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Mini-review

Potassium channels in T lymphocytes: toxins to therapeutic immunosuppressants

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Patch clamp studies in the early eighties revealed the existence of a voltage-gated K^+ (K_v) channel in human lymphocytes that resembled delayed rectifier K^+ channels in electrically excitable cells (DeCoursey et al., 1984; Matteson and Deutsch, 1984; Cahalan et al., 1985; Schlichter et al., 1986). Classical neuronal K^+ channel blockers (4-aminopyridine, tetraethylammonium and quinine) and classical calcium channel blockers (verapamil, diltiazem and nifedipine) blocked this channel at micromolar to millimolar concentrations (Chandy et al., 1984; DeCoursey et al., 1984; Sabath et al., 1986). Despite having widely differing structures, these compounds inhibited cytokine production and T-cell proliferation with a potency parallel to channel block. The fact that these agents were not cytotoxic, acted reversibly, inhibited only if administered within the first 24 h, and did not suppress expression of IL-2 receptors or proliferation by exogenous IL-2, led us to postulate that K^+ channels are required for an early phase of T-cell activation (Chandy et al., 1984). These results suggested that clinically useful immunosuppressants might be developed by selectively targeting lymphocyte K^+ channels, an idea made highly attractive by the excellent track record of channel blockers in the therapeutic management of stroke, epilepsy and cardiac arrhythmias.

The cloning of the gene encoding the lymphocyte K_v channel (Grissmer et al., 1990), and the subsequent isolation of an extended super-family of nearly eighty K^+ channels, each with a unique 'fingerprint' of biophysical and pharmacological properties, paved the way for the development of channel-specific inhibitors. The functional K_v channel in T cells is composed of four identical subunits encoded by the *Kv 1.3*gene, each with six transmembrane segments (S1–S6) and intracellular N- and C-termini. K_v channels open in response to changes in membrane potential across the plasma membrane. The S4 segment functions as the voltage sensor, and the P-loop (located between S5 and S6) and the S6 segment form the channel pore. An intermediate-conductance calcium-activated K^+ channel (IK_{Ca}) is also expressed in human T lymphocytes (Leonard et al., 1992; Grissmer et al., 1993), and is a homotetramer of IKCa1 subunits (Logsdon et al., 1997). Each IKCa1 subunit is organized in a fashion analogous to that of Kv1.3, and is tightly bound via its C-terminus to calmodulin (Fanger et al., 1999). When cytoplasmic Ca^{2+} rises the IKCa1 channel is activated via a mechanism involving Ca^{2+} binding to the EF hands of calmodulin.

1. Toxins that block lymphocyte channels

Many potent polypeptide inhibitors of the lymphocyte K^+ channels have been discovered in scorpion venom (Price et al., 1989; Sands et al., 1989; Deutsch et al., 1991; Lin et al., 1993; Garcia et al., 1994; Grissmer et al., 1994; Kharrat et al., 1996; Lebrun et al., 1997; Koschak et al., 1998; Peter et al., 2000). Charybdotoxin (ChTx, from *Leiurus quinquestriatus*) was the first polypeptide shown to block any K_V channel, inhibiting Kv1.3 with nanomolar affinity. Other scorpion toxins include noxiustoxin, kaliotoxin, margatoxin, agitoxin-2, hongotoxin, HsTx1, maurotoxin, Pi1, Pi2 and

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Table 1
Pharmacology of Kv1.3 and IKCa1. Numbers in brackets correspond to structural formulae in Fig. 2. Values for AgTx2, Pi1, Pi2, Pi3, MTx, HsTx1 and sulfamibenzamidoindane are from published data and the remaining values are based on our published and unpublished results. These values might vary from those obtained from radiolabeled drug-binding or drug-displacement studies for several reasons. First, binding and displacement studies are typically conducted under lower ionic strength conditions, which could affect measured K_d values. Second, displacement studies assume that the radiolabeled compound and its competitors bind with identical configurations. If two competitors interact with the channel with identical intrinsic affinities but different geometries, they may exhibit different abilities to displace the labeled compound resulting in differing K_ds. Third, patch clamp studies allow the measurement of the strength of binding of the inhibitor in the channel's closed, open or inactivated conformations ('state-dependence'), whereas in binding and displacement studies the conformational state of the channel is indeterminate. We therefore feel that patch clamp measurements are likely to yield more consistent and physiologically relevant K_d values

Kv1.3		IKCa1	
Scorpion		*Scorpion*	
ChTx	3 nM	ChTx	5 nM
NTx	1 nM	ChTx-Glu[32]	33 nM
MgTx	110 pM	MTx	1 nM
KTx	650 pM		
AgTx2	200 pM		
Pi1	11 nM		
MTx	150 nM		
HsTx1	12 pM		
Pi2	50 pM		
Pi3	500 pM		
Sea anemone		*Sea anemone*	
ShK	16 pM	ShK	30 nM
BgK	39 nM	BgK	172 nM
ShK-Dap[22]	52 pM	ShK-Dap[22]	2.6 μM
Small molecule		*Small molecule*	
TEA	10 mM	TEA	24 mM
4-AP	190 μM	ketoconazole	30 μM
quinine	14 μM	econazole	12 μM
diltiazem	60 μM	nifedipine	4 μM
verapamil	6 μM	nimodipine	1 μM
CP-339,818 (1)	150 nM	nitrendipine	900 nM
UK-78282 (2)	200 nM	TRAM-3	520 nM
Correolide (3)	90 nM	clotrimazole (6)	70 nM
sulfamidbenzamidoindane (4)	100 nM	TRAM-34 (7)	20 nM

Pi3. Polypeptide inhibitors of the lymphocyte channels were subsequently discovered in extracts from the sea anemones *Stichodactyla helianthus* (ShK) (Pennington et al., 1996; Kalman et al., 1998) and *Bunodosoma granulifera* (BgK) (Cotton et al., 1997). Table 1 summarizes K_d values of these polypeptides and small molecule antagonists for Kv1.3 and IKCa1 channels based on patch clamp studies done under physiological conditions.

ChTx, kaliotoxin, margatoxin, noxiustoxin and agitoxin-2 share a conserved three-dimensional structure stabilized by three disulfide bonds (Fig. 1(A)), with the exceptions of HsTx1, maurotoxin and Pi1 which contain four (Bontems et al., 1991; Johnson et al., 1994; Aiyar et al., 1995; Dauplais et al., 1995; Krezel et al., 1995; Blanc et al., 1997; Delepierre et al., 1997; Savarin et al., 1999). The structures consist of a three-stranded anti-parallel β-sheet with an α-helix lying across strands 2 and 3. In kaliotoxin this orientation is constrained by two disulfide bonds between Cys^{14} and Cys^{18} in the helix and Cys^{33} and Cys^{35}, respectively, in strand 3 of the β-sheet. The loop connecting the α-helix to strand 2 of the β-sheet is extended by two residues in margatoxin and noxiustoxin compared to kaliotoxin and agitoxin-2, and the orientation of this loop is also different in ChTx. These differences are apparent at the left of Fig. 1(A) (indicated by the arrow), and might account for differences in channel-blocking specificity. The channel-binding surface of kaliotoxin as defined by mutagenesis studies lies below the horizontal plane linking residues Leu^{15} and Arg^{31} (Fig. 1(C) and (E)).

The sea anemone toxins, ShK and BgK, are also stabilized by three disulfide bonds but their structures are substantially different from those of scorpion toxins and somewhat different from each other (Tudor et al., 1996; Dauplais et al., 1997). ShK (Fig. 1(B), left) consists of two short α-helices encompassing residues 14–19 (at the top of Fig. 1(B)) and 21–24 (at the bottom), and an N-terminus with an extended conformation up to residue 8 followed by a pair of interlocking turns that

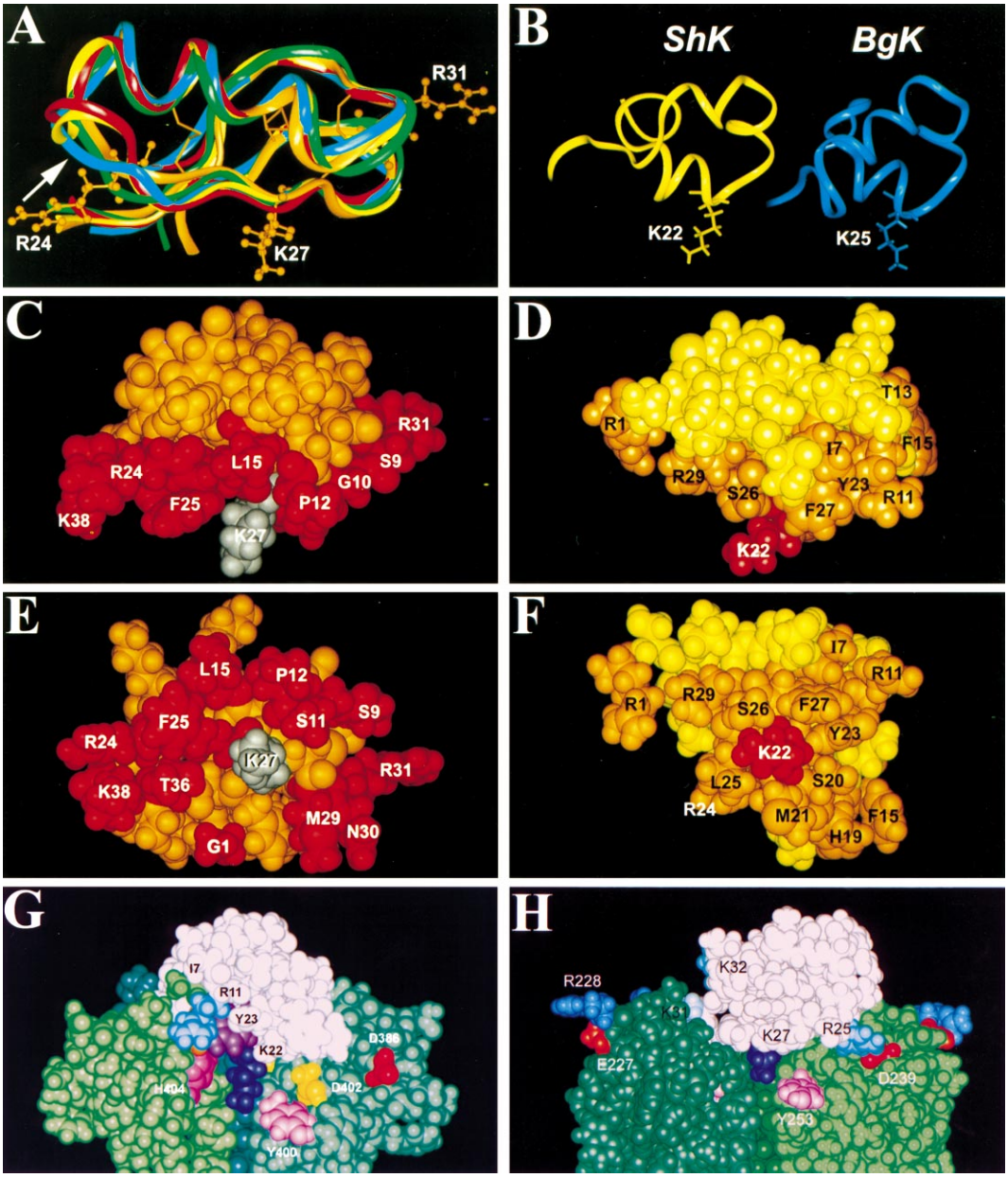

Fig. 1. Toxin structures and docking. A. Comparison of the backbone structures of five scorpion toxins. ChTx (blue), NTx (yellow), MgTx (red), KTx (orange) and AgTx2 (green). B. Backbone structures of BgK and ShK. ShK Lys[22] and BgK Lys[25] are shown. C. Side view of the channel-binding surface (red) and the opposite surface (gold) of KTx. Lys[27] grey. D. Side view of the channel-binding surface ShK (orange) and the opposite surface (yellow). E. Channel-binding surface of KTx Lys[27] (grey). F. Channel-binding surface of ShK. Lys[22] red. G. Docking configuration of ShK (white) in the external vestibule of Kv1.3 (green). The subunit nearest the viewer has been removed for clarity, and the remaining subunits are in different shades of green. ShK Lys[22] (dark blue) protrudes into the Kv1.3 pore. Other side chains are colored as follows: ShK: Ile[7] (light green), Arg[11] (cyan), Ser[20] (orange); Tyr[23] (purple), Kv1.3: Asp[386] (yellow); Asp[402] (red); and His[404] (magenta). We thank Mark Lanigan for help in generating this image. H. Docking configuration of ChTx (white) in IKCa1 (green). The channel subunit nearest the viewer has been removed. Side chains are colored: ChTx: Arg[25] and Lys[32] (cyan); Lys[27] (dark blue); Lys[31] (sky blue); IKCa1: Arg[228] (blue); Glu[227] (orange); Tyr[253] (purple); Asp[239] (red). (Image from Rauer et al., 2000, used with permission).

resembles a 3_{10}-helix. BgK (Fig. 1(B), right) also contains two stretches of α-helix differing slightly in length and position from those of ShK toxin (the equivalent of ShK toxin's first helix [14–19] being longer [9–16] in BgK), but their overall

topologies are quite similar. The channel-binding surface of ShK, based on mutagenesis studies (Pennington et al., 1996; Rauer et al., 1999), lies below the horizontal plane joined by Arg^{1} and Phe^{15} (Fig. 1(D) and (F)). Like the scorpion toxins, ShK contains a central lysine (Lys^{22}), and analogous to Arg^{24} in kaliotoxin, ShK contains a second charged residue (Arg^{11}) necessary for the toxin's interaction with the channel (Kalman et al., 1998; Rauer et al., 1999). Arg^{24} in ShK may also be important for channel binding (Rauer et al., 1999). Thus, the channel-binding surfaces of the scorpion and sea anemone polypeptides, despite their structural dissimilarity, have roughly the same dimensions (25 Å wide and 8 Å deep) and utilize similar residues to interact with the channel.

2. Toxins: molecular probes of channel architecture

In the mid-1990s, our group and those of Chris Miller and Rod MacKinnon used polypeptide toxins as molecular probes and exploited complementary mutagenesis to identify several contact points between scorpion toxins and K_v channels. Coupling energies between interacting pairs of residues were assessed by the semi-quantitative methods of electrostatic compliance and thermodynamic mutant cycle analysis (Stocker and Miller, 1994; Hidalgo and MacKinnon, 1995; Schreiber and Fersht, 1995). Working primarily with kaliotoxin and Kv1.3, we showed that Lys^{27} protruded into the channel pore and lay near the ionic filter (Aiyar et al., 1995,1996). Furthermore, Asp^{386} in Kv1.3 formed a strong electrostatic interaction with Arg^{24} in kaliotoxin. Knowing the distance between Lys^{27} and Arg^{24} from the structure of kaliotoxin, and assuming that the channel has four-fold symmetry, we deduced the distance between the central axis of the channel pore and Asp^{386} to be roughly 14 Å. This placed Asp^{386} residues of opposing subunits ~28 Å apart. By identifying other toxin-channel contact points and knowing the spatial relationships of these toxin residues, we inferred that scorpion toxins bound to a 30 Å wide by 6–8 Å deep vestibule at the external entrance to the Kv1.3 pore (Aiyar et al., 1995, 1996). The Miller and MacKinnon groups independently arrived at similar estimates for the fly Shaker channel (Goldstein et al., 1994; Hidalgo and MacKinnon, 1995; Naini and Miller, 1996; Naranjo and Miller, 1996; Ranganathan et al., 1996). These predicted dimensions for K_v channels proved to be remarkably accurate when compared with the crystal structure of the phylogenetically related bacterial K^+ channel KcsA published 3 years later (Doyle et al., 1998; MacKinnon et al., 1998). Although the KcsA channel has only two transmembrane helices rather than the six found in voltage-gated channels, its structure determination represented a landmark in our understanding of the structure and mechanism of action of K^+ channels. Moreover, the good agreement between the dimensions of the pore and vestibule region inferred from indirect mapping and the crystal structure showed that the KcsA and K_v channels are architecturally conserved in these regions. Although the KcsA channel does not bind scorpion toxins, two groups independently engineered toxin-binding receptors into this channel by substituting residues from *Shaker* or Kv1.3, further demonstrating the conservation in the structures of the K_v and KcsA channels (MacKinnon et al., 1998; Legros et al., 2000).

In early studies, we generated a molecular model of the Kv1.3 channel (Aiyar et al., 1995) based on the estimated dimensions of the vestibule and on a molecular model of the Shaker channel (Durell and Guy, 1992). Kaliotoxin was manually docked in this model by guiding Lys^{27} into the pore such that it would lie near the selectivity filter, and the toxin was then rotated about the central pore axis until Arg^{24} in the toxin aligned with Asp^{386} in Kv1.3 (Aiyar et al., 1995). A refined model of the Kv1.3 vestibule was subsequently developed based on the KcsA structure, and ShK toxin was docked in this model using restrained molecular dynamics simulations (Kalman et al., 1998). In this docked configuration (Fig. 1(G)), ShK-Lys^{22} projected into the pore, ShK-Arg^{11} lay in the vicinity of His^{404} in one Kv1.3 subunit, and two of the remaining His^{404} residues in the tetramer were in close proximity to ShK residues Met^{21} and Arg^{29}. In later mapping studies using ChTx and ShK, we demonstrated that the external vestibule and pore of the IKCa1 channel had an architecture similar to that of K_v and KcsA channels (Rauer et al., 1999). A molecular model of the IKCa1 vestibule was created and ChTx was docked into the channel vestibule (Fig. 1(H)).

3. Structure-based design of selective polypeptide inhibitors

Studies using high-throughput toxin displacement, ^{86}Rb-efflux screening or membrane potential assays led to the identification of a new generation of nanomolar non-polypeptide blockers of Kv1.3, including the dihydroquinoline CP339818 (Nguyen et al., 1996), the piperidine UK78282 (Hanson et al., 1999), the nor-triterpenoid correolide (Felix et al., 1999), phenyloxoazapropylcycloalkanes (Baker et al., 2000) and the sulfimidebenzamidoindanes (Castle et al., 2000) (Fig. 2). More recent design efforts led to the development of a highly selective, triarylmethane nanomolar inhibitor (TRAM-34) of IKCa1 (Wulff et al., 2000) (Fig. 2).

Molecular models of Kv1.3 and IKCa1 and the availability of a crystal structure for KcsA facilitated the design of highly selective polypeptide inhibitors of these channels. Starting with ShK, which blocked Kv1.3, Kv1.1, Kv1.4 and Kv1.6 with low picomolar affinity, we exploited minor differences in the pore sequences and engineered an analog (ShK-Dap^{22}) in which the critical Lys^{22} was replaced by the shorter, positively charged, non-natural residue diaminopropionic acid (Kalman et al., 1998). ShK-Dap^{22} blocks Kv1.3 with high specificity and picomolar potency. Using similar approaches we generated a ChTx analog

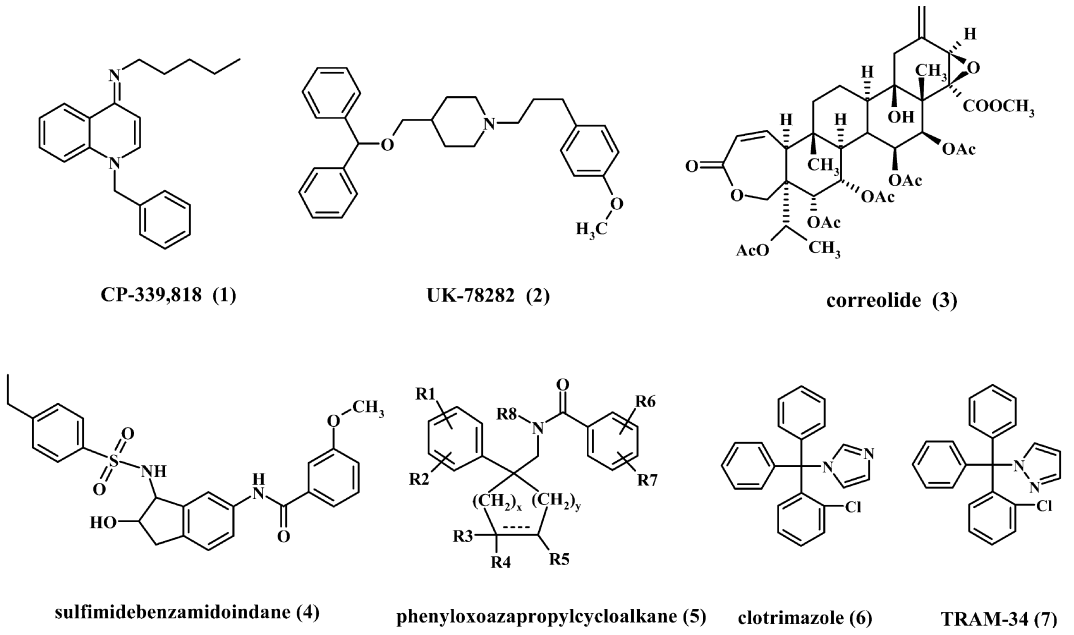

Fig. 2. Structural formulae of small molecule inhibitors of Kv1.3 and IKCa1. (1) CP-339,818: 1-benzyl-4-pentylimino-1,4-dihydroquinoline; (2) UK-78282: 4-(diphenylmethoxymethyl)-1-[3-(4-methoxyphenyl)-propyl]-piperidine; (3) correolide; (4) sulfimidebenzamidoindane: 1-(p-ethylphenyl)sulfimide-2-hydroxy-6-(m-methoxy)benzamido-indane; (5) phenyloxoazapropylcycloalkanes; (6) clotrimazole; (7) TRAM-34: 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole.

(ChTX-Glu^{32}) that inhibits IKCa1 with 30-fold greater potency than Kv1.3 (Rauer et al., 2000). Our design strategy in this case was based on the finding that Lys^{32} in ChTX interacted with a cluster of negatively charged residues in the outer vestibule of Kv1.3 that was not present in IKCa1. By introducing glutamate at position 32 in the toxin, we were able to reduce the affinity of the polypeptide for Kv1.3 via electrostatic repulsion but retain affinity for IKCa1. A related approach we are pursuing is to design peptidomimetics that mimic key residues from these toxins.

4. Defining the function of T-cell K^+ channels using pharmacological probes

Resting human T cells express about ~300–400 Kv1.3 channels and only 8–10 IKCa1 channels, and mitogen activation results in a dramatic transcriptional up-regulation of IKCa1 channel expression (Ghanshani et al., 2000). From the use of pharmacological agents in functional assays a better understanding has emerged of the roles of Kv1.3 and IKCa1 in resting T cells and following their activation. K^+ channels indirectly regulate the Ca^{2+} signal by modulating the membrane potential (Cahalan and Chandy, 1997), but Kv1.3 channels, due to their greater abundance, are more important than IKCa1 channels in regulating the membrane potential of resting T cells. Highly selective blockade of Kv1.3 channels (but not IKCa1) in resting T cells suppresses cytokine production and cell proliferation (Ghanshani et al., 2000). In contrast, IKCa1 but not Kv1.3 blockade suppresses mitogen-stimulated proliferation of pre-activated cells that express roughly equal numbers of Kv1.3 and IKCa1 channels (Ghanshani et al., 2000). Thus, Kv1.3 channels are essential for initiating the human immune response, and IKCa1 channels are required to sustain the activation process.

5. Toxins to novel immunosuppressants

Immunosuppressants are used in the prevention of graft rejection following organ transplants, and in the management of autoimmune diseases. Immunosuppression via highly selective blockade of the T-cell K^+ channels is attractive due to the functionally restricted tissue distribution of Kv1.3 and IKCa1, their important role in T-cell activation, and the availability of specific and potent inhibitors of these channels. Of the small molecule antagonists of Kv1.3, CP-339818 is highly toxic due to blockade of neuronal sodium channels (Wanner et al., 1999), UK-78282 blocks the Kv1.4 channel in the heart and brain (Hanson et al., 1999), and phenyloxoazapropylcycloalkanes and sulfimidebenzamidoindanes block the cardiac Kv1.5 channel (Baker et al., 2000; Castle et al., 2000). Correolide blocks Kv1.2, Kv1.4 and Kv1.5 in addition to Kv1.3 (Felix et al., 1999), has poor oral bioavailability (Koo et al., 1999) and elicits twitches in guinea-pig ileum by stimulating the enteric nervous

system and enhancing neurotransmitter release (Vianna-Jorge et al., 2000). Newer generation correolide analogs are orally bioavailable, and in vivo blockade of Kv1.3 channels by one of these suppressed the delayed-type immune hypersensitivity reaction in mini-pigs (Koo et al., 1999). Therapeutic efficacy of these analogs in animal models for autoimmune disease remains to be shown.

The exquisite selectivity and potency of the polypeptide inhibitors, improvements in their large-scale synthesis, and newer peptide delivery technologies now permit these polypeptides to be considered as viable candidate therapeutic immunosuppressants. In vivo Kv1.3 blockade by margatoxin suppresses delayed-type immune hypersensitivity in mini-pigs (Koo et al., 1997). However, blockade of Kv1.1 channels in the enteric nervous system by this polypeptide causes ileal twitching (Suarez-Kurtz et al., 1999), raising the specter of gastrointestinal side effects. More recently, kaliotoxin has been shown to reduce the clinical severity of experimental adoptive autoimmune encephalomyelitis, a model for human multiple sclerosis (Beeton et al., 2001). Since this toxin blocks both Kv1.3 and Kv1.1 channels, it is not known whether the therapeutic effect is due to immunosuppression (Kv1.3 blockade) or enhanced neurotransmission (Kv1.1 blockade). Future studies with highly selective inhibitors of Kv1.3 and IKCa1 channels, alone or in combination, may test more definitively whether in vivo blockade of the T-cell channels will help in the management of autoimmune diseases and graft rejection.

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