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Cysteinyl leukotriene type I receptor desensitization sustains Ca^{2+}-dependent gene expression

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Abstract

A universal mechanism to turn off a biological response is receptor desensitization, where 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/second messenger pathway, followed by receptor internalization[1]. G protein-coupled cysteinyl leukotriene type I (CysLT1) receptors regulate immune cell function and the receptor is an established therapeutic target for allergies including asthma[2].

Desensitization of these receptors arises predominantly from protein kinase C-dependent phosphorylation of three serine residues in the receptor C-terminus[3]. Physiological concentrations of the receptor agonist LTC_4 evoke repetitive cytoplasmic Ca^{2+} oscillations, reflecting regenerative Ca^{2+} release from stores that is sustained by Ca^{2+} entry through store-operated 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. 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. 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.

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

Stimulation of RBL-1 cells with LTC_4, acting exclusively on CysLT1 receptors[7,8] (Supplementary Fig. 1), led to cytoplasmic Ca^{2+} signals (Fig. 1a) followed by robust expression of c-fos at both mRNA (Fig. 1b-c)[4] and protein levels (Fig. 1d-e). Maximal

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Author contributions.
S.N. performed and analysed Ca^{2+} 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 Ca^{2+} measurements. R.A. and G.B. supplied human nasal polyps. A.P. carried out some patch clamp experiments, discussed the results, supervised the project and wrote the paper.

Materials and Methods (see Supplementary Information for more details).
Cultured RBL-1 cells and acutely isolate human nasal polyps were prepared as described previously[7]. Transfection with siRNA constructs was achieved using the AMAXA system. Patch clamp recordings and cytoplasmic Ca^{2+} measurements followed standard methods[7]. RT-PCR, western blotting and confocal microscopy were carried out as described previously[5]. Error bars are s.e.m.

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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[4,5]. Thapsigargin led to a larger, more sustained Ca^{2+} signal than LTC_4 (Fig. 1a, see also[4,5]) and the rate of Ca^{2+} entry through CRAC channels was ~ 2-fold more for thapsigargin than LTC_4 (Fig. 1f), consistent with patch clamp recordings[7]. 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 striking differences in the extent of CRAC channel activation. These included i) CysLT1 receptors tapped into a different signalling mechanism linking CRAC channel microdomains to c-fos expression; ii) Local Ca^{2+} entry through CRAC channels was larger following receptor activation because LTC_4 hyperpolarized the membrane potential and iii) 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 following physiological levels of receptor stimulation.

Experiments described in Fig. 1g-h revealed an important role for protein kinase C (PKC) in receptor-dependent gene expression. The structurally distinct PKC blockers G06983 and calphostin C abolished c-fos expression (Figure 1g-h). PKC block had a dramatic effect on the Ca^{2+} signal evoked by agonist. Whereas cytoplasmic Ca $^{2+}$ oscillations were routinely observed with LTC_4 (Figure 1i), the response was converted into a large, single, slowly decaying Ca^{2+} spike after PKC inhibition (Fig. 1i,j). Acute stimulation with PMA in the absence of LTC_4 failed to induce significant c-fos expression (data not shown)[9], 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, that raise local Ca^{2+} 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 where desensitization is mediated predominantly by PKC[3] and prevention of desensitization leads to broader Ca^{2+} signals[12]. Inhibition of CysLT1 receptor desensitization is predicted to lead to greater $InsP_3$ production and hence more extensive Ca^{2+} store emptying. Several findings are consistent with this. First, Ca^{2+} release to LTC_4 lasted ~ 5 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[13], was

substantially less after activation of CysLT1 receptors in the presence of PKC block than in control cells (Fig. 2a). Third, $InsP_3$ production, measured using the GFP-PH construct[14], increased to a greater extent when PKC was inhibited (Fig. 2b).

Cytoplasmic Ca^{2+} inhibits CRAC channels through mechanisms of fast and slow inactivation[15]. The prolonged Ca^{2+} release evoked by LTC_4 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 Ca^{2+} chelator EGTA in the cytoplasm rescued gene expression to CysLT1 receptor activation in the presence of PKC block (Fig 2c-d). Ca^{2+}-dependent fast inactivation of CRAC channels is unlikely to contribute here because i) it is unaffected by the slow chelator EGTA[16,17], which reversed the inhibitory effects of PKC block (Fig. 2c-d) and ii) the rate and extent of fast inactivation was unaltered by CysLT1 receptor activation in the presence of PKC downregulation (Fig. 3a). Instead, Ca^{2+}-dependent slow inactivation is likely to be the dominant mechanism because: i) it too is suppressed by cytoplasmic EGTA[18,19]; ii) the Ca^{2+}-dependence of slow inactivation has a K_D of ~0.5 μM and full block occurs at ~ 1 μM (Fig. 3b), which is similar to the peak Ca^{2+} rise evoked by LTC_4 in the presence of PKC inhibitors or following downregulation of PKC (0. 87±01μM); iii) Ca^{2+}-dependent slow inactivation develops with a time course similar to the duration of the prolonged Ca^{2+} rise seen to LTC_4 following loss of PKC activity[18,19].

If prolonged Ca^{2+} release to non-desensitizing CysLT1 receptors leads to slow inactivation of CRAC channels, then development of I_{CRAC} to a subsequent stimulus should be impaired. Pre-activation of CysLT1 receptors reduced I_{CRAC} evoked by thapsigagin but only in the presence of PKC block (Fig. 3c). No such inhibitory effect was seen when cells were dialysed with a strongly buffered Ca^{2+}-containing pipette solution, which prevents the development of slow inactivation (Fig. 3d). Increasing the time between the termination of Ca^{2+} release and subsequent store-operated Ca^{2+} entry should enable some recovery from Ca^{2+}-dependent slow inactivation and this should partially rescue gene expression. When Ca^{2+} influx was evoked a few minutes after Ca^{2+} release, significant, albeit incomplete, rescue of Ca^{2+} entry (Fig. 3e) and c-fos transcription (Fig. 3f) occurred in cells stimulated with LTC_4 in the presence of PKC inhibition. Hence allowing CRAC channels time to recover from Ca^{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[3], although in a few cells we observed that Ca^{2+} oscillations to LTC_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[20]. 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_4 and express functional CRAC channels[7,8]. Stimulation with LTC_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^{2+}-dependent PKCα, β and ζ isozymes[11,21] but only faint expression of PKCδ and ε (Fig. 4a). Overnight PMA exposure significantly reduced PKCα and β expression, but not ζ (Figs 4a and b). Their weak expression made

PKCδ and ε difficult to quantify. Confocal microscopic studies confirmed robust expression of PKCα, β and ζ (Fig. 4c), with barely detectable levels of δ and ε (data not shown). Overnight PMA exposure significantly reduced PKCα and β but not ζ at the cellular level (Fig. 4d). Knock down of PKCα using a targeted siRNA approach (Fig. 4e) resulted in a broadening of the first Ca^{2+} oscillation evoked by LTC_4, indicative of less receptor desensitization, and fewer Ca^{2+} oscillations in each cell (Supplementary Fig. 7). Knockdown of PKCβ had a much weaker effect on the Ca^{2+} oscillations (Supplementary Fig. 7). Knockdown of PKCα or PKC α plus β simultaneously, but not PKCβ alone, reduced LTC_4-driven c-fos expression to an extent similar to that seen in following overnight PMA treatment (Fig. 4f). Stimulation of non-G protein coupled FCεRI receptors in RBL-2H3 cells activates c-fos expression primarily through PKCδ and ε[22]. Although it is possible that these PKC isoforms also contribute to gene expression under our conditions, our results nevertheless suggest a major role for PKCα in G-protein coupled receptor desensitization, and thus coupling to the nucleus.

Collectively, our findings reveal a counter-intuitive function for desensitization of a phospholipase C-coupled receptor. Rather than terminating a response, homologous receptor desensitization is essential for maintaining excitation-transcription coupling. Desensitization of CysLT1 receptors is mediated principally by PKC-dependent phosphorylation[3]. Prevention of receptor desensitization through either acute block or degradation of protein kinase C or after knockdown of PKCα all led to loss of Ca^{2+}-dependent gene expression, despite potentiation of Ca^{2+} release to agonist. Mechanistically, the prolonged Ca^{2+} release phase accelerated Ca^{2+}-dependent slow inactivation of CRAC channels, resulting in loss of Ca^{2+} entry. Because Ca^{2+} microdomains near open CRAC channels drive c-fos expression, the decline in CRAC channel activity abolishes excitation-transcription coupling. The interval between Ca^{2+} oscillations following CysLT1 receptor activation is ~ 25 seconds[4]. Since $InsP_3$ has a short half-life in the cytoplasm (~ 1 second)[23], receptor desensitization will presumably lower $InsP_3$ levels during the interspike interval. Store refilling will occur quickly and CRAC channel activity will be transient following CysLT1 receptor stimulation. The short duration of Ca^{2+} release and thus Ca^{2+} entry, determined by receptor desensitization, will ensure Ca^{2+}-dependent slow inactivation does not develop, since this inhibitory mechanism requires a sustained Ca^{2+} rise for several seconds. It is therefore the kinetics of receptor desensitization and recovery from desensitization within a highly Ca^{2+} sensitive and high gain system that ensures bursts of store-operated Ca^{2+} entry occur that are sufficient for the activation of c-fos expression, without the build-up of the Ca^{2+}-dependent slow inactivation pathway that would abolish the response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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APPENDIX

MATERIALS AND METHODS

Cell Culture and Transfection

Rat basophilic leukemia (RBL-1) cells were bought from ATCC and were cultured (37 °C, 5% CO_2) in Dulbecco's modified Eagle medium with 10% fetal bovine serum, 2 mM L-

glutamine and penicillin-streptomycin, as previously described[24]. Cells were transfected with siRNA against Syk (purchased from Invitrogen; 5′-3′ Sense: CCCUCUGGCAGCUAGUGGAACAUUA, Antisense: UAAUGUUCCACUAGCUGCCAGAGGG) using the Amaxa nucleofection system[25]. siRNA against protein kinase C isozymes were purchased from from Invitrogen;

PKCα

Sense: GCGACACCUGCGACAUGAAUGUUCA

Antisense; UGAACAUUCAUGUCGCAGGUGUCGC

PKCβ

Sense: GGCUAAUGACCAAACACCCAGGCAA

Antisense: UUGCCUGGGUGUUUGGUCAUUAGCC

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 described[26] and used within 6 hours of surgical removal.

I_{CRAC} recordings

Patch-clamp experiments were conducted in the tight-seal whole-cell configuration at room temperature (20-24$_0$C) as previously described[24,26]. Sylgard-coated, fire-polished pipettes had d.c. resistances of 4.2-5.5MΩ when filled with standard internal solution that contained (in mM): Cs_+ glutamate 145, NaCl 8, $MgCl_2$ 1, Mg-ATP 2, Ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N',-tetraacetic acid (EGTA) 10, $CaCl_2$ 4.6 mM (free Ca_{2+} ~ 140 nM), HEPES 10, pH 7.2 with CsOH. In some experiments, weak Ca_{2+} buffer was used instead (0.2 mM EGTA and no added $CaCl_2$). A correction of +10 mV was applied for the subsequent liquid junction potential that arose from this glutamate-based internal solution. The composition of the extracellular solution was (in mM): NaCl 145, KCl 2.8, $CaCl_2$ 10, $MgCl_2$ 2, CsCl 10, D-glucose 10, HEPES 10, pH 7.4 with NaOH.

I_{CRAC} was measured by applying voltage ramps (−100 to +100 mV in 50 msec) at 0.5 Hz from a holding potential of 0 mV. For fast inactivation, step pulses (250 msec duration) were applied from 0 mV to −100 mV every 2 seconds. Currents were filtered using an 8-pole Bessel filter at 2.5 kHz and digitised at 100 μs. Currents were normalised 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 amplifier. For I_{CRAC}, leak currents were subtracted by averaging 2-3 ramp currents obtained just before I_{CRAC} had started to develop, and then subtracting this from all subsequent currents.

Ca_{2+} imaging

Ca_{2+} imaging experiments were carried out at room temperature using the IMAGO CCD camera-based system from TILL Photonics, as described previously[25]. Cells were alternately excited at 356 and 380 nm (20 msec exposures) and images were acquired every 2 seconds. Images were analysed offline using IGOR Pro for Windows. Cells were loaded with Fura 2-AM (2 μM) for 40 minutes at room temperature in the dark and then washed

three times in standard external solution of composition (in mM) NaCl 145, KCl 2.8, $CaCl_2$ 2, $MgCl_2$ 2, D-glucose 10, HEPES 10, pH 7.4 with NaOH. Cells were left for 15 minutes to allow further deesterification. Ca_{2+}-free solution had the following composition (in mM) NaCl 145, KCl 2.8, $MgCl_2$ 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 minutes with EGTA-AM as described[27].

Confocal microscopy

Cells were fixed in 4% paraformaldehyde in phosphate buffer for 30 min at room temperature. All the washes used 0.01% PBS (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4). The cells were blocked with 2% BSA and 10% goat serum for 1 h. Anti-c-fos, -protein kinase Cδ, ε and ζ were used in carrier (0.2% BSA, 1% goat serum) and left overnight at 4°C and were purchased from Cell Signalling (Boston, MA, USA). Anti-protein kinase Cα 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 HandL chain specific (goat) fluorescein conjugate (Alexa Fluor 568, excitation at 578 nm, emission at 603 nm wavelength) from Invitrogen, Paisley, UK. 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 described[27].

Western blotting

Total cell lysates (50 μg) were separated by SDS-PAGE on a 10% gel and electrophoretically transferred to nitrocellulose membrane, as described$_2$. Membranes were blocked with 5% nonfat dry milk in TBS plus 0.1% Tween 20 (TBST) buffer for 1 hour at room temperature. Membranes were washed with TBST three times and then incubated with primary antibody overnight at 4 °C. Anti-PKCδ, ε and ζ antibodies were obtained from New England Biolabs and used at 1:2500 dilution. Anti-PKCα, β and total ERK2 antibodies were purchased from Santa Cruz Biotechnology. PKCα and β antibodies were used at 1:2500 dilution and total ERK antibody was used at a dilution of 1:5000. The membranes were then washed with TBST again and incubated with a 1:2500 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, West Sussex, UK), as described$_{2,4}$. RNA was quantitated spectrophotometrically by absorbance at 260 nm. Total RNA (1 μg) was reverse-transcribed using the iScript$_{TM}$ cDNA Synthesis Kit (Bio-Rad, Hemel Hempstead, UK), according to the manufacturer's instructions. Following cDNA synthesis, PCR amplification was then performed using BIO-X-ACT$_{TM}$. Short DNA Polymerase (Bioline, London, UK) with primers specific for the detection of c-fos were synthesized by Invitrogen, UK. The PCR products were electrophoresed through an agarose gel and visualized by ethidium bromide staining.

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Statistics

Results are presented as means ± S.E.M. Statistical significance was assessed using Student's t test for comparison between two groups or analysis of variance (ANOVA) followed by a post hoc Newman Keuls multiple comparison test for the difference between groups and considered significant at $p < 0.05$ (*); $p<0.01$ (**).

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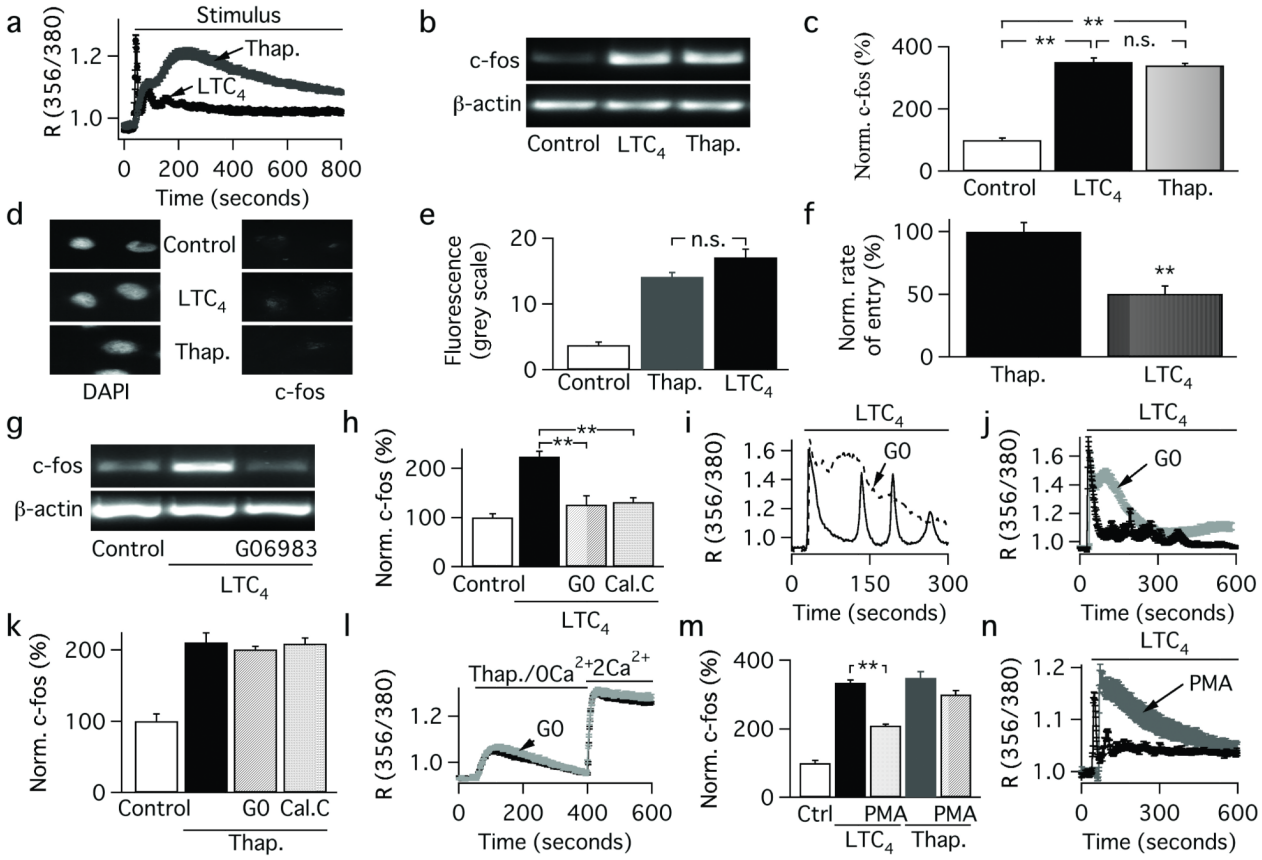

Figure 1. CysLT1 receptor-dependent c-fos expression requires PKC

a, Averaged Ca^{2+} signals to LTC_4 and thapsigargin are compared (>50 cells per graph). b, c-fos expression is compared between control (non-stimulated), 160 nM LTC_4- and 2 μM thapsigargin-stimulated cells. Stimulus was present for 8 minutes. c, Histograms show averaged responses from 3 independent experiments. LTC_4 and thapsigargin groups were different from control ($p<0.001$), but not from one another ($p>0.3$; Anova). d, Ca^{2+} entry rate was measured following readmission of Ca^{2+} to cells stimulated with LTC_4 or thapsigagin in Ca^{2+}-free solution (* denotes $p<0.01$). e, Cells stained with antibody against c-fos protein. f, Aggregate data are compared (n>20 per bar). Thapsigargin and LTC_4 groups were different from control ($p<0.001$) but not from one another ($p=0.11$). g, G06983 (1 μM; 10 min pre-treatment) suppresses LTC_4-induced c-fos expression. h, Histogram comparing the effects of PKC blockers. LTC_4 control group (LTC_4 in absence of PKC block) was different from the other groups ($p<0.01$). There were no significant differences between the other groups. G0 is G06983. i, Single-cell Ca^{2+} signals to LTC_4 are compared for the conditions shown. j Averaged data is compared (> 45 cells for each condition). k, Histogram showing c-fos expression to thapsigargin in 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, Ca^{2+} signals to thapsigargin are unaffected by PKC block. m, Downregulation of PKC (PMA; 500 nM, 24 hours) reduces LTC_4- but not thapsigargin-induced c-fos expression (data from 4 independent experiments). All stimulated groups were significantly different from control ($p<0.01$). For LTC_4 the PMA group was different from the LTC_4 control ($p<0.01$). For thapsigargin, the PMA groups was not different from the thapsigargin control ($p=0.07$). n, PKC downregulation alters the LTC_4-evoked Ca^{2+} signal.

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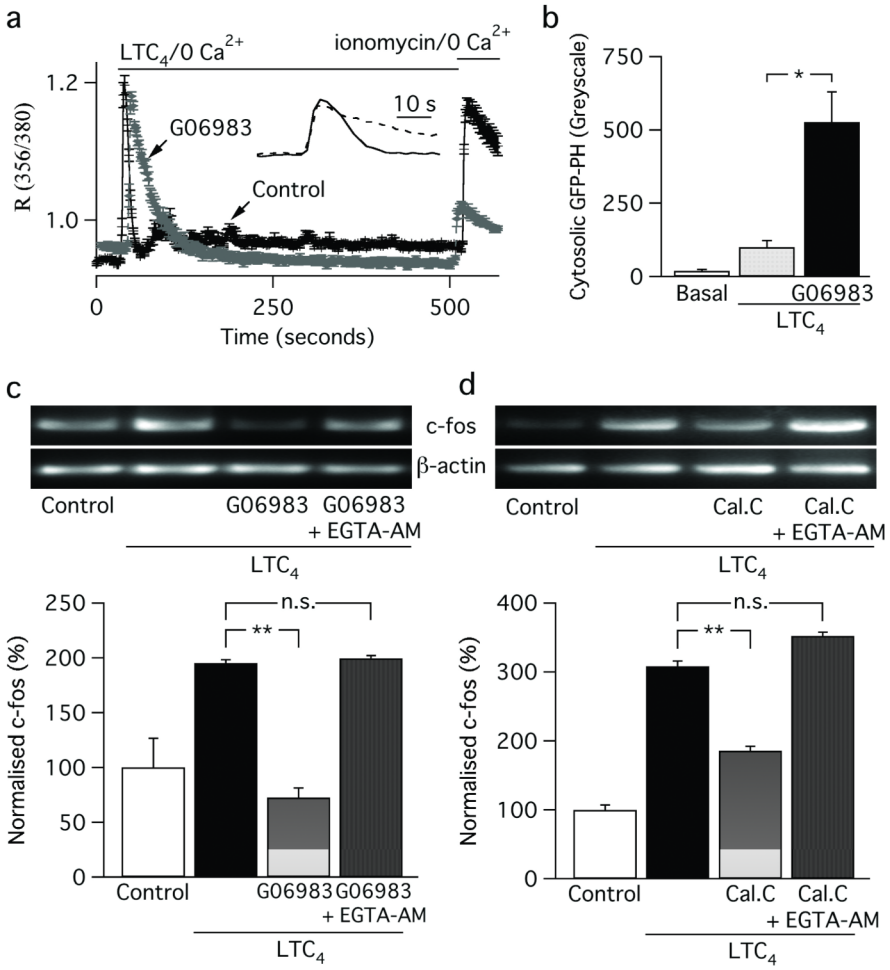

Figure 2. Gene expression to non-desensitizing CysLT1 receptors is rescued by preventing a cytoplasmic Ca^{2+} rise

a, Stimulation with LTC_4 in the presence of G06983 evokes a more sustained Ca^{2+} release response, and this leads to more extensive store depletion (measured through the extent of Ca^{2+} release evoked by 5 μM ionomycin). Both LTC_4 and ionomycin were applied in Ca^{2+}-free external solution. Inset compares the kinetics of Ca^{2+} release. b, Cytosolic GFP-PH levels, a measure of $InsP_3$ levels, rise when CysLT1 receptors are stimulated in the presence of G06983. c, Upper panel, loading cells with the Ca^{2+} chelator EGTA prevents loss of gene expression to agonist when PKC is blocked. Lower panel, aggregate data from five independent gels are summarised. d as in c, but calphostin C was used to block PKC instead. Lower panel, aggregate data from three independent gels are summarised.

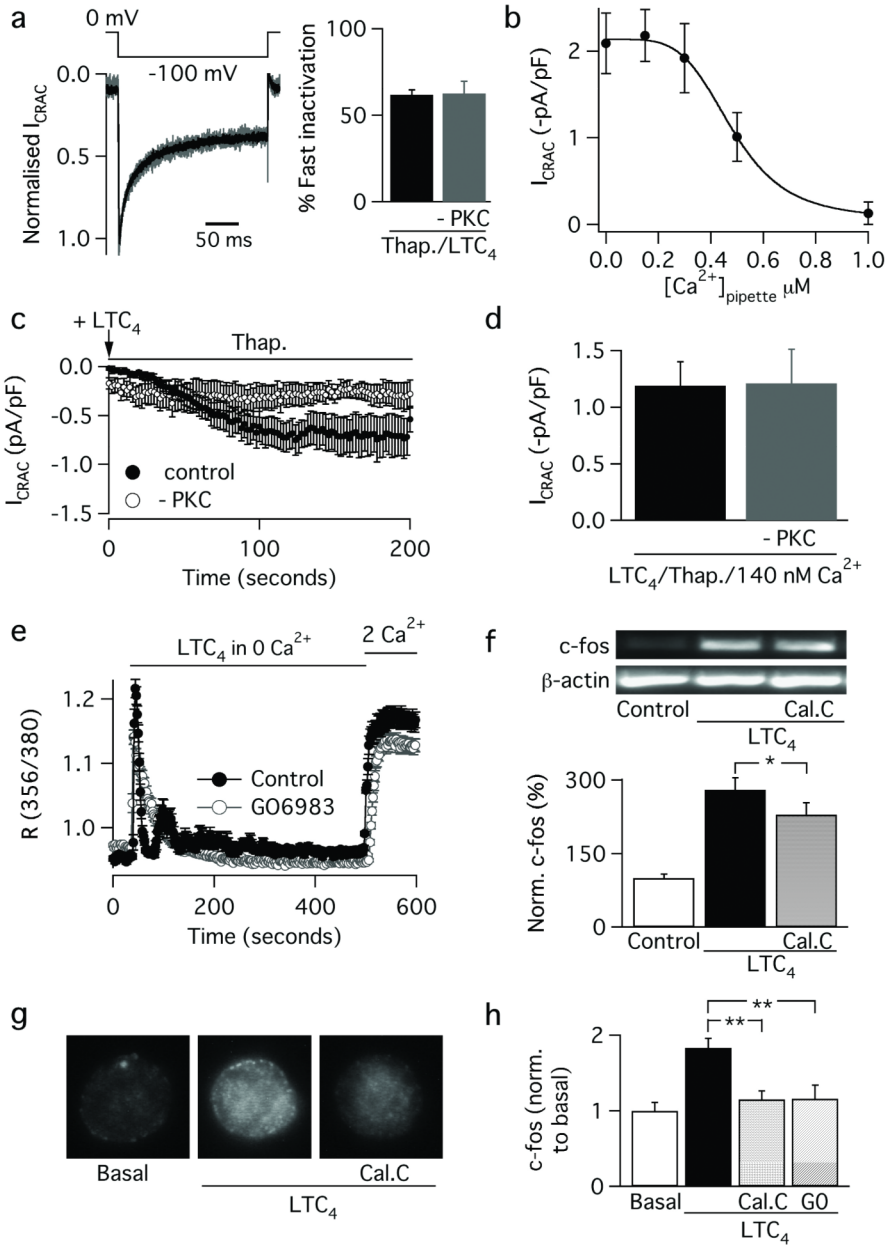

Figure 3. Ca^{2+}-dependent slow inactivation underlies suppression of c-fos expression to non-desensitizing CysLT1 receptors

a, Ca^{2+}-dependent fast inactivation is unaffected by non-desensitizing receptors (labelled – PKC). Cells were stimulated with LTC_4 (160 nM) prior to breaking in with a pipette solution containing thapsigargin and buffered Ca^{2+} (140 nM) and fast inactivation was measured within 60 seconds of break-in. b, dependence of Ca^{2+}-dependent slow inactivation on patch pipette Ca^{2+} concentration. c, Stimulation of non-desensitizing receptors with LTC_4 prior to break-in significantly reduced the size of I_{CRAC} that developed in response to dialysis with thapsigargin in weak buffer (0.2 mM EGTA). d, as c, but cells were dialysed with a pipette solution containing strong Ca^{2+} buffer (10 mM EGTA, 140 nM free Ca^{2+}). e, Store-operated Ca^{2+} entry recovers partially by increasing the time interval between Ca^{2+} release and subsequent Ca^{2+} entry. f, c-fos expression to non-desensitizing receptor stimulation is rescued partially when Ca^{2+} entry occurs several minutes after Ca^{2+} release

has reached completion. g, c-fos expression in human nasal mast cells after CysLT1 receptor activation is suppressed by PKC inhibition. h, Aggregate data is compared (12-17 cells per bar; 3 patients each).

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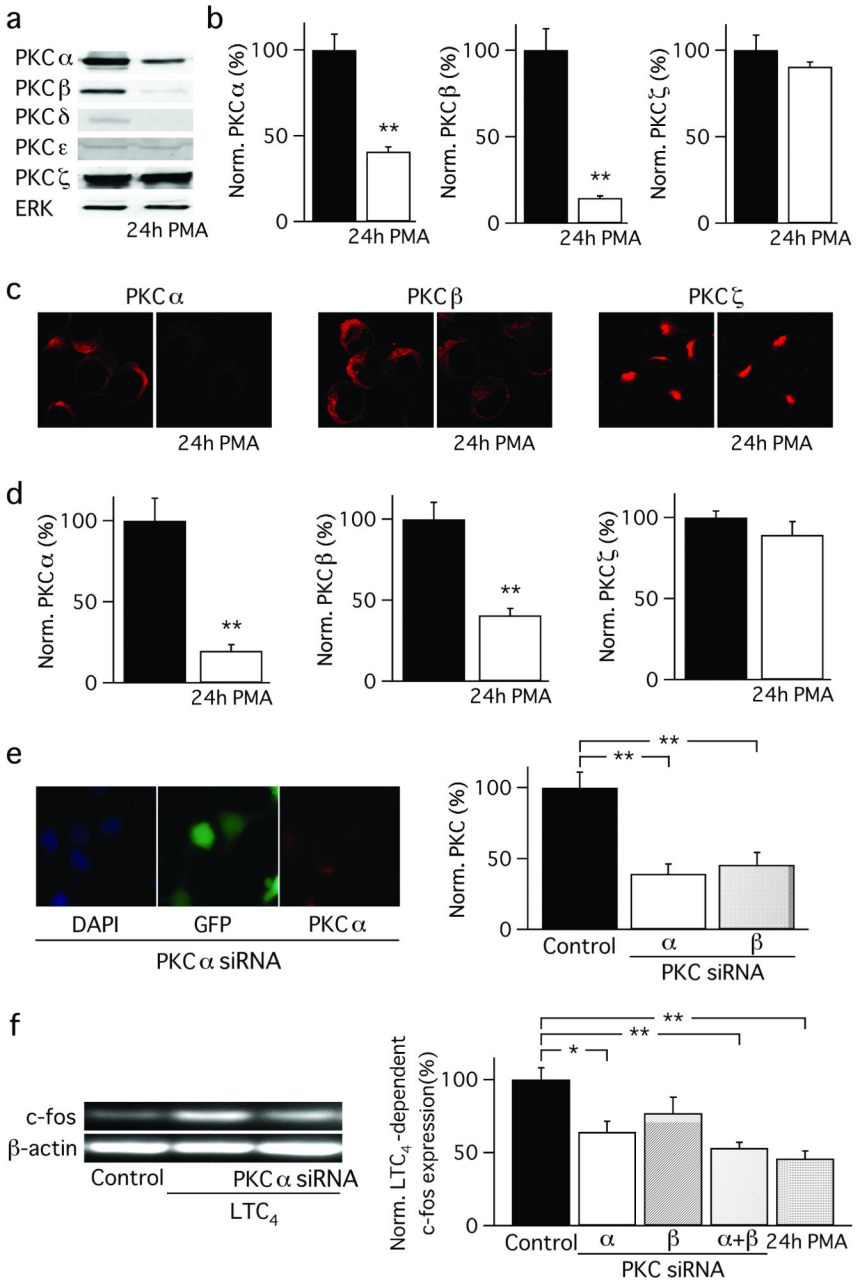

Figure 4.

PKCα regulates CysLT1 receptor-driven c-fos transcription. a, Expression of PKCα, β and ζ (western blot) is shown in control cells and cells exposed to PMA for 24 hours. b, Quantification of data from 3 independent experiments as in a. c, Confocal microscopic images of PKC expression for the conditions shown. Cells were fixed before analysis. d, Quantification of images from experiments as in c. e, siRNA against PKCα or β significantly reduces corresponding protein expression. Left panel: DAPI staining of nuclei (left), GFP expression (indicating transfection; middle) and PKCα expression (right) after siRNA-mediated knockdown. Right panel, aggregate data from 4 experiments are depicted. Both siRNA groups were different from control ($p<0.005$). f, knockdown of PKCα, β and α + β on LTC_4-dependent c-fos expression. Data are compared with mock-transfected cells.

For comparison, 24 hour exposure to PMA is included. All treated groups were significantly different from the LTC_4 control (black bar) group except siRNA β knockdown ($p>0.1$). α+β and 24 h PMA groups had $p<0.01$; α group had $p<0.05$.

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