Cysteinyl leukotriene type I receptor desensitization sustains Ca\(^{2+}\)–dependent gene expression

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Receptor desensitization is a universal mechanism to turn off a biological response; in this process, the ability of a physiological trigger to activate a cell is lost despite the continued presence of the stimulus. Receptor desensitization of G-protein-coupled receptors involves uncoupling of the receptor from its G-protein or second-messenger pathway followed by receptor internalization\(^1\). G-protein-coupled cysteinyl leukotriene type I (CysLT1) receptors regulate immune-cell function and CysLT1 receptors are an established therapeutic target for allergies, including asthma\(^2\). Desensitization of CysLT1 receptors arises predominantly from protein-kinase-C-dependent phosphorylation of three serine residues in the receptor carboxy terminus\(^3\). Physiological concentrations of the receptor agonist leukotriene C\(_4\) (LTC\(_4\)) evoke repetitive cytoplasmic Ca\(^{2+}\) oscillations, reflecting regenerative Ca\(^{2+}\) release from stores, which is sustained by Ca\(^{2+}\) entry through store-operated calcium-release-activated calcium (CRAC) channels\(^4\). CRAC channels are tightly linked to expression of the transcription factor c-fos\(^5\), a regulator of numerous genes important to cell growth and development\(^6\). Here we show that abolishing leukotriene receptor desensitization suppresses agonist-driven gene expression in a rat cell line. Mechanistically, stimulation of non-desensitizing receptors evoked prolonged inositol-trisphosphate-mediated Ca\(^{2+}\) release, which led to accelerated Ca\(^{2+}\)-dependent slow inactivation of CRAC channels and a subsequent loss of excitation–transcription coupling. Hence, rather than serving to turn off a biological response, reversible desensitization of a Ca\(^{2+}\)-mobilizing receptor acts as an ‘on’ switch, sustaining long-term signalling in the immune system.

The paradox of receptor desensitization is how long-term responses can be evoked if the receptor is inactivated. This is a particularly acute problem in immune cells, in which cell differentiation and clonal selection develop over hours in the continued presence of external cues.

Stimulation of rat basophilic leukaemia (RBL-1) cells with LTC\(_4\) acting exclusively on CysLT1 receptors\(^7\) (Supplementary Fig. 1), led to cytoplasmic Ca\(^{2+}\) signals (Fig. 1a) followed by robust expression of c-fos at both messenger RNA (Fig. 1b, c) and protein levels (Fig. 1d, e). Maximal activation of CRAC channels with thapsigargin led to a similar increase in c-fos expression (Fig. 1b–e). Both LTC\(_4\) and thapsigargin induce c-fos expression through the build-up of Ca\(^{2+}\) microdomains near open store-operated CRAC channels\(^8\). Thapsigargin led to a larger, more sustained Ca\(^{2+}\) signal than LTC\(_4\) (Fig. 1a)\(^9\) and the rate of Ca\(^{2+}\) entry through CRAC channels was approximately twofold more for thapsigargin than LTC\(_4\) (Fig. 1f), consistent with patch-clamp recordings\(^2\). The similar increase in Ca\(^{2+}\)-dependent c-fos expression to LTC\(_4\) and thapsigargin was therefore surprising, given the significant difference in CRAC channel activation.

We considered various explanations for why CysLT1 receptor activation and thapsigargin evoked similar c-fos levels despite marked differences in the extent of CRAC channel activation. These included: (1) CysLT1 receptors tapped into a different signalling mechanism linking CRAC channel microdomains to c-fos expression; (2) local Ca\(^{2+}\) entry through CRAC channels was larger after receptor activation because LTC\(_4\) hyperpolarized the membrane potential; and (3) cytoplasmic Ca\(^{2+}\) and protein kinase C (PKC) interacted synergistically to drive gene expression in response to CysLT1 receptor activation. Evidence against these possibilities is presented in Supplementary Figs 2–4. Instead, gene expression showed high sensitivity to Ca\(^{2+}\) entry, enabling CysLT1 receptor activation to couple effectively to c-fos transcription (Supplementary Fig. 5), as well as rapidity and high gain. Combined, this ensures efficient gene expression to bursts of CRAC channel activity after physiological levels of receptor stimulation.

Experiments described in Fig. 1g and h revealed an important role for PKC in receptor-dependent gene expression. The structurally distinct PKC blockers G06983 and calphostin C abolished c-fos expression (Fig. 1g, h). PKC block had a marked effect on the Ca\(^{2+}\) signal evoked by agonist. Whereas cytoplasmic Ca\(^{2+}\) oscillations were routinely observed with LTC\(_4\) (Fig. 1i), the response was converted into a large, single, slowly decaying Ca\(^{2+}\) spike after PKC inhibition (Fig. 1j). Acute stimulation with PMA in the absence of LTC\(_4\) failed to induce significant c-fos expression (data not shown)\(^6\), demonstrating that PKC activity per se was not sufficient to induce c-fos expression in these cells. The PKC inhibitors had no effect on thapsigargin-evoked c-fos expression (Fig. 1k) or cytoplasmic Ca\(^{2+}\) signals (Fig. 1l). Thapsigargin (2 μM) activates CRAC channels maximally (Supplementary Fig. 5) and, by blocking SERCA pumps (which can be located near CRAC channels\(^10\)), reduces the decay of Ca\(^{2+}\) gradients radiating from the plasma membrane. It is possible that other non-receptor-dependent stimuli, which raise local Ca\(^{2+}\) levels less effectively than 2 μM thapsigargin, might activate c-fos in a manner dependent on basal PKC activity, but this activity would be unusual in that it is not stimulated acutely by PMA in the presence of submaximal CRAC channel activation (Supplementary Fig. 4).

In RBL cells, exposure to the phorbol ester PMA for several hours downregulates several PKC isozymes\(^11\). Using this protocol, we found that c-fos expression was substantially reduced in response to CysLT1 receptor stimulation (Fig. 1m), whereas no significant reduction was seen when thapsigargin was used instead (Fig. 1m) or when inactive 4α-phorbol replaced PMA (data not shown). Similar to PKC blockers, the Ca\(^{2+}\) signal to LTC\(_4\) was prolonged after PKC downregulation (Fig. 1n). This prolonged Ca\(^{2+}\) signal did not reflect a change in Ca\(^{2+}\) clearance mechanisms (Supplementary Fig. 6); instead, it is characteristic of loss of receptor desensitization, particularly for CysLT1 receptors, in which desensitization is mediated predominantly by PKC\(^2\) and prevention of desensitization leads to broader Ca\(^{2+}\) signals\(^2\).

Inhibition of CysLT1 receptor desensitization is predicted to lead to greater inositol trisphosphate (Ins\(_3\)) production and hence more extensive Ca\(^{2+}\) store emptying. Several findings are consistent with this. First, Ca\(^{2+}\) release to LTC\(_4\) lasted approximately five times longer when PKC was blocked than in control cells (Fig. 2a, expanded in inset). Second, the amount of Ca\(^{2+}\) remaining within the stores, measured as the ionomycin-sensitive Ca\(^{2+}\) response\(^3\), was substantially less after activation of CysLT1 receptors in the presence of PKC block than in control cells (Fig. 2a). Third, Ins\(_3\) production, measured using the green fluorescent protein–pleckstrin homology
inactivate CRAC channels to suppress agonist-evoked gene expression in the presence of non-desensitizing receptors could therefore alter the duration of the prolonged \( \text{Ca}^{2+} \) release to non-desensitizing CysLT1 receptors. LTC4 control group (LTC4 in the absence of PKC block) was different from the other groups (\( P < 0.01 \)). There were no significant differences between the other groups. G0, G06983, C, calphostin C. i, Single-cell \( \text{Ca}^{2+} \) signals to LTC4 are compared for the conditions shown. j, Averaged data are compared (\( > 45 \) cells for each condition). k, Histogram showing \( c\)-\( fos \) expression to thapsigargin in the presence of PKC blockers. All thapsigargin-treated groups were significantly different from control (\( P < 0.001 \)) but were not significantly different from one another. l, \( \text{Ca}^{2+} \) signals to thapsigargin are unaffected by PKC block. m, Downregulation of PKC (PMA; 500 nM, 24 h) reduces LTC4- but not thapsigargin-induced \( c\)-\( fos \) expression (data from four independent experiments). All stimulated groups were significantly different from control (\( P < 0.001 \)) for the conditions shown. All stimulated groups were significantly different from control (\( P < 0.001 \)). Thap, thapsigargin control (\( P < 0.001 \)). For thapsigargin, the PMA group was different from the thapsigargin control (\( P < 0.001 \)). For LTC4 the PMA group was different from the LTC4 control (\( P < 0.001 \)). For thapsigargin, the PMA group was different from the thapsigargin control (\( P < 0.001 \)). Ctrl, control. n, PKC downregulation alters the LTC4-activated \( \text{Ca}^{2+} \) signal. Error bars show data \( \pm \) s.e.m. NS, not significant.

**Figure 1 | CysLT1-receptor-dependent \( c\)-\( fos \) expression requires PKC**

a, Averaged \( \text{Ca}^{2+} \) signals to LTC4 and thapsigargin (Thap.) are compared (\( > 50 \) cells per graph). \( R \) (356/380), ratio of fluorescence measured at 356 and 380 nm.

b, \( c\)-\( fos \) expression is compared between control (non-stimulated), 160 nM LTC4, and 2 \( \mu \text{M} \) thapsigargin-stimulated cells. Stimulus was present for 8 min.

c, Histograms show averaged responses from three independent experiments. LTC4 and thapsigargin groups were different from control (\( P < 0.001 \)), but not from one another (\( P > 0.3 \), analysis of variance (ANOVA)).

d, Cells stained with antibody against c-Fos protein. DAPI, 4',6-diamidino-2-phenylindole.

e, Aggregate data are compared \( (n > 20 \) per bar). Thapsigargin and LTC4 groups were different from control (\( P < 0.001 \)) but not from one another (\( P = 0.11 \)).

f, \( \text{Ca}^{2+} \) entry rate was measured after readmission of \( \text{Ca}^{2+} \) to cells stimulated with LTC4 or thapsigargin in \( \text{Ca}^{2+} \)-free solution (*\( P < 0.01 \)).

g, G06983 (1 \( \mu \text{M} \); 10 min pre-treatment) suppresses LTC4-induced \( c\)-\( fos \) expression.

h, Histogram comparing the effects of PKC blockers. LTC4 control (LTC4 in the absence of PKC block) was different from the other groups (\( P < 0.01 \)). There were no significant differences between the other groups. G0, G06983, C, calphostin C. i, Single-cell \( \text{Ca}^{2+} \) signals to LTC4 are compared for the conditions shown. j, Averaged data are compared (\( > 45 \) cells for each condition). k, Histogram showing \( c\)-\( fos \) expression to thapsigargin in the presence of PKC blockers. All thapsigargin-treated groups were significantly different from control (\( P < 0.001 \)) but were not significantly different from one another. l, \( \text{Ca}^{2+} \) signals to thapsigargin are unaffected by PKC block.

m, Downregulation of PKC (PMA; 500 nM, 24 h) reduces LTC4- but not thapsigargin-induced \( c\)-\( fos \) expression (data from four independent experiments). All stimulated groups were significantly different from control (\( P < 0.001 \)). For LTC4, the PMA group was different from the LTC4 control (\( P < 0.001 \)). For thapsigargin, the PMA group was not different from the thapsigargin control (\( P = 0.07 \)). Ctrl, control. n, PKC downregulation alters the LTC4-activated \( \text{Ca}^{2+} \) signal. Error bars show data \( \pm \) s.e.m. NS, not significant.

Cytoplasmic \( \text{Ca}^{2+} \) inhibits CRAC channels through mechanisms of fast and slow inactivation. The prolonged \( \text{Ca}^{2+} \) release evoked by LTC4 in the presence of non-desensitizing receptors could therefore inactivate CRAC channels to suppress agonist-evoked gene expression. In support of this, accumulation of the slow \( \text{Ca}^{2+} \) chelator EGTA in the cytoplasm rescued gene expression to CysLT1 receptor activation in the presence of PKC block (Fig. 2c, d). \( \text{Ca}^{2+} \)-dependent fast inactivation of CRAC channels is unlikely to contribute here because (1) it is unaffected by the slow chelator EGTA, which reverses the inhibitory effects of PKC block (Fig. 2c, d), and (2) the rate and extent of fast inactivation were unaltered by CysLT1 receptor activation in the presence of PKC downregulation (Fig. 3a). Instead, \( \text{Ca}^{2+} \)-dependent slow inactivation is likely to be the dominant mechanism because (1) it too is suppressed by cytoplasmic EGTA (Fig. 2c, d), and (2) the \( \text{Ca}^{2+} \)-dependence of slow inactivation has a dissociation constant (\( K_d \)) of \( \sim 0.5 \mu \text{M} \) and full block occurs at \( \sim 1 \mu \text{M} \) (Fig. 3b), which is similar to the peak \( \text{Ca}^{2+} \) rise evoked by LTC4 in the presence of PKC inhibitors or following downregulation of PKC (0.87 \( \pm \) 0.1 \( \mu \text{M} \)). 

If prolonged \( \text{Ca}^{2+} \) release to non-desensitizing CysLT1 receptors leads to slow inactivation of CRAC channels, then development of a calcium-release-activated calcium current (\( I_{\text{CRAC}} \)) to a subsequent stimulus should be impaired. Pre-activation of CysLT1 receptors reduced \( I_{\text{CRAC}} \) evoked by thapsigargin but only in the presence of PKC block (Fig. 3c). No such inhibitory effect was seen when cells were dialysed with a strongly buffered \( \text{Ca}^{2+} \)-containing pipette solution, which prevents the development of slow inactivation (Fig. 3d). Increasing the time between the termination of \( \text{Ca}^{2+} \) release and...
human nasal polyp, which is rich in mast cells. The polyp and associated nasal mucosa are largely self-contained, providing an excellent in vivo system. Mast cells from polyps, acutely isolated and -asso-
cells.

Our attempts to express the PKC-insensitive CysLT1 receptor, in which S313, S315 and S316 had been mutated to alanines, were thwarted by the difficulty of expressing these receptors, although in a few cells we observed that Ca\textsuperscript{2+} oscillations to LTC\textsubscript{4} were observed in the presence of G06983. Both LTC\textsubscript{4} and ionomycin were applied in Ca\textsuperscript{2+}-free external solution. Inset compares the kinetics of Ca\textsuperscript{2+} release. b, Cytosolic GFP–PH levels, a measure of InsP\textsubscript{3} levels, rise when CysLT1 receptors are stimulated in the presence of G06983. c, Top, loading cells with the Ca\textsuperscript{2+} chelator EGTA prevents loss of gene expression to agonist when PKC is blocked. Bottom, aggregate data from five independent gels are summarized. Error bars show data ± s.e.m. NS, not significant.

subsequent store-operated Ca\textsuperscript{2+} entry should enable some recovery from Ca\textsuperscript{2+}-dependent slow inactivation and this should partially rescue gene expression. When Ca\textsuperscript{2+} influx was evoked a few minutes after Ca\textsuperscript{2+} release, significant, albeit incomplete, rescue of Ca\textsuperscript{2+} entry (Fig. 3e) and c-fos transcription (Fig. 3f) occurred in cells stimulated with LTC\textsubscript{4} in the presence of PKC inhibition. Hence, allowing CRAC channels time to recover from Ca\textsuperscript{2+}-dependent inactivation results in partial rescue of agonist-driven gene expression.

Our attempts to express the PKC-insensitive CysLT1 receptor, in which S313, S315 and S316 had been mutated to alanines, were thwarted by the difficulty of expressing these receptors, although in a few cells we observed that Ca\textsuperscript{2+} oscillations to LTC\textsubscript{4} were less frequent (3.1 ± 0.5 versus 5.4 ± 0.4, 4 and 6 cells, respectively) and the initial spike was a little broader (~1.25-fold) than mock-transfected cells.

To place our findings in a physiological context, we turned to the human nasal polyp, which is rich in mast cells. The polyp and associated nasal mucosa are largely self-contained, providing an excellent quasi in vivo human system. Mast cells from polyps, acutely isolated from patients undergoing surgery, respond to LTC\textsubscript{4} and express functional CRAC channels. Stimulation with LTC\textsubscript{4} activated c-Fos protein expression in mast cells isolated from polyps (Fig. 3g) and this was reduced by pre-treatment with either calphostin C or G06983 (Fig. 3h). PKC inhibitors had no inhibitory effect when thapsigargin was used instead.

Western blots revealed the presence of Ca\textsuperscript{2+}-dependent PKC-α, -β and -ε isoforms but only faint expression of PKC-δ and -ε (Fig. 4a).

Overnight PMA exposure significantly reduced PKC-α and -β expression, but not PKC-δ (Fig. 4a, b). Their weak expression made PKC-δ and -ε difficult to quantify. Confocal microscopy studies confirmed robust expression of PKC-α, -β and -ε (Fig. 4e), with barely detectable levels of PKC-δ and -ε (data not shown). Overnight PMA exposure significantly reduced PKC-α and -β but not -ε at the cellular level (Fig. 4d). Knockdown of PKC-α using a targeted short interfering RNA (siRNA) approach (Fig. 4e) resulted in a broadening of the first Ca\textsuperscript{2+} oscillation evoked by LTC\textsubscript{4}, indicative of less receptor desensitization, and fewer Ca\textsuperscript{2+} oscillations in each cell (Supplementary Fig. 7). Knockdown of PKC-β had a much weaker effect on the Ca\textsuperscript{2+} oscillations (Supplementary Fig. 7). Knockdown of PKC-α or PKC-β plus -β simultaneously, but not PKC-β alone, reduced LTC\textsubscript{4}-driven
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Figure 4 | PKC-α regulates CysLT1 receptor-driven c-fos transcription. a. Expression of PKC-α, β and γ (western blot) is shown in control cells and cells exposed to PMA for 24 h. Quantification of data from three independent experiments, as in a, c, shows that PKC-α expression is unaffected by PMA. In contrast, PMA decreases PKC-β and PKC-γ expression. b. Knockdown of PKC-β and PKC-α plus γ on LTC4-dependent c-fos expression. Data are shown as mean and standard error of the mean. c. Knockdown of PKC-α, β and PKC-α plus γ on LTC4-dependent c-fos expression. Data are shown as mean and standard error of the mean. d. Knockdown of PKC-α, β and PKC-α plus γ on LTC4-dependent c-fos expression. Data are shown as mean and standard error of the mean. e. Knockdown of PKC-α, β and PKC-α plus γ on LTC4-dependent c-fos expression. Data are shown as mean and standard error of the mean. f. Knockdown of PKC-α, β and PKC-α plus γ on LTC4-dependent c-fos expression. Data are shown as mean and standard error of the mean.

METHODS SUMMARY

Cultured RBL-1 cells and acutely isolated human nasal polyps were prepared as described previously. Transfection with siRNA constructs was achieved using the AMAXA system. Patch-clamp recordings and cytoplasmic Ca2+ measurements followed standard methods. RT–PCR, western blotting and confocal microscopy were carried out as described previously. Error bars are s.e.m.

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Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Contributions S.-W.N. performed and analysed Ca2+ measurements, RT–PCR, western blots and siRNA knockdown. D.B. performed and analysed patch-clamp recordings. C.N. carried out immunocytochemistry. R.M. helped with Ca2+ measurements. R.A. and G.B. supplied human nasal polyps. A.B.P. carried out some patch-clamp experiments, discussed the results, supervised the project and wrote the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to A.B.P. (anant.parekh@dpag.ox.ac.uk).
METHODS

Cell culture and transfection. RBL-1 cells were bought from ATCC and were cultured in 5% CO2 in Dulbecco’s modified Eagle medium with 10% fetal bovine serum, 2 mM t-glutamine and penicillin-streptomycin, as previously described24. Cells were transfected with siRNA against Syk (purchased from Invitrogen; 5’-3’ sense, CCCUCUCCAGCUAGUGGAAACUAU; antisense, UAAUGUCUCAGUCUGCCAGGG) using the Amaxa nucleofection system24. siRNA against PKC isozymes were purchased from Invitrogen; PKC-α sense, GCGACACCUUGGCAUAAGAAGUUA; antisense, UGAACAUCUACUGGAGGGUGUG; PKC-β sense, GCCUAUUGCAACACCCAGGCC AA; antisense, UUGCCUGGGGUUGUCUGAUUAGCC. Cells were used 36-48 h after plating.

Human tissue. Human polyps were removed from patients with full ethical consent and with approval from the National Research Ethics Service (REC number 07/H0607/104). Polyps were treated as described6 and used within 6 h of surgical removal.

ICRAC recordings. Patch-clamp experiments were conducted in the tight-seal whole-cell configuration at room temperature (20–24°C as previously described24). Sylgard-coated, fire-polished pipettes had resistances of 4.2–5.5 MΩ when filled with standard internal solution that contained (in mM): CsCl 145, MgCl2 1, Mg-ATP 2, EGTA 10, CaCl2 4.6 mM (free Ca2+ ~140 nM), HEPES 10, pH 7.2 with NaOH. In some experiments, weak Ca2+-free solution had the following composition (in mM): NaCl 145, KCl 2.8, MgCl2 2, D-glucose 10, HEPES 10, EGTA 0.1, pH 7.4 with NaOH. ICRAC was measured by applying voltage ramps (~100 to +100 mV in 50 ms) at 0.5 Hz from a holding potential of 0 mV. For fast inactivation, step pulses (250 ms duration) were applied from 0 mV to ~100 mV every 2 s. Currents were filtered using an 8-pole Bessel filter at 2.5 kHz and digitized at 100 μs. Currents were normalized by dividing the amplitudes (measured from the voltage ramps at ~80 mV) by the cell capacitance. Capacitative currents were compensated before each ramp by using the automatic compensation of the EPC-9/2 (HEKA Elektronik) patch-clamp amplifier. For ICRAC, leak currents were subtracted by averaging 2–3 ramp currents obtained just before ICRAC had started to develop, and then subtracting this from all subsequent currents.

Ca2+ imaging. Ca2+ imaging experiments were carried out at room temperature (21–24°C) using the IMAGO CCD camera-based system from TILL Photonics, as described previously24. Cells were alternately excited at 356 and 380 nm (20 ms exposures) and images were acquired every 2 s. Images were analysed offline using IGOR Pro for Windows. Cells were loaded with Fura 2-AM (2 μM) for 40 min at room temperature in the dark and then washed three times in standard external solution of composition (in mM): NaCl 145, KCl 2.8, MgCl2 2, d-glucose 10, HEPES 10, pH 7.4 with NaOH. Cells were left for 15 min to allow further de-esterification. Ca2+-free solution had the following composition (in mM): NaCl 145, KCl 2.8, MgCl2 2, d-glucose 10, HEPES 10, EGTA 0.1, pH 7.4 with NaOH.

EGTA-AM loading. Cells were loaded with EGTA by incubation for 45 min with EGTA-AM as described6.

Confocal microscopy. Cells were fixed in 4% paraformaldehyde in phosphate buffer for 30 min at room temperature. All the washes used 0.01% PBS (137 mM NaCl, 2.7 mM KCl, 8 mM NaH2PO4, 1 mM KH2PO4). The cells were blocked with 2% BSA and 10% goat serum for 1 h. Anti-c-Fos, -PKC-δ, -PKC-ε, and -PKC-ζ antibodies were used in carrier (0.2% BSA, 1% goat serum) and left overnight at 4°C and were purchased from Cell Signalling. Anti-PKC-α and -β were used in carrier (0.2% BSA, 1% goat serum) and left overnight at 4°C and were purchased from Santa Cruz. The secondary anti-rabbit IgG was a Handll chain-specific (goat) fluorescein conjugate (Alexa Fluor 568, excitation at 578 nm, emission at 603 nm wavelength) from Invitrogen. This was used in PBS for 2 h at room temperature. The cells were mounted in Vectashield mounting medium. Images were obtained using a Leica confocal microscope, as described6.

Western blotting. Total cell lysates (50 μg) were separated by SDS–PAGE on a 10% gel and electrophoretically transferred to nitrocellulose membrane, as described24. Membranes were blocked with 5% non-fat dry milk in TBS plus 0.1% Tween 20 (TBST) buffer for 1 h at room temperature. Membranes were washed with TBST three times and then incubated with primary antibody overnight at 4°C. Anti-PKC-δ, -ε and -ζ antibodies were obtained from New England Biolabs and used at 1:2,500 dilution. Anti-PKC-α, -β and total ERK2 antibodies were purchased from Santa Cruz Biotechnology. PKC-α and -β antibodies were used at 1:2,500 dilution and total ERK antibody was used at a dilution of 1:5,000. The memranes were then washed with TBST again and incubated with a 1:2,500 dilution of goat anti-rabbit secondary antibody IgG from Santa Cruz Biotechnology for 1 h at room temperature. After washing with TBST, the bands were developed for visualization using ECL-plus western blotting detection system (GE Healthcare). Gels were quantified using the UN-SCAN-IT software package (Silk Scientific). Total ERK2 is widely used as a control for gel loading. The antibody does not discriminate between phosphorylated (and hence active) and non-phosphorylated ERK2 and therefore detects the total amount of this protein, regardless of whether the kinase has been activated. The extent of PKC was therefore normalized to the total amount of ERK2 present in each lysate, to correct for any differences in amount of cells used for each experiment.

RT-PCR. Total RNA was extracted from RBL cells by using an RNeasy Mini Kit (Qiagen), as described26. RNA was quantified spectrophotometrically by absorbance at 260 nm. Total RNA (1 μg) was reverse-transcribed using the iScriptTM cDNA Synthesis Kit (Bio-Rad), according to the manufacturer’s instructions. Following cDNA synthesis, PCR amplification was then performed using BIO-X-ACTM. Short DNA Polymerase (Bioline) with primers specific for the detection of c-fos were synthesized by Invitrogen. The PCR products were electrophoresed through an agarose gel and visualized by ethidium bromide staining.

Statistics. Results are presented as means ± s.e.m. Statistical significance was assessed using Student’s t-test for comparison between two groups or ANOVA followed by a post-hoc Newman Keuls multiple comparison test for the difference between groups and results were considered significant at *P<0.05, **P<0.01.

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