

Molecular determinants for activation and inactivation of HERG, a human inward rectifier potassium channel

Roland Schönherr and Stefan H. Heinemann*

Max-Planck-Gesellschaft, Arbeitsgruppe Molekulare und zelluläre Biophysik an der Friedrich-Schiller-Universität Jena, Drackendorfer Strasse 1, D-07747 Jena, Germany

1. The human *eag*-related potassium channel, HERG, gives rise to inwardly rectifying K⁺ currents when expressed in *Xenopus* oocytes.
2. The apparent inward rectification is caused by rapid inactivation. In extracellular Cs⁺ solutions, large outward currents can be recorded having an inactivation time constant at 0 mV of about 50 ms with an e-fold change every 37 mV.
3. HERG channel inactivation is not caused by an amino-terminal ball structure, as a deletion of the cytoplasmic amino terminus (HERGΔ2–373) did not eliminate inactivation. However, channel deactivation was accelerated about 12-fold at –80 mV.
4. Mutation of S631 to A, the homologous residue of *eag* channels, in the outer mouth of the HERG pore completely abolished channel inactivation.
5. Activity of HERG channels depended on extracellular cations, which are effective for channel activation, in the order Cs⁺ > K⁺ ≫ Li⁺ > Na⁺. The point mutation S631A strongly reduced this channel regulation.
6. By analogy to functional aspects of cloned voltage-gated potassium channels, rectification of HERG, as well as its kinetic properties during the course of an action potential, are presumably governed by a mechanism reminiscent of C-type inactivation.

The human *eag*-related gene (*HERG*) encodes a potassium channel that is strongly expressed in the heart. The protein belongs to the family of voltage-dependent K⁺ channels which have six putative transmembrane segments. *HERG* has recently been identified as the locus of one form of inherited long-QT syndrome (Curran, Splawski, Timothy, Vincent, Green & Keating, 1995; Trudeau, Warmke, Ganetzky & Robertson, 1995), a disorder with the risk of sudden death due to cardiac arrhythmias. The long-QT phenotype is characterized by an unusually slow repolarization of cardiac action potentials. *HERG* has been cloned and expressed in *Xenopus* oocytes, where it caused depolarization-activated potassium currents (Sanguinetti, Jiang, Curran & Keating, 1995; Trudeau *et al.* 1995). These channels were sensitive towards the class III antiarrhythmic agent E4031, which blocks a rapidly activating repolarizing cardiac K⁺ current ($I_{K(r)}$), supporting the idea that HERG is involved in the generation of this current and thus important for action potential repolarization. Interestingly, the expressed channels allow only minor outward current during depolarizing pulses, but give rise to slow tail

currents with large amplitudes. This behaviour is especially surprising given the high degree of homology to outwardly rectifying members of the *eag* family. The molecular mechanism that prevents potassium outward current during depolarization has not yet been identified. Both inactivation as well as block by internal blocking molecules have been discussed (Sanguinetti *et al.* 1995; Trudeau *et al.* 1995); the latter mechanism has been shown to be responsible for inward rectification of potassium channels with two putative transmembrane segments (Ficker, Taglialatela, Wible, Henley & Brown, 1994; Fakler *et al.* 1994). In this study, we analysed N-terminally truncated HERG channels as well as single-point mutations in the putative pore domain. We provide evidence for an inactivation mechanism that does not involve an N-terminal ball domain, but is critically dependent on a serine residue (S631) in the outer mouth of the pore, which is not conserved in *eag* proteins. Replacement of this residue by alanine abolished inactivation and reduced the activating effect of extracellular cations. The N-terminal domain of HERG was found to be responsible for slow deactivation

* To whom correspondence should be addressed.

kinetics. An N-terminally truncated channel protein revealed a marked acceleration of deactivation compared with the wild type.

METHODS

Generation of channel mutants

HERG channel mutants were created by oligonucleotide-directed mutagenesis using the polymerase chain reaction (PCR) according to standard protocols. A truncated *HERG* gene (*HERG* Δ 2–218) was introduced into the modified expression vector pSPOoD (Villarreal, Herlitz, Koenen & Sakmann, 1991) using the restriction sites *Nco*I and *Eco*RI, resulting in a deleted gene with a unique *Nco*I site covering the start codon. To construct the N-terminal deletion mutant *HERG* Δ 2–373, one PCR primer was designed to introduce a *Nco*I site in position 1115 of *HERG* (nucleotides numbered from start codon). The second primer covered a unique *Bgl*II site. The corresponding PCR product was then cloned into *HERG* Δ 2–218 using *Nco*I and *Bgl*II. The pore mutations were constructed analogous to the deletion mutant *HERG* Δ 2–373 but with mutagenic oligonucleotides covering the *Bgl*II site. Mutations were confirmed by sequencing of the sub-cloned fragments. The SP6 mMessage mMachine Kit (Ambion, Austin, TX, USA) was used for synthesis of capped cRNA.

Oocyte expression and solutions

Stage V oocytes were surgically obtained from *Xenopus laevis* anaesthetized with 0.2% tricaine in ice water. Oocyte incubation and mRNA injection were performed as described previously (Stühmer, Terlau & Heinemann, 1992). Forty nanolitres of mRNA (0.05–0.5 μ g μ l⁻¹) were injected and currents were recorded, starting 12 h after injection. All extracellular solutions contained 1.8 mM CaCl₂ and 10 mM Hepes. They varied in the monovalent cations as follows (mM): 2.5 K⁺ Ringer solution: 2.5 KCl, 115 NaCl; 10 K⁺ Ringer solution: 10 KCl, 107 NaCl; K⁺ Ringer solution: 115 KCl; Na⁺ Ringer solution: 115 NaCl; Li⁺ Ringer solution: 115 LiCl; Cs⁺ Ringer solution: 115 CsCl. The pH was adjusted to 7.2 with the corresponding hydroxides.

Data acquisition

Recordings were made at 20–23 °C with a two-electrode voltage clamp amplifier (Turbo-TEC 01, NPI, Tamm, Germany). Electrodes were filled with 2 M KCl and had resistances between 0.6 and 1.0 M Ω . Stimulation and data acquisition were controlled with the Pulse+PulseFit software (HEKA Elektronik, Lambrecht, Germany) running on a Macintosh Quadra 650 computer. In all experiments, the holding potential was –100 mV. A *P/n* method with a leak holding potential of –90 mV and a scaling factor of –0.2 was used to subtract leak and capacitive currents. Data analysis was performed with the programs PulseFit (HEKA Elektronik) and Igor-Pro (WaveMetrics Inc., Lake Oswego, OR, USA). Data are given as mean values \pm s.d. (n = number of experiments) unless stated otherwise.

RESULTS

Expression of HERG channels in *Xenopus* oocytes gave rise to depolarization-activated currents with significant inward rectification in solutions with high external K⁺ (K_o⁺) concentrations (Fig. 1A). Replacement of K_o⁺ ions by Na_o⁺ abolished both outward and inward currents, whereas small outward currents were found in Li_o⁺. Cs_o⁺ resulted in a strong increase of outward currents compared with

measurements in K_o⁺, but the channels also displayed a significant Cs⁺ permeability, giving rise to inward tail currents. Interestingly, the outward currents revealed a rapid inactivation which had not been previously shown for HERG channels. Inactivation has been discussed as a possible mechanism for inward rectification (Trudeau *et al.* 1995; Sanguinetti *et al.* 1995). We therefore performed experiments in Cs⁺ Ringer solution to further investigate this phenomenon. In double-pulse experiments with variable interpulse duration, the recovery from inactivation was found to be much faster than the time course of deactivation (Fig. 1B). As a result, short pulse interruptions allowed instantaneous outward currents in the second pulse to be greater than in the first pulse. Apparently, the current elicited by the second pulse was much less influenced by activation. This becomes obvious in the double-pulse experiments shown in Fig. 1C. The second-pulse inactivation time constants were significantly smaller than the apparent inactivation time constants of the first pulses and, therefore, gave a more faithful estimate for the real time course of inactivation. Time constants of both pulses were determined by fits to single-exponential functions and plotted *versus* the test potential (Fig. 1D). During both pulses, inactivation showed a similar voltage dependence; between 0 and +80 mV, the secondary inactivation was accelerated compared with the first pulse by a factor of 4.2 ± 0.2 ($n = 4$).

In order to gain information about the mechanism underlying the rapid channel inactivation, we created an N-terminally truncated protein (*HERG* Δ 2–373) carrying only 25 N-terminal residues before the start of the putative S1 transmembrane segment. As shown in Fig. 2A, the resulting channels still gave rise to inactivating outward currents in Cs⁺ Ringer solution characterized by even faster inactivation kinetics than the wild type, thus excluding the possibility of an N-terminal ball domain as a cause of rapid inactivation. The apparently faster inactivation of *HERG* Δ 2–373 can be attributed to an unexpected acceleration of channel activation, allowing a more precise determination of inactivation time constants. In double-pulse experiments analogous to those described in Fig. 1C, we found only slightly faster inactivation time constants in the second pulse compared with the first one. In the range of 0 to +80 mV, time constants were decreased by a factor of 1.4 ± 0.1 ($n = 6$) (experiment not shown). The speed of channel activation was estimated by measuring the time to reach the peak current, as illustrated in Fig. 2C. Both channels exhibited the same voltage dependence of activation but the kinetics were accelerated in *HERG* Δ 2–373 by a factor of 1.5 ± 0.1 (between 0 and +80 mV). Tail currents were recorded in K⁺ Ringer solution (Fig. 2B) and fitted with single-exponential functions in order to assess the deactivation kinetics. As shown in Fig. 2D, the N-terminal deletion caused an acceleration of deactivation by a factor of between 2.6 ± 0.6 (–160 mV) and 12.2 ± 4.0 (–80 mV). The mid-point of the apparent steady-state activation determined from tail current

protocols was shifted from -19.2 ± 2.6 ($n = 12$) to -23.6 ± 2.7 mV ($n = 6$).

To test whether the pore region of *HERG* channels is involved in channel inactivation, we replaced single amino acids in the putative extracellular part of the pore. In order to improve the analysis of inactivation kinetics, the mutations were introduced into the background of *HERG* $\Delta 2-373$, thus preventing a masking effect of the slow activation and deactivation kinetics of wild-type channels. As one target residue we chose S631, which

corresponds to T449 in *Shaker* B channels, known to be critical for the so-called C-type inactivation (López-Barneo, Hoshi, Heinemann & Aldrich, 1993). We created the mutant S631A (VA) because an alanine residue is found in the corresponding positions of the homologous *eag* channels from rat (Ludwig *et al.* 1994) and *Drosophila* (Warmke, Drysdale & Ganetzky, 1991). In a second mutant (IS), we replaced V630 by I. Isoleucine in this position occurs in rat *eag*, whereas valine is found in *Drosophila eag*. As shown in Fig. 3, the mutation S631A completely removed channel inactivation in K⁺ Ringer solution, as well as in Cs⁺ Ringer

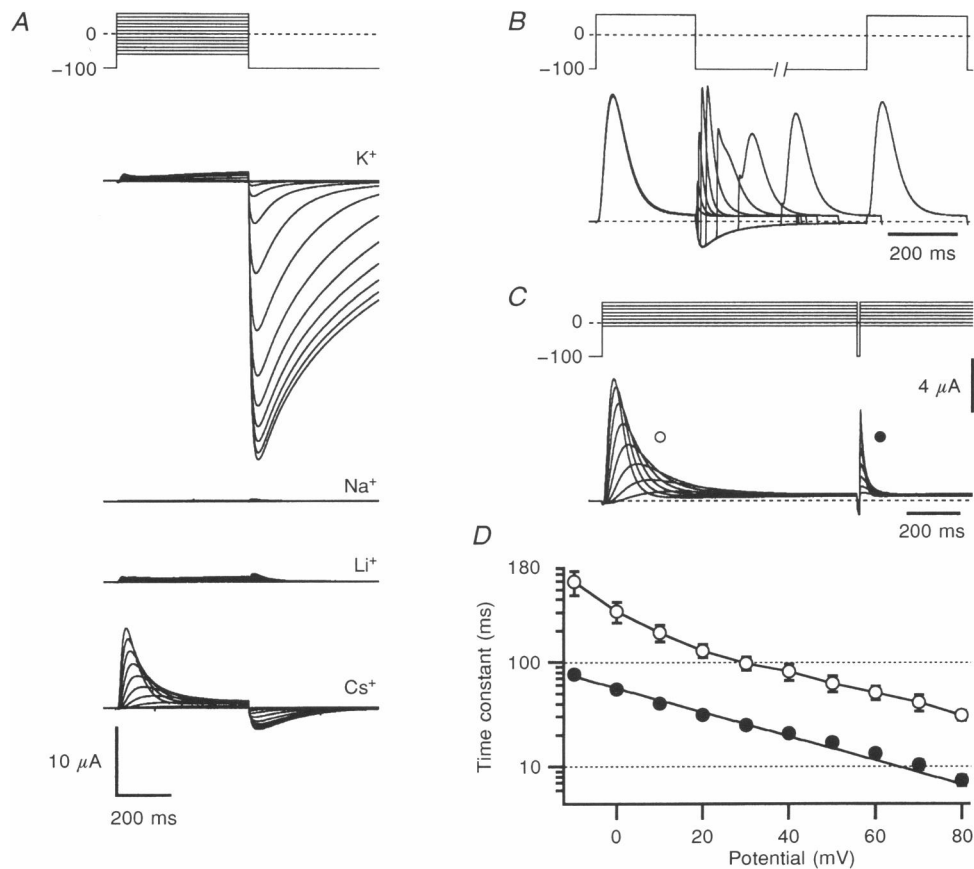


Figure 1. Activation and inactivation of *HERG*

A, *Xenopus* oocytes were voltage clamped at a holding potential of -100 mV and depolarizing pulses between -60 and $+60$ mV were applied. The resulting current responses in K⁺, Na⁺, Li⁺ and Cs⁺ Ringer solutions are shown. *B*, double-pulse experiments in Cs⁺ Ringer solution with test potentials of $+60$ mV and an interpulse potential of -100 mV. The current scaling is as in *C*. The superimposed current responses show that recovery from inactivation is much faster than channel deactivation. In addition, inactivation during the second pulse after short hyperpolarizations is faster than the initial inactivation, indicating that the apparent inactivation kinetics of *HERG* are strongly masked by the slow activation process. *C*, double-pulse experiments with the indicated variable test potentials and an interpulse hyperpolarization of 10 ms at -100 mV; the secondary inactivation gives a more faithful, activation-independent, estimation of the inactivation kinetics. *D*, time constants of apparent inactivation during the first pulse (○) and secondary inactivation during the second pulse (●) plotted versus the test potential. Data points are shown as mean values \pm s.e.m. of 4 experiments. The data points for apparent inactivation are connected by straight lines. The continuous line superimposed on the secondary inactivation time constants is the result of a single-exponential fit characterized by a time constant at 0 mV of 53 ms and an e-fold change every 37 mV.

solution. The same phenotype was found for the double mutation V630I•S631A (IA), whereas the single replacement V630I (VS) did not influence inactivation. The inactivation time constants of this mutant, as determined by single-exponential fits, did not deviate from the control HERG Δ 2–373 (VS) (Fig. 3D). Plotting the relation of the steady-state outward and maximum inward currents versus the potential (Fig. 3B) revealed a loss of inward rectification for those two mutant channels with the S631A replacement. Reversal potentials were determined in Cs⁺ Ringer solution employing tail current protocols (not shown), giving an indirect indication of the relative permeability of Cs⁺ versus K⁺. Since the reversal potentials for the mutants (V630I: -27.9 ± 0.3 mV; S631A: -25.8 ± 0.4 mV; V630I•S631A: -27.3 ± 1.6 mV; $n = 7$) did not deviate significantly from those of HERG Δ 2–373 (-29.7 ± 1.6 mV; $n = 7$), neither of the two pore residues appears to be responsible for the remarkable Cs⁺ permeability of HERG.

Functional availability of HERG K⁺ channels is increased by increasing K_o⁺ concentration despite the lowered driving force for K⁺ ions (Sanguinetti *et al.* 1995; Trudeau *et al.*

1995). Since the residues homologous to S631 were reported to be responsible for channel regulation by K_o⁺ in rat K_v1.4 (Pardo *et al.* 1993) and Shaker B (López-Barneo *et al.* 1993), we tested the effects of external K⁺ and Cs⁺ on the HERG pore mutants (Fig. 4). The mutants HERG Δ 2–373 (VS) and V630I in this background (IS) were strongly activated when Na_o⁺ was replaced by K_o⁺; the largest outward currents were recorded in Cs_o⁺. Representative traces recorded from HERG Δ 2–373 and the mutant S631A (VA) are shown in Fig. 4A. The results for all mutants are summarized in Fig. 4B. Apparently, the effect of Cs_o⁺ was reduced in the two mutants with the replacement S631A (VA and IA), whereas sensitivity towards K_o⁺ remained.

DISCUSSION

The recently cloned HERG channel has been described as an apparent inward rectifier (Warmke & Ganetzky, 1994; Trudeau *et al.* 1995). Inactivation, as well as internal block, have been discussed as potential mechanisms to prevent large outward currents. Using mutated *HERG* clones, we

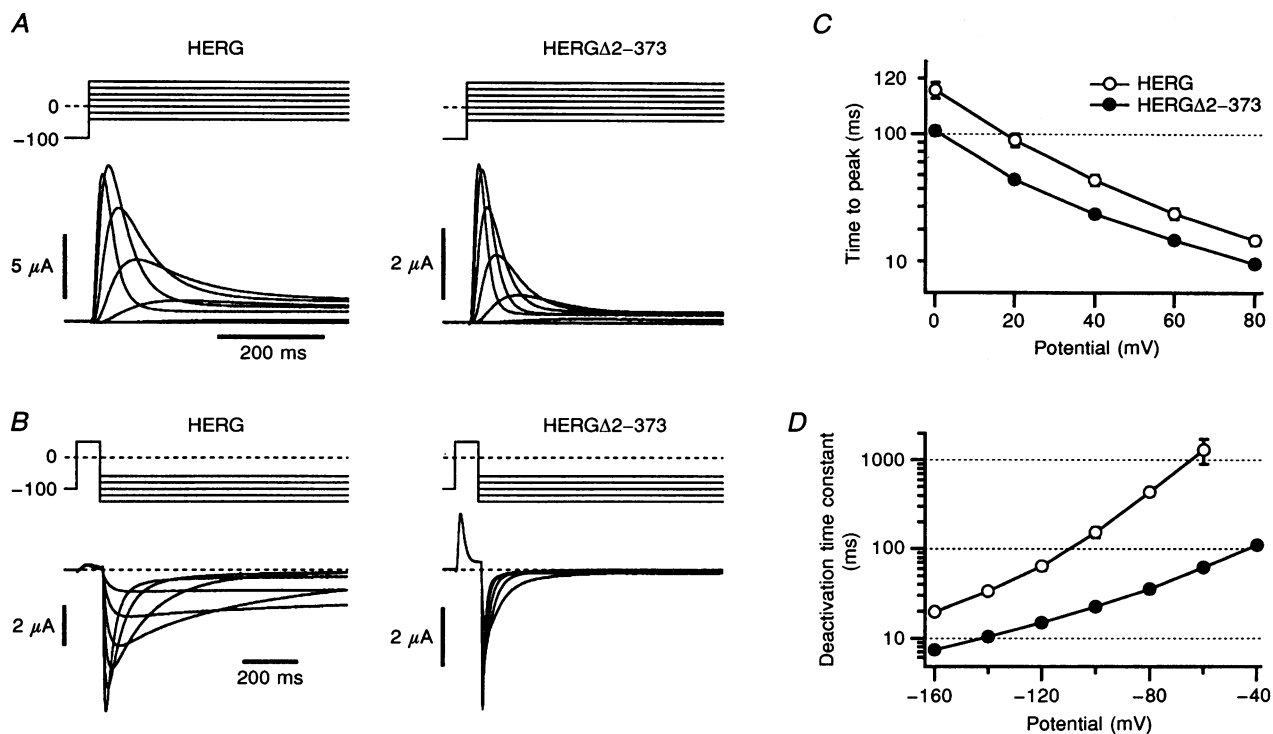


Figure 2. N-terminal deletions accelerate channel deactivation but do not abolish inactivation

A, current recordings in Cs⁺ Ringer solution in response to the indicated potential protocol. HERG Δ 2–373 activates and inactivates faster than HERG, which results in a shortening of the time to peak at the test potentials between 0 and +80 mV. The averaged results of 5 experiments are plotted in *C*. *B*, tail current recordings in K⁺ Ringer solution in response to the indicated potential protocol. HERG Δ 2–373 deactivates much faster than the wild type and the larger peak outward current also indicates a faster activation. *D*, deactivation time constants for the wild type (O, $n = 9$) and HERG Δ 2–373 (●, $n = 7$) as a function of the tail potential. The data points are connected by straight lines.

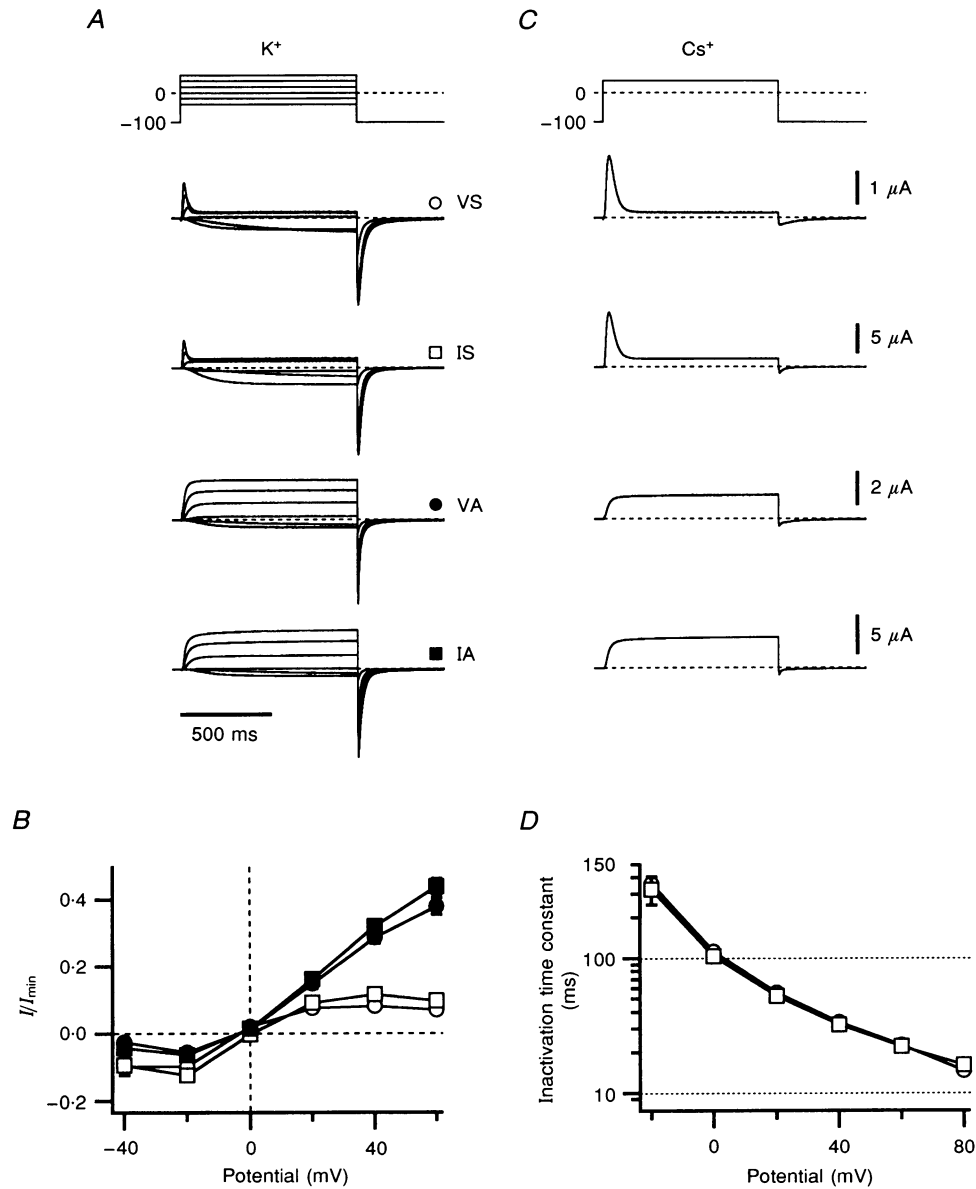


Figure 3. Pore mutations abolish channel inactivation

A, current–voltage recordings in K^+ Ringer solution from oocytes expressing channel mutants based on *HERG* $\Delta 2$ –373. The mutants are characterized by the amino acid residues at the positions V630 and S631, i.e. VS corresponds to the wild-type pore structure, IS to V630I, VA to S631A and IA to V630I•S631A. *B*, current rectification was characterized by plotting the current level at the end of a 1 s test pulse (I) normalized to the maximal peak inward current during the tail hyperpolarization at -100 mV (I_{min}). The symbols correspond to the mutants as indicated in *A*. The data points were connected by straight lines; the averages were taken from 5 (VS), 7 (IS), 5 (VA) and 5 (IA) experiments. *C*, current responses to step depolarizations to +40 mV in Cs^+ Ringer solution, indicating that the mutation S631A (VA) completely abolishes channel inactivation. Data in *C* were obtained from the same oocytes as those in *A*. *D*, time constants of inactivation in Cs^+ Ringer solution for VS (\circ , $n = 7$) and IS (\square , $n = 7$) as a function of the test potential. The mutants VA and IA are not considered in this plot because they did not show any inactivation at +40 mV after depolarizations of 10 s duration.

have now identified an inactivation mechanism which is responsible for the typical properties of HERG potassium channels. Analysis of the deletion mutant HERG Δ 2–373 did not reveal a severe change in inactivation kinetics. We can therefore exclude a ‘ball-and-chain’ inactivation mechanism, as it is typically found in fast-inactivating K⁺ channels like the *Shaker* B channel (Hoshi, Zagotta & Aldrich, 1990). However, the N-terminal domain has a marked slowing influence on the deactivation kinetics. Under physiological conditions, N-terminally truncated HERG channels would not significantly contribute to repolarizing currents in the terminating phase of an action potential, therefore the N-terminal domain of HERG causing the unusually slow deactivation could be an

important target for channel regulation *in vivo*.

For *Shaker* B channels, an inactivation mechanism additional to N-type inactivation has been described (Hoshi, Zagotta & Aldrich, 1991). This so-called C-type inactivation strongly depends on the pore structure as it is markedly altered by mutations in or close to the pore region (Hoshi *et al.* 1991; DeBiasi, Hartman, Drewe, Tagliatela, Brown & Kirsch, 1993; López-Barneo *et al.* 1993). Through variation of a single residue in the outer vestibule of the *Shaker* pore (T449), which is homologous to the mutated residue S631 in HERG, the speed of C-type inactivation could be altered by more than three orders of magnitude (López-Barneo *et al.* 1993). The closest related homologues of HERG, the *eag*

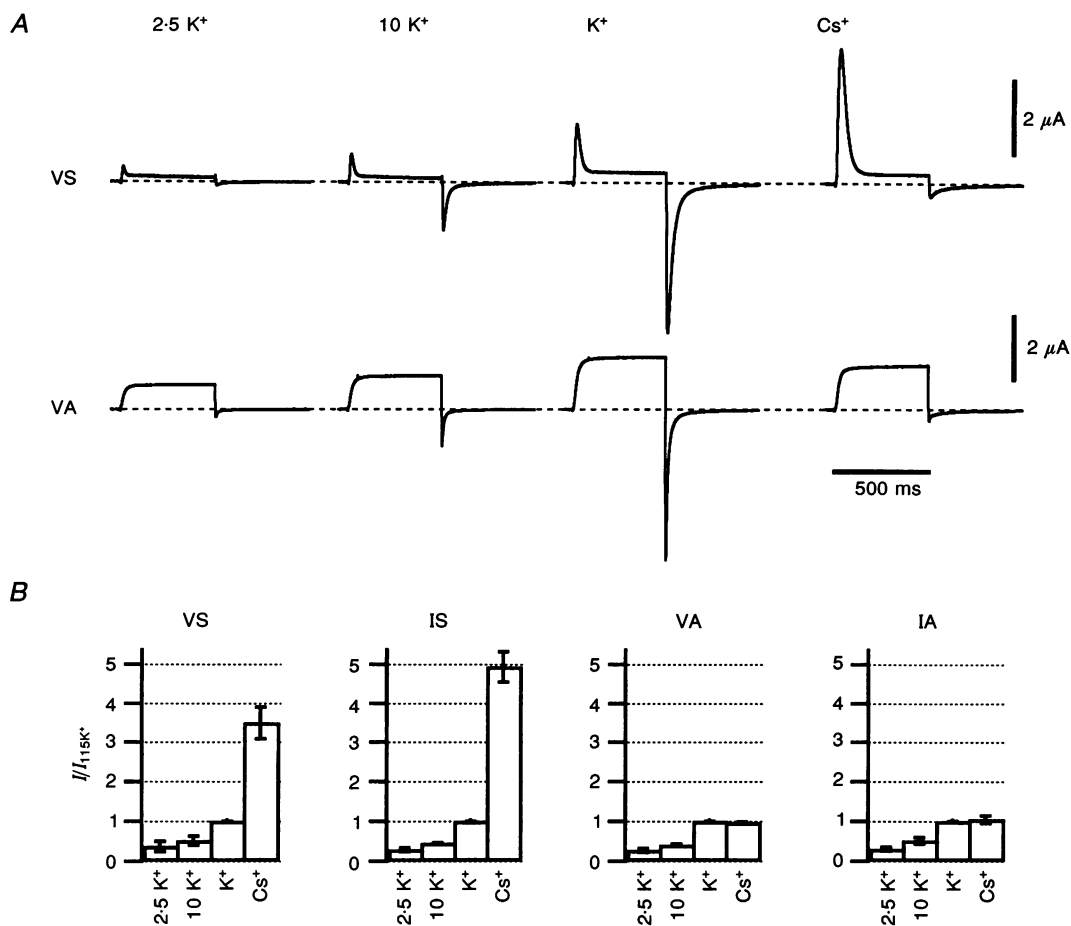


Figure 4. Pore mutations abolish channel regulation by K_o⁺

A, step depolarizations to +50 mV from a holding potential of –100 mV were applied in the extracellular solutions indicated at the top: 2.5 K⁺ Ringer solution, 10 K⁺ Ringer solution, K⁺ Ringer solution (115 mM K⁺) and Cs⁺ Ringer solution (115 mM Cs⁺) (for complete composition of the solutions, see Methods). While the outward currents through HERG Δ 2–373 (VS) strongly depended on the species of extracellular cations, the mutation S631A (VA) almost completely eliminated this effect. The data shown in one row were recorded consecutively in the indicated order from one oocyte each. *B*, maximal outward currents at +50 mV from experiments as shown in *A* normalized to the current value obtained in 115 mM K⁺ Ringer solution for the wild type (VS) and the indicated mutants, abbreviated as in Fig. 3 ($n = 7$).

channels from rat, mouse and *Drosophila*, have an alanine residue in the corresponding position and show no or only slow inactivation. The replacement S631A in HERG resulted in a complete loss of inactivation. In contrast, replacement of the neighbouring valine residue by isoleucine (V630I), which is found in rat *eag*, had no obvious effect on the channel properties. These data suggest that the typical prevention of outward currents during depolarizing pulses is due to a mechanism reminiscent of C-type inactivation and is critically influenced by the residue S631. This is in agreement with the finding that the mutation T449S in *Shaker* B channels resulted in very fast C-type inactivation, whereas the mutant T449A inactivated rather slowly (Schlief, Schönherr & Heinemann, 1996). The importance of the position S631 was also shown by the recent work of Smith, Baukowitz & Yellen (1996). Interestingly, replacement of S631 by a cysteine accelerated HERG inactivation, while the double mutant G628C•S631C did not inactivate.

Trudeau *et al.* (1995) reported that low concentrations of Cs_o⁺ (1 mM) inhibited peak inward currents. This is in accordance with our own findings (data not shown), but by using elevated Cs_o⁺ concentrations we could show that HERG channels have a significant permeability for Cs⁺ ions. This is analogous to *Drosophila eag* channels, which show even more pronounced permeability to Cs⁺ (Brüggemann *et al.* 1993), whereas rat *eag* channels are highly selective for K⁺ (Ludwig *et al.* 1994). It had been speculated that a cysteine residue found in the pore region of *Drosophila eag*, but not in rat *eag*, could be responsible for this difference (Ludwig *et al.* 1994). Our finding of Cs⁺ permeability in the homologous HERG channel argues against this hypothesis, since HERG has a serine residue in this position, which is the same residue as in rat *eag*. We can also exclude a critical function of V630 and S631 in Cs⁺ selectivity of HERG, as mutation of these residues did not alter reversal potentials for Cs⁺.

K_v1.4 potassium channels are modulated by K_o⁺ in the physiological concentration range and the residue K533 was determined to be responsible for this regulation (Pardo *et al.* 1993). A similar regulation is observed in *Shaker* B channels mutated at position T449, anticipating a strong correlation of C-type inactivation and K_o⁺ sensitivity (López-Barneo *et al.* 1993). Also, HERG channels were reported to depend on K_o⁺ (Sanguinetti *et al.* 1995; Trudeau *et al.* 1995). We have shown here that Cs_o⁺ has an even stronger activating effect than K_o⁺; due to the greater driving force for K⁺ efflux in Cs⁺ Ringer solution, large, inactivating, outward currents can be measured in this configuration. The amino acid replacement S631A reduced this strong Cs_o⁺ sensitivity, indicating that this position is important for this kind of channel regulation. Interestingly, S631A had only minor effects on channel regulation by K_o⁺.

The results presented in this study will be important in understanding the contribution of HERG to the formation

of cardiac action potentials. At high firing frequencies, the relatively slow processes of activation and deactivation of HERG are unlikely to be rate determining; HERG channel opening would, therefore, mainly be regulated by the processes of inactivation and recovery from inactivation. This is due to the unusual mechanism whereby deactivation, and to a smaller degree also activation, are slowed by the cytoplasmic N-terminal domain. It is tempting to speculate that this domain itself could be the target of post-translational modifications, which would then regulate the open-channel probability.

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Acknowledgements

We would like to thank M. T. Keating (University of Utah, Salt Lake City, UT, USA) for providing us with the cDNA coding for the wild-type HERG channel and M. Koenen (Max-Planck Institute for Medical Research, Heidelberg, Germany) for the expression vector pSPOoD. Our appreciation is extended to A. Roßner and A. Grimm for technical assistance. This work was in part supported by a Human Frontier Science Program grant awarded to S.H.H.

Author's email address

S. H. Heinemann: ite@rz.uni-jena.de

Received 6 February 1996; accepted 2 April 1996.