

Ph.D. THESES

**IMMUNOPHARMACOLOGICAL INVESTIGATION OF
THE HUMAN LYMPHOCYTE KV1.3 CHANNEL USING
PEPTIDE TOXINS**

MÓZES PÉTER



**UNIVERSITY OF DEBRECEN
MEDICAL AND HEALTH SCIENCE CENTER
DEPARTMENT OF BIOPHYSICS AND CELL BIOLOGY
DEBRECEN, 2001.**

**IMMUNOPHARMACOLOGICAL INVESTIGATION OF
THE HUMAN LYMPHOCTE KV1.3 CHANNEL USING
PEPTIDE TOXINS**

MÓZES PÉTER

**UNIVERSITY OF DEBRECEN
MEDICAL AND HEALTH SCIENCE CENTER**

DEPARTMENT OF BIOPHYSICS AND CELL BIOLOGY

DEBRECEN, 2001.

I. INTRODUCTION

The voltage-gated potassium channel, Kv1.3, plays an important role in T-cell physiology because of its involvement in the regulation of the membrane potential of these cells thereby influencing T-cell activation in vitro. K⁺ channel blocker scorpion toxins together with complementary mutagenesis of Kv1.3 proved to be excellent tools in studying structure-function relations and defining binding sites for the blockers on the ion channel. Recently, Kv1.3 channel blocker Margatoxin has been shown to be effective even in suppressing T-cell function in vivo. The above results suggest that Kv1.3 may be an excellent target for modulating immune system functions by specific and potent blockers.

During the search for more potent K⁺ channel inhibitors the whole venom of scorpion *Pandinus imperator* (PiV) has been applied to the voltage-gated potassium channels of nerve fibers and GH3 cells. The effects of the *Pandinus venom* was shown to be different from other known K⁺ channel scorpion toxins in their marked voltage dependence and poor reversibility of the block on these channels.

Several new K⁺ channel blocking peptides named Pi1-Pi7 were purified to homogeneity from the venom of *Pandinus imperator* and their primary structures were determined. Toxins Pi2 and Pi3 are very closely related in their structure, both contain three disulfide bridges and their sequences differ only in one amino acid at position 7 (proline in Pi2 vs. glutamic acid in Pi3) in the N-terminal region. Pi2 and Pi3 bear 7 and 6 net positive charges, respectively. The three-dimensional structure of Pi2, under the name of PTX K- α , was determined by NMR spectroscopy.

The whole *Pandinus imperator* venom efficiently reduces whole-cell K⁺ currents in human peripheral blood lymphocytes. Preliminary experiments also showed that purified peptide components Pi2 and Pi3 of the venom powerfully blocked Kv1.3 channels in human peripheral blood lymphocytes with approximate K_d values of 50 pM and 500 pM, respectively.

Introduction

The single amino acid difference between Pi2 and Pi3 and the large difference in their blocking potency for Kv1.3 provides excellent background for studying the structure–function relationship of current block by these toxins. Analysis of factors influencing toxin binding to Shaker and Ca²⁺ activated K⁺ channels include long–range non–specific electrostatic interactions, short–range electrostatic interactions and close contact steric interactions. The contribution of these interactions to toxin binding requires the analysis of blocking kinetics, the determination of association and dissociation rates and the ionic strength dependence of the rate constants. We applied this approach to determine the difference between the binding of Pi2 and Pi3 to Kv1.3 channels. In addition to patch–clamp experiments we analyzed the results in the view of differences found between the three dimensional structures of these toxins, as well as between those of relevant charybdotoxin (ChTx) mutants.

II. OBJECTIVES

1. How the *Pandinus imperator* full venom (PiV) influences the function of human lymphocytes voltage–gated K⁺ channels?
2. Which peptide components of the full venom have effect on the ion channels, what is their effect on the channels and in what concentration range do they influence the function of Kv1.3 channels? How do the toxins influence the membrane potential of human lymphocytes?
3. Is there a peptide component which binds to human lymphocyte voltage–gated K⁺ channels irreversibly?
4. Is there any effect of the toxins on the gating of Kv1.3?
5. Which parts of the interaction between the toxin and the channel protein are responsible for the binding and the modification of channel function?
6. What mechanisms are responsible for the binding of the toxin molecule to the channel protein?

III. MATERIALS AND METHODS

Cells: Heparinized human peripheral venous blood was obtained from healthy volunteers. Mononuclear cells were separated and cultured. The culture medium contained 2.5 or 5 µg/ml of phytohemagglutinin.

Results

Electrophysiology: Whole-cell measurements were carried out using Axopatch-200 and Axopatch-200A amplifiers. For data acquisition and analysis the pClamp6 software package was used. T lymphocytes were selected for current recording by incubation with mouse anti-human CD2 followed by selective adhesion to Petri dishes coated with goat anti-mouse IgG antibody. Standard whole cell patch clamp techniques were used. Series resistance compensation up to 85% was used to minimize voltage errors and achieve good voltage clamp conditions ($V_{err} < 5\text{mV}$). Pipettes were pulled from GC 150 F-15 borosilicate glass capillaries. 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, ionic strength=190.5 mM). The high ionic strength external solution was (in mM): 77.5 NaCl, 5 KCl, 48.6 MgCl_2 , 2.5 CaCl_2 , 5.5 glucose, 10 HEPES (pH 7.35, ionic strength=265.9 mM). The measured osmolality of the external solutions was between 302 and 308 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).

Test substances: *Pandinus imperator* venom was obtained from anesthetized animals by electrical stimulation. To separate the peptide toxin components the soluble venom was initially fractionated in a Sephadex G-50 column and the subfractions were further fractionated by high performance liquid chromatography. Solutions were supplemented with 0.1 mg/ml bovine serum albumin to suppress nonspecific binding of the toxins to the walls of the tubes and to the Petri dish. Bath perfusion around the measured cell with different test solutions was achieved using a gravity flow perfusion setup. For the measurement of blocking kinetics of toxins data acquisition was synchronized to fluid exchange using solenoid valves controlled by pClamp6 via digital outputs of Digidata 1200. The reference electrode was connected to the recording chamber with an agar bridge to eliminate junction potential changes during perfusion.

IV. RESULTS

Investigation of the Pandinus imperator venom

Human peripheral lymphocytes display a strong outward whole-cell K^+ current upon adequate depolarization. The voltage-gated K^+ channels open and the whole-cell K^+ current reaches its peak in a few milliseconds. After the peak, because of the inactivation of the channels the amplitude of the whole cell K^+ current decreases to a low steady state level. According to previous studies PiV irreversibly blocks K^+ channels of myelinated frog nerve fiber and GH_3 cells. On these cells the block by PiV is voltage dependent. This implies that the binding site of the toxin is deeply immersed into the transmembrane electric field. According to

Results

our experiments on human peripheral lymphocytes adding 20 ng/ml PiV to the external solution the amplitude of the K⁺ current decreases significantly.

Several blocking experiments were done with different concentrations of PiV in the 0.0001–100 µg/ml range. The dose–response curve could be fitted with the one channel – one toxin molecule model. In this model the remaining fraction of current (I/I_o) can be calculated with the following formula: $I/I_o = K_{df}/(K_{df} + [PiV])$, where I_o is the control peak current, I is the peak current during the application of PiV, K_{df} is the apparent dissociation constant and $[PiV]$ indicates the concentration of the *Pandinus imperator* venom. The dissociation constant is apparent because here the solution contains not only one toxin molecule, but also a full venom with different toxins. The good fit indicates that one toxin molecule binds to one channel protein and blocks the channel. The half blocking concentration of the toxin is 14 ng/ml.

From the full venom 8 different toxin molecules were isolated and named Pi1..Pi8. They contain 27–38 amino acids and their molecular weight is between 3795 and 4370. They contain 3 or 4 disulfide bridges. We investigated the effect of the toxins on the Kv1.3 channel and determined their dissociation constants. Their affinities to Kv1.3 proved to be different to a great extent.

We further investigated toxin component Pi2, because of its high affinity to Kv1.3 channels ($K_{dPi2}=44$ pM). The primary structure of toxin component Pi2 and Pi3 differ only in one amino acid: in Pi3 the 7. amino acid is prolin instead of glutamate. This results in an 18 fold increase of the K_d as determined from the equilibrium block of the channels.

After characterizing the block of whole–cell K⁺ current we investigated the effect of PiV on the gating of the channels. On the voltage dependence of K⁺ conductivity and on the activation and inactivation kinetics of Kv1.3 channels PiV has no effect. However, we found significant effect on the recovery from inactivation of Kv1.3 channels. During the depolarization the amplitude of the whole–cell K⁺ current decreases to a steady state level because the inactivation of the channels. Applying a second test pulse before the channels recover from the inactivated state results a lower peak whole–cell current. The recovered fraction (RF) of the channels can be calculated using following formula:

$$RF = (P_2 - SS_1) / (P_1 - SS_1),$$

where P_1 and P_2 are peak current values observed during the first and second pulses, respectively, and SS_1 is the steady state current at the end of the first pulse. We determined the recovery time constants by plotting the recovered fractions as the function of interpulse interval and fitting a single exponential function: $RF(t) = 1 - \exp(-t/T)$ to the data points. Our results show that the recovery from inactivation of Kv1.3 channels becomes faster in the presence of PiV. This means

Results

that toxin component of PiV remain bound to the inactivated channel and destabilizes this conformation.

Pi2 binds to Kv1.3 with high affinity

Dose dependence of equilibrium K^+ channel block by Pi2 was determined by measuring the peak whole-cell K^+ current at +50mV in control bath solution (I_o) and then in solutions containing Pi2 at different concentrations (I). In order to verify that equilibrium block was reached, short (50ms duration) depolarizing pulses to +50mV were applied every 50s. The peak current in the presence of the toxin (I) was calculated by averaging 3–4 peaks at equilibrium block. From I_o and I the remaining fraction of the current ($R=I/I_o$) was calculated and plotted as a function of toxin concentration. The resulting dose response curve could be fitted by equation $R=K_d/(K_d+[Tx])$ describing a model where one toxin molecule binds to one channel protein. In the above formula R indicates the remaining fraction of the whole-cell current, $[Tx]$ is the extracellular toxin concentration and K_d is the dissociation constant characterizing the drug-channel interaction. The excellent fit obtained supports a mechanism in which one toxin molecule binds to one channel protein and plugs the pore. As the result of the fitting procedure $K_d = 44$ pM was obtained.

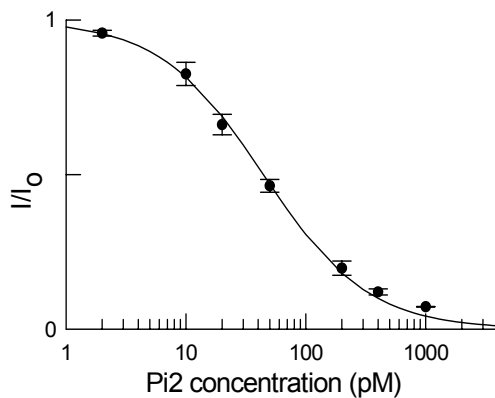


Figure 1.: Dose dependence curve of Pi2. The unblocked fraction of current (I/I_o) was plotted as the function of Pi2 concentration. I_o is the peak current in the control solution and I is the peak current after the application of Pi2. The dose response curve could be fitted by the equation $I/I_o = K_d/(K_d+[Pi2])$, where $[Pi2]$ indicates the concentration of Pi2 toxin and K_d indicates the dissociation constant of Pi2. As the result $K_d = 44$ pM was obtained.

Block by Pi2 and Pi3 is voltage independent

Pappone et al. reported previously a voltage dependent action of the whole soluble venom on delayed rectifier K^+ channels of myelinated frog nerve fiber. Pi2 bears net 7 positive charge and has a lysine in position 24 equivalent to the lysine in position 27 of ChTx, which is critical for interaction with Shaker potassium

Results

channels. However, other charged and uncharged amino acids in several positions influence their binding affinity. To further examine the reasons for the above mentioned difference in the voltage dependence of the K^+ channel block by the *Pandinus imperator* venom we tested the effect of toxins Pi2 and Pi3 purified from the whole venom on the voltage dependence of the block. We determined the remaining fraction of whole-cell current in the presence of 50 pM Pi2 at various membrane potentials. Current voltage relations on Kv1.3 show that the percentage of block by Pi2 is independent of the applied test potential. Similar results proved the voltage independence of the Pi3 block. The voltage independence of the block in both cases implies that the binding site of the highly charged toxin component on Kv1.3 is not deeply immersed into the transmembrane electric field. Alternatively, the rate of obtaining a new equilibrium at different depolarized membrane potentials is not fast enough to show the voltage dependence of the block measured from the peak current.

Association and dissociation of Pi2 and Pi3 is very fast

K^+ channel block by Pi2 and Pi3 is reversible, because after washing toxin treated human T lymphocytes with drug free extracellular solution the whole-cell K^+ current returns to its original amplitude. The very low dissociation constant of Pi2 and the fact that its blocking effect was fully reversible by washing in less than 15 minutes, suggested that the on and off rates of the toxin are very high. Therefore, we examined the association and dissociation rates of Pi2 to the channel protein by determining the wash-in and wash-out kinetics of the drug on the Kv1.3 channels of human T lymphocytes in whole-cell configuration. In order to achieve a good time resolution we used depolarizing pulses lasting for 8 ms only, and applied at every 15 s. This pulsing rate did not induce cumulative inactivation since identical peak amplitudes were obtained after wash-out compared to those measured at the beginning of the wash-in process. The gravity driven flow of extracellular solution was switched on and off by electromagnetic valves controlled by the data acquisition apparatus.

The measurement of the time course of current block by the toxin was started in whole-cell configuration after the peak amplitude stabilized. Whole-cell K^+ currents recorded after switching to an extracellular solution containing 50 pM Pi2 and peak values were plotted as a function of time and fitted to a single exponential function: $A(t)=B \times \exp(-t/T_{ON})+C$, where $A(t)$ indicates the amplitude of the measured current at time t , C is the peak current at equilibrium block and $B=A_{(t=0)}-C$. The time constant (T_{ON}) describing the wash-in kinetics of Pi2 yields 58 ± 7 s ($n=7$). After reaching equilibrium block of the whole-cell current the perfusion was switched back to the drug free control solution, while the voltage-clamp protocol recording the whole-cell current was running continuously.

Results

Peak currents recorded during the wash-out procedure were plotted the same way as above and were also fitted to a single exponential function: $A(t)=B\times(1-\exp(-t/T_{\text{OFF}}))+C$, where $B=A(t=\infty)-C$, $A(t)$ and C were defined above. The resulting time constant (T_{OFF}) was 158 ± 21 s ($n=7$) in this case.

K^+ channel block by Pi3 was also found to be reversible. We determined the time constants of block and unblock by 500 pM Pi3 using the same method as above. For Pi3 the values of T_{ON} and T_{OFF} were 53 ± 3 s ($n=8$) and 80 ± 6 s ($n=7$), respectively. From the measured time constants and assuming a simple bimolecular reaction between the toxin and the channel, k_{ON} , k_{OFF} and K_d were expressed as follows:

$$k_{\text{ON}} = \frac{1 - T_{\text{ON}} \times k_{\text{OFF}}}{T_{\text{ON}} \times [\text{Tx}]}, \quad k_{\text{OFF}} = \frac{1}{T_{\text{OFF}}}, \quad K_d = \frac{k_{\text{OFF}}}{k_{\text{ON}}}.$$

Electrostatic interactions influence the binding of Pi2 and Pi3 to Kv1.3

In order to study the influence of through-solution electrostatic interaction between the cationic toxin and the anionic mouth of the ion channel on the kinetics of ion channel blockade the effect of increasing ionic strength of the extracellular solution was assayed. In high ionic strength solution, the concentration of divalent magnesium ions was raised in the recording solution to 48.6 mM while the osmolarity of the solution was kept constant for undisturbed patch-clamping of human lymphocytes. The isosmotic substitution resulted in a 1.4 fold increase in the ionic strength. We determined the dissociation constants, the on and the off rates of Pi2 and Pi3 in high ionic strength solution and compared them to the ones measured in control solution. In the high ionic strength solution on rates decreased 32 fold for both toxins, when off rates increased 2.8 and 1.4 fold altogether causing 88 and 46 fold increased dissociation constants for Pi2 and Pi3, respectively.

3D structure of Pi3

Based on the parameters obtained from NMR spectroscopy data of Pi2, a model of the three-dimensional structure of Pi3 was generated. This independently generated model was fully compatible with the recently published 3D NMR structure of Pi3.

Pi3 is a natural mutant (Pro-7-Glu) of Pi2, in which the substituted proline for glutamic acid in position 7 does not modify the folding pattern of the molecule. However there is an 18 fold difference between their dissociation constants. This fact can have two different explanations:

Results

1. This amino acid plays an important role in the association to the channel protein and the glutamic acid in Pi3 interacts with a negatively charged amino acid in the channel protein.

2. This negatively charged amino acid interacts with an other side chain in the toxin molecule.

The proline for glutamic acid in position 7 in Pi3 does not modify the folding pattern of the molecule, on the contrary, the distances and angle torsion constraints are quite compatible with the formation of a salt bridge with Lys24. Lys24 of Pi2 and Pi3 is at the equivalent structural position of Lys27 in ChTx, and Lys28 in noxiustoxin. Both lysines were shown to be essential for binding to the K-channels studied.

Comparing the 3D structure of Ser10Asp mutant ChTx to Pi3, the natural mutant of Pi2

Earlier work performed by the group of Miller showed that a mutant of ChTx containing an aspartic acid in position 10, substituting for serine, was capable of recognizing the channel with 1500 fold less affinity, compared to native ChTx. When we aligned the amino acid sequences of these peptides in search for the best similarity, we observed that Ser10 of ChTx corresponds to position 7 in Pi2, where the glutamic acid substitutes for proline in Pi3.

Thus, the three dimensional models of wild type ChTx and its mutant containing aspartic acid were constructed on the basis of literature data and compared to our results. The Asp10 has a spatial geometry compatible with a salt bridge formation with Lys27 in the mutant ChTx.

Pi2 alters the kinetics of recovery from inactivation of Kv1.3 channels

Current block by PiV is associated with a significant enhancement of the rate of recovery from inactivation of Kv1.3. We extended this study to the purified Pi2 and Pi3 peptides of the venom. In the presence of Pi2 the recovery from inactivation of human lymphocyte Kv1.3 channels became faster. During the experiments we applied 1 s long depolarizing pulse pairs with various interepisode intervals ranging from 1 to 60 s. The 1 s long test pulse was enough for reaching steady-state amplitude for the whole-cell current. Time between pulse pairs was 80 s at a holding potential of -120 mV to allow full recovery of the channels. We calculated the fraction of channels recovered from the inactivated state (RF) during the varying interpulse intervals using the formula $RF = (P_2 - SS_1) / (P_1 - SS_1)$, where P_1 and P_2 are peak currents detected during the first and the second pulses in the pair, respectively

Results

and SS_1 is the steady-state amplitude of the current during the first pulse. We determined the recovery time constant by plotting the recovered fractions as the function of interpulse interval and fitting a single exponential function: $RF(t)=1-\exp(-t/T)$ to the data points. Recovery from inactivation was significantly accelerated in the presence of 50 pM Pi2, because the time constant characterizing it decreased from 15.42 ± 1.87 s (control) to 10.42 ± 1.04 s ($p<0.0028$, $n=6$).

One amino acid difference between Pi2 and Pi3 caused an 18-fold difference in their affinity for Kv1.3 as determined from the equilibrium block of the channels. We were interested to know if the ability of Pi2 and Pi3 to speed up recovery from inactivation of Kv1.3 were different. Any such existing difference could be immediately attributed to the mutated amino acid in position 7 (Glu for Pro), between the two toxins.

We have shown that recovery from inactivation was a single exponential process. Therefore, in order to characterize the effect of toxins on the recovery kinetics, it was sufficient to compare RF values obtained at fixed time intervals under control conditions and after the application of Pi2 or Pi3. We have chosen 7 s interpulse interval and applied close to half blocking concentrations of Pi2 (44 pM) and Pi3 (500 pM). We used the 7 s long inter-episode time because by this time the recovered fraction of the current was close to 0.5. We calculated the recovered fractions of the channels in the control extracellular solution and in solutions containing Pi2 and Pi3. The recovered fraction of the K^+ current during the 7 s long inter-episode time was 0.53 ± 0.03 in the absence and 0.56 ± 0.03 in the presence of 500 pM Pi3 ($p<0.099$, $n=6$). Using the same protocol the recovery from inactivation was accelerated by Pi2 resulting in a recovered current fraction 0.53 ± 0.05 compared to the control value of 0.45 ± 0.02 . The latter difference proved to be significant (paired t-test, $p<0.009$, $n=5$). The observed difference in the ability of Pi2 and Pi3 to speed up the recovery from inactivation of Kv1.3 can only be associated to the seventh amino acid in the toxins, which is the only different amino acid in these peptides.

Pi2 depolarizes the cell membrane

Voltage-gated K^+ channels play an important role in the regulation of membrane potential of human lymphocytes. Opening of these channels causes an increase of the K^+ permeability and hyperpolarisation. Blocking these channels results in membrane depolarization, which hinders the activation of lymphocytes. We investigated the effect of Pi2 on the membrane potential of human lymphocytes. While the membrane is depolarized the initial steps of the lymphocyte activation are blocked, this way by influencing the membrane potential of lymphocytes the lymphocyte activation can be specifically blocked. Using the Oxonol dye we

Discussion

determined the membrane potential of human lymphocytes in control solution and in solution containing 50 pM Pi2. Already 1.5 minutes after the administration of Pi2 the depolarization is noticeable. This result corresponds to our patch-clamp measurements.

V. DISCUSSION

In the present work we describe the effect of the full venom and the peptide toxin components of the venom of scorpion *Pandinus imperator* on Kv1.3 voltage-gated K⁺ channel of human T lymphocytes. We further investigated Pi2 as it had the highest affinity among the toxins indicated by a very low dissociation constant and Pi3 because of its very similar structure to Pi2.

In general, channel blockers can be pore blockers or gating modifiers. Pore blockers bind to the channel in 1:1 stoichiometry and plug the pore of the channel impeding the flow of the ionic current. Our data suggest that both Pi2 and Pi3 belong to the pore blocker family. This fact is supported by the following properties of the equilibrium block and the blocking kinetics of the compounds. The dose response curve can be fitted well to a function describing a one channel – one blocker model. We also investigated the wash-in and wash-out kinetics of the toxins and found that both of them follow a single exponential time course. This behavior is characteristic of first order (dissociation) and pseudo first order (association) bimolecular reactions.

We determined the dissociation constant of Pi2 from both the equilibrium block and the on and off rates of the toxin molecules binding to the channel protein. The first approach resulted in a $K_d=44$ pM for Pi2, whereas the calculated K_d from the on and off rates was 29 pM. These data are comparable and support the 1:1 stoichiometry of Pi2 binding to Kv1.3 channels. We also determined the dissociation constant of Pi3 using both methods. The K_d of Pi3 was 795 pM according to the equilibrium method and 970 pM calculated from the on and off rates, again leading to a reasonable agreement of the results.

Discussion

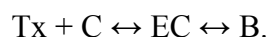
If the binding site of a channel blocker is deeply immersed into the electric field of the cell membrane, and the blocker is a charged particle like Pi2, the block may show voltage dependence. The whole soluble venom of scorpion *Pandinus imperator* showed highly voltage dependent block on voltage-gated K⁺ channels of nerve fibers. Our experiments, however, did not show voltage dependence of the block by Pi2 and Pi3 on voltage-gated K⁺ channels of human T lymphocytes, although both toxins were positively charged pore blockers of the channels. This is not surprising if we consider the time course of channel activation and the on and off rates of the block. The time needed to reach the peak of the current depends on the amplitude of the test pulse but it is always in the ms range. On and off rates determined experimentally were in the order of seconds. The block of Kv1.3 channels by Pi2 and Pi3 may be voltage dependent although this fact is not seen in our results, where we determined the remaining fractions of the K⁺ current from the peak amplitudes measured at different test potentials. It can be assumed that the peak of the current is reached much faster during a test pulse than the time necessary for the development of a new equilibrium of block at the currently applied test potential. Indeed, Goldstein and Miller studied the voltage dependence of current block by several low affinity mutants of ChTx (e.g. Y36A) having high off rate. The voltage dependence of block in those cases could be determined from equilibration of block during a single depolarizing pulse.

The only difference between Pi2 and Pi3 is the presence of a negatively charged amino acid in Pi3 (Glu7), resulting in an 18 fold increase of the K_d as determined from the equilibrium block of the channels. The analysis of blocking kinetics of Kv1.3 channels isolated that the 17-fold decrease of Pi3 on rate was primarily responsible for the increase of K_d, since the change of the off rate was negligible (only 2-fold increase over Pi2). The minimal increase of Pi3 off rate indicates that the Pro to Glu change did not alter the steric interactions between the channel and the toxin. This means that steric hindrance for binding to Kv1.3 was not generated by the amino acid substitution.

Discussion

At the same time, the Pro-7-Glu mutation in Pi3 results in a decrease in the net positive charge of the toxin, which might influence the long-range electrostatic interaction between the channel and the toxin. Several authors discussed previously the importance of through-space electrostatics in toxin binding by introducing charge neutralization or charge reversal mutations either in the channel vestibule or in the toxin. Simple electrostatics predicts that charge alterations should be manifested in the association rate of the toxin. Indeed, mutations decreasing the net charge of ChTx and Iberitoxin decreased the toxin on rates. Similarly, substituting acidic residues for neutral or basic ones near the toxin binding site (position 422 in Shaker) also decreased the association rate of ChTx and toxin Lq2. Our experiments showed that the association (on) rate of Pi3 (bearing net 6 positive charges) was 17-times smaller than that of Pi2 (bearing net 7 positive charges), while the off rates were comparable. So far our results are compatible with the predictions from through space electrostatics. This model, however, also predicts that (i) increasing the ionic strength of the recording solution should decrease the association rate of the toxins, and (ii) the ionic strength dependence of the association rate should be more pronounced for Pi2 (+7 charges) as compared to Pi3 (+6 charges). Our results showed that the on rate of both Pi2 and Pi3 was reduced when the ionic strength was raised. At the same time, the association rate of Pi2 and Pi3 was equally sensitive to the ionic strength of the medium, the 1.4-fold increase in the ionic strength resulted in a 32-fold decrease in the association rate of both toxins. These results pointed out that through-space electrostatics is an important factor for toxin binding but glutamic acid residue at position 7 in Pi3 does not contribute significantly to the overall long-range electrostatic interaction, similarly to ChTx mutations studied by Park and Miller. Thus, through-space electrostatics can not account for the decreased association rate of Pi3.

So what can be the explanation for the reduced on rate of Pi3? To answer this question the model of the three-dimensional structure of Pi3 was generated based on the parameters obtained from NMR spectroscopy data of Pi2. This model showed that glutamic acid in position 7 (Pi3) is likely to form a salt bridge with Lys24. Lys24 of Pi2 and Pi3 is at the equivalent structural position of Lys27 in ChTx that is essential for toxin binding to K^+ channels. In order to examine the effect of the salt bridge between Glu7 and Lys24 we applied the analysis of non diffusion-limited bimolecular toxin-channel interactions used by Escobar et al. and Mullmann et al.. This approach considers a two step reaction for toxin binding:



Discussion

The first step includes diffusion of the toxin (Tx) up to and away from the encounter complex (EC). This step accounts for the local electrical potential and ionic strength dependence of the association rate (see above). The EC represents an infinite number of possible contact orientations formed between the toxin (Tx) and the channel (C) from which the bound state of the toxin (B) is formed in the second step. In non-diffusion controlled reactions this second step is the rate limiting and might include the rearrangement of amino acid side chains, formation of hydrogen bonds and squeezing water molecules and cations out of the vestibule to form the tightly bound (non conducting) toxin-channel complex.

The second (binding) step would allow specific short-range electrostatic interactions between specific basic residues on the toxin and cations in the vestibule. Accordingly, Mullmann et al. argued that neutralization of Lys27 and Arg34 reduces the ability of IbTx mutants to repel cations from the vestibule, which would account for the 10- to 20-fold slower association rate of Lys-27-Gln, Lys-27-Asn and Arg-34-Asn mutants. Our results are similar to these data both qualitatively and quantitatively. Thus, our interpretation of the reduced on rate of Pi3 is that the intramolecular salt bridge formed between Glu7 and Lys24 reduces local positive electrostatic potential around Lys24 resulting in decreased short-range electrostatic interactions during the binding step. This might lead to a decreased association rate by a similar mechanism to the one described above for IbTx mutants.

The ϵ -amino group of Lys-27 in ChTx protrudes slightly into the K^+ conduction pore, where it interacts with conducting ions. Does the salt bridge between Lys24 and Glu7 break up during the binding step to allow Lys24 to interact freely with the ion channel? The overall free energy change calculated for the binding of Pi2 and Pi3, based on the K_d , k_{ON} and k_{OFF} values obtained is 58.5 kJ/mol for Pi2 and 51.4 kJ/mol for Pi3. The difference is in the order of 7 kJ/mol, a value that is not enough to break a salt bridge (approx. 15 kJ/mol). This means that besides the salt bridge, other interactions are implicated in the overall free energy change observed, either through the interaction of the carboxylate of the glutamic acid with some other residues in the channel, or even with the formation of hydrogen bonds or van der Waals interactions between residues of the toxin and the channel. The overall free energy change due to these interactions may easily compensate for the free energy change during the breaking of the salt bridge. It is certainly true that the overall free energy change for Pi2 is higher than Pi3 and thus it goes into the right direction of the expected results, assuming our proposition of salt bridge formation in Pi3 and its breaking during the interaction of the toxin with the channel.

Discussion

The influence of salt bridge formation on binding of Pi3 to Kv1.3 is further substantiated by a comparison of the present problem to the binding of a ChTx mutant to a similar ion channel, the Shaker K⁺ channel. A considerable difference between the affinity of the wild type ChTx and its Ser-10-Asp mutant has been observed to the Shaker K⁺ channel. According to the sequence alignment positions 7 and 24 in Pi2 correspond to positions 10 and 27 in ChTx, respectively. Mutation of Ser to an acidic residue (Asp) in position 10 of ChTx resulted in a 1500 fold increase in the equilibrium dissociation constant for the Shaker channel. At the same time the 3D molecular modeling performed by us pointed out the existence of a salt bridge between the Asp10 and Lys27 positions in the Ser-10-Asp mutant ChTx similarly to the salt bridge between corresponding positions in Pi3. Thus, a similar mechanism, as discussed above for Pi3, can account for the observed high K_d of the Ser-10-Asp mutant ChTx.

Our salt-bridge hypothesis is strongly supported by a recent paper in which the three dimensional structure of Pi3 was determined by NMR spectroscopy and compared to that of Pi2. They determined that the overall folding pattern of Pi3 was very similar to Pi2. In addition, a salt bridge between Glu7 and Lys24 of Pi3 was described. Based exclusively on NMR spectroscopy data the authors attributed the reduced affinity of Pi3 to the intramolecular salt bridge between Glu7 and Lys24.

Since our three dimensional models of Pi2, Pi3 and ChTx showed the possibility of salt bridge formation between acidic residues of position around 7–10 with the lysine in position 24–27, we looked into the literature for other homologues. Recently, a paper by Tytgat et al. proposed a generalized nomenclature for K-scorpion toxin sub families, and listed 49 complete amino acid sequences (now 50). Examination of all these peptides suggests that none of them (except Pi3) would qualify for such salt bridge formation. The only other two toxins with acidic residues around residue 10 are two novel toxins, PBTX1 and PBTX2 purified from scorpions of the genus *Parabuthus* of South Africa. These toxins belong to sub family 11 and were reported to have a low affinity for K⁺ channels, however they contain a valine in the position 26 (equivalent to Lys27 of ChTx), which would make impossible a salt bridge formation in this spatial location.

Recently, we found that the whole soluble venom of the scorpion *Pandinus imperator* accelerates the recovery from inactivation of Kv1.3 channels. We investigated the same effect for purified Pi2, which is the most effective peptide component of the *Pandinus imperator* venom on Kv1.3 channels. We found that Pi2 accelerates the recovery from inactivation of Kv1.3 channels. This means that the toxin remains bound to the inactivated channel and destabilizes this conformation. The ability of Pi2 to speed up the recovery from inactivation of Kv1.3 channels is remarkable.

Discussion

The kinetics of dissociation of Pi2 and Pi3 from Kv1.3 is relatively fast, the characteristic time constants are ~180 s and 58 s, respectively. The fast dissociation provides that during the recovery process substantial number of on–off reactions take place to account for the enhanced recovery from inactivation measured in the presence of Pi2. We compared the ability of Pi2 and Pi3 to speed up the recovery from inactivation of human lymphocyte voltage–gated K⁺ channel. Recovery from inactivation is enhanced by Pi2, but not by Pi3. This means that the single amino acid difference between the two toxins affects the ability of enhancing the recovery rate of Kv1.3. We do not know yet the nature of molecular interactions responsible for this difference. Due to the complexity of C–type inactivation and recovery several mechanisms might be involved. For example toxins bound to the channel pore might alter diversely the extracellular K⁺ modulatory site of recovery from inactivation.

In conclusion we described the effects of two potent and closely related peptide toxins on the Kv1.3 channels in human T lymphocytes and gave an explanation for the observed differences in their channel blocking ability and binding kinetics on the basis of the differences in their 3D structures and by comparing them to structurally related ChTx mutants. Due to its remarkably low dissociation constant of 44 pM, Pi2 may provide a good model for developing a non– peptide drug which could be used to achieve immunosuppressive effect by specifically blocking human lymphocyte voltage–gated K⁺ channels.

VI. SUMMARY

The voltage-gated Kv1.3 potassium channel plays an important role in the activation of lymphocytes. Blocking these channels depolarizes the membrane and consequently inhibits the mitogenic activation of lymphocytes. We used peptide toxin components of the *Pandinus imperator* scorpion venom in order to investigate the interactions between the channel and the blocker. *Pandinus imperator* scorpion toxins Pi2 and Pi3 differ only by a single amino acid residue (neutral Pro7 in Pi2 vs. acidic Glu7 in Pi3). The binding kinetics of these toxins to human Kv1.3 showed that the decreased on rate ($k_{ON} = 2.18 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ for Pi2 and $1.28 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for Pi3) was almost entirely responsible for the increased dissociation constant (K_d) of Pi3 ($K_d = 795 \text{ pM}$) as compared to Pi2 ($K_d = 44 \text{ pM}$). The ionic strength dependence of the association rates was exactly the same for the two toxins indicating that through-space electrostatic interactions can not account for the different on rates. Results were further analyzed on the basis of the three-dimensional structural models of the toxins. A 3D structure of Pi3 was generated from the NMR spectroscopy coordinates of Pi2 by computer modeling. The Pi3 model resulted in a salt bridge between Glu7 and Lys24 in Pi3. Based on this finding our interpretation of the reduced on rate of Pi3 is that the intramolecular salt bridge reduces the local positive electrostatic potential around Lys24 resulting in decreased short-range electrostatic interactions during the binding step. To support our finding, we constructed a 3D model of the Ser-10-Asp Charybdotoxin mutant displaying distinctly reduced affinity for Shaker channels. The mutant Charybdotoxin structure also displayed a salt bridge between residues Asp10 and Lys27 equivalent to the one between Glu7 and Lys24 in Pi3.

VIII. PUBLICATIONS:

Publications on the subject of the thesis:

1. Péter, M., jr., Varga, Z., Panyi, G., Bene, L., Damjanovich, S., Pieri, C., Possani, L.D. and Gáspár, R., jr. *Pandinus imperator* scorpion venom blocks voltage-gated K^+ channels in human lymphocytes. *Biochem. Biophys. Res. Commun.* 242. 621–625, (1998)

IF: 3,161

2. Péter, M., jr., Varga, Z., Hajdu, P., Gáspár, R., jr., Damjanovich, S., Horjales, E., Possani, L.D. and Panyi, G. Effects of toxins Pi2 and Pi3 on human T lymphocyte Kv1.3 channels: the role of Glu7 and Lys24 J. *Membrane Biol.* 179. 13–25, (2001)

IF: 3,187

Publications

Other publications:

1. **Péter, M., jr.**, Hajdu, P., Varga, Z., Damjanovich, S., Possani, L.D., Panyi, G. and Gáspár, R., jr. Blockage of Human T Lymphocyte Kv1.3 Channels by Pi1, a Novel Class of Scorpion Toxin. *Biochem. Biophys. Res. Commun.* 278. 34–37, (2000)

IF: 3,161

2. Varga, Z., **Péter, M. jr.**, Panyi, G., Pieri, C., Csécsey Gy., Damjanovich, S., Gáspár, R. jr. Multiple Binding Sites for Melatonin on Kv1.3. *Biophys. J.* 80. 1280–97, (2001)

IF: 4,58

Book chapters:

1. **Péter, M., jr.**, Varga, Z., Krasznai Z., Panyi, Gy., Gáspár, R. Recording and analysis of membrane potential and ion currents in cultured peripheral human lymphocytes, *In: Practical guide to physical analysis of cell surface receptors*, University Medical School of Debrecen, Hungary, 1998. ISBN: 9639070319

Abstracts on the subject of the thesis:

1. Gáspár, R., jr., **Péter, M., jr.**, Varga, Z., Panyi, Gy., Damjanovich, S. The effect of *Pandinus imperator* scorpion venom on the K⁺ channels of human peripheral blood lymphocytes, *Toxicon*, 36: 1280, (1998)

2. Varga Z., **Péter M. Jr.**, Panyi Gy., Pieri C., Damjanovich S., Gáspár R.: Molecular pharmacological effects of melatonin on K⁺ channels of human T-lymphocytes, *Biophys J.*, 76: A76 (1999)

3. **Péter M. Jr.**, Varga Z., Panyi Gy., Damjanovich S., Possani L. D., Gáspár R.: *Pandinus imperator* Pi2 toxin blocks voltage-gated potassium channels with high affinity, *Biophys J.*, 76: A75 (1999)

4. Varga Z., **Péter M., Jr.**, Panyi Gy., Damjanovich S., Possani L. D., Gáspár R., jr.: A putative salt bridge between Glu7 and Lys24 may explain the different binding kinetics of Pi2 and Pi3 scorpion toxins to Kv1.3, *Biophys. J.*, 78. 97A (2000)