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Exploring the pharmacology of the leukotriene B_4 receptor BLT_1, without the confounding effects of BLT_2

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Abstract

Most previous studies of leukotriene B_4 (LTB_4) pharmacology using primary leukocyte cultures and myeloid cell lines do not differentiate between leukotriene BLT_1 and BLT_2 receptor activation because both receptors are often expressed by these cells. Here we show that in HeLa cells expressing BLT_1 but not BLT_2 receptors, BLT_1 receptor activation resulted in IP_3 mediated calcium release from intracellular stores initially, followed by calcium influx through cell membrane channels. BLT_1 calcium signalling was sensitive to the activity of protein kinase C (PKC), protein kinase A (PKA) and protein-tyrosine kinases (PTKs), as well as changes in membrane cholesterol levels and treatments that are known to disrupt normal membrane physiology and/or lipid rafts. Inhibition of MAP kinases, Rho-associated kinases, or phosphoinositol-3-kinases (PI_3K) had no effect on BLT_1 receptor induced calcium signalling, and the receptor was insensitive to the redox state of the extracellular compartment.

Keywords: Leukotriene B_4; G-protein coupled receptor; BLT_1; Fura-2; Pharmacology; Calcium

1. Introduction

Leukotriene B_4 (LTB_4) is involved in a variety of processes that are important for inflammation, such as chemotactic cell movement, the production of reactive oxygen molecules, and phagocytosis. LTB_4 binds to and activates several different surface receptors: the high-affinity BLT_1 (Owman et al., 1997; Yokomizo et al., 1997), the lower affinity BLT_2 (Kamohara et al., 2000; Tryselius et al., 2000; Wang et al., 2000; Yokomizo et al., 2000), as well as the vanilloid receptor, VR1 (Hwang et al., 2000). LTB_4 also activates the nuclear peroxisome proliferator activating receptor α (PPARα) (Lin et al., 1999).

A great deal has been written regarding the physiological effects of LTB_4 and the majority of this literature was produced using leukocytes with the assumption that they expressed only one LTB_4 receptor, with two different affinity states. It was not until BLT_1 and BLT_2 were cloned that it became clear that this was not the case and, that these receptors have different pharmacological profiles, expression patterns, and signal transduction pathways. The BLT_1 receptor binds radiolabeled LTB_4 with a K_d in the nanomolar or sub-nanomolar range (Owman et al., 1997; Yokomizo et al., 1997) and this receptor is found predominantly in peripheral blood monocytes and granulocytes, as well as in the thymus and spleen (Yokomizo et al., 1997). BLT_2 receptors are expressed more ubiquitously and have a K_d for LTB_4 that is at least 20 times higher (Kamohara et al., 2000; Wang et al., 2000; Yokomizo et al., 2000). Monocytes, neutrophils and eosinophils have all been shown to express both BLT_1 and BLT_2 receptors (Kamohara et al., 2000; Pettersson et al., 2000; Yokomizo et al., 2001).

The signal transduction pathways activated by LTB_4 have been investigated before, but as mentioned above, this has until recently occurred without separating the effects of BLT_1 receptor activation from those arising following the

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activation of BLT_2 receptors. The ex-vivo primary cell cultures used in earlier experiments are also difficult to maintain and transfect. Recombinant cell lines have been used to show that activation of BLT_1 or BLT_2 receptors can induce an increase in intracellular calcium concentration, that both receptors are involved in chemotaxis, and that they inhibit adenylyl cyclase (Yokomizo et al., 2000). These two receptors use different G-proteins, however, as BLT_2 signalling appears to be less sensitive to pertussis toxin (Yokomizo et al., 2000).

Here we have established a monoclonal cell line based on HeLa cells that have been selected for stable expression of BLT_1 receptors. This cell line provides a reliable, robust and easily accessible source of human cells expressing BLT_1, but not BLT_2 receptors. HeLa cells can also transduce BLT_1 calcium signals without the need for transfection of additional components in the signalling cascade. Using these cells we have studied BLT_1 signal transduction to corroborate previous results obtained using ex-vivo cell cultures that endogenously express BLT1, and to increase our understanding beyond what is currently known.

This broad investigation of BLT_1 pharmacology focuses on intracellular calcium, and can be subdivided into three parts: (1) we have examined the origins of the calcium ions that are released into the cytosol following BLT_1 receptor activation, (2) we have studied agents that modify the function of the receptor protein itself and its immediate environment in the membrane, and (3) we have studied how various other signal transduction pathways, via different protein kinases, interact with BLT_1 receptor-induced calcium signalling.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were purchased from Sigma, unless otherwise stated. All tissue culture media and reagents and amphotericin were from Life Technologies (Sweden). 1,1′diheptyl-4,4′-bipyridium dibromide (DHBP), nifedipine, bisindoylmalmeimide (GF109203X), U 73122 1-[6[[17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione, SKF 96365, 2-aminoethoxydiphenylborane (2APB), dantrolene (1-[[[5-(4-nitrophenyl)-2-Furanyl]methylene]amino]-2,4-imidazolinedione), UO126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene), Y-27632 (*trans*-4-[(1*R*)-1-Aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide dihydrochloride) and genestein were from Tocris (UK). Butanol (butan-1-ol) was purchased from Merck, and H-89 (*N*-[2-(*p*-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide·2HCL from Biomol (USA). RP69698 was obtained from Rhône-Poulenc (France). Fluorescent LTB_4 was produced according to Sabirsh et al. (2003).

2.2. Cell culture and transfection

Cultures of HeLa HF1 cells transfected with BLT_1 (HF1pBLT1) or an empty plasmid (HF1pSHAM) were constructed and maintained according to Kotarsky et al. (2001). Sham transfected HeLa HF1 cells did not respond to LTB_4. These cells do, however, express receptors for ATP (P2Xn receptors), thromboxane A_2/prostaglandin H_2 (TP receptors) and histamine (H1 receptors), all of which signal using calcium. These receptors were used as positive controls.

2.3. EGFP tagging of BLT_1

To create BLT_1 receptors that were C-terminal tagged with enhanced green fluorescent protein (EGFP), we used the mammalian expression vector pEAK12 (Edge Biosystems, USA). This vector was used to construct the pEAK-HFTE vector containing a 10× histidine tag (H), a FLAG-tag (F), and the recognition site for the Tobacco Etch Virus (T) protease followed by EGFP (E). The HFTE-cassette was assembled using PCR and cloned into pEAK12. The open reading frame of human $BLTR_1$ was generated using polymerase chain reactions with the following primers:

5 prime: ATATAAGCTTCCACCATGAACACTA-CATCTTCT
3 prime: CAGTGAATTCCCGTTCAGTTCGTT-TAACTTGAGAG

BLT_1 was then cloned into pEAK-HFTE to generate pEAK-BLTR-HFTE, where the ORF of BLT_1 was in frame with, and upstream of, the HFTE-cassette such that all three tags and the protease site were added to the C-terminal end of the expressed receptor protein. The integrity of the construct was confirmed by sequencing with BigDye (Applied Biosystems). HeLa HF1 cells were stably transfected with pEAK-BLTR-HFTE to produce HF1pBLT1-EGFP cells.

2.4. Membrane preparation

Stably transfected monoclonal HF1pBLT1 or HF1pSham cells were grown in tissue culture plates until confluent and then chilled to 4 °C. The cell growth medium was removed and the cells were rinsed once with ice-cold phosphate buffered saline (PBS), before ice-cold Tris–HCl buffer (50 mM Tris base ((hydroxymethyl)-aminomethane), 5 mM $MgCl_2$, 1 mM EGTA (ethylene glycol-*O*,*O*′-bis-[2-aminoethyl]-*N*,*N*,*N*′,*N*′,-tetraacetic acid), pH 7.5) was added to each plate. The cells were then scraped off and homogenised using a motor driven pestle. The cell homogenates were centrifuged at 1000×*g* for 10 min at 4 °C. The resulting supernatant was centrifuged at 100,000×*g* for 30 min at 4 °C. The supernatant was then discarded, additional buffer was added, and the pellet was re-homogenised. The

membrane protein concentration was determined (BCA protein assay, Pierce), and the membrane isolates were portioned into aliquots and frozen at −80 °C until use.

2.5. Intracellular calcium concentration assays of BLT_1 receptor activity

Confluent cultures of monoclonal HF1pBLT1 or HF1pSham cells were loaded with Fura-2-AM (Fura-2 acetoxymethyl ester) and analysed according to Sabirsh et al. (2003). After dye loading, test substances or control solutions were added to alternating wells (all results are shown with final concentrations), and the plates were incubated for 5 min. For substances requiring longer incubation times, the cells were incubated with the test substance or an appropriate control while loading Fura-2-AM, and test substances were re-added after the wash procedure. Some pre-treatments resulted in altered baseline intracellular calcium concentrations, which may have resulted in part from variations in how Fura-2 partitioned within various cell compartments, but we were unable to detect differences in Fura-2 loading as a result of any particular treatment. Using an MTT (methylthiazolyldiphenyl-tetrazolium bromide/thiazolyl blue) cell viability assay, we did not observe any decreases in cell viability following exposure to any of the solutions over the time period used for these experiments (a maximum of 1.5 h).

Results are shown as representative curves from each experiment. Unless otherwise stated, at least three separate experiments were performed in quadruplicate for all treatments. In some cases, the data from all experiments was normalised to the control curves, and 95% confidence intervals (95% CI) were calculated for responses to various treatments. Changes were defined as statistically significant when control responses were outside the boundaries defined by the 95% CI for the treated cells.

2.6. Fluorescence polarisation assays of ligand binding

Fluorescence polarisation assays were performed according to Sabirsh et al. (2003). The effects of various treatments on ligand binding were measured by allowing LTB_4 to compete for LTB_4-FL binding sites in the presence of various test substances. For each treatment, the LTB_4 concentration that inhibited half of the specific binding (IC_{50}) of LTB_4-FL was determined using non-linear regression (Graph Pad, Prism) of specific binding data from at least 8 competitive binding curves. IC_{50} values from the various treatment groups were then compared using one-way analysis of variance followed by Bonferroni comparisons to controls.

2.7. Flow cytometry

HF1pBLT1-EGFP cells were grown (for at least two days) until 90% confluent and then dissociated at 4–16 °C using a calcium and magnesium free PBS solution containing 2.5 mM EDTA (ethylenediaminetraacetric acid tetrasodium). The cells were divided into portions and incubated with methyl β cyclodextrine (MβCD) without cholesterol, MβCD pre-loaded with cholesterol, amphotericin, butanol, or the appropriate control solution for 60 min at 37 °C. The cells were then incubated for 5 min with 4% paraformaldehyde, washed once in PBS before MβCD solutions, amphotericin, or control solutions were re-added. These groups were each divided in half and received either an anti-BLT_1 antibody (clone 14F11), or an isotype control antibody immunoglobulin G1 (IgG1), both covalently coupled to phycoerythrin (a red fluorophore). Following 30 min of incubation at room temperature, the cells were washed and then analysed using a Becton Dickenson FACSVantage flow cytometer. The same number of EGFP positive cells were analysed for each treatment.

3. Results

We have examined the pharmacology of recombinant leukotriene BLT_1 receptors expressed in HeLa cells. As an indication of receptor function we have observed LTB_4 mediated intracellular calcium concentration changes following a variety pharmacological treatments. Our observations are summarised in Table 1.

HF1pBLT1 cells responded robustly and reproducibly with increases in intracellular calcium ($[Ca^{2+}]_i$) following exposure to LTB_4, with an EC_{50} of 9.2 (1.9–43) nM (mean and 95% CI). *Bordetella pertussis* toxin pre-treatment reduced subsequent calcium responses by about 50%. Calcium responses could also be significantly reduced by pre-incubation with the BLT_1 receptor partial agonist, U75302, and abolished using the BLT_1 receptor antagonist RP69698 (Fig. 1). Sham transfected cells did not respond to LTB_4.

3.1. The origin of calcium ions found in the cytosol following BLT_1 activation

Following LTB_4 binding, BLT_1 receptors induced an immediate rise in intracellular calcium concentrations which gradually declined towards baseline levels. We found that the initial BLT_1 receptor-induced increase in intracellular calcium concentration could occur in absence of extracellular calcium (Fig. 2A), so we investigated how BLT_1 receptors are coupled to these intracellular calcium stores. 1,1′diheptyl-4,4′-bipyridium dibromide (DHBP, 100 μM, 5 min incubation) and Dantrolene (50 μM, 5 min incubation), two ryanodine receptor antagonists, had no effect on BLT_1 receptor-induced changes in intracellular calcium concentration (results not shown). In contrast, interfering with the function of the inositol triphosphate (IP_3) receptors, using the membrane-permeable antagonist 2APB (Fig. 2B), or inositol triphosphate synthesis using the phospholipase C (PLC) inhibitor U 73122 (Fig. 2C) completely abolished

Table 1
A summary of the effects of various pharmacological treatments on LTB_4/BLT_1 induced intracellular calcium concentration changes

Treatment	Pharmacological Effect	LTB4 Response
PTX	disruption of G-protein signalling	↓
U75302	BLT_1 partial agonist	↓
RP69698	BLT_1 antagonist	↓↓
Propranolol	phosphatidic acid hydrolase inhibitor	↓
DHBP	ryanodine receptor antagonist	→
Dantrolene	ryanodine receptor antagonist	→
Ca-free medium	abolishes calcium influx	↓
2APB	IP_3 receptor inhibitor	↓↓
U 73122	PLC inhibitor	↓↓
Thapsigargin	Ca^{2+}-ATPas inhibitor	↓↓
NiCl	non-specific Ca^{2+}-channel blocker	↓
Nifedipine	L-type calcium channel blocker	→
SKF 96395	inhibitor of receptor and store operated channels	↓
DNTB	oxidizing agent	→
DTT	reducing agent	→
Butanol	increased membrane fluidity	→
Amphotericin	disrupted membrane physiology	↓
Methyl ß-Cyclodextrin− cholesterol	membrane cholesterol removal	↓
Methyl ß-Cyclodextrin+ cholesterol	membrane cholesterol supplementation	↑
Staurosporine	non-specific protein kinase inhibitor	↑
GF109203X	specific PKC inhibitor	↑
Y-27632	p160ROCK, PKA and PKC inhibitor	↑
Phorbol Myrastate Acetate (PMA)	PKC activator	↓
Forskolin	adenylyl cyclase stimulator	↓
H-89	PKA inhibitor	↓
Wortmannin	phosphoinositol-3 kinase inhibitor	→
Genestein	protein tyrosine kinase inhibitor	↓
UO126	MAP kinase kinase inhibitor (MEK1, MEK2)	→

The pharmacological treatments used in this paper are listed in the first column, and are grouped according to their pharmacological function shown in the second column. The effect of each treatment on LTB_4 induced intracellular calcium concentration changes is shown as either an attenuated response (↓), an enhanced response (↑) or no change (→).

responses to LTB_4. Interrupting diacylglycerol synthesis using the phosphatidic acid hydrolase inhibitor, propranolol, also significantly inhibited calcium responses (Fig. 2D). LTB_4-induced calcium currents originating from intracellular sources were also investigated using thapsigargin, a sarco-endoplasmic reticulum calcium-adenosine triphosphatase inhibitor (Fig. 2E). Pre-treatment with 1 μM thapsigargin and the ensuing depletion of intracellular calcium stores completely abolished subsequent responses to LTB_4, which is consistent with the above results.

When HF1pBLT1 cells in calcium-free extracellular media were stimulated with LTB_4, the secondary phase of the calcium response could be reconstituted by adding extracellular calcium (Fig. 3A), indicating that calcium influx from the extracellular compartment was responsible for this part of the response. This influx could be significantly reduced using nickel ($NiCl_2$) as a non-specific channel blocker (Fig. 3B). LTB_4 induced changes in the 340 nm/380 nm fluorescence ratio of Fura-2 were also inhibited when HF1pBLT1 cells in a calcium and magnesium free buffer containing 100 μM extracellular manganese ($MnCl_2$) were stimulated with LTB_4 (Fig. 3C). Nifedipine, an L-type calcium channel blocker had almost no effect on this calcium influx (results not shown), whereas the influx could be attenuated using SKF 96365, a putative receptor-operated and store-operated channel blocker. Adding SKF 96365 to the cells during the secondary phase also shortened the time required to return to baseline calcium levels (Fig. 3E,F).

When observed over a period of more than 20 min, populations of HF1pBLT1 cells had elevated intracellular calcium levels following the initial responses to LTB_4 stimulation (Fig. 4). This was not observed when stimulating with adenosine triphosphate (ATP) or histamine. Furthermore, this increased baseline did not appear to be sensitive to $NiCl_2$ pre-treatment, as $NiCl_2$ affected only the length of the plateau phase, and not the subsequent baseline increase.

3.2. The local micro-environment and leukotriene BLT_1 receptor function

The extracellular presence of the oxidizer DTNB (5,5′-Dithiobis-[2-nitrobenzoic acid], 0.5 mM, 5 min incubation before exposure to LTB_4) or the reducing agent DTT (dithiothreitol, 4 mM, 5 min incubation), did not have any significant effect on receptor function when measured as receptor-induced changes in intracellular calcium concentration. LTB_4 binding was also unaffected. Positive control experiments using endogenously expressed thromboxane A_2/prostaglandin H_2 (TP) receptors and the thromboxane A_2/prostaglandin H_2 receptor-specific agonist, U44069, to induce calcium release indicated that both DTT and DNTB could affect calcium release triggered by thromboxane A_2/prostaglandin H_2 receptor activation (not shown).

Removing membrane cholesterol, using methyl β-cyclodextrine, attenuated responses to LTB_4, whereas adding cholesterol enhanced responses (Fig. 5). Control experiments with thapsigargin in calcium-free media showed, however, that these treatments affected normal calcium homeostasis, because adding *or* removing cholesterol attenuated both calcium release from intracellular stores and calcium influx. Antibody labelling of cell-surface BLT_1 receptors was proportional to changes in membrane cholesterol levels, but ligand binding was not. The IC_{50} value for LTB_4 displacement of LTB_4-FL bound to BLT_1

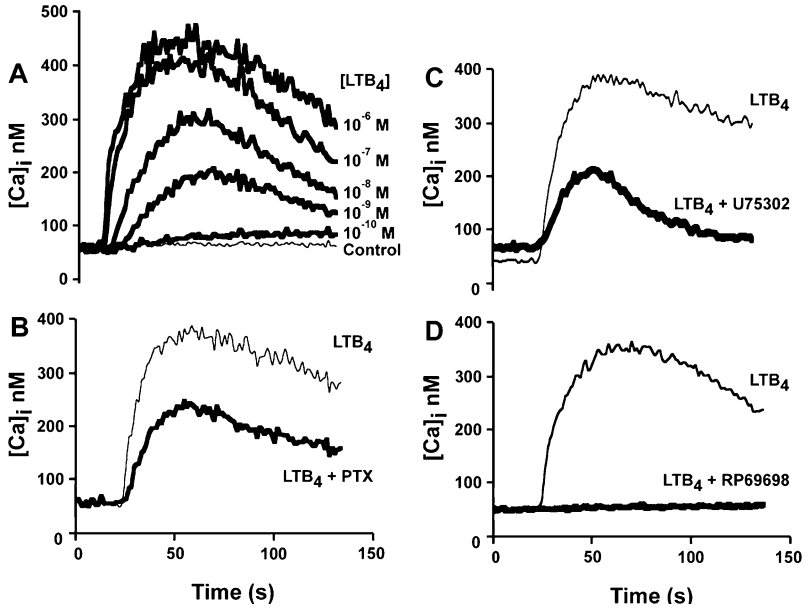

Fig. 1. (A) LTB_4 concentration–response curves, n=4 for each concentration. LTB_4 concentrations were from 10^{-11} to 10^{-6} M (10^{-11} M has been omitted for clarity but was similar to the control response). Normal BLT_1 responses to 100 nM LTB_4 (thin lines) compared to responses following pre-treatment with (thick lines); (B) PTX 100 ng/ml, (>12 h incubation at 37 °C); (C) 10 μM U75302 (BLT_1 selective partial agonist, 5 min incubation); (D) 1 μM RP69698 (BLT_1 antagonist, 5 min. incubation).

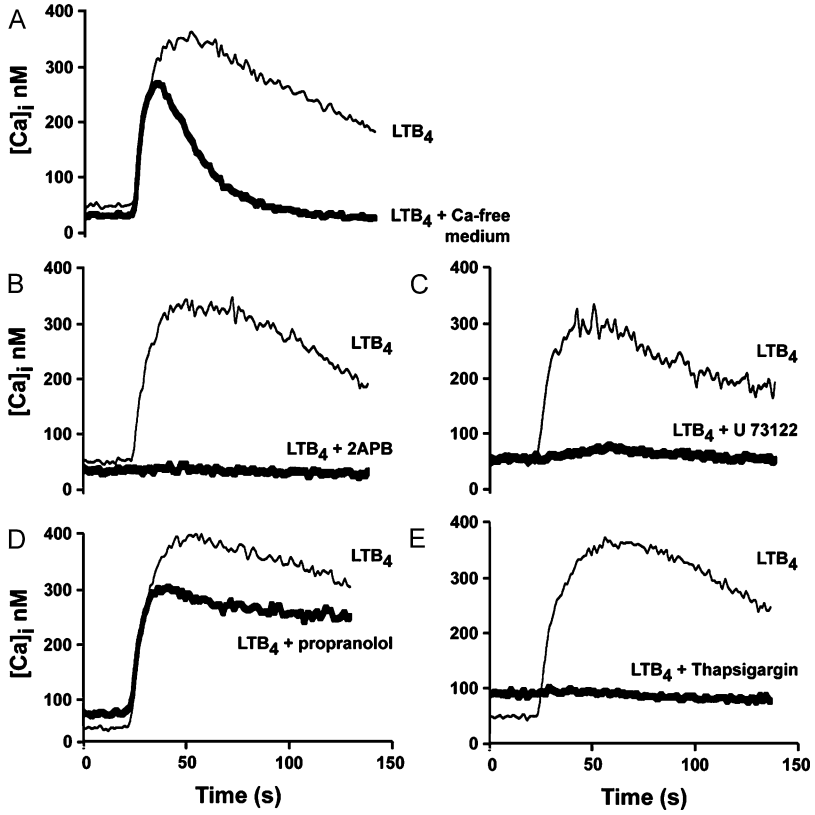

Fig. 2. BLT_1 activation by LTB_4 releases calcium from internal stores. (A) The response to 100 nM LTB_4 in calcium-free medium (thick line) compared to control cells in a buffer containing calcium (thin line). HF1pBLT_1 cell responses to 100 nM LTB_4 (thin lines) were also compared to HF1pBLT_1 cells in normal calcium containing buffer (thick lines) pre-treated with (B) 100 μM 2APB (5 min incubation); (C) 50 μM U 73122, (60 min incubation) (D) 150 μM propranolol (5 min incubation); (E) 1 μM Thapsigargin, (60 min incubation).

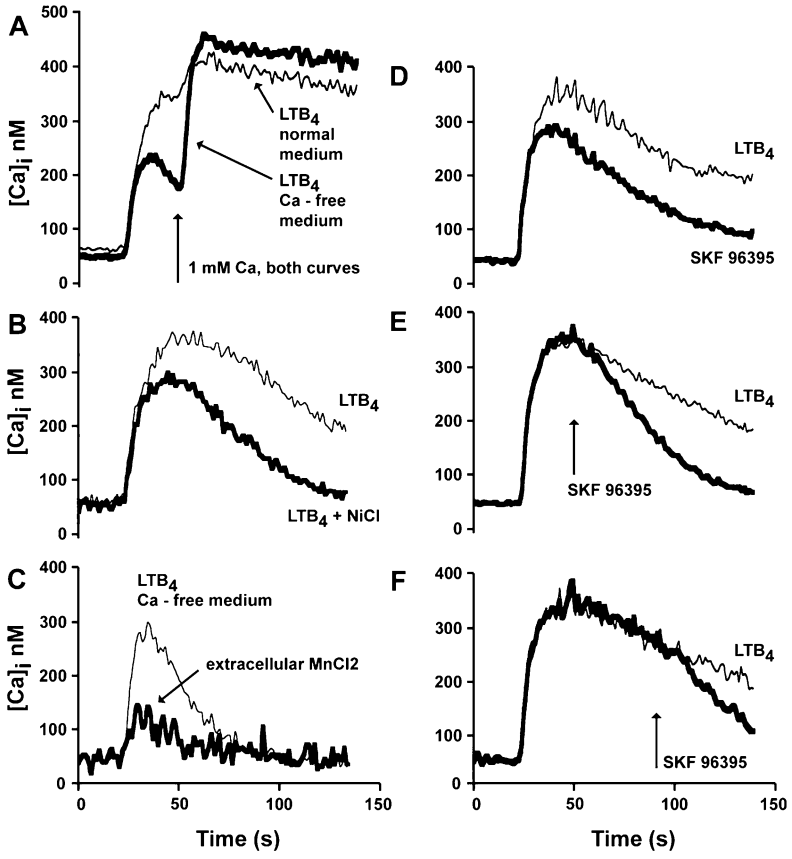

Fig. 3. BLT_1 couples to calcium channels in the cell membrane. (A) Normal HF1pBLT1 cell responses to 100 nM LTB_4 in buffer containing calcium (thin line) were compared to cells stimulated with 100 nM LTB_4 in a calcium-free medium (thick line). Both groups received 1 mM Ca^{2+} at 50 s. Normal HF1pBLT1 cell responses to 100 nM LTB_4 (thin lines) were also compared to cells pre-treated (thick lines) with (B) 5 mM (extracellular) $NiCl_2$, (5 min incubation); (C) 100 μM (extracellular) $MnCl_2$ (in the absence of extracellular magnesium or calcium, 5 min incubation); or (D) 10 μM SKF 96395 (5 min incubation). When 100 μM SKF 96395 was given (thick lines) at 50 (E) and 90 s (F) into the experiment, (injections at indicated time points) the second phase of the response could be shortened.

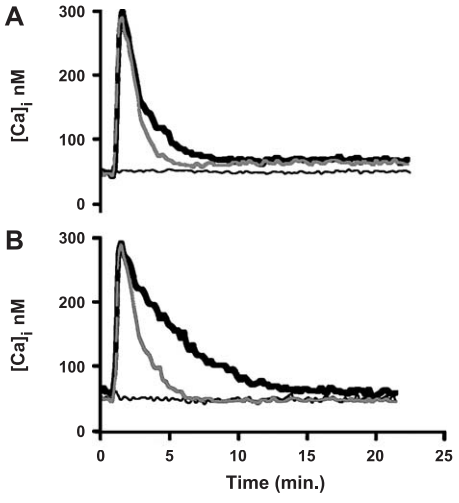

Fig. 4. BLT_1 induced calcium release over longer time intervals following exposure to LTB_4. HF1pBLT1 cells were exposed to 100 nM LTB_4 in normal buffer containing calcium, (A) with (grey line) or without (thick line) 1 mM $NiCl_2$ pre-treatment (a control group that did not receive LTB_4 or NiCl is shown as a thin line). (B) A different response pattern was observed when stimulating HF1pBLT1 cells (in buffer containing calcium) with 100 μM ATP (thick line) or 1 mM histamine (grey line). A buffer control that did not receive ligands is shown as a thin line.

receptors was calculated to be 155 (120–202) nM (mean and 95% CI), and a similar value was obtained using methyl β-cyclodextrine treated membranes (124 (97–158) nM). After pre-treatment with methyl β-cyclodextrine containing cholesterol, the LTB_4 IC_{50} decreased to 86 (65–113) nM ($p<0.01$). This was not a consequence of *LTB_4-FL* binding by methyl β-cyclodextrine because neither of these treatments altered the polarisation of free LTB_4-FL.

Amphotericin B is an antibiotic that can decrease membrane fluidity and disrupt membrane lipid rafts (Simons and Toomre, 2000). Amphotericin significantly decreased calcium responses to LTB_4 in HF1pBLT1 cells (Fig. 5). Endogenously expressed thromboxane A_2/prostaglandin H_2 receptors were also found to be sensitive to this treatment (results not shown). Additional control experiments with thapsigargin (as above) indicated that normal calcium homeostasis was not affected, and flow cytometric analysis of EGFP-tagged receptors showed that the BLT_1 receptor antibody binding epitopes were also unaffected by amphotericin, and majority of the receptors remained on the cell surface. In binding experiments, the LTB_4 IC_{50} decreased from 155 to 88 (71–108) nM ($p<0.01$) in the presence of amphotericin. Amphotericin did not bind to free LTB_4-FL.

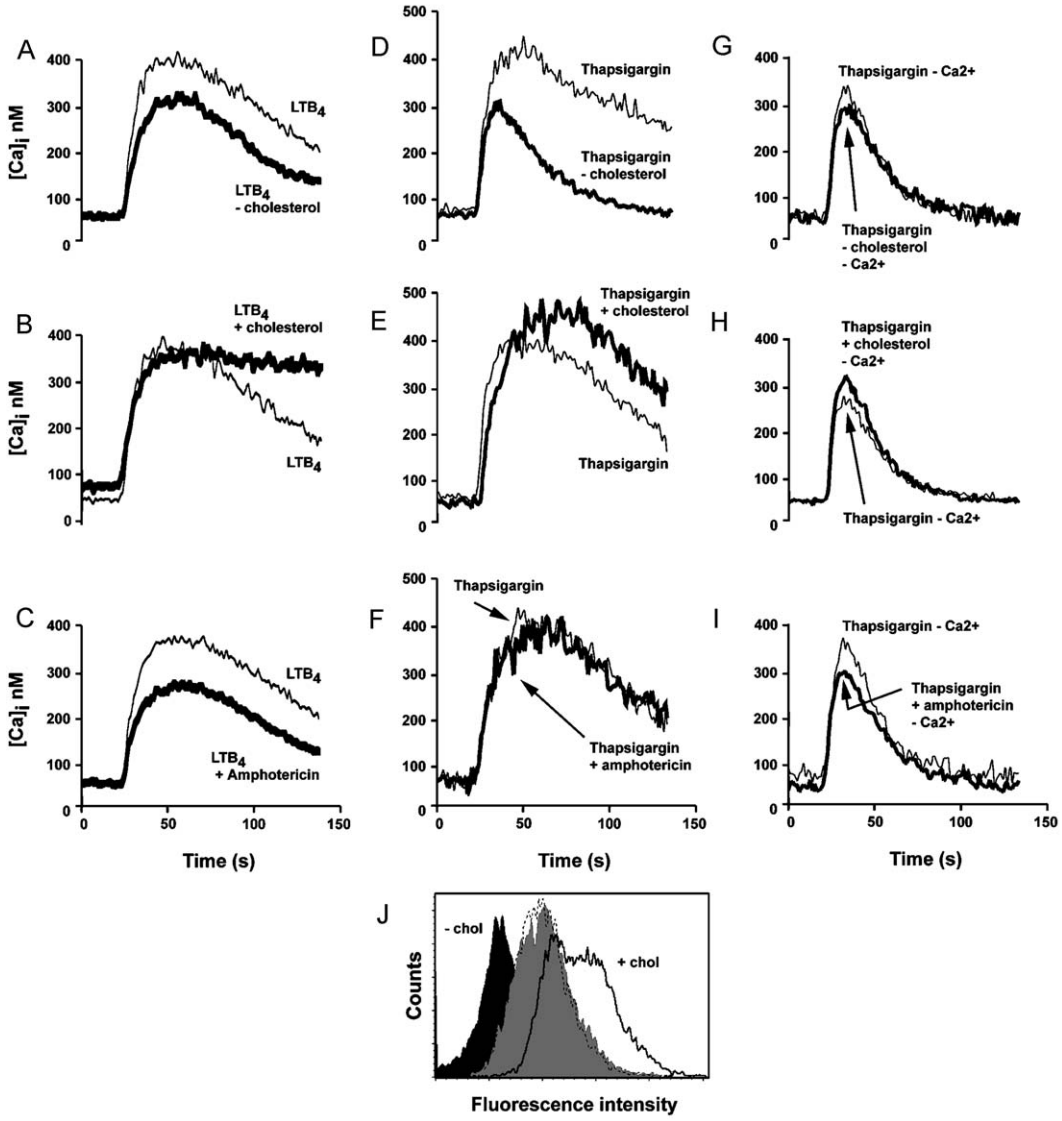

Fig. 5. BLT_1 responses to LTB_4 following chemical modification of the receptor protein or the surrounding microenvironment. HF1pBLT1 cells were stimulated with 100 nM LTB_4 following pre-treatment with (thick lines) (A) 10 μM Amphotericin (60min incubation), (B) 10 mM methyl β-cyclodextrin with cholesterol (60 min incubation), (C) 10 mM methyl β-cyclodextrin without cholesterol (60 min incubation). Control responses from vehicle pre-treated cells are shown as thin lines. (D–F) As with A–C, except that cells were stimulated with 1μM thapsigargin. (G–I) As with D–F except that cells were stimulated in a calcium and magnesium free buffer containing 50 μM EGTA. (J) Flow cytometric analysis of BLT_1 expression following pre-treatment of HF1pBLT1 cells under the same conditions as in A–C. Exposure to LTB_4 induces the production of EGFP in these reporter cells. An equal number of EGFP positive cells were analysed for each treatment (LTB_4 was administered 16 hours prior to each treatment to allow EGFP to accumulate). Antibody binding to BLT_1 receptors is measured as red fluorescence in this green population. ■: methyl β-cyclodextrin without cholesterol, ■: control treatment, □: methyl β-cyclodextrin with cholesterol, dotted line: Amphotericin.

Pre-treatment with butanol (0.25%, v/v), also had no significant effect on leukotriene BLT_1 receptor function when measured as changes in intracellular calcium concentration, and, LTB_4 binding and antibody binding to BLT_1 receptors was also unaffected (results not shown).

3.3. Kinase activity and BLT_1 and calcium release

We also examined how leukotriene BLT_1 receptor signalling via calcium could be affected by six types of kinases: protein kinase C (PKC), protein kinase A (PKA), protein-tyrosine kinases (PTKs), phosphatidylinositol 3-kinases (PI_3K), Rho-dependent kinases and mitogen-activated protein (MAP) kinase kinases.

The PKC activator phorbol 12-myristate 13-acetate (PMA) significantly attenuated LTB_4-induced changes in intracellular calcium concentration (Fig. 6). PKC inhibitors, such as staurosporine or the more specific GF109203x, both promoted higher intracellular calcium concentrations following BLT_1 receptor activation, although this effect was statistically significant only towards the end of the measure-

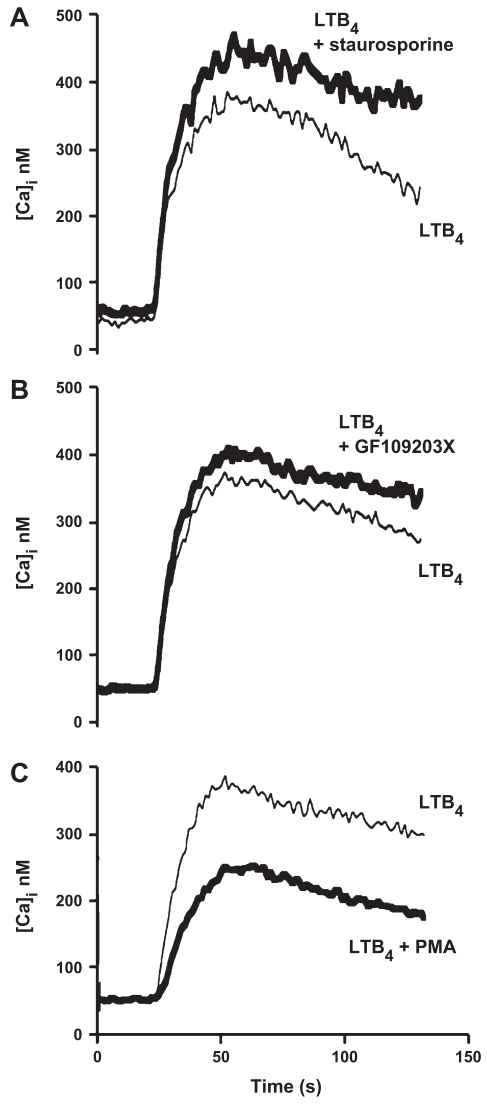

Fig. 6. PKC and BLT_1 function. HF1pBLT1 cells in normal buffer containing calcium were stimulated with 100 nM LTB_4 following pre-treatment (5 min incubation, thick lines) with (A) 1 μM staurosporine, n=24, (B) 200 nM GF109203X, n=16, (C) 100 nM phorbol myristate acetate n=16. Control responses to 100 nM LTB_4 from vehicle treated cells are shown as thin lines.

ment period (>100 s). Both forskolin (which activates adenylyl cyclase and subsequently PKA) and H-89 (a PKA inhibitor) produced comparable decreases in responses to LTB_4 stimulation (Fig. 7). H-89 is, however, known to inhibit a number of kinases (Davies et al., 2000) including Rho-dependent kinases. To further examine the effects of H-89 we used the Rho-dependent-kinase inhibitor Y-27632. At lower (1 μM) concentrations Y-27632 had no effect on LTB_4 induced calcium responses (not shown). At higher (100 μM) concentrations Y-27632 begins to inhibit PKC and cAMP activated kinases, and has almost the same effect as the PKC inhibitor GF109203x. Genistein, an inhibitor of tyrosine specific protein kinases, also had a significant attenuating effect on calcium responses following the activation of BLT_1 receptors. Other kinase inhibitors, such as the PI_3K inhibitor Wortmannin (0.5 μM, 5 min incubation), and the mitogen activated protein (MAP) externally regulated kinase (ERK), or MEK1 and MEK2, inhibitor UO126 (1 μM, 5 min incubation), had no effect on changes in intracellular calcium concentration following BLT_1 receptor stimulation (results not shown).

4. Discussion

We have examined how BLT_1 receptor-induced intracellular calcium concentration changes are affected by various intracellular signalling pathways using HeLa HF1 cells expressing the leukotriene BLT_1 receptor subtype exclusively. There are inherent disadvantages associated with observing BLT_1 in a cell that does not normally express this receptor, but HeLa cells are of human origin, easily transfected, and, they respond naturally to BLT_1 stimulation with robust intracellular calcium concentration changes, while other cell types require transfection with additional signal transduction components (Gaudreau et al., 1998). Furthermore, unlike leukocytes from mice that have had BLT_1 or BLT_2 deleted from their genomes, HF1pBLT1 cells are also available in large numbers. Specific BLT_2 antagonists or RNA interference could be employed to inhibit cellular responses to LTB_4 mediated by BLT_2, but this approach is limited by technical, practical and economic problems. Taken together these factors make BLT_1 transfected HeLa cells a convenient platform for exploring the pharmacology of BLT_1, and for identifying targets for further investigation in the more physiological setting offered by primary leukocyte cultures.

The EC_{50} value for LTB_4 in HF1pBLT1 cells is comparable to the value obtained using eosinophils and measurements of calcium release (Lindsay et al., 1998b). The effect of *B. pertussis* toxin on calcium responses to LTB4 stimulation measured using transfected cells expressing BLT_1 is typically rather small (Haribabu et al., 1999; Yokomizo et al., 1997) and we also found that a significant portion of the calcium response is not mediated via *B. pertussis* toxin sensitive G-proteins.

4.1. The origin of calcium ions found in the cytosol following BLT_1 receptor activation

The source of calcium for the BLT_1 receptor-induced intracellular calcium concentration increases observed in our recombinant HeLa cells was intracellular initially, with store operated or receptor operated calcium influx becoming increasingly dominant after approximately 20 s. LTB_4 has been shown to induce increases in intracellular calcium concentration via IP_3 receptors (Maruyama et al., 1997; Smith et al., 1990), or ryanodine receptors (Kim et al., 1998), depending on the cell type examined

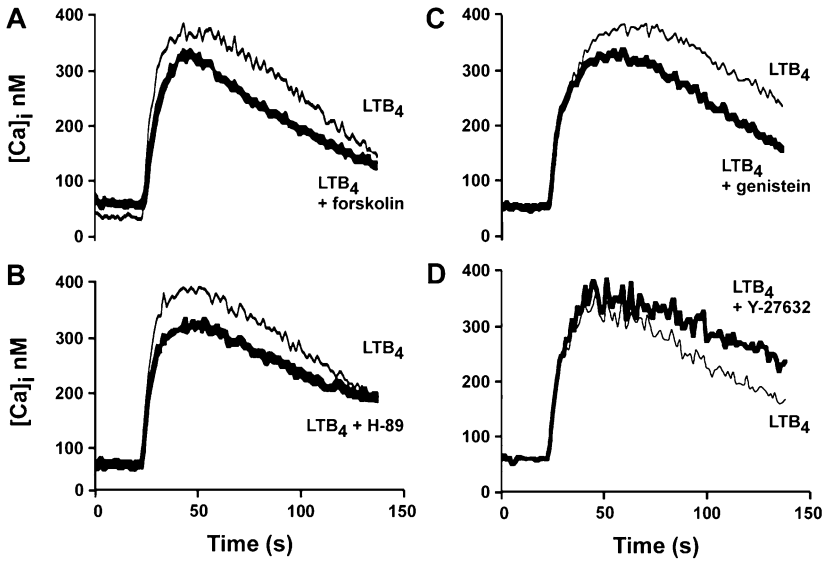

Fig. 7. Various kinases, cAMP, and BLT_1 function. HF1pBLT1 cells in normal buffer containing calcium were stimulated with 100 nM LTB_4 following a 5 min pre-treatment (black lines) with (A) 10 μM forskolin, (B) 50 μM H-89, (C) 100 μM Genistein, (D) 100 μM Y-27632. Control responses to 100 nM LTB_4 from vehicle treated cells are shown as thin lines.

(neutrophils and astrocytoma cells respectively). LTB_4 has also been shown to directly activate ryanodine receptors in a cell-free preparation (Striggow and Ehrlich, 1997). While HeLa cells express ryanodine receptors (Bennett et al., 1996), LTB_4 does not seem to activate them because interfering with IP_3 mediated calcium release could completely block BLT_1 receptor-induced intracellular calcium concentration changes.

We found that the putative receptor-operated channel inhibitor, SKF 96365, could inhibit capacitive calcium influx. Receptor coupling to store-operated channels is thought to occur via the production of diacylglycerol, and a reduction in diacylglycerol following exposure to a phosphatidic acid hydrolase inhibitor, propranolol, could also attenuate calcium responses. Responses following pre-treatment with nickel were almost the same as those observed when using calcium free buffers, indicating that nickel could inhibit calcium influx almost completely. Similar results obtained using neutrophils have been reported by Merritt et al. (1989) and Montero et al. (1991).

Exposure to LTB_4 increased baseline intracellular calcium levels for at least 20 min after the initial response. The increased baseline could not be eliminated using nickel, which suggests that sustained calcium influx is not involved. In the presence of other GPCR agonists, such as histamine, individual HeLa cells have been shown to have repeated "spikes" of calcium release over longer periods of measurement (Bootman et al., 1996). Asynchronous, recurring increases in intracellular calcium levels (spikes) in individual cells could explain the increase in post-LTB_4 baselines, although histamine did not increase baseline calcium levels following the initial response.

4.2. The redox state of the local micro-environment and BLT_1

Many of the cells expressing leukotriene BLT_1 receptors release chemically reactive mediators (oxidizers) intended to incapacitate bacteria or parasites, and G-protein coupled receptors have previously been shown to be susceptible to oxidative modification of their function, expression levels, and ligand affinity (Dorn, 1990). Furthermore, proteins that are sensitive to the redox state of their environment often contain cysteine residues that form disulfide bonds when oxidized (Aslund and Beckwith, 1999). Near the extracellular portion of the third TM helix in BLT_1 three cysteines (cys90, cys93 and cys97), are positioned such that disulphide bonds may form either locally (cys93 and 97) or with the extracellular portions of the receptor (cys90 or 93 with cys168 in extracellular loop 2). Treatment with the sulfhydral oxidizing agent DNTB or the reducing agent DTT at concentrations found to affect the function of endogenously expressed thromboxane A_2/prostaglandin H_2 receptors had no effect on LTB_4 binding, or, BLT_1 receptor function, measured as receptor-induced changes in intracellular calcium concentration. This implies that the sulfhydral redox state of BLT_1 receptors does not change as cells move into areas of inflammation.

4.3. Membrane fluidity and physiology, and BLT_1 receptor function

Changes in membrane composition and normal membrane physiology are important pathologically, and LTB_4 has recently been implicated in the genesis of atherosclerotic lesions (Aiello et al., 2002), a condition that also involves dysfunctional cholesterol homeostasis. If BLT_1 receptors

respond differently in the microenvironment associated with the atherosclerotic lesions this could be important for understanding, and hopefully impeding, lesion formation. To investigate possible effects of the membrane microenvironment on BLT_1 receptor function, methyl β-cyclodextrine was used to extract cholesterol from the cell membranes or, alternatively, to add cholesterol, and amphotericin and butanol were used to disrupt normal membrane physiology.

We found that adding cholesterol enhanced LTB_4 binding, prolonged the increase in intracellular calcium concentration following BLT_1 receptor stimulation and increased the cell surface binding of BLT_1 receptor antibodies. Removing cholesterol using empty methyl β-cyclodextrine had more or less opposite effects, except that ligand affinity remained unchanged relative to controls.

Many heptahelical G-protein-coupled receptors require cholesterol for high affinity binding (e.g. Lagane et al., 2000; Nguyen and Taub, 2002), however the effect of cholesterol modulation on BLT_1 calcium signalling may or may not be a result of direct effects on the receptor. Our results agree well with the recent work by Barabé et al. (Barabe et al., 2002) using neutrophils and peptide chemoattractant fMLP (formyl-methionyl-leucyl-phenylalanine). They found that treating neutrophils with empty methyl β-cyclodextrine attenuated calcium responses to fMLP, but also affected calcium influx in general, implying that calcium channels, rather than fMLP receptors, were affected by the lower levels of cholesterol. It has been shown that BLT_1 receptors bind preferentially to $G\alpha_i$, $G\alpha_o$ (Masuda et al., 2003), or $G\alpha_{16}$ (Gaudreau et al., 1998) depending on the cell system, and these G-proteins cluster in membrane subdomains (Oh and Schnitzer, 2001) and altering the composition of the membrane may therefore affect the availability or function of transducer proteins. Membrane calcium channels have also been shown to be sensitive to cholesterol levels (Lockwich et al., 2000). Antibody labelling of BLT_1 receptors increased when cholesterol was added, but this can be explained by conformational changes in the receptor protein as well as changes in number of receptors on the cell surface.

Amphotericin is also known to affect the integrity and/or function of sub-domains of membrane lipids (Simons and Toomre, 2000). At doses comparable to those used for treating fungal infections in cell cultures (but 100 times lower than clinical doses; Kotwani et al., 2002), we found that amphotericin increased BLT_1 receptor affinity for LTB_4, but reduced BLT_1 receptor-induced calcium release without affecting anti-BLT_1 antibody binding. Additional experiments will be necessary to determine the point (or points) at which amphotericin interferes with the BLT_1 signalling cascade, but the well known effects of amphotericin on membrane physiology, the changes in ligand affinity and the lack of any observed effect on normal calcium homeostasis (in the present model system) imply that BLT_1 receptors may be more directly affected by amphotericin. Using neutrophils, Brom and Konig (Brom and Konig, 1989) noted that amphotericin treatment had no effect on the "high affinity" LTB_4 receptor subset, but increased the proportion of "low affinity" receptors. This does not agree with our data obtained using HeLa cells which express BLT_1 receptors exclusively (unlike neutrophils which also express the lower affinity LTB_4 receptor, BLT_2).

Following pre-treatment with butanol doses shown to be biologically active (Perkins et al., 1995), we observed no change in the ability of BLT_1 receptors to induce intracellular calcium concentration changes, Brom and Konig (Brom and Konig, 1989) have also reported that neutrophil LTB_4 receptors could be shifted into a high affinity state by butanol. In contrast, we did not observe this.

Taken together our data suggests that leukotriene BLT_1 receptors are sensitive to changes in membrane cholesterol levels and treatments that change normal membrane physiology. Given the role of BLT_1 receptors in pathologies involving dysfunctional regulation of cholesterol homeostasis, more experiments to examine the interaction between BLT_1 receptors and cholesterol are certainly warranted.

4.4. Kinase activity and BLT_1 receptor-induced calcium release

Following exposure to LTB_4, BLT_1 receptor signalling is regulated by kinases that phosphorylate the receptor protein (Gaudreau et al., 2002), and/or signal transduction proteins down stream from the receptor (Haribabu et al., 1999; Naccache et al., 1985). This allows different signalling systems to modulate the activity of BLT_1 receptors, and it allows the receptor to modulate its own activity. Understanding these interactions is therefore important for understanding BLT_1 receptor pharmacology, the physiological role of the receptor, and for identifying ways to alter the activity of BLT_1 receptors in pathophysiological situations. The pharmacological tools for studying kinase activity—kinase inhibitors—tend to be non-specific however, although this can be avoided to some extent by using an appropriate dose. Choosing an appropriate dose based on in vitro studies is in turn complicated by the fact that most kinase inhibitors compete with adenosine triphosphate for binding to these kinases, so in vivo doses need to be higher. As pointed out by Davies et al. (2000) kinase inhibitors are thus more useful for revealing which spectrum of kinases are *not* involved in a given physiological process.

Changes in the activity of either phosphoinositol-3 kinases (inhibited by wortmannin) or the MAP kinase kinases MEK-1 and MEK-2 (the activation of which is inhibited by UO126) did not have any effect on the mobilisation of calcium following BLT_1 activation. Similar observations have been made by Haribabu et al. (1999), using BLT_1-transfected RBL cells treated with wortmannin. Lindsay et al. (Lindsay et al., 1998a) have examined several eosinophil responses to LTB_4 (arachidonic release, aggregation and H_2O_2 generation) and were also unable to

observe changes following treatment with a MEK-1/2 inhibitor. So, while BLT_1 does activate MAP kinases (Kotarsky et al., 2001) and phosphoinositol-3 kinases, there is no apparent cross talk between these pathways and BLT_1 induced calcium release in HeLa cells.

We found that PKC activity prior to BLT_1 receptor activation was inversely related to the magnitude of LTB_4 induced calcium responses, which is in agreement with many experiments using leukocyte cultures (e.g. Naccache et al., 1985; Teixeira et al., 1997).

Neutrophils also express several different receptors that, when activated, increase intracellular cyclic adenosine monophosphate (cAMP) concentrations, and these receptors have been shown to inhibit the activity of other chemotactic factors, such as fMLP (Tintinger et al., 2001). Changes in neutrophil cAMP levels have also been shown to modulate calcium signalling pathways (Tintinger et al., 2001) and de novo LTB_4 production (Flamand et al., 2000, 2002). Inhibiting PKA using H-89 had a small, but reproducible, inhibitory effect on BLT_1 receptor-induced intracellular calcium concentration changes. Activation of adenylyl cyclase using forskolin also had a similar effect, which was unexpected given that this presumably leads to the activation of a variety of downstream signalling proteins, including PKA.

The interaction between cAMP and PKA appears to be finely tuned, and different cell lines respond differently to this treatment. Thapsigargin induced calcium influx in HL-60 cells for example is more vigorous following pretreatment with forskolin (Song et al., 1998). Results very similar to ours have, however, been reported using lymphocytes stimulated with thapsigargin (de la Rosa et al., 2001). In these cells both forskolin and H-89 inhibited the calcium influx that followed thapsigargin induced calcium release from intracellular stores. It may be that small changes in the activity of PKA, up or down, cause the influx of intracellular calcium to decrease from a maximal level. This behaviour has been proposed based on observations from an excitable cell model (Obrietan and van den Pol, 1997). Another possible explanation is that PKA activity may increase mitochondrial uptake of calcium, leading to a perceived decrease in intracellular calcium concentrations, even if the calcium influx is also increased (Fernando et al., 1998).

H-89 does, however, inhibit a number of other protein kinases besides PKA (notably mitogen and stress activated kinase 1 and the rho-associated kinase, ROCK-II) [Davies, 2000 #4780], and lower H-89 doses had no effect on BLT_1-induced calcium release. To investigate whether non-specific effects could explain the effects of H-89 treatment, we used Y-27632 which, at low doses, is specific for Rho-associated protein kinases, whereas higher doses also inhibit PKA and PKC [Davies, 2000 #4780]. Low doses of Y-27632 had no effect on calcium responses, suggesting that the observed effects of H-89 are not due to inhibition of Rho-associated kinases. At higher doses Y-27632 inhibited PKC, producing responses that were very similar to those observed following treatment with the PKC-specific inhibitor GF 109203x.

We observed that genistein, an inhibitor of a range of tyrosine kinases, attenuated the LTB_4-induced increase in intracellular calcium concentration. Various studies have reported that tyrosine kinase activity attenuates increases in intracellular calcium concentration and, Dryden et al. (1992) have reported observations similar to ours using neutrophils, although LTB_4 induced intracellular calcium concentration changes have also been reported to be unaffected by tyrosine kinase activity (Naccache et al., 1990). These interactions are interesting given the importance of tyrosine kinases for leukocyte physiology.

The leukotriene receptors, BLT_1 and BLT_2, are often co-expressed on many of the cells commonly used to study the pharmacology of LTB_4. These results represent the first investigation of BLT_1 receptor signalling without the confounding effects of BLT_2 activation. It is important to separate these two LTB_4 receptor subtypes in terms of physiological function because this is one of the first steps towards identifying targets for pharmacological intervention at the receptor level. Our data from recombinant cells corroborates and also extends data from previous studies using ex vivo leukocyte preparations.

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