Probing pore topology and conformational changes of Kir2.1 potassium channels by cysteine scanning mutagenesis

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Received 13 May 1998; revised version received 29 July 1998

Abstract **Using cysteine (Cys) scanning mutagenesis of the inward rectifier K^+ channel Kir2.1, we investigated its pore structure and putative conformational changes. In the background of the Kir2.1 mutant C149F which showed no sensitivity towards Cys-modifying reagents, Cys residues were introduced at 10 positions in the H5 pore region. Out of six functional mutants, T141C and F147C showed clear changes in current amplitude when Cys-modifying reagents were applied from the external side. These results suggest that the corresponding sections of the H5 pore region face to the external side which is in contrast to the results previously obtained for voltage-gated K^+ (Kv) channels. Using the mutants T141C and F147C, we investigated whether or not Kir2.1 channels show state-dependent conformational changes of the pore structure. Substantial alterations of the holding potential or external K^+ concentration, however, did not cause any significant change in the speed of channel modification upon application of Cys-specific reagents, suggesting that Kir2.1 channels do not undergo conformational changes, in contrast to C-type inactivating Kv channels.**

Key words: Potassium channel; Inward rectifier; Cysteine; Pore topology; Mutagenesis

1. Introduction

Cysteine scanning mutagenesis is a powerful approach for structure-function studies of proteins [1]. For ion channels, it has proved particularly useful in determining protein topology [2,3] and conformational changes associated with channel gating such as activation or inactivation [4,5]. The principle of this method is as follows. Cys residues are introduced at various positions of the target region, and the change of channel function (usually the current amplitude) inducted by Cys-modifying reagents is monitored. The modification reflects the accessibility of the reagents to the introduced Cys residues giving insight into the protein topology. Alterations in the speed of modification under various conditions (e.g. various channel conformational states) are indicative of underlying protein conformational changes.

In the case of voltage-gated K^+ (Kv) channels, it was reported that mutation of an amino acid residue in the center of the pore-forming H5 region (T441) changes the sensitivity to tetraethylammonium applied from the inside [6]. Based on this result, the central part of the H5 region was thought to face to the cytoplasmic side. A Cys scanning mutagenesis study by Pascual et al. [2] supported this hypothesis.

For the cloned inward rectifier K^+ channel Kir2.1 [7], it was reported that an amino acid residue in the putative cytoplasmic chain, E224, affects ion permeation, suggesting that this region is also involved in forming the pore [8,9]. Such a structure is hard to imagine by simple analogy with Kv channels, and it implies that the pore structures of these two K^+ channel families may differ significantly. Therefore, the first aim of our Cys-scanning mutagenesis study was to map the topology of the H5 region of Kir2.1 channels.

After removal of the N-terminal inactivation, Kv channels, such as *Shaker*, show slow inactivation upon depolarization [10]. This process, referred to as C-type inactivation, is strongly influenced by K^+ at the external mouth of the pore [11,12]. In addition, it was reported that residue T449 at the extracellular end of the H5 region is important for the speed of C-type inactivation [11], and that a dynamic rearrangement occurs at the external mouth of the pore during C-type inactivation [4].

For inward rectifier K^+ channels (Kir channels) no obviously protein-intrinsic gating mechanism like the voltage-dependent activation and inactivation of Kv channels has been defined. Instead, these channels appear to be mainly gated by the highly sensitive block by cytoplasmic Mg^{2+} and polyamines [13,14]. However, the unblocked (active) voltage range changes when $[K^+]_o$ is changed [7,15,16]. In addition, outward current through Kir2.1 channels disappears completely upon removal of external K^+ [17]. These observations clearly showed that the 'gating' of Kir2.1 channels depends on $[K^+]_o$, but it is not clear how much of this dependence arises from $K^+{}_o$-dependent conformational changes and how much from interactions of K^+ with the cytosolic blocking ions. The point mutation at the site corresponding to T449 in *Shaker* channels, R148Y, caused a dramatic change of the external K^+ dependence [15]. Thus, it was conceivable that, in addition to ion-ion interactions, conformational changes similar to those reported for C-type inactivation of Kv channels may contribute to the $K^+{}_o$-dependent gating of Kir2.1 channels. The second aim of this paper was to test this hypothesis.

2. Materials and methods

2.1. Molecular biology

cRNA was transcribed in vitro from the linearized plasmid DNA using an RNA transcription kit (Stratagene). Point mutants were made with the Sculptor Kit (Amersham) using oligonucleotide DNA primers and single-stranded template DNA. The mutations were confirmed by sequencing the primer and the surrounding regions. The electrophysiological properties of the mutants were confirmed to be

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PII: S0014-5793(98)01038-2

identical using two independent mutant clones. Oocytes were isolated and treated with collagenase (2 mg/ml) as described previously [7], and injected with 50 nl of cRNA solution. Oocytes were incubated for 2–5 days at 17°C after injection of cRNA.

2.2. Two-electrode voltage clamp

Macroscopic currents were recorded with a two-electrode voltage clamp amplifier (OC-725B-HV, Warner Co., Hamden, CT, USA). Data acquisition and analysis were done on an 80486-based computer using Digidata 1200 and pCLAMP program (Axon Instruments, Foster City, CA, USA). Intracellular glass microelectrodes were filled with 3 M KCl or 3 M K-acetate with 10 mM KCl. The resistance ranged from 0.2 to 0.8 MΩ. The standard bath solution contained 90 mM KCl, 3 mM $MgCl_2$, and 5 mM HEPES (pH 7.4). To lower $[K^+]_o$, KCl was replaced with *N*-methylglucamine-Cl (Fig. 3A) or with NaCl (Fig. 3C). All recordings were carried out at room temperature (23 ± 2°C). Cys-modifying reagents, chloramine T (ChT, Sigma), [2-(trimethylammonium) ethyl] methane-thiosulfonate bromide (MTSET, Toronto Research, North York, Canada), sodium (2-sulfo-natoethyl) methane-thiosulfonate (MTSES, Toronto Research) and 2-(aminoethyl) methane-thiosulfonate hydrobromide (MTSEA, Toronto Research) were dissolved in the bath solution just before use [1], and one-fifth volume of 5 times concentrated stock solution was applied to the bath and was mixed immediately by thorough pipetting. For internal application, approximately 100 nl (one tenth of the oocyte volume) was injected into the oocytes by a third pipette while recording under two-electrode voltage clamp.

The *n* values of each experiment are indicated in the text. In the cases where modification of the current amplitude was observed, the time constant of modification and the amplitude of the remaining current were calculated by fitting the time course with a single exponential function. The percentage of the remaining Kir2.1 current was calculated after subtracting the leak current, which was assumed to be 1 μA from the recording of non-cRNA injected oocytes. Means and standard deviations of the percentage of remaining current and of the modification time constant are indicated.

3. Results and discussion

3.1. Pore topology of Kir2.1 channels

As the Kir2.1 channel subunits have one Cys at the external mouth of the pore (C149, see Fig. 2A), the effects of Cys-modifying reagents such as ChT, MTSET, MTSES, and MTSEA as well as of Cd^{2+} ions were examined on wild-type channels as a first step. The current amplitude was decreased by a high dose (1 mM) of ChT (percentage of remaining current: 69 ± 6.3%, time constant of current modification: 19 ± 7.0 s, $n = 7$). The effect of ChT on wild-type channels did not affect the current-voltage relationship (Fig. 1A). MTSET, MTSES and MTSEA (each 100 μM) did not show a clear effect ($n = 5$, each). 1 mM Cd^{2+} caused a slight decrease of the current amplitude (96 ± 2%, 6.4 ± 2.3 s, $n = 5$) (data not shown). These results suggest that C149 is not close enough to the permeation pathway to cause marked alterations of ion permeation upon chemical modification. When C149 was mutated to Phe (C149F), the current was no longer affected by 1 mM ChT (Fig. 1B) ($n = 7$); application of 100 μM MTSET, MTSES, or MTSEA or of 1 mM Cd^{2+} did not modify the current either ($n = 7$, each) (data not shown), suggesting that C149 is the only externally accessible site of Kir2.1 channels whose modification causes moderate changes in the current amplitude.

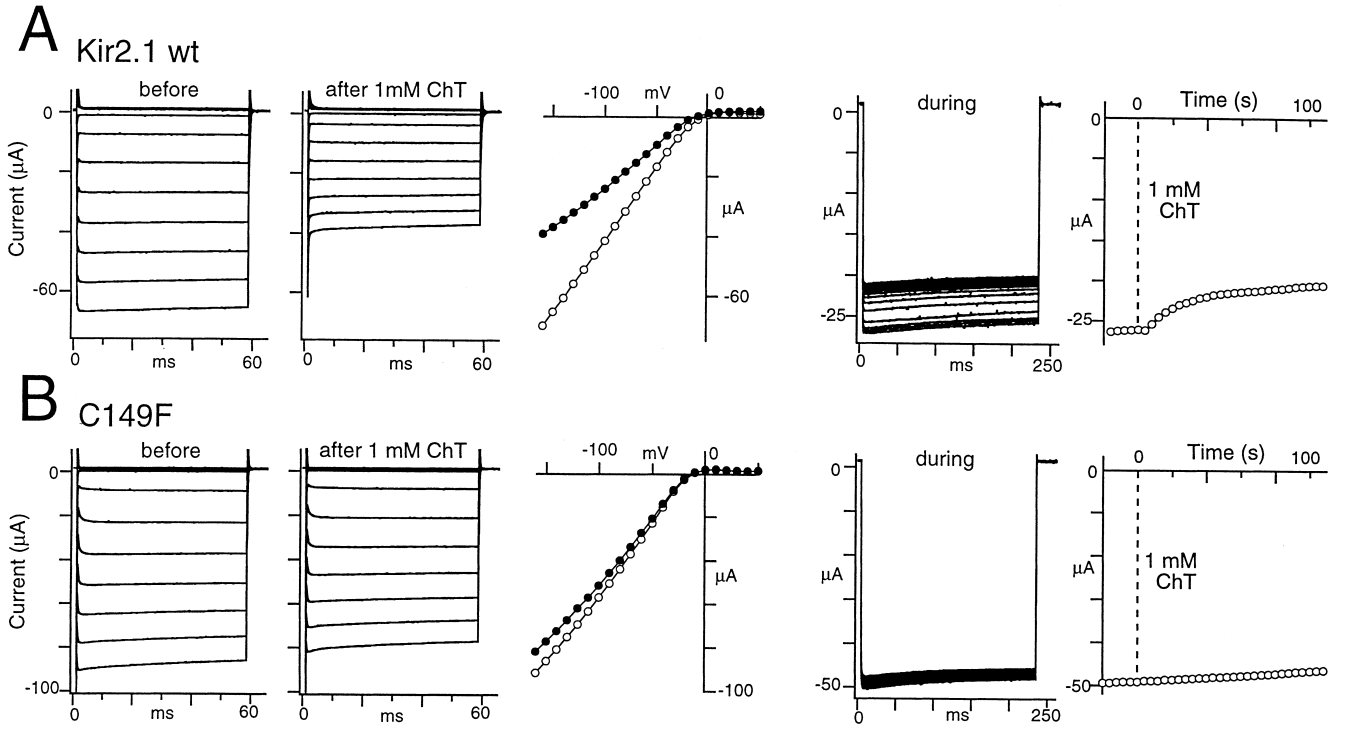

Fig. 1. Effect of ChT on Kir2.1 and mutant C149F. A, first and second column: Current traces under voltage clamp in 90 mM K^+ solution before and after application of 1 mM ChT. Step pulses (from +50 mV down to −160 mV, every 10 mV) were applied from the holding potential of 0 mV. Current traces of every 20 mV (+50 mV to −150 mV) are shown here. Third column: The current-voltage plot indicates that the voltage dependence did not clearly change upon application of ChT (control: open circles; after ChT: filled circles). Fourth column: Changes of Kir2.1 current in 90 mM K^+ solution during application of 1 mM ChT. Same step pulses to −80 mV for 200 ms were applied repeatedly every 4 s from the holding potential of 0 mV. Current traces of every 8s are shown here. Fifth column: The current amplitudes at the end of the step pulses were plotted against time. 1 mM ChT was applied at time 0. B: Same plot as in A for mutant C149F. Only a slight run-down was observed.

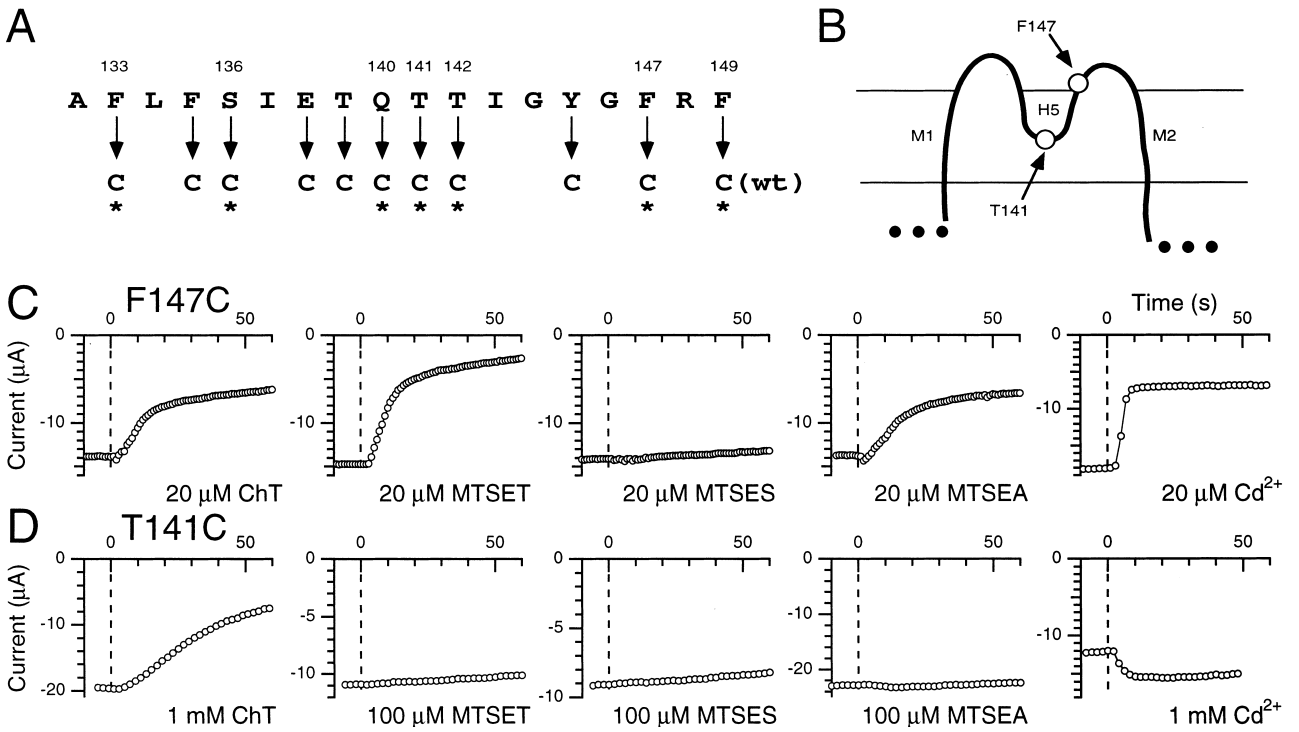

Fig. 2. Cys scanning mutagenesis and current modification by Cys-specific reagents. A: The amino acid sequence of the H5 pore region. B: The standard scheme of the structure of Kir channels. The introduced cysteines are indicated by arrows, and the functional mutants are marked by asterisks. Mutant C149F was used as an insensitive background. C, D: Time course of current modification of F147C (C) and T141C (D) by the indicated Cys-modifying reagents. Currents were measured in 90 mM K^+_o. Test pulses were given to −120 mV from a holding potential of 0 mV. Pulse duration and repetition intervals were 6 ms and 1 s for F147C (except for Cd^{2+} experiment) and 50 ms and 2 s for T141C (and Cd^{2+} experiment of F147C). The indicated Cys-specific reagents were applied at time 0. Note that the reagents were applied at lower concentrations for F147C due to the greater sensitivity.

Using this insensitive mutant C149F as a background, we carried out Cys scanning mutagenesis, introducing Cys at the positions F133, F135, S136, E138, T139, Q140, T141, T142, Y145, F147 (Fig. 2A). Among the 10 mutants, six (F133C, S136C, Q140C, T141C, T142C, F147C) showed functional expression (marked by asterisks in Fig. 2A).

Their sensitivities to the external application of ChT, MTSET, MTSES, MTSEA, and Cd^{2+} were investigated (Table 1). F133C, S136C, Q140C, T142C did not show a very clear modification of the current amplitude by these reagents. F147C (Fig. 2C), however, was clearly modified by these reagents applied from the outside. T141C (Fig. 2D) was also clearly modified by external ChT and Cd^{2+}. While F147C was modified by the reagents at relatively low concentrations, modification of T141C required higher concentrations. This lower accessibility of C141 is expected given the putative lo-

Table 1
Summary of the effects of externally applied Cys-modifying reagents (1 mM ChT, 100 μM MTSEA, MTSES, MTSET, 1 mM Cd^{2+}) on Cys mutants

	F133C	S136C	Q140C	T141C	T142C	F147C
Cht	n.d ($n=5$)	n.d ($n=5$)	54 ± 3.0% 88 ± 16 s ($n=5$)	21 ± 6.5% 76 ± 16 s ($n=7$)	n.d. ($n=7$)	33 ± 5.3% 1.7 ± 0.6 s ($n=8$)
MTSET	n.d. ($n=5$)	n.d. ($n=7$)	n.d. ($n=6$)	n.d. ($n=37$)	n.d. ($n=5$)	2.9 ± 1.8% 6.1 ± 1.9 s ($n=6$)
MTSES	n.d. ($n=5$)	n.d. ($n=7$)	n.d. ($n=6$)	n.d. ($n=21$)	n.d. ($n=5$)	16 ± 4.6% 8.2 ± 4.2 s ($n=5$)
MTSEA	n.d. ($n=5$)	n.d. ($n=5$)	n.d. ($n=7$)	n.d. ($n=14$)	n.d. ($n=5$)	28 ± 1.2% 4.7 ± 3.4 s ($n=6$)
CD^{2+}	n.d. ($n=10$)	n.d. ($n=8$)	106 ± 2.7% 4.8 ± 1.8 s ($n=9$)	131 ± 10% 2.8 ± 0.9 s ($n=17$)	92 ± 3.4% 2.3 ± 1.1 s ($n=10$)	6.2 ± 4.4% 1.8 ± 0.9 s ($n=6$)

The percentage of the remaining current after modification and the time constant of the modification speed are shown. Values over 100% indicate an increase of the current by modification. The *n* values are shown in parentheses. n.d. (not detected) means that there were no clear triggered changes of the current amplitude upon application of the reagents. MTSET, MTSES and MTSEA caused a slight decrease of T141C current in a limited number of batches of oocytes, but those were judged to be exceptional.

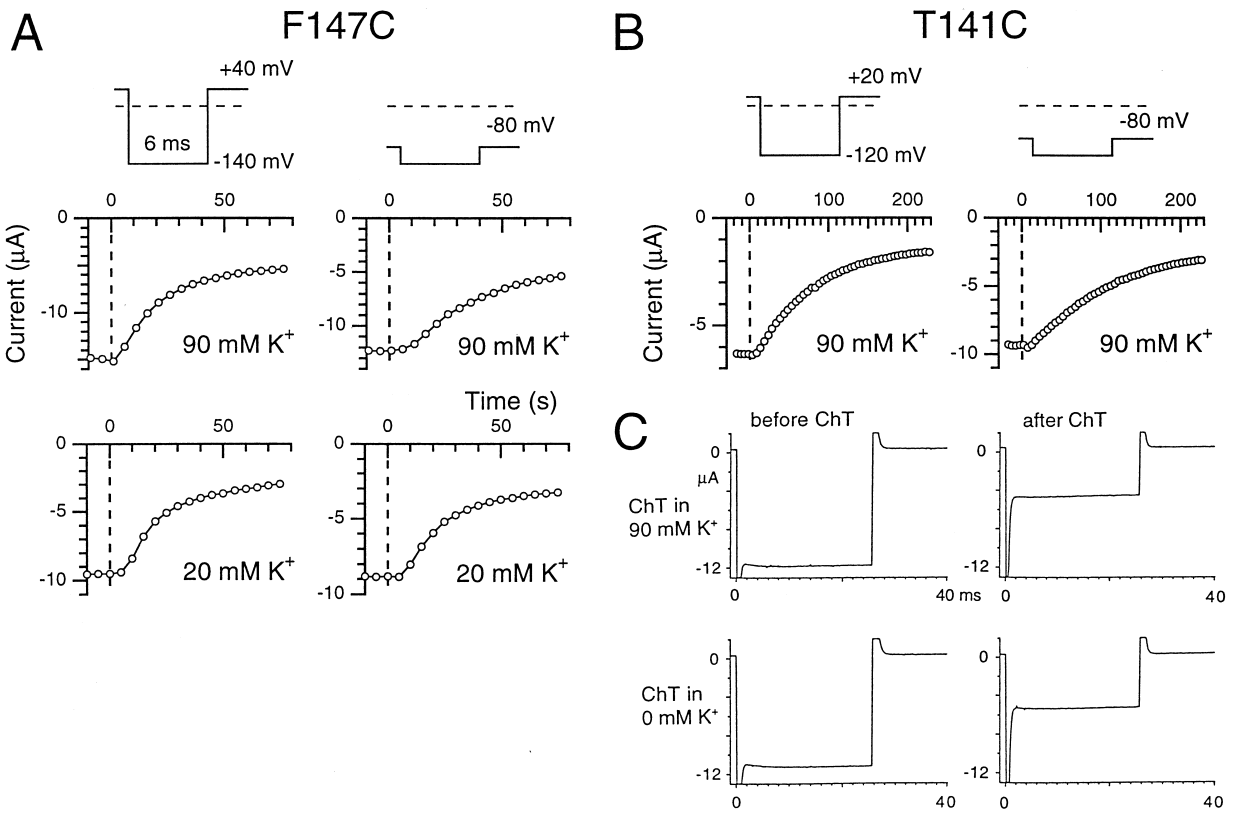

Fig. 3. Examination of state-dependent residue accessibility. A: Comparison of the speed of F147C channel modification by 10 μM MTSET under different external K^+ concentrations (90 mM K^+ (upper), 20 mM K^+ (lower)) or holding potentials (+40 mV (left), −140 mM (right)). Test pulses to −140 mV were elicited for 6 ms every 5 s. B: Comparison of the speed of T141C channel modification by 0.5 mM ChT under different holding potential (+20 mV (left), −80 mV (right)) in 90 mM K^+. Test pulses to −120 mV were elicited for 6 ms every 5 s. C: Modification of T141C channels was achieved by application of 1 mM ChT for only 30 s, either in 90 mM or in 0 mM K^+ solution. The plotted current traces were obtained in 90 mM K^+ solution before application and after wash-out of ChT. A step pulse to −120 mV from the holding potential of 0 mV was applied. The difference of the two traces shows the effect of ChT.

cation of that residue in the pore region (Fig. 2B). Interestingly, application of Cd^{2+} resulted in an increase in current through T141C, while current was efficiently reduced in F147C. To examine the sensitivities of T141C to these reagents from the inside, we tried internal application while recording from an inside-out macropatch, but due to the intense run-down of the current upon excision, clear conclusions could not be obtained. Thus, we could not determine whether T141 is accessible from the inside or not. The assured conclusion is that T141 is highly accessible from the outside judging from the quick modification by external Cd^{2+} ($\tau = 2.8$ s).

The accessibility of T141 of Kir2.1 from the outside was unexpected by analogy to Kv channels in which the corresponding amino acid residue (T441, *Shaker*) was reported to be accessible from the inside but not from the outside [2,6]. Although Kurz et al. [18] described a slight modification of a corresponding mutant of Kv2.1 (T372C) by external Cd^{2+}, the accessibility is judged to be extremely low and not comparable to that of Kir2.1, since the modification of the current was reported to take minutes. Thus, the high accessibility of T141 of Kir2.1 from the outside clearly differs from the properties of Kv channels. External accessibility of the corresponding site of Kir1.1 channels was also shown by Schwalbe et al. [19] by means of an extracellular glycosylation analysis. Thus, the pore structure of inward rectifier channels seems to be quite different from that of Kv channels, although these two families have a common K^+ selective filter sequence, composed of the motif GYG. Yang et al. [20] reported that R148 and E138 of Kir2.1 form a salt bridge which is unique for the inward rectifiers. It is conceivable that the H5 region of Kir2.1 forms the selective filter at the outer entrance and does not penetrate into the membrane, and the R-E salt bridge stabilizes this rather loose structure. Such a structural difference to those of Kv channels could be caused by the lack of the stabilizing outer shell region of the segments S1–S4 [7].

Recently, the 3D structure of KcsA, a potassium channel from *Streptomyces lividans* [21], was determined. Although KcsA is a two-transmembrane type channel similar to Kir channels, the primary structure of the H5 pore region of the KcsA channel is rather close to that of Kv channels, and it also lacks the R-E salt bridge. Thus, the revealed pore structure of KcsA might be more similar to that of Kv channels than to that of Kir channels. Only the determination of the 3D pore structure of Kir channels will give direct insight regarding the unique accessibility of T141 from the external side.

Further evidence for the protein topology of Kir1.1 channels was gained by Schwalbe et al. [22], who reported that a Thr residue introduced at position 259 in the putative C-terminal cytoplasmic region was glycosylated by external tunicamycin. This result suggested that this residue is accessible from the extracellular side. To test whether Kir2.1 also has

a similar structure, we introduced Cys into the corresponding and the surrounding residues. All these mutants, G257C, I258C, D259C, R260C, and I261C, were functional, but the current could not be modified by Cys-specific reagents applied from the outside ($n = 3$, each) (data not shown). Thus, in contrast to the report for Kir1.1 [22], the putative C-terminal region of Kir2.1 was not shown to be located on the extracellular side. However, the negative results in this study leave open the possibility that these residues are located outside and are too far from the permeation pathway to cause modification of the current amplitude.

3.2. Probing for conformational changes in the pore region

As a next step, we carried out experiments aiming to detect conformational changes in Kir2.1 channels analogous to those associated with C-type inactivation of Kv channels. For this approach we used mutants T141C and F147C. F147C was thought to support this aim because the corresponding site in *Shaker* channels (M448) [16] was shown to exhibit a state-dependent accessibility to ChT [5]. In addition, this residue is next to T449 which was used to monitor conformational changes at the external mouth of the pore during C-type inactivation [4]. As the macroscopic conductance of Kir2.1 channels depends on $[K^+]_o$ and on the membrane potential [15,16], both parameters were systematically altered and the speed of Cys modification was measured. In Fig. 3A,B experiments are shown for mutants F147C and T141C in which current amplitude was assayed every 5 s by brief (6 ms) pulses to negative voltages (−120 or −140 mV); between these pulses the membrane potential was clamped to depolarized (left) or hyperpolarized (right) values. Although these different protocols resulted in strongly different macroscopic conductance of the channels, no clear changes in the speed of current modification were observed upon application of 10 μM MTSET (Fig. 3A) (14.8 ± 7.8 s, $n = 7$ (90 mM K^+, +40 mV); 17.4 ± 6.8 s, $n = 7$ (90 mM, −80 mV); 15.8 ± 5.9, $n = 7$ (20 mM, +40 mV); 19.0 ± 7.8 s, $n = 8$ (20 mM, −80 mV)), or 0.5 mM ChT (Fig. 3B) (187 ± 35 s, $n = 7$ (+20 mV); 172 ± 52 s, $n = 6$ (−80 mV). Fig. 3C shows an example for T141C in which the extent of current reduction by application of ChT in the presence and absence of external 90 mM K^+ was compared. ChT was applied for 30 s in 90 mM K^+, or in 0 mM K^+ (90 mM Na^+) and current magnitude was tested in 90 mM K^+ before and after this application. Analysis of the remaining current yielded no clear difference between the two cases ($31 \pm 8.0\%$, $n = 5$, 90 mM K^+; $35 \pm 8.5\%$, $n = 5$, 0 mM). Therefore, these results do not provide evidence which suggests conformational changes in the pore region of Kir2.1 channels during voltage- or K^+_o-induced 'gating'.

From these results, it was speculated that the activity of inward rectifier K^+ channels is not determined by protein conformational changes but by the interaction of external K^+ and cytoplasmic blockers, such as Mg^{2+} and polyamines [13,14], with the channel. Once K^+ at the external mouth of the pore is depleted, the channel might stop conducting, not due to a conformational change but by the inability to expel cytoplasmic blockers by means of electrostatic repulsion. The two phenomena which appear to be related to each other, C-type inactivation of Kv channels and K^+_o-dependent activity regulation of Kir2.1, therefore seem to be caused by different mechanisms.

Acknowledgements: This work was supported by an international grant from HFSP (to Y.K. and to S.H.H.) and a grant to the priority area 'Channel and Transporter Correlation' from the Ministry of Education, Science, Sports and Culture, Japan (to Y.K.).

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