The $K^+$ channels $K_{Ca3.1}$ and $K_{v1.3}$ as novel targets for asthma therapy

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

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 Kv1.3 and the intermediate conductance Ca\(^{2+}\)-activated K\(^+\) channel KCa3.1 (also known as IKCa1 or SK4). Kv1.3 is expressed in IFN\(\gamma\)-producing T cells while KCa3.1 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 hypothesise that KCa3.1- and/or Kv1.3-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 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 Kv1.3 and KCa3.1 in the treatment of asthma.
Asthma pathophysiology

Asthma affects 10% of westernised populations and is an important cause of morbidity and mortality at all ages (Asher et al., 2006; Masoli et al., 2004). It is a complex disease characterised 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 remains 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; Ebina et al., 1993; Brightling et al., 2005), and inflammatory cell activation with “over-active” 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 & Adcock, 2003). However, for approximately 10% of patients steroids are of poor efficacy for reasons, which 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 randomised controlled trials (Berry et al., 2006; Wenzel et al., 2009). Similar disappointment has occurred with the use of anti-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.

The $K^+$ channels $K_v1.3$ and $K_{Ca3.1}$ 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 which do not have/fire action potentials such as leukocytes are generally regarded as non-excitatable cells. However, molecular biology and patch-clamp analyses in recent years have shown that non-excitatable cells such as lymphocytes express a complex mix of ion channels carrying $K^+$, $Cl^-$, $Ca^{2+}$ and non-selective combinations of cations (Bradding, 2005; Chandy et al., 2004). 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 (Fanger et al., 2001; Ghanshani et al., 2000; 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 & Penner, 1992). For example in T cells (Fig. 1), the voltage-gated $K^+$ channel $K_v1.3$ and the $Ca^{2+}$-activated $K^+$ channel $K_{Ca3.1}$ regulate $Ca^{2+}$-influx through the calcium-release activated $Ca^{2+}$ (CRAC) channel, which consists of the $Ca^{2+}$-sensor STIM1 and the pore forming protein CRACM1 (Orai1) (Feske et al., 2006; Vig et al., 2006; Prakriya et al., 2006; Yeromin et al., 2006; Lis et al., 2007; Zhang et al., 2005). The $Ca^{2+}$- influx results in the increase in cytosolic $Ca^{2+}$ concentration necessary for the translocation of NFAT to the nucleus and the initiation of new transcription ultimately resulting in cytokine secretion and T cell proliferation (Dolmetsch et
However, this crucial influx of \( \text{Ca}^{2+} \) is only possible if the T cell can keep its membrane potential negative by a counterbalancing \( \text{K}^+ \) efflux through \( \text{K}_{v}1.3 \) and/or \( \text{K}_{\text{Ca}}3.1 \) (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, \( \text{K}_{v}1.3 \) and \( \text{K}_{\text{Ca}}3.1 \) 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 \( \text{Ca}^{2+} \) signalling processes. We therefore hypothesise that \( \text{K}_{\text{Ca}}3.1 \)- and/or \( \text{K}_{v}1.3 \)-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.

**\( \text{K}_{v}1.3 \)**

Both \( \text{K}_{v}1.3 \) and \( \text{K}_{\text{Ca}}3.1 \) have a well-developed pharmacology and have been shown previously to be amenable to drug therapy. Functional \( \text{K}_{v}1.3 \) channels are opened by membrane depolarisation, 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 \( \text{K}_{v}1.3 \). These include ShK (\( K_d \) 11 pM) a 35 amino acid polypeptide derived from the Caribbean Sea anenome *Stichodactyla helianthus* and margatoxin (\( K_d \) 110 pM) 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 \( \text{K}_{v}1.3 \) is greatly enhanced by the substitution of the critical Lys\(^{22} \) in ShK
with diaminopropionic acid (ShK-Dap22) (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).

**\( K_{Ca3.1} \)**

\( K_{Ca3.1} \) 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. \( K_{Ca3.1} \) 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 (PKA) 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, \( K_{Ca3.1} \) activity is increased by the nucleoside diphosphate kinase B (NDPK-B), which phosphorylates \( K_{Ca3.1} \) on histidine 358 (Srivastava et al., 2006). In contrast, histidine 358 is dephosphorylated by the mammalian protein histidine phosphatase (PHPT-1), which directly binds to the \( K_{Ca3.1} \) protein and negatively regulates T cell \( Ca^{2+} \) flux by decreasing \( K_{Ca3.1} \) activity (Srivastava et al., 2008). \( K_{Ca3.1} \) 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 \( \text{K}\text{Ca}_3.1 \) function. Charybdotoxin is a 37-amino acid peptide isolated from the venom of the scorpion \textit{Leiurus quinquestriatus} and blocks \( \text{K}\text{Ca}_3.1 \) with a \( K_d \) of 5 nM, but also blocks the large conductance \( \text{K}^+ \) channel \( \text{K}\text{Ca}_{1.1} \) (\( \text{BK}_{\text{Ca}} \)) and \( \text{K}_{\text{v}1.3} \) with similar potency (Chandy \textit{et al.}, 2004; Wulff \textit{et al.}, 2007). Another more potent, but less commonly used peptidic \( \text{K}\text{Ca}_3.1 \) blocker is maurotoxin (\( K_d \) 1 nM) from the venom of the Tunisian scorpion \textit{Scorpio maurus} (Kharrat \textit{et al.}, 1996; Castle \textit{et al.}, 2003). In contrast to charybdotoxin, maurotoxin does not affect \( \text{K}\text{Ca}_{1.1} \) but instead potently inhibits the voltage-gated \( \text{K}_{\text{v}1.2} \) 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 \( \text{K}\text{Ca}_3.1 \) with a \( K_d \) of 20 nM. TRAM-34 blocks \( \text{K}\text{Ca}_3.1 \) by binding to internal residues below the selectivity filter, in contrast to charybdotoxin which binds to the external pore (Wulff \textit{et al.}, 2000). ICA-17043 (\( K_d \) 11 nM) is another small molecule blocker with high specificity for \( \text{K}\text{Ca}_3.1 \) (Stocker \textit{et al.}, 2003). Interestingly, \( \text{K}\text{Ca}_3.1 \) channels can be activated by a number of benzimidazolones and benzothiazoles, which increase the \( \text{Ca}^{2+} \) sensitivity of these \( \text{Ca}^{2+}/\text{calmodulin} \)-gated channels. The “classic” activator 1-ethyl-2-benzimidazolinone (1-EBIO) activates heterologously expressed \( \text{K}\text{Ca}_3.1 \) with an \( EC_{50} \) of 30 \( \mu \text{M} \), and achieves maximal \( \text{K}^+ \) currents at 100 \( \mu \text{M} \) in the presence of 100 nM free \( \text{Ca}^{2+} \), which is below the resting \( [\text{Ca}^{2+}]_i \) of most cell types (Pedersen \textit{et al.}, 1999). A more potent \( \text{K}\text{Ca}_3.1 \) activator is the recently described benzothiazole SKA-31 (naphtho[1,2-\( d \)]thiazol-2-ylamine) which activates \( \text{K}\text{Ca}_3.1 \) with an \( EC_{50} \) of 250 nM (Sankaranarayanan \textit{et al.}, 2009). The structures of PAP-1, TRAM-34, ICA-17043, EBIO and SKA-31 are shown in Fig.2. and the selectivity of TRAM-34, PAP-1 and SKA-31 in table 1.
Cellular Expression and Function of Kv1.3 and KCa3.1

In this section we will give a brief summary of what is currently known about the expression and (patho)physiological function of Kv1.3 and KCa3.1 in T cells, mast cells, epithelial cells, airway smooth muscle cells, and fibroblasts.

**T cells:** Human T cells express both Kv1.3 and KCa3.1. 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<sub>CM</sub>) and CCR7-CD45RA- effector memory T cells (T<sub>EM</sub>) in both the CD4 and the CD8 compartment express ~250 Kv1.3 and less than 20 KCa3.1 channels per cell (Wulff et al., 2003; Beeton et al., 2003). Following activation naïve and T<sub>CM</sub> cells transcriptionally up-regulate KCa3.1 to 500 channels per cell without any change in Kv1.3 expression (Fig. 3). In contrast, CCR7- T<sub>EM</sub> cells exclusively increase Kv1.3 expression to 1500 to 2000 channels per cell following activation. This differential expression of Kv1.3 and KCa3.1 in CCR7+ versus CCR7- T cells has important functional consequences. Naïve and T<sub>CM</sub> cells are initially affected by Kv1.3 blockers but quickly become insensitive to them because they up-regulate KCa3.1 during activation and then rely on KCa3.1 for proliferation and cytokine secretion (Ghanshani et al., 2000; Wulff et al., 2003). In contrast, CCR7- T<sub>EM</sub> cells solely rely on Kv1.3 for their activation processes and Kv1.3 blockers like ShK(L5) and PAP-1 potently inhibit their Ca<sup>2+</sup> 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). Kv1.3 blockers have therefore been proposed for the selective suppression of T<sub>EM</sub> cells, while KCa3.1 blockers are regarded as more useful for immune responses that are carried by CCR7+ naïve and T<sub>CM</sub> cells. In pre-activated T cells, KCa3.1 channels are localised evenly throughout the
T cell plasma membrane, but rapidly redistribute to the immunological synapse following antigen presentation, where they co-localise with CD3 and F-actin (Nicolaou et al., 2007). Similar findings have been reported in T_{EM} cells for K_{v}1.3 which co-localises at the immunological synapse with K_{v}β2, SAP97 (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_{v}1.3 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_{v}1.3 blockers strongly inhibit the IL-2 and IFN_{γ} production of T cells from the synovial fluid of patients with RA but have little effect on IL-4 and TNF_{α} production (Beeton et al., 2006), might suggest that K_{v}1.3 is not an ideal target in asthma. However, there is good evidence of IFN_{γ} over-expression by asthmatic T cells (Krug et al., 1996; Brightling et al., 2002), and activation of Th1-dependent pathways such as the CXCR3/CXCL10 axis (Brightling et al., 2005; Miotto et al., 2001).

**Mast cells:** While K_{v}1.3 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 in humans (Duffy et al., 2001; Duffy et al., 2004; Kaur et al., 2005). In addition, Shumilina et al. have described the presence of K_{Ca}3.1 in mouse bone-marrow derived mast cells (Shumilina et al., 2008). K_{Ca}3.1 channels open following IgE-dependent activation (Duffy et al., 2001; Duffy et al., 2005; Kaur et al., 2005; Duffy et al., 2007) resulting in acute plasma membrane hyperpolarisation ([Fig.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; Duffy et al., 2004; Shumilina et al., 2008). In consequence, block of K\(_{Ca}3.1\) channels in human lung mast cells (HLMC) 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). Since 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 this is biologically relevant.

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 (SCF), and the complex milieu of chemokines present in asthmatic ASM conditioned media (Cruse et al., 2006) (Fig. 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 \(\beta_2\)-adrenoceptor (Duffy et al., 2005), the adenosine A\(_{2A}\) receptor (Duffy et al., 2007), and EP\(_2\) prostanoid receptor (Duffy et al., 2008). The effects occur rapidly, and are not modulated by analogues of cAMP or forskolin, suggesting 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 (Duffy et al., 2007; Duffy et al., 2008; Gebhardt et al., 2005).

**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 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 on-going 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 \( K_v 1.3 \) and \( K_{Ca}3.1 \) are expressed by epithelial cell lines (Grunnet et al., 2003; Devor et al., 1999). In particular, \( K_{Ca}3.1 \) 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.

**Airway smooth muscle (ASM) and fibroblasts:** The central physiological abnormality in asthma is bronchial hyperresponsiveness (BHR), which results in airflow obstruction in response to bronchospastic stimuli (Boulet, 2003; Boushey et al., 1980). The ASM in asthma is therefore highly dysfunctional, and in addition demonstrates both hypertrophy and hyperplasia (Ebina et al., 1990; Ebina et al., 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., 2004; Johnson et al., 2001; Burgess et al., 2003; Brightling et al., 2005; Roth et al., 2004). We were the first to demonstrate that $K_{Ca3.1}$ is expressed by both normal and asthmatic human ASM (Shepherd et al., 2007). $K_{Ca3.1}$ expression is increased by both basicFGF and TGFβ, and $K_{Ca3.1}$ inhibition with TRAM-34 attenuates human ASM proliferation (Shepherd et al., 2007). This up-regulation of $K_{Ca3.1}$ in ASM is reminiscent of the $K_{Ca3.1}$ up-regulation that occurs in mouse, rat and pig vascular or coronary SM during the remodelling associated with restenosis and atherosclerosis (Kohler et al., 2003; Tharp et al., 2006; Tharp et al., 2008; Toyama et al., 2008). We envisage that $K_{Ca3.1}$ mediates important biological effects in ASM of asthmatic subjects and that $K_{Ca3.1}$ 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 $K_{Ca3.1}$ (Rane, 1991; Pena & Rane, 1999) and charybdotoxin prevents FGF-induced fibroblast proliferation. Whether primary human airway fibroblasts express $K_{Ca3.1}$ has not been reported, however, we believe it is highly likely that $K_{Ca3.1}$ plays an important role in the fibrogenic activity of human airway fibroblasts.

**Other cells**

$K_{Ca3.1}$ is also expressed by other cells of potential importance to asthma. Human endothelial cell expression of $K_{Ca3.1}$ was increased by both basicFGF and VEGF, two growth factors implicated in the angiogenesis which characterises human asthma (Shute et al., 2004; Siddiqui et al., 2007). Blockade of $K_{Ca3.1}$ with charybdotoxin and TRAM-34 inhibited human endothelial cell
proliferation \textit{in vitro}, while TRAM-34 inhibited angiogenesis in mice in an \textit{in vivo} matrigel plug assay (Grgic \textit{et al.}, 2005). Inhibition of K\textsubscript{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\textsubscript{Ca}3.1 is expressed by human and mouse macrophages and K\textsubscript{Ca}3.1 knock-out or pharmacological inhibition has been shown to suppress macrophage activation and migration (Toyama \textit{et al.}, 2008; Schmid-Antomarchi \textit{et al.}, 1997). K\textsubscript{Ca}3.1 has not been described to-date in eosinophils.

\textbf{Roles in disease}

Pharmacological blockers of both K\textsubscript{v}1.3 and K\textsubscript{Ca}3.1 have been tested in many disease models. Compounds which block K\textsubscript{v}1.3 suppress effector memory T cell function \textit{in vitro} and effectively treat memory T cell-mediated immune reactions such as delayed-type hypersensitivity (DTH) in rats and minipigs (Schmitz \textit{et al.}, 2005; Koo \textit{et al.}, 1997; Beeton \textit{et al.}, 2005), as well as experimental autoimmune encephalomyelitis (EAE) (Beeton \textit{et al.}, 2001), experimental autoimmune diabetes (Beeton \textit{et al.}, 2006), pristane-induced arthritis (Beeton \textit{et al.}, 2006) and allergic contact dermatitis (ACD) in rats (Azam \textit{et al.}, 2007), without causing any toxic side effects (Beeton \textit{et al.}, 2006). In all these disease models K\textsubscript{v}1.3 blockers seem to have selectively suppressed T\textsubscript{EM} cell functions as suggested by a recent two-photon \textit{in vivo} imaging study, which showed that K\textsubscript{v}1.3 blockers inhibited DTH and suppressed T\textsubscript{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 \textit{et al.}, 2008). In keeping with this observation, K\textsubscript{v}1.3 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\textsubscript{EM} cell mediated effector phase of the disease (Azam \textit{et al.}, 2007).

\(K\textsubscript{Ca}3.1\) blockers which inhibit the activation and migration of naïve T cells, and many structural and inflammatory cells \textit{in vitro}, and have been shown to treat EAE in mice and to prevent vascular restenosis after systemic delivery in rats (Kohler \textit{et al.}, 2003) and after local delivery in pigs (Tharp \textit{et al.}, 2008). The \(K\textsubscript{Ca}3.1\) blocker TRAM-34 further reduces atherosclerosis development in ApoE\textsuperscript{-/-} mice by inhibiting both vascular smooth muscle cell proliferation and T cell and macrophage activity (Toyama \textit{et al.}, 2008). Of relevance to asthma, the \(K\textsubscript{Ca}3.1\) knockout mouse displays an attenuated IgE-dependent systemic anaphylactic response (Shumilina \textit{et al.}, 2008). Furthermore, it is reported on the website of the pharmaceutical company Icagen Inc. that the orally active \(K\textsubscript{Ca}3.1\) 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 \(K\textsubscript{r}1.3\) and \(K\textsubscript{Ca}3.1\)**

\(K\textsubscript{r}1.3\): A key issue for any long-term therapy is a favourable balance between efficacy and safety. In addition to CCR7 \(T\textsubscript{EM}\) cells, \(K\textsubscript{r}1.3\) is also expressed in the central nervous system, kidney, liver, skeletal muscle, platelets, macrophages, testis, and osteoclasts, raising the possibility that \(K\textsubscript{r}1.3\) 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/kg/d orally) and a 28-day toxicity study with ShK-L5 (500 μg/kg/d s.c.) in both male and female rats (Beeton \textit{et al.}, 2006). [Please note that PAP-1 effectively prevents autoimmune diabetes in
diabetes prone BB/Wor rats at the same dose and that ShK-L5 suppresses DTH at 10 μg/kg and treats EAE at 100 μg/kg]. Both blockers failed to induce any histopathological changes in any tissue examined, including those reported to express Kv1.3. PAP-1 and ShK-L5 also did not induce any changes in hematological or serum chemistry parameters. Both blockers further did not delay influenza virus clearance in rats suggesting that Kv1.3 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/kg/d for 28-days to rhesus macaques. The treatment again did not induce any changes in blood chemistry or hematology 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 Kv1.3 blockers may be due in part to channel redundancy and also because Kv1.3 blockers may not inhibit Kv1.3-containing heteromultimers (e.g., in the CNS) with the same affinity as Kv1.3 homotetramers in T cells.

**K_{Ca}3.1:** Similar to Kv1.3, 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
erthrocytes and lymphocytes (Begenisich et al., 2004), a reduced 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., 2008). Pharmacological blockade of \( \text{K}_{\text{Ca}}3.1 \) 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/kg/d did
not induce any changes in blood chemistry, hematology 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 \( \text{K}_{\text{Ca}}3.1 \) 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; Ataga et al., 2008).

**Summary**

In summary, \( \text{K}_v1.3 \) and \( \text{K}_{\text{Ca}}3.1 \) 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 \( \text{K}_v1.3 \) or \( \text{K}_{\text{Ca}}3.1 \) blockade in humans. In turn
this will help determine the primary outcomes for clinical trials. For example, if eosinophilia is the predominant feature which is inhibited, then the rate of exacerbations should be the primary outcome (Green et al., 2002), whereas if bronchial hyperresponsiveness or remodelling is the predominant feature which improves, then measurement of these as the primary outcome would be more appropriate. The studies to-date with \( K_v 1.3 \) 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

PB has undertaken contract research and acted as a consultant for Icagen Inc.. H.W. 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 \( K_v 1.3 \) blockers for the treatment of multiple sclerosis and psoriasis.
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Table 1

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

<table>
<thead>
<tr>
<th>Channel</th>
<th>TRAM-34</th>
<th>PAP-1</th>
<th>SKA-31</th>
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<tbody>
<tr>
<td>$K_v$1</td>
<td>$K_v$1.1</td>
<td>9.5 $\mu$M</td>
<td>65 nM</td>
</tr>
<tr>
<td></td>
<td>$K_v$1.2</td>
<td>4.5 $\mu$M</td>
<td>250 nM</td>
</tr>
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<td></td>
<td>$K_v$1.3</td>
<td>5 $\mu$M</td>
<td>2 nM</td>
</tr>
<tr>
<td></td>
<td>$K_v$1.4</td>
<td>7.5 $\mu$M</td>
<td>75 nM</td>
</tr>
<tr>
<td></td>
<td>$K_v$1.5</td>
<td>7 $\mu$M</td>
<td>45 nM</td>
</tr>
<tr>
<td></td>
<td>$K_v$1.6</td>
<td>n.d.</td>
<td>62 nM</td>
</tr>
<tr>
<td>$K_v$3</td>
<td>$K_v$3.1</td>
<td>30 $\mu$M</td>
<td>3 $\mu$M</td>
</tr>
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<td></td>
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</tr>
<tr>
<td>$K_v$4.2</td>
<td>$K_v$4.2</td>
<td>6 $\mu$M</td>
<td>1.2 $\mu$M</td>
</tr>
<tr>
<td>$K_v$11</td>
<td>$K_v$11.1</td>
<td>20 $\mu$M</td>
<td>5 $\mu$M</td>
</tr>
<tr>
<td>$K_{IR}$</td>
<td>$K_{IR}$2.1</td>
<td>$&gt;$ 20 $\mu$M</td>
<td>15 $\mu$M</td>
</tr>
<tr>
<td>$K_{Ca}$</td>
<td>$K_{Ca}$1.1</td>
<td>25 $\mu$M</td>
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<td>20 $\mu$M</td>
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<td>28 $\mu$M</td>
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<td></td>
<td>$K_{Ca}$3.1</td>
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</tr>
<tr>
<td>$Na_v$</td>
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<td>20 $\mu$M</td>
<td>7 $\mu$M</td>
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<tr>
<td></td>
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<td>7 $\mu$M</td>
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<tr>
<td>$Ca_v$</td>
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<td>12 $\mu$M</td>
<td>5 $\mu$M</td>
</tr>
</tbody>
</table>

$K_v$ - 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).
Figure legends

Figure 1

Involvement of K_v1.3, K_Ca3.1 and CRAC (Orail 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 C_γ (PLC_γ) downstream of the tyrosine kinases LCK and ZAP70. PLC_γ catalysis 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 nuclear factor of activated T cells (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_Ca3.1 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 plasma membrane and activated 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_Ca3.1 channels in response to Ca^{2+} binding to calmodulin (CAM). [The resting intracellular Ca^{2+} concentration in a 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].
Figure 2
The chemical structures of PAP-1, TRAM-34, ICA-17043, 1-EBIO and SKA-31.

Figure 3
$K_v1.3$ versus $K_{Ca3.1}$ channel numbers per cell in naïve, $T_{CM}$ and $T_{EM}$ CD4$^+$ T cells before and after activation.

Figure 4
Opening of $K_{Ca3.1}$ channels (A) and hyperpolarisation 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 ASM supernatant (S/N) is inhibited by the $K_{Ca3.1}$ blockers TRAM-34 and charybdotoxin (ChTX) (A), but not the $K_{Ca1.1}$ 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). DMSO 0.1% was present in all conditions. Reproduced from Cruse et al. 2006.