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STUDY OF K^+ CHANNELS IN NATIVE AND EXPRESSION SYSTEMS

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1 INTRODUCTION

The primary human T lymphocytes initiate and direct the specific response of the immune system to antigenic challenge. The trigger of this signal cascade is the activation of the T cell receptor/CD3 complex (TCR/CD3) that is followed by the initiation of the protein kinases (PK) and the production of inozitol trisphosphate (IP_3) second messenger. Finally, the free cytosolic Ca^{2+} level is elevated and the protein kinase C (PKC) activates. Besides these biochemical processes several physical factors play role in the transfer of the proliferative tramsmembrane signal towards the cytoplasm and genes.

So far a great deal of ion channels (Na^+, K^+, Ca^{2+}, Cl^-) have been identified in lymphocytes, although some of them could have been characterized adequately, including their concentration in the cell membrane and loci on the chromosomes. The predominant ion channel of human T cells is the *n*-type, depolarization activated K^+ channel which was named Kv1.3 based on its amino acid sequence homology. The presence of *n'* and *l*-type K^+ channels with different pharmacological and biophysical properties that of the *n*-type was only described in mouse lymphocytes. Ca^{2+} activated K^+ channels can be found in human leukemia and human preipherial T cells. The variability of K^+ channels in T cells is also confirmed by the results of molecular biological research.

Several lines of evidences underlie the essential role of K^+ channels in the proliferation of T cells:

- The potassium conductance of resting T cells increases after the mitogen induced hyperpolarization.
- The number of voltage-gated K^+ channels in the membrane elevates manifold upon mitogen activation.
- The conventional inhibitors of K^+ channels, such as tetraethil-ammonium (TEA), charybdotoxin (ChTx), noxiustoxin (NxTx), margatoxin (MgTx), prevent the activation and proliferation *in vitro*, and even *in vivo*.

At present it is not clear through what mechanisms the potassium channels influence the T cell activation. Since K^+ diffusion potential predominantly determines the membrane potential of lymphocytes, the K^+ channel blockers are likely to depolarize the membrane, thereby preventing the elevation in the intracellular Ca^{2+} level. Consequently, the K^+ channels support the initiation of Ca^{2+} signal by maintaining a hyperpolarized potential difference.

Kv1.3, that is responsible for the *n*-type current in T cells belongs to the *Shaker* family of K^+ channels. *Shaker* channels are tetrameric molecules with six putitative α-helical transmembrane segments (TM) linked by extra - and intracellular loops: the fifth and the sixth (S5-S6) segments flank the pore region and make up the conducting pore, whereas the first four (S1-S4) TM's are supposed to scaffold the activation gate of channels.

Shaker channels open on membrane depolarization and then get into a non-conducting, inactivated state. The activation of Kv channels is thought to be a conformational change initiated in the first four segments of the channel protein, while the inactivation can occur by two mechanisms: the N-type and the C-type inactivation. The former, which is the faster one, can be characterized by the so-called ball-and-chain model that assumes an inactivation particle binding to the inner mouth of channel pore. The latter is the result of a complex, less-understood molecular process sensitive to the external cations and the amino acid composition of the channel pore. The Kv1.3 channel possesses a special feature: only the C-type mechanism is responsible for their inactivation so they are ideal for investigating this complex procedure. There are two accepted theories for the molecular mechanism of C-type inactivation. An earlier model described the C-type inactivation as a consequence of a complete collapse of the ion conductivity pore . A later model restricts conformational changes to the selectivity filter of the potassium channels only: the C-inactivated channels are not permeable to K^+ although they retain significant Na^+ permeability.

The oligopeptide loop that connects the S5 and S6 segments of each subunit form the pore region of the K^+ channels. Here can be found the selectivity filter that is responsible for the K^+-

selectivity. Furthermore, inhibitors which plug the pore of the conduction pathway interact with the side chains of amino acids of this region.

To reveal the structure-function of ion channels different peptide and non-peptide blockers are used with complementary mutagenesis on the channel protein. The former group of blockers involves the venom of various animal species (scorpion, snake etc.) which bind to the K^+ channel proteins with high affinity.

Majority of peptide inhibitors binds into the extracelular vestibule of the channel pore, and prevents the pass of K^+ ions through the conductive pore (pore blocker). Mutagenesis approach of toxins with known sequence and the channel protein facilitated to disclose the topology of the pore and the role of individual amino acids.

Due to the extraordinary blocking mechanism hanatoxin (HnTx) excels from the other peptide blockers, which was isolated from the venom of a spider. HnTx does not dock into the channels pore, however, it modifies the channel gating by binding to the S3 segments of the *Shaker* channels, thereby inhibits the K^+ current. The bond of a HnTx molecule shifts the voltage-dependence of activation (gating-modifier).

Besides peptide blockers the non-peptide compounds are useful tools to study the channel function, the gating mechanisms. Because these molecules possess much simpler structure, distinct sites of action and blocking mechanism they are suitable to map the structure-function relation despite to the lower affinity to the ion channels,.

The commonly used non-peptide blocker of K^+ channels is the tetraethyl-ammonium (TEA) which distinct the N- and C-type inactivation of *Shaker* channels. TEA has both intra- and extracellular binding site in the channel pore: it binds to the open pore of *Shaker* channels and prevents the flow of K^+ ions and slow the C-inactivation.

In general, the cell membrane is a thin, lipid bilayer sheet in which various proteins, glikoproteins, cholesterol, sphyngolipids etc. diffuse and interact with each other. During the

lifetime of a cell the composition of plasma membrane can change due to different reasons such as diet, diseases, development and aging. The molecules comprising/building up the membrane obviously influence the structure and physical properties of the plasma membrane (fluidity, curvature, stress, stiffness etc.) as well as the function of the embedded receptors. Both lipid-protein interaction at the lipid interface of the receptor proteins and the membrane-induced deformation "forces" due to the altered physical parameters of the membrane can give rise to the altered operation of receptors; moreover these are also necessary to maintain proper functioning of the transmembrane receptors, or even to form -helices.

Cholesterol, the prominent component of the mammalian plasma membrane, modifies the dynamic properties of the outer leaflet. Cholesterol was shown to be essential for integrity and functionality of the nACh receptor. Activity of Na^+-K^+-ATPase in bovine epithelial cells was decreased either by cholesterol loading or depletion. Altered lipid-protein interactions were thought to be responsible for the former; the latter effect was attributed to the changes in the membrane structure. Manipulation of cholesterol content and lipid composition in the cell membrane affects the function of ion channels: cholesterol enrichment suppressed the activity of VRAC channels (Volume-Regulated Anion Current) while cholesterol depletion was stimulatory; the equilibrium between the closed and the open state was altered without influence on the permeability properties. Single-channel experiments on K_{Ca} channels in vascular smooth cells or reconstituted into bilayers demonstrated that the modification of lipid composition and cholesterol content of the plasma membrane altered the kinetic properties of channel gating due to the lateral elastic stress generated by the treatments.

Recent evidences propose that specialized microdomains, commonly referred as lipid rafts, exist in cell membranes. These rafts are rich in cholesterol and sphingolipid, and localize various proteins - including ion channels - playing essential role in triggering of different intracellular pathways, while other proteins are excluded from these compartments. Nowadays Kv2.1 and Kv1.5

have also been shown to target to lipid rafts unlike Kv4.2 which does not show clusterization in the membrane. Cholesterol removal resulted in a change of steady-state parameters of inactivation for Kv2.1 and those of the activation and inactivation for Kv1.5, thus, the collapse of the diverse raft system resulted in the reduction of signaling through native pathways.

Two-pore K^+ channels

Recently a new family of K^+ channels with two pore (2-P) domains, four transmembrane (TM) segments, an extended extracellular loop and intracellular N- and C-termini has been identified in various species. Presently more and more members of this family have been discovered due to the genome project, they can be found in pancreatic cells as well as in neurons, either in excitable or in non-excitable cells. The cloned members of this family are not voltage-gated, non-inactivating (except TWIK-2 with slow inactivation kinetics), insensitive to conventional potassium channel inhibitors (4-AP, TEA, various peptide blockers) and are likely to play a role in setting the membrane potential through a leak current. 2-P channels share very low amino-acid sequence homology (25-40%) among each other and show unusual basic electrophysiological properties: there are inward-, Goldman-Hodgkin-Katz- (GHK or open) and outward-rectifiers. With respect to the pharmacology acid-, stretch- and PUFA-sensitive (**p**oly**u**nsaturated **f**atty **a**cid) channels can be distinguished, and various anesthetics, neuromodulators- and protective agents (bupivacaine, halothane, riluzole etc.) can inhibit or, contrary, activate these channels. Recently the neuroprotective sipatrigine has been shown to be a very potent and specific blocker of human TREK-1 and TRAAK-1 (IC_{50} value of 4 and 10 μM, respectively). In addition, it was shown that a single point mutation in TWIK-1 at residue 69 (C69S) prevented homodimerization of subunits. Furthermore, the forming of heterodimers of single subunits from different subfamilies was also verified. Based on the facts mentioned above, the 2 pore-domain channels are considered to function as background K^+ currents in a huge variety of cells.

Screening a mouse heart cDNA library a new type of two pore K^+ channels was isolated by Kim et al: the cTBAK-1 (**c**ardiac **T**wo-pore **B**ackground $\mathbf{K}^+$ channel). cTBAK-1 as well as mouse TASK-1 are extremely acid-sensitive in the narrow physiological range and GHK-rectifying K^+ channels, although the former has nine additional amino acids in the N-terminus. This small "extra" chain of amino acid residues at N-terminus does not result in a significant difference between the two channel types: their intrinsic gating mechanism is not affected. Moreover, there are no real effective inhibitors of the 2-P cTBAK-1 channel, except the very potent anandamide which is an effective, selective and direct blocker of TASK-1 in the submicromolar range.

2 OBJECTIVES

1 The composition of the plasma membrane changes due to the various physiological and patophysiological factors that results in the modification of the physicochemical and the structural properties of the membrane. The appropriate condition of the cell membrane is necessary for membrane proteins to function. In our study we demonstrate that manipulation of cholesterol content in the membrane of activated T lymphocytes affected the kinetic and steady-state parameters of activation and inactivation for Kv1.3.

2 Toxin Pi1, a recently described peptide toxin having four disulfide bridges instead of the usual three in scorpion toxins. We examined whether the presence of the extra disulfide bond influences the affinity of Pi1 to the Kv1.3.

3 Fluoxetine (*FL*) and D-norpropoxyphene (NORP) block the current of cTBAK-1 channels differently, the latter modified the selectivity of the channel pore. Here we have used a drug- and mutagenesis-based approach to study the effect of both drugs on cTBAK-1.

3 MATERIALS AND METHODS

***Cells*:** T cells were separated by Ficoll density gradient centrifugation from heparinized human peripheral venous blood. The lymphocytes were activated with phytohemagglutinin

Oocytes were obtained from female *Xenopus laevis* frogs. Prior to injection cells were selected according to condition and size, and incubated in ND-96 supplemented with antibiotics.

Electrophysiology: T lymphocytes were selected for current recording by selective antibody adhesion to Petri dishes. Whole-cell measurements were carried out using Axopatch-200 and Axopatch-200A amplifiers connected to personal computers using Axon Instruments Digidata 1200 data acquisition boards. For data acquisition and analysis the pClamp8 software package was used. The bath solution was (in mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 5.5 glucose, 10 HEPES (pH 7.35, 305 mOsm). The pipette solution was (in mM): 140 KF, 11 K_2EGTA, 1 $CaCl_2$, 2 $MgCl_2$, and 10 HEPES (pH 7.20, ~295 mOsm).

Oocytes were injected with 50 nl of cRNA at concentration of 0.1 ng/nl. The currents were measured by means of two-electrode voltage-clamp 2-4 days after injection using GeneClamp 500 amplifier. Electrodes were filled with 3 M KCl. The bath solutions were: low K^+ containing ND-96, high-potassium (HK) solution, HK solution containing 300 μM $BaCl_2$ (Ba^{2+} solution). Na^+-free solution was achieved by substitution of Na^+ for TRIS (TRIS solution). The bath solutions were exchanged by a gravity driven perfusion system, the excess fluid was removed by continuous suction.

Prior to analysis whole cell current traces were digitally filtered (3 point boxcar smoothing). Non-linear least squares fits were done using the Marquardt-Levenberg algorithm. Fits were evaluated visually, as well as by the residuals and the sum of squared differences between the measured and calculated data points. We applied off-line leak subtraction if the leak current was not negligible with respect to the peak current.

Cholesterol manipulation: Cultured lymphocytes were incubated in Hanks' solution containing different concentrations of Methylated-β–Cyclodextrin/Cholesterol or Cyclodextrin. In control experiments MβCD/C or MβCD was not present in the solution.

Molecular biology: The mutations Y105F and F211Y were introduced into the pGEMHE/cTBAK-1 vector using standard PCR technique. The results were analysed by enzyme restriction digest thanks to appearance of these silent mutation sites. For *in vitro* transcription of pGEMHE/mcTBAK-1, expression vectors were linearized with *Nhe I*. Then capped cRNAs were synthesized from the linearized plasmids.

4 RESULTS AND DISCUSSION

1 Cholesterol modifies the gating of Kv1.3 in human T lymphocytes

Cholesterol loading and removal by cyclodextrins was monitored using two independent functional parameters: fluorescence polarization anisotropy and current density normalized to cell capacitance. We argue that the change in these parameters confirms that both MβCD/C and MβCD treatments were effective.

The fast activation of outward current through Kv1.3 channels is followed by a relatively slow decay of current corresponding to the C-type inactivation of this channel. The decaying part of the curves was fitted with a single exponential function for both treated and control cells ($I(t) = I_0 \times \exp(-t/\tau_{in,i}) + C$, I_0: amplitude of current, $\tau_{in,i}$: inactivation time constant for treatment "i", C: steady-state value of whole-cell current) to obtain the time constant describing the inactivation kinetics. Exposure of T lymphocytes to MβCD (0.95 and 1.425 mg/ml) decreased the time constant of inactivation relative to the control while the cholesterol enrichment slows down (1 and 1.5 mg/ml MβCD/C) the inactivation of current. The steady-state value of current over peak increased significantly upon cholesterol loading, which point toward the change of rate constants depicting

the open-inactivated transition of Kv1.3 channels that is in coincide with the prolonged inactivation of the current.

Activation kinetics of the Kv1.3 channels has been determined from the current traces evoked by short depolarizations to + 50 mV. Elevation of membrane cholesterol level using 1 and 1.5 mg/ml MβCD/C concentrations slowed the activation of current that could be described by a single exponential rising function in accordance with the Hodgkin-Huxley (HH) model ($I(t) = I_a \times (1-\exp(-t/\tau_a))^4 + C$) where I_a is the amplitude of the activating current component; τ_a is the activation time constant of the current; C: current amplitude at –120 mV). The Kv1.3 current of the lymphocytes treated with MβCD exhibited the same activation properties as the control cells.

For some cholesterol enriched cells treated with 1 and 1.5 mg/ml MβCD/C the activation became biphasic.). In this case the activation curves could be fitted with a sum of two exponential terms, and we described the activation with parameter ***R***, which gives the ratio of the amplitudes of the fast component of current ($I_{a,f}$) over the sum of the fast and slow ($I_{a,s}$) current components ($I_{a,f} /(I_{a,f} + I_{a,s})$), $\tau_{a,f}$ and $\tau_{a,s}$ are the activation time constant of the fast and slow activating current components, respectively. The "fast" time constants ($\tau_{a,f}$) for these treatments increased significantly, while the "slow time constant" and ***R*** for both treatments had statistically the same value; the latter showed voltage-independence

Cholesterol depletion and loading may influence the equilibrium parameters of voltage-dependent either activation or inactivation, or both. The voltage dependence of normalized whole-cell conductance (G_N, G_N-V curve) describes the equilibrium distribution of the ion channels between the closed and open states at a given membrane potential, meanwhile the equilibrium parameters of the recovery from the inactivated state (the **R**ecovered **F**raction (RF)) plotted versus membrane potential gives the ratio of the pools of closed and inactivated channels at the defined test potential. The parameters of Boltzmann function fitted to data points were used to evaluate the changes upon different treatments. The midpoint of voltage dependence of steady-state activation

for control cells was ($V_{½,c}$) - 27.5 ± 1.2 mV (n=24) while for treatments with 1.5 mg/ml and 1 mg/ml MβCD/C the midpoint were shifted toward the positive voltages: - 20.2 ± 2.0 mV and -20.9 ± 1.3 mV (n=7), respectively. The slope factor (s_a) for loaded cells showed significantly increased with respect to the control value. In contrary, depletion of cholesterol did not alter either of the parameters of steady-state activation. Neither cholesterol elevation in the membrane nor depletion from it resulted in a significant change of the equilibrium parameters of the recovery from the inactivation.

In summary, we presented that manipulation of cholesterol content in human T lymphocytes membrane profoundly affected the inactivation and activation gating of the predominant voltage-gated K^+ channel, Kv1.3. The increased viscosity of the membrane upon cholesterol enrichment can give explanation for the slowdown of activation kinetics: the increased "frictional force" between alkyl chains of phospholipids and the channel as well as between -helices retards the conformational change in the S1-S4 transmembrane region upon activation.

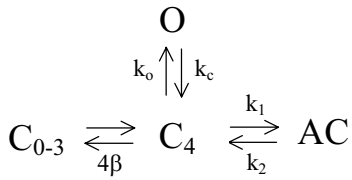
To explain the biphasic behavior of Kv1.3 activation we set up two models. According to the first, there exist two Kv1.3 channel populations located in different lipid environment with different voltage-dependent gating. If the slowly activating component represents channels with right-shifted voltage-dependent activation curve, our expectation is that the more depolarized potentials we use, the smaller the fraction of the slowly activating channel population will be. On the other hand, we may expect to experience biphasic decay for the inactivation of Kv1.3 currents. However, our results show that parameter ***R*** is independent of the test potential applied, which argues against this scenario.

Alternatively, we proposed a model for the activation of Kv1.3 that considers an additional closed state (AC: activated closed state, Scheme 1) and built on the work by Zagotta et al. (Zagotta et al. *J.Gen.Physiol.* 1990.). Consequently, we imagine that the appearance of the AC state is the consequence of the altered k_1 and k_2 rate constants (see Scheme 1) due the elevation in the

membrane viscosity: this state might be present in normal, physiological circumstances but the change in the ratio of the rate constants (k_1 and k_2) allow to detect it.

Kv1.3 channels undergo a slow C-type inactivation, which is believed to be the result of a complex conformational change in the S5-S6 segments and the P region. In contrast to the S1-S4 segments, the S5 and S6 together with the P region face to the protein environment, thus they are not directly affected by the change in the plasma membrane composition other than through the interaction between the individual -helices themselves. Hence, we argue that the interaction between the side chains of the residues on the S5-S6 segments and the first four ones retards the rearrangement necessary for the development of inactivation.

Scheme 1



2 What is the role of the fourth disulfide bridge in Pi1?

Toxin Pi1 purified from the venom of *Pandinus imperator* differs from the other fractions: it is stabilized by four disulfide bridges. Pi1 applied extracellulary inhibits the whole-cell current of T lymphocytes (1) it does not alter both kinetic and steady-state parameters of activation and inactivation; (2) inhibition is voltage independent; (3) considering 1 toxin molecule : 1 channel stochiometry the half blocking concentration is 11.4 nM.

Comparison of the 11.4 nM half blocking concentration of Pi1 to preliminary K_d values of 50 pM and 0.5 nM, published for Pi2 and Pi3, respectively shows that the four disulfide bridge containing Pi1 is the least effective toxin in the group. The observed differences in the Kv1.3 blocking ability of Pi1 and the other Pi's are most likely due differences in side chains and surface charge. The change of Tyr to Gly at position 11 and replacement of Arg with Cys at position 35

may play key roles because docking of the Pi toxins to a K^+ channel model showed that these amino acids are exposed at critical positions compared to the binding surface of the channel. The Arg to Cys change at position 35 also induces a large change in the local surface charge, because the positively charged Arg side chain is replaced by the negatively charged carboxyl-terminal in Pi1.

In summary, the fourth disulphide bridge in Pi1 did not influence the binding ability of the toxin to the Kv1.3 channels in human T lymphocytes. The less effective Kv1.3 block by Pi1 compared to Pi2 and Pi3 is due to differences in amino acid side chain properties at positions 11 and 35. Pi1 might be still an advantageous Kv1.3 blocker against its hindered channel binding ability, because the fourth disulfide bridge may confer extra stability to this peptide.

3 Drug- and mutagenesis-induced changes in the selectivity filter of a 2-pore background K^+ channel

The current of cTBAK-1 channels expressed in *Xenopus laevis* oocytes was K^+ selective and Goldmann-Hodgkin-Katz (GHK) rectified. In present study we demonstrated that fluoxetine (*FL*) and D-norpropoxyphene (*NORP*) affect the current of cTBAK-1 in different manner. Furthermore, utilizing mutagenesis experiments we proved that modification of certain amino acid residues in the selectivity filter of cTBAK-1 resulted in phenotypes possessing distinct pharmacological and basic properties.

NORP proved to act in a complex manner on the current of cTBAK-1: (1) inhibition of the outward current (IC_{50}=168 μM); (2) potentiation of the inward current; (3) change in the reversal potential (E_{rev}) (EC_{50} = 122 μM). The incomplete inhibition of the outward current component is due to a *NORP* induced change in selectivity of cTBAK-1. The ion-substitution experiments revealed that application of *NORP* elevated the permeability of the pore for Na^+ over K^+.

We investigated the block of *FL* in ND-96 on the mcTBAK-1 whole-cell currents using voltage-ramp protocols and we found that (1) both outward and inward currents were inhibited but not to the same degree, (2) the washout of the compound was not complete after several minutes, and most interestingly (3) the current-voltage relation curve was not shifted. In this series of experiments *FL* was very useful to demonstrate the specific effect of *NORP*, i.e. modification of the selectivity of cTBAK-1.

The hallmark of K^+-selective channels is their highly conservative P-region (signature sequence) that is considered to determine the permeation properties. Recently Ulens et al. have reported that *NORP* induces change in selectivity and gating of HERG, but not of Kv1.1 channels in *Xenopus* oocytes (Ulens et al. *Cardiov. Res.* 1999.). The selectivity filter of a cTBAK-1 channel contains two GYGH and two GFGD sequence motifs which represent a "transition" between the HERG (GFGN) and Kv1.1(GYGD) channels. Using PCR, we introduced mutations into the selectivity filter of the first and the second P-region to study the putitative *NORP*-channel interaction. In the first P-region, we mutated the Y in position 105 to F while in the second P-region (position 211), F was changed for Y to mimic the pore arrangement of HERG and Kv1.1 channels, respectively.

Mutations introduced into the first and second selectivity generated two basically different phenotypes. The change Y for F in the GYGH motif of the first pore resulted in a tetrameric construct that was unable to maintain a K^+-selective channel. *NORP* is incapable of changing E_{rev} since the mutation disrupted the ion-selectivity filter, thereby increasing Na^+ permeability. Contrary, the mutant F211Y shared the same electrophysiological properties as the wild-type but it became less sensitive to *NORP*.

In conclusion, we have taken advantage of the specific manner of action of two drugs used in human medicine, to produce different phenotypes of the cloned 2-pore mcTBAK-1 K^+ channel expressed in *Xenopus* oocytes. To date, and to our knowledge, no drugs have been reported that

could modify the selectivity of this emerging family of leak K^+ channels. Based on a pharmacological approach on the one hand, on a mutagenesis study on the other hand, this work also highlights the significance of the crucially important GYGD sequence in this family of 2-P channels.

5 Summary

1 We demonstrated that the modification of the cholesterol content in the T lymphocyte membrane altered the kinetic and steady-state parameters of the gating of Kv1.3. The activation and inactivation slows upon increase of the cholesterol content, while the depletion of cholesterol from the plasma membrane does not have significant effect on either. Furthermore, the elevation of cholesterol level resulted in the biphasic activation of Kv1.3 current. We proposed a model that explains the biphasic activation with the appearance of an activated-closed state due to the change in rate constants. Further experiments are necessary to reveal the physiological relevance of our findings, especially, the possibility of compartmentalization of Kv1.3 in lipid rafts.

2 Pi1, a toxin purified from the venom of *Pandinus imperator* is stabilized by four disulfide bridges. We showed that the fourth disulphide bridge in Pi1 does not influence the binding ability of the toxin to the Kv1.3 channels in human T lymphocytes. The less effective Kv1.3 block by Pi1 compared to Pi2 and Pi3 is due to differences in amino acid side chain properties at positions 11 and 35, both of them play crucial role in the formation of channel-toxin complex.

3 We have taken advantage of the specific manner of action of two drugs used in human medicine (fluoxetine and D-norpropoxyphene), to produce different phenotypes of the cloned 2-pore mcTBAK-1 K^+ channel expressed in *Xenopus* oocytes. To date, and to our knowledge, no drugs have been reported that could modify the selectivity of this emerging family of leak K^+ channels. Based on a pharmacological approach on the one hand, on a mutagenesis study on the other hand, this work also highlights the significance of the crucially important GYGD sequence in this family of 2-P channels.