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REVIEW

The K^+ channels $K_{Ca}3.1$ and $K_v1.3$ as novel targets for asthma therapy

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Asthma affects 10% of the UK population and is an important cause of morbidity and mortality at all ages. Current treatments are either ineffective or carry unacceptable side effects for a number of patients; in consequence, development of new approaches to therapy are important. Ion channels are emerging as attractive therapeutic targets in a variety of non-excitable cells. Ion channels conducting K^+ modulate the activity of several structural and inflammatory cells which play important roles in the pathophysiology of asthma. Two channels of particular interest are the voltage-gated K^+ channel $K_v1.3$ and the intermediate conductance Ca^{2+}-activated K^+ channel $K_{Ca}3.1$ (also known as $IK_{Ca}1$ or SK4). $K_v1.3$ is expressed in IFNγ-producing T cells while $K_{Ca}3.1$ is expressed in T cells, mast cells, macrophages, airway smooth muscle cells, fibroblasts and epithelial cells. Both channels play important roles in cell activation, migration, and proliferation through the regulation of membrane potential and calcium signalling. We hypothesize that $K_{Ca}3.1$- and/or $K_v1.3$-dependent cell processes are one of the common denominators in asthma pathophysiology. If true, these channels might serve as novel targets for the treatment of asthma. Emerging evidence lends support to this hypothesis. Further validation through the study of the role that these channels play in normal and asthmatic airway cell (patho)physiology and *in vivo* models will provide further justification for the assessment of small molecule blockers of $K_v1.3$ and $K_{Ca}3.1$ in the treatment of asthma.
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Keywords: asthma; $K_{Ca}3.1$; $K_v1.3$; ion channel

Abbreviations: ASM, airway smooth muscle; ACD, allergic contact dermatitis; BHR, bronchial hyperresponsiveness; EAE, experimental autoimmune encephalomyelitis; HLMC, human lung mast cell

Asthma pathophysiology

Asthma affects 10% of westernized populations and is an important cause of morbidity and mortality at all ages (Masoli *et al.*, 2004; Asher *et al.*, 2006). It is a complex disease characterized by airway inflammation, airway wall remodelling and bronchial hyperresponsiveness (BHR). Exactly how these three key features interact and whether they are dependent on each other for their occurrence remain unknown. There is continued debate about the most important cell type mediating the airway changes in asthma, but critical analysis of the current evidence indicates that most if not all elements of the asthmatic airway are dysfunctional. There is epithelial dysfunction with failure of healing and overproduction of growth factors and pro-inflammatory cytokines (Holgate *et al.*, 1999), mucous gland hyperplasia with associated mucus hypersecretion (Carroll *et al.*, 2002), airway smooth muscle (ASM) dysfunction with resulting hypertrophy, hyperplasia, BHR and cytokine secretion (Ebina *et al.*, 1990; 1993; Brightling *et al.*, 2005), and inflammatory cell activation with 'overactive' mast cells (Bradding *et al.*, 2006), T cells (Robinson *et al.*, 1992), eosinophils (Bradding *et al.*, 1994), and neutrophils (Carroll *et al.*, 2002). The current cornerstone of asthma management is the use of inhaled corticosteroids, which are efficacious in about 90% of patients (Barnes and Adcock, 2003). However, for approximately 10% of patients, steroids are of poor efficacy for reasons that are not understood. These severe or refractory patients are difficult to treat, suffer great morbidity and use up a disproportionate fraction of healthcare resources (Wenzel, 2005). Novel treatments for asthma targeting the inflammatory response are emerging, but to date, these have been disappointing. An example is the use of anti-TNFα strategies, which, although promising in small pilot studies, have proved ineffective in larger randomized controlled trials (Berry *et al.*, 2006; Wenzel *et al.*, 2009). Similar disappointment has occurred with the use of anti-interleukin (IL)-4 (O'Byrne, 2006). There is therefore an unmet clinical need for new asthma drugs with different mechanisms of action and/or adverse-effect profiles.

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The K^+ channels $K_v1.3$ and $K_{Ca}3.1$ as potential novel therapeutic targets for asthma

Cells such as muscle and nerves fire action potentials and are known as excitable cells. The role of ion channels in propagating these electrical impulses is well described. In contrast, cells that do not have/fire action potentials such as leukocytes are generally regarded as non-excitable cells. However, molecular biology and patch-clamp analyses in recent years have shown that non-excitable cells such as lymphocytes express a complex mix of ion channels carrying K^+, Cl^-, Ca^{2+} and non-selective combinations of cations (Chandy *et al.*, 2004; Bradding, 2005). These channels are expressed at different levels depending on the cell subset and the state of activation and differentiation. Influx of extracellular Ca^{2+} is an essential requirement for the activity of many cellular processes (Berridge *et al.*, 2000). K^+ channels play an important role in Ca^{2+} signalling through their ability to maintain a negative membrane potential during cell activation (Ghanshani *et al.*, 2000; Fanger *et al.*, 2001; Duffy *et al.*, 2004), which enhances Ca^{2+} influx through inward-rectifier Ca^{2+} channels due to an increased electrical driving force for Ca^{2+} entry (Hoth and Penner, 1992). For example, in T cells (Figure 1), the voltage-gated K^+ channel $K_v1.3$ and the Ca^{2+}-activated K^+ channel $K_{Ca}3.1$ regulate Ca^{2+} influx through the calcium-release activated Ca^{2+} channel, which consists of the Ca^{2+}-sensor stromal interaction molecule 1 and the pore-forming protein CRACM1 (Orai1) (Zhang *et al.*, 2005; Feske *et al.*, 2006; Prakriya *et al.*, 2006; Vig *et al.*, 2006; Yeromin *et al.*, 2006; Lis *et al.*, 2007). The Ca^{2+} influx results in the increase in cytosolic Ca^{2+} concentration necessary for the translocation of nuclear factor of activated T cells (NFAT) to the nucleus and the initiation of new transcription, ultimately resulting in cytokine secretion and T cell proliferation (Dolmetsch *et al.*, 1997; 1998; Lewis, 2001). However, this crucial influx of Ca^{2+} is only possible if the T cell can keep its membrane potential negative by a counterbalancing K^+ efflux through $K_v1.3$ and/or $K_{Ca}3.1$ (Lin *et al.*, 1993; Chandy *et al.*, 2004). Both channels are therefore regarded as attractive new targets for immunosuppression (Chandy *et al.*, 2004).

In addition to T cells, $K_v1.3$ and $K_{Ca}3.1$ are widely distributed amongst immune and structural airway cells, where they play key roles in cellular activation, proliferation and migration by regulating membrane potential and Ca^{2+} signalling processes. We therefore hypothesise that $K_{Ca}3.1$- and/or $K_v1.3$-dependent cell processes are one of the common denominators in asthma pathophysiology. If true, these channels might serve as novel targets for the treatment of asthma.

$K_v1.3$

Both $K_v1.3$ and $K_{Ca}3.1$ have a well-developed pharmacology and have been shown previously to be amenable to drug therapy. Functional $K_v1.3$ channels are opened by membrane depolarization, with half maximal opening occurring at –40 mV to –35 mV (Cahalan *et al.*, 1985; Grissmer *et al.*, 1990). With cell depolarization, a conformational change moves the voltage sensor in the S4 transmembrane domain and opens the channel pore (Larsson *et al.*, 1996). There are several potent and relatively selective inhibitors of $K_v1.3$. These include ShK (K_d 11 pM), a 35-amino acid polypeptide derived from the Caribbean Sea anemone *Stichodactyla helianthus*, and margatoxin (K_d 110 pM), which is derived from the scorpion *Centruroides margaritatus* (Chandy *et al.*, 2004). Both bind to the outer mouth of the channel and physically obstruct ion conduction. Once bound, their dissociation is very slow so that their effects may persist for several hours. The specificity of ShK for $K_v1.3$ is greatly enhanced by the substitution of the critical Lys^{22} in ShK with diaminopropionic acid (ShK-Dap^{22}) (Kalman *et al.*, 1998) or by attachment of L-phosphotyrosine to the N-terminus (ShK(L5)) (Beeton *et al.*, 2005). These analogues are remarkably stable in cell culture systems and *in vivo*. PAP-1 [5-(4-phenoxybutoxy)psoralen] is the first relatively specific small molecule blocker of $K_v1.3$ (K_d 2 nM) (Schmitz *et al.*, 2005). A further useful tool for the study of $K_v1.3$ is a fluorescein-6-carboxylic acid (F6CA)-labelled analogue of ShK. F6CA-ShK binds with high affinity to $K_v1.3$ channels and can be used to detect them in T cells using flow cytometry (Beeton *et al.*, 2003).

$K_{Ca}3.1$

$K_{Ca}3.1$ channels have a similar topological structure to $K_v1.3$, but rather than containing a voltage sensor in the S4 domain, they bind calmodulin tightly near the C-terminus, which serves as the Ca^{2+} sensor. $K_{Ca}3.1$ channels are thus opened by a rise in cytosolic free Ca^{2+} $[Ca^{2+}]_i$ due to Ca^{2+}-calmodulin-mediated cross-linking of subunits in the channel tetramer (Fanger *et al.*, 1999). Channel function is reported to be increased by membrane-associated protein kinase A through phosphorylation of either the channel protein itself or a closely associated accessory protein in oocytes and T84 cells (Gerlach *et al.*, 2000). In $CD4^+$ T cells, $K_{Ca}3.1$ activity is increased by the nucleoside diphosphate kinase B, which phosphorylates $K_{Ca}3.1$ on histidine 358 (Srivastava *et al.*, 2006). In contrast, histidine 358 is dephosphorylated by the mammalian protein histidine phosphatase, which directly binds to the $K_{Ca}3.1$ protein and negatively regulates T cell Ca^{2+} flux by decreasing $K_{Ca}3.1$ activity (Srivastava *et al.*, 2008). $K_{Ca}3.1$ modulation in T cells is thus one of the rare examples of histidine phosphorylation/dephosphorylation influencing a biological process in mammals.

There are several tools for the study of $K_{Ca}3.1$ function. Charybdotoxin is a 37-amino acid peptide isolated from the venom of the scorpion *Leiurus quinquestriatus* and blocks $K_{Ca}3.1$ with a K_d of 5 nM but also blocks the large conductance K^+ channel $K_{Ca}1.1$ (BK_{Ca}) and $K_v1.3$ with similar potency (Chandy *et al.*, 2004; Wulff *et al.*, 2007). Another more potent but less commonly used peptidic $K_{Ca}3.1$ blocker is maurotoxin (K_d 1 nM) from the venom of the Tunisian scorpion *Scorpio maurus* (Kharrat *et al.*, 1996; Castle *et al.*, 2003). In contrast to charybdotoxin, maurotoxin does not affect $K_{Ca}1.1$ but instead potently inhibits the voltage-gated $K_v1.2$ channel (K_d 100 pM). Structural modification of the azole antimycotic clotrimazole (K_d 70–250 nM) has resulted in the generation of the small molecule TRAM-34, which specifically blocks $K_{Ca}3.1$

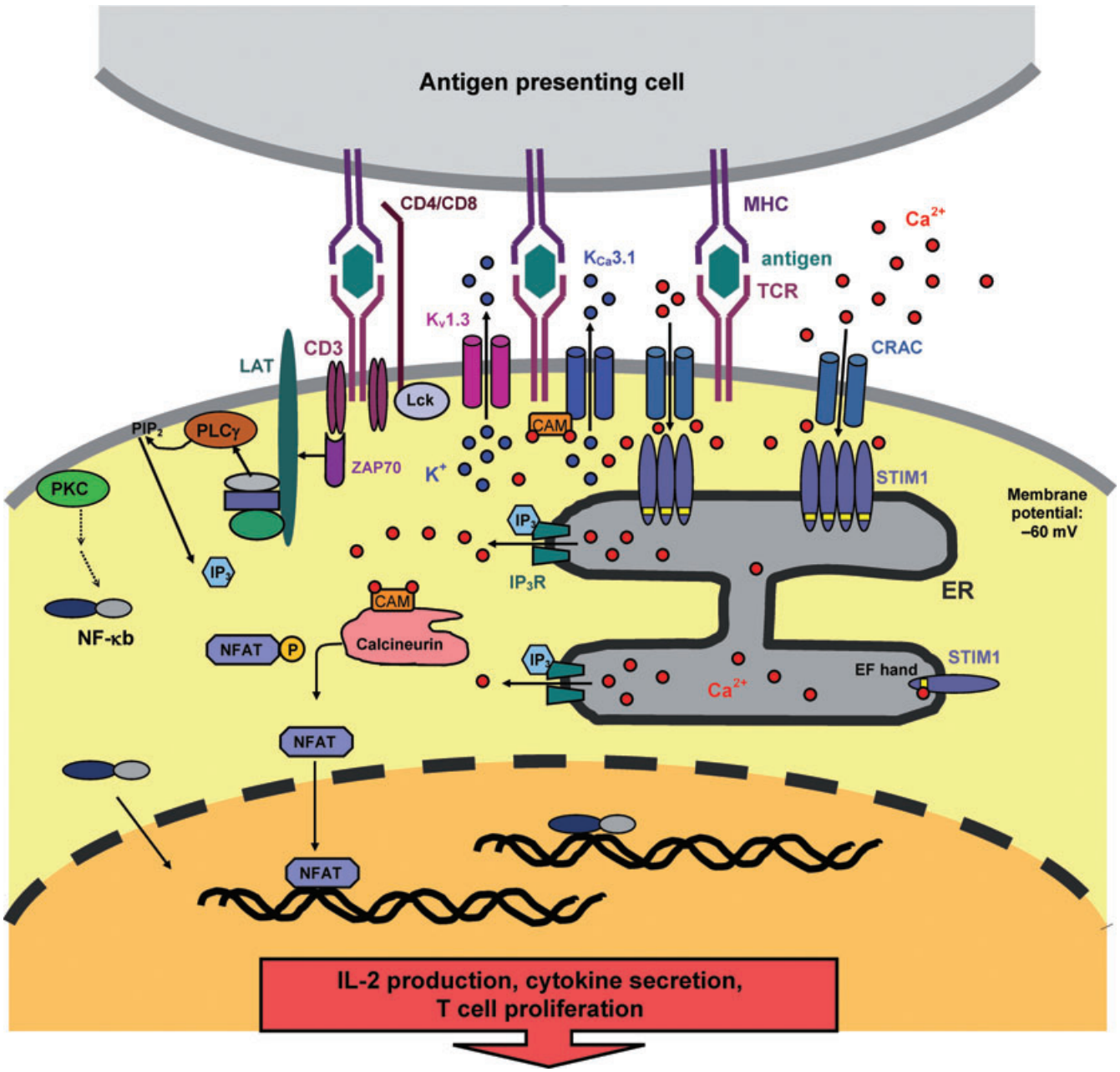

Figure 1 Involvement of $K_v1.3$, $K_{Ca}3.1$ and CRAC (Orai 1) in the activation of a T cell by an antigen-presenting cell. Engagement of the T-cell receptor–CD3 complex through an antigenic peptide presented in the context of major histocompatibility complex (MHC) class II leads to the activation of phospholipase Cγ (PLCγ) downstream of the tyrosine kinases LCK and ZAP70. PLCγ catalyses the hydrolysis of the membrane phospholipid PIP_2 to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 opens the IP_3 receptor (IP_3R) in the membrane of the endoplasmatic reticulum (ER), resulting in the release of Ca^{2+} from intracellular stores. The rise in intracellular Ca^{2+} activates the phosphatase calcineurin, which then dephosphorylates the transcription factor NFAT, enabling it to translocate to the nucleus and to bind to the promoter of cytokine genes such as interleukin 2 (IL-2). CRAC, $K_v1.3$ and $K_{Ca}3.1$ critically regulate Ca^{2+} signalling. Depletion of internal Ca^{2+} stores is 'sensed' by the EF-hand containing stromal interaction molecule 1 (STIM1), which redistributes and clusters into sites adjacent to the plasma membrane and activates CRAC channels. The ensuing Ca^{2+} influx through CRAC channels depolarizes the T cell and reduces Ca^{2+} entry through the 'inward'-rectifier CRAC. The driving force for Ca^{2+} entry is restored by membrane hyperpolarization brought about by the opening of $K_v1.3$ channels in response to membrane depolarization and the opening of $K_{Ca}3.1$ channels in response to Ca^{2+} binding to calmodulin (CAM). (The resting intracellular Ca^{2+} concentration in T cells is 50–100 nM and rises to about 1 μM during T cell activation. The extracellular Ca^{2+} concentration is 1–2 mM).

with a K_d of 20 nM. TRAM-34 blocks $K_{Ca}3.1$ by binding to internal residues below the selectivity filter, in contrast to charybdotoxin, which binds to the external pore (Wulff *et al.*, 2000). ICA-17043 (K_d 11 nM) is another small molecule blocker with high specificity for $K_{Ca}3.1$ (Stocker *et al.*, 2003). Interestingly, $K_{Ca}3.1$ channels can be activated by a number of benzimidazolones and benzothiazoles, which increase the Ca^{2+} sensitivity of these Ca^{2+}/calmodulin-gated channels. The

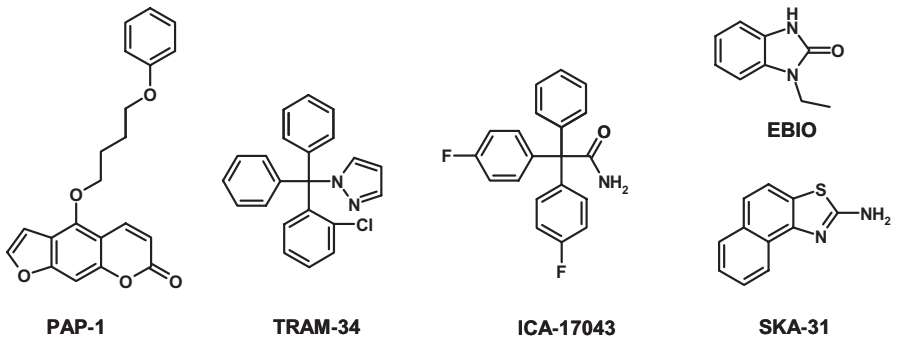

Figure 2 The chemical structures of PAP-1, TRAM-34, ICA-17043, 1-EBIO and SKA-31.

Table 1 The relative ion channel selectivity of TRAM-34, PAP-1 and SKA-31

Channel		*TRAM-34*	*PAP-1*	*SKA-31*
K_v1	$K_v1.1$	9.5 μM	65 nM	>50 μM
	$K_v1.2$	4.5 μM	250 nM	>25 μM
	$K_v1.3$	5 μM	2 nM	>25 μM
	$K_v1.4$	7.5 μM	75 nM	n.d.
	$K_v1.5$	7 μM	45 nM	>25 μM
	$K_v1.6$	n.d.	62 nM	n.d.
K_v3	$K_v3.1$	30 μM	3 μM	>25 μM
	$K_v3.2$	n.d.	1 μM	>25 μM
$K_v4.2$	$K_v4.2$	6 μM	1.2 μM	>50 μM
K_v11	$K_v11.1$	20 μM	5 μM	>50 μM
K_{IR}	$K_{IR}2.1$	>20 μM	15 μM	n.d.
K_{Ca}	$K_{Ca}1.1$	25 μM	2.5 μM	>50 μM
	$K_{Ca}2.1$	20 μM	10 μM	3 μM*
	$K_{Ca}2.2$	20 μM	5 μM	2 μM*
	$K_{Ca}2.3$	28 μM	5 μM	3 μM*
	$K_{Ca}3.1$	20 nM	10 μM	250 nM*
Na_v	$Na_v1.2$	20 μM	7 μM	>25 μM
	$Na_v1.4$	7 μM	7 μM	>25 μM
	$Na_v1.5$	n.d.	10 μM	>25 μM
Ca_v	$Ca_v1.2$	12 μM	5 μM	>50 μM

Values marked by an asterisk (*) are EC_{50} values for channel activation. All other values are IC_{50} values for channel inhibition. n.d., not done.
K_v, voltage gated K+ channels; K_{IR}, inwardly rectifying K^+ channels; K_{Ca}, Ca^{2+}-activated K^+ channels; Na_v, voltage gated Na^+ channels; Ca_v, voltage gated Ca^{2+} channels. For further information on ion channel nomenclature see Alexander *et al.* (2008).

'classic' activator 1-ethyl-2-benzimidazolinone activates heterologously expressed $K_{Ca}3.1$ with an EC_{50} of 30 μM and achieves maximal K^+ currents at 100 μM in the presence of 100 nM free Ca^{2+}, which is below the resting $(Ca^{2+})_i$ of most cell types (Pedersen *et al.*, 1999). A more potent $K_{Ca}3.1$ activator is the recently described benzothiazole SKA-31 [naphtho(1,2-*d*)thiazol-2-ylamine], which activates $K_{Ca}3.1$ with an EC_{50} of 250 nM (Sankaranarayanan *et al.*, 2009). The structures of PAP-1, TRAM-34, ICA-17043, EBIO and SKA-31 are shown in Figure 2, and the selectivity of TRAM-34, PAP-1 and SKA-31 is shown in Table 1.

Cellular expression and function of $K_v1.3$ and $K_{Ca}3.1$

In this section we will give a brief summary of what is currently known about the expression and (patho)physiological function of $K_v1.3$ and $K_{Ca}3.1$ in T cells, mast cells, epithelial cells, ASM cells, and fibroblasts.

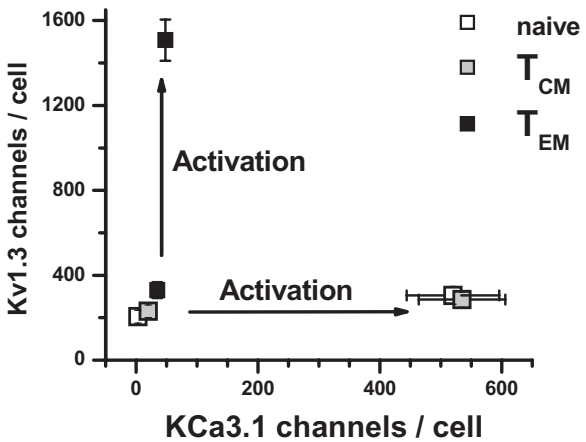

Figure 3 $K_v1.3$ versus $K_{Ca}3.1$ channel numbers per cell in naïve, central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) $CD4^+$ T cells before and after activation.

T cells

Human T cells express both $K_v1.3$ and $K_{Ca}3.1$. However, the relative expression of the two channels depends on the activation and differentiation states of the cells and correlates with the expression of the chemokine receptor CCR7 and the phosphatase CD45RA. In the resting state, $CCR7^+CD45RA^+$ naïve T cells, $CCR7^+CD45RA^-$ central memory T cells (T_{CM}) and $CCR7^-CD45RA^-$ effector memory T cells (T_{EM}) in both the CD4 and the CD8 compartment express ~250 $K_v1.3$ and less than 20 $K_{Ca}3.1$ channels per cell (Beeton *et al.*, 2003; Wulff *et al.*, 2003). Following activation, naïve and T_{CM} cells transcriptionally up-regulate $K_{Ca}3.1$ to 500 channels per cell without any change in $K_v1.3$ expression (Figure 3). In contrast, $CCR7^-$ T_{EM} cells exclusively increase $K_v1.3$ expression to approximately 1500 channels per cell following activation. This differential expression of $K_v1.3$ and $K_{Ca}3.1$ in $CCR7^+$ versus $CCR7^-$ T cells has important functional consequences. Naïve and T_{CM} cells are initially affected by $K_v1.3$ blockers but quickly become insensitive to them because they up-regulate $K_{Ca}3.1$ during activation and then rely on $K_{Ca}3.1$ for proliferation and cytokine secretion (Ghanshani *et al.*, 2000; Wulff *et al.*, 2003). In contrast, $CCR7^-$ T_{EM} cells solely rely on $K_v1.3$

for their activation processes, and $K_v1.3$ blockers like ShK(L5) and PAP-1 potently inhibit their Ca^{2+} flux following TCR ligation, and their IFNγ, IL-2 and IL-17 production as well as their proliferation (Beeton *et al.*, 2006; Azam *et al.*, 2007). $K_v1.3$ blockers have therefore been proposed for the selective suppression of T_{EM} cells, while $K_{Ca}3.1$ blockers are regarded as more useful for immune responses that are carried by $CCR7^+$ naïve and T_{CM} cells. In pre-activated T cells, $K_{Ca}3.1$ channels are localized evenly throughout the T cell plasma membrane, but rapidly redistribute to the immunological synapse following antigen presentation, where they co-localize with CD3 and F-actin (Nicolaou *et al.*, 2007). Similar findings have been reported in T_{EM} cells for $K_v1.3$, which co-localizes at the immunological synapse with $K_v\beta2$, synapse-associated protein 97, ZIP (PKC ζ-interacting protein, $p56^{lck}$-associated p62 protein), $p56^{lck}$ and CD4 (Beeton *et al.*, 2006).

Whether a $K_v1.3$ or a $K_{Ca}3.1$ blocker would be more useful for suppressing T cells in asthmatic airways is currently not clear because both Th1 cells (Krug *et al.*, 1996) (which are presumably of a T_{EM} phenotype) and Th2 cells (which have been reported to express high levels of $K_{Ca}3.1$ (Fanger *et al.*, 2000) are implicated in the immunopathology of asthma (Robinson *et al.*, 1992). The fact that $K_v1.3$ blockers strongly inhibit the IL-2 and IFNγ production of T cells from the synovial fluid of patients with RA but have little effect on IL-4 and TNFα production (Beeton *et al.*, 2006) might suggest that $K_v1.3$ is not an ideal target in asthma. However, there is good evidence of IFNγ over-expression by asthmatic T cells (Krug *et al.*, 1996; Brightling *et al.*, 2002) and of activation of Th1-dependent pathways such as the CXCR3/CXCL10 axis (Miotto *et al.*, 2001; Brightling *et al.*, 2005).

Mast cells

While $K_v1.3$ is not expressed in human or mouse mast cells, we have identified $K_{Ca}3.1$ expression in human lung, blood-derived and bone marrow-derived mast cells (Duffy *et al.*, 2001; 2004; Kaur *et al.*, 2005). In addition, Shumilina *et al.* (2008) have described the presence of $K_{Ca}3.1$ in mouse bone-marrow derived mast cells. $K_{Ca}3.1$ channels open following IgE-dependent activation (Duffy *et al.*, 2001; 2005; 2007; Kaur *et al.*, 2005) resulting in acute plasma membrane hyperpolarization (Figure 4) and enhanced Ca^{2+} influx from the extracellular fluid, but with no effect on Ca^{2+} release from internal stores (Duffy *et al.*, 2001; 2004; Shumilina *et al.*, 2008). In consequence, block of $K_{Ca}3.1$ channels in human lung mast cells (HLMCs) with charybdotoxin attenuates HLMC histamine release in response to IgE-dependent activation (Duffy *et al.*, 2001). Similarly, in mouse bone marrow-derived mast cells cultured from $K_{Ca}3.1$ knockout mice, degranulation in response to IgE-dependent activation is reduced by ~50%, although IL-6 secretion is not affected (Shumilina *et al.*, 2008). Because secretion is only partially dependent on channel opening, $K_{Ca}3.1$ can be considered to increase the gain of an immunological stimulus. Although histamine release is not completely abrogated by $K_{Ca}3.1$ knockout, the $K_{Ca}3.1$ knockout mouse nevertheless has less severe systemic anaphylactic reactions (Shumilina *et al.*, 2008), indicating that this is biologically relevant.

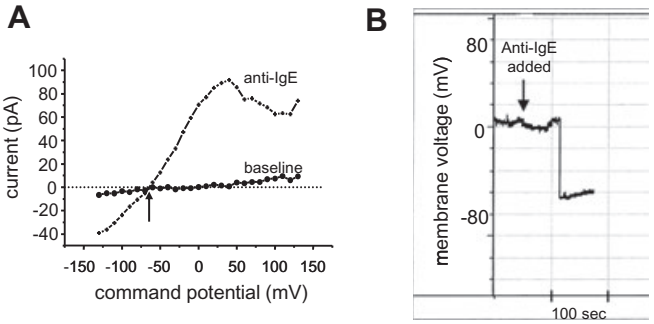

Figure 4 Opening of $K_{Ca}3.1$ channels (A) and hyperpolarization of the plasma membrane (B) in a human peripheral blood-derived mast cell following IgE-dependent activation. Graphs reproduced with permission from Duffy *et al.* (2001); Copyright 2001. The American Association of Immunologists, Inc.

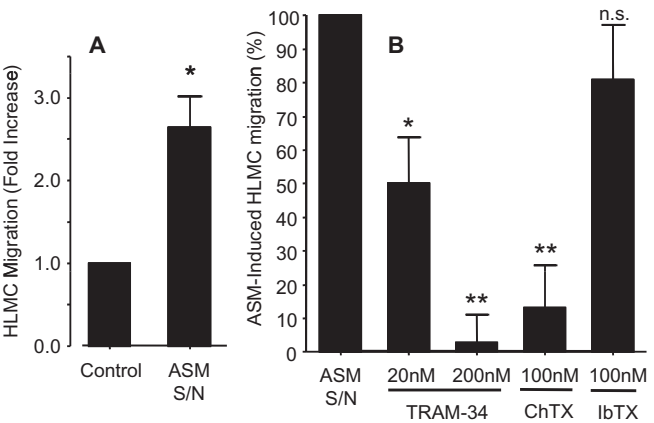

Figure 5 Mast cell migration in response to airway smooth muscle (ASM) supernatant (S/N) (A) is inhibited by the $K_{Ca}3.1$ blockers TRAM-34 and charybdotoxin (ChTX), but not the $K_{Ca}1.1$ blocker iberiotoxin (IbTX) (B). $n = 4$ donors. $^*P < 0.05$, $^{**}P < 0.01$. ASM S/N–dependent migration in (A) is represented as 100% in (B). Dimethyl sulfoxide (DMSO) 0.1% was present in all conditions. Reproduced from Cruse *et al.* (2006).

The growth of bone marrow-derived mast cells in $K_{Ca}3.1$ knockout mice or HLMC in the presence of $K_{Ca}3.1$ blockers is normal (Cruse *et al.*, 2006; Shumilina *et al.*, 2008). However, blockade of $K_{Ca}3.1$ with charybdotoxin or TRAM-34 markedly attenuates HLMC chemotaxis to the chemokine CXCL10, stem cell factor, and the complex milieu of chemokines present in asthmatic ASM-conditioned media (Cruse *et al.*, 2006) (Figure 5). The mechanisms behind this are likely to involve interference with the regulation of cell volume and inhibition of detachment of the rear cell body during migration as described in other cell types (Schwab *et al.*, 2006).

We have observed that $K_{Ca}3.1$ is regulated in HLMC by the β_2-adrenoceptor (Duffy *et al.*, 2005), the adenosine A_{2A} receptor (Duffy *et al.*, 2007) and the EP_2 prostanoid receptor (Duffy *et al.*, 2008). The effects occur rapidly and are not modulated by analogues of cAMP or forskolin, suggesting that they occur through a G_s-coupled membrane-delimited mechanism (Duffy *et al.*, 2005). Activation of these receptors closes $K_{Ca}3.1$, which may explain in part how they inhibit both mast cell secretion and migration (Gebhardt *et al.*, 2005; Duffy *et al.*, 2007; 2008).

Epithelium

The airway epithelium is at the interface with the external environment and is the first structure to interact with noxious stimuli such as allergens, viruses, and pollutants. Not only does the columnar epithelium tend to shed from the basal layer, the airway epithelium is also functionally abnormal in asthma (Holgate *et al.*, 1999; Puddicombe *et al.*, 2000). Epithelial repair normally involves up-regulation of the epidermal growth factor (EGF) receptor, which drives the repair response. In asthmatic epithelium, the proliferative repair response is impeded, but other consequences of EGF receptor activation remain intact. Thus, there is ongoing release of pro-inflammatory cytokines which may promote cellular recruitment, and there is release of profibrogenic growth factors which may drive the remodelling response (Holgate *et al.*, 1999; Puddicombe *et al.*, 2000). Both $K_v1.3$ and $K_{Ca}3.1$ are expressed by epithelial cell lines (Devor *et al.*, 1999; Grunnet *et al.*, 2003). In particular, $K_{Ca}3.1$ expression has been reported in Calu-3 cells (Devor *et al.*, 1999). The proposed role for $K_{Ca}3.1$ in epithelium is to reduce HCO_3^- secretion and to increase Cl^- secretion (Devor *et al.*, 1999). We predict that $K_{Ca}3.1$ will contribute to the secretion of pro-inflammatory cytokines and mucus by epithelial cells through its ability to potentiate Ca^{2+} influx.

ASM and fibroblasts

The central physiological abnormality in asthma is BHR, which results in airflow obstruction in response to bronchospastic stimuli (Boushey *et al.*, 1980; Boulet, 2003). The ASM in asthma is therefore highly dysfunctional, and in addition demonstrates both hypertrophy and hyperplasia (Ebina *et al.*, 1990; 1993). Whether the ASM in asthma is fundamentally different to that in normal subjects due to either genetic or acquired factors is not known. However, *in vitro* several profound phenotypic differences are evident (Johnson *et al.*, 2001; 2004; Burgess *et al.*, 2003; Roth *et al.*, 2004; Brightling *et al.*, 2005). We were the first to demonstrate that $K_{Ca}3.1$ is expressed by both normal and asthmatic human ASM (Shepherd *et al.*, 2007). $K_{Ca}3.1$ expression is increased by both basic fibroblast growth factor (FGF) and TGFβ, and $K_{Ca}3.1$ inhibition with TRAM-34 attenuates human ASM proliferation (Shepherd *et al.*, 2007). This up-regulation of $K_{Ca}3.1$ in ASM is reminiscent of the $K_{Ca}3.1$ up-regulation that occurs in mouse, rat, and pig vascular or coronary smooth muscle during the remodelling associated with restenosis and atherosclerosis (Kohler *et al.*, 2003; Tharp *et al.*, 2006; 2008; Toyama *et al.*, 2008). We envisage that $K_{Ca}3.1$ mediates important biological effects in the ASM of asthmatic subjects and that $K_{Ca}3.1$ blockade might at least partially prevent ASM remodelling.

Fibroblasts, specifically myofibroblasts, contribute to the deposition of collagen beneath the airway epithelium in asthma (Brewster *et al.*, 1990). Fibroblast cell lines express a K_{Ca} channel with the biophysical properties of $K_{Ca}3.1$ (Rane, 1991; Pena and Rane, 1999), and charybdotoxin prevents FGF-induced fibroblast proliferation. Whether primary human airway fibroblasts express $K_{Ca}3.1$ has not been reported, however, we believe it is highly likely that $K_{Ca}3.1$ plays an important role in the fibrogenic activity of human airway fibroblasts.

Other cells

$K_{Ca}3.1$ is also expressed by other cells of potential importance to asthma. Human endothelial cell expression of $K_{Ca}3.1$ was increased by both basicFGF and VEGF, two growth factors implicated in the angiogenesis which characterizes human asthma (Shute *et al.*, 2004; Siddiqui *et al.*, 2007). Blockade of $K_{Ca}3.1$ with charybdotoxin and TRAM-34 inhibited human endothelial cell proliferation *in vitro*, while TRAM-34 inhibited angiogenesis in mice in an *in vivo* matrigel plug assay (Grgic *et al.*, 2005). Inhibition of $K_{Ca}3.1$ may therefore be expected to prevent or reverse the angiogenesis evident in asthmatic airways.

Macrophages have also been implicated in asthma, although their role remains poorly defined (Holgate, 2008). $K_{Ca}3.1$ is expressed by human and mouse macrophages, and $K_{Ca}3.1$ knockout or pharmacological inhibition has been shown to suppress macrophage activation and migration (Schmid-Antomarchi *et al.*, 1997; Toyama *et al.*, 2008). $K_{Ca}3.1$ has not been described to date in eosinophils.

Roles in disease

Pharmacological blockers of both $K_v1.3$ and $K_{Ca}3.1$ have been tested in many disease models. Compounds that block $K_v1.3$ suppress T_{EM} function *in vitro* and effectively treat memory T cell-mediated immune reactions such as delayed-type hypersensitivity (DTH) in rats and minipigs (Koo *et al.*, 1997; Beeton *et al.*, 2005; Schmitz *et al.*, 2005), as well as experimental autoimmune encephalomyelitis (EAE) (Beeton *et al.*, 2001), experimental autoimmune diabetes (Beeton *et al.*, 2006), pristane-induced arthritis (Beeton *et al.*, 2006) and allergic contact dermatitis (ACD) in rats (Azam *et al.*, 2007), without causing any toxic side effects (Beeton *et al.*, 2006). In all these disease models $K_v1.3$ blockers seem to have selectively suppressed T_{EM} cell functions as suggested by a recent two-photon *in vivo* imaging study, which showed that $K_v1.3$ blockers inhibited DTH and suppressed T_{EM} cell enlargement and motility in inflamed tissue but had no effect on homing to or motility in lymph nodes of naive and central memory T cells (Matheu *et al.*, 2008). In keeping with this observation, $K_v1.3$ blockers did not prevent antigen presentation and memory T cell development in oxazolone-induced ACD in rats but effectively inhibited ear swelling during the T_{EM} cell-mediated effector phase of the disease (Azam *et al.*, 2007).

$K_{Ca}3.1$ blockers that inhibit the activation and migration of naïve T cells, and many structural and inflammatory cells *in vitro*, have been shown to treat EAE in mice and to prevent vascular restenosis after systemic delivery in rats (Kohler *et al.*, 2003) and after local delivery in pigs (Tharp *et al.*, 2008). The $K_{Ca}3.1$ blocker TRAM-34 further reduces atherosclerosis development in $ApoE^{-/-}$ mice by inhibiting both vascular smooth muscle cell proliferation and T cell and macrophage activity (Toyama *et al.*, 2008). Of relevance to asthma, the $K_{Ca}3.1$ knockout mouse displays an attenuated IgE-dependent systemic anaphylactic response (Shumilina *et al.*, 2008). Furthermore, it is reported on the web site of the pharmaceutical company Icagen Inc (Durham, NC, USA). that the orally active $K_{Ca}3.1$ blocker ICA-17043 (Senicapoc) inhibits the late airway response and the development of BHR following

allergen challenge in a sheep model of asthma (http://www.icagen.com/randd/memorydisorders.html).

Safety of targeting $K_v1.3$ and $K_{Ca}3.1$

$K_v1.3$

A key issue for any long-term therapy is a favourable balance between efficacy and safety.

In addition to $CCR7^-$ T_{EM} cells, $K_v1.3$ is also expressed in the central nervous system, kidney, liver, skeletal muscle, platelets, macrophages, testis and osteoclasts, raising the possibility that $K_v1.3$ blockers could have adverse side effects. To investigate this possibility, the Wulff and Chandy laboratories performed 28-day and 6-month toxicity studies with PAP-1 (50 $mg \cdot kg^{-1} \cdot day^{-1}$ orally) and a 28-day toxicity study with ShK-L5 (500 $\mu g \cdot kg^{-1} \cdot day^{-1}$ s.c.) in both male and female rats (Beeton *et al.*, 2006). (Please note that PAP-1 effectively prevents autoimmune diabetes in diabetes-prone BB/W or rats at the same dose and that ShK-L5 suppresses DTH at 10 $\mu g \cdot kg^{-1}$ and treats EAE at 100 $\mu g \cdot kg^{-1}$.) Both blockers failed to induce any histopathological changes in any tissue examined, including those reported to express $K_v1.3$. PAP-1 and ShK-L5 also did not induce any changes in haematological or serum chemistry parameters. Both blockers further did not delay influenza virus clearance in rats, suggesting that $K_v1.3$ blockers truly selectively inhibit T_{EM} cells and do not affect the function of naïve and T_{CM} cells (Matheu *et al.*, 2008). In collaboration with Dr Aftab Ansari at the Primate Center of Emory University, the Wulff laboratory also administered PAP-1 at 25 $mg \cdot kg^{-1} \cdot day^{-1}$ for 28 days to rhesus macaques. The treatment again did not induce any changes in blood chemistry or haematology and did not affect the development of a protective T_{CM} response following nasal flu vaccination (Pereira *et al.*, 2007). However, in keeping with a role of T_{EM} cells in suppressing chronic viral infections, PAP-1 treatment caused a reactivation of CMV virus, which however, did not result in any symptoms of CMV disease but was detectable by PCR. Before performing these experiments we thoroughly tested PAP-1 for *in vitro* toxicity and found that it is not cytotoxic, not phototoxic, and is negative in the Ames test, which assesses mutagenic potential. Most importantly, PAP-1 exhibits excellent selectivity over other ion channels as well as various receptors and transporters (Schmitz *et al.*, 2005). The relative safety of $K_v1.3$ blockers may be due in part to channel redundancy and also because $K_v1.3$ blockers may not inhibit $K_v1.3$-containing heteromultimers (e.g. in the central nervous system (CNS)) with the same affinity as $K_v1.3$ homotetramers in T cells.

$K_{Ca}3.1$

Similar to $K_v1.3$, $K_{Ca}3.1$ seems to be relatively safe as a therapeutic target. Two independently generated $K_{Ca}3.1^{-/-}$ mice (Begenisich *et al.*, 2004; Si *et al.*, 2006) were both viable, of normal appearance, produced normal litter sizes, did not show any gross abnormalities in any of their major organs and exhibited rather mild phenotypes: impaired volume regulation in erythrocytes and lymphocytes (Begenisich *et al.*, 2004), a reduced endothelial-derived hyperpolarising factor (EDHF) response together with a mild –7-mmHg increase in blood pressure (Si *et al.*, 2006), and subtle erythrocyte macrocytosis and progressive splenomegaly (Grgic *et al.*, 2009). Pharmacological blockade of $K_{Ca}3.1$ also seems to be safe and well tolerated. TRAM-34 exhibits an excellent selectivity over other ion channels and was 'clean' in a Hit Profiling screen on 32 neuronal receptors and transporters (Wulff *et al.*, 2000; Toyama *et al.*, 2008). Daily administration of TRAM-34 at 120 $mg \cdot kg^{-1} \cdot day^{-1}$ did not induce any changes in blood chemistry, haematology or necropsy of major organs in a 28-day toxicity study in mice or rats (Toyama *et al.*, 2008). There have also been no reports about toxicity for the structurally related $K_{Ca}3.1$ blocker ICA-17043 (Senicapoc), which was developed by Icagen Inc. and which entered clinical trials as an orphan drug for sickle cell anemia (Stocker *et al.*, 2003). ICA-17043 was found to be both effective and safe in Phase-2 clinical trials (Ataga *et al.*, 2008), but the phase-III trials were stopped in 2007 due to a lack of efficacy in reducing sickling crises. ICA-17043 recently re-entered clinical trials and is currently being evaluated for asthma in two phase-II proof-of-concept trials. Dose-escalating studies with ICA-17043 in 28 otherwise healthy patients with sickle cell disease did not increase blood pressure or lead to electrocardiogram changes (Ataga *et al.*, 2006; 2008).

Summary

In summary, $K_v1.3$ and $K_{Ca}3.1$ regulate many diverse cell processes of relevance to asthma. As such, they offer the potential for the development of a truly novel approach to the treatment of this disease. Further validation of these targets is required to define which aspects of the asthmatic process are most likely to be attenuated by $K_v1.3$ or $K_{Ca}3.1$ blockade in humans. In turn, this will help determine the primary outcomes for clinical trials. For example, if eosinophilia is the predominant feature that is inhibited, then the rate of exacerbations should be the primary outcome (Green *et al.*, 2002), whereas if BHR or remodelling is the predominant feature that improves, then measurement of these as the primary outcome would be more appropriate. The studies to date with $K_v1.3$ and $K_{Ca}3.1$ blockers are encouraging, and the lack of any toxicity with ICA-17043 when administered to humans with sickle cell disease or of TRAM-34 and PAP-1 administered to rodents and primates suggests real therapeutic potential for human disease.

Conflicts of interest

Peter Bradding has undertaken contract research and acted as a consultant for Icagen Inc. Heike Wulff is an inventor on the University of California-owned patents claiming TRAM-34 and PAP-1 as immunosuppressants. Her laboratory has received student fees from Icagen Inc., and she is co-founder of Airmid Inc, a company aiming to develop $K_v1.3$ blockers for the treatment of multiple sclerosis and psoriasis.

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