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Interaction of the scorpion toxin discrepin with Kv4.3 channels and A-type K^+ channels in cerebellum granular cells

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ABSTRACT

Background: The peptide discrepin from the α-KTx15 subfamily of scorpion toxins preferentially affects transient A-type potassium currents, which regulate many aspects of neuronal function in the central nervous system. However, the specific Kv channel targeted by discrepin and the molecular mechanism of interaction are still unknown.
Methods: Different variant peptides of discrepin were chemically synthesized and their effects were studied using patch clamp technique on rat cerebellum granular cells (CGC) and HEK cells transiently expressing Kv4.3 channels.
Results: Functional analysis indicated that nanomolar concentrations of native discrepin blocked Kv4.3 expressed channels, as previously observed in CGC. Similarly, the apparent affinities of all mutated peptides for Kv4.3 expressed channels were analogous to those found in CGC. In particular, in the double variant [V6K, D20K] the apparent affinity increased about 10-fold, whereas in variants carrying a deletion (ΔK13) or substitution (K13A) at position K13, the blockage was removed and the apparent affinity decreased more than 20-fold.
Conclusion: These results indicate that Kv4.3 is likely the target of discrepin and highlight the importance of the basic residue K13, located in the α-helix of the toxin, for current blockage.
General significance: We report the first example of a Kv4 subfamily potassium channel blocked by discrepin and identify the amino acid residues responsible for the blockage. The availability of discrepin variant peptides stimulates further research on the functions and pharmacology of neuronal Kv4 channels and on their possible roles in neurodegenerative disorders.

1. Introduction

K^+ channels responsible for A-type currents are of particular interest because they regulate firing frequency, spike initiation and waveform in excitable cells. They are thought to contribute to specialized functions like learning, memory and behavior. Physiological studies have revealed that A-type currents exhibit a wide range of biophysical and pharmacological properties, according to their cellular function, as well as their molecular heterogeneities. In the heart, activation of the channels responsible for the A-type currents modulates the action potential duration and its shape. In the CNS, they play a critical role by regulating many aspects of neuronal function, and therefore, their pharmacological modulation potentially represents a powerful means of controlling CNS disorders, such as epilepsy, dementia, anxiety, pain, depression and stroke (for review, see references [1,2]). The characterization of new toxins, which could target A-type channels, is expected to reveal more about the role of K^+ channels in neuronal tissues. The K^+ channel specific peptides were divided in several families based on structural and functional properties; the most widely studied are the α-KTxs now subdivided into 26 sub-families ([3], see also UniProt databank), among which the subfamily α-KTx15 is the one reported to affect the transient I_A currents [4–6]. Our group contributed by investigating the structure and pharmacology of K^+ channels expressing transient currents in cerebellum granular cell (I_A-CGC) by means of synthetic peptides corresponding to various segments or variants of scorpion toxins [7–10]. Discrepin isolated from the venom of the Venezuelan scorpion *Tityus discrepans* belongs to the sub-family α-KTx15 [11] and its 3D-structure was determined by NMR [12]. The members of the α-KTx15 subfamily share very high sequence similarities (up to 98%). Discrepin (α-KTx15.6) displays only around 50% of sequence identity with the remaining members of the subfamily (Fig. 1). It has been shown that discrepin and different variants of this toxin can block A-type currents of rat CGC [13].

Cerebellum granular cells are located at the input stage of the cerebellum and play a critical role for regulating inputs coming from mossy fibers. The primary cell culture of cerebellum-neurons is widely used as a model of CNS, due to their abundance and the fact that they represent a highly homogeneous neuronal population [14]. They exhibit

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A

Peptide	Amino acid sequence	Identity (%)
Discrepin	ZIDTNVKCSGSSKCVKICIDRYNTRGAKCINGRCTCYP	100
[V6K, D20K]	ZIDTNKKCSGSSKCVKICIKRYNTRGAKCINGRCTCYP	
[R25A]	ZIDTNVKCSGSSKCVKICIDRYNTAGAKCINGRCTCYP	
[S12L,D20K,T35L]	ZIDTNVKCSGSLKCVKICIKRYNTRGAKCINGRCLCYP	
[K13Del]	ZIDTNVKCSGSS-CVKICIDRYNTRGAKCINGRCTCYP	
[K13A]	ZIDTNVKCSGSSACVKICIDRYNTRGAKCINGRCTCYP	
[D20K]	ZIDTNVKCSGSSKCVKICIKRYNTRGAKCINGRCTCYP	
Aa1	ZNETNKKCQGGS-CASVCRRVIGVAAGKCINGRCVCYP	48
BmTx3	ZVETNVKCQGGS-CASVCRKAIGVAAGKCINGRCVCYP	51
AmmTx3	ZIETNKKCQGGS-CASVCRKVIGVAAGKCINGRCVCYP	51
AaTx1	ZIETNKKCQGGS-CASVCRRVIGVAAGKCINGRCVCYP	51
AaTx2	ZVETNKKCQGGS-CASVCRRVIVVAAGKCINGRCVCYP	48
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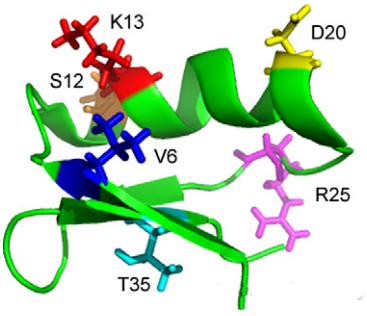

Fig. 1. (A) Sequence alignment of the α-KTx15 sub-family and synthetic variant peptides [V6K, D20K], R25A, [S12L, D20K, T35L], K13A, ΔK13 and D20K. The members of the α-KTx15 subfamily discovered formerly include: Aa1 from *Androctonus australis* [7]; BmTx3 from *Buthus martensi* Karch [15]; AmmTx3 from *Androctonus mauretanicus mauretanicus* [16]; AaTx1 and AaTx2 from *A. australis* putative isoforms of Aa1 [21]; and discrepin from *T. discrepans* [10]. Most of them share very high amino acid sequence similarities (around 97% identity). Discrepin with systematic number α-KTx15.6 displays only around 50% of sequence identity with the remaining members of the subfamily and around 30% with toxins of other α-KTx subfamilies. (B) Tridimensional structure of discrepin. Amino acid residues taken into account in this study are highlighted.

some components of the voltage-dependent outward K^+ currents following depolarization. One of the main components is the transient current (A-type current), which is characterized by fast activating and inactivating kinetics and block by 4-aminopyridine (4-AP). The A-type currents in cerebellum granular cells are thought to be generated by voltage-dependent K^+ channels of sub-type Kv4 of the *Shal* subfamily [15,16]. Despite the fact that α-KTx15 are known to affect I_A currents, they have not been extensively used as a pharmacological tool to study Kv4 channels [17].

In order to find which K^+ channel was involved in the blocking activity of discrepin, we describe the electrophysiological characteristics of this toxin on Kv4.3 channels transfected in HEK cells. For a deeper understanding of the molecular mechanism of the blocking effect, new variants of discrepin were chemically synthesized, assayed and analyzed on I_A-CGC and Kv4.3 currents. Our aim has been to discover, from natural sources or synthetic peptides, high affinity ligands that can be used as tools on studies related to K^+ channels and their pathologies in the brain. The rationale used to select the new synthetic discrepin variants reported here was based on the sequence similarities with the other members of the sub-family α-KTx15, where important differences are located at position K13 (present only in discrepin) and R25 (occupied by A25 in other members of the subfamily). The double variant [V6K, D20K] was chosen based on the blocking activities observed with the single basic residues V6K and D20K in a previous work [13]. Finally, the synthesis of the triple mutant [S12L, D20K, T35L] was based on sequence similarities (both D20 and T35 are absent in the other members of the subfamily) and also supported by a simulation of docking discrepin with Kv4.3 channel (Picco et al. unpublished results).

2. Material and methods

2.1. Peptide synthesis

Variants of discrepin, the deleted K13 amino acid (ΔK13), K13A, R25A, the double variant [V6K, D20K] and the triple variant [S12L, D20K, T35L] were chemically synthesized using solid-phase techniques with fluoren-9-ylmethoxycarbonyl (Fmoc) methodology on an Applied Biosystems 433A peptide synthesizer. Fmoc-Pro-TrtA-PEG resin (Watanabe Chemical Industries, Ltd., Hiroshima, Japan) and pyroglutamic acid (Peptide Institute Inc., Osaka, Japan) were used to generate the correct C- and N terminal residues of such peptides, respectively. Cleavage and deprotection of the two synthetic peptides from the resin were performed using a chemical mixture composed of 0.25 g crystalline phenol, 0.2 g imidazole, 1 ml thioanisol, and 0.5 ml 1,2-ethanedithiol in 20 ml TFA [18]. The resin was removed by filtration, and the deprotected peptides in solution were precipitated using cold ethyl ether. The precipitated peptides were washed twice with cold ether to remove the remaining oxygen scavengers and the amino acid side-chain protecting groups. The cystine reduced synthetic peptides were dried under a flow of nitrogen and dissolved in 20% aqueous acetonitrile. The peptides were purified under acidic conditions by RP-HPLC (Waters 600, with dual wavelength detector model 2847, Milford, MA, USA) on a semi-preparative C_{18} column (5C18MS, 10 × 250 mm, Nacalai Tesque, Japan) to 90–95% homogeneity using a 60 min linear gradient from 20 to 60% aqueous acetonitrile containing 0.1% TFA, at flow of 2 ml/min. The six-free cysteine residues were allowed to oxidize in the presence of air for 24 h at room temperature in a 0.2 M aqueous Tris-base solution containing 1 mM reduced glutathione and 0.1 mM oxidized glutathione at pH 8.0. The folded synthetic peptides were purified by the same RP-HPLC system using an analytical C_{18} column (5C18MS, 4.6 × 250 mm, Nacalai Tesque, Japan) subjected to a 20 min linear gradient from 10 to 30% aqueous acetonitrile containing 0.1% TFA, at flow 1 ml/min. The mass identities of the peptides, ΔK13, K13A, R25A, the double variant [V6K, D20K] and the triple variant [S12L, D20K, T35L] were verified by electrospray ionization mass spectrometry using a Finnigan LCQ^{DUO} ion trap mass spectrometer (San Jose, CA, USA).

2.2. Cell cultures

Experiments were performed on cerebellum granular cells in primary culture obtained from 8-day-old Wistar rats. Dissociated cell cultures were prepared by trypsin digestion and mechanical trituration, following the procedure of Levi et al. [14]. Cells were plated at a density of 2.5 × 10^6 per dish, on 35 mm plastic dishes or on glass coverslips, coated with 10 mg/ml poly-L-lysine and kept at 37 °C in humidified 95% air/5% CO_2 atmosphere. Experiments were performed 5 to 12 days after plating.

2.3. Heterologous expression of potassium channel

Human embryonic kidney (HEK) cells were grown in DMEM supplemented with 10% fetal bovine serum. Cells were transfected with the expression vector pcDNA3, containing the full-length cDNA coding for the human Kv4.3 channel (kind gift of Prof. Bahring, University of Hamburg-Eppendorf, Hamburg, Germany), using the Effectene Transfection Kit (QIAGEN). For transfection, 1 μg of Kv4.3 pcDNA and 10 μl of Effectene were used. Cells were co-transfected with pcI-CD8 and the success of transfection was tested using CD-antigen coated microspheres (Dynabeads Dynal, Invitrogen, USA). Transient expression was tested electrophysiologically between 24 and 72 h after transfection. Only cells that showed the expression of CD8 receptor by capturing the CD8-antigen covered microspheres were used for electrophysiological experiments.

2.4. Patch-clamp measurements

Ionic currents were recorded in whole-cell patch-clamp technique configuration [19], as described by our group [7]. Patch pipettes were made from borosilicate glass (CLARK Electro-medical Instruments) and fire polished to obtain resistances between 5 and 8 MΩ for CGC and between 2 and 5 MΩ for HEK cells. Cell responses were amplified and filtered at 2 kHz by an AxoPatch 200 (Axon Instruments). Both stimulation and data acquisition were performed with a 16-bit AD/DA converter controlled by a PC with a custom acquisition program (Gepulse, users.ge.ibf.cnr.it/pusch/programs-mik.htm). The remaining linear responses after analogical compensation were digitally subtracted with a standard P/4 protocol [20]. Access resistance was always less than 12 MΩ, and cell membrane capacitance and series resistance were carefully compensated (between 80 and 90%). The whole-cell currents elicited by a 200 ms-long voltage steps between −60 and 80 mV in increment of 20 mV from a holding potential (HP) of −90 mV were acquired at a sampling time of 200 μs. Junction potentials were not usually corrected but did not exceed 5 mV. The current was quantified as the maximal outward current (I peak). Data were stored on hard disk for subsequent analysis and curve fitting. The composition of the pipette filling solution was the following (in mM): 90 KF, 30 KCl, 2 $MgCl_2$, 2 EGTA, 5 NaCl, 10 HEPES, and 30 Glucose, pH 7.35. The external standard solution, designed to suppress Ca^{++} currents, was (in mM): 135 NaCl, 2.5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 0.2 $CdCl_2$, 10 HEPES, and 10 Glucose, pH 7.35. Discrepin variants were manually added with a graduate syringe in the chamber containing 200 μl of external solution. All experiments were carried out at room temperature (23 ± 2 °C).

2.5. Data analysis and curve fitting

Data analysis and curve fitting were conducted using IGOR Pro v. 5.05 software (WaveMetrics). The experimental points of dose–response curve versus peptide concentrations were fitted to a Hill equation, $I/I_{max} = 1 / [1 + (IC_{50} / C)^n]$, where I is the peak of the current, I_{max} is the peak of the maximal current (i.e. the current obtained without the addition of peptide), C is the concentration of peptide, IC_{50} is the concentration of peptide that blocks 50% of the maximal current and n is the Hill coefficient. To analyze the voltage dependence of activation the peak currents, evoked by depolarizing steps to potential between −60 and +80 mV with 20 mV increment, were fitted to the following normalized Boltzmann equation: $I(V) = G_{max}\ (V - V_{rev})\ /\ \{1 + exp[-(V - V_{0.5})\ /\ k]\}$, where G_{max} is the maximal conductance, V_{rev} is the reversal potential for the K^+ current (estimated as -98 mV), $V_{0.5}$ is the membrane potential for half activation and k is the slope factor.

For the analysis of the voltage dependence of inactivation, peak currents evoked by a 50 mV test pulse after a 200 ms prepulse to conditioning voltages between −120 and 0 mV with 10 mV intervals, were fitted to the following normalized Boltzmann equation: $I(V) = I_{max}\ /\ \{1 + exp[-(V - V_{0.5})\ /\ k]\}$, where I_{max} is the maximal current to the step (40 mV) measured after prepulse to −120 mV.

3. Results

3.1. Chemical synthesis of discrepin variants

Fig. 1 shows the amino acid sequence of the various peptides synthesized for this work and a short list comparing them with other peptide toxins known to affect the function of A-type K^+ currents. The discrepin variants differed in their overall assembly and cleavage yields. For example, the yields of the crude synthetic peptides after chromatographic purification before folding were 20 and 34% for ΔK13 and [V6K, D20K], respectively. These percentages correspond to the final amount of product, initiated with 0.1 mmol of peptidyl resin and expected to produce theoretically *ca.* 250 mg of final peptide. The final yields after chromatographic purification of the linear peptide and refolding for the discrepin variants were 0.05 and 4% for ΔK13 and [V6K, D20K], respectively. Fig. 2 shows the overlapping chromatograms of the purification of the crude (discontinuous line) and folded (continuous line) double variant [V6K, D20K]. The chromatogram shows that the crude product of variant [V6K, D20K] (discontinuous line) contains synthetic byproducts and incorrectly assembled peptides that were removed before the folding process. The oxidized peptide (continuous line) has a shorter elution time than the reduced [V6K, D20K]. The formation of cysteines reduces the exposition of hydrophobic side chains on the surface of [V6K, D20K]. The pure folded discrepin variants were proved to have the expected molecular masses. The experimentally determined molecular weights versus the theoretical masses were: for ΔK13 4048.8 and 4048.6 Da; for K13A 4119.0 and 4119.6 Da; for D20K 4190.4 and 4189.9 Da; for R25A 4091.9 and 4091.7; for [V6K, D20K] 4218.9 and 4218.1; and for the triple variant [S12L, D20K, T35L] 4228.1 and 4228.8 Da, respectively. Therefore, these results show a good agreement between the experimentally determined molecular weights and those expected for each synthesized peptide.

3.2. Functional analysis of discrepin and variant peptides block

3.2.1. Discrepin blocks Kv4.3 channels

Experiments were performed to identify the K^+ channel type involved in the block of I_A-CGC currents by discrepin. Since these particular currents in granular cells are mainly generated by the Kv4 potassium subfamily [21,22], electrophysiological experiments were performed on Kv4.3 channels. Macroscopic outward K^+ currents were measured in HEK cells transfected with the Kv4.3 clone using the patch-clamp technique in the whole-cell configuration. Fig. 3 shows the effects of native discrepin on I_A-CGC and Kv4.3-HEK currents. Discrepin was able to block K^+ currents of Kv4.3 channels similarly to that observed in CGC. This graphic illustrates a family of control and toxin-modified K^+-currents recorded by stepping the membrane from −60 to +80 mV in 20 mV increments from a holding potential of −90 mV. As in CGC, Kv4.3-mediated currents were blocked by 200 nM of toxin at all membrane potentials. Peak currents measured in control solution and after the addition of discrepin were plotted versus membrane potential. The IV curves for both CGC and Kv4.3 showed a similar behavior; the

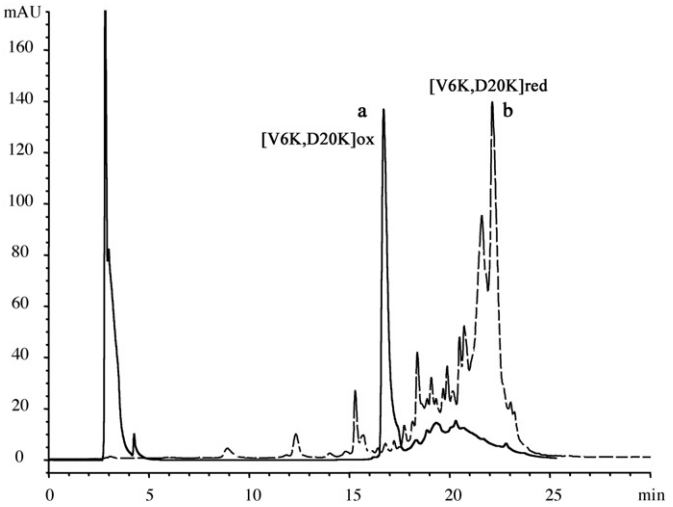

Fig. 2. Purification and folding of synthetic peptides. Crude unfolded (discontinuous line, b) and folded (continuous line, a) discrepin variant [V6K, D20K] were separated by HPLC in a C18 reverse column, using a linear gradient from 10% to 30% solution B (0.10% TFA in acetonitrile), run for 20 min (effluent at 1 ml/min).

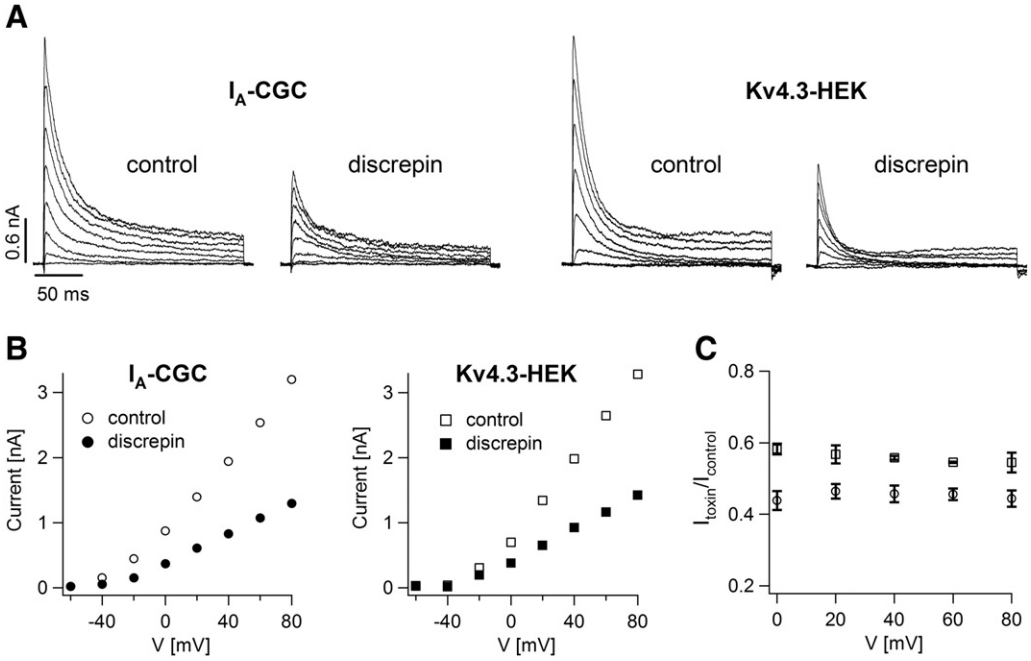

Fig. 3. Comparison of discrepin block on I_A currents of CGC and Kv4.3 channels. (A) Families of current traces from a granular cell and a HEK cell transfected with the Kv4.3 clone. Currents were elicited by voltage steps from −60 to +80 mV in 20 mV increments from a HP of −90 mV in control solution and in the presence of 200 nM of discrepin. (B) I/V relationship of the I_A current calculated from current traces shown in A for both CGC and Kv4.3 channel. Experimental points for I_A current were measured at the peak value and plotted versus the membrane potential. (C) Peak currents measured in the presence of discrepin were normalized to those obtained in control solution and plotted versus membrane potential. Data are mean values ± S.E. obtained from at least 4 experiments.

toxin blocked about 50% of the current in a voltage-independent manner, as shown in Fig. 3C.

3.2.2. Variant peptide blockage on K^+ currents in CGC and HEK cells

In order to get insights into the molecular mechanism of discrepin binding to K^+ channels, the new synthetic variants K13A, ΔK13, R25A, D20K, [V6K, D20K] and [S12L, D20K, T35L] were tested on I_A-CGC and Kv4.3-HEK currents. Discrepin variants were applied to the extracellular solution at a concentration of 160 nM and their effect on K^+ currents was assayed at the step potential of +40 mV (Fig. 4). Similarly to the results obtained in control experiments with native discrepin, the variant peptides mainly blocked the I_A currents in CGC, leaving almost unchanged the steady-state I_d currents. Variant peptides affected I_A-CGC (Fig. 4A) and Kv4.3 currents in HEK cells in a similar manner (Fig. 4B). Different current blockage efficiencies were found for the single variants, where [V6K, D20K] showed the strongest effect whereas the variant K13A did not show any blocking activity at this toxin concentration. Vice versa, the substitution of the charged arginine at position 25 with the neutral amino acid alanine increased the block of currents, whereas the triple variant [S12L, D20K, T35L] did not show any difference compared to the native discrepin. These results indicated that the positive charge K13 of discrepin was critical in the recognition process between toxin and K^+ channels. The variant ΔK13 tested on I_A-CGC confirmed this hypothesis.

In order to compare the affinities of native discrepin and the different variant peptides for K^+ channels in granular cells and expressed Kv4.3 channels, both CGC and HEK cells were exposed to different toxin concentrations and the peak currents were normalized to the current in absence of peptide (control). The different peptides affected K^+ conductance in a dose-dependent manner. Dose–response curves were obtained for K13A, ΔK13, R25A, D20K, [V9K, D20K] and [S12L, D20K, T35L] variants interacting with CGC (Fig. 5A) and Kv4.3 channels (Fig. 5B). The mean values were fitted to the Hill equation and IC_{50} values for I_A-CGC and Kv4.3-HEK currents were summarized in Table 1. The block of K^+ permeation is consistent with a 1:1 stoichiometry of peptide binding to the channels (Hill coefficient = 1). The good agreement between affinities obtained for native and variant peptides confirmed that the Kv4.3 channel subunit is a likely target for the action of discrepin.

3.2.3. Mechanism of block by discrepin variant on Kv4.3 channels

Further experiments were carried out on transfected HEK cells to elucidate the blocking mechanism of discrepin on Kv4.3 and the possible effect of increasing of positively charged residues in both cell lines. A major difference between the block observed for I_A-CGC and for Kv4.3-HEK was in the recovery after washout of the toxin (Fig. 6). As observed in previous work [10], discrepin blocked I_A-CGC in an almost irreversible manner. All peptide variants investigated here did not remove this peculiarity. The irreversible reduction of the current induced by [V9K, D20K] peptide is best shown in Fig. 6A: the steady-state inhibition of the current was reached rapidly (half time <30 s), but full recovery was not obtained at any time during prolonged washout. Differently from CGC, the block of Kv4.3-HEK currents was completely reversible for native and variant peptides (Fig. 6B).

Previous studies have shown that discrepin blocks A-type K^+ channels in CGC in the closed state and channel block is not influenced by channel opening [11]. Our analysis performed on Kv4.3 channels expressed in HEK cells with WT discrepin confirmed the result obtained on CGC (data not shown). Moreover, the increment of the toxin charges did not modify this mechanism of block. The double variant [V6K, D20K] was applied to CGC (Fig. 6C) and HEK cells (Fig. 6D) at the holding potential keeping the channels in the closed state. The first test pulse (+40 mV) applied at 5 min after toxin addition greatly reduced the current in both CGC and HEK cells. Exponentials were fitted to the decay of trace and extrapolated to the beginning of the voltage step to estimate the degree of blockage before the opening of the channel. The subsequent test pulse delivered 1 min later elicited the same current, suggesting that the open state of the channel was not necessary for toxin block and that the open state did not increase the affinity of the variant peptide for the channel.

Finally, we compared the biophysical channel properties in control conditions and after toxin application, in order to evaluate possible effects

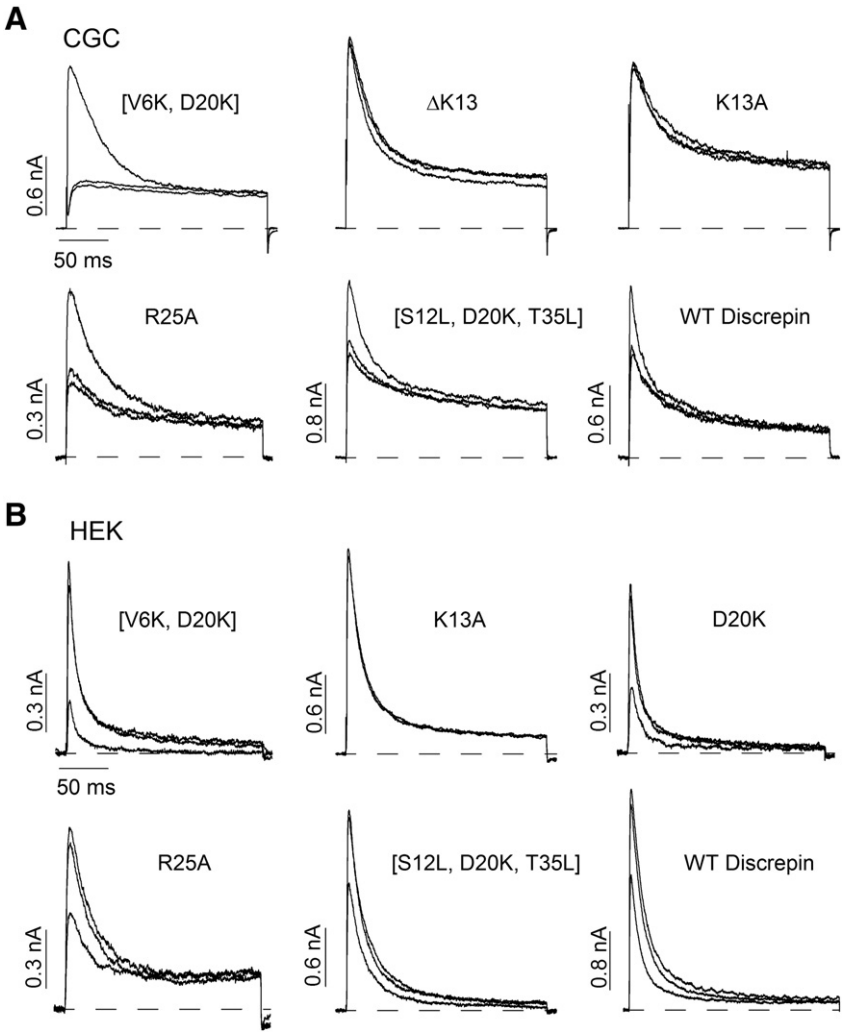

Fig. 4. Discrepin variant peptide block of A-type K^+ currents of CGC and Kv4.3 channels. Currents recorded at 40 mV in control solution, in the presence of 160 nM of WT and variant peptides of discrepin and after recovery with standard solution in CG cells (A) and in Kv4.3 channels transfected in HEK cells (B) (HP = −90 mV).

on channel gating. Fig. 7 compares the features of I_A-CGC and Kv4.3-HEK for the double variant [V6K, D20K] interaction, but similar results were obtained for the other variant peptides tested in this work (data not shown). The double variant did not influence the voltage dependence of activation and steady-state inactivation for both I_A-CGC (Fig. 7A) and Kv4.3-HEK (Fig. 7B). Table 2 summarizes the respective parameters of the voltage dependence. The channel kinetics, activation (time-to-peak for the transient currents) and fast inactivation time constants were evaluated, but no effects on the kinetic parameters were induced by the peptide for both current types. Also, the recovery from fast inactivation did not change significantly after the application of the toxin.

4. Discussion

The aims of this work were, first, to identify the K^+ channel subunit involved in the recognition process of the discrepin toxin and second, to investigate the possible role of topologically well-defined basic residues of discrepin in the blockage of A-type K^+-currents.

CGC express Kv4 channels and a number of studies have suggested that the Kv4.3 subunit is the major component of transient I_A currents in the mammalian CNS [21,22]. The comparison of the currents mediated by Kv4.3 channels heterologously expressed in HEK cells with I_A-CGC confirmed that discrepin blocks both channel types with highly similar affinities (see Table 1); in both cases the double variant [V6K, D20K] presents the highest affinity (IC_{50} 20 and 43 nM for I_A-CGC and Kv4.3-HEK, respectively) whereas the peptide K13A partially removes the current block (IC_{50} 3070 and 4200 nM for I_A-CGC and Kv4.3-HEK, respectively). This work has further pointed out that, similar to CGC, the voltage dependence of activation and the steady-state inactivation of Kv4.3 channels were not influenced by the presence of either native discrepin or the double variant peptide [V6K, D20K] (Table 2; Fig. 6); therefore, the increment of positive charges in this discrepin variant does not influence the voltage dependence of current block. Moreover, similar to what has been found for K^+ channels in CGC [9], a closed-state blocking mechanism was observed also for Kv4.3 channels expressed in HEK cells. These features suggest that the block of discrepin likely involves the external mouth of the pore of the channel with a mechanism of blockade that resembles that described by MacKinnon and Miller [23]. We conclude that the K^+ channels targeted by discrepin in CGC mainly involve Kv4.3 subunits. Interestingly, the only difference observed in the effects of discrepin and variant peptides was that K^+ current inhibition was irreversible for I_A-CGC, but reversible for Kv4.3-HEK. This behavior may be explained by the regulatory action of K^+ channel interacting proteins KChIPs on Kv4 channels in CGC that are absent in HEK cells [24]. Recently, it has been shown that two variants of dipeptidyl peptidase-like protein 6 (DPP6) co-assemble with Kv4 channel α-subunits and KChIPs to form channel complexes reproducing the properties of A-type K^+ currents of CGC [25]. In particular, the presence of DPP6K produces a negative shift in the steady-state inactivation curve of Kv4 channels [26]. The absence

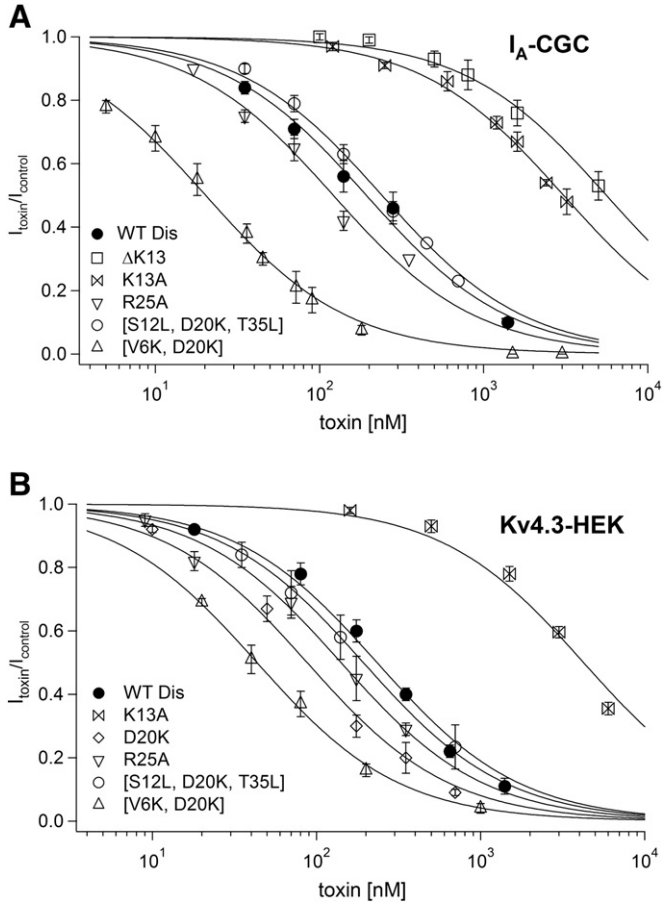

Fig. 5. Dose–response curves for native discrepin and variant peptides. Dose–response curves for WT and the variant peptides of discrepin obtained from CG cells (A) and HEK cells transfected with Kv4.3 channel (B). Mean values were obtained from at least three experiments and were fitted with the Hill equation. Hill coefficient = 1 for all peptides. For IC_{50} values see Table 1.

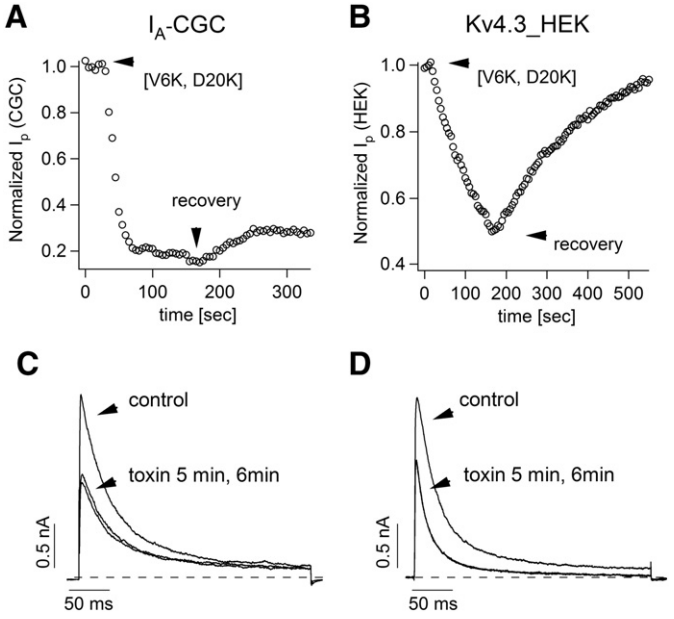

Fig. 6. The block of K^+ currents was reversible in HEK cells but not in CGC. (A, B) Time course of the double variant [V6K, D20K] response and current recovery after washout in CGC (A) and HEK cells transfected with Kv4.3 channels (B). Depolarizing pulses to 40 mV were repeated every 5 s. The peptide was applied between the two arrows. (C, D) Peptide block of closed channels is not influenced by channel opening. Current traces for CGC (C) and HEK cells (D) at +40 mV in control conditions and after 5 and 6 min of exposure to the double variant [V6K, D20K] (HP = −90 mV).

of the subunits could explain the difference observed in our work between the voltage dependence of the steady-state inactivation of Kv4.3 and I_A-CGC (Table 2), as well the reversibility of channel blockade. The hypothesis that auxiliary subunits may account for the differences between the effects of α-KTx15 toxins on Kv4 channels in heterologous cells and neurons, is also supported by the study of Maffie et al. [27]. The authors reported that, in heterologous cells, high-affinity blockade of Kv4.2 and Kv4.3 channels by AmmTX3, another member of the α-KTx15 sub-family of scorpion toxins, requires the presence of the associated proteins DPP6 and DPP10. The co-expression of these proteins with the channels made AmmTX3 blockade comparable to that observed in cerebellar granule neurons.

Table 1
IC_{50} values obtained from the fit of dose–response curves for WT discrepin and variant peptides.

Peptide	IC_{50} (nM)	
	CGC	Kv4.3
Discrepin	190 ± 30	234 ± 17
V6K, D20K	20 ± 1	43 ± 2
D20K	96 ± 21	88 ± 7
R25A	121 ± 9	135 ± 20
S12L, D20K, T35L	240 ± 12	204 ± 24
K13 deletion	5595 ± 280	ND
K13A	3070 ± 104	4200 ± 480

ND means not determined.

It has been reported that α-KTx peptides show distinct interacting surfaces with different types of K^+ channels [28]. The most extensively studied α-KTx subfamilies (α-KTx 1, 2 and 3) display the ability of distinguish between the large family of Kv channels and the maxi-K channel. The interaction surface of these scorpion toxin subfamilies with maxi-K and Kv channels was thought to depend on several amino acid side-chains mainly located on the beta-sheet face of the toxins [29]. Also in the α-KTx15 subfamily toxins show different interaction with different K^+ channels. BmTx3 inhibited preferentially Kv4.1 channels (IC_{50} = 105 nM) and was less effective on Kv4.2 and Kv4.3 [17], whereas AmmTX3 interacts with both Kv4.2 and Kv4.3 channels [27].

With the exception of R25, which corresponds to A24 in the other members of the α-KTx15 subfamily, almost all of the residues forming the β-face of discrepin are conserved. V6 located on the first β-strand also contributes to the surface, and in a previous work, we showed that the most evident effect (enhancement of the blocking activity) was obtained when an additional positive charge was introduced to replace this hydrophobic residue (V6K) [13]. Vice versa, the electrostatic surface of the α-helix side of discrepin displays great variability of residues between the different α-KTx15 sequences. Discrepin is the only peptide of this subfamily presenting the insertion of a positively charged amino acid (K13) and an acidic residue (D20) substituting for R19 in the other members of the subfamily. This was the main reason for synthesizing the new variants, whose effects are reported here. The affinity of the double variant [V6K, D20K] for both Kv4.3 and CGC K^+ channels was observed to be higher (Table 1), confirming the previous hypothesis that the increment of positive charges at the surface of discrepin enhances the electrostatic interaction with the channel vestibule. In fact, this double mutated variant peptide has nine positive charges whereas variants ΔK13, K13A and R25A have six positive residues. Contrary to a recent structural study in which the authors underlined the electrostatic interaction between charybdotoxin and KcsA channel [30], the substitution of the charged R25 with the neutral alanine residue increased the blocking effect of the toxin. This result

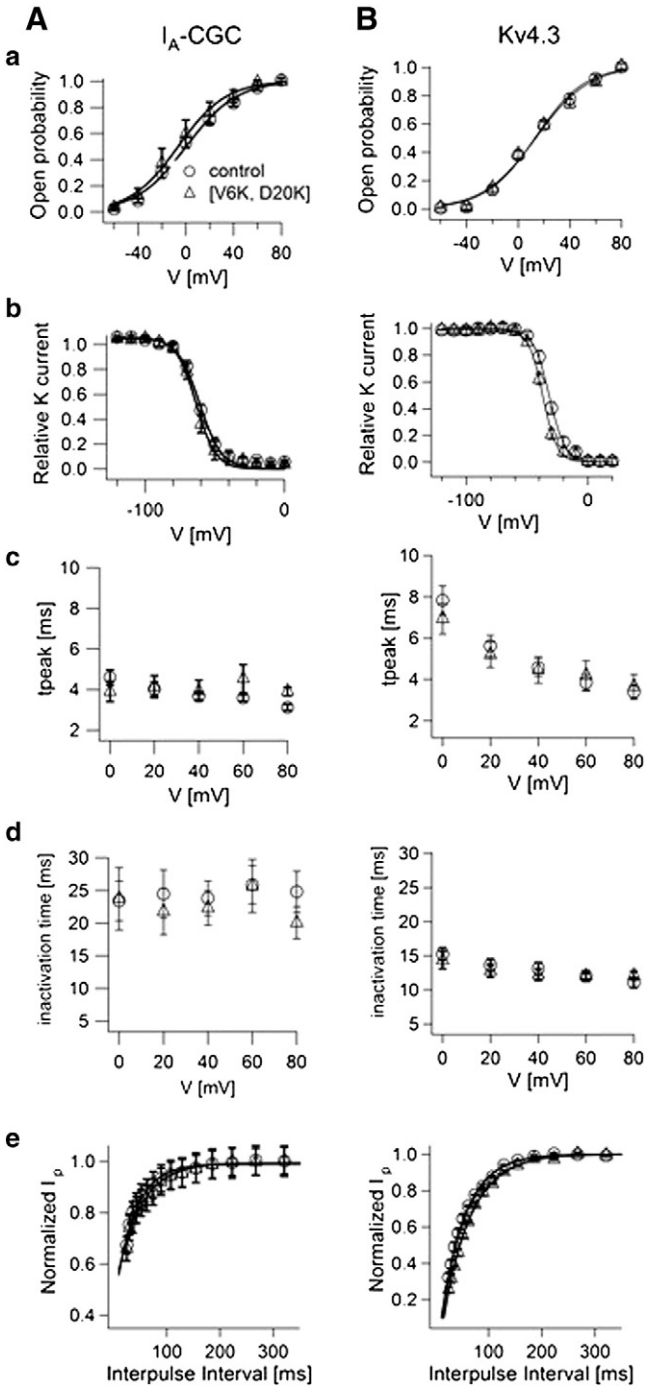

Fig. 7. Comparison of the biophysical features of double variant [V6K, D20K] on (A) CGC cells and (B) Kv4.3. (a) Open probability of K^+ channels *vs* test potential in control condition and after addition of 80 nM peptide. Data were obtained calculating the ratio I/(V − Vrev) and fitting to a Boltzmann distribution, as described in Material and methods section. (b) Steady-state inactivation curves before and after exposure to 80 nM peptide. Data represent peak currents at 50 mV after a 250 ms prepulse to the indicated potentials (from −120 to 0 mV). The values of Boltzmann fits for both I_A-CGC and Kv4.3 are shown in Table 2. (c) Time-to-peak for transient current from HP −90 mV. (d) Inactivation time constant vs depolarizing pulses. (e) Recovery from inactivation. Normalized peak current (I_p) plotted versus interpulse interval at −100 mV. Data were fitted to a single exponential curve and yielding the following values: (CGC = open circles) 36.5 ± 3.5 ms and 45.4 ± 4.4 ms; (Kv4.3 = open triangles) 44.7 ± 1.5 ms and 51.9 ± 1.2 ms for control and [V6K, D20K], respectively. All data are mean values ± S.E. obtained from at least 6 experiments.

demonstrates that not only electrostatic interactions occur in the docking of discrepin to Kv4.3 channels. The triple variant [S12L, D20K, T35L] did not yield any detectable variation in the affinity of discrepin

Table 2
Biophysical parameters for voltage dependence of activation and steady-state inactivation obtained from currents of CGC cells and Kv4.3.

	$V_{0/5}$ act (mV)	k_{act}	$V_{0/5}$ inact (mV)	k_{inact}
CGC (control, n = 10)	0 ± 2	21 ± 2	−61 ± 2	−7.8 ± 0.8
CGC (discrepin, n = 6)	−6 ± 3	20 ± 2	−61 ± 2	−7.1 ± 0.7
CGC ([V6K, D20K], n = 8)	−3 ± 3	21 ± 1	−60 ± 3	−7.2 ± 0.7
Kv4.3 (control, n = 12)	9 ± 2	21 ± 2	−33 ± 2	−6.0 ± 0.3
Kv4.3 (WT dis, n = 9)	7 ± 2	20 ± 1	−37 ± 3	−5.8 ± 0.6
Kv4.3 ([V6K, D20K], n = 8)	7 ± 2	21 ± 1	−35 ± 2	−6.2 ± 0.7

suggesting that residues S12 and T35 are not important for docking to the receptor.

Here we found that K13 plays a crucial role in the blocking of Kv channels by discrepin. ΔK13 and K13A variant peptides showed strongly impaired I_A current block, i.e. the apparent affinity decreased from the nanomolar to the micromolar range (Fig. 6, Table 1). This result demonstrates the involvement of the charged residue present in the α-helix and the specificity of discrepin in the α-KTx15 subfamily in docking to the Kv4.3 channel.

In this context, the availability of the discrepin variant peptides reported in this work makes them a new important tool for investigating different I_A-type K^+ channels. The mutated peptides will certainly stimulate further research on the functions and pharmacology of neuronal Kv4 channels and their possible roles in disease states, such as epilepsy, chronic pain, neurodegenerative disorders and autism.

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