Functional K\textsubscript{Ca}3.1 K\textsuperscript{+} channels are required for human lung mast cell migration

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Background: Mast cell recruitment and activation are critical for the initiation and progression of inflammation and fibrosis. Mast cells infiltrate specific structures in many diseased tissues such as the airway smooth muscle (ASM) in asthma. This microlocalisation of mast cells is likely to be key to disease pathogenesis. Human lung mast cells (HLMC) express the Ca\textsuperscript{2+} activated K\textsuperscript{+} channel K\textsubscript{Ca}3.1 which modulates mediator release, and is proposed to facilitate the retraction of the cell body during migration of several cell types. A study was undertaken to test the hypothesis that blockade of K\textsubscript{Ca}3.1 would attenuate HLMC proliferation and migration.

Methods: HLMC were isolated and purified from lung material resected for bronchial carcinoma. HLMC proliferation was assessed by counting cells at various time points following drug exposure. HLMC chemotaxis was assayed using standard Transwell chambers (8 \ \mu\text{m} pore size). Ion currents were measured using the single cell patch clamp technique.

Results: K\textsubscript{Ca}3.1 blockade with triaryl methane-34 (TRAM-34) did not inhibit HLMC proliferation and clotrimazole had cytotoxic effects. In contrast, HLMC migration towards the chemokine CXCL10, the chemoattractant stem cell factor, and the supernatants from tumour necrosis factor \alpha stimulated asthmatic ASM was markedly inhibited with both the non-selective K\textsubscript{Ca}3.1 blocker charybdotoxin and the highly specific K\textsubscript{Ca}3.1 blocker TRAM-34 in a dose dependent manner. Although K\textsubscript{Ca}3.1 blockade inhibits HLMC migration, K\textsubscript{Ca}3.1 is not opened by the chemotactic stimulus, suggesting that it must be involved downstream of the initial receptor-ligand interactions.

Conclusions: Since modulation of K\textsubscript{Ca}3.1 can inhibit HLMC chemotaxis to diverse chemoattractants, the use of K\textsubscript{Ca}3.1 blockers such as TRAM-34 could provide new therapeutic strategies for mast cell mediated diseases such as asthma.

Mast cells are major effector cells in many different inflammatory and fibrotic diseases including asthma, pulmonary fibrosis, and rheumatoid arthritis.\textsuperscript{1} In these diseases there is evidence of increased mast cell numbers within specific tissue structures,\textsuperscript{2,3} where they contribute to the immunopathology through the release of a plethora of pleiotropic autacoids, proteases, and cytokines. For example, in asthma, mast cells infiltrate the airway smooth muscle (ASM) bundles in the airways of asthmatic subjects, but not those of either patients with eosinophilic bronchitis or normal subjects.\textsuperscript{2} This event is likely to be key in the development of the disordered ASM physiology of asthma. Similarly, in rheumatoid disease there is accumulation of mast cells in the pannus at the edge of articular cartilage erosion.\textsuperscript{3} It follows from this that targeting mast cell migration to these sites may offer a novel means of treating diseases in which mast cells play a role.

We have recently shown that ASM cell derived CXCL10 (also known as interferon-\gamma inducible protein of 10 kDa (IP-10)) induces human lung mast cell (HLMC) chemotaxis.\textsuperscript{4} CXCL10 induces HLMC migration by binding to the chemokine receptor CXCR3 which is preferentially expressed on mast cells within the ASM of asthmatic subjects.\textsuperscript{5} Inhibition of the CXCL10/CXCR3 axis could therefore provide a new and effective treatment for asthma. However, in addition to CXCL10, the ASM produces several other mast cell chemoattractants including stem cell factor (SCF),\textsuperscript{6} transforming growth factor \beta (TGF\beta),\textsuperscript{7} and the chemokines CXCL8, CXCL12 and CCL11.\textsuperscript{8} A more general approach to preventing mast cell migration may therefore demonstrate better efficacy in the treatment of asthma and other mast cell mediated diseases than targeting of specific chemokines.

Ion channels are emerging as interesting therapeutic targets in both inflammatory and structural non-excitable cells.\textsuperscript{8,9} HLMC, human bone marrow derived and human peripheral blood derived mast cells express the intermediate conductance Ca\textsuperscript{2+} activated K\textsuperscript{+} channel, K\textsubscript{Ca}3.1 (also known as IKCa1).\textsuperscript{10–12} This channel maintains a negative membrane potential during cell activation, thus increasing the driving potential for Ca\textsuperscript{2+} influx through store operated calcium channels.\textsuperscript{13} Blockade of K\textsubscript{Ca}3.1 therefore attenuates HLMC degranulation while opening it enhances it.\textsuperscript{10–11} In addition to their role in cell activation, ion channels carrying K\textsuperscript{+} and Cl\textsuperscript{−} have been implicated in many diverse cellular processes including proliferation, chemotaxis, and apoptosis. K\textsubscript{Ca}3.1 channels are required for lysophosphatidic acid induced microglial cell migration\textsuperscript{14} and evidence also exists for the involvement of K\textsubscript{Ca}3.1 in both T and B cell proliferation.\textsuperscript{15}

In this study we have examined the hypothesis that K\textsubscript{Ca}3.1 is important for HLMC migration and proliferation. To test this we examined the effects of K\textsubscript{Ca}3.1 blockade with the relatively non-selective K\textsubscript{Ca}3.1 blocker charybdotoxin and the highly specific K\textsubscript{Ca}3.1 blocker triaryl methane-34 (TRAM-34) on HLMC migration induced by CXCL10, SCF, and ASM. We also studied the effect of the K\textsubscript{Ca}3.1 blockers TRAM-34 and clotrimazole on HLMC proliferation.

METHODS

Reagents

The following were purchased: recombinant human (rh)SCF, rhIL-6, rhIL-10, rhCXCL10 (R&D, Abingdon, UK).

Abbreviations: ASM, airway smooth muscle; HLMC, human lung mast cell; IL, interleukin; SCF, stem cell factor; TGF-\beta, transforming growth factor \beta; TNF-\alpha, tumour necrosis factor \alpha; TRAM-34, triaryl methane-34.
1-ethyl-2-benzimidazolinone (1-EBIO), charybdotoxin, iberiotoxin (Sigma, Poole, Dorset, UK); mouse IgG1 mAb YB5.B8 (anti-CD117) (Cambridge Bioscience, Cambridge, UK); sheep anti-mouse IgG1 Dynabeads (Dynal, Wirral, UK); DMEM/ Glutamax/Hepes, antibiotic/antimycotic solution, MEM non-essential amino acids and foetal calf serum (FCS) (Life Technologies, Paisley, UK). TRAM-34 was a generous gift from Dr Heike Wulff (University of California Irvine, California, USA).

HLMC purification
HLMC were dispersed enzymatically from macroscopically normal lung obtained within 1 hour of resection for lung cancer and purified using immunofinity magnetic selection (Dynabeads) as described previously. The final mast cell purity, assessed using metachromatic staining, was >99% with cell viability >98% (monitored by exclusion of trypan blue). HLMC were cultured in DMEM/Glutamax/Hepes containing 1% antibiotic/antimyotic solution, 1% non-essential amino acids, 10% FCS, 100 ng/ml SCF, 50 ng/ml IL-6, and 10 ng/ml IL-10.

HLMC proliferation assay
Following purification, HLMC were resuspended in DMEM/Glutamax/Hepes containing SCF (100 ng/ml), interleukin (IL)-6 (50 ng/ml), and IL-10 (10 ng/ml) at a concentration of 0.25×10⁶ cells/ml as described previously. Clotrimazole and TRAM-34 were added in the concentration range 10–1000 nM. Control wells containing either 0.1% dimethyl sulfoxide (DMSO) or culture medium alone were also established. Metachromatic cells were counted after 1 and 4 weeks in culture using Kimura stain.

HLMC chemotaxis
HLMC chemotaxis assays were performed using the Transwell system (BD Biosciences, Oxford, UK) with 24 well plates as described previously. CXCL10 or SCF was placed in the lower wells (omitted in negative control) at a concentration of 100 ng/ml, interleukin (IL)-6 (50 ng/ml), and IL-10 (10 ng/ml) at a concentration of 0.25×10⁶ cells/ml as described previously. Clotrimazole and TRAM-34 were added in the concentration range 10–1000 nM. Control wells containing either 0.1% dimethyl sulfoxide (DMSO) or culture medium alone were also established. Metachromatic cells were counted after 1 and 4 weeks in culture using Kimura stain.

Patch clamp electrophysiology
The whole cell variant of the patch clamp technique was used. Patch pipettes were made from borosilicate fibre containing glass (Clark Electromedical Instruments, Reading, UK) and their tips were heat polished resulting in resistances of typically 4–6 MΩ. The standard pipette solution contained (in mM): KCl, 140; MgCl₂, 2; HEPES, 10; Na⁺ATP, 2; GTP, 0.1; pH 7.3. The standard external solution contained (in mM): NaCl, 140; KCl, 5; CaCl₂, 2; MgCl₂, 1; HEPES, 10; pH 7.3. For recording, mast cells were placed in 35 mm dishes containing standard external solution.

Whole cell currents were recorded using an Axoclamp 200A amplifier (Axon Instruments, Foster City, CA, USA), and currents evoked by applying voltage commands to a range of potentials (120 to +130 mV) in 10 mV steps from a holding potential of −20 mV. The currents were digitised (sampled at a frequency of 10 kHz), stored on computer, and subsequently analysed using pClamp software (Axon Instruments). Capacitance transients were minimised using the capacitance neutralisation circuits on the amplifier. Correction for series resistance was not routinely applied. In some experiments continuous membrane currents were recorded at a constant holding potential of +40 mV, data being digitised at 200 Hz and recorded using Axoscope (Axon Instruments). Experiments were performed at 27°C, the temperature being controlled by a Peltier device. Experiments were performed with a perfusion system (Automate Scientific Inc, San Francisco, CA, USA) to allow solution changes, although drugs were added directly to the recording chamber.

Data presentation and statistical analysis
Data are presented as mean (SE) values from separate donors performed in duplicate. Confidence intervals were calculated at 95% using Graphpad Prism 4 software. The inhibition of chemotaxis is presented as the percentage migration compared with the positive control after the subtraction of the negative control from all conditions. Differences between
data sets were analysed using a paired two tailed Student’s t test (Microsoft Excel v2003). p values of <0.05 were considered statistically significant.

All human subjects gave written informed consent and the study was approved by the Leicestershire Research Ethics Committee.

RESULTS

HLMC proliferation

HLMC proliferate in long term culture. Clotrimazole, a blocker of K<sub>Ca</sub>3.1 (K<sub>d</sub> 70 nM) and inhibitor of cytochrome P450, produced a dose dependent reduction in HLMC number after 4 weeks in culture, with complete cell death evident at 1000 nM (fig 1A). In contrast, TRAM-34, a highly specific blocker of K<sub>Ca</sub>3.1 (K<sub>d</sub> 20 nM) without any effect on cytochrome P450, had no effect on HLMC at 1 or 4 weeks (fig 1B). This suggests that K<sub>Ca</sub>3.1 is not involved in HLMC proliferation and that the effects of clotrimazole were “toxic” and independent of its effect on K<sub>Ca</sub>3.1. This is in contrast to the role of K<sub>Ca</sub>3.1 in human T lymphocyte proliferation.

HLMC chemotaxis

Because of its apparent toxic effect on HLMC, clotrimazole was not used in the chemotaxis assays. For blockade of K<sub>Ca</sub>3.1 during chemotaxis we used TRAM-34 and another K<sub>Ca</sub>3.1 blocker, charybdotoxin (K<sub>d</sub> 5 nM).

The migration of HLMC in response to 100 ng/ml CXCL10 was 2.2 (0.2) fold greater than that of the control (no CXCL10, n = 6; 95% CI 1.7 to 2.7, p = 0.001, fig 2), in keeping with previous experiments. CXCL10 induced migration was inhibited dose dependently by TRAM-34. Thus, with 200 nM TRAM-34 migration was reduced by 80 (7)% (n = 6; 95% CI 62 to 99, p = 0.0001, fig 3). Consistent with this, CXCL10 induced HLMC migration was inhibited by 78 (14)% with the addition of 100 nM charybdotoxin (n = 6; 95% CI 42 to 113, p = 0.002, fig 3).

To determine whether the inhibition of HLMC migration by K<sub>Ca</sub>3.1 blockade was restricted to an interaction with G protein coupled chemotactants or a general phenomenon,
Effects of CXCL10 on HLMC KCa3.1 activation

We have recently shown that the Gαs coupled β3 adrenoceptor influences KCa3.1 gating. To assess whether the Gαs coupled CXCL10 receptor CXCR3 influences KCa3.1 function we attempted to record KCa3.1 currents following the addition of 100 ng/ml CXCL10 to HLMC (n = 23 cells from four separate donors). In spite of being able to record KCa3.1 currents in these cells following the addition of the KCa3.1 specific channel opener 1-ethyl-2-benzimidazolinone (1-EBIO), we did not see any evidence of either the transient or sustained opening of KCa3.1 in response to CXCL10 (fig 6).

DISCUSSION

In this study we have shown for the first time that blockade of the K+ channel KCa3.1 markedly attenuates the chemotactic response of ex vivo HLMC to the chemokine CXCL10, the mast cell growth factor SCF, and conditioned media from asthmatic ASM. In contrast to its effects on acute mitogen induced lymphocyte proliferation, KCa3.1 does not appear to be important for HLMC proliferation in long term culture.

The redistribution of mast cells within human tissues is likely to be key in the initiation and propagation of a variety of diseases.235 Inhibiting their migration within tissues may therefore offer a truly novel approach for the treatment of mast cell driven pathobiology. As an example, we have recently shown that the CXCL10/CXCR3 axis may be a critical determinant of mast cell distribution within the human lung.3 Thus, inhibition of CXCL10/CXCR3 induced chemotaxis is an attractive target for asthma therapy. However, while targeting CXCL10/CXCR3 may prove fruitful, CXCL10 also produces several other chemoattractants including SCF, TGFβ and other chemokines.5–7 These may play their own part in mast cell recruitment by ASM under certain conditions and, in addition, are probably important for the microlocalisation of mast cells within other tissue compartments in unrelated diseases such as tubulointerstitial renal disease and rheumatoid arthritis.8–10 The ability of KCa3.1 blockade profoundly to inhibit mast cell migration in response to two diverse chemoattractants—CXCL10 which activates the G protein coupled receptor CXCR3 and SCF which activates the tyrosine kinase receptor CD117—therefore suggests that KCa3.1 has great potential as a therapeutic target for mast cell mediated disease in humans. Moreover, blockade of KCa3.1 almost completely attenuates HLMC migration towards the complex milieu of chemoattractants present in TNFα stimulated asthmatic ASM cell supernatants, strengthening the hypothesis that functional KCa3.1 channels are an absolute requirement for HLMC migration.

To inhibit HLMC migration we used charybdotoxin and TRAM-34, two distinct molecules which block the KCa3.1 pore at different sites.19–20 Charybdotoxin is a 37 amino acid peptide derived from the venom of the scorpion Leiurus quinquestriatus and blocks KCa3.1 with a Kd for channel block of 5–10 nM.19–20 It also blocks the voltage gated K+ channel Kv1.3 and the large conductance KCa,KCa1.1. However, HLMC do not express KCa1.1 currents or mRNA,10,22 iiberiotoxin should be ineffective. Indeed, iiberiotoxin did not attenuate the migration of HLMC to either CXCL10 or SCF (2.0 (0.6) fold in the control compared with 1.8 (0.5) fold with 100 nM iiberiotoxin). Background control migration was not inhibited by charybdotoxin or TRAM-34.

Since HLMC migration to SCF and CXCL10 was inhibited with similar efficacy, we next examined the effects of TRAM-34 and charybdotoxin on KCa3.1 currents following the addition of 1-EBIO to the recording chamber. (A) Continuous recording of current at +40 mV in an HLMC showing a stable baseline with minimal basal current. No response was seen to the addition of CXCL10 where indicated, but a rapid increase in outward current occurred within 90 seconds of adding 1-EBIO to the recording chamber. (B) Current-voltage curves from another cell at rest 3 minutes after the addition of CXCL10 and 3 minutes after the addition of 1-EBIO showing development of a typical KCa3.1 whole cell current following the latter. We have recently shown that the K+ channel KCa3.1, also known as BKCa blocker iiberiotoxin (1-EBIO). Background control migration was not inhibited by charybdotoxin or TRAM-34.

Since HLMC migration to SCF and CXCL10 was inhibited with similar efficacy, we next examined the effects of KCa3.1 blockade on HLMC migration towards the complex milieu of chemoattractants present in cell supernatants from stimulated asthmatic ASM. The ASM supernatants induced HLMC migration to a greater extent than either CXCL10 or SCF (2.0 (0.2) fold compared with 1.8 (0.5) fold with 100 nM iiberiotoxin). Background control migration was not inhibited by charybdotoxin or TRAM-34.

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because it has been estimated previously that, for complete channel block to be achieved, drugs need to be present at 5–10 times the \( K_d \). The ability of these two potent but pharmacologically distinct \( K_{Ca3.1} \) blockers to inhibit HLMC migration to a similar extent at 10 times the \( K_d \) therefore indicates that the mechanism behind this is indeed \( K_{Ca3.1} \) blockade. In addition, the \( K_{Ca3.1} \) blocker ibetioxato was without effect, further suggesting that the effects observed are specific to \( K_{Ca3.1} \).

The role of \( K_{Ca3.1} \) in HLMC and T cell mediated secretion is to maintain the negative membrane potential during cell activation, counteracting the tendency for \( Ca^{2+} \) influx to depolarise the cell membrane. Thus, \( K_{Ca3.1} \) increases the driving force for \( Ca^{2+} \) influx because store operated \( Ca^{2+} \) channels conduct larger currents at negative membrane potentials.\(^{10,11,13} \) However, the role of this channel in cell migration is predicted to be different. A \( K_{Ca3.1} \) channel with some properties of \( K_{Ca3.1} \) has been demonstrated in glial cells and has been proposed to facilitate the retraction of the rear body of the migrating cell,\(^{24,25} \) despite appearing to be more highly expressed at the leading edge of lamellipodia in migrating MDCK-F (transformed renal epithelial cells), NIH-3T3 fibroblasts, and human melanoma cells.\(^{26} \) The mechanism controlling their seemingly selective activation at the rear body of the cell is unclear, although the localised \( Ca^{2+} \) concentration is likely to be critical. In addition, migration appears to require the opening and closing of the channels, most probably due to calcium oscillations, since both \( K_{Ca3.1} \) channel blockers and the opener 1-EBIO all inhibit chemotaxis in these cells.\(^{14,16} \) The intermittent activation of the \( K_{Ca3.1} \)-like channel in these cells is therefore believed to facilitate the swelling and shrinking of the cell body required for cell migration,\(^{27,28} \) and would explain why functional \( K_{Ca3.1} \) are important for migration in HLMC.

CXCL10 increases intracellular \( Ca^{2+} \) transiently in HLMC through the release of \( Ca^{2+} \) from internal stores but does not activate \( Ca^{2+} \) influx from the extracellular fluid.\(^{4} \) We have shown in this study that CXCL10 does not directly open \( K_{Ca3.1} \), which is in keeping with our previous finding that influx of extracellular \( Ca^{2+} \) is the critical requirement for \( K_{Ca3.1} \) opening during HLMC activation.\(^{16} \) In addition, SCF does not open \( K_{Ca3.1} \).\(^{14} \) This indicates that the gating of \( K_{Ca3.1} \) during HLMC migration is downstream of the chemoattractant stimulus, most probably related to adhesive signals required for the migratory process. These data also show that, while the \( K_{Ca3.1} \) channel in HLMC is known to be directly coupled to the \( G_{m} \) dependent \( \beta_2 \) adrenoceptor,\(^{17} \) it is not coupled to the \( G_{m} \) dependent CXCR3 receptor.

We have previously shown that HLMC proliferate in long term culture. Following an initial decrease in cell number over the first week, HLMC proliferate so that by 4 weeks there can be up to four times the starting number.\(^{16} \) Interestingly, in contrast to published observations in T and B cells\(^{29,30} \) and endothelial cells,\(^{31} \) TRAM-34 had no significant effect on HLMC proliferation. TRAM-34 inhibits mitogenesis of preactivated human T cells at concentrations several orders of magnitude higher than those required for channel blocking.\(^{27,28} \) Blockade of \( K_{Ca3.1} \) has shown promise in several diseases/disease models. Clotrimazole was very effective in ameliorating active human rheumatoid arthritis but caused unacceptable side effects due to inhibition of cytochrome P450.\(^{32} \) TRAM-34 prevents vascular restenosis after balloon angioplasty in rats through its ability to inhibit neointimal vascular smooth muscle proliferation and without any undue toxicity.\(^{29} \) Another specific \( K_{Ca3.1} \) blocker, 4-phenyl-4H-pyran (K\( _d \) 8 nM), reduces infarct volume in a rat model of subdural haematoma, suggesting a possible use in the management of traumatic brain injury.\(^{33} \) Clinical trials are also underway to study the effects of \( K_{Ca3.1} \) inhibition in sickle cell anaemia. These studies of \( K_{Ca3.1} \) blockage are encouraging, and the lack of obvious toxicity of TRAM-34 suggests real therapeutic potential for human disease. With regard to mast cell mediated disease, however, further mechanistic studies of \( K_{Ca3.1} \) blockade using rodent models are unlikely to be informative because \( K_{Ca3.1} \) currents have not been observed in rodent mast cells.\(^{34} \)

In summary, we have shown for the first time that blockade of the \( K^+ \) channel \( K_{Ca3.1} \) markedly attenuates the chemotactic response of ex vivo HLMC to both the chemokine CXCL10 and the mast cell growth factor SCF, as well as TNF\( \alpha \) stimulated asthmatic ASM supernatants. This suggests that blocking \( K_{Ca3.1} \) has great potential as a target for the treatment of asthma and other inflammatory diseases in which mast cells play a role.

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**REFERENCES**

LUNG ALERT

Lung cancer is more common but less often fatal in women

Lung cancer is on the rise, especially in women. Previous studies have suggested that women have a higher relative risk of developing lung cancer. This large follow up study from the International Early Lung Cancer Action Programme (ELCAP) investigators was designed to assess the risk of lung cancer related to smoking in women compared with men. Survival after diagnosis was also evaluated, using the data from the present study combined with those from the original ELCAP study.

An additional 6296 women and 8139 men (all asymptomatic) were screened with baseline CT scans. All patients with lung nodules underwent immediate biopsy or surgical resection. A panel of five expert lung pathologists reported the histological samples. Lung cancer was diagnosed in 111/6296 women (1.7%) and 93/8139 men (1.1%). Combining the two series together, lung cancer was diagnosed in 156/7498 women (2.1%) and 113/9427 men (1.2%). This gave an odds ratio of cancer in women of 1.9 (95% CI 1.5 to 2.5) corrected for age and pack-year smoking history. It was also noted that survival was better in women, regardless of the stage of lung cancer and after matching for cell type and treatment. The hazard ratio for a fatal outcome in women was 0.48 (95% CI 0.25 to 0.89).

This is an interesting result, but the authors could not find any specific reason for the difference in survival. It has been suggested that lung cancer in women may be less aggressive or relatively more curable than in men, and this needs to be evaluated further.

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