Potassium Channel Blockade by the Sea Anemone Toxin ShK for the Treatment of Multiple Sclerosis and Other Autoimmune Diseases

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Abstract: Expression of the two lymphocyte potassium channels, the voltage-gated channel Kv1.3 and the calcium activated channel IKCa1, changes during differentiation of human T cells. While IKCa1 is the functionally dominant channel in naïve and "early" memory T cells, Kv1.3 is crucial for the activation of terminally differentiated effector memory (T_{EM}) T cells. Because of the involvement of T_{EM} cells in autoimmune processes, Kv1.3 is regarded as a promising target for the treatment of T-cell mediated autoimmune diseases such as multiple sclerosis and the prevention of chronic transplant rejection. ShK, a 35-residue polypeptide toxin from the sea anemone, *Stichodactyla helianthus*, blocks Kv1.3 at low picomolar concentrations. ShK adopts a central helix-kink-helix fold, and alanine-scanning and other mutagenesis studies have defined its channel-binding surface. Models have been developed of how this toxin effects K^+-channel blockade and how its docking configuration might differ in ShK-Dap22, which contains a single side chain substitution that confers specificity for Kv1.3 blockade. ShK, ShK-Dap22 and the Kv1.3 blocking scorpion toxin kaliotoxin have been shown to prevent and treat experimental autoimmune encephalomyelitis in rats, a model for multiple sclerosis. A fluoresceinated analog of ShK, ShK-F6CA, has been developed, which allows the detection of activated T_{EM} cells in human and animal blood samples by flow cytometry and the visualization of Kv1.3 channel distribution in living cells. ShK and its analogs are currently undergoing further evaluation as leads in the development of new biopharmaceuticals for the treatment of multiple sclerosis and other T-cell mediated autoimmune disorders.

INTRODUCTION

Marine organisms are a rich source of novel peptides and proteins, many with therapeutic potential, and sea anemones are no exception. Early studies of sea anemones focused on polypeptides that modulate sodium channel activity [1] and proteins known as actinoporins that cause cell lysis [2], but more recently a novel family of potassium channel blockers has generated considerable interest. The first representative of this family to be isolated and characterized thoroughly was ShK, from the anemone *Stichodactyla helianthus* [3, 4]. This large, carpet-type sea anemone is sessile and tends to associate with clown fish. It is very common to the Caribbean, living in shallow areas with medium to strong currents and its beautiful green colour derives from a symbiotic colonization of algae. The presence of potent neurotoxins is thought to be primarily for defence against its major predator, the spiny lobster.

A number of other K^+ channel blockers have since been isolated from sea anemones, including AsKS (kaliseptine) [5], BgK [6, 7], HmK [8] and AeK [9]. Another family of polypeptides isolated from anemones has also been found to act on K^+ channels; the first members to be characterized were BDS-I and -II, which were isolated some time ago before their target was defined, but have recently been shown to block Kv3.4 [10]. The related toxin APETx1 specifically inhibits human ether-a-go-go-related gene (HERG, Kv11.1) channels and shares 54% homology with BDS-I [11], although these polypeptides share no sequence homology with other K^+ channel toxins from sea anemones. The focus of this article is ShK, a K^+ channel blocker that shows considerable promise in the treatment of autoimmune diseases, in particular multiple sclerosis (MS).

Figure **1** compares the amino acid sequence of ShK [3] with that of related anemone-derived K^+ channel blockers. The known sequences fall into two groups, with ShK and HmK lacking the basic four-residue insert found in the N-terminal half of AeK, AsKS and BgK but having two single-residue inserts in the C-terminal half. The location of the three disulfide bridges [12], which are common to all members of this family [7], is also shown in (Fig. **1**). The solution structure, determined by NMR spectroscopy [13, 14], represents a novel fold consisting of two short α-helices encompassing residues 14-19 and 21-24, and an N-terminus with an extended conformation up to residue 8 followed by a pair of interlocking turns that resembles a 3_{10}-helix (Fig. **2**). It contains no β-sheet. Its structure is thus quite distinct from the α/β fold found in scorpion K^+ channel blocker such as charybdotoxin (ChTX) [15] and margatoxin (MgTX) [16], but is similar to the structure determined subsequently for BgK toxin [17].

INTERACTION WITH THE POTASSIUM CHANNEL

The targets of these toxins are K^+ channels, which in mammals are encoded by a total of 76 genes ([18], and

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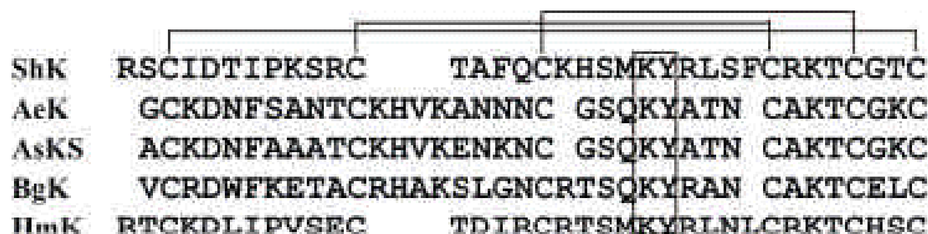

Fig. (1). Amino acid sequences of the sea anemone toxins ShK [3], AeK [9], AsKS or kaliseptine [5], BgK [6, 7] and HmK [8]. The locations of the three disulfide bridges [7, 12] are indicated by the horizontal lines. The boxed residues are the Lys-Tyr diad discussed in the text.

http://www.iuphar-db.org/iuphar-ic/). According to the number of transmembrane segments and the number of pore loops, these channels can be divided into five structural classes. The two most widely studied of these in terms of their physiological function and pharmacology are the inward-rectifier channels with two transmembrane (2TM) segments and the six-transmembrane (6TM) channels, which include the voltage-activated K^+ channels (Kv) and the related small-conductance and intermediate-conductance Ca^{2+}-activated K^+ channels (K_{Ca}). In the 6TM channels the region between the fifth and sixth transmembrane segments (the pore loop) forms the ion conduction pathway and four subunits come together to form a functional channel tetramer. The Kv channels are voltage-activated, opening in response to membrane depolarization, while the K_{Ca} channels are activated by increases in the cytosolic calcium concentration "sensed" by the Ca^{2+} binding protein calmodulin, which is tightly bound to the C-terminus of the IKCa1 protein [19].

The surface of ShK involved in binding to Kv channels has been probed using alanine scanning and selected toxin analogs [20, 21]. Two residues, Lys22 and Tyr23, are crucial for activity, as also found subsequently for BgK toxin [17]. Other residues in ShK also contribute to the K^+ channel-binding surface, with Arg11 being important for activity against Jurkat T-lymphocytes but not for rat brain binding [20], and Ile7, Ser20 and Phe27 contributing to rat brain binding [21]. More recently, functional assays on mKv1.3 stably expressed in L929 cells identified His19, Ser20 and Arg24 as important [22], although the role of His19 may be at least partly structural [14] as its significance was inferred using an H19K mutant rather than H19A because the latter did not fold in reasonable yield [22]. Arg11 was found to be only moderately important for Kv1.3 binding in this study.

It appears that Lys22 and Tyr23 in the anemone toxins represent a conserved diad of residues that is essential for K^+-channel blockade by a range of polypeptide toxins. This motif, which is also found in structurally unrelated scorpion toxins such as ChTX, consists of a lysine and a neighbouring aromatic residue separated by ~ 7 Å [17]. Based on mutational analysis of the snake toxin α-dendrotoxin, Gasparini *et al*. [23] proposed that this motif be more broadly defined as a lysine and a neighbouring hydrophobic residue. The same diad is also important in the κ-conotoxins [24, 25], even though these toxins adopt yet another class of structure, the ICK motif [26].

A recent study on Pi1, a 35-residue scorpion toxin, that acts on both small-conductance Ca^{2+}-activated and Kv channels, however, suggests that the integrity of the functional diad may not be a pre-requisite for the recognition and binding of this toxin to Kv1.2 channels [27]. [A24, A33]-Pi1 and P-Pi1 (a Pi1 analog phosphorylated on Tyr33) are lethal by intracerebroventricular injection in mice and both compete with ^{125}I-apamin for binding to SK channels of rat brain synaptosomes (IC_{50} values of 30 and 10 nM, respectively) and block rat Kv1.2 (IC_{50} values of 22 μM and 75 nM, respectively), whereas they are inactive on Kv1.1 or Kv1.3 channels at μM concentrations. Thus, although both analogs are less active than Pi1 *in vivo* and *in vitro*, toxin residues other than Lys24 and Tyr33 must also play a role in Pi1 action. Simulations of Pi1 docking with Kv1.2 suggest a possible role for other basic residues (Arg5, Arg12, Arg28 and Lys31) in toxin binding.

These polypeptide toxins block K^+ channels by binding to a shallow vestibule at the outer entrance to the ion conduction pathway and occluding the entrance to the pore. Extensive studies a decade ago [28-32] employed the scorpion toxins ChTX, agitoxin and kaliotoxin (KTX) as molecular probes and exploited complementary mutagenesis to identify contact points between the scorpion toxins and Kv channels. Coupling energies between interacting pairs of residues were assessed by the semi-quantitative methods of electrostatic compliance and thermodynamic mutant cycle analysis [29-31, 33] in order to obtain estimates of inter-residue distances. The approximate Kv channel dimensions inferred from these indirect toxin mapping methods proved to be reasonably accurate when compared with the crystal structure of the bacterial K^+ channel KcsA, published in 1998 [34]. Although the KcsA channel is a proton-activated channel [35] with only two transmembrane segments rather than the six found in voltage-gated channels from higher organisms, its structure determination represented a landmark in our understanding of the structure and mechanism of action of K^+ channels, as acknowledged by the 2003 Nobel Prize in Chemistry shared by Rod MacKinnon and Peter Agre. Moreover, the good agreement between the dimensions of the pore and vestibule region inferred from indirect mapping and the crystal structure indicated that the KcsA and Kv channels are architecturally conserved in these regions. Although KcsA does not bind scorpion toxins, substitution of as few as three residues from *Shaker* into this channel was sufficient to generate nM affinity [36]. Legros *et al.* [37] generated even higher affinity KcsA-Kv1.3 chimeras by making more extensive substitutions in the P region.

Similar mutational strategies have been employed to understand the molecular basis for channel blockade by ShK,

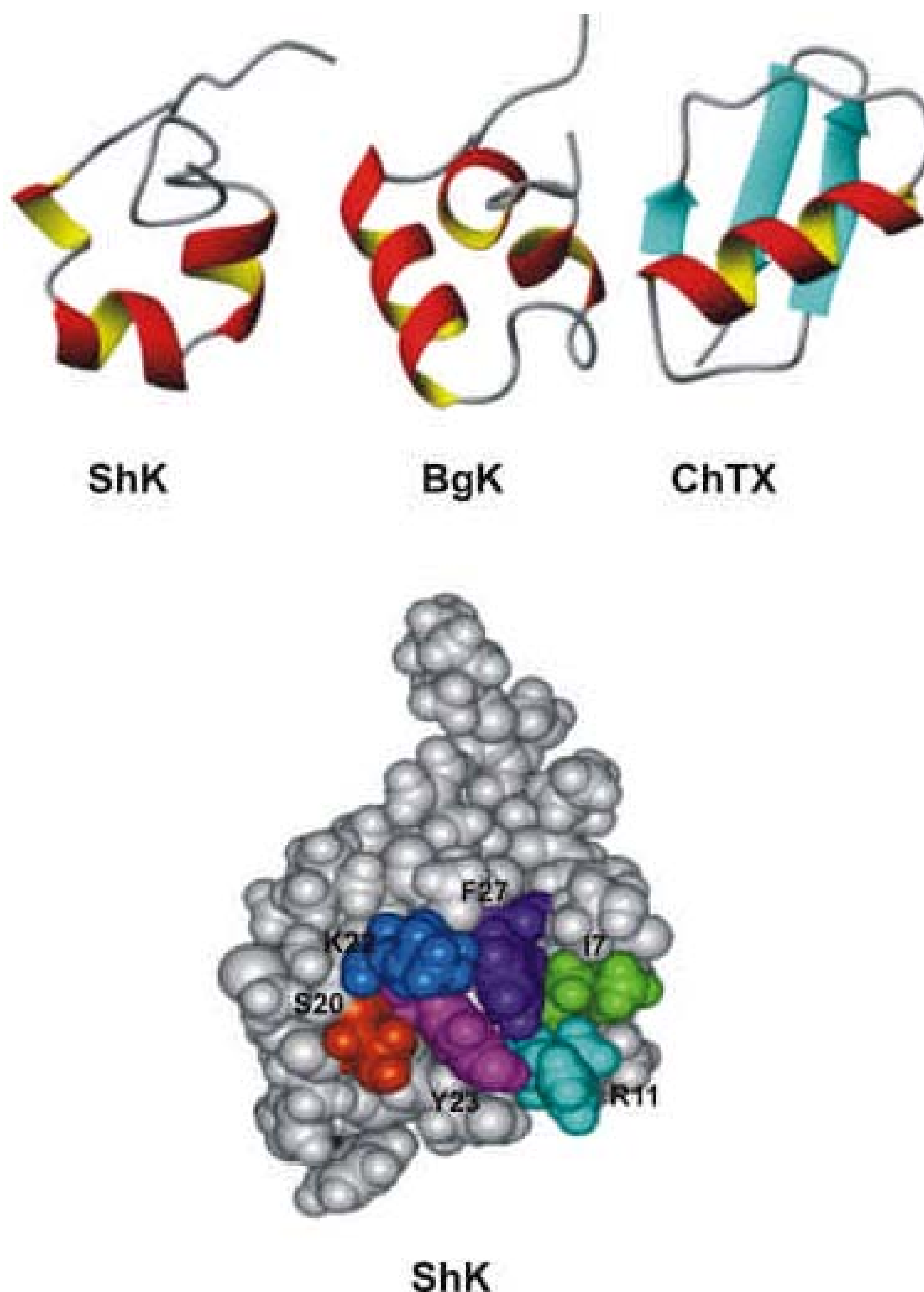

Fig. (2). A. Ribbon diagrams of the closest-to-average NMR-derived structures of ShK [13, 14], BgK [17] and charybdotoxin [15]. B. CPK representation of ShK with residues identified by Pennington . [21] as being important for K^+ channel binding coloured as follows: Ile7 green, Arg11 cyan, Ser20 orange, Lys22 blue, Tyr23 magenta and Phe27 purple.

although the availability of the KcsA crystal structure allowed a much more reliable model of the pore-vestibule region of Kv1.3 to be constructed. ShK was initially docked with a crude model using restrained molecular dynamics simulations guided by data from mutant cycle analyses [38]. In this docked configuration, ShK-Lys22 projected into the ion conduction pathway but its side chain did not make direct contact with the side chains of Tyr400 and Asp402 in Kv1.3. Arg11 of ShK lay in the vicinity of His404 in one Kv1.3 subunit, and two of the remaining His404 residues in the tetramer were in close proximity to ShK residues Met21 and Arg29. Subsequently, the channel model was refined [39, 40] and ShK was docked using a larger number of restraints from complementary mutational analyses, as shown in (Fig. **3**).

Tyr23 is a conserved residue across the family of K^+-channel blocking toxins from sea anemones and, with Lys22, constitutes the conserved diad found in sea anemone and other polypeptide K^+-channel blockers [17]. In the ShK-Kv1.3 model, Tyr23 sits within 5 Å of Gly401, Asp402 and His404 from the same subunit to which Arg11 is coupled.

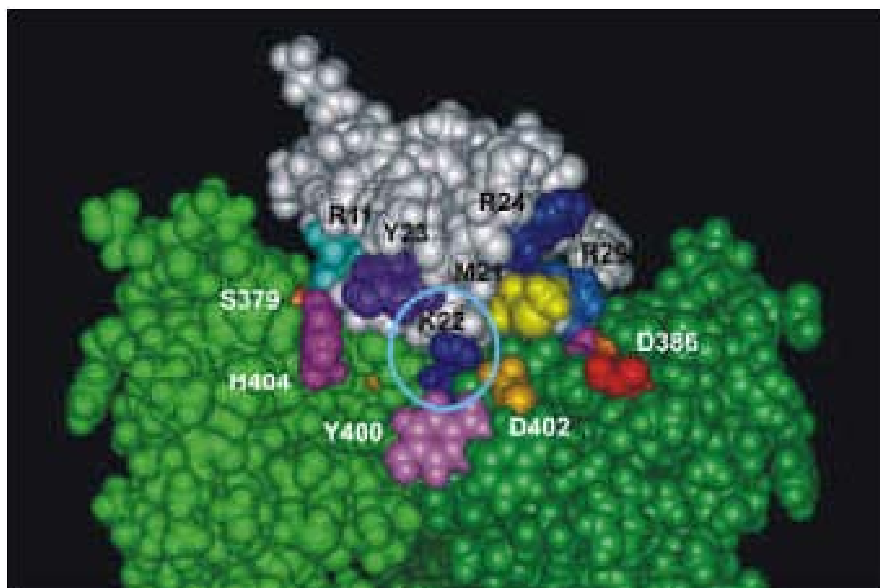

Fig. (3). Side view of ShK docked into the Kv1.3 model [22]. The channel subunit nearest the viewer has been removed. Toxin and channel side chains are coloured as follows: ShK and ShK-Dap22 Arg11 cyan, Lys22 and Arg24 blue, Tyr23 purple, Met21 yellow; Kv1.3 Asp386 red, Asp402 gold, His404 magenta and Ser379 orange. In this view the side chains of Tyr23 and Arg11 of ShK are partially obscured. The channel subunits are coloured different shades of green. This diagram is modified from (Fig. **2**) of Lanigan *et al*. [40].

Indeed, the Kv1.3-binding surface of ShK incorporates most of the residues reported by Pennington *et al*. [21] and Rauer *et al*. [39] to be part of the essential binding surface. Thus, although the crystal structures of both voltage- and calcium-gated K^+ channels have been solved more recently [41-43], we believe the Kv1.3 model based on KcsA provides an adequate basis for docking studies and the design of further experiments in the case of toxins that act by blocking the pore-vestibule region.

MORE SELECTIVE ANALOGS FOR KV1.3

ShK blocks Kv1.3 (K_d 11 pM) and the related Kv1-family channels Kv1.1 (K_d 16 pM), Kv1.4 (K_d 312 pM) and Kv1.6 (K_d 165 pM) with high affinity in electrophysiological experiments [38]. In the course of probing the importance of the chain length at position 22 in ShK, the critical Lys22 was replaced by the shorter, positively charged, non-natural residue 1, 3-diaminopropionic acid (Dap). It was thought that the Dap ammonium group might target the unique ring of His404 residues located at the outer entrance to the Kv1.3 pore [38]. In fact ShK-Dap22 appears to be both a highly potent and highly selective blocker of Kv1.3 [38]. ShK and ShK-Dap22 bind Kv1.3 with very similar potencies, implying that there is no significant energetic penalty associated with the substitution at position 22. In electrophysiological experiments, however, blockade by ShK-Dap22 is as easily reversible as blockade by MgTX or ChTX, while the native ShK is more "sticky" and is extremely hard to wash off. In contrast to ShK, which is similarly potent against Kv1.1 and only ~15-fold less potent against Kv1.6, ShK-Dap22 is 80- and 450-fold less potent against these same channels.

ShK-Dap22 has a similar solution structure to that of the native toxin (Fig. **4**), but with some differences in the relative orientations of key binding side chains [38]. Using a combination of molecular modeling and experimentally derived distance constraints the docking of ShK-Dap22 with Kv1.3 was also investigated [40]. Surprisingly, ShK-Dap22 appears to make different interactions from those of the native toxin [40].

A recent binding study revealed a previously unsuspected affinity for ShK-Dap22 to Kv1.1-Kv1.2 hetermultimeric channels that had not been anticipated from experiments conducted with the homomeric channels. Garcia and co-workers [44] found that heteromultimeric Kv1.1-Kv1.2 channels exhibited a 200-fold higher affinity for ShK-Dap22 than Kv1.1 homomultimers. In addition, ShK-Dap22 was found to be about 10^3-fold less effective in inhibiting human T-cell activation than ShK itself. The authors concluded that ShK-Dap22 would not have the same *in vivo* immunosuppressant efficacy as other Kv1.3 blockers such as MgTX or ShK and was not as selective as previously thought [38].

ShK-F6CA is a fluorescein-labelled analog of ShK, which has potential applications as a diagnostic for Kv1.3^{high} T_{EM} cells [45], as discussed below. ShK-F6CA was 160-fold less effective (K_d 4.0 ± 0.3 nM) than ShK in blocking Kv1.1 channels stably expressed in L929 cells and showed 83-fold greater affinity for Kv1.3 over Kv1.1. The enhanced specificity of ShK-F6CA for Kv1.3 over Kv1.1 might be explained by the fact that F6CA is negatively charged. When ShK is docked into Kv1.3 [40], Arg1 is near Asp375-Asp376-Pro377-Ser378-Ser379 on the channel. If ShK sits in the Kv1.1 vestibule with the same geometry as it does in Kv1.3, the presence of three glutamates in the corresponding sequence in Kv1.1 (Glu350-Glu351-Ala352-Glu353-Ser354) may electrostatically repel the F6CA moiety (even though this corresponds to only one extra acidic side chain in Kv1.1). Negatively charged channel residues in

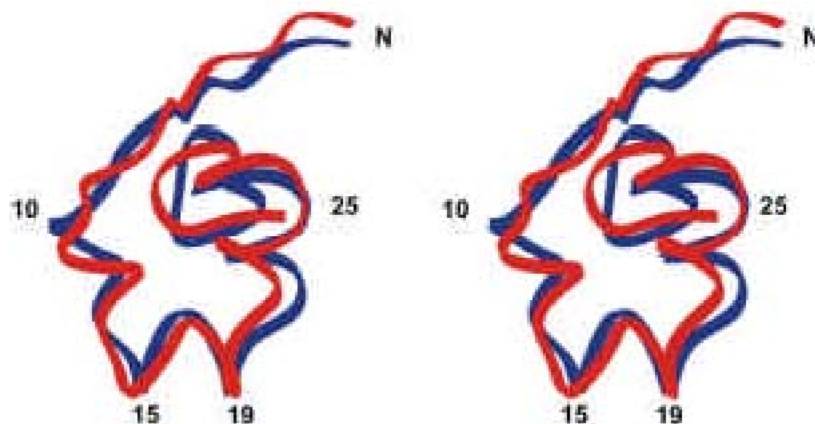

Fig. (4). Comparison of solution structures of ShK and ShK-Dap22. Ribbon diagrams of the closest-to-average structures for ShK-Dap22 (red) and ShK (blue) are superimposed over the backbone heavy atoms of residues 3-33, excluding residue 22. This diagram is modified from Figure **8B** of Kalman *et al*. [38].

neighbouring loops (e.g. S3-S4 and S1-S2) may also contribute to the reduced potency of ShK-F6CA for Kv1.1.

POTASSIUM CHANNELS IN T CELLS

In 1984 DeCoursey *et al.* [46] and Matteson *et al.* [47] discovered a voltage-gated K^+ channel in peripheral blood human T cells that was called *n*-type channel at that time and later identified as Kv1.3 [48, 49]. George Chandy first realized that this channel might constitute a novel potential target for immunosuppression and demonstrated that K^+ channel blockers like 4-aminopyridine, tetraethylammonium chloride and quinine could inhibit PHA-stimulated human T-cell proliferation and prevent protein synthesis and cytokine secretion [46, 50, 51]. However, it soon became apparent that K^+ channel expression in T lymphocytes depended on their activation and differentiation state and also on the species from which they were derived. Mouse T cells were found to have two additional voltage-gated K^+ channels, types *n*' and *l* [52, 53], and expression of the three Kv channels varied during T-cell differentiation in the thymus [54] and also changed following activation [53, 55]. The first hint that altered K^+ channel expression might also be involved in autoimmunity came from a study showing that T cells from *lpr* mice, which develop an autoimmune disease resembling human lupus erythematosus, expressed unusually high levels of the *l*-type channel (Kv3.1 in IUPHAR nomenclature) [56]. When the intermediate-conductance calcium-activated K^+ channel IKCa1 was subsequently discovered in human and murine T and B cells [57-59], and its expression was found to increase with activation, it became increasingly clear that the roles of the different K^+ channels had to be dissected out carefully with specific inhibitors for each channel.

Using the scorpion toxin ChTX, several groups in the early 90s corroborated earlier studies conducted with less selective compounds and demonstrated that K^+ channels indeed played a crucial role in human T-cell activation [60-62]. However, ChTX inhibits both Kv1.3 [60, 63, 64] and IKCa1 [58, 61] at low nanomolar concentrations and does not allow the functional contributions of the two channels to be distinguished. Investigators at Merck first showed with the scorpion toxins MgTX and noxiustoxin (neither of which affects IKCa1), that Kv1.3 blockade alone seemed to be sufficient to inhibit human T-cell proliferation [62], but observed that the effect of Kv1.3 blockers was not as robust as the effect of the calcineurin inhibitor FK506 and could be overcome by CD28 stimulation. They also provided the first evidence that Kv1.3 blockade can suppress immune responses *in vivo* by demonstrating that continuous infusion of MgTX prevented delayed-type hypersensitivity to tuberculin in Yucatan mini-pigs [65]. Contrary to mouse T cells, on which Kv1.3 blockers showed little or no effect [55, 65, 66], MgTX also potently inhibited the proliferation of mini-pig T cells.

Through a combination of electrophysiology and molecular biology, Ghanshani *et al.* [67] later established that mitogenic activation of peripheral blood human T cells leads to a rapid transcriptional up-regulation of IKCa1 through the protein kinase C pathway, and employed a panel of selective Kv1.3 and IKCa1 blockers to probe the functional contributions of Kv1.3 and IKCa1 to human T-cell activation. While the proliferation of resting human T cells was suppressed by the Kv1.3 blockers MgTX, ShK-Dap22 and correolide, and not by the IKCa1 blockers clotrimazole and TRAM-34 [68], the proliferation of T cells that had been pre-activated for 48 hr and were then exposed to K^+ channel blockers was insensitive to Kv1.3 but sensitive to IKCa1 blockade. Together with a subsequent Ca^{2+} imaging study [69], these results suggested that resting T cells relied on Kv1.3, but that IKCa1 was the functionally dominant K^+ channel in activated T cells. From this and other studies [58, 62], it also became clear that, contrary to mouse T cells [52, 55, 65], human T cells expressed only Kv1.3 and IKCa1.

POTASSIUM CHANNEL EXPRESSION CHANGES DURING HUMAN T-CELL DIFFERENTIATION

Both Kv1.3 and IKCa1 indirectly regulate Ca^{2+} entry during T-cell activation by modulating the membrane potential [62, 70-74]. Engagement of the T-cell receptor during an immune response triggers a Ca^{2+} influx through a calcium channel called CRAC (calcium release activated

calcium channel) [74, 75], resulting in the increase in cytosolic Ca^{2+} concentration necessary for translocation of NFAT (nuclear factor of activated T cells) to the nucleus and the initiation of new transcription, ultimately resulting in cytokine secretion and T-cell proliferation [74, 76]. This crucial Ca^{2+} influx is only possible if the T cell can keep its membrane potential negative by a counterbalancing K^+ efflux through Kv1.3 and/or IKCa1 [62, 74, 76-78]. Blockade of these two K^+ channels depolarises the T-cell membrane and inhibits Ca^{2+} signaling, thus preventing T-cell activation [62, 74, 76-78]. However, which of these two K^+ channels, Kv1.3 or IKCa1, is the key player in these events depends on the activation and differentiation state of the particular T cell.

One key characteristic of the mammalian immune system is that it can develop immunological memory. Following repeated encounters with the same antigen, be it a pathogen or an autoantigen in the context of an autoimmune process, both $CD4^+$ and $CD8^+$ T cells differentiate from naïve T cells into increasingly reactive memory T cells (Fig. **5**). In humans this differentiation is accompanied by a change in the expression of the phosphatase CD45RA and the chemokine receptor CCR7 [79, 80]. Naïve T cells, which have not previously encountered antigen, are positive for both markers, migrate to lymphoid organs in the search for antigen, and require a relatively high-strength stimulus in order to be induced to proliferate. Central memory T cells (T_{CM}) have a lower activation threshold than naïve T cells, but still bear the lymph node homing receptor CCR7 and lack immediate effector function (Fig. **5A**). Terminally differentiated $CCR7^-$ effector memory T cells (T_{EM}), in contrast, can home directly to inflamed tissues and rapidly produce and secrete large amounts of effector cytokines in the case of $CD4^+$ cells, or contain perforin granules in the case of $CD8^+$ cells [79].

In parallel with the differentiation from naïve into T_{EM} cells, the expression pattern of the lymphocyte K^+ channels, Kv1.3 and IKCa1, changes drastically (Fig. **5B**), as has been shown recently in a study employing a combination of fluorescent microscopy and patch-clamp [81]. Resting T cells of all three subsets were found to express about 300 Kv1.3 and very few IKCa1 channels. Following activation, naïve and T_{CM} cells up-regulated IKCa1 100-fold to ~500 channels per cell with little or no change in Kv1.3 expression [81], corroborating the earlier study by Ghanshani *et al.* [67] performed on unseparated T cells. T_{EM} cells, in contrast, increased Kv1.3 to ~1500 channels per cell with no increase in IKCa1 expression. These findings suggested that the two channels might play very different roles in naïve and T_{CM} cells versus T_{EM} cells, and that their differential expression pattern could potentially be exploited for both therapeutic and diagnostic purposes. T_{EM} cells were extremely sensitive to Kv1.3 blockade. ShK inhibited their proliferation with an EC_{50} of 100 to 400 pM [81], whereas naïve and T_{CM} cells were initially sensitive to Kv1.3 blockade (ShK: EC_{50} of 4 nM) but quickly up-regulated IKCa1 despite the presence of a Kv1.3 blocker and then became insensitive to Kv1.3 and sensitive to IKCa1 blockade [78, 81, 82]. In the light of these findings it also became apparent why the reported potencies of Kv1.3 blockers had often varied so much in proliferation assays. In a typical person, naïve and T_{CM} cells constitute the majority of the peripheral blood T cells while T_{EM} cells are less frequent. However, their percentages can vary considerably among different individuals (1-30%), and their low activation threshold [79] and high sensitivity to Kv1.3 blockade can render unseparated T cells from blood donors with a lot of T_{EM} cells much more sensitive to Kv1.3 suppression than T cells from donors with few T_{EM} cells.

In contrast to their effect on T cells, Kv1.3 blockers seem to have little effect on the innate immune system, as recently shown by Shah *et al.* [83]. In agreement with previous studies, MgTX inhibited T-cell proliferation, cytokine secretion and $CD8^+$ T-cell mediated cytolysis, but showed

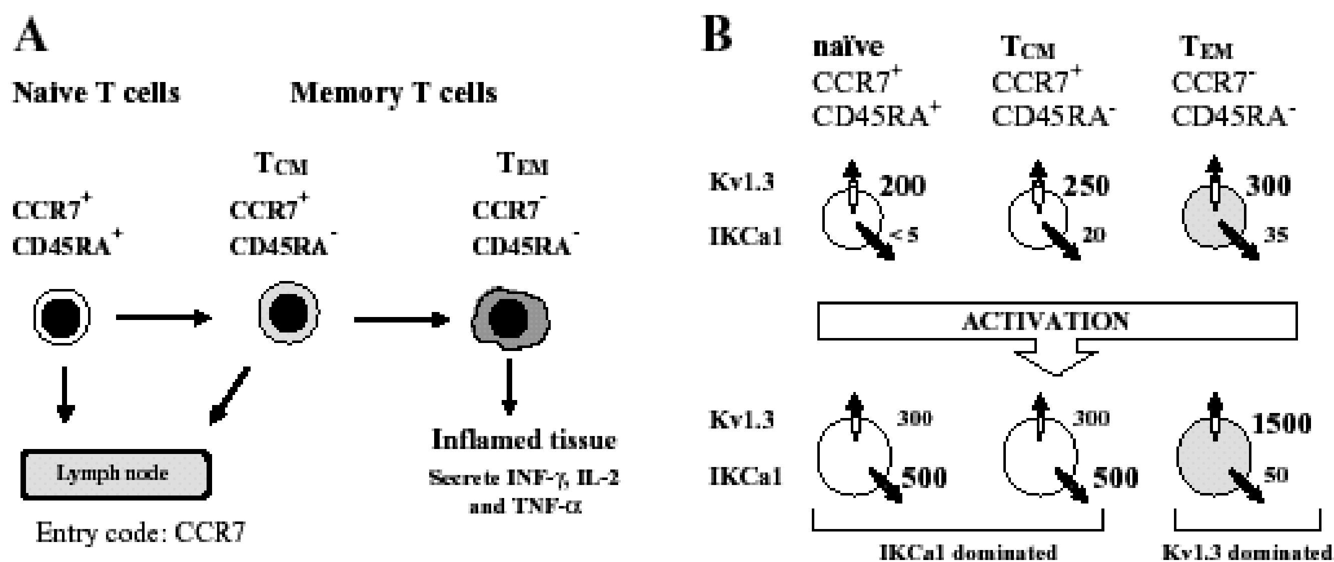

Fig. (5). A. Based on the expression of the chemokine receptor CCR7 and the phosphatase CD45RA, three types of T cells can be distingushed in both the $CD4^+$ and the $CD8^+$ subset: naive, central memory (T_{CM}) and effector memory (T_{EM}) T cells [79]. Expression of CCR7 allows $CCR7^+$ cells to enter lymph nodes. B. Schematic presentation of the average Kv1.3 and IKCa1 channel numbers in naïve, T_{CM} and T_{EM} T cells before and after activation. Data are taken from [81].

no effect on cytolysis of antibody-opsonized target cells by human monocytes. In agreement with these results, the authors found no functional Kv1.3 expression in human monocytes in electrophysiological experiments.

Based on the functional dominance of IKCa1 in naïve and T_{CM} T cells, it seems reasonable to postulate that IKCa1 blockers might be useful for the suppression of acute immune responses, while Kv1.3 blockers might constitute valuable new therapeutics for the treatment of autoimmune diseases mediated by memory T cells, such as multiple sclerosis.

KV1.3 BLOCKERS AND MULTIPLE SCLEROSIS

Multiple sclerosis is a T-cell mediated chronic inflammatory disease of the central nervous system that is characterized by focal areas of demyelination, resulting in disabling neurological deficits [84]. Myelin antigen-specific $CD4^+$ T cells appear to be crucial to the pathogenesis of MS because they can be isolated from the blood and the cerebrospinal fluid of MS patients [85, 86] and because of their ability to induce experimental autoimmune encephalymyelitis (EAE) in rodents and primates [87-89]. Although myelin-antigen specific T cells are also present in the blood of healthy controls, their activation state in MS patients is different [90]. Myelin-reactive T cells from MS patients secrete larger amounts of IL-2, IFN-γ and TNF-α than T cells from controls, and are predominantly of a memory phenotype [91-94]. Pharmacological strategies to selectively suppress the function of myelin-reactive T cells, therefore, have been long regarded as desirable for the treatment of MS.

Recently, Chandy's group showed that the majority of myelin-reactive T cells in the blood of MS patients were $Kv1.3^{high}$ T_{EM} cells [81], whereas myelin-reactive T cells from healthy controls or control antigen-specific T cells from MS patients were naïve or T_{CM} cells. They further demonstrated that the Kv1.3 blocker ShK potently inhibited the proliferation of myelin-reactive MS patient T cells and suggested that selective suppression of autoreactive T_{EM} cells with Kv1.3 blockers might constitute a novel therapy for MS and other memory T-cell mediated autoimmune diseases such as type-1 diabetes mellitus [95-97] and psoriasis [98].

In proof of this concept, the Kv1.3 blocking toxins KTX [99], ShK and ShK-Dap22 [100] have been shown to prevent and treat adoptive transfer EAE in rats, an animal model for MS. In this model, a myelin basic protein specific $CD4^+$ T-cell line is injected into the abdomen of Lewis rats ('adoptive transfer') following *in vitro* activation with myelin basic protein. Within 5-7 days, the disease-inducing T cells invade the brain and cause a MS-like disease characterized by severe paralysis, incontinence, brain edema and demyelination. If large enough numbers of cells are injected the disease can even be lethal. ShK injected subcutaneously at 80 μg/kg completely prevented disease in a trial where all control animals died from EAE, and it significantly ameliorated disease severity in several trials when administered after the first onset of symptoms (Fig. **6**). Administration of ShK did not cause any obvious side effects in these experiments. In an accompanying electrophysiological study of the K^+ channel expression pattern of naïve rat T cells and 20 myelin reactive T-cell lines that are used to induce adoptive transfer EAE, Beeton *et al.* [100] further showed that chronically activated rat memory T cells (corresponding to human T_{EM} cells) expressed the same $Kv1.3^{high}$ phenotype as human T_{EM} cells. Naïve rat T cells resembled naïve human T cells in up-regulating IKCa1. Interestingly, the number of Kv1.3 channels expressed in the myelin-reactive T cells correlated roughly with their encephalitogencity [100, 101]. In keeping with this K^+ channel expression pattern, the Kv1.3 inhibitors KTX, MgTX, ShK and ShK-Dap22 potently suppressed antigen-driven proliferation of chronically activated rat memory T cells, while IKCa1 blockade suppressed the proliferation of naïve rat T cells [100]. Contrary to mouse T cells, rat T cells thus resemble human T cells in terms of their K^+ channel expression pattern, demonstrating that rat models are useful for testing the effectiveness of Kv1.3 blockers while mouse models are unsuitable [55, 65, 82].

Taken together, the facts that myelin-reactive T cells from patients with MS are predominantly $Kv1.3^{high}$ T_{EM} cells and that Kv1.3 blockade effectively treats an animal model of MS strongly suggest that Kv1.3 in autoreactive T_{EM} cells constitutes a promising new target for the therapy of MS and possibly other T-cell mediated autoimmune diseases. This idea seems even more plausible in light of the recent demonstration that Kv1.3 blockade can also effectively prevent memory T-cell mediated periodontal bone resorption in a rat model [102], suggesting that Kv1.3 blockers might also be useful for preventing bone loss in inflammatory bone resorptive disorders such as periodontal disease or rheumatoid arthritis. Compared to other immunosuppressive therapies, selective suppression of T_{EM} cells in autoimmune disease with Kv1.3 blockers should constitute a more selective approach than general immunosuppression (e.g with cyclosporin or FK506), but a less selective approach than antigen-specific vaccination strategies. However, this might be an advantage in diseases like MS where epitope spreading (the liberation of new antigens and the rise of T-cell clones with new specificities during the course of the disease) occurs. Along these lines it is also interesting to note that vaccination strategies, although very effective in animals, have recently failed in humans for both MS and type-1 diabetes [103, 104].

POSSIBLE DIAGNOSTIC APPLICATIONS OF POLYPEPTIDE BLOCKERS

The findings that high Kv1.3 expression is a marker for activated T_{EM}, which are involved in the pathogenesis of autoimmune diseases, has enhanced interest in a faster and more convenient means than patch-clamp for determining Kv1.3 levels in human and animal blood samples and tissues. Optimal for this purpose would be a fluorescent reagent that could be used in flow cytometry and combined with existing antibodies directed against various lymphocyte surface markers. In the absence of a monoclonal antibody directed against an extracellular epitope of the Kv1.3 channel protein, Beeton *et al.* recently used ShK-F6CA [45], a fluorescein-labelled analog of ShK, to detect $Kv1.3^{high}$ T_{EM} cells. Because of its extremely high affinity to Kv1.3, ShK-

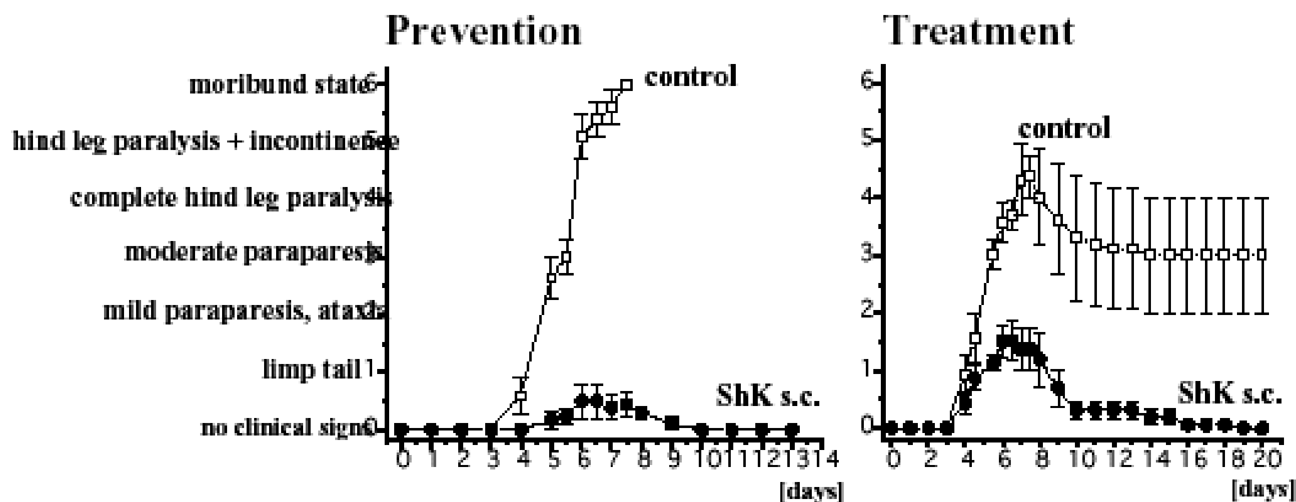

Fig. (6). ShK prevents and treats adoptive-transfer EAE in Lewis rats. *Prevention trial:* Encephalitogenic rat T cells were activated *in vitro* with MBP in the presence or absence of ShK and adoptively transferred on day 0 [100]. Rats were then injected with ShK at 80 µg/kg from day 0 until day 4. *Treatment trial:* EAE was induced by adoptive transfer of encephalitogenic T cells and rats were injected with ShK for 3 days after the onset of clinical signs on day-4. Adapted from [100].

F6CA was used in much the same way as an antibody and allowed reliable and reproducible differentiation of Kv1.3^{high} activated T_{EM} cells (expressing 1500-2000 Kv1.3 channels per cell) from resting T_{EM} cells and from resting and activated naïve and T_{CM} cells (expressing 200-400 Kv1.3 channels per cell). ShK-F6CA or another fluorescently-labelled Kv1.3 blocking toxin like HgTX [105] might therefore be useful as diagnostic agents for screening blood samples, body fluids or tissue samples for the presence of activated T_{EM} cells, and could thus help to determine the contribution of T_{EM} cells to pathological processes or help assess the effectiveness of T-cell suppressing therapies in autoimmune diseases.

PROSPECTS FOR POLYPEPTIDE THERAPEUTICS

Immunosuppressants such as cyclosporin A are used in the prevention of graft rejection following organ transplants, but tissue damage in liver and kidney limits its use [106]. As described above, immunosuppressants also have a role in the management of autoimmune diseases such as MS, rheumatoid arthritis, psoriasis, systemic lupus erythematosus and type 1 diabetes mellitus. There is clearly a need for novel, less toxic, immunosuppressive agents. However, what are the prospects that polypeptides could satisfy this need?

There are of course several proteins, which are major pharmaceuticals, including erythropoietin, interferon, TNF receptor, granulocyte colony stimulating factor and insulin, as well as several therapeutic antibodies [107]. Moreover, the calcium channel blocker ω-conotoxin MVIIA, known as Prialt or Ziconotide, is now awaiting FDA approval for the treatment of chronic pain [108, 109]. These examples, to name just a few [110], demonstrate that problems of administration and bioavailability can be overcome by formulation where the therapeutic indication is compelling and the protein or polypeptide is clearly superior to alternative, non-proteinaceous agents. It is noteworthy here that the protein interferon-β is one of the current treatments for MS [111], and that another, glatiramer acetate (Copaxone), is a peptide heteropolymer made up of the constituent amino acids in myelin polymerized in a random order (Glu, Tyr, Ala, Lys) [112]. Both compounds are most commonly administered by subcutaneous injection.

ShK and ShK-Dap22 have low toxicity in rodents [38] but they lack oral bioavailability and have short half-lives *in vivo* (about 20 min in rats following intraperitoneal or subcutaneous injection [100]). However, their high affinity for target K^+ channels may allow therapeutically useful levels to accumulate at the required sites of action despite the short half-life of circulating peptide. Newer developments such as transdermal administration [113] offer a means of overcoming poor oral bioavailability, although not necessarily short plasma half-life. Subcutaneous depot formulations with biodegradable polymers composed of differing ratios of lactide/glycolide to effect slow continuous release have proven to be extremely effective for delivering peptide therapeutics [114]. More detailed analyses of the absorption, distribution, metabolism and excretion of ShK and its polypeptide analogs are needed to identify the major causes of their poor bioavailability. If, for example, excretion via the kidney is responsible for ShK's short plasma half-life, conjugation to polyethylene glycol could represent one possible approach to prolonging plasma half-life and enhancing stability [115]. Encapsulation is another [116]. However, if proteolytic degradation is a significant problem *in vivo*, then steps could be taken to engineer a more resistant analog, which nonetheless retains potency and specificity. As we have a detailed knowledge of the structures of ShK and ShK-Dap22 and their key residues, we are in a strong position to undertake such a program. The ability of ShK and ShK-Dap22 to cross the blood-brain barrier [117] also needs to be assessed, although this barrier is compromised in MS patients [118].

A significant determinant of how far ShK or one of its analogs will progress towards the clinic will be the ability of

small molecule drugs to match or exceed the K^+ channel subtype specificity of the polypeptides. The final section of this article therefore, summarizes recent developments in the field of small molecule Kv1.3 blockers.

SMALL MOLECULE ANALOGS

As a means of overcoming the limitations of peptides as therapeutics, we have explored low molecular weight analogs, both peptidic and non-peptidic, of ShK. Three peptidic analogs were designed that retained key functional groups from the native toxin and were stabilized in a native-like conformation by the introduction of non-native cross links [119]. In one of these, the native sequence was truncated and then stabilized by the introduction of additional covalent links (a non-native disulfide and two lactam bridges), while in the other two non-native structural scaffolds stabilized by disulfide and/or lactam bridges were modified to include key amino acid residues from the native toxin (Fig. **7**). These analogs were tested *in vitro* for their ability to block Kv1.3 in *Xenopus* oocytes and their solution structures were determined using ^{1}H NMR spectroscopy. The truncated and stabilized analog was inactive, apparently due to a combination of slight deviations from the native structure and alterations to side chains required for binding. One of the peptide scaffolds was also inactive because it failed to adopt the required structure, but the other had a K_d of 92 µM. This active peptide incorporated mimics of the key residues Lys22 and Tyr23, plus an Arg residue that could mimic Arg11 or Arg24 in the native toxin. Modification of this peptide could produce a more potent peptidic analog which would be useful for further *in vitro* and *in vivo* studies of the effect of blocking Kv1.3, and might also be helpful in mapping the interactions with the pore and vestibule of this K^+ channel that are required for potent blockade. The modifications introduced to stabilize the structural scaffold, in our case lactam bridges, may also enhance bioavailability by making the peptide more resistant to proteolysis.

Towards the goal of therapeutically useful analogs of ShK, we have also synthesized a number of type-III peptidomemtics [120] of ShK that are smaller and more "drug-like". Baell and co-workers [121] developed a R17-K22-Y23 mimetic as a first generation Kv1.3 blocker. Although a relatively weak binder (K_d around 95 µM), this compound is highly optimisable, with synthetic junction points suited to combinatorial development, and with flexible side chains highly suited to conformational constraint. This mimetic also possesses fused 5-6 heterocyclic ring systems, which appear to constitute versatile, drug-like, synthetically-friendly scaffolds for mimicry of both continuous and discontinuous binding epitopes.

The recently reported substituted tetraphenylporphyrin derivatives represent a different kind of peptide mimetic [122]. These compounds mimic toxins by making use of a porphyrin ring as a scaffold to position charged groups at the optimal distance to form salt bridges with Asp residues in each Kv channel subunit. They bind to Kv1.x channels such as *Shaker* and Kv1.3 with nM affinities, and partially block the conductance of the channels in a reversible fashion. Labelling these compounds with fluorescent dyes might also provide useful tools for visualizing Kv1.3 channels in different tissue samples.

Non-peptidic Kv1.3 blockers identified prior to 2002 have been reviewed recently [78, 82] and will not be discussed in detail here. Most of these compounds lack selectivity for Kv1.3 and block the other Kv1-family channels, Kv1.1, Kv1.2 and Kv1.5, with nearly equal

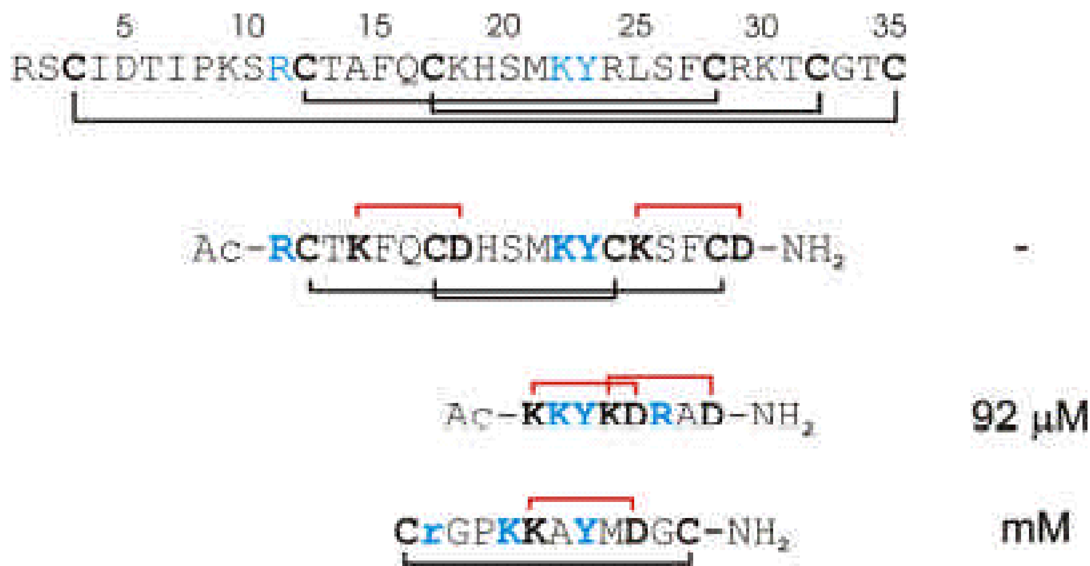

Fig. (7). Primary structures of peptide analogs of ShK [119]. Disulfide bonds and lactam bridges are represented by bold lines below and above the sequences, respectively, with the residues linked in each case shown in bold font. Residues designed to mimic Arg11, Lys22 and Tyr23 are in blue; the lower case for Arg in the last peptide shown denotes D-Arg. This diagram is modified from Figure 1 of Lanigan *et al*. [119]. K_d values measured on Kv1.3 stably expressed in *Xenopus* oocytes are indicated; in these assays native ShK toxin had a K_d of 4.5 ± 0.9 pM.

affinity. However, the di-substituted cyclohexyl derivatives of PAC (4-phenyl-4-(3-(2-methoxyphenyl)-3-oxo-2-azaprop-1-yl)cyclohexanone [123] and the chalcone derivatives of the benzofuran khellinone [124] constitute promising new leads that could be improved upon. With the 5-phenylalkoxypsoralen Psora-4, Vennekamp et *al.* [125] recently identified the first small molecule that inhibits Kv1.3 with single nanomolar potency (K_d 3 nM). Similar to ShK, Psora-4 potently suppresses the proliferation of human and rat T_{EM} cells. All three classes of compounds are distinguished from previously described peptidic and small molecule Kv1.3 blockers by their Hill coefficients of 2, indicating that two drug molecules bind per Kv1.3 channel tetramer. The chalcones are especially promising, despite their relatively low potency (~400 nM), because they are the first class of small molecules that shows selectivity for Kv1.3 over Kv1.2 and over the cardiac K^+ channel Kv1.5. However, none of the small molecules, with the exception of Psora-4, has yet reached single or sub-nanomolar affinity and they do not yet constitute real alternatives to ShK and its analogs as pharmacological tools or potential drugs.

CONCLUSIONS

The voltage-gated K^+ channel Kv1.3 has emerged as a promising new target for the treatment of T cell mediated autoimmune diseases. The availability of the sea anemone toxin ShK, which blocks this channel with picomolar affinity, has been instrumental in this development. ShK thus constitutes an attractive drug candidate. Although native ShK blocks several types of K^+ channel, analogs such as ShK-Dap22 and ShK-F6CA are more potent and selective blockers than any low molecular weight analogs characterized to date. While it remains an important goal to develop low molecular weight, more drug-like, blockers of Kv1.3, the exquisite potency of ShK and high selectivity of some of its polypeptide analogs continue to drive efforts to improve the bioavailability and stability *in vivo* of these polypeptides. This work will benefit from the experience gained in bringing other polypeptides and proteins into the clinic, and from the increasing industry acceptance of biopharmaceuticals as drugs. Ongoing studies into the efficacy of ShK in animal models relevant to human MS will provide a clearer picture of the clinical potential of this polypeptide, but the current prospects are very encouraging.

ACKNOWLEDGEMENTS

We thank numerous colleagues, past and present, who have contributed to our knowledge of ShK and its therapeutic potential.

ABBREVIATIONS

ChTX = Charybdotoxin

EAE = Experimental autoimmune encephalomyelitis

IKCa1 = Intermediate-conductance calcium-activated K^+ channel

K_{Ca} = Ca^{2+}-activated K^+ channel

Kv = Voltage-gated K^+ channel

KTX = Kaliotoxin

MgTX = Margatoxin

MS = Multiple sclerosis

ShK = *Stichodactyla helianthus* K^+ channel toxin

T_{CM} = Central memory T cell

T_{EM} = Effector memory T cell

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