The S631A Mutation Causes a Mechanistic Switch in the Block of hERG Channels by CnErg1

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ABSTRACT We have studied the interaction of CnErg1, a member of the γ-KTX subfamily of scorpion toxins with the inactivation-deficient S631A hERG channel. In the background of this mutation, we observed a mechanistic switch from turret block, characteristic of the action of γ-KTXs on Kv11-type channels, to pore plugging, characteristic of α-KTX block of Kv1-type channels. We suggest this reflects destabilization of the outer pore (turret region) of hERG allowing access of the toxin molecule to directly plug the conduction pathway.

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The KTX family of peptide toxins from scorpion venoms has evolved over hundreds of millions of years to act with high specificity and selectivity on many members of the diverse K^+ channel superfamily. By far the most extensively characterized group of these peptides are the α-KTX subfamily, with over 70 members, whose actions have been described on a variety of mostly *Shaker* (Kv1) type channels. Despite the diversity of structural folds within the subfamily, it was suggested that these peptides share common key elements: The functional dyad, which contains a positively charged residue whose side chain plugs the conduction pathway separated by ~6–7 Å from a hydrophobic group; and a ring of basic amino-acid residues which contribute to a multipoint interaction of the toxin and channel (1).

The mechanism of classical block of Kv1-type channels by the α-KTX subfamily is best described for charybdotoxin (CTX) block of *Shaker*. In this case, the ε-amino group of a lysine residue (K27) is thought to approximate to a tethered K^+ ion which extends into the selectivity filter and competes with K^+ ions at the outermost K^+ binding site. Complete block therefore occurs through direct plugging of the conductance pathway and is sensitive to external [K^+] (2). Additionally, experimentally determined time- and rate-constants have shown that CTX block of *Shaker* is well described by a simple, diffusion-limited bimolecular interaction,

$$T + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} TC, \qquad \text{(Scheme 1)}$$

where T and C are free toxin and channel, and TC is the toxin-blocked channel.

More recently, another subfamily, the γ-KTXs, has been described. These toxins are specific for Kv11-type (*ether-á-go-go* related) channels and are thought to have a unique mechanism of interaction and block (3). The mechanism of action of the γ-KTX subfamily is best understood for CnErg1 (γ-KTX 1.1), the first of this subfamily to be identified. A characteristic feature of the action of CnErg1 on hERG is incomplete block of macroscopic current even at concentrations orders-of-magnitude higher than the K_d value (4). In a previous publication (4), we showed that this is due to the kinetics of the toxin channel interaction as outlined below,

$$T + C \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} TC^* \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} TC, \qquad \text{(Scheme 2)}$$

where T and C are free toxin and channel, respectively; TC^* is a full conductance toxin-channel encounter complex; and TC is the toxin-blocked channel. In this kinetic scheme, incomplete block occurs due to rapid fluctuations between the toxin-blocked channel and a full conductance toxin-channel encounter complex, i.e., k_{-2} is relatively large compared to k_{+2}. In this two-step reaction, the rate-limiting factor is the lifetime of the encounter complex, TC^*.

The binding site for CnErg1 on hERG is thought to be formed, at least in part, by the extracellular linker between the S5 transmembrane helix and the pore helix (S5P linker) (5), which is also thought to be critically involved in voltage-dependent inactivation in hERG (6). We therefore initially set out to use CnErg1 to help answer a longstanding question from the literature: What are the structural rearrangements involved in voltage-dependent inactivation in hERG? We reasoned that by using toxin footprinting (7) to probe structural differences in the S5P inactivation domains between wild-type and S631A hERG (an inactivation-deficient mutant), we might gain insight into the series of molecular

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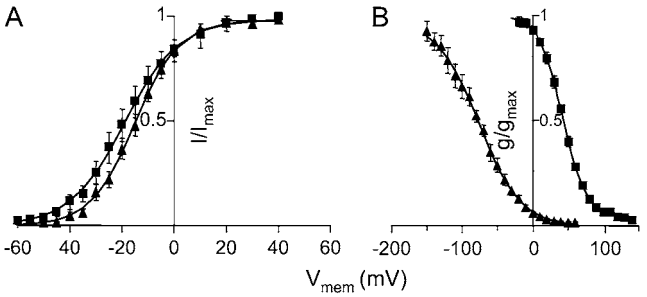


FIGURE 1 Voltage-dependent gating in wild-type and S631A hERG. (*A*) $V_{0.5}$ for activation was not significantly different in wild-type (▲) and S631A (■) hERG channels (−16.4 ± 1.5 mV and −18.7 ± 3.1 mV, respectively). (*B*) S631A shifts $V_{0.5}$ for inactivation from −82.7 ± 5.7 mV for wild-type (▲) to 43.2 ± 2 mV for S631A hERG (■). Solid lines are best fits of the Boltzmann equation to the data. Data are mean ± SE; $n \geq 5$.

events that lead to hERG inactivation. If the S631A mutation had disrupted a distinct aspect of the inactivation pathway, this system could provide a unique opportunity to get a snapshot of the hERG channel protein arrested in a preinactivation state. Thus, we investigated the mechanism of block of S631A hERG channels by CnErg1 using whole cell voltage-clamp electrophysiology.

The S631A mutation, in the linker region between the pore helix and the S6 transmembrane domain in hERG channels, causes an ~120-mV depolarized shift in the $V_{0.5}$ of inactivation, while the voltage dependence of activation is unaltered (Fig. 1). However, our steady-state binding data immediately indicated fundamental differences in the mechanism by which CnErg1 blocks S631A compared to wild-type hERG channels: mutation from serine to alanine at position 631 caused an ~20-fold increase in the affinity of CnErg1 for hERG; but more significant, however, was a change in the maximum block. A fit of the Hill equation to the dose-response curve for CnErg1 binding to wild-type hERG gave a maximum block of 93.5%, while a similar fit to the data for S631A showed that CnErg1 completely blocks macroscopic current in S631A channels (Fig. 2 *A*). Further differences in the mechanism of block are identified though examining the relationship between on-rate and toxin concentration. For CnErg1 block of wild-type hERG, the lifetime of a toxin-channel encounter complex was the rate-limiting step (see Scheme 2 above, and (4)), consequently there is a nonlinear relationship between k_{+1} [toxin] and [toxin] (Fig. 2 *B*). On the other hand, a similar analysis of CnErg1 binding to S631A hERG showed a linear relationship (Fig. 2 *C*), indicating that the rate of diffusion of the toxin molecule to its binding site on the channel protein is the rate-limiting factor. In this case, the bimolecular interaction can be well described by a simple one-step kinetic system such as that shown in Scheme 1 (2). Both of these features, complete block and diffusion-limited binding, are characteristic of block of Kv1-type channels by α-KTX toxins.

This raises the intriguing question as to whether CnErg1, as well as blocking wild-type hERG via its usual γ-KTX-like mechanism (where block is defined by the kinetics of the interaction), switches to classical Kv1-type block in the background of the S631A mutation. To further test if this were the case we examined the sensitivity of block to external $[K^+]$. If the initial indications were correct, and the S631A mutation does alter the mechanism to a pore plug, analogous to block of Kv1-type channels, then the extent of block would be sensitive to external $[K^+]$. Fig. 3 illustrates that this was indeed the case. While block of wild-type hERG by CnErg1 was insensitive to $[K^+]$, block of S631A hERG was significantly reduced when external $[K^+]$ was raised. An increase in extracellular $[K^+]$ from 5 to 100 mM caused an ~10-fold decrease in binding affinity. Closer inspection of the kinetics revealed this change in affinity was entirely due to a decreased association rate resulting from increased competition for occupancy of the K^+ binding site as external $[K^+]$ was increased. Dissociation rates on the other hand were independent of extracellular $[K^+]$ (see Table, Fig. 3). This is consistent with the binding site for K^+ in the selectivity filter being inaccessible to ions in the extracellular space once the toxin has plugged the pore.

In summary, the S631A mutation appears to switch the mechanism of block by CnErg1 of hERG to a pore plug, and all of the characteristics defining the block are common with α-KTx block of Kv1-type channels. Is this an evolutionary

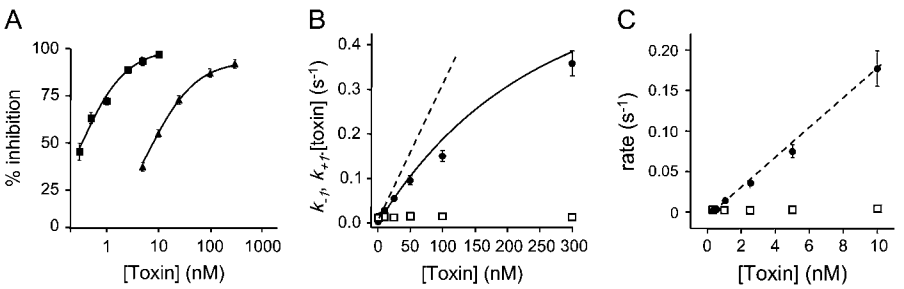
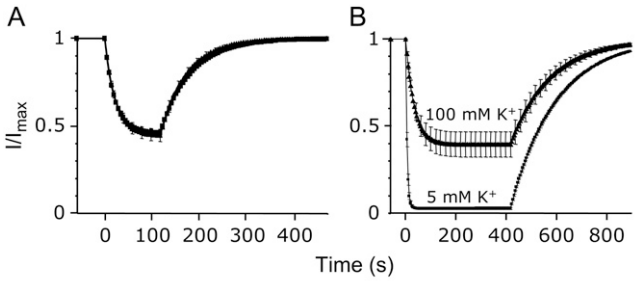


FIGURE 2 (*A*) Block of wild-type (▲) and S631A (■) hERG by CnErg1. A fit of the Hill equation to the data showed the IC_{50} decreased from 6.5 nM for wild-type hERG to 300 pM for S631A while the maximum block increased from 93.5% to 100%. (*B*,*C*) Summary of [toxin] dependence of the association (k_{+1}.[toxin], ●) and dissociation (k_{-1}, □) rate constants for CnErg1 block of wild-type (*B* reproduced from (4)) and S631A (*C*) hERG currents. Data points show mean ± SE for n = 5–14 experiments and the dashed line shows a straight line of best fit to the association rate constant data at low toxin concentrations (*B*) or the entire dataset (*C*). The solid line in panel *B* represents the modeled data based on Scheme 2 (4).



	wildtype		S631A	
$[K^+]_o$ (mM)	5	100	5	100
% block	54.8±1.6	56.3±3.6	96.9±.7	60.4±7.2
k_{+1} ($M^{-1}s^{-1}x10^6$)	2.9±0.2	2.4±.2	18±2	2.3±.1
k_{-1} (s^{-1})	.02±.0005	.02±.001	.006±.0009	.007±.0008
K_d (nM)	6.4±.3	7.9±1.2	.35±.07	2.9±0.3

FIGURE 3 External $[K^+]$ dependence of block of (*A*) wild-type and (*B*) S631A hERG by 10 nM CnErg1. Increasing external $[K^+]$ from 5 mM to 100 mM did not affect CnErg1 block of wild-type hERG (data overlap) while block of S631A was markedly reduced. (*Table*) Summary of the effects of external $[K^+]$ on rates and equilibrium binding constants of CnErg1 binding to wild-type and S631A hERG. Data are mean ± SE; n = 4–15.

throwback to a time before the γ-KTX subfamily split from the remaining KTXs, suggesting that the pore plug is an ancient and fundamental characteristic of KTX toxins which has become redundant in block of Kv11-type channels or turret block? Alignments of CnErg1 with α-KTXs show the well-conserved lysine involved in plugging the pore in α-KTX block (Lys^{27} in CTX) is absent in CnErg1 (5), suggesting this may not be the case. A more likely explanation is a fortuitous alternative match of toxin and channel structures in the background of the S631A mutation. The obvious question this raises is the identity of the plug. We previously presented the structure of the CnErg1 molecule and highlighted the presence of two lysine residues, Lys^{13} and Lys^{25}, whose position relative to neighboring hydrophobic residues satisfied the requirements to form a functional dyad (8). Although the mechanism of γ-KTX block of Kv11-type channels is now more fully understood, and is thought not to involve a dyad or pore plug, it is worth reconsidering these residues as the likely candidates for plugging the selectivity filter in S631A hERG channels. Further mutagenesis work should answer this question but it is not critical to the issue of a mechanistic switch presented in this letter.

An offshoot of this work is a cautionary reminder about the use of mutagenesis in inferring details of wild-type function, and in toxin footprinting. S631A is a widely used inactivation-deficient mutant in the study of hERG kinetics and state-dependent drug binding. Turret block of Kv11-type channels is thought to involve binding to the S5P linkers, slightly distal from the selectivity filter; and these same S5P domains, which form the turret, sterically prevent direct access to the selectivity filter (9). In the S631A mutant, there must be sufficient destabilization of the conformation of the outer pore that the toxin does have direct access to plug the selectivity filter. This cannot solely be due to side-chain alterations at the 631 position since CnErg1 does not block the related EAG channel (5), which has an alanine at this position. Therefore, in using S631A as a background for further investigation of inactivation in hERG we must bear in mind that the structures of the extracellular domains of the channel are likely to be different from those in the wild-type channel before drawing any mechanistic conclusions.

In summary, we have shown for the first time, to our knowledge, that a single point mutation on the hERG channel protein can switch the mechanism of block by the toxin CnErg1 from turret block to pore-plug and we suggest that this is due to a structural rearrangement of the extracellular S5P domains allowing direct access of the toxin to the selectivity filter.

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