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RESEARCH PAPER

Effect of verapamil on the action of methanethiosulfonate reagents on human voltage-gated $K_v1.3$ channels: implications for the C-type inactivated state

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BACKGROUND AND PURPOSE

Voltage-gated $K_v1.3$ channels appear on T-lymphocytes and are characterized by their typical C-type inactivation. In order to develop drugs stabilizing the C-type inactivated state and thus potentially useful in treatment of autoimmune diseases, it is important to know more about the three-dimensional structure of this inactivated state of the channel.

EXPERIMENTAL APPROACH

The patch-clamp technique was used to study effects of methanethiosulphonate (MTS) compounds on currents through wild-type human $K_v1.3$ (*h*$K_v1.3$) and two mutant channels, *h*$K_v1.3$ V417C and *h*$K_v1.3$ H399T-V417C, in the closed, open and inactivated states.

KEY RESULTS

Extracellular application of 2-aminoethyl methanethiosulphonate (MTSEA) irreversibly reduced currents through *h*$K_v1.3$ V417C channels in the open and inactivated, but not in the closed state, indicating that a modification was possible. Co-application of verapamil prevented this reduction. Intracellular application of MTSEA and [2-(trimethylammonium)ethyl] methanethiosulphonate (MTSET) also modified the mutant channels, whereas extra- and intracellular application of sodium (2-sulfonatoethyl)methanethiosulphonate (MTSES) and intracellular application of MTSET did not.

CONCLUSIONS AND IMPLICATIONS

Our experiments showed that the binding site for MTS compounds was intracellular in the mutant channels and that the V417C mutant channels were modified in the open and the inactivated states, and this modification was prevented by verapamil. Therefore, the activation gate on the intracellular side of the selectivity filter must be open during inactivation. Furthermore, although the S6 segment is moving further apart during inactivation, this change does not include a movement of the side chain of the amino acid at position 417, away from lining the channel pore.

Abbreviations

CTX, charybdotoxin; GFP, green fluorescent protein; MTS, methanethiosulphonate; MTSEA, 2-aminoethyl methanethiosulphonate; MTSES, sodium (2-sulfonatoethyl)methanethiosulphonate; MTSET, [2-(trimethylammonium)ethyl] methanethiosulphonate; TEA, tetraethyl ammonium; wt, wild type

Introduction

The human voltage-gated potassium channel $hK_v1.3$ (nomenclature follows Alexander *et al.*, 2009) belongs to the *Shaker*-related potassium channel family (Grissmer *et al.*, 1990) and is known to be involved in T-lymphocyte activation (Chandy *et al.*, 1984; DeCoursey *et al.*, 1984). This channel consists of four subunits, each containing six transmembrane segments S1–S6. The segments S1–S4 form the voltage sensor and the segments S5 and S6 form the central pore through which potassium ions can pass. Many substances have been found that can bind to S5 and S6 and thus block the current through the $hK_v1.3$ channel. For example, tetraethyl ammonium (TEA) or peptide toxins like charybdotoxin bind to the extracellular vestibule between S5 and S6 (Kavanaugh *et al.*, 1991; Kaczorowski and Garcia, 1999), thereby plugging the pore physically, whereas compounds like verapamil bind to the intracellular side of the channel (Rauer and Grissmer, 1996; 1999), resulting in a reduction of current flow through the channel.

From electrophysiological experiments, three states of the $K_v1.3$ channel, closed (C), open (O) and inactivated (I), have been described (DeCoursey, 1995). At hyperpolarized potentials, for example, all channels stay in the non-conducting C state and a depolarization leads to the conducting O state of the channel. Prolonged depolarization leads to another non-conducting state named the C-type inactivated I state. This C-type inactivation is typical of $hK_v1.3$ channels and distinguishes it from other members of the *Shaker*-related potassium channels.

Some models based on functional studies had been trying to explain the conformational changes of the channel that occur when going from the closed to the open state. Liu *et al.* (1997) showed, by studies with methanethiosulphonate compounds and Cd^{2+} binding, that the opening was due to changes occurring mainly at the intracellular site of the *Shaker* channel, i.e. that there is a gate at the intracellular site of the channel which opens when the channel is activated, changing from the C to the O state (trap door motion). This gate was composed of the four intracellular S6 transmembrane segments of each subunit making a 'bundle-crossing' (Holmgren *et al.*, 1998), resembling the top of a teepee in the C state so that the permeation pathway for the ions was blocked. The 'bundle crossing' was composed of a highly conserved proline sequence making a bend within S6 (del Camino *et al.*, 2000). To open the channel, the S6 transmembrane helices swing open either by using this proline sequence or using a highly conserved glycine sequence as a hinge (Yellen, 2002).

Based on crystallographic data, additional models had been published that could be used as a scaffold for the open and closed conformation of the channel. For example, a model of the *KcsA* channel was published by Doyle *et al.* (1998), presumably in the closed state (Yellen, 2002). The first model for a voltage-gated, six transmembrane segment, potassium channel was published by Long *et al.* (2005), showing the $K_v1.2$ channel in the open conformation. The structural data from the $K_v1.2$ channel agree well with the data gained by functional analysis (Tombola *et al.*, 2005), confirming the changes in S5 and S6 when the channel opens.

A crystal model of the C-type I state was published for *KcsA* channels and was written by Cuello *et al.* (2010), suggesting that inactivation occurs due to a narrowing at positions 2 and 3 in the selectivity filter, restricting the ion permeation pathway in the C-type I state. This model is supported by functional analysis through K^+ depletion experiments and a change in selectivity when K^+ is removed from the channel (López-Barneo *et al.*, 1993; Kiss and Korn, 1998), indicating a partial collapse of the selectivity filter (Yellen, 1998), although other measurements suggest that the C-type I state of the channel is not identical to the K^+-removed state of the channel (Jäger *et al.*, 1998).

Independent of a restriction of the selectivity filter, earlier experiments, however, supported the idea that inactivation also involves structural rearrangements close to or even at the external vestibule of the channel (Liu *et al.*, 1996). This idea came from experiments with extracellularly applied TEA that showed that inactivation could not occur when TEA blocked current flow (Grissmer and Cahalan, 1989; Choi *et al.*, 1991), thereby keeping the 'inactivation gate' open (Yellen *et al.*, 1994).

No matter what the underlying mechanism is for C-type inactivation in $hK_v1.3$ channels, this state would be ideal for the development of a potent and $hK_v1.3$-selective drug, as the C-type I state is typical of $K_v1.3$ channels. In order to develop such a drug through rational drug design, it is necessary to know the 3D structure of the inactivated state of the $K_v1.3$ channel, in relation to that of the closed and open states.

In our present study, we have used mutant $hK_v1.3$ channels with the valine at position 417, a part of the S6 segment in the channel changed to a cysteine. The change to a cysteine was chosen in order to enable treatment with methanethiosulphonate (MTS) compounds which interact with cysteines by building a disulphide bond (Akabas *et al.*, 1994). We hoped thus to find out if the side chain of this cysteine was lining the pore and was accessible to MTS compounds and, further, if this cysteine participated in the inactivation of the channel. We chose this amino acid residue as it (at 417) is closer to the selectivity filter than the amino acid at position 470 in the *Shaker* channel ($K_v1.3$ position 420) studied by Liu *et al.* (1997) and because we thought that the proposed changes in the selectivity filter during inactivation (see above) could also affect amino acids close to the selectivity filter (here amino acid at position 417, *Shaker* position 467). Data obtained using MTS compounds have helped to understand the movement of some residues in S4 according to changes in the membrane potential in voltage-gated sodium channels (Yang *et al.*, 1996). They were also useful in determining the position of the activation gate in *Shaker* channels (Liu *et al.*, 1997), which is between position 477 and 482 in the channel or in order to determine the pathway of ions through the omega pore in *Shaker* channels (Tombola *et al.*, 2007).

In our study, we have investigated the influence of MTS compounds on currents through wild type (wt) and mutant $hK_v1.3$ channels and found that two such compounds, 2-aminoethyl methanethiosulphonate (MTSEA) and [2-(trimethylammonium)ethyl] methanethiosulphonate (MTSET), irreversibly blocked currents through mutant $hK_v1.3$ channels in the O and I states, but not in the C state. Moreover, co-application of verapamil prevents the reduction in current following these MTS compounds.

Methods

Cell culture

The COS-7 cell line was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany, Cat.-No. ACC 60). The cells were grown in Dulbecco's modified Eagle's medium with high glucose (Invitrogen, Carlsbad, CA, USA, Cat.-No. 41966) containing 10% fetal bovine serum (Thermo Fisher Scientific, Bonn, Germany, Cat.-No. CH30160.02/03) in a humidified incubator at 37°C and 10% CO_2.

Electrophysiology

All experiments were performed at room temperature (18–22°C) and the whole-cell recording mode of the patch-clamp technique (Hamill *et al.*, 1981) was used for all experiments. The electrophysical measurements were performed in an external bath solution (Na-Ringer solution; Na-Ri), with high $[Na^+]_{out}$, containing 160-mM NaCl, 4.5-mM KCl, 2-mM $CaCl_2$, 1-mM $MgCl_2$ and 5-mM HEPES. The internal pipette solution contained 155-mM KF, 10-mM K-EGTA, 10-mM HEPES and 1-mM $MgCl_2$. Osmolarity was 290–320 mOsm for the external and internal solutions and the pH was adjusted to 7.4 for the bath and to 7.2 for the pipette solution with NaOH and KOH respectively. Electrodes were pulled from glass capillaries (Science Products, Hofheim, Germany) in three stages and then fire polished to a resistance of 2–4 MΩ. Data were acquired with an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) connected to a Dell computer running Patchmaster/Fitmaster v2.00 data acquisition and analysis software (HEKA Elektronik). All currents were filtered by a 2.9-kHz Bessel filter and recorded with a sampling frequency of 1 kHz. Capacitative and leak currents were subtracted and a series resistance compensation of 75–85% was used for currents exceeding 2 nA.

The holding potential was −120 mV if not mentioned otherwise. Further data analysis, i.e. exponential fits to data points, was performed with the analysis function of the software Igor Pro 3.12 (WaveMetrics, Lake Oswego, OR, USA) which also calculated the respective time constant τ for each measurement.

Molecular biology

The *h*K_v1.3 wt plasmid was a generous gift from Prof Dr O. Pongs (Institut für Neuronale Signalverarbeitung, Zentrum für Molekulare Neurobiologie, Hamburg, Germany). It contains the *h*K_v1.3 potassium channel gene in a pRc/CMV vector (Invitrogen, Carslbad, CA, USA) with a CMV promoter for protein expression in mammalian cells. The V417C and H399T-V417C mutants were originally generated in our laboratory by Dr Tobias Dreker (4SC AG, Martinsried, Germany) and Dr Sylvia Prütting (4SC AG), respectively, by introducing the corresponding point mutation in the cloned *h*K_v1.3 gene with the QuickChange site-directed mutagenesis kit (Stratagene, Amsterdam, the Netherlands). COS-7 cells were transfected using the FuGene 6 transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany). When cells were grown to a confluence of ~80%, they were co-transfected with ~1 µg *h*K_v1.3 DNA and ~0.5 µg of green fluorescent protein (eGFP-N1) DNA (eGFP-N1 Plasmid-DNA, Clontech Laboratories, Inc, Palo Alto, CA, USA). The cells were used after 1–4 days after transfection, as sufficient protein was expressed for the electrophysiological measurements at this time.

Modelling

The three-dimensional model of the MTSEA molecule was created automatically by the Dundee Prodrug2 Server (Schuettelkopf and van Aalten, 2004) on the basis of the chemical structure of this molecule. Afterwards, a pdb-file of the three-dimensional model of MTSEA was created. This pdb-file was uploaded together with the pdb-file of the crystallization data of *h*K_v1.2 channel (pdb-file 2A79, Long *et al.*, 2005) that served as a scaffold for the *h*K_v1.3 mutant channel, into the Swiss-PDB Viewer. The β-subunit and the t1-domains of the channel were removed using the Swiss-PDB Viewer so the model only contains the α-subunit of the channel. For clarity, S1 to S4 and all the side chains of the amino acids were also removed, with the exception of the side chains of the amino acids at positions 417 and 412.

The MTSEA molecule was brought into close contact to the residue of the amino acid at position 417 in the channel by hand.

Materials

The MTS compounds [MTSEA, Cat.-no. A609100; sodium (2-sulfonatoethyl)methanethiosulphonate (MTSES), Cat.-no. S672000; MTSET, Cat.-no. T795900] were purchased from Toronto Research Chemicals, Inc. (North York, Canada), with stock solutions (1 M) prepared in distilled water and stored at –20°C. They were diluted to the final concentration in the external bath or internal pipette solution before application. The phenylalkylamine verapamil was purchased from Sigma-Aldrich Co. (Munich, Germany), with stock solutions prepared in dimethyl sulfoxide (DMSO) and diluted to the final concentration in the external bath solution before application. The DMSO content of the final solutions was always less than 0.1%.

Results

In this study, we investigated the influence of different MTS compounds on the current through *h*K_v1.3 wt and *h*K_v1.3 V417C mutant channels in order to find out if these compounds, as cysteine-modifying reagents, would react with the cysteine introduced at position 417 in the mutated channel, compared with their action on *h*K_v1.3 wt channels.

Influence of externally applied MTSEA on the wt and mutant hK_v1.3 V417C channels

We first assessed the actions of MTSEA on the wt *h*K_v1.3 channel. We elicited currents through *h*K_v1.3 wt in the whole-cell configuration of the patch-clamp by depolarizing steps from a holding potential of –120 mV to +40 mV for 200 ms every 30 s (Figure 1A). The elicited current showed that the channels activated rapidly, reaching a peak of ~5.6 nA within 20 ms and declined to ~3.5 nA during the 200 ms voltage step, resembling typical currents through wt *h*K_v1.3 channels. Between two voltage steps (between trace a and trace b in Figure 1A,B) we applied 1-mM MTSEA to the bath solution.

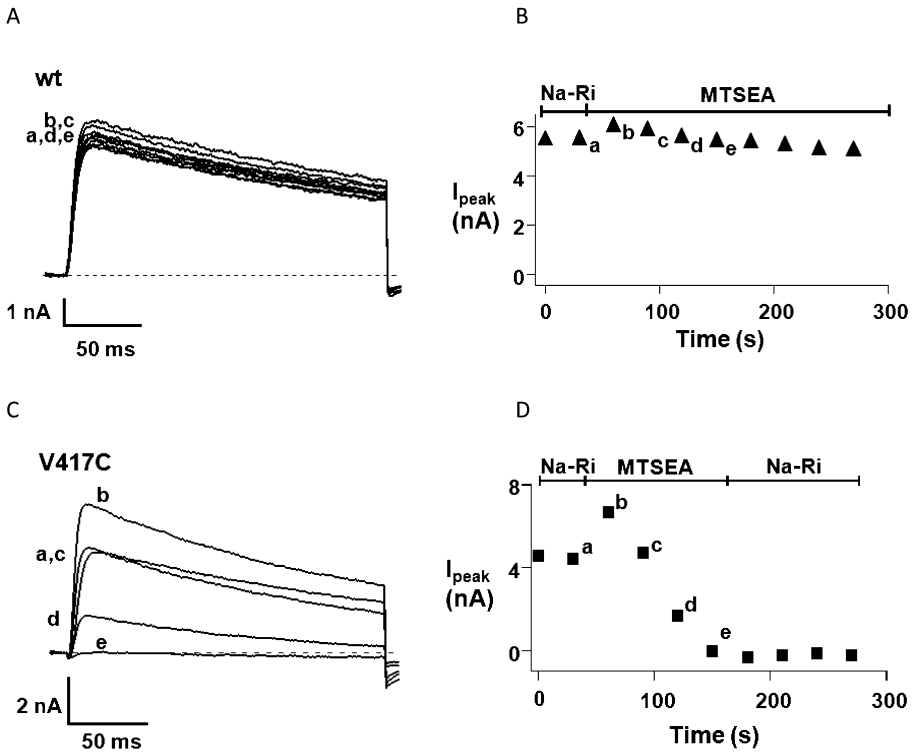

Figure 1

Effect of externally applied MTSEA on currents through *h*K_v1.3 wt and *h*K_v1.3 V417C mutant channels. (A,C) Currents were elicited with 200-ms depolarizing steps from a holding potential of –120 mV to +40 mV every 30 s before (trace a) and after (traces b–e) application of 1-mM MTSEA to the bath solution. (B,D) Peak currents of the traces shown in A and C plotted against the time during the experiment. MTSEA, 2-aminoethyl methanethiosulphonate; Na-Ri, Na-Ringer solution without methanethiosulphonate compound; wt, wild type.

This resulted in a small reduction of peak current of less than 10%, comparing the peak current before the application of MTSEA and the last peak current in Figure 1B.

To test whether the introduction of a cysteine at position 417 in *h*K_v1.3 V417C channels resulted in a different response to externally applied MTSEA, compared with that of the wt channel, we performed the identical experiment with the mutant channel (Figure 1C). The elicited currents through *h*K_v1.3 V417C channels showed the same characteristics as currents through wt *h*K_v1.3 channels (Figure 1C, trace a), indicating that the V417C mutation had hardly any effect on the gating, compared with the wt channels. Again, we added 1-mM MTSEA to the bath solution during two voltage steps (between trace a and trace b in Figure 1C,D). This resulted in a rapid reduction of current to zero within 90 s. This reduction of current was irreversible as after washout with Na-Ri solution without MTSEA, the current remained at zero (Figure 1D). Thus, externally applied MTSEA modified *h*K_v1.3 V417C mutant channels but not wt *h*K_v1.3 channels. However, these experiments did not show which state of the mutant channels (O, C or I) was modified by MTSEA.

Influence of externally applied MTSEA on wildtype and mutant h*K$_v$1.3_V417C channels in the C state*

We first tested the effects of MTSEA on the C state of the wt *h*K_v1.3 channel. The currents were elicited by the same voltage step protocol as described for Figure 1A, again using the whole-cell configuration of the patch-clamp technique. MTSEA (1 mM) was washed into the bath solution after a voltage step (after trace a, Figure 2A,B) and kept there for 5 min while the voltage step protocol was paused for 5 min. During the pause, the membrane potential was kept at –120 mV to make sure all channels were in the C state. After resuming the voltage steps, the size of the peak current was similar to the current recorded before MTSEA (Figure 2B). However, a small reduction in peak current (about 15%) from one trace to another after the pause in Figure 2B was observed for the traces in the presence of MTSEA (traces b–e in Figure 2A,B). This value is similar to the reduction in current already described for Figure 1B where MTSEA was added during two voltage steps.

We then performed the identical experiment with the mutant channel (Figure 2C,D), again adding 1-mM MTSEA to the bath solution after trace a in Figure 2C. However, as observed for current through wt channels (Figure 2A,B), the first current trace through the mutant *h*K_v1.3 V417C channels after 3 min in MTSEA and without pulsing (trace b, Figure 2C,D) showed the same size of peak current as before pausing the pulsing (trace a, Figure 2C,D). Although the size of the peak current was unchanged, indicating that MTSEA was unable to modify currents through *h*K_v1.3 V417C mutant channels in the C state, the time course of current decay (trace b in Figure 2C) during the voltage step was drastically changed, i.e. the current declined to zero during the 200-ms

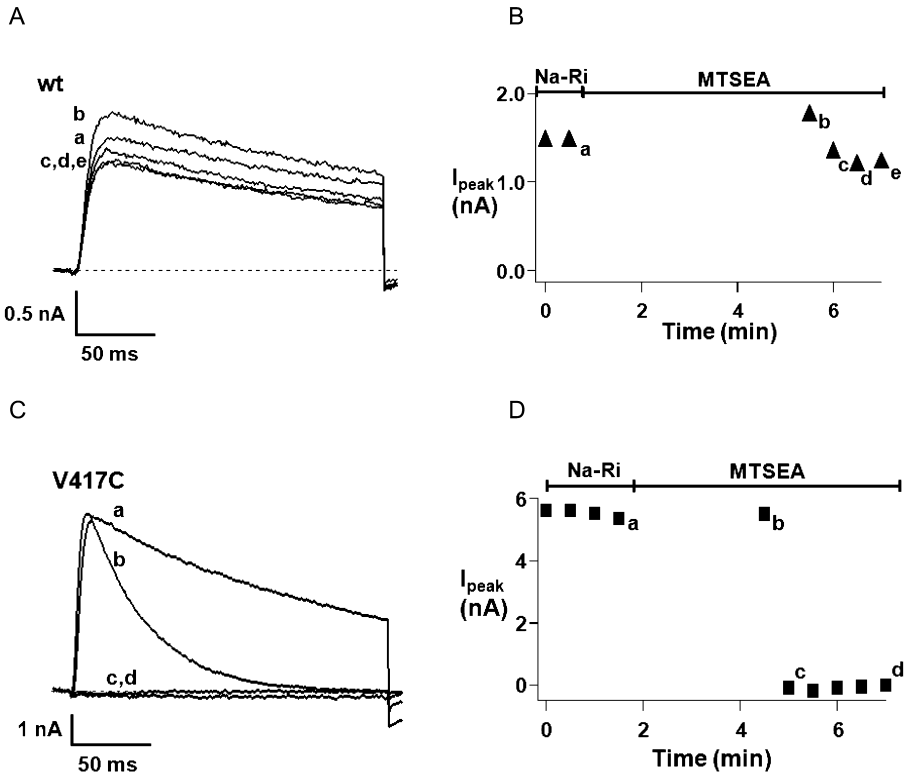

Figure 2

Effect of externally applied MTSEA on currents through *h*K_v1.3 wt and *h*K_v1.3 V417C mutant channels in the closed and open state. (A,C) Currents were elicited by 200-ms depolarizing voltage steps from a holding potential of −120 mV to +40 mV every 30 s before (trace a) and after (traces b–d) application of 1-mM MTSEA to the bath solution. Between traces a and b, no pulses were recorded and the membrane potential was held at −120 mV. (B,D) Peak currents of the traces shown in A and C plotted against the time during the experiment. MTSEA, 2-aminoethyl methanethiosulphonate; Na-Ri, Na-Ringer solution without methanethiosulphonate compound; wt, wild type.

voltage step and all the following voltage steps did not elicit any current through the mutant channels. In order to get a value of both time courses, i.e. before and after application of MTSEA, we fitted an exponential function to trace a and trace b in Figure 2C and obtained a time course with a time constant of 205 and 35 ms respectively.

From Figure 2D, it is clear that the peak current in MTSEA was completely reduced between two pulses 30 s apart (between trace b and c), but it did not change between trace a and trace b. This indicated that MTSEA did not modify the C state of the channel and only after the channels were opened they could be modified by MTSEA. Alternatively, the fact that we observed the same peak current in Figure 2C before (trace a) and after (trace b) the pause could be explained assuming that MTSEA could modify the closed channel; however, the effect would only be visible when the channel is opened. To distinguish whether the mutant channels were modified in the C state versus the O state, we performed an experiment where we applied MTSEA in the bath solution and kept it there for 3 min while the membrane potential was constantly held at −120 mV to make sure all mutant channels stay in the C state. After 3 min, we replaced the bath solution by a Na-Ri-solution in order to remove MTSEA and waited another 2 min before starting the voltage-step protocol (Figure 3B). If MTSEA had modified the C state of the mutant channel, this modification is not to be reversed as MTSEA forms a covalent bond with the cysteine. The first trace after the 5-min period without pulsing (trace b, Figure 3A,B) showed the same peak current as recorded for the trace before the washing process (Figure 3B). In contrast to the previous experiment where MTSEA was present during pulsing, no fast decay in current during the first 200-ms depolarizing pulse could be observed (trace b, Figure 3A) and the following traces (traces b–d) had the same shape as the traces recorded before the application of MTSEA.

The fact that the first trace after the washing process (trace b, Figure 3A) did not resemble trace b in Figure 2C and that in the following traces currents induced through voltage steps could still be observed indicated that MTSEA was unable to modify the C state of the mutant channel. The fast decay of current in trace b in Figure 2C must therefore be the effect of MTSEA on the O or I state of mutant *h*K_v1.3 V417C channels.

Effect of externally applied MTSEA on hK_v*1.3 V417C mutant and* hK_v*1.3 H399T-V417C mutant channels*

In order to find out if the observed current reduction in Figure 1C,D is due to the effect of externally applied MTSEA on the O state of the channel and not the I state of the channel, we pursued two different strategies to minimize

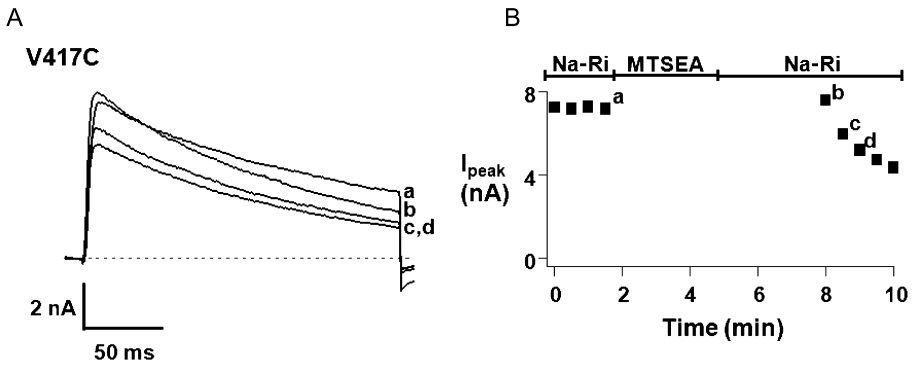

Figure 3

Effect of external application and removal of MTSEA on currents through *h*K_v1.3 V417C mutant channels in the closed state. (A) Currents were elicited by 200-ms depolarizing voltage steps from a holding potential of –120 mV to +40 mV every 30 s before (trace a) and after (traces b–d) application and removal of 1-mM MTSEA to the bath solution. Between traces a and b, no pulses were given and the membrane potential was kept at –120 mV. (B) Peak currents of the traces shown in A plotted against the time during the experiment. MTSEA, 2-aminoethyl methanethiosulphonate; Na-Ri, Na-Ringer solution without methanethiosulphonate compound.

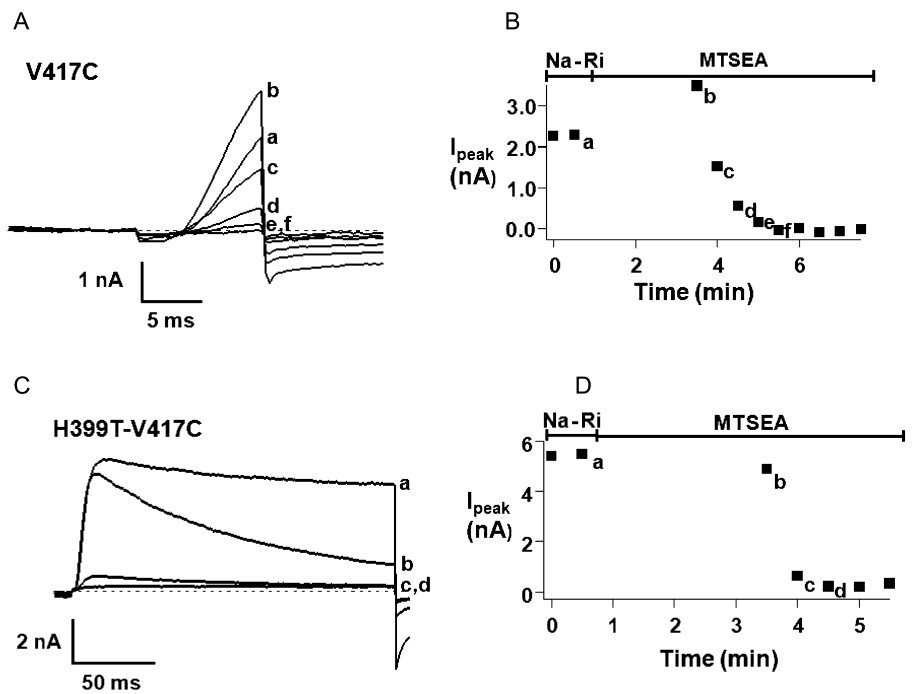

Figure 4

Effect of externally applied MTSEA on currents through *h*K_v1.3 V417C mutant channels (A,B) and *h*K_v1.3 H399T-V417C mutant channels (C,D) in the open state. (A,C) Currents were elicited by depolarizing voltage steps from a holding potential of –120 mV to +40 mV every 30 s for 10 ms in A and for 200 ms in C before (trace a) and after (traces b–f) application of 1-mM MTSEA to the bath solution. Between traces a and b, no pulses were recorded and the membrane potential was held at –120 mV. (B,D) Peak currents of the traces shown in A and C plotted against the time during the experiment. MTSEA, 2-aminoethyl methanethiosulphonate; Na-Ri, Na-Ringer solution without methanethiosulphonate compound.

inactivation during MTSEA treatment. First, we used very short (10 ms) pulses during which C-type inactivation cannot occur and second, we used a double-mutant *h*K_v1.3 H399T-V417C channel that showed similar inactivation properties (data not shown) compared with the *h*K_v1.3 H399T mutant channel, i.e. a reduced C-type I state compared with wt (Rauer and Grissmer, 1996; Dreker and Grissmer, 2005) and *h*K_v1.3 V417C mutant channels (data not shown). The results of both experimental approaches are shown in Figure 4. Even 10-ms pulses (Figure 4A,B) were enough for MTSEA to reduce the current through *h*K_v1.3 V417C mutant channels, indicating that the channels do not need to undergo C-type inacti-

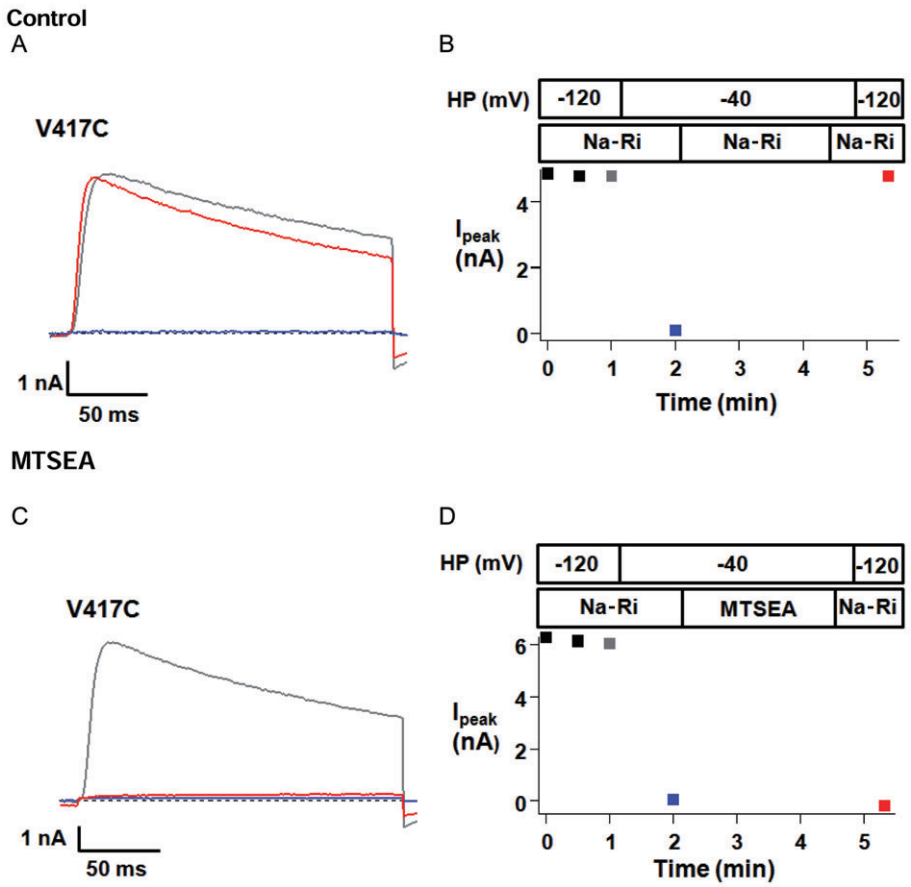

Figure 5

Effect of externally applied MTSEA on the inactivated state of *h*K_v1.3 V417C mutant channels. (A,C) Currents were elicited by 200-ms depolarizing voltage steps from a holding potential of –120 mV to +40 mV every 30 s. After the grey trace, the membrane potential was set to –40 mV to make sure the channels were in the inactivated state and a depolarizing step from this holding potential to +40 mV was given (blue trace). After the blue trace was recorded, the solution was replaced by Na-Ri or MTSEA for A or C respectively. After 2 min, the bath solution was changed to fresh Na-Ri and the membrane potential was set to –120 mV again from which the next depolarizing voltage step to +40 mV was recorded (red trace). Corresponding changes in the holding potential (HP) and solution changes are given in the boxes on top of B and D. (B,D) Peak currents of the traces shown in A plotted against the time during the experiment. MTSEA, 2-aminoethyl methanethiosulphonate; Na-Ri, Na-Ringer solution without methanethiosulphonate compound.

vation in order to be modified by MTSEA. This idea was confirmed by experiments with the double-mutant *h*K_v1.3 H399T-V417C channels (Figure 4C,D) demonstrating an identical MTSEA-modification pattern compared with the single mutant *h*K_v1.3 V417C channels. From these experiments, we conclude that the O state of the *h*K_v1.3 V417C channel is sufficient for MTSEA modification.

Effect of externally applied MTSEA on hK_v*1.3 V417C mutant channels in the I state*

To find out if the cysteine introduced at position 417 in mutated *h*K_v1.3 V417C channels was also accessible in the I state of the channel, we carried out another experiment where we applied MTSEA only to inactivated channels.

Here, we used the same protocol in the same cell, first without and then with MTSEA. The currents were elicited through mutated *h*K_v1.3 V417C channels by depolarizing pulses from a holding potential of –120 mV to +40 mV for 200 ms every 30 s (grey trace). Then the membrane potential was set to –40 mV after the pulse protocol had been stopped to make sure all channels were in the I state. After 1 min at –40 mV, a 200-ms voltage step from this holding potential to +40 mV was recorded (Figure 5A, blue trace), showing no current indicating that all mutant channels were in the I state. After this voltage step, the cell was washed twice with fresh Na-Ri-solution for 2 min while the membrane potential was maintained at –40 mV. After the second wash, the membrane potential was changed to –120 mV in order for the channels to recover from the I state. Twenty seconds after changing the membrane potential, a 200-ms voltage step from this holding potential of –120 mV to +40 mV was recorded (Figure 5A, red trace), showing a peak current of almost 100% compared with the trace recorded before the first wash (Figure 5A, grey trace). This indicated that 20 s at –120 mV would be enough time for the channels to recover from inactivation induced by the change in the holding potential to –40 mV.

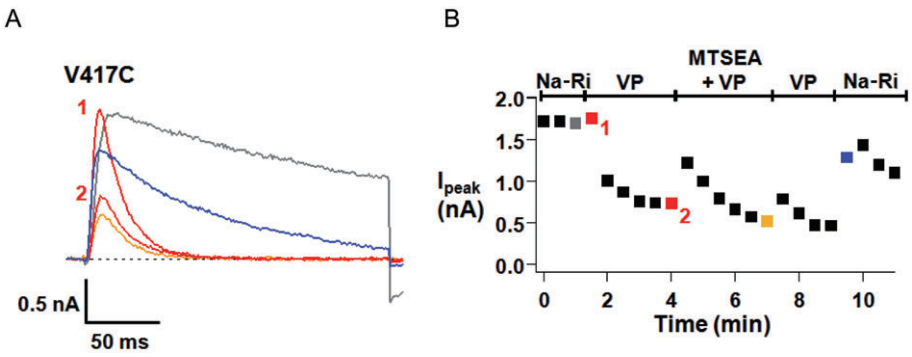

Figure 6

Protection against the effects of MTSEA (1 mM) by application of 100-μM verapamil (VP) to the bath solution in *h*K_v1.3 V417C mutant channels. (A) Currents were elicited by 200-ms depolarizing voltage steps from a holding potential of −120 mV to +40 mV every 30 s before and after application of the substances (as seen in B, top) to the bath solution. (B) Peak currents of the traces shown in A plotted against the time during the experiment. MTSEA, 2-aminoethyl methanethiosulphonate; Na-Ri, Na-Ringer solution without methanethiosulphonate compound.

Cells were exposed to MTSEA using the same protocol and the same changes in the holding potential. Instead of washing-in Na-Ri after the voltage pulse from −40 mV to +40 mV, 1-mM MTSEA was washed into the bath solution and kept there for 2 min while the membrane potential was at −40 mV. In order to wash out MTSEA, a Na-Ri-solution was used. The trace before the washing process (Figure 5C, grey trace) resembled the corresponding trace in the control experiment (Figure 5A, grey trace). Also, the traces where a voltage pulse from −40 mV to +40 mV was recorded (Figure 5A, blue trace and Figure 5C, blue trace) correspond to each other, indicating that most of the *h*K_v1.3 V417C channels were in the I state. In contrast to the control experiment, where a complete recovery from inactivation was observed (Figure 5A, red trace), the channels treated with MTSEA in the I state did not recover at all (Figure 5C, red trace), indicating that MTSEA could modify channels in the I state.

From the experiments described so far, it was clear that MTSEA could modify mutant *h*K_v1.3 V417C channels in the O and I states but not in the C state, and that the modification resulted in an irreversible reduction in current.

Verapamil prevents modification of hK_v*1.3 V417C mutant channels by MTSEA*

Because we wanted to find a substance which could prevent MTSEA to modify the mutant channels, we selected verapamil as one of the binding sites for verapamil in this channel is the alanine at position 413 (Dreker and Grissmer, 2005), which is very close to position 417, the putative site of modification by MTSEA in the mutant channel. Therefore, we elicited currents through the mutant channels as described for Figure 1C and added all substances (verapamil without and with MTSEA; for solution changes see top of Figure 6A) to the bath solution between two depolarizing steps (Figure 6).

After obtaining a control current through the *h*K_v1.3 V417C mutant channels (Figure 6A, grey trace), 100-μM verapamil was added to the bath solution. This resulted in the characteristic effect of verapamil on current through K_v1.3 channels: an acceleration of the current decay during the depolarization and a reduction in the peak current amplitude from trace to trace until the peak current amplitude has reached a new steady state (Jacobs and DeCoursey, 1990; DeCoursey, 1995; Rauer and Grissmer, 1996). In a second step, we applied a mixture of 1-mM MTSEA and 100-μM verapamil to the bath solution for 150 s (Figure 6B). Subsequent testing showed that current reduction by this mixture was only marginal. In addition, after washout of verapamil and MTSEA, the current almost fully recovered (Figure 6A, blue trace).

As currents through *h*K_v1.3 V417C mutant channels were still elicited after the washout of verapamil and MTSEA, the irreversible block by MTSEA (see Figure 1D) due to reaction with cysteine 417 must have been prevented by verapamil.

Effect of internally applied MTSEA on hK_v*1.3 V417C mutant channels*

As verapamil can pass through the membrane and act on the inner site of the channel (Rauer and Grissmer, 1996), we wanted to know if MTSEA could also modify the channel when applied internally. This would indicate that externally applied MTSEA could pass through the membrane to reach an internally accessible binding site in the channel.

Currents through the mutant channels were elicited by the same protocol described for Figure 1C; however, the first trace (trace a, Figure 7A,B) was recorded 6 min after breaking into the cell in order to rule out diffusion limiting effects. During the first 6 min after breaking into the cell, the membrane potential was continuously held at −120 mV to make sure all channels were in the closed state.

The recorded traces were similar to currents through *h*K_v1.3 V417C mutant channels in Figure 2C where MTSEA was applied externally and then the pulse protocol was paused, although here the current reached zero after the second trace whereas in Figure 2C one voltage pulse was enough to completely block the current through the mutant channels.

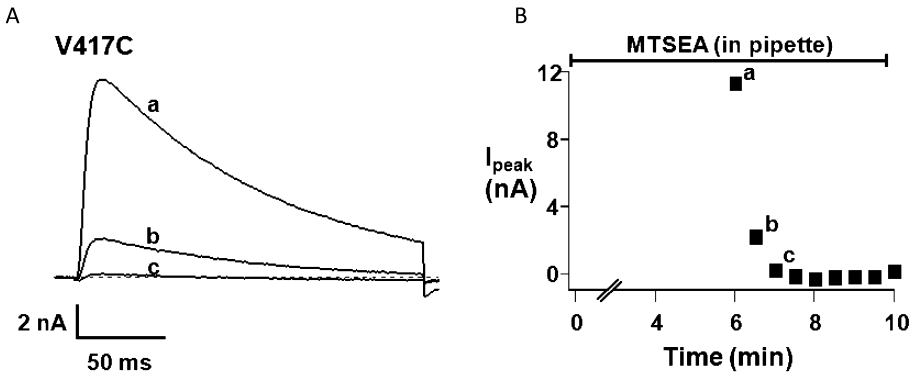

Figure 7

Effect of internally applied MTSEA (1 mM) on currents through *h*K$_v$1.3 V417C mutant channels. (A) Currents were elicited by 200-ms depolarizing voltage steps from a holding potential of −120 mV to +40 mV every 30 s. (B) Peak currents of the traces shown in A plotted against the time after breaking into the cell. MTSEA, 2-aminoethyl methanethiosulphonate.

The reduction of current during the voltage pulses (traces a–c and following in Figure 7B) indicated that MTSEA could also modify *h*K$_v$1.3 V417C mutant channels when applied internally. To determine whether MTS compounds interact with internally or externally accessible sites of the channel, we used charged MTS compounds (MTSET and MTSES carrying a positive or a negative charge, respectively), which are unable to pass through the cell membrane. When applied extracellularly, both charged MTS compounds (MTSET and MTSES) did not rapidly reduce current through V417C mutant channels. During the first 5 min after application, extracellularly applied MTSET led to a current reduction of 29% and extracellular MTSES led to a current reduction of 25% (data not shown). This indicated that the binding site for MTSET and MTSES could not be reached from the extracellular side of the channel.

Effect of internally applied MTSET and MTSES on h*K$_v$1.3 wt and* h*K$_v$1.3_V417C mutant channels*

To test the effects of internally applied MTSET or MTSES, we first tested the positively charged MTSET (5 mM), internally applied to wt *h*K$_v$1.3 channels. We elicited the currents in the whole-cell recording mode of the patch-clamp technique by depolarizing steps from a holding potential of −120 mV to +40 mV every 30 s. The first trace was recorded 10 min after breaking into the cell (Figure 8A,B) and the membrane potential was kept at −120 mV during these 10 min. Within 10 min after starting the pulse protocol, no significant reduction in current (Figure 8A,B) was observed, indicating that internally applied MTSET, similar to externally applied MTSEA (compare Figures 1A,D and 2A,B), did not modify wt *h*K$_v$1.3 channels.

We then used the same protocol and MTSET in the same concentration with *h*K$_v$1.3 V417C mutant channels. Starting the pulse protocol resulted in a reduction of current (Figure 8C,D) similar to externally applied MTSEA on the mutant channels, although this current decay was slower compared with internally applied MTSEA (Figure 7B) because here the current reached zero after the fifth trace whereas in Figure 7B it reached zero after the second trace.

In the next set of experiments, we tested the effect of the negatively charged MTSES (5 mM). With wt channels, intracellular application of MTSES did not result in a current decay (data not shown) and the traces resembled Figure 8A where MTSET was applied intracellularly to wt channels.

With *h*K$_v$1.3 V417C mutant channels, internally applied MTSES (5 mM) induced no significant decay in current within 5 min (Figure 8E,F). After 5 min, 70% of the peak current recorded in the first trace after starting the pulse protocol could still be elicited, whereas for internally applied MTSEA after 1 min (Figure 7B) and for internally applied MTSET after 2.5 min (Figure 8C and D) no current could be elicited. This indicated that internally applied MTSES had hardly any effect on mutant *h*K$_v$1.3 V417C channels.

In summary, our experiments showed that extracellularly applied MTSEA and intracellularly applied MTSET induced a rapid current reduction in *h*K$_v$1.3 V417C mutant channels, whereas the current reduction for these channels treated with MTSES was only marginal. MTSEA irreversibly blocked the I and O states of the channel whereas the C state of the channel could not be modified by extracellularly applied MTSEA. In wt channels, none of the MTS compounds induced a rapid reduction in current.

Discussion

The aim of this study was to find out whether the cysteine introduced at position 417 in the mutant channel could be modified by externally and/or internally applied MTS compounds and which state of the channel was accessible for modification. We found that MTS compounds did not reduce current through wt *h*K$_v$1.3 channels. This indicated that the naturally occurring cysteines in this channel, especially the cysteine at position 412 (*Shaker* position 462), which is close to position 417 (*Shaker* position 467), cannot be modified by MTS compounds or that the modification was possible but did not reduce current.

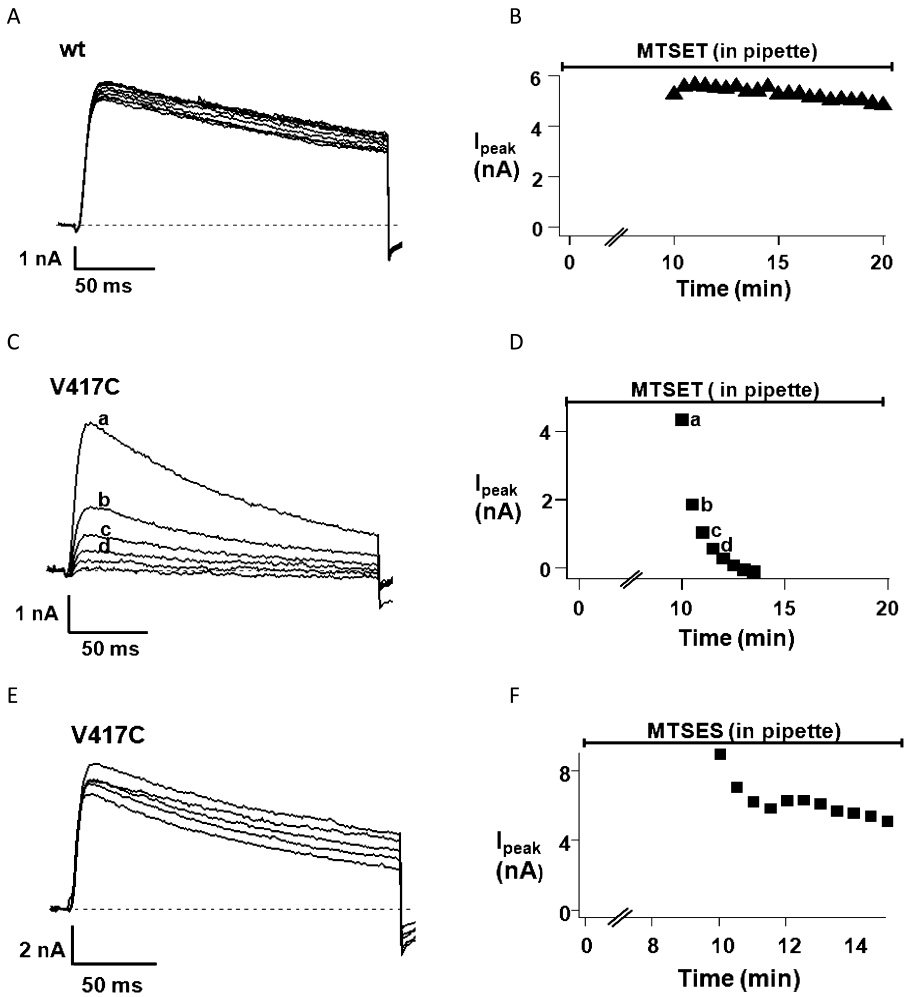

Figure 8

Effect of internally applied MTSET (5 mM) and MTSES (5 mM) on currents through *h*K_v1.3 wt and *h*K_v1.3 V417C mutant channels. (A,C,E) Currents were elicited by 200-ms depolarizing voltage steps from a holding potential of –120 mV to +40 mV every 30 s. For clarity, only every other trace is shown in A and E. (B,D,F) Peak currents of the traces shown in A, C, and E plotted against the time after breaking into the cell. MTSES, sodium (2-sulfonatoethyl)methanethiosulphonate; MTSET, [2-(trimethylammonium)ethyl] methanethiosulphonate; wt, wild type.

MTS compounds reach the cysteine at position 417 in hK_v*1.3 V417C from the intracellular side of the channel*

The extracellular and intracellular applications of MTSEA (Figures 1C,D and 7A,B) resulted in an irreversible current reduction in the mutant channel, indicating that the cysteine introduced at position 417 was modified by MTSEA. This irreversible current reduction confirmed earlier measurements in HCN1 channels (Xue and Li, 2002) and must be due to the covalent binding of MTSEA to the cysteine-417 in the mutant channel.

As MTSEA can pass the membrane, these experiments did not help to elucidate whether MTS compounds act from the intra- or the extracellular side of the channel. For this purpose, charged MTS compounds (the positively charged MTSET and the negatively changed MTSES) were used. Because MTSET blocked the current through mutant channels only when applied intracellularly (Figure 8C,D), this indicated that the binding site is indeed intracellular and that extracellularly applied MTSEA must pass the membrane to reach its internal binding site. Similar results have been obtained for the membrane-permeable verapamil and the permanently charged, membrane-impermeable N-methyl verapamil to confirm the internal binding site of verapamil (Rauer and Grissmer, 1996). Both molecules contain a central nitrogen, which can either be neutral, as in verapamil, or permanently positively charged as in N-methyl verapamil. Verapamil may physically occlude the internal vestibule of the channels by blocking the ion permeation pathway with its central nitrogen (Wulff *et al.*, 2009). For MTSEA and MTSET, we propose a similar mechanism, because both molecules contain a nitrogen, which is neutral or positively charged for MTSEA or permanently positively charged in MTSET. We suppose that this charged nitrogen in MTSET and MTSEA, similar to that in verapamil, is blocking the ion conduction pathway through the V417C mutant channels. This hypothesis would also explain why MTSES, which does

not carry a nitrogen, did not modify the *h*K_v1.3 V417C mutant channels.

Internally applied MTSES did not reduce current through mutant channels compared with internally applied MTSEA and MTSET. A simple explanation for this difference could be the different efficacy of MTSES compared with MTSEA and MTSET to modify simple thiols (Stauffer and Karlin, 1994). Alternatively, the difference could be due to the negative charge of MTSES. Naturally occurring negative charges in the channel could prevent MTSES from reaching its binding site due to electrostatic repulsion. Although no amino acids carrying a negative charge can be found within 16 Å of position 417 in the channel, it is conceivable that Glu343 (K_v1.2 Glu327) being part of S5 and further away from the selectivity filter than cysteine-417 in the *h*K_v1.3 V417C channel might, through electrostatic repulsion, prevent MTSES from reaching its binding site. Alternatively, the MTSES-molecule could be too large or not flexible enough to reach its binding site although MTSES is smaller than MTSET (Akabas *et al.*, 1992). Another possibility for the different results obtained for MTSES is that MTSES led to a modification but this modification did not affect the current because MTSES lacks the amino group (see above).

Although we cannot exclude any of these possibilities as explanations of the inability of MTSES to reduce current, we favour the idea that somehow the amino group in the MTS compounds, as in verapamil, is important for the block of the channel. Verapamil is known to bind to position 413 (Dreker and Grissmer, 2005) in the channel, close to position 417. Taken together, the experiments clearly demonstrated that the binding site for MTS compounds in the mutant channel must be intracellular.

MTS compounds did not modify the C state of the hK_v*1.3 V417C mutant channels*

Our experiments described in Figure 2C,D/Figure 3A,B clearly indicated that the C state of the channel could not be modified by MTSEA. This result confirms experiments made with the *Shaker* channel where the amino acid at position 470 (*h*K_v1.3 position 420) and position 474–477 (*h*K_v1.3 position 424–427) could not be modified by MTS compounds in the C state whereas a modification in the O state was possible (Liu *et al.*, 1997). Positions further away from the selectivity filter towards the inside of the cell, position 482–486 (*h*K_v1.3 position 432–436), could be modified in the O and C state of the channel. In addition, using tetrabutylammonium, an open-channel blocker, the authors concluded that at least the cysteines at positions 470 and 474 must be behind an activation gate which is moved away when the channel is in the O, activated state. In the C state, this gate is closed and the cysteines behind this gate (position 470 and 474–477 in the *Shaker* channel) cannot be modified, whereas the cysteines outside the gate can be modified when the channel is closed. As the mutation we introduced at position 417 in the *h*K_v1.3 channel is deeper in the cavity, i.e. closer to the selectivity filter compared with position 470 in the *Shaker* (position 420 in *h*K_v1.3) channel, it seems likely that this position is protected by the activation gate and that access to the cysteine at position 417 in the *h*K_v1.3 mutant channel is only possible when the gate is open.

MTS compounds modify the O and the I states of the hK_v*1.3 V417C mutant channels*

Our experiments with MTSEA (Figure 5C,D) indicated that MTSEA could modify the I state of the channel. The fact that the I state could be modified has implications for the position of the activation gate during inactivation and for the availability of the side chain of the cysteine at position 417 during inactivation. Cuello *et al.* (2010) proposed that during inactivation, the activation gate must be open. This model is confirmed by our data because we know that modification of cysteine-417 can only take place when the activation gate is open and MTSEA can gain access to this residue. The activation gate must still be in the open position during inactivation, otherwise we would have not observed a modification in the I state of the channel. In addition, the proposed movement of S6 during inactivation (Cuello *et al.*, 2010) does not change the position of the side chain at position 417, part of S6, so that it is no longer lining the pore, because the channel was still blocked.

Verapamil prevents the MTSEA modification in hK_v*1.3 V417C mutant channels*

The binding site of MTSEA in the mutant *h*K_v1.3 V417C channel is close to position 413, one of the interaction sites of verapamil (Dreker and Grissmer, 2005). An application of verapamil hindered MTSEA to modify the channel. A similar result was shown for mutant *Shaker* channels (positions 470 and 474, *h*K_v1.3 positions 420 and 424), which could be prevented from MTSEA modification by the open channel blocker tetrabutylammonium (Liu *et al.*, 1997). As verapamil is known to block the O and I states of the channel (DeCoursey, 1995), it could prevent MTSEA from reaching its binding site, presumably by binding to position 413 (Dreker and Grissmer, 2005), thereby at least partially covering position 417 and suggesting a functionally significant overlap in the binding sites for verapamil and MTSEA. Our results indicate that the side chain of the amino acid at position 417 must face the pore in the O, and also in the I, states of the channel. In contrast, the cysteine at position 412 must either face away from the pore because modification was not possible or the cavity in this part of the channel is too narrow for MTSEA to fit in.

Model of the interaction of MTS-reagents with position 417 in the channel

We designed a model (Figure 9) showing one MTSEA molecule in the channel in order to better visualize the interaction between the MTS compound and the introduced cysteine. The left panels (Figure 9) show one MTSEA molecule in close contact with the amino acid at position 417 and the covalent bond between the amino acid and the MTSEA molecule can be seen on the right.

In the top left panel, there is enough space for another three MTSEA molecules to sit within the central pore interacting with the cysteine at position 417 in the channel. It becomes clear that a modification of all four cysteines at position 417 in the four subunits would result in an obstruction of the central current pathway. From Figure 9, it is also clear that the amino acid residue at position 412 is not facing the pore, explaining why external MTSEA appli-

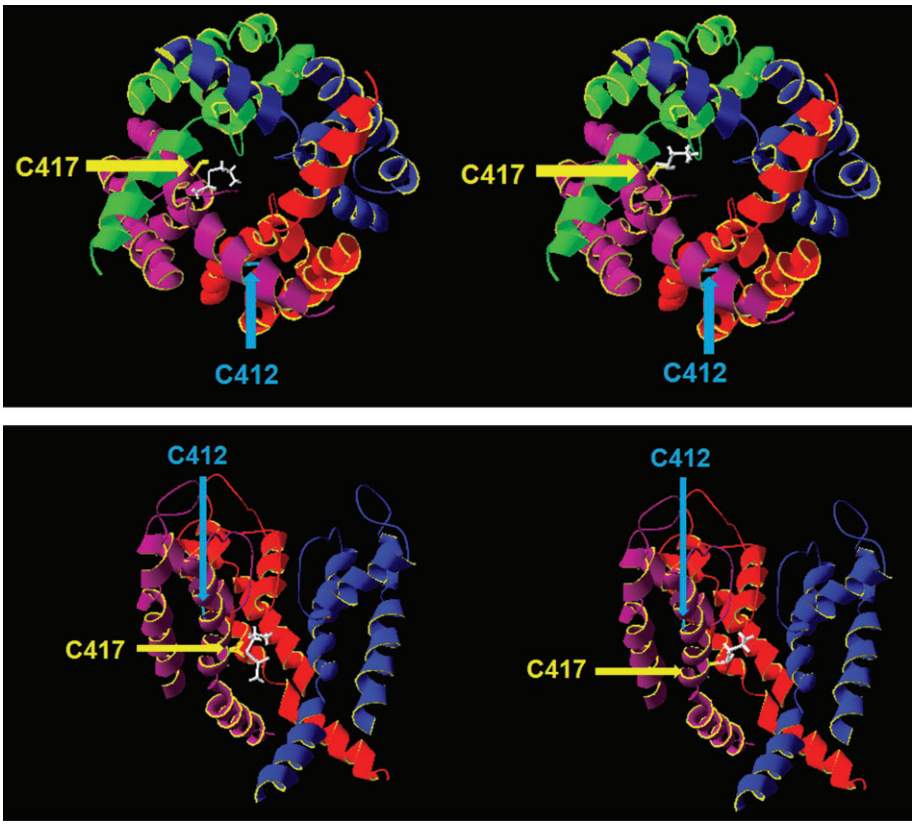

Figure 9

3D Model of the *h*K_v1.3 V417C mutant channel [the K_v1.2 channel (PDB-File: 2A79) was used as a scaffold] containing S5 and S6. Each subunit is drawn in another color (green, red, blue and magenta respectively). Upper figure: view through the pore of the channel from the intracellular side; lower figure: lateral view with the extracellular part showing upward. Side chains of amino acids are not shown except for the side chain of the amino acid at position 412 (light blue, arrow) and 417 (yellow, arrow). The MTSEA molecule is drawn in white before (left) and after (right) modification. The sulfinic acid which forms after modification is not shown in the right panel. MTSEA, 2-aminoethyl methanethiosulphonate.

cation could not reduce current through wt channels (see Figure 1A,B) because MTSEA could not reach this target, cysteine-412.

In conclusion, the cysteine at position 412 in the wt *h*K_v1.3 channel cannot be modified by MTS compounds and is not lining the pore or the pore at this position is too narrow for MTS compounds to fit. For *h*K_v1.3 V417C channels, MTS compounds act from the intracellular side of the channel and the side chain of the cysteine introduced at position 417 is lining the pore and is available for modification when the channel is in the O or in the I state. The MTSEA-induced modification could be prevented by verapamil. During inactivation, the activation gate must be open, thereby confirming the model of Cuello *et al*. (2010). The movement of S6 during the inactivation process (Cuello *et al*., 2010) does not include a movement of the side chain of the cysteine at position 417 in a way that it is not lining the pore any more. This single amino acid modification demonstrated the accessibility of amino acids during different conformations of the *h*K_v1.3 channel. This has implications for the 3D structure of the channel in the C, O and specifically in the C-type I state – a state specific for *h*K_v1.3 – thereby facilitating the rational design of drugs useful for the treatment of autoimmune diseases.

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Conflict of interest

None.

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