Review TRENDS in Pharmacological Sciences Vol.25 No.5 May 2004

K^+ channels as targets for specific immunomodulation

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The voltage-gated Kv1.3 channel and the Ca^{2+}-activated IKCa1 K^+ channel are expressed in T cells in a distinct pattern that depends on the state of lymphocyte activation and differentiation. The channel phenotype changes during the progression from the resting to the activated cell state and from naïve to effector memory cells, affording promise for specific immunomodulatory actions of K^+ channel blockers. In this article, we review the functional roles of these channels in both naïve cells and memory cells, describe the development of selective inhibitors of Kv1.3 and IKCa1 channels, and provide a rationale for the potential therapeutic use of these inhibitors in immunological disorders.

In the 1950s, James Gowans' research group defined the central role of lymphocytes in immune responses, and in the 1960s the seminal work of Jacques Miller and Robert Good and their colleagues elucidated the crucial role of thymus-derived lymphocytes (T cells). Ionic movements associated with mitogen activation of lymphocytes were first reported in the 1970s [1], but ion channels and electrophysiological studies were far removed from the consciousness of immunologists during this period, being the domain of those investigating excitable cells (nerves and muscles). This barrier was breached in 1984 with the first patch-clamp studies on K^+ channels in lymphocytes [2–4], and during the past two decades there has been a steady growth of publications in this research area (Figure 1a).

In addition to characterizing the biophysical properties of the voltage-gated K_V channel in T cells, we showed that these channels are important for T-cell activation by demonstrating that several chemically unrelated K^+ channel blockers suppressed proliferation and cytokine production with potencies paralleling channel blockade [2,5]. The serendipitous discovery in 1989 that charybdotoxin (ChTX), a peptide from the venom of the scorpion *Leiurus quinquestriatus*, blocks the K_V channel at nanomolar concentrations [6] led to the isolation of increasingly potent and selective channel inhibitors from scorpion venom, sea anemone extracts and plants [7]. The pace of discovery accelerated with the demonstration that the *KCNA3* (*Kv1.3*; http://www.iuphar-db.org/iuphar-ic/KV1x.html) gene encodes the lymphocyte K_V channel [8,9]. An intermediate-conductance Ca^{2+}-activated K^+ channel was identified in T cells in 1992 [10–12], and shown to be a product of the *KCNN4* (*IKCa1*, $K_{Ca}3.1$; http://www.iuphar-db.org/iuphar-ic/KCa.html) gene in 1997 [13]. Subsequent studies by our group identified calmodulin as the Ca^{2+} sensor of the IKCa1 channel [14]. The salient features of both channels were summarized in a recent review [15].

Following the discovery that K^+ channels are essential for T-cell function, several other K^+ channels have been implicated in the proliferation of a wide variety of normal and malignant cells (Figure 1b). In this review, we define the physiological roles of Kv1.3 and IKCa1 channels in T cells, describe the development of increasingly selective channel inhibitors and discuss the rationale for their possible use as therapeutic immunomodulators.

Physiological roles of Kv1.3 and IKCa1 channels in T cells

Molecular interactions at the immunological synapse

The T-cell-mediated immune response is initiated by recognition of processed antigenic peptide bound to major histocompatibility complex (MHC) proteins on antigen-presenting cells (APCs) by the antigen-specific multi-subunit T-cell receptor (TCR)–CD3 complex on T cells (Figure 2). Within minutes the TCR–CD3 complex and accessory proteins cluster at the zone of contact with the APC in a region termed the 'immunological synapse' (IS). Antigen-induced redistribution of key receptors and signaling molecules at the IS facilitates transduction of signals that activate T cells. A recent fluorescence resonance energy transfer (FRET) study in lymphocytes transfected with Kv1.3 channels showed that the channel was in close proximity to CD3 [16]. Biochemical experiments [17–22] also suggest that the Kv1.3 channel is part of a signaling complex that includes enzymes [tyrosine kinase $p56^{lck}$ and protein kinase C (PKC)], adaptor proteins [Kvβ2 and hDlg (human homolog of the *Drosophila* discs large tumor suppressor protein), PSD-95 (postsynaptic density 95), ZIP-1 (Zrt/Irt-like protein) and ZIP2] and the accessory protein CD4. Tyrosine phosphorylation of Kv1.3 channels and modulation of Kv1.3 channel currents by $p56^{lck}$ further suggests that the clustering of Kv1.3 channels and $p56^{lck}$ could provide a mechanism for regulating channel function [17–19,21,22].

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 0165-6147/$ - see front matter doi:10.1016/j.tips.2004.03.010

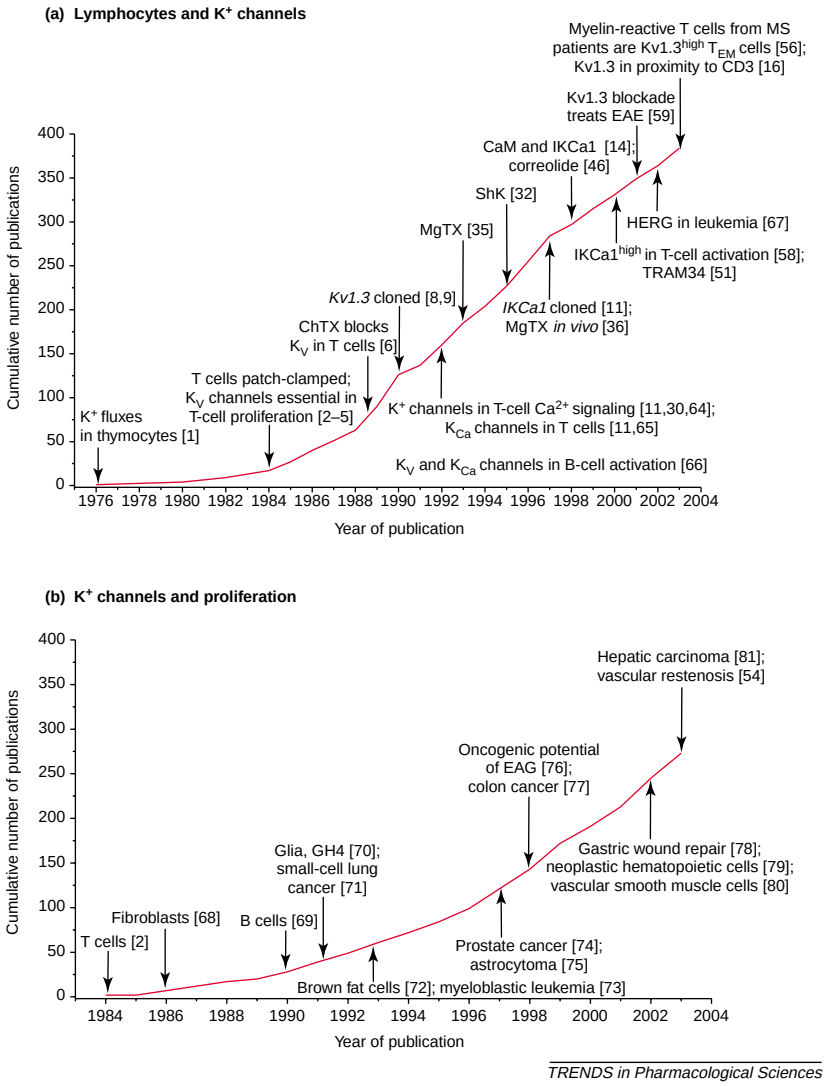

Figure 1. The cumulative number of publications as of November 2003 on **(a)** 'lymphocytes and K^+ channels' and **(b)** 'K^+ channels and proliferation'. Key discoveries in both fields are highlighted. Abbreviations: CaM, calmodulin; ChTX, charybdotoxin; EAE, experimental autoimmune encephalomyelitis; EAG, ether a-go-go; IKCa1, intermediate-conductance Ca^{2+}-activated K^+ channel subtype 1; HERG, human ether-à-go-go-related gene; Kv1.3^{high}, high levels of Kv1.3 channels; MgTX, margatoxin; MS, multiple sclerosis; ShK, *Stichodactyla helianthus* toxin; T_{EM} cells, effector memory T cells. (Data are from [1–6,8,9,11,14,16,30,32,35,36,46,51,54,56,58,59,64–81].)

Clustering interactions of Kv1.4 channels with PSD-95 inhibit the internalization of Kv1.4 channels [23] and the same could be true for Kv1.3 channels. Because CD4 and CD3 aggregate at the IS, it is conceivable that the envisaged CD4–Kv1.3 channel complex localizes at the IS and regulates early events in T-cell activation. A recent study of cytotoxic T cells transfected with Kv1.3 channels supports this notion by demonstrating clustering of Kv1.3 channels at the site of interaction of cytotoxic T cells with the target cell [24]. The Kv1.3 channel has also been reported to be physically and functionally coupled to β1-integrins [25] that stabilize the IS and are also important in lymphocyte adhesion and migration.

K^+ channels and Ca^{2+} signaling

The activation events following T-cell engagement with antigen and the role of Kv1.3 and IKCa1 channels in this signaling cascade are shown in Figure 2 [15,22]. Antigen recognition leads to the activation of tyrosine kinases and phospholipase C (PLC), resulting in the generation of inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol, which induce the release of Ca^{2+} from internal stores, and the activation of PKC. Depletion of internal Ca^{2+} stores causes voltage-independent Ca^{2+} release-activated Ca^{2+} (CRAC) channels to open in the membrane, and the ensuing Ca^{2+} influx sustains elevated levels of cytosolic Ca^{2+}. The physiological significance of the Ca^{2+} signal mediated by CRAC channels is highlighted by their absence in severe-combined immunodeficiency patients [26,27] and by the requirement of sustained Ca^{2+} influx for 75% of new gene expression via the phosphatase calcineurin [27,28]. Coordinated activity of Ca^{2+}- and PKC-dependent signaling pathways culminates in cell proliferation.

Kv1.3 and IKCa1 channels regulate Ca^{2+} signaling events via the control of membrane potential [15,22]. Ca^{2+} influx through CRAC channels is reduced at depolarized potentials and consequently membrane depolarization attenuates the Ca^{2+} signal. The driving force for Ca^{2+} entry is restored by membrane hyperpolarization brought

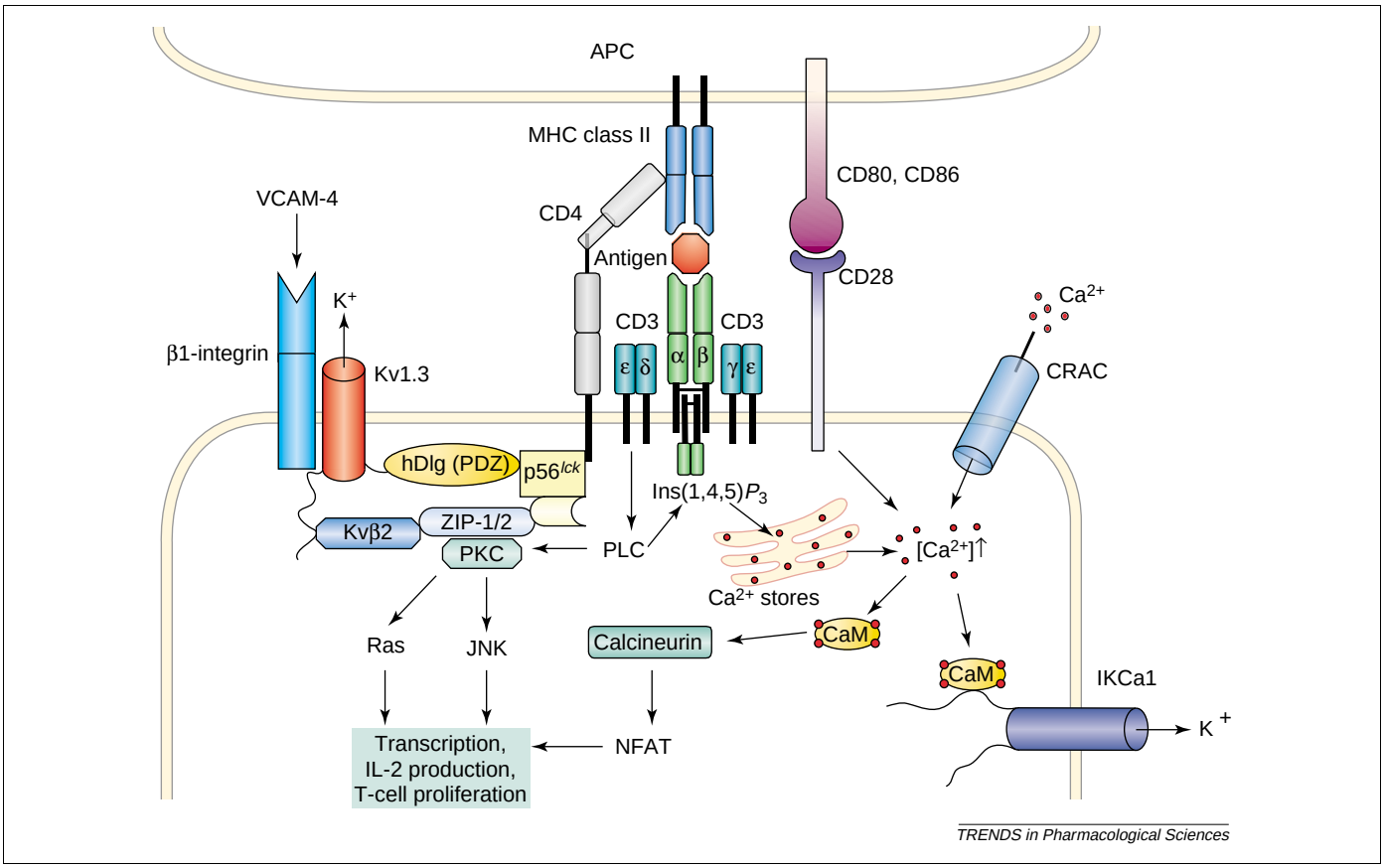

Figure 2. The involvement of voltage-dependent Kv1.3 channels, intermediate-conductance Ca^{2+}-activated IKCa1 channels and voltage-independent Ca^{2+} release-activated Ca^{2+} (CRAC) channels in the activation of a $CD4^+$ T cells by an antigen-presenting cell (APC) is shown. Engagement of the T-cell receptor–CD3 complex through an antigenic peptide presented in the context of major histocompatibility complex (MHC) class II activates phospholipase C (PLC), which leads to the activation of protein kinase C (PKC) and the production of inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3], which liberates Ca^{2+} from intracellular stores. Simultaneous activation of CD28 by the co-stimulatory molecules CD80 or CD86 further amplifies the resulting Ca^{2+} signal. The rise in the intracellular concentration of Ca^{2+} activates the phosphatase calcineurin, which then dephosphorylates the transcription factor nuclear factor of activated T cells (NFAT), enabling it to accumulate in the nucleus and bind to the promoter of the gene encoding interleukin 2 (IL-2). Parallel activation of the c-JUN N-terminal kinase (JNK) and Ras by PKC results in the activation of other transcription factors and initiates transcription of various genes and finally T-cell proliferation. CRAC, Kv1.3 and IKCa1 channels regulate Ca^{2+} signaling. Depletion of internal Ca^{2+} stores causes CRAC channels in the membrane to open, and the ensuing Ca^{2+} influx sustains elevated levels of cytosolic Ca^{2+}. Ca^{2+} influx through CRAC channels is reduced following membrane depolarization. The driving force for Ca^{2+} entry is restored by membrane hyperpolarization brought about by the opening of Kv1.3 channels in response to membrane depolarization and the opening of IKCa1 channels as a consequence of elevated concentrations of cytosolic Ca^{2+}. Selective blockade of K^+ channels leads to membrane depolarization, inhibits Ca^{2+} influx and shuts down cytokine production and cell proliferation. Abbreviations: CaM, calmodulin; hDlg, human homolog of the *Drosophila* discs large tumor suppressor protein; VCAM-4, vascular cell adhesion molecule 4; ZIP-1, Zrt/Irt-like protein.

about by the opening of Kv1.3 channels in response to membrane depolarization and the opening of IKCa1 channels as a consequence of elevated concentrations of cytosolic Ca^{2+}. Selective blockade of K^+ channels leads to membrane depolarization, inhibition of Ca^{2+} influx and inhibition of cytokine production and cell proliferation.

Pharmacology of Kv1.3 and IKCa1 channels

The roles of Kv1.3 and IKCa1 channels have been defined in T cells with the help of structurally diverse peptides, small molecules and metal ions that block these channels with potencies ranging from picomolar to millimolar values. Figures 3a and 4 display the chemical structures of selected peptide and small organic inhibitors, whereas Tables 1 and 2 provide lists of Kv1.3 and IKCa1 channel modulators in order of decreasing potency. For Kv1.3, but not IKCa1, channels the potency of peptide inhibitors exceeds by orders of magnitude the potency of the small organic inhibitors described thus far, as shown graphically in plots of blocker potency versus the year of publication for both channels (Figures 3b,c).

Peptide blockers

Although the use of ChTX (Figure 3a) has demonstrated a crucial role for K^+ channels in T-cell activation [11,29,30], it inhibits both Kv1.3 [6,29,31] and IKCa1 [11,12] channels at low nanomolar concentrations and does not distinguish between the functional contributions of the two channels. The most potent Kv1.3 inhibitor, ShK from the Caribbean sea anemone *Stichodactyla helianthus* [32], blocks the channel under physiological conditions with a K_d value of 11 pM [33] and exhibits >1000-fold selectivity over IKCa1 channels (Tables 1,2). ShK contains 35 amino acids held together by three disulfide bonds (Figure 3a) [33,34]. Margatoxin (MgTX) (Table 1) from the venom of *Centruroides margaritatus* is also used widely as a Kv1.3 channel inhibitor [35,36]. The most potent inhibitor of IKCa1 channels maurotoxin (MTX), from the venom of *Scorpio maurus* ($K_d = 1$ nM), exhibits >150-fold selectivity over Kv1.3 channels (Tables 1,2) [37,38]. MTX contains

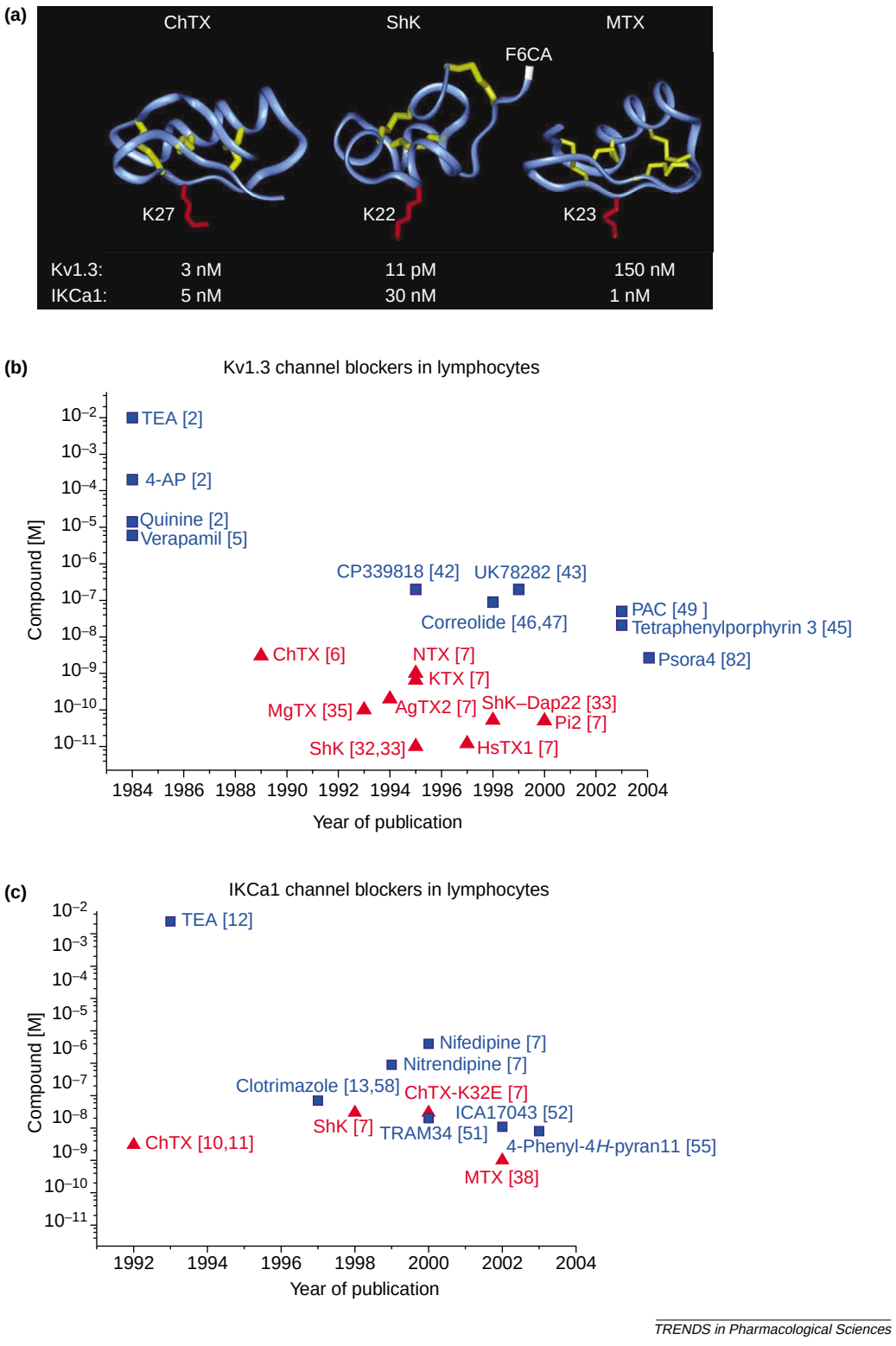

Figure 3. **(a)** Backbone structures of charybdotoxin (ChTX), *Stichodactyla helianthus* toxin (ShK) and maurotoxin (MTX), which are peptide inhibitors of voltage-dependent Kv1.3 channels and intermediate-conductance Ca^{2+}-activated IKCa1 channels. The crucial lysine that occludes the channel pore is highlighted in red in each structure: Lys27 in ChTX; Lys22 in ShK; and Lys23 in MTX. The disulfide bonds are highlighted in yellow. Arg1 in ShK, to which the fluorophore fluorescein-6-carboxylic acid (F6CA) is attached, is highlighted in white. Such fluorophores attached to specific inhibitors can be used to visualize these K^+ channels. **(b,c)** Plots of potency versus year of publication for peptide (red; triangles) and small-molecule (blue; squares) blockers of Kv1.3 (b) and IKCa1 (c) channels in lymphocytes are shown. Abbreviations: AgTX2, agiotoxin-2; 4-AP, 4-aminopyridine; ChTX-K32E, charybdotoxin derivative with glutamate at position 32 in place of the native lysine; HsTX1, *Heterometrus spinnifer* toxin 1; KTX, kaliotoxin; MgTX, margatoxin; NTX, noxiustoxin; PAC, 4-phenyl-4-[3-(2-methoxyphenyl)-3-oxo-2-azaprop-1-yl]cyclohexanone; Pi2, *Pandinus imperator* toxin 2; ShK–Dap22, *Stichodactyla helianthus* toxin with diaminopropionic acid introduced at position 22 in place of the native lysine; TEA, tetraethylammonium chloride. See Chemical names. (Data are from [2,5–7,10–13,32,33,35,38,42,43,45–47,49,51,52,55,58,82].)

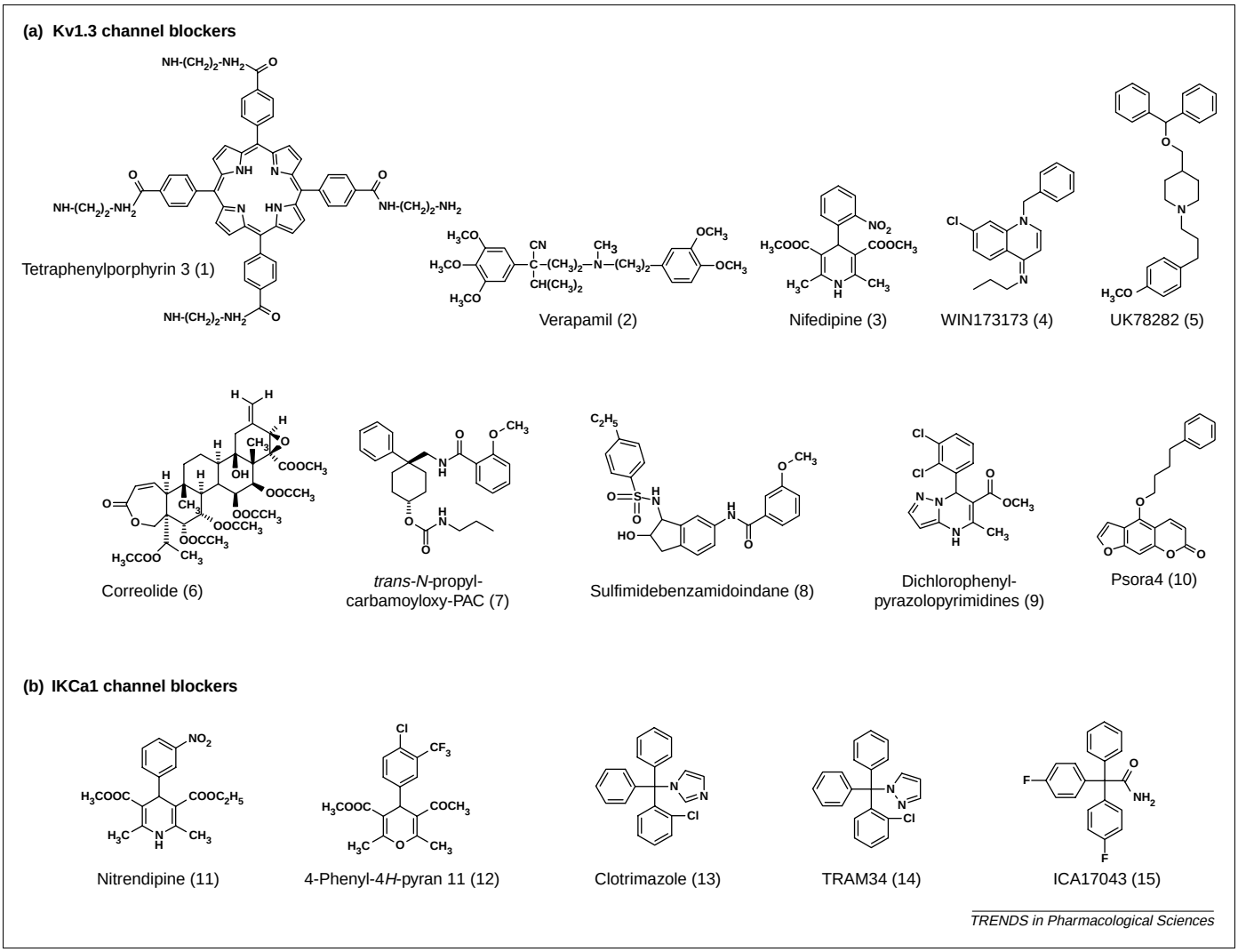

Figure 4. Structures of small-molecule Kv1.3 channel and IKCa1 channel blockers. See Tables 1 and 2 for their K_d values on the respective channels.

34 amino acids held together by four disulfide bonds (Figure 3a) [39]. Despite differences in their structural fold, most toxins that block K^+ channels use a crucial lysine to occlude the channel pore [7] (Figure 3a).

The lack of channel-specific antibodies that bind to extracellular epitopes on Kv1.3 or IKCa1 channels has precluded visualization of these channels in live cells. However, the affinity of peptide inhibitors of Kv1.3 channels is extremely high, and their ability to interact with all four subunits of the tetramer has made them attractive as fluorophore-tagged tools for channel visualization. Attachment of fluorescein-6-carboxylic acid (F6CA) through a nine-atom hydrophilic linker to Arg1 of ShK (Figure 3a) yielded a potent and specific inhibitor of Kv1.3 channels (Table 1) that detected, using flow cytometry, high levels of Kv1.3 channels ($Kv1.3^{high}$) in chronically activated T cells [40]. Single Kv1.3 channels were also visualized in Jurkat T cells with hongotoxin conjugated to Cy5 via a spinster Cys19 on the 'backside' of the peptide [41].

Small-molecule Kv1.3 channel blockers

L-type Ca^{2+} channel blockers of the dihydropyridine (e.g. nifedipine; compound 3 in Figure 4) and phenylalkylamine (verapamil; compound 2 in Figure 4) classes were the first small molecules found to inhibit lymphocyte K^+ channels with low micromolar affinity [5,7]. Verapamil, dilitazem and nifedipine inhibit IL-2 secretion and T-cell proliferation at concentrations between 10 and 50 μM [5], potencies that are consistent with K^+ channel blockade. The first small-molecule Kv1.3 channel blockers with nanomolar affinity were the iminodihydroquinolines WIN173173 (see Chemical names; compound 4 in Figure 4) and CP339818 [42], and the benzhydryl piperidine UK78282 (compound 5 in Figure 4) [43]. However, the iminodihydroquinolines also block Na^+ channels [44] and UK78282 blocks Kv1.4 channels [43], and thus further development of these compounds was abandoned. The tetraphenylporphyrins [45] are promising drug candidates, the most potent (compound 1 in Figure 4) blocking Kv1.3 channels with a K_d of 20 nM in binding studies (Table 1), but their selectivity remains to be determined. The attachment of one or more fluorophores to these compounds might constitute interesting alternatives to fluorophore-tagged peptide inhibitors [45] because they mimic toxins by using a porphyrin ring as a scaffold to position charged groups at the optimal distance to form salt bridges with aspartate residues in the outer vestibule of each of the four Kv1.3 channel subunits. Other blockers with low

Table 1. K_d values of peptide and small-molecule modulators of Kv1.3 channels[a–c]

Inhibitor	K_d value	Inhibitor	K_d value
Stichodactyla helianthus toxin	11 pM	*Parabuthus* toxin 3	492 nM
Heterometrus spinnifer toxin 1	12 pM	*Parabuthus* toxin 1	800 nM
ShK–F6CA	48 pM	Resiniferatoxin	3 μM
Pandinus imperator toxin 2	50 pM	Nifedipine (3)	5 μM
ShK–Dap22	52 pM	Nitrendipine (11)	5 μM
Hongotoxin	86 pM	Ibu8	5 μM
Margatoxin	110 pM	Phencyclidine	5 μM
Agiotoxin-2	200 pM	Verapamil (2)	6 μM
Pandinus imperator toxin 3	500 pM	H37	10 μM
Kaliotoxin	650 pM	Hg^{2+}	10 μM
Noxiustoxin	1 nM	Quinine	14 μM
Psora4 (10)	3 nM	Cicutotoxin	18 μM
Charybdotoxin	3 nM	La^{3+}	20 μM
Titystoxin-Kα	4 nM	Trifluoperazine	20 μM
Pandinus imperator toxin 1	11 nM	Capsaicin	26 μM
Tetraphenylporphyrin 3* (1)	20 nM	Diltiazem	27 μM
Bunodosoma granulifera toxin	39 nM	Progesterone	30 μM
trans-N-propyl-carbamoyloxy-PAC (7)	50 nM	κ-Hefutoxin	40 μM
Correolide (6)	90 nM	Luteolin	50 μM
Sulfamidbenzamidoindane (8)	100 nM	Flecainide	60 μM
Maurotoxin	150 nM	4-AP	190 μM
CP339818	150 nM	Zn^{2+}, Co^{2+}	200 μM
WIN173173 (4)	200 nM	Ba^{2+}, Cd^{2+}	2 mM
UK78282 (5)	200 nM	TEA	10 mM
Dendrotoxin	250 nM	Mn^{2+}	20 mM
PAC	270 nM		

[a]Inhibitors are shown in order of decreasing potency. All K_d values with the exception of those marked with an asterisk (* = binding data) were determined through whole-cell patch-clamp or two-electrode voltage-clamp on the cloned channels expressed in mammalian cells or oocytes. The numbers in brackets correspond to structures in Figure 4.
[b]Abbreviations: 4-AP, 4-aminopyridine; ShK–Dap22, *Stichodactyla helianthus* toxin with diaminopropionic acid introduced at position 22 in place of the native lysine; ShK–F6CA, *Stichodactyla helianthus* toxin–fluorescein-6-carboxylic acid; *trans-N*-propyl-carbamoyloxy-PAC, *trans-N*-propyl-carbamoyloxy-4-phenyl-4-[3-(2-methoxyphenyl)-3-oxo-2-azaprop-1-yl]cyclohexanone; TEA, tetraethylammonium chloride.
[c]See Chemical names.

micromolar to submicromolar potency for Kv1.3 channels include correolide (compound 6 in Figure 4), *trans-N*-propylcarbamoyloxy-PAC (compound 7 in Figure 4), sulfamidebenzamidoindanes (compound 8 in Figure 4), dichlorophenylpyrazolopyrimidines (compound 9 in Figure 4) and the 5-(4-phenylbutoxy)psoralen Psora4 (compound 10 in Figure 4) [15,82]. The binding sites for these drugs on Kv1.3 channels and the mechanism of channel block have been described in a recent review [15].

In 1998, scientists at Merck (http://www.merck.com/) discovered correolide, a pentacyclic nortriterpene, in extracts of the Costa Rican tree *Spachea correa* [46,47]. Correolide blocks Kv1.3 channels with a K_d of ~100 nM and inhibits mitogen-induced proliferation of T cells, and an analog with more favorable pharmacokinetic properties suppresses *in vivo* delayed-type hypersensitivity immune responses in Yucatan mini-pigs [48]. However, correolide might not be a suitable drug candidate because it blocks other K_V1 channels with potencies equivalent to those that block Kv1.3 channels, and its molecular complexity makes synthesis of new analogs both challenging and expensive. The Merck group recently identified the chemically simpler cyclohexyl-substituted benzamides [PACs (4-phenyl-4-[3-(2-methoxyphenyl)-3-oxo-2-azaprop-1-yl] cyclohexanones)] in $^{86}Rb^+$ flux experiments [49]. These compounds are distinguished from other Kv1.3 channel inhibitors by their Hill coefficient of 2 and interesting structural isomer activity profile [49]. The parent compound PAC is not selective for Kv1.3 channels, but substitutions introduced at position 1 of the cyclohexanone ring yield *cis* or *trans* isomer pairs with differing properties [49]. The *cis*-isomers are not selective for Kv1.3 channels, whereas the *trans*-isomers show two- to sixfold selectivity for Kv1.3 channels over Kv1.1 and Kv1.2 channels and demonstrate a well-defined structure–activity relationship. The most potent compound in this series (compound 7 in Figure 4) blocks Kv1.3 channels with a K_d of 50 nM. Although the *trans*-PACs appear promising, their specificity for Kv1.3 channels needs improvement and their *in vivo* immunomodulatory activity has yet to be reported.

The most potent small-molecule inhibitor of Kv1.3 channels is Psora4 (compound 10 in Figure 4). Psora4 blocks the channel in a use-dependent manner with a Hill coefficient of 2 and an EC_{50} value of 3 nM, by preferentially binding to the C-type inactivated state of the channel [82]. It exhibits 17–70-fold selectivity for Kv1.3 channels over closely related Kv1 family channels (Kv1.1, Kv1.2, Kv1.4 and Kv1.7), with the exception of Kv1.5 channels (EC_{50} = 7.7 nM), and shows no effect on HERG (human ether-à-go-go-related gene), Kv3.1 or Ca^{2+}-activated K^+ channels (IKCa1, SK1–SK3 and BK_{Ca}) or the neuronal $Na_V1.2$ channel. The limited selectivity of Psora4 for Kv1.3 channels over the cardiac channel Kv1.5 channel precludes its use as an immunomodulator, although future analogs might exhibit the requisite selectivity and oral bioavailability to make it a useful therapeutic.

Small-molecule IKCa1 channel blockers

The azole antimycotic clotrimazole (compound 13 in Figure 4) and the dihydropyridines nitrendipine

Table 2. K_d values of peptide and small-molecule modulators of IKCa1 channels[a–c]

Inhibitor	K_d value	Inhibitor	K_d value
Maurotoxin	1 nM	Nimodipine	1 μM
Charybdotoxin	5 nM	ShK–Dap22	2.6 μM
4-Phenyl-4*H*-pyran 11 (12)	8 nM	Nifedipine	4 μM
ICA17043 (15)	11 nM	Econazole	12 μM
TRAM34 (14)	20 nM	Ketoconazole	30 μM
Stichodactyla helianthus toxin	30 nM	Verapamil	28 μM
ChTx–Glu32	33 nM	Berberine	50 μM
TRAM39	60 nM	Cetiedil	79 μM
Clotrimazole (13)	70 nM	4-AP	> 1 mM
Budonosoma granulifera toxin	172 nM	La^{3+}	2 mM
TRAM3	520 nM	Ba^{2+}	10 mM
DiS-C2	700 nM	Cd^{2+}	5 mM
Nitrendipine (11)	900 nM	TEA	24 mM
Activator			
Dichloro-EBIO	1 μM		
Riluzole	3 μM		
EBIO	80 μM		
Zoxazolamine	98 μM		
Chlorzoxazone	100 μM		
Theophylline	1 mM		
Caffeine	1 mM		

[a]Inhibitors and activators are shown in order of decreasing potency. All K_d values were determined through whole-cell patch-clamp or two-electrode voltage-clamp on the cloned channels expressed in mammalian cells or oocytes. The numbers in brackets correspond to structures in Figure 4.
[b]Abbreviations: 4-AP, 4-aminopyridine; ChTx–Glu32, charybdotoxin derivative with glutamate at position 32 in place of the native lysine; dichloro-EBIO, 5,6-dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one; ShK–Dap22, *Stichodactyla helianthus* toxin with diaminopropionic acid introduced at position 22 in place of the native lysine; TEA, tetraethylammonium chloride.
[c]See Chemical names.

(compound 11 in Figure 4) and nifedipine (compound 3 in Figure 4) were the first potent small-molecule inhibitors of IKCa1 channels [15]. Clotrimazole is particularly interesting because it ameliorates rheumatoid arthritis in patients [50], but it was abandoned because of adverse effects resulting from blockade of cytochrome P450 enzymes. Using clotrimazole as a template, our group [51] and chemists at Icagen (http://www.icagen.com/) [52] independently developed selective IKCa1 channel inhibitors that lack cytochrome P450 blocking activity. We designed a pyrazole-substituted triarylmethane called TRAM34 (compound 14 in Figure 4) that exhibits >100-fold selectivity for IKCa1 channels over other K^+ channels [51,53]. TRAM34 did not produce obvious toxic side-effects when administered to rats in a trial that demonstrated its effectiveness in preventing vascular restenosis following balloon angioplasty [54]. The Icagen group developed another clotrimazole analog called ICA17043 (compound 15 in Figure 4), which is now in Phase II clinical trials for sickle cell anemia [52]. More recently, scientists at Bayer (http://www.bayer.com/) used nifedipine as a template to develop a novel 4-phenyl-4*H*-pyran (compound 12 in Figure 4) that blocks IKCa1 channels with a K_d of 8 nM [55] and reduces infarct volume in a rat subdural hematoma model, suggesting that it could have use in the treatment of traumatic brain injury. Lastly, several activators of IKCa1 channels have been identified (Table 2) but the therapeutic utility of these compounds in immunomodulation remains unclear.

K^+ channels in T cells: targets for immunomodulation

The immunomodulatory effects of channel blockers depend on the expression levels of Kv1.3 and IKCa1 channels, which change dramatically when T cells transition from resting to activated cells, and during differentiation from the naïve to the memory state [15,40,56]. Figure 5a shows the three human T-cell subsets that are distinguished based on the expression of the chemokine receptor CCR7 and the phosphatase CD45RA [57]: naïve cells ($CCR7^+CD45RA^+$); central memory cells (T_{CM}) ($CCR7^+CD45RA^-$); and effector memory cells (T_{EM}) ($CCR7^-CD45RA^-$).

Kv1.3 and IKCa1 channels in naïve, T_{CM} and T_{EM} cells

In the quiescent state, cells belonging to all three T-cell subsets express ~250 Kv1.3 channels and 5–35 IKCa1 channels per cell [56] and, because Kv1.3 channels dominate functionally in quiescent cells, ShK and Psora4 suppress their activation [56,82], whereas TRAM34 has no effect [58]. Activation induces differential expression of K^+ channels in these subsets, leading to an altered channel phenotype and consequently to altered responsiveness to Kv1.3 and IKCa1 channel blockers. IKCa1 channels are upregulated to ~500 per cell in naïve and T_{CM} cells [56] via PKC-dependent transcription of the gene encoding the IKCa1 channel [58]. Transcriptional upregulation is detectable three hours after antigen stimulation and is independent of Ca^{2+} signaling because its blockade does not prevent IKCa1 channel augmentation [56,58]. IKCa1 channel levels are augmented even if the initial activation of naïve and T_{CM} cells is suppressed by ShK or cyclosporin A (agents that attenuate Ca^{2+} signaling or activation of calcineurin) because IKCa1 channel upregulation is only minimally dependent on Ca^{2+} [15,56]. As a consequence of IKCa1 channel upregulation, naïve and T_{CM} cells escape further Kv1.3 channel inhibition and become sensitive to IKCa1 channel blockade [15,56]. By contrast, when T_{EM} cells are activated, Kv1.3 channel expression is increased in these cells to 1500 per cell with little change in IKCa1 channel levels [40,56], and consequently ShK ($IC_{50} = 400$ pM) and Psora4 ($IC_{50} = 25$ nM) persistently suppress T_{EM} proliferation, whereas TRAM34 ($IC_{50} > 6$ μM) is ineffective [15,56,82]. Increased Kv1.3 channel expression is a result of new Kv1.3 channel tetramers being inserted into the cell membrane and is dependent on both Ca^{2+}- and PKC-dependent signaling cascades [40]. Flow cytometry with ShK–F6CA (Table 1) can detect this Kv1.3 channel upregulation, and the results from flow cytometry correlate well with measurements obtained in patch-clamp studies (Figure 5b). Because flow cytometry is more rapid than patch-analysis and samples bulk populations, it could have use in screening tissues for $Kv1.3^{high}$ T_{EM} cells that have been implicated in autoimmune disorders. The functional dominance of IKCa1 channels in activated naïve and T_{CM} cells versus Kv1.3 channels in T_{EM} cells provides a powerful way to manipulate the activity of these subsets by administration of specific IKCa1 and Kv1.3 channel inhibitors.

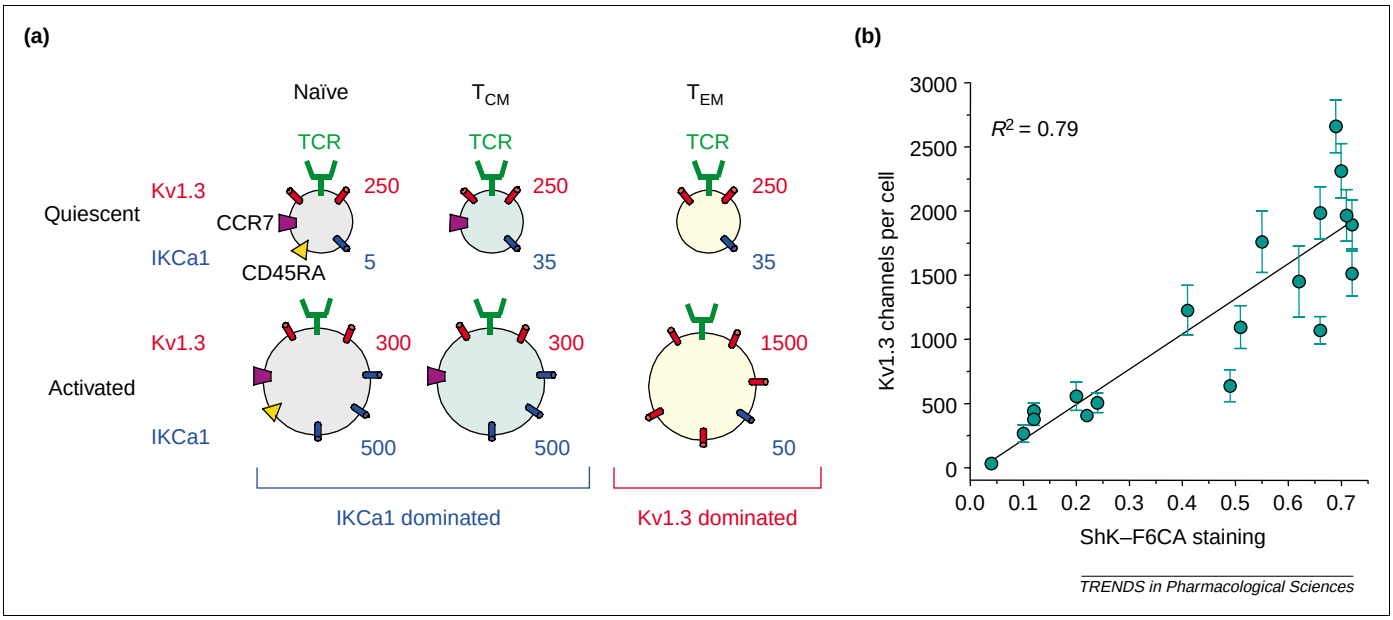

Figure 5. **(a)** Schematic showing the average numbers of Kv1.3 channels and IKCa1 channels per cell in naïve ($CCR7^+CD45RA^+$), central memory (T_{CM}) ($CCR7^+CD45RA^-$) and effector memory (T_{EM}) ($CCR7^-CD45RA^-$) cells. Naïve and T_{CM} cells increase IKCa1 channel expression following activation, whereas T_{EM} cells increase Kv1.3 channel expression. **(b)** Plot of Kv1.3 channel numbers per cell determined by whole-cell patch-clamp in different T-cell populations versus difference (D) values of *Stichodactyla helianthus* toxin (ShK)–fluorescein-6-carboxylic acid (F6CA) staining obtained by flow cytometry; these data were obtained from [40]. The D value is a measure of the difference in fluorescence intensity between ShK–F6CA-stained cells and background fluorescence from unstained cells of the same population. Abbreviation: TCR, T-cell receptor.

IKCa1 and Kv1.3 channel blockers as possible therapeutics

Naïve and T_{CM} cells are likely to be involved in immune-mediated acute rejection of transplanted organs and acute graft-versus-host disease. Because these cells are initially sensitive to Kv1.3 channel blockers and then become dependent on IKCa1 channels, initial combination therapy with Kv1.3 and IKCa1 channel blockers followed by IKCa1 channel blockade alone might be beneficial in the management of these clinical problems. Furthermore, cyclosporin A, a widely used immunosuppressant, synergizes with TRAM34 in T-cell proliferation assays [51], and combining these compounds could reduce the level of toxic side-effects that complicate cyclosporin A therapy.

In a recent study we demonstrated that the majority of pathogenic myelin-reactive T cells from patients with multiple sclerosis (MS) are $Kv1.3^{high}IKCa1^{low}$ T_{EM} cells [56], whereas T cells that are specific for control antigens in these patients express the naïve and/or T_{CM} cell phenotype. Myelin-specific T cells from healthy individuals or from normal rats are naïve and/or T_{CM} cells, but can be converted into $Kv1.3^{high}$ T_{EM} cells by repeated activation *in vitro* by myelin antigens [56,59]. These findings suggest that pathogenic myelin-reactive T cells acquire the $Kv1.3^{high}$ T_{EM} phenotype as a consequence of repeated *in vivo* exposure to myelin antigens during the course of disease. Activated autoreactive T_{EM} cells probably contribute to MS by migrating to inflamed tissues where they secrete interferon γ and tumor necrosis factor α. In keeping with this idea, adoptive transfer of $Kv1.3^{high}$ rat memory T cells into naïve recipients causes severe experimental autoimmune encephalomyelitis (EAE), a model for MS [59]. Selective targeting of these disease-causing cells with Kv1.3 channel blockers prevents and reverses EAE in this rat model [59], and no side-effects are observed despite the expression of Kv1.3 channels in the brain, arteries [60], bladder [61] and epithelia [62]. Together, these findings lead to the prediction that Kv1.3 channel blockers will ameliorate symptoms of MS and other autoimmune disorders mediated by T_{EM} cells in humans. Kv1.3 channel blockers have also been shown recently to be effective in preventing inflammatory bone resorption in experimental periodontal disease [63], and these blockers could also have use in managing chronic graft rejection and chronic graft-versus-host disease that are probably sustained by chronically activated T_{EM} cells [83]. A Kv1.3 channel-based therapeutic approach would have an advantage over agents that cause generalized immunomodulation because naïve and T_{CM} cells would escape inhibition through upregulation of IKCa1 channels, leaving the bulk of the immune response intact.

Concluding remarks

Much work remains to be done to bring IKCa1 and Kv1.3 channel blockers to the clinic. The situation with small-molecule IKCa1 channel blockers is encouraging because ICA17043 is already in Phase II clinical trials for sickle cell disease and TRAM34 has been used successfully in an *in vivo* animal trial [54]. Both these agents and the newly described 4-phenyl-4*H*-pyran need to be evaluated in animal models of organ graft rejection, acute active EAE and rheumatoid arthritis.

Existing small-molecule Kv1.3 channel inhibitors have not shown the requisite selectivity, potency or oral bioavailability to make them viable drug candidates. By contrast, peptide inhibitors such as ShK have exquisitely high potency for Kv1.3 channels and once bound to the channel on lymphocytes are only slowly released, but have short circulating half-lives [59]. A slow-release depot formulation or implantable pump might render these

peptides therapeutically useful. Although ShK was effective in adoptive EAE trials [59], its efficacy needs to be assessed in models that exhibit the relapsing-remitting clinical course observed in MS and its toxicity profile has to be determined. Acute active EAE, the workhorse of drug-screening researchers, might not be useful for this purpose because it involves an acute immune response mediated by naïve and T_{CM} cells, and IKCa1, but not Kv1.3, channel inhibitors might be effective in this model of MS.

In conclusion, Kv1.3 and IKCa1 channels are emerging as important targets for therapeutic manipulation of selective lymphocyte subsets and also as possible diagnostic targets for the detection of pathogenic $Kv1.3^{high}$ T_{EM} cells in autoimmune disorders. The therapeutic promise of this avenue of research remains to be realized but the future does look encouraging.

Chemical names

CP339818: *N*-[1-(phenylmethyl)-4(1*H*)-quinolinylidene]-1-pentanamine monohydrochloride
diS-C2: 3-ethyl-2-[(1*E*,3*E*,5*E*)-5-(3-ethyl-2(3*H*)-benzothiazolylidene)-1,3-pentadienyl]-benzothiazolium iodide
H37: 4,9-diethoxy-7*H*-furo[3,2-*g*] [1]benzopyran-7-one
Ibu8: 8-methoxy-5-methyl-2-(1-methylethyl)-furo[3,2-*c*] quinolin-4(5*H*)-one
ICA17043: 4-fluoro-α-(4-fluorophenyl)-α-phenyl-benzeneacetamide
Psora4: 4-(4-phenylbutoxy)-7*H*-furo[3.2-*g*][1]benzopyrane-7-one
TRAM3: (2-chlorophenyl)diphenylmethanol
TRAM34: 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole
TRAM39: (2-chlorophenyl)diphenylacetonitrile
UK78282: 4-[(diphenylmethoxy)methyl]-1-[3-(4-methoxyphenyl)propyl]-piperidine
WIN173173: *N*-[7-chloro-1-(phenylmethyl)-4(1*H*)-quinolinylidene]-1-propanamine monohydrochloride

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