is Ca^{2+} entry that drives these responses. In cultured mast cells local Ca^{2+} influx through CRAC channels stimulates 5-lipoxygenase through activation of protein kinase C.⁷ A similar mechanism is used in human polyps because treatment with the protein kinase C inhibitor G06983 suppressed LTC_4 production in response to thapsigargin (Fig 1, C).

Do human mast cells also respond to LTC_4 ? To test this, we applied LTC_4 (640 nmol/L) to Fura-2-loaded mast cells isolated from polyps. Robust Ca^{2+} signals were seen (Fig 1, D), which were suppressed by the cysteinyl leukotriene type I receptor antagonist montelukast (100 nmol/L).

Is sufficient cysteinyl leukotriene generated in a polyp to activate mast cells? To address this, we stimulated a nasal polyp with thapsigargin, collected the supernatant, and then applied this to a separate population of mast cells that had been isolated from the polyp and loaded with Fura-2. Supernatant evoked a robust

Ca^{2+} signal (14/14 cells), which was suppressed by montelukast (Fig 1, E).

Because we did not have a pure fraction of mast cells from the nasal polyps, we were concerned that a cell type other than a mast cell might be the major source of the cysteinyl leukotrienes that generate the Ca^{2+} signals seen in Fig 1, E. To ensure that human mast cells were able to produce sufficient cysteinyl leukotrienes to evoke Ca^{2+} signals, we turned to the human mast cell line HMC-1, a popular model for studying mast cell activation. Supernatant taken from a population of HMC-1 cells stimulated with thapsigargin (in the presence of external Ca^{2+}) evoked robust Ca^{2+} signals in RBL-1 cells, and these were substantially reduced by montelukast (Fig 1, F). On the other hand, supernatant taken from HMC-1 cells stimulated with thapsigargin but in the absence of external Ca^{2+} did not generate a Ca^{2+} response in the RBL-1 reporter cells (data not shown). Collectively, these results establish that Ca^{2+} influx through CRAC channels in human mast cells drives the production of cysteinyl leukotrienes, including LTC_4 , and that the leukotrienes thus generated are able to activate mast cells by generating a cytoplasmic Ca^{2+} signal.

Cysteinyl leukotrienes, acting on cysteinyl leukotriene type I receptors, are the dominant paracrine signal

In addition to secreting cysteinyl leukotrienes, mast cells and RBL-1 cells release other factors that could be involved in paracrine signaling, including ATP, histamine, serotonin, and platelet-activating factor. We have previously shown that after the response to ATP had desensitized, cells still responded to the mast cell-derived paracrine signal. Moreover, the response to the supernatant was lost after knockdown of the cysteinyl leukotriene type I receptor, leaving responses to ATP intact.¹⁶ Further evidence against a major role for ATP is shown in Fig E1 (available in this article's Online Repository at www.jacionline.org). This figure also shows that histamine, serotonin, and platelet-activating factor did not evoke Ca^{2+} signals, whereas supernatant was effective.

Although montelukast blocks cysteinyl leukotriene type I receptors, it also inhibits the ATP binding cassette (ABC) transporter responsible for LTC_4 export from the cytoplasm, albeit at higher concentrations. To determine whether this latter action could affect our findings, we measured the extent of cysteinyl leukotriene secretion to 2 $\mu\text{mol/L}$ thapsigargin in the absence and then presence of a concentration of montelukast (50 nmol/L) that fully blocked paracrine signaling. Montelukast had no inhibitory effect on cysteinyl leukotriene secretion (Fig 2, A), ruling out an action on leukotriene export.

We also examined the effects of the structurally distinct receptor antagonist MK-571 on paracrine signaling in RBL-1 cells. MK-571 blocks cysteinyl leukotriene type I but not type II receptors.^{18,19} Pre-exposure of cells to 1 $\mu\text{mol/L}$ MK-571 completely abolished the Ca^{2+} signals evoked by 160 nmol/L LTC_4 (Fig 2, B). Importantly, MK-571 also abolished Ca^{2+} signals evoked by supernatant collected from a population of cells stimulated with thapsigargin (Fig 2, C).

As pointed out earlier, our enzyme immunoassay detection system does not allow us to distinguish unequivocally between the secretion of LTC_4 , LTD_4 , and LTE_4 . LTD_4 , LTE_4 , or both could therefore be responsible for the Ca^{2+} signals, with the role of LTC_4 simply to act as a metabolic precursor. To determine

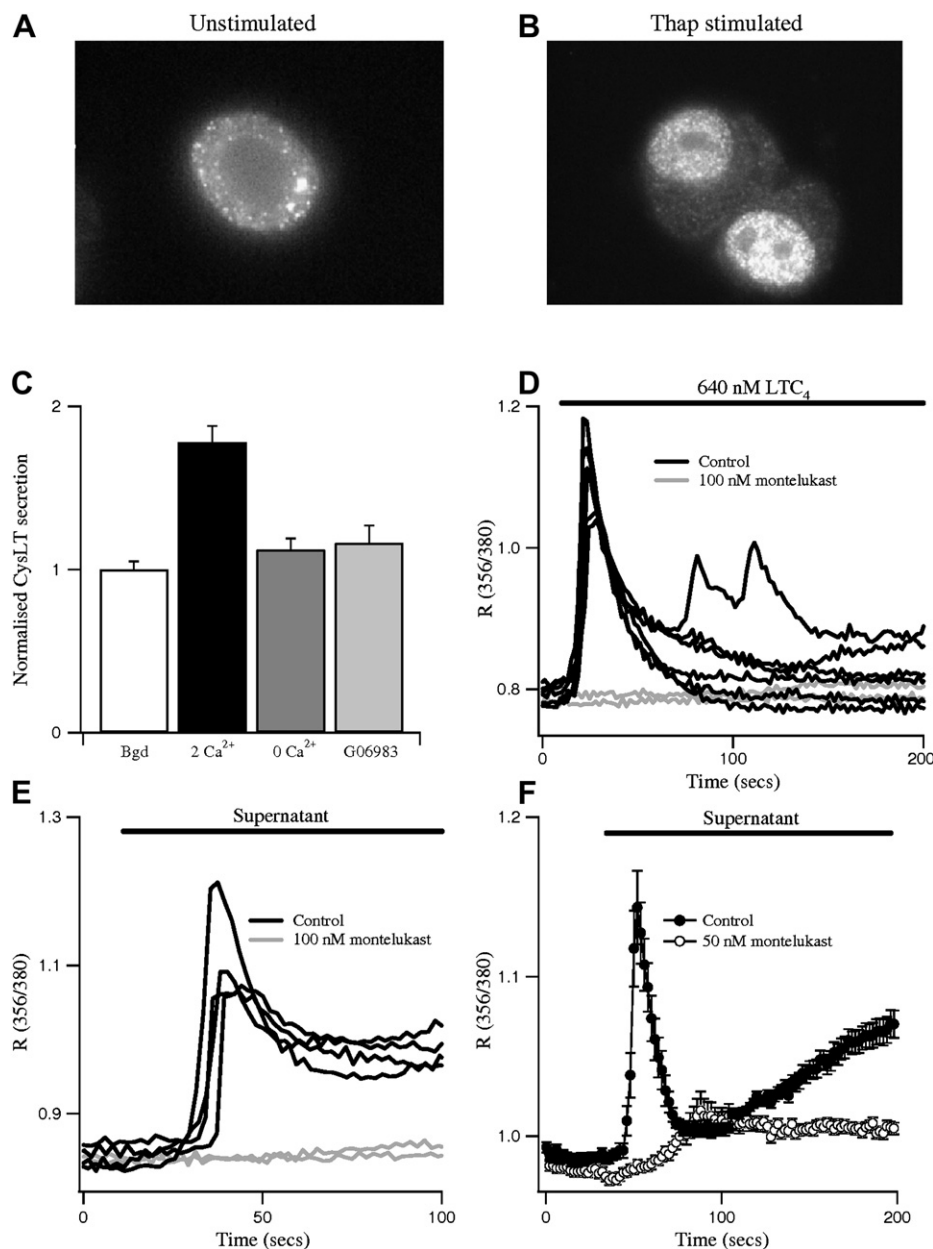


FIG 1. Nasal polyp-derived human mast cells generate and respond to leukotrienes. **A**, 5-Lipoxygenase distribution in an unstimulated human mast cell (typical of 5/5 cells). **B**, 5-Lipoxygenase migrated to the nucleus after thapsigargin exposure (6/6 cells). **C**, Thapsigargin-stimulated LTC₄ production in nasal polyps ($n = 3$ for each bar). *Bgd*, background levels (non-stimulated cells). **D**, Ca²⁺ signals to LTC₄ in human mast cells (*black lines*). **E**, Thapsigargin-stimulated polyp-derived supernatant evoked a Ca²⁺ increase in a second pool of Fura-2-loaded human mast cells (*black lines*). **F**, Supernatant from HMC-1 cells evoked Ca²⁺ responses in RBL-1 cells that were reduced by montelukast. *CysLT*, Cysteinyl leukotriene.

whether LTC₄ was effective, we blocked its conversion to LTD₄ and LTE₄ by inhibiting the enzyme γ -glutamyl transpeptidase with acivicin.²⁰ Supernatant taken from thapsigargin-stimulated RBL-1 cells pretreated with 250 μ M acivicin for 30 minutes evoked Ca²⁺ signals in Fura-2-loaded cells that were indistinguishable from those seen in corresponding controls taken in the absence of the inhibitor (Fig 2, *D*). Hence LTC₄ metabolism is not essential for paracrine signaling. It has been suggested that an additional cysteinyl leukotriene receptor might be expressed on human mast cells that is activated by LTE₄.^{21,22} To

test whether LTE₄ might contribute to paracrine signaling in our system, we applied it directly onto Fura-2-loaded RBL-1 cells. Only a transient and small Ca²⁺ signal occurred (Fig 2, *E*) that was very distinct from that evoked by supernatant (Fig 2, *C*), LTC₄ (Fig 2, *B*), or LTD₄.¹⁶ Montelukast (Fig 2, *E*) and MK-571 (data not shown) fully suppressed the response to LTE₄. These findings are consistent with our previous work that has shown that knockdown of the cysteinyl leukotriene type I receptor fully abolishes the response to the mast cell-derived paracrine signal.¹⁶

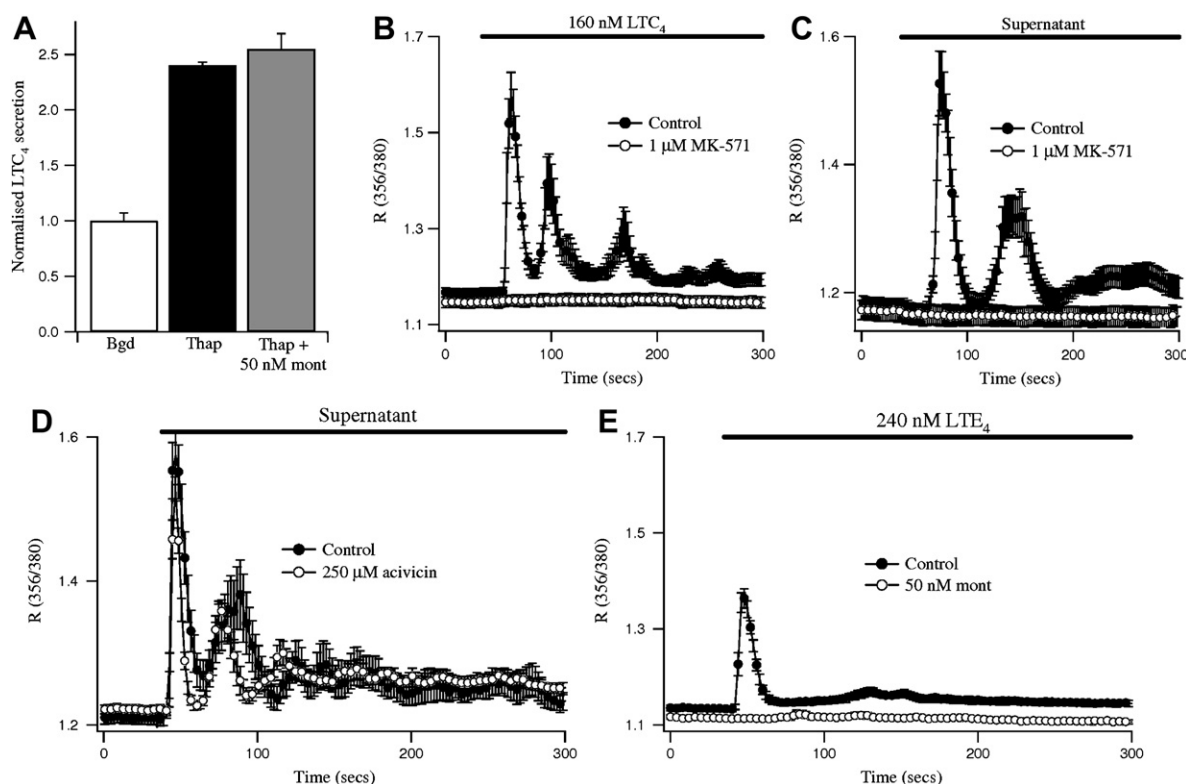


FIG 2. LTC₄ is involved in paracrine signaling. **A**, LTC₄ secretion is unaffected by montelukast. *Bgd*, background levels (non-stimulated cells); *Thap*, thapsigargin. **B**, Ca²⁺ signal to LTC₄ is suppressed by MK-571. **C**, Response to supernatant is blocked by MK-571. **D**, Acivicin has no effect on supernatant-driven responses. **E**, LTE₄ evokes a small Ca²⁺ signal that is blocked by montelukast.

Hypothesis

The presence of a feed-forward loop between CRAC channels and cysteinyl leukotrienes has important implications for therapeutic interventions aimed at managing nasal mast cell activity. Partial inhibition of CRAC channels combined with partial inhibition of cysteinyl leukotriene type I receptors should be as effective at blocking mast cell activation as full block of either CRAC channels or the receptors. To test this, we first established the concentration range over which montelukast inhibited LTC₄-dependent Ca²⁺ signals. We then identified the concentrations over which the lanthanum ion (La³⁺), a CRAC channel blocker, suppressed Ca²⁺ entry-dependent LTC₄ production and subsequent paracrine signaling. We then chose submaximal concentrations of each antagonist and examined their effectiveness in blocking LTC₄ and paracrine signal-dependent mast cell activation. Because human mast cells generate and respond to LTC₄ in a manner similar to RBL-1 cells, we used the latter cell preparation because this provided both a greater number of cells to generate paracrine signals and a more homogeneous cell population.

Montelukast suppresses paracrine signaling

Application of LTC₄ to RBL-1 cells generated an oscillatory Ca²⁺ signal (Fig 3, A). This signal was blocked by pretreating the cells with montelukast. To establish the sensitivity of this response to montelukast, we stimulated mast cells with 160 nmol/L LTC₄ in the presence of different concentrations of montelukast. Increasing the antagonist concentration produced a dose-dependent block (Fig 3, B-D). Full block was seen at 50 nmol/L

montelukast, and the inhibitory concentration of 50% (IC₅₀) was calculated to be approximately 8 nmol/L.

Block of CRAC channels suppresses LTC₄ production

CRAC channels are blocked by the trivalent cation La³⁺.⁸ To identify the IC₅₀ for La³⁺ block, we activated CRAC channels by stimulating cells with thapsigargin in Ca²⁺-free solution to deplete the stores and then applied 2 mmol/L external Ca²⁺. This latter maneuver resulted in a cytoplasmic Ca²⁺ increase caused by Ca²⁺ entry through CRAC channels. Fig 4, A, compares the Ca²⁺ entry-dependent cytoplasmic Ca²⁺ signal in control cells with those exposed to different concentrations of La³⁺. Increasing La³⁺ concentrations resulted in a slower rate of increase of Ca²⁺ concentration and smaller overall amplitude. We analyzed the rate of increase of the Ca²⁺ signal (which arose on Ca²⁺ readmission) because this is a good indicator of Ca²⁺ channel activity. Aggregate data are summarized in Fig 4, B. The results could be fitted with a Hill-type equation, yielding an IC₅₀ of 0.98 μmol/L.

Using a protocol similar to that described in Fig 1, E, we stimulated RBL-1 cells with thapsigargin and collected the supernatant.¹⁶ Application of this supernatant to RBL-1 cells evokes a large Ca²⁺ signal, which is suppressed by cysteinyl leukotriene receptor type I antagonists. A typical response to supernatant is shown in Fig 4, C. If mast cells were pre-exposed for 2 minutes to 3 μmol/L La³⁺, a concentration that substantially blocks CRAC channels (Fig 4, B), and then the supernatant was

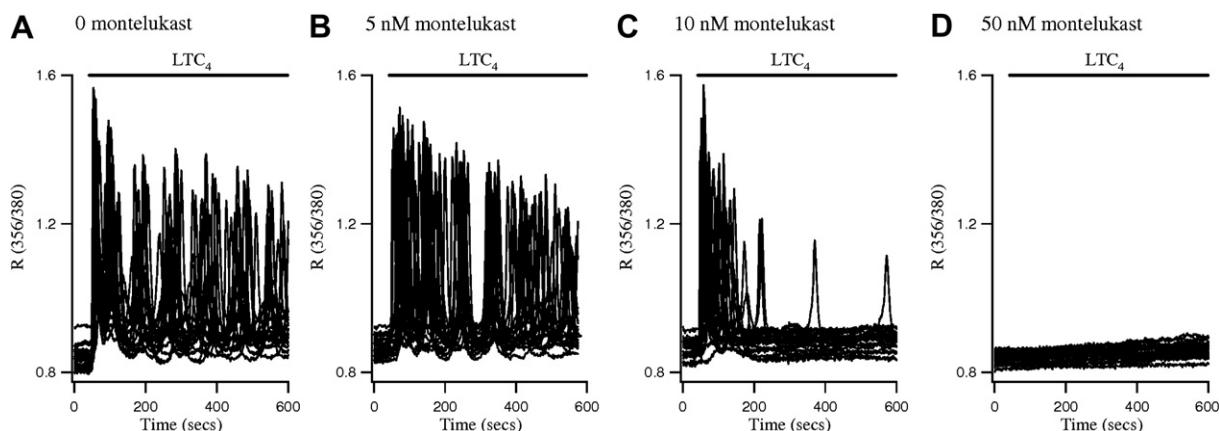


FIG 3. Montelukast blocks LTC₄-triggered Ca²⁺ signals. **A**, LTC₄ (160 nmol/L) applied to Fura-2-loaded RBL-1 cells evoked oscillatory Ca²⁺ signals in all cells (>100 cells). **B** through **D**, Increasing montelukast concentration produced a dose-dependent block of Ca²⁺ oscillations. Each concentration was tested on more than 50 cells. Each trace shows 10 independent cells.

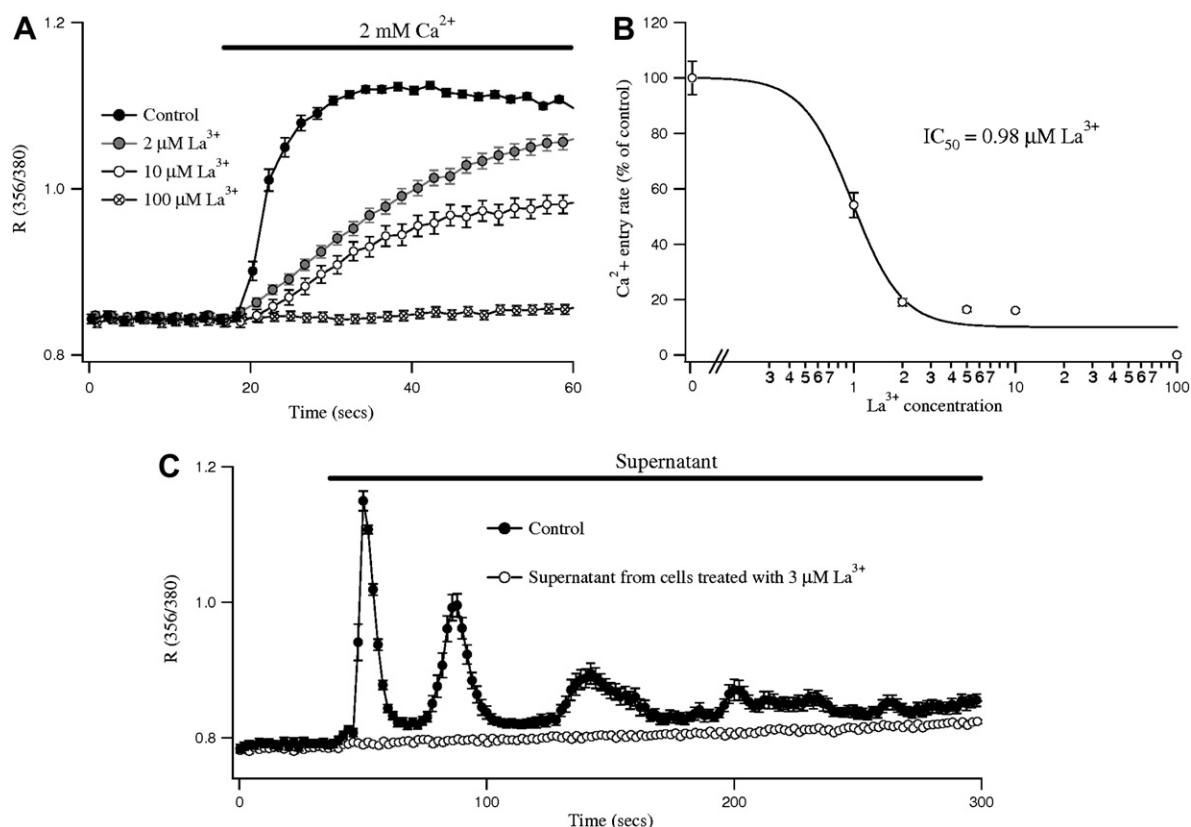


FIG 4. La³⁺ blocks CRAC channels, LTC₄ production, and paracrine signaling. **A**, Ca²⁺ entry through CRAC channels (activated by exposure to thapsigargin in Ca²⁺-free solution followed by readmission of Ca²⁺) was blocked by La³⁺ in a dose-dependent way. **B**, Dose-inhibition curve for La³⁺. **C**, Supernatant from thapsigargin-stimulated RBL-1 cells exposed to La³⁺ did not evoke Ca²⁺ signals in control RBL-1 cells.

collected, it was totally ineffective in generating a Ca²⁺ signal in RBL-1 cells (Fig 4, C). La³⁺ does not interfere with the ability of LTC₄ to activate leukotriene receptors,²³ ruling out an action of the trivalent on receptor-effector coupling. Hence Ca²⁺ influx through CRAC channels is essential for the production of cysteinyl leukotrienes that are released from RBL-1 cells.

Effect of partial block of CRAC channels combined with partial block of cysteinyl leukotriene type I receptors

We tested directly whether the combination of submaximal concentrations of each blocker was more effective than a higher concentration of either alone.

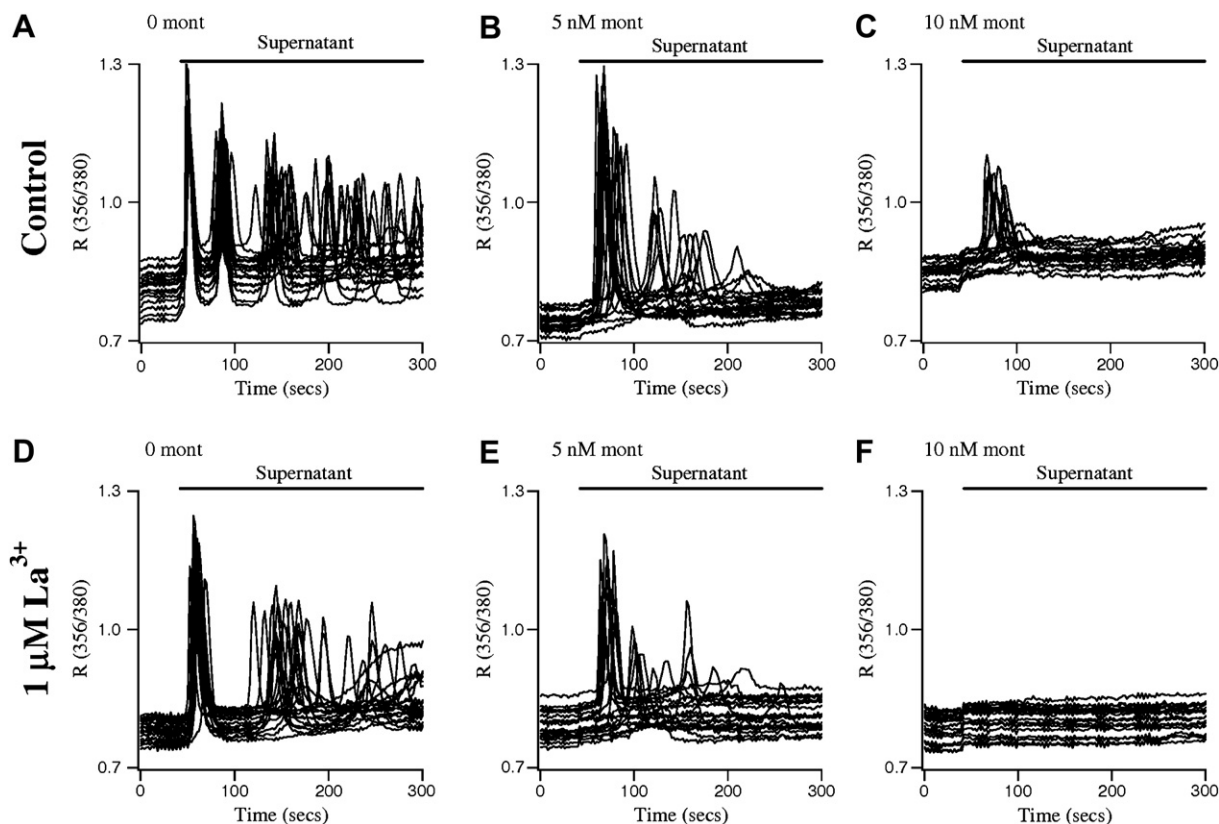


FIG 5. Partial block of CRAC channels and cysteinyl leukotriene type I receptors suppresses LTC_4 -driven paracrine signaling. **A** through **C**, Montelukast (*mont*) inhibits Ca^{2+} signals evoked by supernatant from thapsigargin-stimulated RBL-1 cells. **D**, Supernatant from thapsigargin-treated cells exposed to 1 $\mu\text{mol/L}$ La^{3+} evoked Ca^{2+} signals in control RBL-1 cells. **E**, Low dose of montelukast reduced the size of this Ca^{2+} signal further. **F**, Combination of 1 $\mu\text{mol/L}$ La^{3+} (applied to supernatant-generating cells) and 10 nmol/L montelukast (to Fura-loaded cells) abolished paracrine signaling.

Stimulation with supernatant evoked the typical oscillatory Ca^{2+} signal in control RBL-1 cells (Fig 5, A), but the responses were reduced if the cells were pre-exposed to 5 nmol/L montelukast (Fig 5, B), and they were smaller still, although not abolished, with 10 nmol/L montelukast (Fig 5, C). We then exposed the cells to 1 $\mu\text{mol/L}$ La^{3+} (close to the IC_{50} concentration for CRAC channel block; Fig 4, B), stimulated them with thapsigargin, collected the supernatant, and then applied this to a separate population of Fura-2–loaded cells. When this supernatant was applied to cells in the absence of montelukast, the Ca^{2+} response was reduced (Fig 5, D) when compared with that seen in the absence of La^{3+} (Fig 5, A). Importantly, supernatant from cells treated with 1 $\mu\text{mol/L}$ La^{3+} showed a smaller response to 5 nmol/L montelukast (Fig 5, E) compared with that seen in supernatant from cells not exposed to La^{3+} (Fig 5, B). Strikingly, this same supernatant was totally ineffective in 10 nmol/L montelukast (Fig 5, F, compared with Fig 5, C). Hence the combination of the IC_{50} concentration of La^{3+} together with the IC_{50} concentration of montelukast is considerably more effective at blocking Ca^{2+} signals than a higher concentration of either blocker alone. These results are summarized in Fig 6, which plots the percentage of cells responding to the various conditions depicted. Whereas all Fura-2–loaded RBL-1 reporter cells responded to supernatant (taken from either control cells or cells exposed to 1 $\mu\text{mol/L}$ La^{3+}), the response was reduced by approximately 30% when the reporter cells were pretreated with 10 nmol/L montelukast before

application of control supernatant. However, the combination of 10 nmol/L montelukast (on the reporter cells) and 1 $\mu\text{mol/L}$ La^{3+} (on the cells used to generate supernatant) rendered no cell responsive.

Combined targeting of the 5-lipoxygenase and cysteinyl leukotriene type I receptor

Because Ca^{2+} influx through CRAC channels activates 5-lipoxygenase to generate LTC_4 , we reasoned that submaximal concentrations of a 5-lipoxygenase blocker combined with a submaximal concentration of montelukast should be effective at damping down paracrine activation of mast cells. To test this, we used the 5-lipoxygenase inhibitor zileuton. The IC_{50} for zileuton block of cysteinyl leukotriene secretion was 500 nmol/L (see Fig E2 in this article's Online Repository at www.jacionline.org). Supernatant taken from mast cells pretreated with this submaximal concentration of zileuton only partially reduced the Ca^{2+} response in Fura-2–loaded cells (see Fig E2). However, 500 nmol/L zileuton together with 5 nmol/L montelukast (applied to Fura-2–loaded cells) reduced the response further (see Fig E2), and the combination of 500 nmol/L zileuton with 10 nmol/L montelukast almost totally suppressed the response (see Fig E2). Hence a combination of a submaximal concentration of zileuton with a submaximal concentration of montelukast is more effective than either alone (Fig 6).

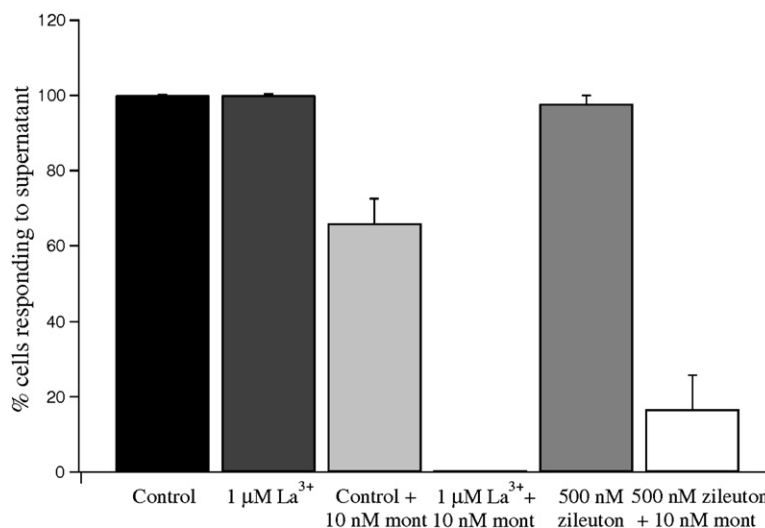


FIG 6. The combination of montelukast (*mont*) and a CRAC channel inhibitor effectively suppresses paracrine signaling. The percentage of cells generating a Ca^{2+} signal to each condition was compared. *Control*, Supernatant taken from cells exposed first to thapsigargin ($2 \mu\text{mol/L}$ for 4 minutes) and then 2 Ca^{2+} ; $1 \mu\text{M}$ La^{3+} , supernatant taken from stimulated cells that were pretreated with La^{3+} ; *Control + 10 nM mont*, supernatant taken from control cells that was then applied to montelukast-treated cells; $1 \mu\text{M}$ La^{3+} + 10 nM mont, supernatant from cells exposed to La^{3+} and then applied to montelukast-treated cells; 500 nM zileuton and 10 nM mont, supernatant taken from cells pre-exposed to zileuton that was then applied to Fura-2-loaded cells treated with montelukast.

DISCUSSION

Our main findings are that a positive feedback cycle exists in human nasal polyps that involves an interplay between CRAC channels and cysteinyl leukotriene type I receptors and that a combination therapy that targets both proteins is effective in suppressing mast cell activation. In both rodent¹⁶ and human mast cells, a feed-forward loop exists in that Ca^{2+} entry through open CRAC channels drives the synthesis and secretion of LTC_4 , which then feeds back in a paracrine manner to generate Ca^{2+} signals that sustain cysteinyl leukotriene production. A high concentration of a CRAC channel blocker prevented the generation of cysteinyl leukotrienes, and a high concentration a cysteinyl leukotriene receptor type I antagonist suppressed the ability of the secreted LTC_4 to activate resting mast cells. Importantly, the combination of a moderate concentration of a CRAC blocker with a modest dose of the cysteinyl leukotriene receptor type I antagonist was effective in fully blocking paracrine activation of mast cells. This is relevant from a clinical intervention perspective because it means a lower concentration of drugs could be used, reducing the incidence of side effects and drug dependence.

Could this combination therapy be of use against nasal polypsis? Activated mast cells are found in high numbers in patients with nasal polyps,²⁴ and increased levels of histamine²⁵ and cysteinyl leukotrienes are associated with polyposis.²⁶ Moreover, we have now shown that mast cells in human polyps respond to LTC_4 by generating robust Ca^{2+} signals, which stimulate LTC_4 production. Mast cell activation would be regenerative, and this could prolong the response. In addition to mast cells, there is also a substantial increase in the number of eosinophils in nasal polyps.³ Eosinophils express the 5-lipoxygenase enzyme⁵ and cysteinyl leukotriene type I receptors.^{27,28} Although we have focused on mast cells, the combination therapy we have identified would also target eosinophils and any other cell type in a polyp that produces cysteinyl leukotrienes, responds to cysteinyl leukotrienes, or both.

How might cysteinyl leukotriene receptor activation stimulate mast cells? We have recently shown that low concentrations of LTC_4 evoke robust expression of c-fos,²³ a transcription factor that plays a key role in the expression of proinflammatory genes.²⁹ Cysteinyl leukotriene receptor-driven expression of c-fos will therefore have important functional consequences during the late phase of the mast cell response. The initial Ca^{2+} signal in response to cysteinyl leukotriene type I receptor activation is sufficient to drive the synthesis and secretion of leukotrienes.¹⁶

Could this Ca^{2+} signal also affect histamine degranulation, an early step in the mast cell response? Initial work reported that the 5-lipoxygenase product 5-hydroperoxyicosatetraenoic acid enhanced histamine degranulation in human basophils³⁰ and that lipoxygenase inhibitors suppressed histamine degranulation in response to receptor activation.³¹ In skin, but not lung, mast cells, a 5-lipoxygenase blocker inhibited histamine secretion in response to antigen.³² Patch clamp recordings in RBL-1 cells revealed that pretreatment with 5-lipoxygenase blockers reduced CRAC current activation, but the inhibitors were much less effective when applied after the current had developed.³³ This might reflect a state-dependent block of CRAC channels by these drugs or that they have targets in addition to 5-lipoxygenase. Subsequent work with more selective 5-lipoxygenase blockers revealed that histamine degranulation in response to antigen was unaffected, despite suppression of leukotriene generation.^{34,35} The reason for this discrepancy is not entirely clear but could be attributed to off-target effects of the different inhibitors used. Studies *in vivo* or on excised human tissue have revealed that leukotriene antagonists and antihistamines act additively, suggesting they function independently.³⁶⁻³⁸ Future work measuring exocytosis in single cells by tracking membrane capacitance should help address whether leukotrienes affect histamine degranulation.

Is a combination therapy involving CRAC channels and leukotriene receptors currently available? Cysteinyl leukotriene type I

antagonists, including montelukast and zafirlukast, are in clinical use for treating aspirin-induced asthma and are of therapeutic benefit in allergen-induced nasal congestion³⁹ and allergic rhinitis,^{40,41} although one study did not show any beneficial effect of a leukotriene antagonist on nasal allergy to grass and ragweed pollen.⁴²

The pharmacology of CRAC channels is still in development, and no channel blocker is currently available. However, the future looks promising. We have recently characterized the effects of a new inhibitor, the Synta compound, which is an effective CRAC channel blocker.⁴³ CalciMedica have reported the synthesis of a CRAC channel inhibitor that is effective in various animal models of T cell–driven immune disorders (<http://www.calcimedica.com/ScienceTech.html>). Hence the combination of such a CRAC channel blocker with montelukast, for example, might provide an alternative noninvasive treatment for managing nasal polyposis and other mast cell–dependent disorders. Further investigation is needed to determine whether this is a viable approach *in vivo*.

Key messages

- In mast cells a positive feedback cycle occurs in which calcium signals generate leukotrienes, which then activate mast cells by generating calcium signals.
- Block of the channels and leukotriene receptors suppresses mast cell activation, suggesting a new therapy targeting both components could be effective in managing allergies.

REFERENCES

- Radene F, Lamblin C, Vandezande L-M, Tillie-Leblond I, Darras J, Tonnel A-B, et al. Quality of life in nasal polyposis. *J Allergy Clin Immunol* 1999;103:79-84.
- Bateman ND, Fahy C, Woolford TJ. Nasal polyps: still more questions than answers. *J Laryngol Otol* 2003;117:1-9.
- Pawankar R. Nasal polyposis: an update. *Curr Opin Allergy Clin Immunol* 2003;3:1-6.
- Ozen Z, Mumbuc S, Sari I, Baglam T, Karatas E, Kanlikama M. Cysteinyl leukotriene receptor expression in aspirin-sensitive nasal polyposis patients. *J Otorhinolaryngol Relat Spec* 2007;69:176-80.
- Adamjee J, Suh YJ, Park HS, Choi JH, Penrose JF, Lam BK, et al. Expression of 5-lipoxygenase and cyclooxygenase pathway enzymes in nasal polyps of patients with aspirin-intolerant asthma. *Allergy Inflamm Res* 2006;209:392-9.
- Chang WC, Parekh AB. Close functional coupling between CRAC channels, arachidonic acid release and leukotriene secretion. *J Biol Chem* 2004;279:29994-9.
- Chang W-C, Nelson C, Parekh AB. Ca²⁺ influx through CRAC channels activates cytosolic phospholipase A2, leukotriene C4 secretion and expression of c-fos through ERK-dependent and independent pathways in mast cells. *FASEB J* 2006;20:2381-3.
- Parekh AB, Putney JWJ. Store-operated calcium channels. *Physiol Rev* 2005;85:757-810.
- Hoth M, Penner R. Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* 1992;355:353-6.
- Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel S-V, Tanasa B, et al. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature* 2006;441:179-85.
- Vig M, Peinelt C, Beck A, Koomoa DL, Rabah D, Koblan-Huberson M, et al. CRACM1 is a plasma membrane protein essential for store-operated calcium entry. *Science* 2006;312:1220-3.
- Zhang SL, Yeromin AV, Zhang XH-F, Yu Y, Safrina O, Penna A, et al. Genome-wide RNAi screen of calcium influx identifies genes that regulate Ca release-activated Ca channel activity. *Proc Natl Acad Sci U S A* 2006;103:9357-62.
- Vig M, DeHaven WI, Bird GS, Billingsley JM, Wang HJ, Rao PE, et al. Defective mast cell effector functions in mice lacking the CRACM1 pore subunit of store-operated calcium release-activated calcium channels. *Nat Immunol* 2008;9:89-96.
- Baba Y, Nishida K, Fujii Y, Hikida M, Kurosaki T. Essential function for the calcium sensor STIM1 in mast cell activation and anaphylactic responses. *Nat Immunol* 2008;9:81-8.
- Chang WC, DiCapite J, Singaravelu K, Nelson C, Halse V, Parekh AB. Local calcium influx through calcium release-activated calcium (CRAC) channels stimulates production of an intracellular messenger and an intercellular pro-inflammatory signal. *J Biol Chem* 2008;283:4622-31.
- DiCapite JL, Shirley A, Nelson C, Bates G, Parekh AB. Intercellular calcium wave propagation involving positive feedback between CRAC channels and cysteinyl leukotrienes. *FASEB J* 2009;23:894-905.
- Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 2001;294:1871-5.
- Lynch KR, O'Neill GP, Liu Q, Im D-S, Sawyer N, Metters KM, et al. Characterization of the human cysteinyl leukotriene cysLT1 receptor. *Nature* 1999;399:789-93.
- Mellor EA, Maekawa A, Austen KF, Boyce JA. Cysteinyl leukotriene receptor 1 is also a pyrimidinergic receptor and is expressed by human mast cells. *Proc Natl Acad Sci U S A* 2001;98:7964-9.
- Bandeira-Melo C, Hall JC, Penrose JF, Weller PF. Cysteinyl leukotrienes induce IL-4 release from cord blood-derived human eosinophils. *J Allergy Clin Immunol* 2002;109:975-9.
- Paruchuri S, Jiang Y, Feng C, Francis SA, Plutzky J, Boyce JA. Leukotriene E4 activates peroxisome proliferator-activated receptor gamma and induces prostaglandin D2 generation by human mast cells. *J Biol Chem* 2008;283:16477-87.
- Maekawa A, Kanaoka Y, Xing W, Austen KF. Functional recognition of a distinct receptor preferential for leukotriene E4 in mice lacking cysteinyl leukotriene 1 and 2 receptors. *Proc Natl Acad Sci U S A* 2008;105:16695-700.
- DiCapite J, Ng S-W, Parekh AB. Decoding of cytoplasmic Ca²⁺ oscillations through the spatial signature drives gene expression. *Curr Biol* 2009;19:853-8.
- Drake-Lee A, Price J. Mast cell ultrastructure in the inferior turbinate and stroma of nasal polyps. *J Laryngol Otol* 1997;111:340-5.
- Drake-Lee A, McLaughlin P. Clinical symptoms, free histamine and IgE in nasal polyps. *Int Arch Allergy Appl Immunol* 1982;69:268-71.
- Chao SS, Graham SM, Brown CL, Kline JH, Hussain I. Cysteinyl leukotriene 1 receptor expression in nasal polyps. *Ann Otol Rhinol Laryngol* 2006;115:394-7.
- Figueroa DJ, Breyer RM, Defoe SK, Kargman S, Daugherty BL, Waldburger K, et al. Expression of the cysteinyl leukotriene 1 receptor in normal human lung and peripheral blood leukocytes. *Am J Respir Crit Care Med* 2001;163:226-33.
- Figueroa DJ, Borish L, Baramki D, Philip G, Austin CP, Evans JF. Expression of cysteinyl leukotriene synthetic and signalling proteins in inflammatory cells in active seasonal allergic rhinitis. *Clin Exp Allergy* 2003;33:1380-8.
- Lee Y-N, Tuckerman J, Nechushtan H, Schutz G, Razin E, Angel P. c-Fos as a regulator of degranulation and cytokine production in FCεRI-activated mast cells. *J Immunol* 2004;173:2571-7.
- Peters SP, Siegel MI, Kagey-Sobotka A, Lichtenstein LM. Lipoxygenase products modulate histamine release in human basophils. *Nature* 1981;292:455-7.
- Marone G, Kagey-Sobotka A, Lichtenstein LM. Effects of arachidonic acid and its metabolites on antigen-induced histamine release from human basophils *in vitro*. *J Immunol* 1979;123:1664-7.
- Cohan V, McKenzie-White J, Triggiani M, Massey W, Kagey-Sobotka A, Lichtenstein LM. Heterogeneity of human mast cells and basophils. *Biochem Pharmacol* 1989;38:4455-9.
- Glitsch MD, Bakowski D, Parekh AB. Activation of the store-operated Ca²⁺ current ICRAC is compromised by inhibitors of the lipoxygenase family of enzymes. *J Physiol* 2002;539:93-106.
- Kusner EJ, Buckner CK, Dea DM, DeHaas CJ, Marks RL, Krell RD. The 5-lipoxygenase inhibitors ZD2138 and ZM230487 are potent and selective inhibitors of several antigen-induced guinea-pig pulmonary responses. *Eur J Pharmacol* 1994;257:285-92.
- Warner JA, Lichtenstein LM, MacGlashan D Jr. Effects of a specific inhibitor of the 5-lipoxygenase pathway on mediator release from human basophils and mast cells. *J Pharmacol Exp Ther* 1988;247:218-22.
- Rouquet A, Dahlen B, Kumlin M, Ihre E, Anstren G, Binks S, et al. Combined antagonism of leukotrienes and histamine produce predominant inhibition of allergen-induced early and late phase obstruction in asthmatics. *Am J Respir Crit Care Med* 1997;155:1856-63.
- Ellis JL, Udem BJ. Role of cysteinyl leukotrienes and histamine in mediating intrinsic tone in isolated human bronchi. *Am J Respir Crit Care Med* 1994;149:118-22.
- Bjorck T, Dahlen SE. Leukotrienes and histamine mediate IgE-dependent contractions of human bronchi: pharmacological evidence obtained with tissues from asthmatic and non-asthmatic subjects. *Pulm Pharmacol* 1993;6:87-96.
- Knapp HR. Reduced allergen-induced nasal congestion and leukotriene synthesis with an orally active 5-lipoxygenase inhibitor. *N Engl J Med* 1990;323:1745-8.
- Philip G, Williams-Herman D, Patel P, Weinstein SF, Alon A, Gilles L, et al. Efficacy of montelukast for treating perennial allergic rhinitis. *Allergy Asthma Proc* 2007;28:296-304.
- Van Hoecke H, Vanderbulcke L, Van Cauwenberge P. Histamine and leukotriene receptor antagonism in the treatment of allergic rhinitis: an update. *Drugs* 2007;67:2717-26.
- Flowers BK, Proud D, Kagey-Sobotka A, Lichtenstein LM, Naciero RM. The effect of a leukotriene antagonist on the early response to antigen. *Otolaryngol Head Neck Surg* 1990;102:219-24.
- Ng S-W, DiCapite JL, Singaravelu K, Parekh AB. Sustained activation of the tyrosine kinase Syk by antigen in mast cells requires local Ca²⁺ influx through Ca²⁺ release-activated Ca²⁺ channels. *J Biol Chem* 2008;283:31348-55.

METHODS

Cell culture

RBL-1 cells were purchased from the American Type Culture Collection. Cells were cultured (at 37°C for 5% CO₂) in supplemented Dulbecco modified Eagle medium (with 10% FBS, 2 mmol/L L-glutamine, and penicillin and streptomycin), as described previously.^{E1} For Ca²⁺ imaging experiments, cells were passaged (with trypsin) onto glass coverslips and used 36 to 72 hours after plating. HMC-1 human mast cells were a generous gift from Dr Joseph Butterfield (Mayo Foundation) and were grown in Iscove medium with 10% iron-supplemented calf serum and 1.2 mmol/L α -thioglycerol.

Human mast cells

Human mast cells were obtained from nasal polyps with full patient consent and with approval from the National Research Ethics Service (REC no. 07/H0607/104). Nasal tissue was minced with fine scissors, mechanically agitated, enzymatically treated at 37°C with collagenase and trypsin, centrifuged twice at 1000g, and then resuspended in supplemented Dulbecco modified Eagle medium. Cells were used within 3 to 6 hours of isolation. Mast cells were identified by means of (1) staining with c-kit,^{E2} (2) staining with toluidine blue,^{E2} (3) staining for 5-lipoxygenase, (4) cell capacitance of between 6 and 8 pF, and (5) rapid degranulation (1–3 pF within 150 seconds) after whole-cell dialysis with inositol trisphosphate in 0.1 mmol/L ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid plus guanosine triphosphate. For Ca²⁺ imaging experiments, cells were plated onto glass coverslips. In our biochemical measurements of LTC₄ production, which reflect the entire cell population in the polyp, other cells, particularly eosinophils, in addition to mast cells will contribute to the response. Nevertheless, mast cells are abundant in the polyps,^{E2} accounting for between 40% to 60% of the purified population in our study, and will make a significant contribution to the overall response.

Ca²⁺ imaging

Ca²⁺ imaging experiments were carried out at room temperature by using the IMAGO CCD camera-based system from TILL Photonics (Gräfelfing, Germany), as described previously.^{E3} Cells were alternately excited at 356 and 380 nm (20-ms exposures), and images were acquired every 2 seconds. Ratios were calculated offline, and data were imported to IGOR Pro (Wave Metrics, Lake Oswego, Ore) for further analysis. RBL-1 cells were loaded with Fura-2-AM (4 μ mol/L) for 45 minutes and human mast cells for 30 minutes at room temperature in the dark and then washed 3 times in standard external solution of the following composition: 145 mmol/L NaCl, 2.8 mmol/L KCl, 2 mmol/L CaCl₂, 2 mmol/L MgCl₂, 10 mmol/L D-glucose, and 10 mmol/L HEPES (pH 7.4) with NaOH. Cells were left for 15 minutes to allow further de-esterification. Ca²⁺-free solution had the following composition: 145 mmol/L NaCl, 2.8 mmol/L KCl, 2 mmol/L MgCl₂, 10 mmol/L D-glucose, 10 mmol/L HEPES, and 0.1 mmol/L ethyleneglycol-

bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid (pH 7.4) with NaOH. The rate of Ca²⁺ influx was obtained by measuring the initial slope of the fluorescence increase after readmission of Ca²⁺ to cells with depleted stores. Ca²⁺ signals are plotted as R (356/380), which denotes the 356/380 nm ratio.^{E3}

Isolation of supernatant

Extracellular solution bathing-stimulated cells were collected, as described previously.^{E2} In brief, populations of RBL-1, HMC-1, or cells from human polyps were stimulated with thapsigargin in Ca²⁺-free solution for 4 minutes and washed extensively in Ca²⁺-free solution alone for a further 4 minutes, and then external Ca²⁺ was readmitted. After 4 minutes, extracellular solution was collected, centrifuged for 2 minutes, and then stored on ice for immediate use. Centrifugation was particularly important for the HMC-1 cells, because these cells grew in a suspension.

LTC₄ assay

Nasal polyps were minced, as described above, and then left to rest for 3 to 4 hours in supplemented Dulbecco modified Eagle medium. Cells were centrifuged at 1000g, and culture medium was removed. After stimulation with thapsigargin, the supernatant was collected, and LTC₄ levels were measured by means of enzyme immunoassay (Cayman Chemicals, Ann Arbor, Mich), as described previously.^{E2}

Immunohistochemistry

Cells were fixed in 4% paraformaldehyde in phosphate buffer for 30 minutes at room temperature and after stimulation with thapsigargin. All the washes used 0.01% PBS (PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 8 mmol/L Na₂HPO₄, and 1 mmol/L KH₂PO₄). The cells were blocked with 2% BSA and 10% goat serum for 1 hour. 5-Lipoxygenase was visualized by using a monoclonal mouse IgG1 antibody (used at a dilution of 1:3000; BD Transduction Laboratories, Oxford, United Kingdom). Anti-5-lipoxygenase was used in carrier (0.2% BSA and 1% goat serum) and left overnight at 4°C. Images were obtained by using the IMAGO CCD camera-based system from TILL Photonics with a 100 $\times$ objective.

REFERENCES

- E1. Chang W-C, Nelson C, Parekh AB. Ca²⁺ influx through CRAC channels activates cytosolic phospholipase A₂, leukotriene C₄ secretion and expression of c-fos through ERK-dependent and independent pathways in mast cells. *FASEB J* 2006;20:2381-3.
- E2. DiCapite JL, Shirley A, Nelson C, Bates G, Parekh AB. Intercellular calcium wave propagation involving positive feedback between CRAC channels and cysteinyl leukotrienes. *FASEB J* 2009;23:894-905.
- E3. Moreau B, Straube S, Fisher RJ, Putney JW, Parekh AB. Ca²⁺-calmodulin-dependent facilitation and Ca²⁺ inactivation of Ca²⁺ release-activated Ca²⁺ channels. *J Biol Chem* 2005;280:8776-83.

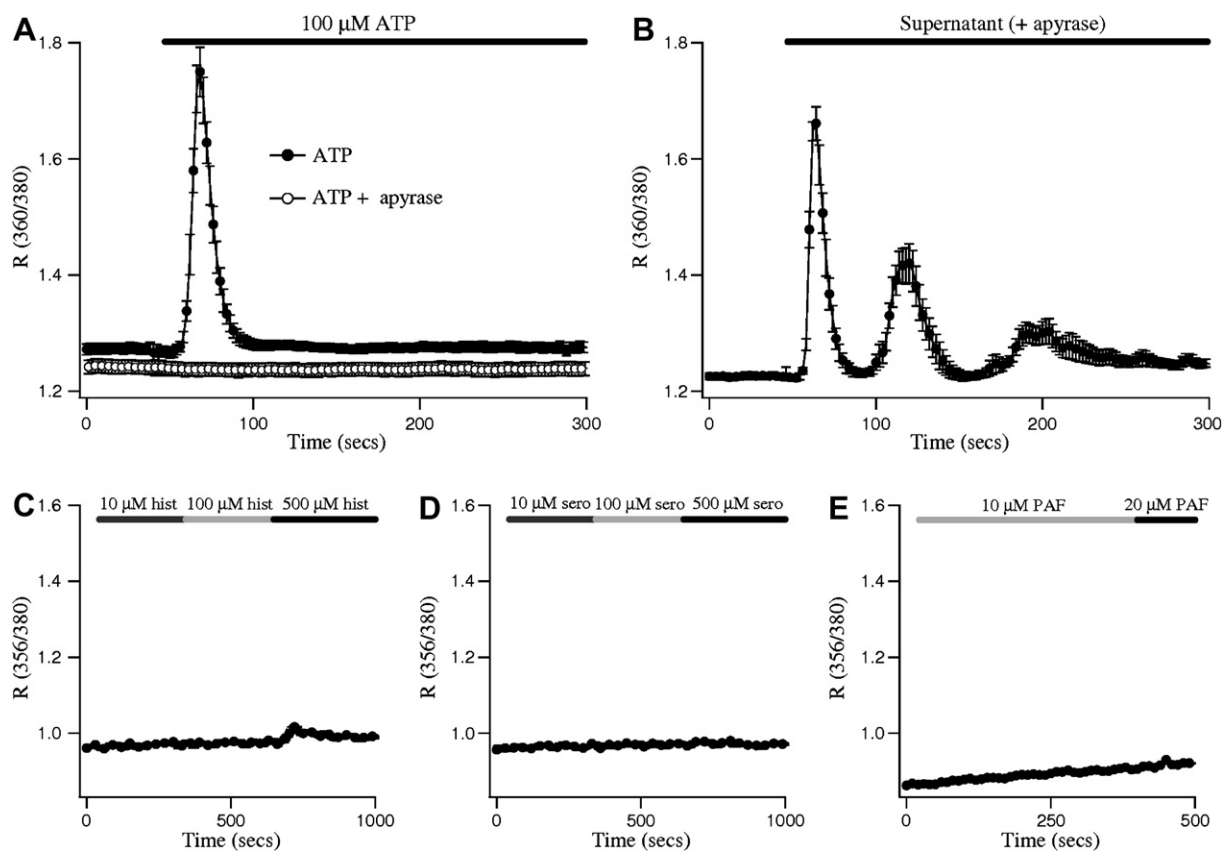


FIG E1. Effects of other mast cell-derived signals on cytoplasmic Ca^{2+} . **A**, ATP evoked a transient increase in Ca^{2+} levels in RBL-1 cells, and this was prevented by mixing the ATP with apyrase (10 units/mL; 40 minutes). **B**, Supernatant was effective, despite treatment with apyrase ($n > 30$ cells for Fig E1, A and B). **C** through **E**, Histamine (*hist*), serotonin (*sero*), and platelet-activating factor (*PAF*) were all ineffective ($n > 10$ cells for each condition).

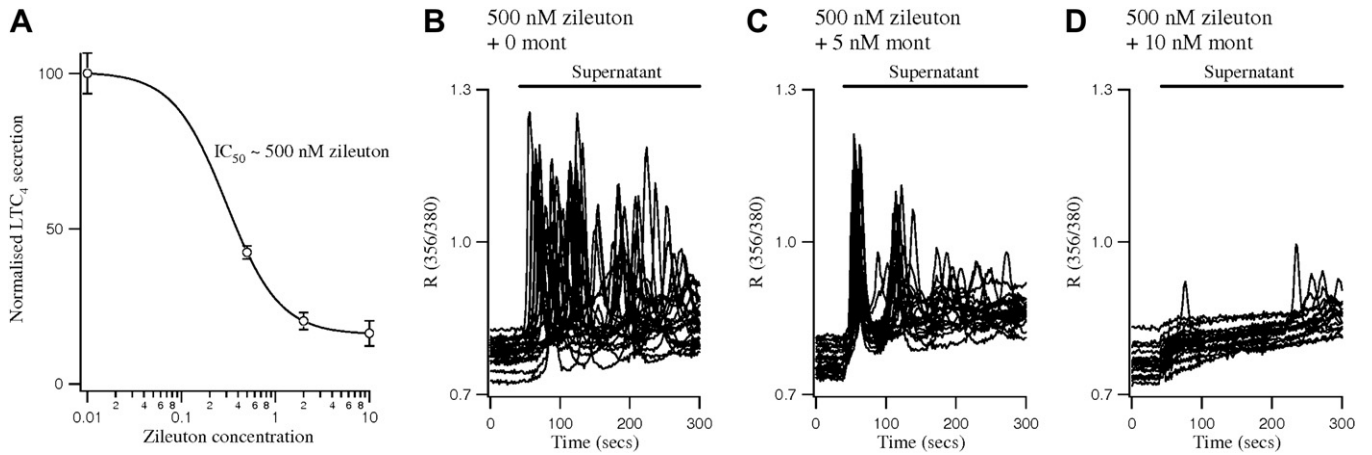


FIG E2. The combination of zileuton and montelukast (*mont*) abolishes mast cell Ca^{2+} signals. **A**, Dose-inhibition relationship for zileuton on LTC_4 secretion. **B** through **D**, The supernatant response is affected by the combination of zileuton (applied to cells used to generate supernatant) and montelukast (applied to the Fura-2-loaded reported cells).