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Engagement of the EP_2 prostanoid receptor closes the K^+ channel $K_{Ca}3.1$ in human lung mast cells and attenuates their migration

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Human lung mast cells (HLMC) express the Ca^{2+}-activated K^+ channel $K_{Ca}3.1$, which plays a crucial role in their migration to a variety of diverse chemotactic stimuli. $K_{Ca}3.1$ activation is attenuated by the β_2-adrenoceptor and the adenosine A_{2A} receptor through a G_s-coupled mechanism independent of cyclic AMP. Prostaglandin E_2 promotes degranulation and migration of mouse bone marrow-derived mast cells through the G_i-coupled EP_3 prostanoid receptor, and induces LTC_4 and cytokine secretion from human cord blood-derived mast cells. However, PGE_2 binding to the G_s-coupled EP_2 receptor on HLMC inhibits their degranulation. We show that EP_2 receptor engagement closes $K_{Ca}3.1$ in HLMC. The EP_2 receptor-specific agonist butaprost was more potent than PGE_2 in this respect, and the effects of both agonists were reversed by the EP_2 receptor antagonist AH6809. Butaprost markedly inhibited HLMC migration induced by chemokine-rich airway smooth muscle-conditioned media. Interestingly, PGE_2 alone was chemotactic for HLMC at high concentrations (1 μM), but was a more potent chemoattractant for HLMC following EP_2 receptor blockade. Therefore, the G_s-coupled EP_2 receptor closes $K_{Ca}3.1$ in HLMC and attenuates both chemokine- and PGE_2-dependent HLMC migration. EP_2 receptor agonists with $K_{Ca}3.1$ modulating function may be useful for the treatment of mast cell-mediated disease.

Key words: Chemotaxis · Ion channel · $K_{Ca}3.1$ · Mast cell · Prostaglandin E_2

Introduction

Mast cells are tissue-dwelling cells derived from bone marrow progenitors. They are present in all organs throughout the human body, both at mucosal surfaces and within connective tissues. Mast cells play a major role in tissue homeostasis, host defence and the pathophysiology of many diverse diseases [1]. These include pulmonary fibrosis, rheumatoid disease and atherosclerosis, but they are most commonly associated with allergic disease due to their activation by allergen [2]. In many diseases mast cells re-locate to specific compartments within tissue. This is typified in asthma where mast cells migrate into the airway epithelium [3], airway smooth muscle (ASM) [4] and submucosal glands [5]. This places activated mast cells in direct contact with these dysfunctional airway elements, allowing the specific delivery of detrimental cell–cell signals. Drugs that inhibit this tissue relocation by preventing mast cell migration may prove particularly effective in the treatment of mast cell-mediated disease.

Human mast cells express the intermediate conductance Ca^{2+}-activated K^+ channel $K_{Ca}3.1$, which plays a critical role in their migration to diverse chemotactic stimuli [6], and to a lesser extent in their degranulation [7, 8]. Drugs that directly block this channel or which close it indirectly therefore have potential as novel therapies for mast cell-dependent disease. $K_{Ca}3.1$ in human

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lung mast cells (HLMC) is closed by both salbutamol and adenosine *via* the β_2-adrenoceptor and A_{2A} adenosine receptors, respectively [9, 10], which are both G_s-coupled G protein-coupled receptors (GPCR). In keeping with a critical role for $K_{Ca}3.1$ in HLMC migration, adenosine also inhibits HLMC chemotaxis *via* the A_{2A} receptor [10].

PGE_2 is a prostanoid with four specific GPCR, designated EP_{1-4}. EP_2 and EP_4 couple to G_s, EP_3 couples predominantly to G_i although two isoforms also couple to G_s, while EP_1 receptors mobilise intracellular Ca^{2+}, probably through G_q [11]. PGE_2 therefore has diverse biological activities depending on the receptors it interacts with and the cells expressing them. With respect to mast cells, mouse mast cells express the EP_3 receptor whose activation induces both degranulation and chemotaxis [12, 13]. Human cord blood-derived mast cells, express both EP_2 and EP_3 receptors. In these cells, PGE_2 enhances degranulation and PGD_2 production in cells primed by IL-4, an effect mediated through the EP_3 receptor. However, following FcεRI-dependent activation PGE_2 inhibits human cord blood-derived mast cell LTC_4, PGD_2, IL-5 and TNF-α production *via* the EP_2 receptor but has no effect on degranulation [14]. In contrast, several studies have shown that PGE_2 consistently inhibits degranulation and eicosanoid production by HLMC, an effect mediated *via* the EP_2 receptor [15–17]. This receptor also appears to dominate PGE_2-dependent signalling on mast cells within the human asthmatic lung as PGE_2 markedly attenuates both the early and late phase airway response to allergen challenge [18].

Since $K_{Ca}3.1$ in HLMC is closed by G_s-coupled β_2-adrenoceptors and A_{2A} adenosine receptors, we hypothesised that activation of the G_s-coupled EP_2 receptor would also close $K_{Ca}3.1$ in these cells. Furthermore, if PGE_2 was to close $K_{Ca}3.1$, then it should inhibit HLMC chemotaxis. To test this hypothesis we have used the patch-clamp technique to investigate the effects of PGE_2 on HLMC ion channel function, and investigated the effect of PGE_2 on HLMC migration induced by asthmatic ASM-conditioned medium.

Results

PGE_2 alone does not open $K_{Ca}3.1$

We initially examined whether PGE_2 opens $K_{Ca}3.1$ in HLMC under resting baseline conditions. HLMC are typically electrically silent at rest and it is possible that PGE_2 could open either $K_{Ca}3.1$ or another channel as reported previously for adenosine [10]. No significant change in current amplitude was observed in seven cells following the addition of 10^{-5} M PGE_2 (current changed from 1.22 ± 0.54 to 1.42 ± 0.85 pA at +40 mV; $p = 0.678$).

PGE_2 closes $K_{Ca}3.1$ in the presence of the $K_{Ca}3.1$ opener 1-EBIO

We then examined whether PGE_2 closes $K_{Ca}3.1$. Because PGE_2 might potentially inhibit many IgE-dependent cell activation pathways that could reduce cytosolic-free Ca^{2+}, and thus reduce $K_{Ca}3.1$ activity indirectly, we concentrated on studying the effects of PGE_2 on $K_{Ca}3.1$ currents that were induced by the $K_{Ca}3.1$ opener 1-EBIO [8–10]. This compound opens $K_{Ca}3.1$ with a half-maximal value of about 30 μM for heterologously expressed $K_{Ca}3.1$, with a maximal effect at about 300 μM [19]. 1-EBIO is specific for $K_{Ca}3.1$ in HLMC and opens it by enhancing the channels sensitivity to $[Ca^{2+}]_i$ [19]. Thus at 100 μM EBIO, maximal K^+ currents are achieved in the presence of 100 nM free Ca^{2+}, which is below the resting $[Ca^{2+}]_i$ of most cell types including HLMC [8].

In cells in which $K_{Ca}3.1$ had been activated by 1-EBIO, addition of PGE_2 (10^{-8}–10^{-5} M) produced a rapid (within 30 s) dose-responsive inhibition of channel activity with an associated positive shift in membrane potential (Fig. 1A–E). PGE_2 10^{-5} M suppressed the $K_{Ca}3.1$ current in >90% of cells (Fig. 1B). Thus, addition of 10^{-5} M PGE_2 reduced $K_{Ca}3.1$ membrane current at +40 mV from 155.4 ± 20.9 to 92.6 ± 13.7 pA ($p = 0.0001$, $n = 22$ cells)(Fig. 1B), with a corresponding shift in reversal potential (Vm) from -69.5 ± 2.8 to -56.3 ± 3.9 mV ($p = 0.0004$) (Fig. 1C). Half maximal suppression (IC_{50}) of $K_{Ca}3.1$ by PGE_2 occurred at approximately 4.0×10^{-7} M (calculated from six cells). Importantly, the effect of PGE_2 was partially reversed within 1 min by removing it from the recording solution (current post PGE_2 49.6 ± 20.8 pA, post wash 103.4 ± 34.5 pA, $p = 0.046$; Vm post PGE_2 -44.1 ± 11.7 mV, post wash -72.1 ± 6.81 mV, $p = 0.028$, $n = 5$) (Fig. 1D and E), indicating that non-specific "rundown" was not responsible for the effects seen.

$K_{Ca}3.1$ modulation by PGE_2 is mediated *via* EP_2 receptors

To examine whether the effects of PGE_2 were mediated via EP_2 prostanoid receptors we examined the effects of EP_2 receptor agonists/antagonists. First, we examined the effects of the selective EP_2 receptor agonist butaprost. Butaprost mimicked the effects of native PGE_2 in a dose-dependent manner (Fig. 2A–D). At a concentration of 10^{-5} M, butaprost reduced the $K_{Ca}3.1$ current from 146.0 ± 18.3 pA to 61.2 ± 6.1 pA ($p = 0.00006$, $n = 20$) (Fig. 2B) with a corresponding shift in reversal from -67.5 ± 1.4 to -54.4 ± 3.2 mV ($p = 0.00006$) (Fig. 2C). Half maximal suppression (IC_{50}) of $K_{Ca}3.1$ by butaprost occurred at approximately 2.1×10^{-7} M ($n = 6$ cells) (Fig. 2D).

The suppression of $K_{Ca}3.1$ by 10^{-5} M PGE_2 was partially reversed by the competitive EP_1 and EP_2 receptor antagonist AH6809 (Fig. 3A–C). Thus, in experiments studying AH6809 at a concentration of 10^{-5} M, current at +40 mV was 112 ± 14.3 pA post PGE_2, increasing to 160.9 ± 23.2 pA post AH6809 ($p = 0.011$, $n = 10$ cells) (Fig. 3B). There was however no significant shift in reversal potential in these experiments explained by the fact that significant $K_{Ca}3.1$ currents remained following PGE_2 application (Vm post PGE_2 -60.9 ± 2.1 mV, post AH6809 -63.6 ± 1.8 mV, $p = 0.17$) (Fig. 3C). We also examined the effect of AH6809 on the selective EP_2 agonist butaprost. The suppression of $K_{Ca}3.1$ by

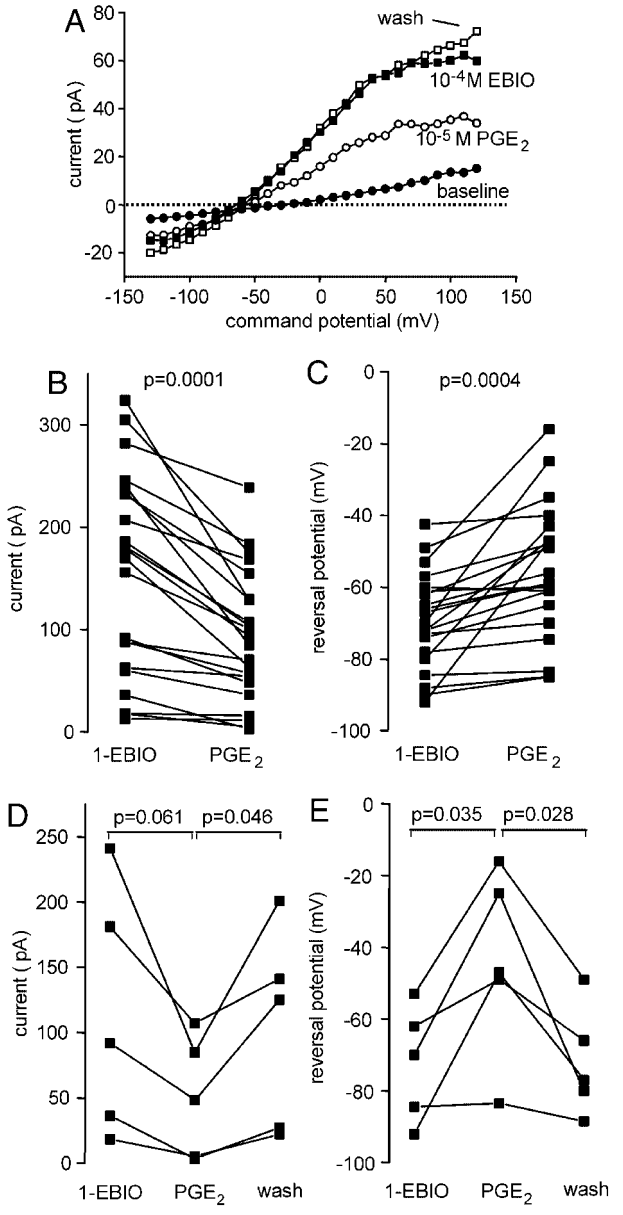

Figure 1. PGE_2 closes $K_{Ca}3.1$ in HLMC in a consistent and reversible manner. (A) Current–voltage curve demonstrating suppression of a 1-EBIO-induced $K_{Ca}3.1$ current by 10^{-5} M PGE_2 and partial reversal of the effect following removal of PGE_2 (wash). (B) Suppression of $K_{Ca}3.1$ current measured at +40 mV by 10^{-5} M PGE_2 ($n = 22$ cells). (C) Shift in whole-cell current reversal potential (Vm) by 10^{-5} M PGE_2 ($n = 22$ cells). (D) $K_{Ca}3.1$ current measured at +40 mV after addition of 1-EBIO, suppression by 10^{-5} M PGE_2 and then reversibility of suppression following removal of PGE_2 (wash) ($n = 5$ cells). (E) Whole-cell current reversal potential (Vm) after the addition of 1-EBIO, a depolarising positive shift in response to 10^{-5} M PGE_2, and then reversibility following removal of PGE_2 (wash) ($n = 5$ cells).

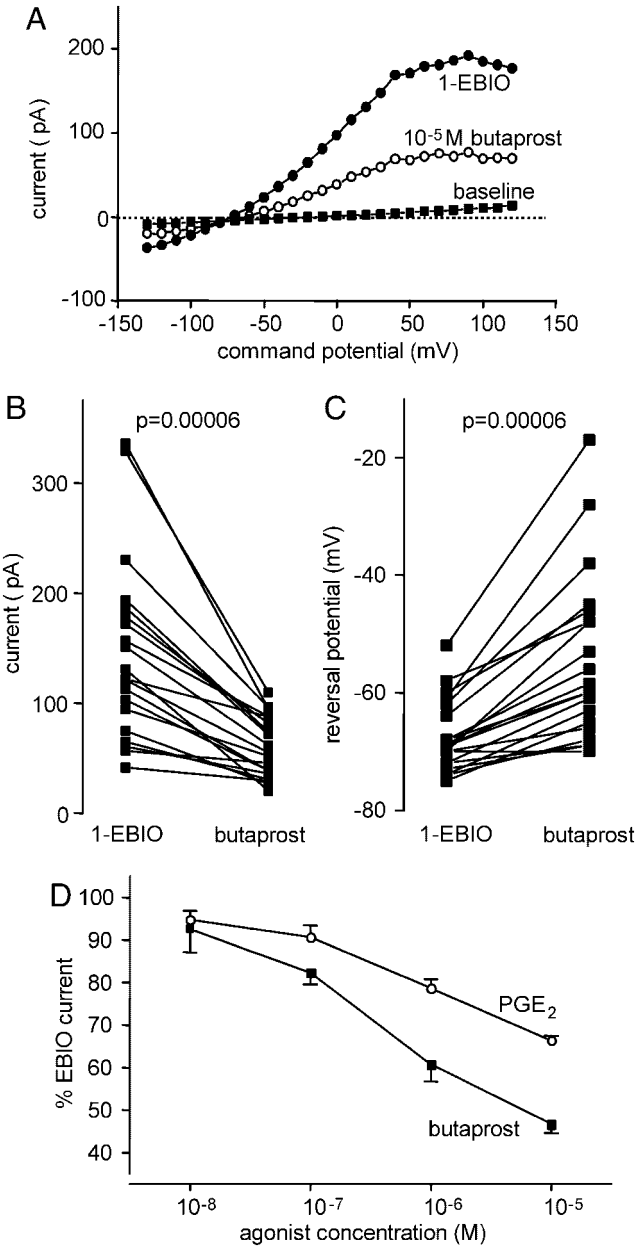

Figure 2. The EP_2 receptor agonist butaprost closes $K_{Ca}3.1$ in HLMC. (A) Current–voltage curve demonstrating suppression of a 1-EBIO-induced $K_{Ca}3.1$ current by 10^{-5} butaprost. (B) Suppression of $K_{Ca}3.1$ current measured at +40 mV by 10^{-5} M butaprost ($n = 20$ cells). (C) Shift in whole-cell current reversal potential (Vm) by 10^{-5} M butaprost ($n = 20$ cells). (D) Concentration–response curves for $K_{Ca}3.1$ suppression by PGE_2 and the EP_2 agonist butaprost (mean ± SEM of five cells for each).

10^{-5} M butaprost was also partially reversed by 10^{-5} M AH6809 (Fig. 3D–F). Current at +40 mV was 58.5 ± 8.3 pA post butaprost, increasing to 111.6 ± 15.9 pA post AH6809 ($p = 0.0004$, $n = 12$) (Fig. 3E). There was also a significant shift in reversal potential (Vm post butaprost −48.1 ± 5.8 mV, post AH6809 −61.0 ± 3.7 mV, $p = 0.0004$) (Fig. 3F). In the presence of AH6809, PGE_2 had no effect on $K_{Ca}3.1$ currents that had been induced by 1-EBIO (current at +40 mV 67.0 ± 17.2 pA post 1-EBIO, 66.2 ± 15.7 post AH6809, 62.7 ± 13.7 pA post PGE_2, $n = 7$)(Fig. 4A). Similarly, $K_{Ca}3.1$ currents did not appear in resting cells to which AH6809 was added prior to PGE_2 (current 6.9 ± 0.9 pA at baseline, 5.8 ± 0.6 pA post AH6809, 6.3 ± 1.1 pA post PGE_2, $n = 6$) (Fig. 4B). The EP_1 and EP_3 receptor agonist 17-phenyl trinor PGE_2 did not close $K_{Ca}3.1$ after activation with

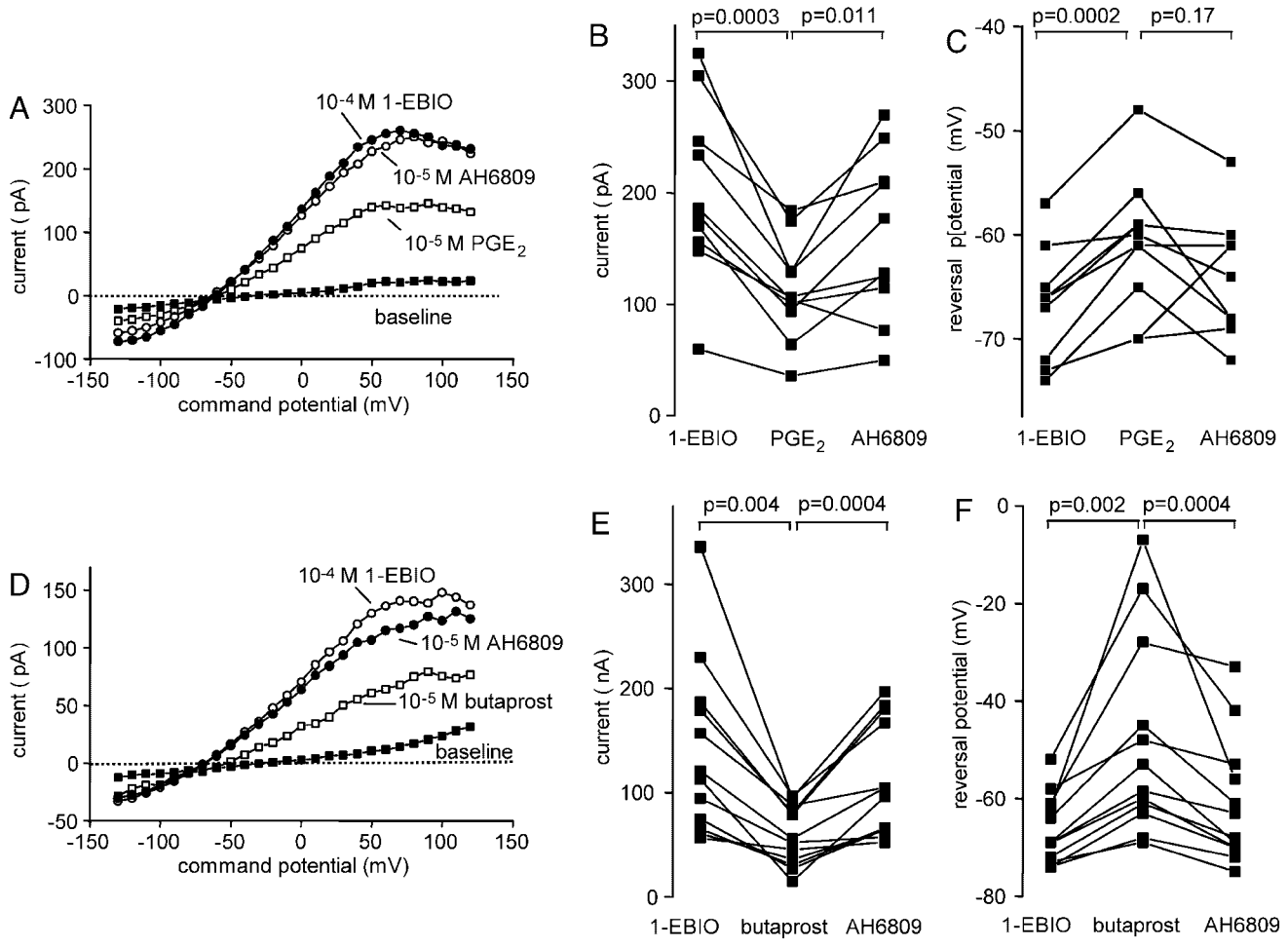

Figure 3. The effect of the $EP_1/_2$ receptor antagonist AH6809 on PGE_2- and butaprost -dependent closure of $K_{Ca}3.1$. (A) Current–voltage curve demonstrating reversibility of $K_{Ca}3.1$ suppression by PGE_2 following administration of the $EP_{1/2}$ receptor antagonist AH6809. (B) $K_{Ca}3.1$ current measured at +40 mV after addition of 1-EBIO, suppression by 10^{-5} M PGE_2 and then reversibility of suppression following addition of AH6809 ($n = 10$ cells). (C) Whole-cell current reversal potential (Vm) after the addition of 1-EBIO, a depolarising positive shift in response to 10^{-5} M PGE_2, and then following addition AH6809 ($n = 10$ cells). (D) Current–voltage curve demonstrating reversibility of $K_{Ca}3.1$ suppression by butaprost following administration of AH6809. (E) $K_{Ca}3.1$ current measured at +40 mV after addition of 1-EBIO, suppression by 10^{-5} M butaprost and then reversibility of suppression following addition of AH6809 ($n = 12$ cells). (F) Whole-cell current reversal potential (Vm) after the addition of 1-EBIO, a depolarising positive shift in response to 10^{-5} M PGE_2, and then reversibility following addition AH6809 ($n = 12$ cells).

1-EBIO (current at +40 mV post 1-EBIO 128.3±94.9 pA, post 17-phenyl trinor PGE_2 127.8±90.4 pA, $p = 0.9567$, $n = 6$) and did not open $K_{Ca}3.1$ in resting cells (baseline current at +40 mV 5.70±0.88 pA, current post 17-phenyl trinor PGE_2 5.36±0.949 pA, $p = 0.202$, $n = 5$). These results exclude a role for EP_1, EP_3 or EP_4 receptors in $K_{Ca}3.1$ modulation. Taking the data together, it can be concluded that suppression of $K_{Ca}3.1$ by PGE_2 is mediated by the EP_2 receptor.

PGE_2 closes $K_{Ca}3.1$ following IgE-dependent activation

Lastly, we confirmed whether PGE_2-dependent regulation of $K_{Ca}3.1$ was relevant to $K_{Ca}3.1$ channels that had been opened by anti-IgE-dependent activation. Anti-IgE (1:1000 dilution) opened $K_{Ca}3.1$ in 5/5 cells tested (baseline current at +40 mV 5.8±1.3 pA, baseline Vm −17.8±3.2 mV; post anti-IgE current 54.2±7.6 pA, Vm −62.4±3.0 mV). PGE_2 (10^{-5} M) suppressed the current to 34.3±4.9 pA post PGE_2, $p = 0.005$) (Fig. 5A and B) and produced an associated positive shift in Vm to −52.2±4.1 mV, $p = 0.051$) (Fig. 5C). This suppressive effect of PGE_2 was partially reversed by AH6809 (current 48.3±6.9 pA, $p = 0.011$; Vm −66.6±4.0 mV, $p = 0.041$) (Fig. 5A and C).

PGE_2 suppresses HLMC migration through the EP_2 receptor

Conditioned medium from asthmatic ASM, which has been activated with TNFα, IFNγ and IL-1β, mediates HLMC chemotaxis predominantly *via* the CXCL10/CXCR3 pathway with additional contributions from ligands for CXCR1 and CXCR3 [20]. Inhibition

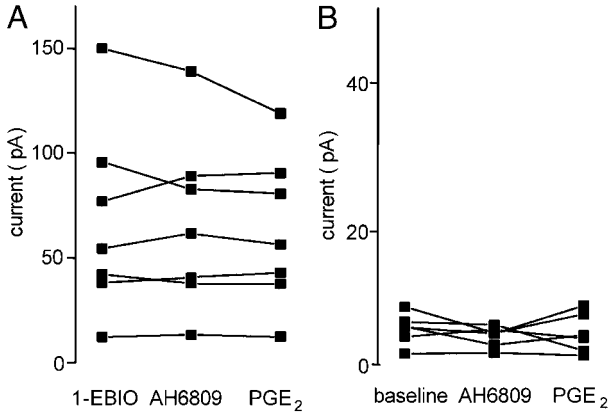

Figure 4. PGE_2 is ineffective in the presence of the AH6809. (A) $K_{Ca}3.1$ current measured at +40 mV after addition of 1-EBIO, stability following addition of AH6809, and failure to suppress following subsequent addition of 10^{-5} M PGE_2. (B) $K_{Ca}3.1$ current measured at +40 mV in resting cells, after addition of AH6809, and then following subsequent addition of 10^{-5} M PGE_2 showing failure of $K_{Ca}3.1$ to open.

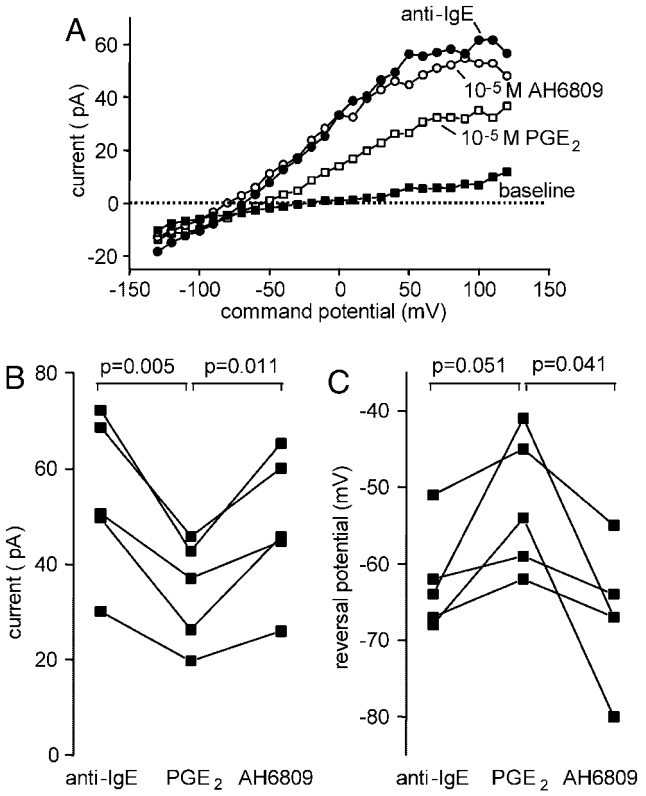

Figure 5. The effect of 10^{-5} M PGE_2 on $K_{Ca}3.1$ currents elicited by anti-IgE-dependent mast cell activation. (A) A representative HLMC demonstrating the development of a $K_{Ca}3.1$ current following anti-IgE-dependent activation, suppression of this by 10^{-5} M PGE_2, and reversal of the PGE_2-induced suppression by the $EP_{1/2}$ receptor antagonist AH6809. (B) $K_{Ca}3.1$ current measured at +40 mV after addition of anti-IgE, suppression by 10^{-5} M PGE_2 and then reversibility of suppression following addition of AH6809 ($n = 5$ cells). (C) Whole-cell current reversal potential (Vm) after the addition of anti-IgE, a depolarising positive shift in response to 10^{-5} M PGE_2, and then reversibility following addition AH6809 ($n = 5$ cells).

of $K_{Ca}3.1$ by channel blockers markedly suppresses this HLMC chemotaxis [6], as do molecules that close $K_{Ca}3.1$ such as adenosine [10]. Migration of HLMC using conditioned medium from asthmatic airway smooth muscle was 2.8 ± 0.9 fold that of medium control ($n = 4$, $p = 0.046$) (Fig. 6A) and this was not inhibited significantly by PGE_2 (Fig. 6B). However, the selective EP_2 agonist butaprost produced marked inhibition of HLMC migration to ASM-conditioned medium (Fig. 6B). Interestingly, PGE_2 was chemotactic on its own, but much less potent than in mouse bone marrow-derived mast cells [13] (Fig. 6C). This HLMC chemotactic activity of PGE_2 was markedly increased in the presence of $EP_{1/2}$ blockade by AH6809 10^{-5} M (Fig. 6D). HLMC migration to both ASM-conditioned medium and PGE_2 itself is therefore attenuated by the EP_2 receptor.

PGE_2 attenuates histamine release from cultured HLMC

PGE_2 inhibits the degranulation of HLMC freshly isolated from lung tissue. Because the cells used in this study were cultured in stem cell factor (SCF), IL-6 and IL-10, we investigated whether PGE_2 also inhibits release from these HLMC. In keeping with observations in freshly isolated HLMC, PGE_2 inhibited histamine release dose dependently over the concentration range 10^{-9}–10^{-6} M (control mean ± s.e.m. net histamine release $19.8 \pm 3.7\%$ versus $7.5 \pm 3.3\%$ with 10^{-6} PGE_2, $n = 4$ donors, $p = 0.046$).

$K_{Ca}3.1$ is not modulated by G_i or G_q-coupled receptors

We have now identified three distinct G_s-coupled receptors that close $K_{Ca}3.1$ in HLMC when exposed to the relevant ligands, and in the case of the β_2-adrenoceptor, the inverse agonist ICI 118551 opens the channel [9]. In contrast, the G_i-coupled CXCR3 receptor does not couple directly to $K_{Ca}3.1$ [6]. We have therefore investigated further G_i and G_q agonists known to have biological effects on HLMC. Platelet activating factor (PAF, 10^{-7} M) (G_i), lysophosphatidic acid (LPA, 10^{-5} M) ($G_{q,12/13,i}$) and UTP (10^{-4} M)(G_q) did not open $K_{Ca}3.1$ in resting HLMC ($n = \geq 6$ cells for each agonist) and did not close $K_{Ca}3.1$ that had been opened by 1-EBIO ($n = \geq 5$ cells for each agonist)(data not shown).

Discussion

In this study we have examined the effects of EP_2 receptor activation on $K_{Ca}3.1$ ion channel function in HLMC. In keeping with the known effects of the β_2-adrenoceptor and A_{2A} adenosine receptor [9, 10], the G_s-coupled EP_2 receptor also closes this channel reversibly. Consistent with this effect on the channel, EP_2 receptor activation attenuates HLMC migration, and in conse-

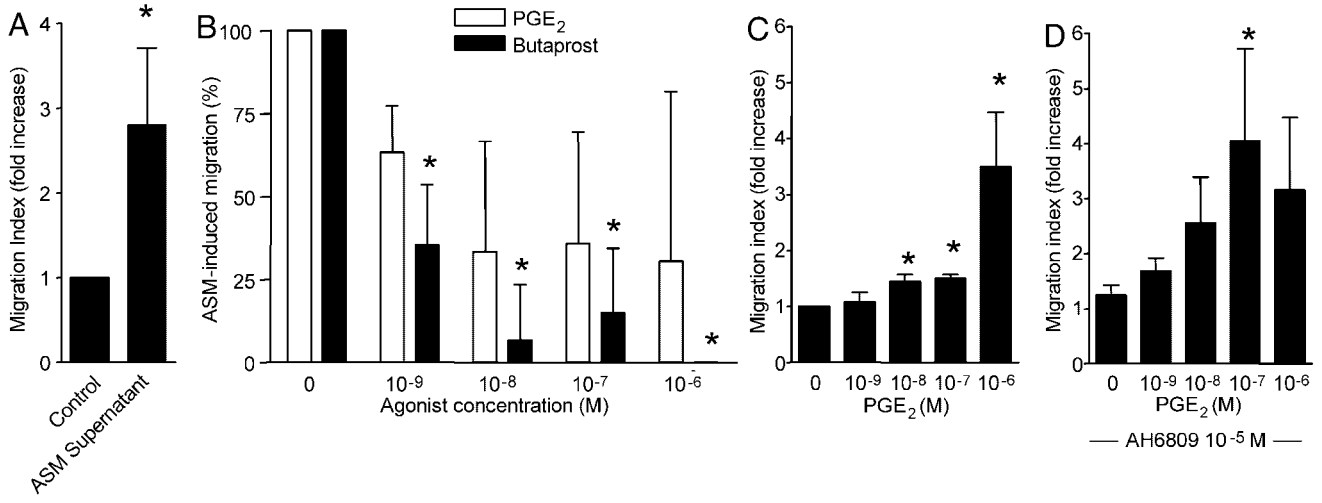

Figure 6. Inhibition of HLMC chemotaxis through EP_2 receptor activation. (A) HLMC migration using conditioned medium from asthmatic airway smooth muscle as the chemotactic stimulus. (B) HLMC migration is attenuated significantly by butaprost (n = 4) but not PGE_2 ($n = 3$) in the presence of ASM-conditioned medium. $^*p < 0.05$ compared with control (no PGE_2 or butaprost). (C) PGE_2 is chemotactic when present in isolation. $^*p < 0.05$ compared to control (no PGE_2), $n = 4$. (D) PGE_2-dependent chemotaxis is enhanced in the presence of the $EP_{1/2}$ receptor antagonist AH6809 (10^{-5} M). $^*p < 0.05$ compared to control (no AH6809), $n = 4$.

quence masks the potential chemotactic activity of PGE_2 on HLMC, which is particularly evident when the EP_2 receptor is blocked pharmacologically.

PGE_2 closed $K_{Ca}3.1$ reversibly following IgE-dependent mast cell activation demonstrating physiological relevance. PGE_2 also closed $K_{Ca}3.1$ channels that had been activated by the $K_{Ca}3.1$ opener 1-EBIO. The inhibition of $K_{Ca}3.1$ by PGE_2 was reversed both by removing it from the recording solution, and by the addition of the competitive EP_1 and EP_2 receptor antagonist AH6809 indicating a receptor-mediated mechanism. Since the effects of PGE_2 were mimicked by the specific EP_2 receptor agonist butaprost but not the EP_1 agonist 17-phenyl trinor PGE_2, and no effects of native PGE_2 were seen in the presence of the $EP_{1/2}$ receptor antagonist AH6809, we have firm evidence that the suppression of $K_{Ca}3.1$ by PGE_2 is mediated via the EP_2 receptor.

Because 1-EBIO opens $K_{Ca}3.1$ directly in resting cells by increasing its affinity for Ca^{2+} [19], it suggests that there is tight coupling between the EP_2 receptor and the channel rather than modulation of intracellular signalling pathways. The ability of PGE_2 to close $K_{Ca}3.1$ is in keeping with our previous observations that this channel is closed by G_s-coupled β_2-adrenoceptors [9] and G_s-coupled adenosine A_{2A} receptors [10]. These effects on $K_{Ca}3.1$ are not mimicked by cAMP analogues or the activator of adenylate cyclase forskolin [9], and considering they are seen in whole-cell configuration of the patch-clamp technique, indicate that the most likely mechanism of action is membrane-delimited involving the $G_{\alpha s}$ or $\beta\gamma$ subunits of these GPCR. This view is further supported by the observation that the β_2-adrenoceptor inverse agonist ICI-118551 actually opens $K_{Ca}3.1$ [9], but G_i agonists such as CXCL10 and PAF, which lower intracellular cAMP, do not directly activate this channel [6].

The ability of GPCR to modify $K_{Ca}3.1$ appears to be limited to G_s-coupled receptors as we have not found any evidence that G_i, G_q, or $G_{12/13}$-coupled receptors modify $K_{Ca}3.1$ activity. In particular blockade of EP_1 and EP_2 in this study did not uncover any G_i-coupled EP_3 effects, and GPCR agonists active on human mast cells such as CXCL10 ($G_{\alpha i}$) [6], PAF (G_i), LPA ($G_{q,\ -12/13\ \text{and}\ i}$), and UTP ($G_{\alpha q}$ P2Y2 receptor) do not open or close $K_{Ca}3.1$ in HLMC. It is well established using cell attached, inside-out and outside patch-clamp recording that most classes of GPCR including $G_{\alpha s}$ couple directly to ion channels through membrane-delimited mechanisms [21,22]. This may lead to either channel opening or channel closing depending on the channel and receptor, and may utilise either the G_α component or specific combinations of $\beta\gamma$ subunits [21, 22]. A further level of specificity between receptor and channel is likely to be achieved by the close approximation of these proteins in tight membrane-restricted signalling complexes. For example, the β_2 adrenoceptor that can modify both $K_{Ca}1.1$ and voltage-gated Ca^{2+} channel gating is associated with these two channels in a macromolecular complex held together by A-kinase-anchoring proteins [23]. It is therefore interesting to speculate that $K_{Ca}3.1$ also localises with the various $G_{\alpha s}$ receptors that modify its function. This and the exact mechanism by which $G_{\alpha s}$ modifies $K_{Ca}3.1$ function will be an important area for future research.

Several molecules that attenuate HLMC secretion including PGE_2, adenosine and β_2-adrenoceptor agonists increase intracellular cAMP [24]. The generally held view is that this increase in intracellular cAMP couples to inhibition of secretion, supported by the observation that cAMP analogues and non-specific inhibitors of adenylate cyclase can also attenuate secretion from HLMC [24]. However, no mechanism has been identified in mast cells that explains how increases in cAMP inhibit the secretory pathway, and the exclusive role of cAMP in the inhibition of

other systems such as smooth muscle relaxation has been challenged [25]. Opening of $K_{Ca}3.1$ enhances IgE-dependent Ca^{2+} influx and degranulation to a submaximal stimulus [8], and its blockade by charybdotoxin attenuates this [7]. Thus, while cAMP plays some role in the inhibition of mast cell mediator release, the demonstration that PGE_2 also closes $K_{Ca}3.1$ supports the view that cAMP-independent, $K_{Ca}3.1$-dependent mechanisms also contribute in part to EP_2-dependent inhibition of HLMC mediator release.

In many diseases the recruitment of mast cells to key tissue structures appears critical for their pathophysiological effects [4, 26, 27]. For example, the contribution of mast cells to the disordered airway physiology of asthma is undoubtedly facilitated by their migration into the airway epithelium [3], submucosal glands [5] and ASM [4]. Inhibition of their migration and subsequent microlocalisation within these structures might therefore offer a novel approach to therapy. Blockade of $K_{Ca}3.1$ markedly inhibits HLMC migration in response to a number of diverse chemotactic stimuli including conditioned medium from activated asthmatic ASM [6]. The ability of PGE_2 to close $K_{Ca}3.1$ suggested that it should also inhibit HLMC migration. However, its ability to inhibit HLMC migration in response to ASM-conditioned medium was variable and did not reach statistical significance. In contrast, the selective EP_2 receptor agonist butaprost, which was more potent at closing $K_{Ca}3.1$ than PGE_2, produced a marked and consistent inhibition of HLMC migration. PGE_2 was also chemotactic at high concentrations when used alone, but was more potent as a chemoattractant when EP_2 receptors were blocked. This indicates that there are competing pro-migratory and anti-migratory signals when PGE_2 is present, mediated by the EP_2 receptor (inhibitory) and most probably the EP_3 receptor (pro-migratory). These findings are in marked contrast to those in mouse bone marrow-derived mast cells in which PGE_2 in isolation is a potent chemoattractant. This chemotactic activity is mediated through the EP_3 receptor, and the lack of inhibition can be attributed to the absence of EP_2 receptors in mouse bone marrow-derived mast cells [13]. In human cord blood-derived mast cells, which express both EP_2 and EP_3 receptors, PGE_2 is not a chemoattractant [13], compatible with an inhibitory role for the EP_2 receptor. In human lung therefore, PGE_2 is not likely to act as a HLMC chemoattractant, in keeping with its anti-inflammatory activities in this tissue [18].

In humans PGE_2 inhibits the early and late asthmatic airway response to allergen challenge [18]. The ability of PGE_2 to close $K_{Ca}3.1$ provides a mechanism through which it is able to achieve these effects. It will be of great interest to investigate whether PGE_2 has similar effects on lung T-cell $K_{Ca}3.1$ function, cytokine secretion and migration, and whether it can inhibit $K_{Ca}3.1$-dependent ASM proliferation [28]. Drugs that block $K_{Ca}3.1$ are also in development as anti-inflammatory treatments [29]. Our demonstration that the EP_2 receptor closes $K_{Ca}3.1$ in HLMC provides further support for the development of specific EP_2 receptor agonists for the treatment of mast cell-mediated pulmonary disease.

Materials and methods

Reagents

We used the following reagents: SCF, IL-6 and IL-10 (R&D, Abingdon, UK); goat polyclonal anti-human IgE, PGE_2, AH6809, butaprost, PAF, LPA, UTP (Sigma, Poole, Dorset, UK); 17-phenyl trinor PGE_2 (Cayman Chemical Company, Ann Arbor, Michigan, US); mouse IgG_1 mAb YB5.B8 (anti-CD117) (Cambridge Bioscience, Cambridge, UK); sheep anti-mouse IgG_1 Dynabeads (Dynal, Wirral, UK); Dulbecco's Modified Essential Medium (DMEM)/glutamax/HEPES, antibiotic/antimycotic solution, MEM non-essential aminoacids, and fetal calf serum (Life Technologies, Paisley, Scotland, UK).

Human mast cell purification and culture

All human subjects gave written informed consent, and the study was approved by the Leicestershire Research Ethics Committee. HLMC were dispersed and purified from macroscopically normal lung ($n = 14$ donors) obtained within 1 h of resection for lung cancer using immunomagnetic affinity selection as described previously [30]. Final mast cell purity determined by Kimura stain was $>99\%$ and viability determined by Trypan blue was $>97\%$. HLMC were cultured in DMEM/glutamax/HEPES containing antibiotic/antimycotic solution, non-essential amino acids, 10% fetal calf serum, 100 ng/mL SCF, 50 ng/mL IL-6, and 10 ng/mL IL-10 for up to 10 wk as described previously [8, 31].

Electrophysiology

The whole-cell variant of the patch-clamp technique was used [7,32]. Patch pipettes were made from borosilicate fibre-containing glass (Clark Electromedical Instruments, Reading, UK), and their tips were heat polished, typically resulting in resistances of 4–6 MΩ. The standard pipette solution contained (in mM) KCl, 140; $MgCl_2$, 2; HEPES, 10; Na^+-ATP, 2; GTP, 0.1 (pH 7.3). The standard external solution contained (in mM) NaCl, 140; KCl, 5, $CaCl_2$, 2; $MgCl_2$,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 were evoked by applying voltage commands to a range of potentials 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. Experiments were performed at 27°C, and the temperature controlled by a Peltier device. Experiments were performed

with a perfusion system (Automate Scientific, San Francisco, CA) to allow solution changes, although drugs were added directly to the recording chamber.

PGE_2 was dissolved in ethanol to give a stock solution of 10^{-2} M. Thus, at the maximal concentration PGE_2 used (10^{-5} M), the concentration of ethanol in the recording chamber was 0.1%. This concentration of ethanol did not affect $K_{Ca}3.1$ currents when tested in isolation.

HLMC chemotaxis

HLMC chemotaxis assays were performed using the Transwell system (BD Biosciences, Oxford, UK) with 24-well plates as described previously [6, 20]. Conditioned medium from asthmatic ASM that had been activated with TNFα, IL-1β and IFNγ was placed in the lower wells as described previously [20], with appropriate cytokine containing medium in the negative control. PGE_2 was added to the bottom wells in the concentration range 10^{-6}–10^{-3} M. A total of 1×10^5 HLMC in 100 μL were added to the top well. After incubating the cells for 3 h at 37°C, we counted the number of HLMC in the bottom well using Kimura stain in a haemocytometer. HLMC migration was calculated as the fold increase of migrated cells in the test wells compared with the negative control (no chemoattractant in the lower well) as described previously [6, 20].

Data presentation and statistical analysis

Data are expressed as mean ± SEM. unless otherwise stated. Differences between groups of data were explored using Student's paired or unpaired *t*-test (two-tailed) as appropriate.

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Abbreviations: **ASM:** airway smooth muscle · **EP:** E prostanoid receptor · **1-EBIO:** 1-ethyl-2-benzimidazolinone · **GPCR:** G protein-coupled receptor · **HLMC:** human lung mast cell · **LPA:** lysophosphatidic acid · **PAF:** platelet activating factor · **PG:** prostaglandin

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