A Novel Current Pathway Parallel to the Central Pore in a Mutant Voltage-gated Potassium Channel*

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Voltage-gated potassium channels are proteins composed of four subunits consisting of six membrane-spanning segments S1-S6, with S4 as the voltage sensor. The region between S5 and S6 forms the potassium-selective ion-conducting central α pore. Recent studies showed that mutations in the voltage sensor of the Shaker channel could disclose another ion permeation pathway through the voltage-sensing domain (S1-S4) of the channel, the ω -pore. In our studies we used the voltage-gated hKv1.3 channel, and the insertion of a cysteine at position V388C (Shaker position 438) generated a current through the α -pore in high potassium outside and an inward current at hyperpolarizing potentials carried by different cations like Na⁺, Li⁺, Cs⁺, and NH₄⁺. The observed inward current looked similar to the ω -current described for the R1C/S Shaker mutant channel and was not affected by some pore blockers like charybdotoxin and tetraethylammonium but was inhibited by a phenylalkylamine blocker (verapamil) that acts from the intracellular side. Therefore, we hypothesize that the hKv1.3_V388C mutation in the P-region generated a channel with two ion-conducting pathways. One, the α -pore allowing K⁺ flux in the presence of K⁺, and the second pathway, the σ -pore, functionally similar but physically distinct from the ω -pathway. The entry of this new pathway (σ -pore) is presumably located at the backside of Y395 (Shaker position 445), proceeds parallel to the α -pore in the S6-S6 interface gap, ending between S5 and S6 at the intracellular side of one α -subunit, and is blocked by verapamil.

The human voltage-gated potassium channel Kv1.3 is a member of the *Shaker*-related potassium channel family (1) and can be distinguished from the other members because of its characteristic C-type inactivation (2, 3). With the crystal structure data on the *h*Kv1.2 channel (4), another member of the *Shaker*-related family, we have a good picture about the architecture of the classical voltage-gated potassium channels. The channels consist of four subunits composed of six membrane-spanning segments, termed S1–S6. The region between the segments S5 and S6, with the pore helix (P) in-between (S5-P-S6), forms, together with three other subunits, the central ion conduction pathway, the α -pore, which is potassium-selective. The S4 segment is known as the voltage sensor, and the helices



S1-S4 form the voltage-sensing domain, which surrounds the pore domain and controls the gates (1, 5). Recent studies showed that mutations in the voltage sensor of the Shaker channel or of the voltage-gated sodium channel Nav1.2 can disclose another ion permeation pathway, the ω -pore, a pathway through the voltage-sensing domain (S1-S4) of the channel (5–9). The substitution of the first S4 arginine (Arg-1) in the Shaker channel with smaller amino acids like serine or cysteine created a pathway for a leak current selective for monovalent cations, the ω -pore. This conductance pathway in the *Shaker* channel was located distinct from the central K⁺-conducting α -pore and ran through the voltage-sensing domain of the channel. The ω -current through the voltage-sensing domain was only observed at hyperpolarizing potentials when S4 was at rest and the α -pore closed (5–7). In our studies we investigated a Shaker family member, the voltage-gated hKv1.3 channel, with a single point mutation V388C (Shaker position 438) located in the pore helix. The hKv1.3_V388C mutant channel showed a current behavior similar to the ω -current (5–7), however, with different properties. Due to these differences and due to our new proposed current pathway we will refer to our observed current as the " σ -current" and the pathway as the " σ -pore."

EXPERIMENTAL PROCEDURES

Molecular Biology—The *h*Kv1.3 wild-type plasmid was a generous gift from Prof. Dr. O. Pongs (Institut für Neurale Signalverarbeitung, Zentrum für Molekulare Neurobiologie, Hamburg, Germany). The channel mutant was generated by introducing the corresponding point mutation in the cloned *h*Kv1.3 gene in a pRc/CMV vector (Invitrogen) with a CMV promoter for protein expression in mammalian cells with the QuikChange site-directed mutagenesis kit (Stratagene). COS-7 cells (DSMZ no. ACC 60, Braunschweig, Germany) were transfected using the FuGENE 6 transfection reagent (Roche Applied Science). Cells were grown to ~80% confluence and co-transfected with ~1 μ g of *h*Kv1.3 or *h*Kv1.3_V388C and ~0.5 μ g of eGFP-C3 (Clontech) DNA. Two days after transfection sufficient protein for electrophysiological measurements was expressed.

Solutions and Chemicals—The measurements were performed in an external bath solution, $[160 \text{ Na}^+ + 4.5 \text{ K}^+]_o$ containing 160 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 4.5 mM KCl 4.5, and 5 mM HEPES, or high $[X^+]_o$ containing 164.5 mM XCl, 2 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES. Osmolarity was 290–320 mOsm, and the pH was adjusted to 7.4 with NaOH or XOH, respectively. X stands for K, Rb, Li, Cs, and NH₄. The internal pipette solution contained 145 mM KF or NaF, 2 mM

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MgCl₂, 10 mM EGTA, and 10 mM HEPES. Osmolarity was 290 – 320 mOsm, and the pH was adjusted to 7.2 with KOH or NaOH, respectively. Verapamil was dissolved in dimethyl sulfoxide as stock solution and diluted to the adequate concentrations in the external bath solution before application. The dimethyl sulfoxide fraction in the final solution was always <2%. (\pm)-Verapamil was obtained as hydrochloride salt from Sigma-Aldrich. CTX³ and KTX (Bachem, Bubendorf, Switzerland) were dissolved in external bath solution containing 0.1% BSA (Sigma-Aldrich). TEA chloride was purchased from Fluka (Taufkirchen, Germany) and dissolved in external bath solution to the respective final concentration.

Electrophysiology-The patch clamp measurements were performed in the whole cell recording mode (10, 11). Experiments were carried out at room temperature (18-22 °C), and the external bath solutions as well as the diluted drug solutions were applied using a syringe-based perfusion system. Electrodes were pulled from glass capillaries (Science Products, Hofheim, Germany) in three stages and fire-polished to resistances of 2-4 megohms. Data were acquired with an EPC-9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany) connected to a Dell computer running Patchmaster/Fitmaster 2.0 data acquisition and analysis software. All currents were filtered by a 2.9-kHz Bessel Filter and recorded with sampling frequencies of 1–10 kHz. Capacitative and leak currents were principally not corrected for. Only the current records shown in Fig. 5*E*, where current through the wild-type hKv1.3 channels in the absence and presence of verapamil was recorded, were subtracted for capacitative and leak current using the P/10 procedure. All voltage ramp protocols were preceded by a 100-ms prepulse to the starting potential to avoid complications associated with the slow "activation" of the σ -current. Further data analysis was performed using the Igor Pro 3.1 (WaveMetrics) software package. If not otherwise mentioned the holding potential was -120 mV.

Molecular Model—The molecular representations of the Kv1.2 structure were made in PyMOL (DeLano Scientific) and Viewer Lite 5.0 (Accelrys Software).

Modeling-Kv1.3 S4/S5/S6 was modeled based on the Kv1.2 PD structure (2A79) by the Modeler 9v2 package (12). The model was inserted in preequilibrated palmitoyloleoyl phosphatidylethanolamine bilayer patches based on Ref. 13 using INFLATEGRO (14). After insertion the mutation was done at position 388 in four monomers by Yasara software (15). All systems were solvated with simple point charge water molecules (16). Molecular dynamics simulations were performed using the GROMACS 4.0.7 package (17, 18) and OPLSAA force field (19). In the simulations, all bond lengths were constrained by shake (20) so that an integration time step of 2 fs could be chosen (21). Systems were simulated at a temperature of 300 K, maintained separately for protein, lipids (where present) and water by a Berendsen thermostat (22) with a time constant of 0.1 ps. Pressure coupling was done using a Parrinello-Rahman (23, 24) employing 1 bar reference pressure and a time constant of 2 ps. Semiisotropic pressure coupling was used to allow

bilayer fluctuations in the membrane plane. Electrostatic interactions were calculated using particle mesh Ewald (PME) summation (25, 26), and twin range cutoffs of 1.0 nm and 1.4 nm were applied for computing the van der Waals interactions. The simulation was run for 20 ns. The graphical images were rendered using PyMOL. Ten snapshots were chosen randomly from the last 2 ns of the equilibrated trajectory, and the channel pore was calculated using CAVER 2.1 software (27).

RESULTS

The single mutation V388C (*Shaker* position 438) in the pore-helix showed an extraordinary current behavior compared with the wild type but similar to the ω -current (5–7), however, with different properties. In the following we characterize the electrophysiological and pharmacological properties of this extraordinary current.

The Cysteine Mutation at Position Val-388 in hKv1.3 Displays an Inward Current Similar to the ω -Current—Fig. 1A shows typical ramp currents through *h*Kv1.3_wt channels in $[160 \text{ Na}^+ + 4.5 \text{ K}^+]_a$ and $[164.5 \text{ K}^+]_a$ as external bathing solutions. As expected for a voltage-gated K⁺ channel, hardly any current was observed at potentials ranging from -200 to -60mV because the channels were closed. As channels opened at potentials more positive than -40 mV an outward current was observed in [160 Na⁺ + 4.5 K⁺]_o and an inward current in [164.5 K⁺], that turned into an outward current around 0 mV. In both bathing solutions ramp currents through hKv1.3_V388C mutant channels (Fig. 1B) showed only little outward current at potentials more positive than 0 mV. In $[160 \text{ Na}^+ + 4.5 \text{ K}^+]_o$ at potentials more negative than -100mV a large inward current appeared (Fig. 1B), which resembled currents through inwardly rectifying K_{ir} channels (28). Two experimental results argue against possible endogenously expressed K_{ir} channels as an explanation for this inward current. First, the inward current was insensitive to 50 μ M Ba²⁺ (Fig. 1*C*, *n* = 4), which should have been sufficient to completely block K_{ir} inward currents at voltages more negative than -80 mV in $[160 \text{ Na}^+ + 4.5 \text{ K}^+]_o$ (28). Second, in [164.5 K^+]_o we observed a decrease in current amplitude instead of an increase (Fig. 1B).

In [164.5 K⁺]_o the mutant channels (Fig. 1*B*) showed similar ramp currents compared with the wild type (compare Fig. 1, *A* and *B*) at least at potentials more negative than 0 mV with the little inward current dip starting at around -40 mV (see *inset* for a better view) and only little inward current at potentials ranging from -200 to -100 mV. At more depolarizing potentials the ramp currents of the *h*Kv1.3_V388C mutant channel showed only little outward current in [160 Na⁺ + 4.5 K⁺]_o and [164.5 K⁺]_o bathing solutions compared with the wild type.

We considered two possible explanations for the small size of the outward currents with hKv1.3_V388C. First, the α -pore was nonfunctional or the mutant channel was in the inactivated state. To determine whether the mutant channel showed C-type inactivation in the different bathing solutions and whether it is possible to measure current through the α -pore and the new observed inward current at hyperpolarizing potentials in one protocol we performed measurements as shown in Fig. 1, D–I. In the hKv1.3_V388C mutant channel in [160



³ The abbreviations used are: CTX, charybdotoxin; KTX, kaliotoxin; PDB, Protein Data Bank; TEA, tetraethylammonium; wt, wild type.



FIGURE 1. *h*Kv1.3_V388C mutant channels promote an inward current at potentials more negative than -100 mV. *A*-*C*, ramp currents through *h*Kv1.3_wt (*A*) and *h*Kv1.3_V388C mutant channels (*B* and *C*) in 160 Na⁺ + 4.5 K⁺ (4.5 K⁺_o) or 164.5 K⁺ external bath solutions. The currents were elicited with 400-ms voltage ramps from -200 to +80 mV every 30 s from a holding potential of -80 mV. *C*, effect of 50 μ M Ba²⁺ in 160 Na⁺ + 4.5 K⁺ on ramp currents through *h*Kv1.3_V388C channels. *D*-*I*, currents through α - and σ -pores of wild-type (*D*, and *G*), *h*Kv1.3_V388C (*E* and *H*), and *h*Kv1.3_V388C/W384F (*F* and *I*) mutant channels elicited with 100-ms depolarizing pulses from the holding potential of -120 mV to +40 mV followed by 100-ms hyperpolarizing pulses to -180 mV in [160 Na⁺ + 4.5 K⁺]_o (*G*-*I*).

Na⁺ + 4.5 K⁺]_o (Fig. 1*E*) we observed an outward current at 40 mV through the α -pore that inactivated \sim 20-fold faster ($\tau_{\text{inact}_V388C} \sim$ 10 ms) than the wild type ($\tau_{\text{inact}_wt} \sim$ 200 ms) as shown in Fig. 1*D* and an inward current at -180 mV. Therefore, it is most likely that the lack of outward current in the ramp in Fig. 1*B* was due to the fast inactivation we observed in Fig. 1*E*.

In comparison, the *h*Kv1.3_V388C mutant in [164.5 K⁺]_o (Fig. 1H) showed slower inactivation at 40 mV compared with that in $[160 \text{ Na}^+ + 4.5 \text{ K}^+]_o$, and at hyperpolarizing potentials we could observe a current that deactivated slower compared with *h*Kv1.3_wt (Fig. 1*G*), however, with little sustained inward current as seen in $[160 \text{ Na}^+ + 4.5 \text{ K}^+]_o$. Therefore, we conclude that the decaying phase of the current during hyperpolarization (Fig. 1*H*) was due to the current flowing through the α -pore. From the above described current behavior we conclude that potassium ions are able to carry current through the α -pore of the hKv1.3_V388C mutant channel, demonstrating that the channels are functionally expressed. To test whether the inward current was also present in a channel without α -current we introduced a second mutation in the hKv1.3 V388C mutant channel. The replacement of Trp with a Phe at position 384 should lead to channels without α -currents under physiological

conditions as described for the identical mutation in the *Shaker* W434F mutant channel (29, 30). As expected for the *h*Kv1.3_W384F/V388C channel we could not observe current through the α -pore (Fig. 1*F*). Although a transient was observed in Fig. 1*F*, it likely represents capacitive current because (i) the traces were not leak-subtracted, (ii) similar sized transients were observed in Fig. 1, *D* and *E*, and (iii) the transient was independent of the composition of the external (Fig. 1*I*) and internal solution (data not shown). Even though we could not observe current through the α -pore of the *h*Kv1.3_W384F/V388C channel, we were able to observe an inward current at -180 mV in [160 Na⁺ + 4.5 K⁺]_o as shown in Fig. 1*F* as in the *h*Kv1.3_V388C mutant channel (Fig. 1*D*) or in [164.5 K⁺]_o (Fig. 1, *H* and *I*).

Apparently, the *h*Kv1.3_V388C mutant channel conducted an inwardly rectifying current carried by sodium ions at potentials more negative than -100 mV even in a double mutant channel with no α -current. This and the fact that the current resembled the ω -current in the R1C/S *Shaker* mutant channel (5–7) might indicate that the uncommon inward current might also pass the channel through a pathway other than through the central α -pore.







FIGURE 2. Effect of KTX and CTX on σ - and α -current in the *h*Kv1.3_V388C mutant channel. *A* and *B*, ramp currents through the *h*Kv1.3_V388C mutant channel in [160 Na⁺ + 4.5 K⁺]_o (*A*) and in[164.5 K⁺]_o (*B*) before and after extracellular application of 1 μ M KTX and with [KF]_i as internal solution. *B*, first trace after application of 1 μ M KTX. Ramp currents were elicited as described in legend to Fig. 1. *C*, effect of 200 nM CTX on currents through the α - and σ -pores of *h*Kv1.3_V388C mutant channels, elicited with 100-ms depolarizing pulses from the holding potential of -120 mV to +40 mV followed by a 100-ms hyperpolarizing pulse to -180 mV every 30 s.

Inward Currents Were Not Affected by Pore-blocking Drugs Acting at the External Vestibule of the Channel-Because the V388C mutation in the hKv1.3 channel is far away from the ω -pore in the voltage-sensing domain and the current could be observed in the Kv1.3_W384F/V388C double mutant with no α -current, we considered two possible explanations for our observed inward current. The hKv1.3_V388C mutation could produce an abnormal opening of the central K⁺ pore, which is normally closed at voltages more negative than -40 mV, in combination with a malfunction of the selectivity filter. Alternatively, the inward current could flow through a pathway different from the central α -pore. We tested the first possibility with α -pore-blocking substances like KTX and CTX, peptide toxins that are known to block the extracellular mouth of the central pore (31-34) in the open state and are also able to block the α -pore in the C-type inactivated state (33). The α -pore of the *h*Kv1.3_wt channels would normally be blocked by KTX in the picomolar range ($IC_{50} = 650 \text{ pM}$) (35). The application of $1 \mu M KTX in [160 Na^+ + 4.5 K^+]_{a}$ (Fig. 2A) showed no blocking effect of KTX on the σ -current. However, the application of 1 μ M KTX in [164.5 K⁺]_o resulted in a block of the K⁺ inward current (Fig. 2B) through the α -pore of the hKv1.3_V388C mutant channel. We initially wondered why KTX did not

reduce the outward current; however, when comparing the time course of inactivation of the mutant V388C channel shown in Fig. 1, D and E, it is easy to imagine that the channel did, during the first 300 ms of the 400-ms voltage ramp (showing the inward current), almost completely inactivate. Therefore, the outward current in the ramp current shown in Fig. 2B cannot go through that channel. We conclude that the outward current in Fig. 2B is either a nonspecific leak current or flows through some other endogenous channels in the cell, for example through chloride channels.

Additional studies with CTX, which normally blocks the α -pore of *h*Kv1.3_wt channels in the nanomolar range (IC₅₀ = 3 nm) (35) at 40 mV showed results similar to those in the studies with KTX. The application of 200 nM CTX in [160 $Na^+ + 4.5 K^+]_o$ (Fig. 2*C*, n = 3) resulted in a block of the peak outward current through the K⁺-conducting α -pore at 40 mV but did not change the amplitude and kinetic of the inward current at -180 mV. Therefore, high doses of CTX and KTX did not affect the inward current carried by sodium in [160 $Na^{+} + 4.5 K^{+}]_{a}$ but inhibited the potassium current through the α -pore (Fig. 2, A-C). We considered the idea that the inward current also flows through the α -pore and that the lack of effect of CTX on the inward current might be attributed to a reduction in toxin-channel interaction at hyperpolarizing potentials. However, the observation that after hyperpolarization the fast inactivating outward current at depolarized potentials was still blocked by CTX suggested that even during hyperpolarization CTX was still bound to the α -pore of the channel. Because of these findings we suggest that the sodium inward current in [160 Na⁺ + 4.5 K⁺]_o was generated by ions flowing through a pathway distinct from the central K⁺ channel pore. We suppose, due to the different location of the mutation in the *h*Kv1.3 channel compared with the ω -current promoting R1C/S mutation in the Shaker channel that the pathway of ion conduction for V388C was not through the ω -pore.

To prove this hypothesis further we performed experiments with extra- and intracellularly applied TEA, which can block the α -pore from the extra- or the intracellular side (36, 37). First, we determined the effect of extracellularly applied 30 mM TEA on α - and σ -current measured in different bathing solutions (Fig. 3*A*,*B*; *n* = 4). External TEA had an effect similar to that of KTX (Fig. 2, *A* and *B*), no reduction in σ -current at potentials more negative than -100 mV in $[160 \text{ Na}^+ + 4.5 \text{ K}^+]_o$ (Fig. 3*A*), but a reduction of inward α -current between -30 and 0 mV in $[164.5 \text{ K}^+]_o$ (Fig. 3*B*). Extracellularly applied TEA blocked current through the α -pore but not the σ -current. This result strengthened the hypothesis that the σ -current moved along the channel through a different pathway.

For the experiments with intracellular applied TEA we first had to determine whether there is also a Na⁺ outward current detectable in the *h*Kv1.3_V388C mutant channel. Therefore, we performed experiments shown in Fig. 3*C*. It shows Na⁺ tail currents through the *h*Kv1.3_V388C mutant channels with [NaF]_{*i*} as internal solution and [164.5 Na⁺]_o without K⁺ as external solution at different potentials. We performed the experiments in the absence of K⁺ to exclude possible disturbances caused by K⁺ currents through the α -pore of the *h*Kv1.3_V388C mutant channels. As shown in Fig. 3*C* we could



FIGURE 3. **Effect of extra- and intracellularly applied TEA on currents through** $hKv1.3_V388C$ **mutant channels.** *A* and *B*, ramp currents through $hKv1.3_V388C$ mutant channels elicited in $[160 Na^+ + 4.5 K^+]_o$ (*A*) and $[164.5 K^+]_o$ (*B*) bathing solution with either 400-ms voltage ramps from -200 to +80 mV, every 30 s from a holding potential of -80 mV (*A*) or with 200-ms voltage ramps from -200 mV to +40 mV, every 30 s and a holding potential of -120 mV (*B*) to minimize the effect of decreasing α -pore current amplitude caused by inactivation, in the absence and presence of 30 mM extracellularly applied TEA. *C* and *F*, family of Na⁺ currents through $hKv1.3_V388C$ mutant channels elicited by a 200-ms prepulse from the holding potential of -80 to -200 mV followed by 100-ms voltage steps from -100 to +40 mV in 10-mV increments every 30 s in $[164.5 Na^+]_o$ with $[NaF]_i$ (*C*) or $[NaF + 10 mM TEA]_i$ (*F*) as internal solution. *D* and *G* to determine the reversal potential. Tail current amplitudes (I_{Na+}) 5 ms after the hyperpolarizing pulse from the experiment shown in *D* and *G* were plotted against the voltage.

observe outward currents at potentials more positive than 0 mV and because of the absence of K⁺ we could classify them as Na⁺ currents. For Na⁺ currents we would expect a reversal potential of ~ 0 mV because of the similar concentrations of Na⁺ in the intra- and extracellular solutions. To confirm the idea of Na⁺ outward currents we measured the tail current amplitude 5 ms after the hyperpolarizing pulse from the experiment shown in Fig. 3D, plotted these tail current amplitudes against the voltage, and could observe in this and similar experiments (n = 3) a reversal potential close to 0 mV. We assume that most of the current that reversed at zero are Na⁺ currents rather than leak currents because of the clear time dependence that we observed. After this experiment we wanted to see whether intracellular TEA might block the Na⁺ currents and to determine whether the observed Na⁺ current was going through the α -pore or passing the channel over an alternative pathway. To test this we repeated the above described experiments with 10 mM TEA applied intracellularly through the pipette solution and in the absence of K⁺. In control experiments testing the effect of TEA_{*i*} on current through the α -pore in wild-type channels (data not shown) we determined an IC₅₀ value of $\sim 1 \text{ mM}$

for TEA_i to block outward currents at 40 mV, close to the reported IC₅₀ value of ~0.6 mM (38). Therefore, we would expect that 10 mM TEA_i that we used in the pipette solution should block approximately 90% of the current through the α -pore. The results of these experiments are shown in Fig. 3, *F* and *G* (n = 4), and like in the experiments without TEA (Fig. 3, A-C) we could observe Na⁺ outward currents in the presence of intracellularly applied TEA. We supposed that the sodium currents were not passing through the α -pore because then the currents should be blocked by TEA from the internal site. Because we could still observe Na⁺ currents, this suggested that Na⁺ passed the channel through a different pathway not identical to the α -pore.

Ion Selectivity of the σ -Current—To characterize the σ -pore we examined which ions could generate the σ -current. A replacement of the main anion chloride by aspartate in the bathing solution as shown in Fig. 4A did not alter the inward current, indicating that the current was presumably not Cl⁻selective. To test the possibility of a proton current we used [160 Na⁺ + 4.5 K⁺]_o bathing solutions with varying pH_o, keeping the internal solution constant. This resulted in a reduction





FIGURE 4. **Ion conduction in the** *hKv1.3_V388C* **mutant channels.** Ramp currents through *hKv1.3_V388C* mutant channels were elicited as described in the legend to Fig. 1 in different external bathing solutions. The main anions (*A*) and cations (*D* and *E*) in the bathing solution or the pH of the external bathing solution (*B* and *C*), or in the absence and presence of 5 mm di- or trivalent cations in [160 Na⁺ + 4.5 K⁺]_o (*F*) are shown at each current trace.

of inward current with pH_o 5.5 (Fig. 4B) and no change in current amplitude with pH_a 8.0 (Fig. 4C). The decrease in inward current with pH_o 5.5 is inconsistent with a proton current because the increase in [H⁺]_a at pH_a 5.5 should have increased proton current rather than decreased it. Therefore, we conclude that the σ -current is not carried by protons. An exchange of cations in the bathing solution altered the inward current (Fig. 4, D-F). The change from [160 Na⁺ + 4.5 K⁺]_a to [Li⁺]_a resulted in no big difference in current amplitude, but the change to $[NH_4^+]_o$ or $[Cs^+]_o$ led to a marked decrease in current amplitude at potentials more negative than -100 mV. Ions like K^+ and Rb^+ , which can pass through the α -pore in the wild-type channel, only generated a tiny inward current in the mutant channel. From these experiments we calculated the ratios (I_{x+}/I_{Na+}) of current amplitudes (I) as a measure of ion conductance using the size of the ramp currents (Fig. 4, D and *E*) at -180 mV. The calculations resulted in an ion permeation efficiency with the following order: Li $^{\scriptscriptstyle +}$ (1.06) \sim Na $^{\scriptscriptstyle +}$ (1) > ${\rm NH_4^+}$ (0.61) > Cs^+ (0.3) > K^+ (0.15) \sim Rb^+ (0.11). It was remarkable that ions with a low affinity to the α -pore like Na⁺ and Li^+ generated a larger σ -current than ions with higher affinity like K^+ and Rb^+ . Therefore, we suppose that the σ -pore is either selective against K⁺ and Rb⁺ or that the occupancy of K^+ or Rb⁺ within the α -pore restricts the σ -pore although one could argue that 4.5 mM K⁺, present in the 160 mM Na⁺ solution, might already occupy the α -pore, thereby preventing σ -current. Under these conditions one might expect an increase in σ -current when the external K⁺ concentration is changed below 4.5 mm. This assumption, however, strongly depends on the affinity of the potassium binding site to be occupied. We assume that at a concentration of 4.5 mM external K⁺ this binding site is not occupied. We have performed experiments without $\rm K^+$ in the external solution and did not see an increase in $\sigma\text{-current}.$ For example the ramp currents in Fig. 4D in 160 mm Na^+ + 4.5 mm K^+ compared with 164.5 mm Li^+ without K^+ had identical amplitudes. Therefore, reducing external K^+ below 4.5 mm had little effect on $\sigma\text{-current}$ amplitude.

Barium ions are known to enter the α -pore and to fit into the selectivity filter, thereby preventing current flow through the α -pore (39). In the *h*Kv1.3 channel Ba²⁺ can even be trapped in the α -pore (40). If occupancy of the α -pore prevents ion flux through the σ -pore, then the addition of Ba²⁺ to the external solution should have an impact on the current at potentials more negative than -100 mV. This was indeed the case (Fig. 4*F*) because Ba²⁺ reduced the inward current (n = 5). To determine whether this effect is specific for Ba²⁺ or whether divalent ions in general have a blocking effect we tested several other divalent cations like Ni²⁺ (Fig. 4*F*, n = 6), Mg²⁺, and Ca²⁺ (n = 4, experiments not shown) at a concentration of 5 mM in the bathing solution. The experiments showed no decrease in current amplitude, suggesting that the observed effect was specific for Ba²⁺.

Next, we examined whether trivalent ions like La^{3+} and Gd^{3+} have an effect on the inward current at potentials ranging from -200 to -100 mV. Adding 5 mM La^{3+} (Fig. 4*F*, n = 4) or Gd^{3+} (experiment not shown, n = 4) to $[160 \text{ Na}^+ + \text{ K}^+]_o$ resulted in a drastic decrease of the inward current.

Block of the σ -Current by Verapamil—Because α -poreblocking substances acting from the outside were not able to block the σ -current we examined whether verapamil, a substance that can permeate through the membrane acting from the inside (41), could affect the inward current. We applied several verapamil concentrations to the [160 Na⁺ + 4.5 K⁺]_o



solution and observed a concentration-dependent blocking effect (Fig. 5A). From these data we calculated the dose-response curve shown in Fig. 5*B*), yielding an IC₅₀ value of 2.3 μ M at -180 mV for blocking the σ -pore. For comparison, we performed similar measurements in the wild-type channel as shown in Fig. 5E but with a different protocol to open the α -pore. From these data we calculated a dose-response curve (shown in Fig. 5F) yielding an IC $_{50}$ of 3.5 $\mu{\rm M}$ at 40 mV for blocking the α -pore of the wild-type channel. Verapamil block takes place from the intracellular site of the cell (10, 41, 42) with a binding site in the water-filled cavity below the selectivity filter and a binding pocket in the S6-S6 interface gap (42). Verapamil may sit at exactly this position, thereby blocking the σ -pore. To determine whether verapamil occupies one single binding site in the mutant channel as in the wild type we performed measurements in [164.5 K^+]_a as shown in Fig. 5C. We observed a block of the α -pore in the mutant channel, yielding an IC₅₀ of 5.7 μ M (Fig. 5D). The similarity of the IC₅₀ values for the verapamil block of current through both the σ -pore and the α -pore suggests that verapamil occupies a single binding site.

One special feature of the verapamil block of the α -pore in the *h*Kv1.3 channel is that this block is state-dependent, *i.e.* verapamil only blocks the open and inactivated conformation of the α -pore of *h*Kv1.3 wild-type channels (43). Therefore, we tested whether blocking the σ -pore with verapamil is possible without opening or inactivating the α -pore. Consequently, we performed the experiment shown in Fig. 5G in which Na⁺ currents through hKv1.3_V388C mutant channels before and after application of 50 µM verapamil were compared. Under the conditions as described in the legend to Fig. 5G the α -pore was kept close and, under these conditions, we know that verapamil is not able to block current through the α -pore (43). Application of 50 µM verapamil resulted in a clear block of current through the σ -pore (n = 3). Presumably, verapamil can bind in the S6–S6 interface gap when the α -pore of the channel is closed, already blocking the current through the σ -pore, and after opening of the α -pore verapamil can bind additionally at the MTT motif (Met-390–Thr-392) in the inner pore region (10) and also block the α -current. Alternatively, the channel is in $[160 \text{ Na}^+ + 4.5 \text{ K}^+]_a$ at potentials more negative then -80 mVstill in the inactivated state where verapamil can bind. In both possible explanations for verapamil binding, the α -pore is nonconducting so the σ -current has to go through a pathway besides the K⁺-conducting α -pore, *i.e.* through the σ -pore.

DISCUSSION

The replacement of the valine by a cysteine at position 388 (*Shaker* position 438) in the *h*Kv1.3 channel resulted in a sustained current at potentials more negative than -100 mV in $[160 \text{ Na}^+ + 4.5 \text{ K}^+]_o$ but displayed normal current behavior in $[164.5 \text{ K}^+]_o$ compared with the *h*Kv1.3_wt channel (Fig. 1*B*). Based on the normal current behavior in the *h*Kv1.3_V388C mutant channel in $[164.5 \text{ K}^+]_o$ compared with the *k*Kv1.3_wt channel and the functional similarity to the ω -current we hypothesize that the V388C mutation in the P-region generated a channel with two ion-conducting pathways, the α -pore and the σ -pore.



FIGURE 5. The inward current through the σ -pore can be blocked by verapamil. A, effect of extracellularly applied verapamil on ramp currents through hKv1.3_V388C mutant channels elicited as described in the legend to Fig. 1 in $[160 \text{ Na}^+ + 4.5 \text{ K}^+]_o$. B, dose-response curves for verapamil to block currents through the σ -pore of the hKv1.3_V388C mutant channels. Data points were obtained from ramp currents shown in A at -180 mV (at least five independent measurements for each concentration). The line was fitted to the *data points* using a modified Hill function $I_{\text{norm}} = (1 - c)/(1 + IC_{50}/C_{drug}) + c$ with I_{norm} defined as the ratio of the current in the presence (I_{drug}) and absence (l_0) of verapamil. C_{drug} represents the concentration of verapamil in the external bath solution, and *c* is the fraction of verapamil-insensitive current, presumably leak current. Values for IC₅₀ and c were 2.3 μ M and 0.22, respectively. C, effect of extracellularly applied verapamil on ramp currents through hKv1.3_V388C channels in [164.5 K⁺]_o elicited as described in legend to Fig. 3B. D, dose-response curves for verapamil to block currents through the α -pore of the hKv1.3_V388C mutant channels. Data points were obtained from the ramp currents shown in C at -25 mV (at least four independent measurements for each concentration). The line was generated as described in B with an IC₅₀ of 5.7 μ M (c = 0). E, effect of extracellularly applied verapamil on steady-state peak currents through the α -pore of hKv1.3_wt channels elicited with 200-ms depolarizing pulses from the holding potential to 40 mV every 30 s in [160 Na⁺ + 4.5 K⁺]_o. F, dose-response curves for verapamil to block currents through the α -pore of the hKv1.3_wt channels. Data points were obtained from steady-state peak currents shown in E at 40 mV (at least six independent measurements for each concentration). The line was generated as described in *B* with an IC₅₀ of 3.5 μ M (c = 0). *G*, Na⁺ currents through the σ -pore of hKv1.3_V388C mutant channels before and after application of 50 μ M verapamil. Currents were elicited by two 200-ms voltage pulses to -200 mV 30 s apart from a holding potential of -80 mV without a depolarizing prepulse to keep the α -pore closed.





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FIGURE 6. **Proposed location of the entry of the** *σ***-pore from the extracellular side within the pore domain.** *A*, conventional top view from the extracellular side of four subunits from S4 to S6 of Kv1.2 (4) (PDB entry 2A79). *B*, view of the four subunits shown in *A* tilted about 13 degrees toward the lower right quadrant as can be visualized by the *purple* potassium ions to show the entry of the *σ*-pore and the orientation of the enlargement in *C* (wt) and *D* (V388C). Please note the enlargement of the entryway into the *σ*-pore by the change of the amino acid from valine (*C*) to cysteine (*D*). Val-388, *red*; tyrosine (Tyr-395) from GYG, *green*; Trp-384, *brown*, K⁺, *purple*. The verapamil binding site in the MTT motif (Met-390–Thr-392) is shown in *yellow* and the position Ala-413 in S6, *orange*.

ω-Current Compared with σ-Current—The σ-current in [160 Na⁺ + 4.5 K⁺]_o was mainly due to sodium ions moving inward. This current showed many similarities to the ω-current flowing through the voltage-sensing domain of the R1C/S mutated *Shaker* channel (5–7): (i) both currents could only be observed in a potential range where the α-pore was normally closed (Fig. 1, *B* and *E*); (ii) the ω- and the σ-current could be carried by different monovalent cations like Li⁺ and Cs⁺; (iii) α-pore blockers (agiotoxin2 in the R1C/S mutant *Shaker* channel, CTX and KTX in the *h*Kv1.3_V388C mutant channel), reduced current through the α-pore but had no effect on the ω-or σ-current, respectively. We therefore conclude that the

σ-current, similar to the ω-current, penetrates the channel protein through a pathway distinct from the α-pore.

Because the ω -current is carried mainly by K⁺ (6) whereas our observed σ -current was mainly a Na⁺ current and because of the different location of the mutation in the pore helix of the *h*Kv1.3 channel compared with the ω -current-promoting R1C/S mutation in the S4 transmembrane segment of the *Shaker* channel, we propose that the pathway of our observed σ -current is distinct from the ω -pore (see below).

Restriction of the Novel Pathway (σ -Pore) by α -Pore-entering Ions—In contrast to the ω -current, K⁺ and Rb⁺ were not or were hardly able to carry σ -current (Figs. 1B and 4, D and E).



FIGURE 7. Proposed location of the *σ*-pore pathway through the membrane within the pore domain. *A*, conventional side view of four subunits from S4 to S6 of Kv1.2 (4) (PDB entry 2A79). *B*, view of only three subunits shown in *A* for the Kv1.3_V388C mutant channel with the proposed *σ*-current pathway shown as a *pink bar* through the verapamil binding site in the S6–S6 interface gap between two adjacent subunits. *Color code* is as described in the legend to Fig. 6.

Apparently, ions like Rb^+ or K^+ , that pass the α -pore best, were less able to permeate through the σ -pore. An intriguing explanation for this phenomenon might be that the binding by K⁺ and Rb^+ to the α -pore modifies the area close to the selectivity filter (44, 45), thereby restricting parts of the σ -pore. This hypothesis could also explain the experiments with 5 mM Ba^{2+} (Fig. 4*F*) because Ba^{2+} was able to enter the α -pore and could even be trapped there (39, 40). Alternatively, the σ -pore could be selective for both mono- and divalent cations based in part on size because Ba^{2+} has a much larger ionic radius (1.35 Å) than the other divalent cations tested (0.65–0.99 Å), and the monovalent cations giving small σ -current (K⁺, Rb⁺) are much larger (1.33–1.45 Å) than Na⁺ or Li⁺ (0.68–0.95 Å). Molecular modeling in combination with the CAVER 2.1 software allowed the calculation of the σ -pore dimension yielding a minimal radial distance of our proposed pathway of ~ 1.5 Å, *i.e.* a minimal diameter of \sim 3 Å, bigger than a sodium ion. It seems that the pathway is big enough with sufficient space between side chains for an ion to pass the entire length of the σ -pore. A comparable pathway running in parallel to the central α -pore was proposed from molecular dynamics simulations in KcsA channels (46). KcsA channels have a high water permeability in the absence of K^+ . The authors claim that water would not be able to pass through the selectivity filter due to a restriction of the selectivity filter and that these water molecules did not travel along the central channel axis but between two adjacent subunits of the selectivity filter, suggesting a new alternative pathway for water molecules in the situation when the obvious path along the channel central pore is not available due to the collapse of the filter (46). In conclusion, at least water molecules could travel along the channel axis in a cavity behind the selectivity filter similar to our proposed σ -pore.

Block of the σ -Current by Verapamil—The experiments with verapamil gave us additional information about the location of the novel pathway. Verapamil was able to block the σ -pore and the α -pore (Fig. 5, A and C) with similar affinities. We interpreted these results that verapamil is occupying the same binding site to block the α -pore and the σ -pore. We know from previous studies that the amino acids Met-390, Thr-391, and Thr-392 close to the selectivity filter in the inner vestibule of the *m*Kv1.3_wt channel are important for verapamil binding (10). There is an additional important amino acid for verapamil binding in the S6 helix (42), the amino acid at position 413. The verapamil binding site to block current through the α -pore was therefore located in the S6–S6 interface gap between two adjacent subunits. We conclude that verapamil sits at exactly this position also to block the σ -pore.

Verapamil blocks current through the α -pore of the *h*Kv1.3_wt channel in a state-dependent manner (43), *i.e.* verapamil is only able to block the α -pore in the open and inactivated state of the channel but is unable to block the α -pore in the closed state of the channel. Because we observed a block of the σ -pore even when the α -pore was closed we interpreted this as additional evidence that the σ -pore is distinct from the α -pore. In addition, this finding has also implications for the interpretation of the state-dependent verapamil block of the α -pore. It seems that verapamil can bind the channel when the α -pore is closed, and for blocking current through the α -pore the channel has to undergo a structural change thereby moving verapamil in a position to block the α -pore. It is conceivable that verapamil can bind in the S6–S6 interface gap, blocking the σ -pore while the α -pore is closed. Upon depolarization, the channel changes its structure so verapamil is able to reach the MTT motif (Met-390-Thr-392) thereby blocking the α -pore.

 σ -Current Is Not Due to Na⁺ Permeation through the α -Pore— In different Shaker channels Na⁺ permeability through the α -pore has been described in the open conformation (A463C) (47) or through the α -pore in the C-type inactivated state (wild type and W434F) (29, 30). In the A463C Shaker mutant channel the Na⁺ permeability was best in the absence of intra- and extracellular K⁺, and the Na⁺ currents through the α -pore were blocked by intracellular K⁺ with a K_d of 6 mM (47). Studies on the Shaker wild-type and the W434F mutant channel showed Na⁺ permeability only in the absence of K⁺ on both sides of the membrane (29, 30). The Shaker wild-type channel was able to permeate even Li⁺ in the C-type inactivated state (30). This might explain why Li⁺ could also permeate through the hKv1.3_V388C mutant channel like Na⁺ in our case because the hKv1.3_V388C mutant channel showed a strong inactivation in the outward current through the α -pore (Fig. 1E). Several experiments, however, argue against Na⁺ permeability through the α -pore of the *h*Kv1.3_V388C mutant chan-





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FIGURE 8. **Proposed location of the exit of the** *σ***-pore to the intracellular side within the pore domain.** *A*, conventional bottom view from the intracellular side of four subunits from S4 to S6 of Kv1.2 (4) (PDB entry 2A79). *B*, view of the four subunits shown in *A* tilted about 12 degrees toward the lower right quadrant as can be visualized by the *purple* potassium ions to show the exit of the *σ*-pore and the orientation of the enlargement in *C* (wt) and *D* (V388C). Please note the proposed exit from the *σ*-pore into the intracellular side between the S5 and S6 segment of one subunit (*light blue*). *Color code* is as described in the legend to Fig. 6.

nel. The Na⁺ currents in the *Shaker* wild-type and its A463C or W434F mutant channels displayed deactivation, *i.e.* a decrease in current amplitude during a voltage step to -100 mV after a depolarization, whereas the V388C and W384F/V388C mutant channels showed an increasing Na⁺ current at hyperpolarizing potentials in [160 Na⁺ + 4.5 K⁺]_o and no deactivation (Fig. 1, *E* and *F*). Furthermore, the experiments on the *Shaker* wild-type and mutant channels were mainly performed in the absence of K⁺, the observed Na⁺ current was only or best seen in the

absence of K⁺ and was blocked by intracellular K⁺. In our experiments we used 145 mM intracellular K⁺, and under these conditions a Na⁺ permeation through the *Shaker* channels was not possible; but in the *h*Kv1.3_V388C and *h*Kv1.3_W384F/V388C mutant channels we observed Na⁺ currents in the absence (Fig. 3, *C* and *F*) and presence (Fig. 1, *D* and *F*) of intracellular K⁺. Additionally, studies on the *h*Kv1.3_wt channel showed that in the absence of K⁺ on both sides of the membrane the *h*Kv1.3_wt channel was not able to conduct Na⁺ (30).



Moreover, our experiments with intracellularly applied TEA and extracellularly applied KTX and CTX argue against the possibility of a current through the α -pore of the hKv1.3_V388C mutant channel. In addition, studies on the *Shaker* channel showed that CTX is also able to bind the inactivated state (33), so if the σ -current would run through the α -pore in the C-type inactivated state it should have been also blocked by CTX; however, this was not the case because the σ -current was not affected while the α -pore was blocked. Due to these findings we believe that the inward current at potentials more negative than -100 mV is a current through the alternative σ -pore distinct from the α -pore and not a current through a Na⁺-permeable α -pore in the *h*Kv1.3_V388C mutant channel.

Because the W434F mutation in the *Shaker* channel is located in the pore helix close to our V388C mutation (*Shaker* position 438) in the *h*Kv1.3 channel, at the entry of the σ -pore of the *h*Kv1.3_V388C mutant channel, the Na⁺ current observed in the W434F *Shaker* mutant channel might be at least in part interpreted as a current, which passes the channel protein by an alternative pathway distinct from the α -pore, like the σ -pore.

Speculative Involvement of the Voltage Sensor in the Genera*tion of* σ *-Current*—To observe σ *-currents at negative voltages* the voltage sensor of the mutant channel might need to be in the resting state, although this observation could also be explained by the inability of K^+ to go through the σ -pore and does not necessarily need to involve the voltage sensor to be in the resting state, especially given the results of the experiments where internal K^+ was replaced by Na⁺ (Fig. 3, *C*–*H*). However, two results argue for the voltage sensor to be involved: (i) the time course of the " σ -current activation" at negative potentials (Figs. 1*E*, 2*C*, and 3, *C* and *F*); and (ii) the time course of the " σ -current deactivation" (Fig. 3, D and G). Although it looks as if the time course of σ -current activation seems to be too slow to be solely explained by voltage sensor deactivation, several interpretations are possible to explain the slow time course of σ -current activation including that in this mutant channel voltage sensor deactivation might be very slow. Alternatively, the slow time course of σ -current activation depends not only on voltage sensor deactivation but on other structural rearrangements within the channel protein that are slower compared with voltagesensor deactivation. However, without a comparison between α - and σ -pore gating, the role of the voltage sensor in the timedependent opening and closing of the σ -current remains speculative.

Location of the Novel Pathway (σ -Pore)—The V388C mutation in the *h*Kv1.3_channel is located at the back side of the selectivity filter. The experiments with verapamil indicated to us that the σ -current had to pass the S6–S6 interface gap where the verapamil binding site is located (42). Based on this information we suppose that the entryway of the σ -pore is located between the back side of the Tyr-395 of the GYG motif of the selectivity filter and the Trp-384 of the pore helix. The exchange of the valine by cysteine, removing the two methyl groups of the valine at position 388, enlarges the space in between Tyr-395 and Trp-384 and may now allow the passage of ions (Fig. 6, *C* and *D*). The pathway proceeds in plane parallel to the α -pore to the binding pocket of verapamil in the S6–S6



FIGURE 9. Proposed location of the σ -pore compared with the α - and ω -pores mapped on the Kv1.2 crystal structure (4) (PDB entry 2A79).

interface gap (Fig. 7*B*) and ends between S5 and S6 at the intracellular side of one subunit (Fig. 8, *C* and D). For allowing the ions to exit the σ -pore S4 has to be in the resting state because then the concerted movement of S5 and S6 (48, 49) might enlarge the space between them and presumably opens the exit of the σ -pore. Our results suggest that the V388C mutation in the *h*Kv1.3 channel opens another pathway in parallel to the α -pore and different from the ω -pore. A comparison of the different pathways is shown in Fig. 9. The new pathway can conduct different ions and can be blocked by verapamil.

The finding of a novel and unexpected ion pathway behind the central pore, *i.e.* an ion-permeating cavity in the channel running in parallel to the central pore defines the structure of that area under physiological conditions. The flexibility of the selectivity filter of the central pore in the absence and presence of permeant ions result in the opening and/or closing (constricting) of the novel ion pathway. This finding gives mechanistic insights into the structural rearrangement behind the central pore when permeant ions occupy the central pore of the channel.

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A Novel Current Pathway Parallel to the Central Pore in a Mutant Voltage-gated Potassium Channel

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