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Biophysical and pharmacological aspects of K^+ channels in T lymphocytes

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Abstract Voltage-gated Kv1.3 and Ca^{2+}-activated IKCa1 K^+ channels play a pivotal role in antigen-dependent activation and proliferation of lymphocytes. These channels primarily determine the membrane potential of T cells, and thus regulate the magnitude of the Ca^{2+} signal required for efficient gene transcription and subsequent proliferation. Although these facts are generally well described and acknowledged, some recent discoveries have motivated research in this field, which is reviewed herein along with the basic biophysical characterization of Kv1.3 and IKCa1. The discovery of T cell subset-specific expression of Kv1.3 points towards the potential therapeutic use of high affinity and high specificity Kv1.3 inhibitors as specific immunosuppressors in the management of autoimmune diseases, such as Multiple Sclerosis. In meeting the demands for an ideal immunosuppressor, several laboratories have discovered potent natural Kv1.3-specific inhibitors and engineered peptides which have a better pharmacological profile based on the biophysical characterization of the interaction surface between the channel pore and the toxins. In contrast to the generally accepted permissive role of Kv1.3 during lymphocyte activation, the discovery of the localization of Kv1.3 in the immunological synapse might open new opportunities in the regulation of T cell activation by this channel species.

Abbreviations $[Ca^{2+}]_i$: cytosolic free Ca^{2+} concentration; CaM: calmodulin; ChTx: charybdotoxin; CLSM: confocal laser scanning microscopy; CRAC: calcium-release activated Ca^{2+} channel; CTLs: cytotoxic T cells; FRET: fluorescence resonance energy transfer; IP_3: 1,4,5-inositol trisphosphate; IS: immunological synapse; KcsA: prokaryotic K^+ channel from *Streptomyces lividans*;; Kv1.3/FLAG: FLAG epitope-tagged Kv1.3; KvAP: prokaryotic voltage-dependent K^+ channel from *Aeropyrum pernix*; PKC: protein kinase C; ShK: *Stichodactyla helianthus* peptide; TCR/CD3: T cell receptor/CD3 complex; T_{CM}: central memory T cell; T_{EM}: effector memory T cell

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Overview of lymphocyte activation, role of ion channels

The generation of an efficient immune response requires the clonal expansion of lymphocytes recognizing a given antigen specifically. Antigens are presented to the T cells by professional antigen presenting cells; both antigen presentation and recognition are mediated by membrane proteins. Major histocompatibility complex proteins (MHC) of antigen presenting cells loaded with processed antigens are sampled by the T cell receptor/CD3 complex (TCR/CD3) of T cells. Specific interaction between the presented antigen and the TCR/CD3 complex activates a variety of transmembrane signaling pathways involving non-receptor tyrosine kinases. The initial events depend primarily on the activation of two Src-family kinases Lck and Fyn. Phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMS) allows further kinases to be recruited and activated by different phosphorylation cascades. Some of the protein kinase pathways, such as the Ras/MAP pathway, contribute directly to the regulation of gene transcription required for the proliferation of T cells. The other consequence of the specifically increased tyrosine kinase activity is the activation of phospholipase C-γ (PLCγ) (Fig. 1). This enzyme cleaves a membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, to yield diacylglycerol and 1,4,5-

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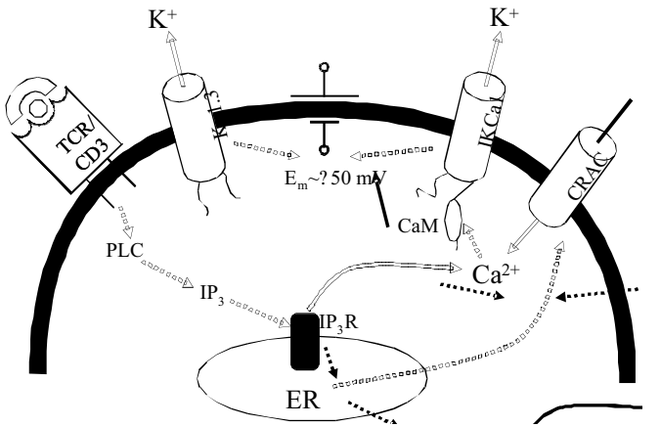

Fig. 1 Ion channels participating in the T cell receptor-mediated signal transduction. Stimulation of the T cell receptor complex (TCR/CD3) with antigenic peptide (octagon, left) activates phospholipase Cγ (PLCγ) which generates inositol 1,4,5 triphosphate (IP_3). IP_3 binds to the IP_3 receptor (IP_3R) located in the endoplasmic reticulum (ER) membrane and releases Ca^{2+}. Depletion of the ER Ca^{2+} store activates Ca^{2+} release-activated Ca^{2+} channels (CRAC) resulting in a Ca^{2+} influx. The activity of Kv1.3 and IKCa1 channels provide the electrical driving force for Ca^{2+} influx by maintaining a negative membrane potential. The activity of Kv1.3 is regulated by the membrane potential whereas IKCa1 is activated by a rise in the cytosolic free Ca^{2+} concentration via binding of Ca^{2+} to calmodulin (CaM) permanently associated with the channel. Arrows show ion fluxes, dotted arrows show regulatory interactions. Additional transmembrane signaling events were emitted for figure clarity.

inositol trisphosphate (IP_3). At this point the two additional signaling pathways of lymphocyte activation diverge. Diacylglycerol activates the protein kinase C (PKC) pathway, particularly through protein kinase Cθ, which leads to the phosphorylation of several intracellular substrates leading to the activation of another transcription factor, NFκB thereby triggering specific gene transcription. The second pathway, initiated by the generation of IP_3, evokes a biphasic increase in the concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$). Not surprisingly, the Ca^{2+} signal also converges to the activation of a transcription factor NF-AT (nuclear factor of activated T cells). This is mediated by the activation of the Ca^{2+}–calmodulin dependent phospahatase calcineurin, which dephosporylates NF-AT thereby enabling it to accumulate in the nucleus and bind to the promoter element of the interleukin-2 (IL-2) gene. Following the activation of the IL-2 gene T cells are committed to proliferate even in the absence of the antigen.

The first phase of the Ca^{2+} signal is directly coupled to the generation of IP_3 as IP_3 releases Ca^{2+} from endoplasmic reticuluum Ca^{2+} stores via binding to its designated receptor, the IP_3 receptor (reviewed in (Lewis, 2001)) (Fig. 1). The IP_3 receptor is considered an intracellular ligand-gated ion channel; therefore, this is the first occurrence of the involvement of an ion channel in lymphocyte activation. The second, sustained phase of the Ca^{2+} signal is the consequence of Ca^{2+} entry into T cells from the extracellular space (Fig. 1). The plasma membrane ion channel type being responsible for Ca^{2+} influx is the calcium-release activated Ca^{2+} channel, or CRAC for short (Zweifach and Lewis, 1993). The CRAC channel is activated by emptying of the intracellular Ca^{2+} stores, thus, it belongs to the armory of diverse channels being responsible for store-operated calcium entry (Lewis, 2001). Apart from the significant scientific interest, neither the molecular identification, nor the mechanism of activation of the CRAC channel has been elucidated. The most important biophysical characteristics of the CRAC channel distinguishing it from other store-operated channels is its extreme Ca^{2+} selectivity (Zweifach et al., 1993; Kozak et al., 2002; Prakriya and Lewis, 2002).

An additional feature of CRAC channels is that once activated the current is mainly determined by the electrochemical driving force for Ca^{2+}, i.e., the gating of the channel is voltage independent, but the magnitude of the current will be sensitive to the membrane potential of the cells (Zweifach et al., 1993). This, in combination with the inward rectification results in larger Ca^{2+} current at negative membrane potentials. The depolarizing inward Ca^{2+} current through CRAC channels must be counterbalanced by a cation efflux through K^+ channels in order to maintain the electrical driving force for Ca^{2+} entry and sustain Ca^{2+} signaling required for efficient signal transduction (reviewed in (Cahalan et al., 2001; Panyi et al., 2004c)). The membrane potential of T cells is primarily determined by the activity of two types of K^+ channels: the voltage-gated Kv1.3 (Matteson and Deutsch, 1984; Decoursey et al., 1984) and Ca^{2+}-activated IKCa1 channel (Grissmer *et al*., 1993) (Fig. 1). This relationship between CRAC current and K^+ channel activity makes the proliferation of lymphocytes sensitive to pharmaceutical interference with K^+ channels.

The contribution of Kv1.3 and IKCa1 channels to the membrane potential control of human T cells is not equal; it is related to the T cell subtype and the activation state of the cells. The K^+ conductance of resting T cells is dominated by the voltage-gated Kv1.3 channel (reviewed in (Cahalan et al., 2001; Panyi et al., 2004c)). The number of Kv1.3 channels is ~ 200-300/cell whereas fewer than 30 IKCa1 channels are expressed in these cells. Several experiments demonstrated that ion channel blockers selective for Kv1.3 over IKCa1, such as margatoxin and noxiustoxin, depolarize resting T cells, inhibit Ca^{2+}

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signaling and consequently, the mitogen-induced proliferation (Leonard et al., 1992; Lin et al., 1993). Due to the low number of IKCa1 channels present in these cells the selective block of these channels by the clotrimazole derivative TRAM-34 inhibited the proliferation of resting T cells at 250-500 higher concentrations than required for IKCa1 block (Wulff et al., 2000; Ghanshani et al., 2000). Once T cells are activated they transcriptionally upregulate the expression of IKCa1 by PKC-dependent pathways (~500 channel/cell) whereas the expression of Kv1.3 increases only modestly (~1.5-fold) (Ghanshani *et al*., 2000). Accordingly, the proliferation of pre-activated T cells becomes sensitive to IKCa1 block, the distinguished role of this channel type in the regulation of store-depletion-induced Ca^{2+} influx in these cells has been documented (Fanger *et al*., 2001). This general picture about the activation-induced change in the expression of Kv1.3 and IKCa1 has been refined recently as discussed later in the review (Wulff *et al*., 2003b).

Properties of the Kv1.3 channel

The first electrophysiological evidence about the existence of voltage-gated K^+ channels in human T cells was provided more than two decades ago (Matteson et al., 1984; Decoursey et al., 1984). The channel described at that time was classified as the normal or n-type K^+ channel. Cloning of the channel gene in 1992 (Cai et al., 1992; Attali et al., 1992) allowed its classification based on homology into the *Shaker* family of voltage-gated channels (Kv1) and it has been referred to as the Kv1.3 channel (Chandy, 1991).

A functional channel is composed of four identical, pore-forming alpha subunits (Fig. 2). Co-expression of auxiliary β-subunits alters several biophysical properties of Kv1.3 gating (Vicente *et al*., 2005). Each subunit is composed of six transmembrane α-helices connected by intra- and extracellular loops (Fig. 2). Basic structural elements of Kv1.3 are supposed to be similar to those of other voltage-gated K^+ channels including the bacterial homologues (KcsA, KaVP) used to determine the crystal structure of these channels (Doyle et al., 1998; Jiang et al., 2003). The extracellular loop between the 5^{th} and 6^{th} transmembrane segments (S5 and S6) along with segments of the S6 helix from each subunit form the pore through which potassium ions cross the membrane. The main structural elements of the ion conduction pore are the selectivity filter, located outermost in the permeation pathway followed by a large water-filled cavity and the activation gate formed

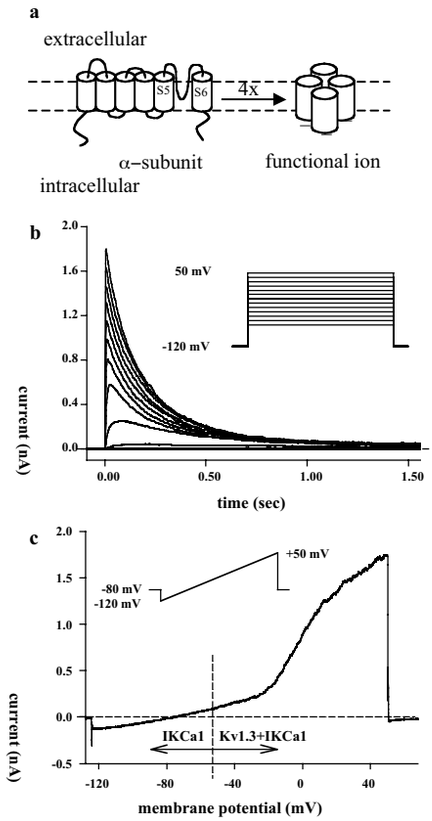

Fig. 2 Basic biophysical properties of Kv1.3 and IKCa1. (a) A functional K^+ channel is composed of four identical pore forming α-subunits, each consisting of 6 transmembrane α-helices connected by intra- and extracellular loops. The loop connecting the 5^{th} and 6^{th} helices along with segments of the 6^{th} helix from each α-subunit forms the permeation pathway (pore) for K^+ ions and this region also serves as the receptor for various blockers. (b) Whole-cell K^+ currents in a mitogen-activated T cell. Currents were elicited by test pulses of 2-second duration to increasing voltages from a holding potential of –120 mV as indicated by the inset. (c) Whole-cell K^+ current in a mitogen-stimulated T-cell recorded using a pipette solution containing 1μM Ca^{2+}. Currents were elicited by voltage ramps of 200-ms duration from –120 mV to +50 mV. The linear part of the trace at negative voltages (left of the vertical dashed line) represents voltage-independent K^+ current through IKCa1 channels. Both IKCa1 and Kv1.3 currents are measured at membrane potentials more positive than the threshold for Kv1.3 activation (right of the vertical dashed line).

by the "bundle crossing" of S6 helices. The selectivity filter contains the signature sequence of K^+ channels. Carbonyl oxygens of critically positioned amino acids in the selectivity filter act as surrogate water to guide K^+ across the narrowest part of the pore (Doyle *et al*., 1998). The selectivity sequence of Kv1.3 is $K^+ > Rb^+ >> NH_4^+ >> Cs^+ >> Na^+$, overall, Kv1.3 is ~1000-fold selective for K^+ as compared to Na^+ (Cahalan *et al*., 1985). Amino acid side chains in the outer vestibule, especially in the turret region, make

important contacts with peptide-toxin inhibitors of the channels thereby contributing to high affinity binding (Rodriguez de la Vega and Possani, 2004; Giangiacomo et al., 2004).

As Kv1.3 is a voltage-gated K^+ channel (Fig. 2), the S4 helix containing positively charged amino acid side chains is considered as the voltage sensor. Although the correct topology of the S4 in the transmembrane electric field is still debated (Jiang et al., 2003; Cuello et al., 2004; Bezanilla, 2005), its outward movement couples changes in the membrane potential to pore opening in a cooperative manner (Pathak *et al*., 2005). The activation threshold of Kv1.3 channels is between −50 mV and −60 mV, and the open probability of the channel increases steeply with depolarization (Cahalan et al., 1985; Pahapill and Schlichter, 1992). The midpoint of the voltage-dependence of steady-state activation measured in whole-cell patch-clamp configuration in human T cells is between −40mV and −30mV and the slope factor characterizing the steepness of the conductance-voltage relationship is around 10 mV. The voltage-dependence of steady-state inactivation of Kv1.3 has a midpoint between −60mV and −70mV and the slope factor of ~10 mV. The biophysical properties of Kv1.3 define a membrane potential "window" at which the channels can be open at steady-state (Pahapill et al., 1992). This window overlaps with the resting membrane potential of T cells, which is between −50 mV and −70 mV (reviewed in (Grinstein and Dixon, 1989) and (Lewis and Cahalan, 1995)), thereby underlining the importance of Kv1.3 in the membrane potential control of T cells. As an experimental proof of this, transfection of the Kv1.3 channel gene alone into CHO cells was sufficient to shift the resting membrane potential to ~–50mV, as compared to the –5 - –20 mV membrane potential measured in non-transfected cells (Defarias *et al*., 1995). The membrane potential window is shifted to depolarized potentials by ~15 mV if measurements were carried out in a more physiological cell-attached patch configuration (Pahapill et al., 1992).

Upon depolarization of the membrane Kv1.3 channels open rapidly (Fig. 2). The activation time constant of the current becomes shorter with depolarization reaching values of 1-1.5 ms at positive voltages (Cahalan *et al*., 1985). The short rising phase of the whole-cell current is followed by a slow decay during prolonged depolarization as a consequence of inactivation (Fig. 2). Members of the Shaker K^+ channel family can inactivate via two distinct inactivation mechanisms, the N-type and the so-called "slow inactivation" referring to the fact it is generally slower than the fast N-type inactivation. The former is a consequence of the occlusion of the open pore by an N-terminal tethered cytoplasmic 'ball' (Hoshi *et al*., 1990), whereas the latter is related to the constriction or collapse of the selectivity filter near the extracellular mouth of the pore (Liu et al., 1996; Loots and Isacoff, 1998). Slow inactivation in response to a depolarization is believed to involve two, usually sequential, conformational responses (De Biasi et al., 1993; Olcese et al., 1997; Yang et al., 1997; Harris et al., 1998; Loots et al., 1998). The first, P-type inactivation, is the closing of the gate at the extracellular end of the pore. The closure of the P-gate includes the postulated collapse of the selectivity filter (with a concomitant change in selectivity) (Starkus *et al*., 1997). P-type inactivation is followed by C-type inactivation, in which a further conformational change in this region stabilizes both the non-conducting (i.e., P-inactivated) state and the conformation of the voltage sensors (Olcese et al., 1997). Kv1.3 channels lack the N-terminal ball peptide but inactivate by the slow P/C-type mechanism. The inactivation time constant for the whole cell currents at a large depolarization (i.e. +50 mV) is ~200 ms measured in peripheral blood lymphocytes (Decoursey *et al*., 1985) as well as in heterologously expressed Kv1.3 (Panyi *et al*., 1995).

Studying of slow inactivation of Kv1.3 in lymphocytes led to important general conclusions regarding P/C-type inactivation of Shaker-family channels. The first operational definition of slow inactivation (referred to as C-type at that time) came from the analysis of the interaction of extracellular tetratetylammonium (TEA) with Kv1.3 (Grissmer and Cahalan, 1989b). TEA is a small-molecule pore blocker of Kv1.3 and when bound, prevents inactivation resulting in a slowing of the inactivation kinetics proportionally to the extent of current block. This, along with the slowing of the inactivation in the presence of high extracellular K^+ concentration was originally claimed as "hallmarks" for C-type inactivation (Grissmer and Cahalan, 1989a; López-Barneo et al., 1993). Other studies reported that slow inactivation is state-dependent and voltage-independent and can occur from the open state of the channel or from a closed (silent) state before channel opening (Decoursey, 1990; Marom and Levitan, 1994). The analysis of the inactivation kinetics of whole-cell currents produced by homo-and heterotetrameric channels composed of kinetically-tagged mutant (A413V) and wild-type subunits showed that a cooperative interaction between individual subunits determine the rate of C-type inactivation (Panyi *et al*., 1995). This result was confirmed in Shaker pointing

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out the general applicability of the observation (Ogielska *et al*., 1995). The same A413V subunits and the analysis of the inactivation kinetics was used to characterize the formation of heterotetrameric channels between endogenous wild-type Kv1.3 subunits and heterologously expressed mutant subunits in Jurkat T lymphocytes (Panyi and Deutsch, 1996). It was concluded that subunit assembly is a random process and that tetramers expressed in the plasma membrane do not dissociate and reassemble.

Inactivation limits the amount of K^+ conductance available for the regulation of membrane potential. Several factors were shown to influence the inactivation kinetics of Kv1.3 including temperature (Lee and Deutsch, 1990), phosphorylation by receptor (Bowlby *et al*., 1997) and non-receptor tyrosine kinases (Cook and Fadool, 2002), interaction with blockers (Robe and Grissmer, 2000; Varga et al., 2001) and the ionic composition of the extracellular solution (Grissmer et al., 1989a; Levy and Deutsch, 1996a; Levy and Deutsch, 1996b). A remarkable property of Kv1.3 inactivation, distinguishing it from other Shaker channels, is its sensitivity to the extracellular pH (pH_o). As opposed to the acceleration of inactivation kinetics of *Shaker* channels at acidic pH_o (López-Barneo et al., 1993; Trapani and Korn, 2003; Starkus et al., 2003) inactivation of Kv1.3 is slowed in low pH_o solutions (Deutsch and Lee, 1989). Regarding the molecular mechanism of the latter effect it was shown (Somodi *et al*., 2004) that at low pH_o the electric field of a protonated histidine (H399), which is located at the extracellular mouth of the ion conducting pore, creates an electrostatic potential barrier for K^+ that hinders its exit from the binding site controlling inactivation (Baukrowitz and Yellen, 1995; Kiss and Korn, 1998; Harris et al., 1998), thereby slowing the inactivation kinetics of the current.

In summary, the biophysical properties of Kv1.3 channels provide the basis for effective clamping of the membrane potential close to the activation threshold of the channels. In addition, the extremely high electrical resistance of the T cell membrane (10-20 GΩ) allows the membrane potential to be regulated by a small number of Kv1.3 channels expressed in T cells.

Properties of the IKCa1 channel

The IKCa1 channel expressed in human T cells is encoded by the hKCa4 gene (Logsdon *et al*., 1997). The unitary conductance of the channel in symmetrical K^+ solution is 33-35 pS. Based on this and the characteristic pharmacological profile, i.e., the current is insensitive to the small-conductance Ca^{2+}-activated K^+ channel blocker apamin (Grissmer *et al*., 1993) but can be inhibited by clotrimazole (Logsdon et al., 1997; Ishii et al., 1997) and its engineered analogue TRAM-34 (K_d= 20 nM) (Wulff *et al*., 2000), IKCa1 is an intermediate conductance Ca^{2+}-activated K^+ channel, also known as $K_{Ca}3.1$. Based on the amino acid sequence the proposed membrane topology of the channel is similar to that of voltage-gated K^+ channels: it is composed of four non-covalently linked subunits, each subunit consisting of 6 transmembrane helices (Fig. 1). The pore architecture of IKCa1 is similar to that of Kv1.3 as revealed by the similar interaction surface of these channels with peptide toxins (Rauer *et al*., 1999). Permeability ratios, relative to K^+, determined from reversal potential measurements are: K^+ (1.0) > Rb^+ (0.96) > NH_4^+ (0.17) > Cs^+ (0.07) (Grissmer *et al*., 1993). Although the IKCa1 current shows inward rectification the activation of the channels is voltage-independent (Fig. 2). This latter property is in agreement with the presence of fewer positively charged amino acids in positions corresponding to the voltage sensor S4 helix in voltage-gated K^+ channels (Logsdon et al., 1997; Ishii et al., 1997).

The IKCa1 channel is activated by the rise of the intracellular free Ca^{2+} concentration above ~100 nM (Grissmer *et al*., 1993). This means that these channels are silent at the resting state of the cells, but can readily be activated during transmembrane signaling involving $[Ca^{2+}]_i$ changes. The Ca^{2+} concentration-dependence of the IKCa1 conductance is very steep, it is characterized by a Hill coefficient of 4 and a midpoint of ~300 nM. The channels are fully activated by 1 μM $[Ca^{2+}]_i$, in the pipette solution at which most of the pharmacological experiments are conducted. The original description of the channel gene indicated the lack of the consensus sequence for Ca^{2+}-binding EF-hand motifs (Logsdon *et al*., 1997). Several lines of evidence support that calmodulin (CaM) is the Ca^{2+} sensor of IKCa1 (Fanger *et al*., 1999). CaM was found to interact strongly with the cytoplasmic carboxyl-tail of hIKCa1 in a yeast two-hybrid system. Interestingly, the association of CaM with the channel does not require Ca^{2+} indicating that CaM is permanently associated with the channel. The concerted action of the four CaM molecules, one per each subunit, leads to channel opening upon increase in $[Ca^{2+}]_i$. Since binding of CaM to the channel is immediate to the S6 helical segment participating in the formation of the activation gate of K^+ channels, the Ca^{2+}-induced conformational change in the CaM/channel complex might be transmitted directly to the opening of the channel (Fanger *et al*., 1999).

Neue Datei AV.indd Abs1:519 06.09.2005 10:16:49

The biophysical properties of IKCa1, in principle, argue for an important positive feedback regulation of the Ca^{2+} signal in T cells by these channels, i.e., Ca^{2+} influx through CRAC channels activates IKCa1 and the increased K^+ conductance of the membrane supports further Ca^{2+} entry by maintaining a permissive membrane potential. However, as mentioned earlier, the K^+ conductance provided by IKCa1 becomes important only in cells which upregulated IKCa1 following T-cell receptor-dependent/mitogen-induced activation.

Peptide blockers of Kv1.3 and their potential use to achieve specific immunosuppression

Based on their chemical structure molecules inhibiting Kv1.3 and IKCa1 channels are classified as inorganic ions, small-molecule inhibitors (reviewed in (Wulff et al., 2003a)) and peptide blockers. Similarly to other ion channels, peptide blockers of Kv1.3 were isolated from animals, mostly from the venom of scorpions (Price et al., 1989; Leonard et al., 1992). Scorpion toxins recognizing K^+ channels are composed of 23 to 64 amino acids. Their structural hallmark is the presence of a cysteine-stabilized α/β motif in which disulphide bridges covalently link the antiparallel β-sheets located on the toxin's surface interacting with the channels to the α-helical segment on the opposite side of the molecule (extensively reviewed in (Rodriguez de la Vega et al., 2004; Giangiacomo et al., 2004)) (Fig. 3). The blocking mechanism of scorpion toxins is quite simple: the peptides bind tightly into the external vestibule of the channels with 1:1 toxin-channel stoichiometry and occlude the ion conduction pore (Goldstein and Miller, 1993). The disulphide-bridge stabilized rigid structure of the peptides allowed to obtain molecular dimensions of the external vestibule of several voltage-gated K^+ channels, including that of Kv1.3, in the absence of the crystal structure-based coordinates. Based on the NMR-derived dimensions of kaliotoxin and margatoxin, the complementary mutagenesis of Kv1.3 and these toxins, combined with electrostatic compliance and thermodynamic mutant cycle analyses revealed a shallow external vestibule of Kv1.3 (~ 28-32 Å wide at its outer margin, ~28-34 Å wide at its base, ~ 4-8 Å deep) (Aiyar *et al*., 1995). The dimensions obtained in this and other studies agree well with those of the prokaryotic KcsA and KvAP channels derived from X-ray crystallography (Doyle et al., 1998; Jiang et al., 2003). The molecular dimension and the recognition of interacting sites in the toxins and in the channels serve as a basis for structure-guided development of Kv1.3 blockers aiming better pharmacological profile (see below).

The most potent natural toxin blockers of Kv1.3 channels are characterized by equilibrium dissociation constants in the low nM and pM range. These include charybdotoxin (ChTx, ~0.5-1 nM, (Sands et al., 1989; Price et al., 1989)), noxiustoxin (NTx 1 nM(Grissmer *et al*., 1994)), margatoxin (MgTx, 50 pM (Garcia-Calvo *et al*., 1993)), kaliotoxin (KTx, 650 pM (Grissmer *et al*., 1994)), Pi1, Pi2 and Pi3 toxins from *Pandinus imperator* (11 nM, 44 pM and 795 pM, respectively (Péter et al., 1998; Péter et al., 2000; Péter et al., 2001)) hongotoxin 1(HgTx1, 86 pM (Koschak *et al*., 1998)) HsTx1 isolated form *Heterometrus spinnifer* venom (12 pM (Lebrun *et al*., 1997)) and the recently characterized anuroctoxin from *Anuroctonus phaiodactylus* (730 pM (Bagdany *et al*., 2005)) and OSK1 form *Orthochirus scrobiculosus* (14 pM

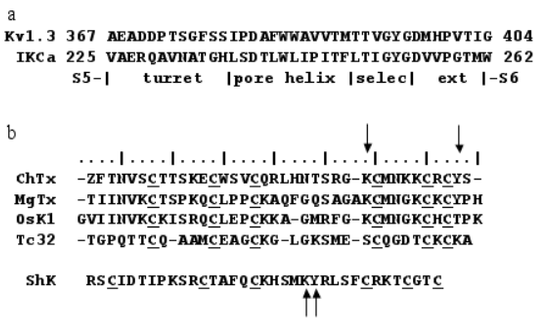

Fig. 3 Primary sequence of the pore region of T cell K^+ channels and their peptide blockers. (a) The primary sequence of the pore region of human Kv1.3 and human IKCa1 channels. The bottom row marks different functional segments in this region; "selec" corresponds to the selectivity filter. (b) Aligned primary sequence of selected scorpion toxins and the sequence of the sea anemone peptide ShK. Arrows indicate the amino acids in "diad position", underlined cysteines form the disulphide bridges stabilizing the structure of the peptides.

(Mouhat *et al*., 2005)). In addition to the scorpion toxins one of the most potent and most extensively studied blockers of Kv1.3, ShK, was isolated for the sea anemone *Stichodactyla helianthus*, whose dissociation constant for Kv1.3 is ~11 pM (Kalman *et al*., 1998). The structure of sea anemone toxins is substantially different from those of scorpion toxins, however, the interaction surface between the toxins and the Kv1.3 pore are similar (Dauplais *et al*., 1997). This common interaction surface contains a central, positively charged amino acid residue that protrudes into the pore, and a neighboring aromatic residue placed ~7 Å from the α carbon of the central lysine. (Dauplais *et al*., 1997). The side chain of the critical

Neue Datei AV.indd Abs1:520 06.09.2005 10:16:50

lysine is thought to protrude into the selectivity filter and interact with the residues of the signature sequence (GYG, a highly conserved sequence of amino acids forming the selectivity filter in K^+ channels) thereby "plugging" the permeation pathway (Fig. 3). This essential diad of critically spaced amino acid residues seems to be required for the recognition of K^+ channels, however, this theory has been challenged recently by the isolation of a novel scorpion toxin (Tc32) that has neither central lysine nor aromatic residue but blocks Kv1.3 with high affinity (Batista *et al*., 2002) (Fig. 3). Other reports suggested that other amino acid residues surrounding the functional diad make important contacts with specific residues in the turret region of the channels forming a ring of basic residues (Mouhat et al., 2004; Bagdany et al., 2005)

The discovery of the physiological roles of K^+ channels in T-cell activation was also aided mainly by scorpion toxins. As two types of K^+ channels exist in the plasma membrane of T cells the delineation of their individual contribution to T cell activation processes required specific blockers of Kv1.3 and IKCa1. Pioneering studies led to the discovery of peptide blockers having good selectivity for Kv1.3 over IKCa1, e.g. margatoxin and noxiustoxin and to the recognition that depending on the activation status of the T cells the contribution of the channels to the membrane potential control is different, as discussed earlier in this review.

One major disadvantage of natural toxin blockers is that they are not specific for a given type of ion channel, they also inhibit other Kv1 family channels and Ca^{2+} activated K^+ channels with variable, but high potency. Based on the molecular models of the Kv1.3 and IKCa1 pore and the crystal structure of KcsA highly selective peptide inhibitors of these channels have been generated recently. For example, replacement of the diad lysine (Lys^{22}) in ShK (Fig. 3) with a shorter, positively charged non-natural amino acid diaminopropionic acid (ShK-Dap^{22}) increased dramatically the selectivity of the natural ligand against other members of the Kv1 family as well as against IKCa1 (Kalman *et al*., 1998). Molecular-dynamics simulation of docking to the Kv1.3 vestibule suggested that ShK- Dap^{22} having a shorter side chain docks in different orientation and therefore possesses new interactions with the vestibule of Kv1.3 only, but not with other members of the Kv1 family, which may account for the increased selectivity of ShK- Dap^{22} (Lanigan *et al*., 2002). Another successful attempt to improve the pharmacological profile of a Kv1.3 inhibitor was the development of mutant OSK1 toxins (Mouhat *et al*., 2005). The analysis of the primary sequence of OSK1 (Fig. 3) indicated that at positions 16 and 20 wild-type OSK1 contains side chains with "inverted charges" with respect to the conserved ones in other toxins of the α-KTx3 toxin family. Mutation of Glu^{17} to lysine and Lys^{20} to aspartate yielded a toxin ([Lys^{16},Asp^{20}]-OSK1) which had increased affinity towards Kv1.3 (K_d=3 pM) and at the same time, its selectivity over the other Kv channels and IKCa1 also increased. Although none of the mutated residues of OSK1 participate directly in the interaction with Kv1.3, molecular dynamics simulations suggested that mutating these amino acid residues presumably alters the structure of OSK1, thereby allowing more favorable interactions with the channel, for example, between Arg^{24} of the mutant toxin with Gly^{380} of Kv1.3 (mouse clone). Structure-based drug design also yielded a ChTx mutant, which exhibited ~20-fold higher affinity for IKCa1 over Kv1.3 (Rauer *et al*., 2000). A cluster of negatively charged amino acids in the outer vestibule of the Kv1.3 interacting with Lys^{22} of ChTx (Fig. 3) was identified but a similar sequence was missing in IKCa1. This allowed a selective disruption of ChTx-Kv1.3 interaction by introducing negatively charged amino acids into position 22 in ChTx. In agreement with the expectations ChTx-Glu^{22} retained its affinity for IKCa1 but its potency to block all types of Kv channels possessing the cluster of negative charges was greatly reduced.

What motivates the efforts in increasing the affinity and selectivity of Kv1.3 inhibitors? This could be understood in the light of the discovery of a T cell subset whose proliferation primarily depends on the activity of Kv1.3 channels and participates in the pathogenesis of autoimmune diseases (Wulff *et al*., 2003b). This T cell subset is called effector memory T cell (T_{EM}) which nomenclature corresponds to its (patho)physiological function (Sallusto *et al*., 2004). As compared to naïve (cells had not encounter antigens yet) or central memory T cells (T_{CM} – cells arrested at intermediate stages of differentiation preceding T_{EM} (Sallusto *et al*., 2004)) T_{EM} cells do not home to lymphoid organs in order to be activated by antigen presenting cells, rather, they execute immediate effector functions in peripheral organs, secrete large amounts of cytokines and display immediate cytotoxicity. It turned out that T_{EM} cells upon activation with *specific* antigens increase the expression of Kv1.3 channels (~300→~1500/cell) dramatically, whereas the expression of IKCa1 channels remains relatively constant thereby obeying a $Kv1.3^{high}IKCa1^{low}$ channel phenotype (Wulff *et al*., 2003b). In contrast, naïve and T_{CM} cells respond to

Neue Datei AV.indd Abs1:521 06.09.2005 10:16:51

antigenic stimulation by an increase in the IKCa1 expression (<30→500/cell) accompanied with a modest increase in the Kv1.3 expression thereby the channel phenotype of these cells is Kv1.3^{low}IKCa1^{high} (Wulff *et al*., 2003b)

The group of Chandy has shown recently that myelin-reactive T_{EM} isolated from patients suffering from the neurodegenerative autoimmune disease Multiple Sclerosis (MS) selectively up-regulate Kv1.3 channels (Kv1.3^{high}IKCa1^{low} channel phenotype) upon acute *in vitro* stimulation with myelin antigen (Wulff *et al*., 2003b). Functionally, myelin specific T cells in MS lesions are terminally differentiated effector memory T cells (Kivisakk *et al*., 2004). The following lines of evidence suggest a strong correlation between the disease-causing nature of the T_{EM} cells and their ion channel expression: *i)* stimulation of MS patient T cells with irrelevant antigens such as insulin peptide, ovalbumin or with conventional mitogens did not induce the Kv1.3^{high}IKCa1^{low} channel phenotype, *ii)* short term myelin antigen–activated (three antigenic stimulations) patient T cells expressed significantly higher numbers of Kv1.3 channels/cell than did myelin antigen–activated T cells from healthy controls, *iii)* control cells stimulated repeatedly (seven to ten antigenic stimulations) with myelin antigen acquire the Kv1.3^{high}IKCa1^{low} T_{EM} phenotype, *iv)* a similar T cell sub-type having Kv1.3^{high} channel phenotype was observed in experimental autoimmune encephalomyelitis (EAE), a rat model for MS mediated by chronically activated myelin basic protein specific T cells (Beeton et al., 2001a; Beeton et al., 2001b).

The great therapeutic potential of Kv1.3-specific blockers comes from the *selective* inhibition of the proliferation of T_{EM} cells as naïve and T_{CM} cells escape from Kv1.3 block-mediated inhibition of proliferation (Wulff *et al*., 2003b) by transcriptional upregulation of IKCa1 (Ghanshani *et al*., 2000). This differential effect was shown for ShK, ShK-Dap^{22} (Wulff *et al*., 2003b), and ShK(L5) (Beeton *et al*., 2005) among the peptid blockers and for PSORA-4, the most Kv1.3 selective small-molecule inhibitor of Kv1.3 (Vennekamp *et al*., 2004). In this respect Kv1.3-specific blockers fulfill the requirements of an ideal immunosuppressor by selectively inhibiting the proliferation of cells mediating the autoimmune reactions whereas not affecting T cell proliferation required for normal immune reactions. These *in vitro* results are supported by extensive *in vivo* experiments in rats. Beeton et al. showed that the symptoms of EAE were significantly ameliorated using selective Kv1.3 inhibitors, furthermore, the adoptive transfer of EAE to healthy animals could be prevented by *in vitro* treatment of myelin-specific chronically activated T cells by selective Kv1.3 inhibitors (Beeton et al., 2001a; Beeton et al., 2001b). This, in combination with the lack of toxicity of the selective Kv1.3 inhibitors ((Beeton et al., 2001a; Beeton et al., 2001b; Beeton et al., 2005) places these compounds in a very prestigious position for clinically applicable selective immunosuppression (Wulff et al., 2003a; Chandy et al., 2004; Panyi et al., 2004b; Panyi et al., 2004c).

Molecular interactions and lateral organization of Kv1.3 in the membrane

Besides its interaction with blockers, the activity of the Kv1.3 channel can be modulated by several factors, including their regulation with protein kinases. Experiments demonstrated more than a decade ago that both protein kinase A (PKA) and protein kinase C downregulate the activity of Kv1.3 in Jurkat cells (Jurkat T lymphoma cells resemble in many aspects the properties of human peripheral T cells, but see below), and that PKC-dependent modulation is a prerequisite for the regulation of Kv1.3 by PKA (Payet and Dupuis, 1992). The phosphorylation of the channel by both kinases in Jurkat T cells has also been demonstrated (Cai and Douglass, 1993). On the contrary, phosphorylation by PKA (Chung and Schlichter, 1997a) and PKC (Chung and Schlichter, 1997b) upregulates Kv1.3 channel activity in human T lymphocytes by modifying the voltage-dependence of steady-state activation and inactivation of the channel. These latter studies, however, agreed with the results obtained in Jurkat cells that PKC-dependent phosphorylation acts as a master switch in the complex regulation of Kv1.3 activity by serine/threonine phosphorylation. The fact that the sensitivity of Kv1.3 to PKC- and PKA-mediated regulation is partially lost when expressed in HEK-293 cells pointed towards the importance of specific proteins/cellular environment that exists in the native cells but may not be present in the host cell (Martel et al., 1998).

More is known about the regulation of Kv1.3 by tyrosine phosphorylation. It is well documented that activation of receptor tyrosine kinases, e.g. that of the epidermal growth factor and the insulin receptor, suppresses the Kv1.3 current (Bowlby et al., 1997; Colley et al., 2004). However, signal transduction of T cells is mediated by non-receptor tyrosine kinases recruited upon TCR/CD3 stimulation, thus the regulation of Kv1.3 by these kinases have more physiological relevance in T cells. Transfection of a constitutively active non-receptor tyrosine kinase (v-

Neue Datei AV.indd Abs1:522 06.09.2005 10:16:52

src) along with Kv1.3 into HEK-293 cells caused a large increase in the tyrosine phosphorylation of the channel protein accompanied by the reduction of the macroscopic currents recorded in cell-attached patch configuration (Holmes et al., 1996). Application of a tyrosine phosphatase inhibitor (pervanadate) increased Kv1.3 phosphorylation by endogenous kinases with the corresponding reduction in the current amplitude. A subsequent study using the same approach also showed that v-Src-induced suppression of Kv1.3 current was not mediated through decreased channel protein expression or interference with its targeting to the plasma membrane. In addition, v-Src co-expression also slowed C-type inactivation and sped up the deactivation of the residual Kv1.3 current. The complex regulation of current amplitude and inactivation kinetics required the phosphorylation of Kv1.3 at multiple tyrosine residues (Fadool et al., 1997). The suppression of Kv1.3 current by non-receptor tyrosine kinase-mediated phosphorylation was also shown in Jurkat cells upon activation of the Fas receptor-mediated apoptosis program either by direct stimulation of the Fas receptor (Szabo et al., 1996) or by ceramide, a lipid metabolite synthesized upon Fas receptor ligation (Gulbins et al., 1997). Both studies confirmed the role of the Src-like non-receptor tyrosine kinase Lck in the inhibition of Kv1.3 using Lck-deficient JCaM1.6 cells. In contrast, a Fas-receptor activation-mediated upregulation of the Kv1.3 conductance has been recently reported which was attributed to the activation of caspase 8, a proteolytic enzyme playing a central role in the apoptotic process of T cells (Storey et al., 2003).

What can be the platform for protein kinase-mediated regulation of Kv1.3? As discussed above, during physiological stimulation with antigens non-receptor tyrosine kinases are recruited and activated at the TCR/CD3 complex, thus a spatial segregation of the signaling events is predicted. It has turned out recently, that the interaction of professional antigen presenting cells with T cells takes place at a specialized intercellular contact, called immunological synapse (IS), where the encounter causes proteins to segregate into micrometer-scale domains (Monks et al., 1998; Davis, 2002). The center of the supramolecular activation clusters (cSMACs) in T-cell side of the synapse contains TCR/CD3, CD28 and CD2 membrane proteins and the recruited Lck or PKCθ cytosolic protein kinases, whereas the periphery of the IS is enriched in the adhesion molecule LFA-1 (reviewed in (van der Merwe, 2002)). The redistribution of the CD4 coreceptor to the cSMACs and its tight physical interaction with TCR/CD3 has also been demonstrated using live FRET (fluorescence resonance energy transfer) imaging (Zal et al., 2002). This is particularly interesting since the cytoplasmic tail of CD4 (and CD8) coreceptors associate with Lck (Thome et al., 1995).

Several lines of biochemical evidence suggested that Kv1.3 might also be part of this signaling complex (Fig. 4). First, Kv1.3 is effectively regulated by Lck and PKC (see above). Second, although the cytoplasmic domain of Kv1.3 channel does conform to known SH3 binding consensus motifs, which may facilitate its binding to Lck tyrosine kinase, an adaptor protein called hDlg (human homologue of the Drosophila discs large tumor suppressor protein) might couple Lck to Kv1.3 thereby allowing efficient tyrosine phosphorylation of the channel (Hanada et al., 1997). The C-terminus of Kv1.3 contains a recognition site for the PDZ domain of the scaffolding protein hDlg (and PSD-95); and hDlg in Jurkat cells was shown to interact with Lck (Hanada et al., 1997). Third, the N-terminus of Kv1.3 is associated with the accessory protein $Kv\beta_2$ (McCormack et al., 1999); and $Kv\beta_2$ may also link K^+ channels to signaling molecules such as PKC and Lck using ZIP1 and ZIP2 adaptor proteins (Gong et al., 1999). Thus, Kv1.3 could be physically

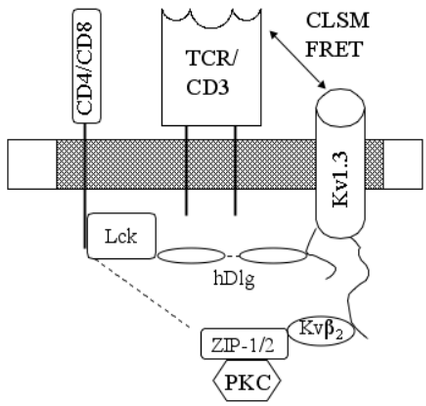

Fig. 4 Kv1.3 channels in the signaling complex of T cells. Fluorescenc resonance energy transfer (FRET) showed the molecular proximit between T cell receptor/CD3 complex (TCR/CD3) and Kv1.3 Colocalization of CD3 and Kv1.3 was shown in isolated Jurkat cell along with recruitment of Kv1.3 into the immunological synapse forme between cytotoxic and target cells using confocal laser scannin microscopy (CLSM). The shaded membrane area represents lipid rafts Kv1.3 might be coupled to the Src-family kinase Lck through hDl (human homologue of the Drosophila discs large tumor suppresso protein) and through $Kv\beta_2$ accessory and ZIP-1/2 adaptor proteins. Th latter protein may also recruit protein kinase C (PKC) into the complex.

coupled to Lck through both its N- and C-termini.

The physical association of Kv1.3 with molecules participating in the signal transduction of T cells, in particular with the TCR/CD3 complex, has been shown recently in Jurkat cells (Panyi et al., 2003) (Fig. 4). The spatial distribution of epitope-tagged Kv1.3 channels in the cell membrane was analyzed using electron microscopy following the labeling of the channels at their FLAG epitope using colloidal gold-conjugated monoclonal antibodies. The analysis of electron microscopic images showed that the observed distribution of Kv1.3 channels significantly differed from the predictions given by the stochastic Poisson distribution, thereby indicating the preferential localization of these channels in certain areas of the membrane. The non-random distribution of Kv1.3 might reflect its physical association with other membrane proteins. Motivated by the previously suggested association of Kv1.3 with TCR/CD3 (see above) confocal laser scanning microscopy (CLSM) and cross correlation analysis of the fluorescence signals were applied to investigate this scenario. The analysis of CLSM images taken in both Kv1.3- and CD3-labeled cells showed that Kv1.3/FLAG channels and CD3 molecules accumulated in largely overlapping membrane areas. As CLSM resolves fluorescently labeled species on a scale from 0.2 to several micrometers (Nagy et al., 2001) the co-distribution of the studied molecules was concluded within this distance range. A close vicinity or molecular association of the labeled Kv1.3 and CD3 proteins was verified at much higher spatial resolution (2-10 nm) using FRET (Matyus, 1992; Szollosi et al., 1998). The physiological relevance of these observations obtained in isolated Jurkat T cells was further investigated in a model system which mimicked normal T cell functions (Panyi et al., 2004a). Human peripheral blood cytotoxic T cells (CTLs) specific for antigens of a given target cell were generated and the distribution of CD3 and CD8 molecules and the transfected Kv1.3/FLAG was determined using CLSM upon formation of the immunological synapse between CTL and target cells. This IS is also called a "kiss of death" which leads to, e.g., the elimination of virally infected cells in the body. The IS of the CTLs is similar to that of other T cells except it also contains a secretory domain delivering the lethal hit to the target cells (Stinchcombe et al., 2001).

In agreement with the results obtained in single Jurkat cells Kv1.3 showed a patchy distribution in CTLs not engaged with target cells or conjugated with non-specific target cells. On the contrary, the engagement of CTLs with specific target cells resulted in the accumulation of Kv1.3 in the IS along with the expected re-distribution of CD3 and CD8 molecules. Kv1.3 channels either accumulated directly in the contact area between the cells or surrounded the IS like a belt. The two arrangements of Kv1.3 with respect to the IS might reflect redistribution of the channel during the maturation of the IS, as it was shown for other molecules as well (Grakoui et al., 1999; Bromley et al., 2001; Davis, 2002). The intimate relationship between Kv1.3 and CD3 was also modestly maintained as reported by the FRET efficiency measured in CLSM (Panyi et al., 2004a) using the acceptor photobleaching technique (Bastiaens et al., 1996). As a plausible mechanism being responsible for the rearrangement of Kv1.3 into the IS, the partition of Kv1.3 channels into specialized lipid microdomains of the membrane, commonly referred to as lipid rafts, was also shown (Panyi et al., 2004a) (Fig. 4). This latter observation agrees well with other reports obtained using confocal microscopy (Bock et al., 2003) or by electrophysiology (Hajdu et al., 2003).

The recruitment of Kv1.3 into the IS most likely provides the molecular proximity between protein kinases and Kv1.3 required for efficient regulation of the Kv1.3 conductance and consequently, that of the cell activation processes (Fig. 4). On the other hand, the activity of Kv1.3 in the IS might reciprocally regulate the function of the IS itself (Matko, 2003; Panyi et al., 2004b) either by membrane potential-driven conformational changes in membrane proteins mediating antigen recognition processes (Vereb et al., 2003), or by the activation of the function of cell adhesion molecules by local K^+ efflux through Kv1.3 (Artym and Petty, 2002).

Conclusions

The voltage-dependence of steady-state activation and inactivation of Kv1.3 determines a membrane potential window at which the ion channels are active at steady-state, whereas IKCa1 channels are mostly silent in the resting state of cells but can be activated by a rise in the intracellular Ca^{2+} concentration above 100 nM. These biophysical properties of Kv1.3 and IKCa1 and the extremely high electrical resistance of the T cell membrane allows an effective control of the membrane potential by a small number of ion channels present in unstimulated T cells. Activation by specific antigens induces a T cell subset-specific change in the expression of ion channels. While naïve and central

Neue Datei AV.indd Abs1:524 06.09.2005 10:16:54

memory T cells upregulate IKCa1, effector memory T cells increase the expression of Kv1.3 and their proliferation becomes exclusively Kv1.3 dependent. As effector memory T cells are thought to be responsible for the damage of the central nervous system in Multiple Sclerosis, the specific inhibition of the proliferation of this T cell subset by high affinity Kv1.3 inhibitors holds a great therapeutic potential. The X-ray crystallographic determination of the pore architecture of prokaryotic K^+ channels boosted structure-guided design of high affinity and high selectivity Kv1.3 inhibitor molecules based on natural toxin templates isolated from scorpions and sea anemone. The analysis of influential contact points between the toxins and the channel vestibule along with molecular dynamics simulation of the docking isolated a pair of critical residues important for K^+ channel recognition and other contacts conferring selectivity. The regulation of Kv1.3 by protein kinases is also well described. The discovery of the physical association of Kv1.3 and CD3 molecules underlines the importance of the molecular environment permitting physiological regulation of the membrane conductance for K^+ by protein kinases. The recruitment of Kv1.3 into the immunological synapse argues for the possibility of mutual regulation of the function of the IS and that of Kv1.3 during the first steps of T cell activation.

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Neue Datei AV.indd Abs1:526 06.09.2005 10:16:56

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