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MUTATIONS IN THE P-REGION OF A MAMMALIAN POTASSIUM CHANNEL (RCK1): A COMPARISON WITH THE *SHAKER* POTASSIUM CHANNEL

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Summary: Deletion mutants of *Shaker* K channels lacking the middlemost 2 residues of the amino acid sequence GYGD in their P-region lose K-selectivity and become functionally similar to voltage-activated Ca channels. Ca channel characteristics are also conferred on voltage-activated Na channels when residues K in domain III and/or A in domain IV in the P-region of the Na channel are replaced by E residues . In this study, we have investigated a possible evolutionary connection between voltage-activated K and Na channels. Mutant monomeric and multi-heteromeric RCK1 K channel cDNAs were made to match the residues at equivalent positions in the P-region of the Na channel. We found that in contrast to mutant *Shaker* K channels, the same mutants in RCK1 K channels did not yield functional expression. Therefore possible Na channel-like ion conduction properties conferred on K channels could not be demonstrated. However, our results show that the same mutations in highly homologous channels can produce different effects and point to hitherto unknown structural differences in the P-region of these homologous K channels. Therefore we conclude that extrapolation of the structural and functional importance of residues should be done with caution, even when ion channels belong to the same family.

The essential features that distinguish the pore regions (P-regions) in voltage-activated K channels from these in cyclic nucleotide-gated (CNG) ion channels have recently been identified (1). The different ion conduction properties can now be explained in terms of the middlemost 2 residues of the amino acid sequence GYGD (Gly-**Tyr**-**Gly**-Asp) that are present in K-selective channels and absent in CNG channels (Figure 1). *Shaker* K channel deletion mutants lacking residues YG have also been found to be functionally similar to voltage-activated Ca channels (1). The GE (Gly-Glu) pair present in the P-region of 3 of the 4 Ca channel domains matches the remaining GD (Gly-Asp) pair in the K channel deletion mutant. With respect to the equivalent amino acid residues of the P-region in voltage-activated Na channels, it has been shown that mutations K to E (Lys to Glu) in domain III, and A to E (Ala to Glu) in domain IV confer Ca channel-like ion conduction properties on the Na channel (2). Given the experimental evidence that the P-

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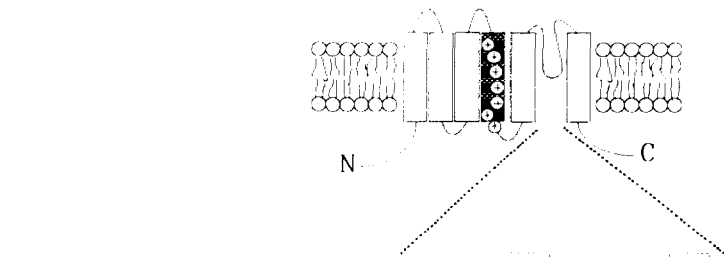

Shaker IR/RCK1		PDAFWWAVVT/SMTTVGYGDMT/YP
Del		-G D
CNG		----------G E-----
Ca brain I		----------M E-----
II		----------G E-----
III		----------G E-----
IV		----------G E-----
Na brain I		----------Q D-----
II		----------G E-----
III		----------F K-----
IV		----------S A-----
RCK1 mutants	Del D	G D
	Del E	-G E
	Del K	-G K
	Del A	-G A

Figure 1. Proposed membrane topology and sequence alignment of the P-region from different cloned cation channels. The amino acid sequence corresponding to the P-region of WT *Shaker* IR/RCK1 K channels is shown together with a schematic diagram of the proposed membrane topology of a subunit of voltage-activated K channels (top). *Shaker* IR channels have 2 T (Thr) residues, whereas RCK1 channels have a S (Ser) and Y (Tyr) residue (boxed residues). The amino acid sequences of the "Del" mutants are characterized by the absence of the middlemost 2 residues **YG** of the sequence GYGD (underlined in bold). Full and interrupted lines in the sequences represent respectively identical and different amino acid compositions. Alignment of the sequences of CNG ion channels and voltage-activated Ca and Na channels is done as in ref (1).

regions of K, Ca, and CNG ion channels are possibly related, the aim of our study was to investigate a possible evolutionary relation between voltage-activated K and Na channels, and to determine the residues governing ion selectivity. To achieve this objective, we have made deletion mutants (designated hereafter as "Del") lacking the middlemost 2 residues **YG** of the sequence GYGD in the P-region of a delayed rectifier RCK1 (Kv1.1) K channel. The next residue in the sequence, D (Asp), was either left unaffected or mutated to E (Glu), K (Lys), or A (Ala). These mutations were performed based on a primary sequence alignment with the P-region of the Na channel (see also Figure 1). The 4 different mutant monomeric K channel cDNAs ("Del D, Del E, Del K, and Del A") were also concatenated into multimeric constructs to manipulate the subunit composition of the functional channels (3,4), and such that residues at equivalent positions in the P-region

of tetrameric K channels match the ones in the P-region of the Na channel. We have tried to express these constructs in *Xenopus laevis* oocytes and investigated if these mutations confer Na channel-like ion conduction properties on K channels.

Methods

Site-directed mutagenesis on the RCK1 K channel was performed using the "Transformer" system (Clontech). Mutations were verified by dideoxy DNA sequencing (Sequenase Version 2.0, USB). Construction of multimeric K channel cDNAs into a high expression vector, pGEM-HE, was as previously described (5). After *in vitro* transcription, 50 nl of mRNA was injected in *Xenopus laevis* oocytes. The oocytes were incubated in ND-96 solution (in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 Hepes, pH 7.5, supplemented with 50 mg/liter gentamycin sulfate) at 18 °C for 1-4 days. Whole-cell currents from oocytes were recorded with a two-microelectrode voltage-clamp amplifier. Voltage and current electrodes (1 megaohm) were filled with 3 M KCl solution. The external solution contained either (in mM): 100 NaCl, 0.1 $MgCl_2$, 0.05 $CaCl_2$, 5 Hepes, pH 7.5, or 100 NaCl, 0.1 $MgCl_2$, 5 EGTA, 5 Hepes, pH 7.5. Current records were sampled at 4 ms intervals after low-pass filtering at 0.1 kHz and analyzed on an IBM-compatible computer. Linear components of capacity and leak currents were subtracted digitally. All electrophysiological experiments were performed at room temperature (19-22 °C). For each construct, several independent clones were sequenced, transcribed and expressed. "*Shaker* IR Del D" cDNA represents a voltage-activated *Shaker* K channel in which the N-type inactivation has been removed ("IR"), the middlemost 2 residues **YG** of the sequence **GYGD** have been deleted ("Del"), and the deletion is followed by residue "D".

Results and Discussion

Figure 2 shows whole-cell currents recorded from *Xenopus laevis* oocytes injected with mRNA coding for *Shaker* IR Del D and RCK1 Del D mutants. The oocytes were held at -90 mV and clamped for 1 s to test potentials

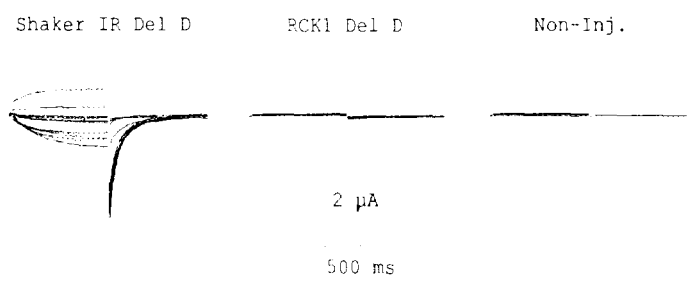

Figure 2. Whole-cell recordings from oocytes. Macroscopic currents recorded from *Xenopus laevis* oocytes injected with mRNA coding for *Shaker* IR Del D and RCK1 Del D mutants. In contrast to the functional expression observed for *Shaker* IR Del D channels, no time-dependent currents were observed for the RCK1 Del D mutant. Non-injected oocytes displayed only very small endogenous currents not significantly different from the oocytes injected with RCK1 Del D. Holding potential -90 mV. Test potentials from -80 to +10 mV.

	Functional Expression
Shaker IR Del D	+
Del E	+
RCK1 Del D	-
Del E	-
Del K	-
Del A	-
RCK1 Dimer Del D - Del E	-
RCK1 Tetramer Del D - Del E - Del K - Del A	-
RCK1 Dimer WT - Del E	-
RCK1 Tetramer WT - WT - WT - Del E	-

Figure 3. Summary of functional expression of mutant channels. Functional expression observed in *Shaker* IR Del D and E channels (1), in contrast to RCK1 mutant channels. Monomeric, dimeric and tetrameric constructs did not yield functional currents. Likewise, dimers and tetramers composed of WT and mutant RCK1 subunits failed to produce functional expression.

ranging from -80 to +10 mV. In contrast to the *Shaker* IR Del D mutant, the RCK1 Del D mutant did not yield functional expression. The reversal potential of the current generated by the *Shaker* IR Del D channel was within 10 mV of zero and reveals that these ion channels are no longer selective for K ions. This result confirms the findings by Heginbotham *et al.* (1992). Like the RCK1 Del D mutant, also RCK1 Del E, K and A mutants did not produce functional currents. As a control, non-injected oocytes only displayed very small endogenous currents in the tested voltage range.

In an effort to rescue the non-functional monomeric RCK1 deletion mutants and to try to understand why *Shaker* IR K channels tolerate the YG deletion in contrast to RCK1, we have made several other constructs combining mutant and/or wild-type (WT) monomeric cDNAs into dimeric and tetrameric constructs (Figure 3). Dimers containing either 2 deletion mutants (Del D-Del E) or 1 deletion mutant combined with a WT subunit (WT-Del E) did not produce functional currents. Also tetramers composed of 4 deletion mutants (Del D-Del E-Del K-Del A) or 3 WT subunits and 1 deletion mutant (3 WT-Del E) were non-functional. This is a surprising result for the reason that the overall sequence homology between the *Drosophila Shaker* K channel protein and its mammalian counterpart, the RCK1 (Kv1.1) K channel protein from the rat brain, is 82 % (6). The highest degree of identity is found in the transmembrane segments S1 to S6, as well as in the P-region between domain S5 and S6, and the cytoplasmic N-terminus domain preceding the S1 segment. Since ion selectivity is very similar in all K channels, it is reasonable to look for conservation in the primary sequence of

the P-region that could underlie the common K-selectivity. Figure 1 shows 19 residues situated between the proline residues (P, Pro) of the P-region in *Shaker* and RCK1 K channels. Between the 2 channels, only 2 residues on 19 are different (i.e. 90 % homology): twice a T (Thr) residue in *Shaker* opposite to a S (Ser) and Y (Tyr) residue in RCK1. This difference between *Shaker* and RCK1 could be a possible explanation for the lack of functional expression in RCK1 mutants. While the difference between a T and S residue is small (both have aliphatic hydroxyl side chains), the difference between a T and Y residue is substantial: Y has an aromatic side chain with more bulky dimensions than the side chain of T. This could occlude the permeation pathway of the RCK1 channel when the middlemost **YG** residues of the sequence G**YG**D are deleted. Interestingly, studies have been reported wherein one has matched the sequence of the P-region of the *Shaker* channel to the RKC1 channel and conversely. It was found that replacement of the first T residue by S in the P-region of *Shaker* does neither influence the functional expression nor alter the K-selectivity of the channel (7). In contrast, replacement of the last Y residue by T in the P-region of RCK1 results in a loss of functional expression (5). Alternatively, since the entire region from the S45 loop to the end of the S6 domain may line the aqueous conduction pathway (8-10), it is also possible that residues in this region (and not in the P-region) specific to *Shaker* or RCK1 are crucial for functional expression. To date, we have unfortunately no idea how our mutations affect the 3-dimensional structure of the channel protein. Therefore, improper folding and/or assembly of the subunits, disturbed gating and/or channel opening of the mutant RCK1 channel protein, are other possible explanations for the lack of expression which we cannot rule out. The observation that heteromeric mutant channels with 2 or 3 WT subunits still do not yield functional expression is remarkable, and stresses the structural importance of residues YG in the P-region of RCK1 different from the one in *Shaker*. Interestingly, Heginbotham *et al.* (1992) have observed that in *Shaker* IR Del channels, the presence of an acidic residue (D or E) is necessary for producing functional currents. This however still does not explain why RCK1 Del D and E channels do not yield functional currents. The lack of functional expression for RKC1 mutants, and not for *Shaker* mutants, is also not restricted to mutations in the P-region. It has been reported that *Shaker* K channels tolerate neutralization of the 3rd or 4th charged residue in the voltage sensor, S4, whereas the same mutants in RCK1 are fatal for functional expression (11,12). Since the *Shaker* IR Del D channel is blocked by divalent cations (K_{iCa} 38 μM, K_{iMg} 418 μM (1)), we performed an additional series of experiments with zero Ca solution in the bath (with 5 mM EGTA + 0.1 mM Mg). Also under this condition, no functional expression was observed.

As a positive control, RCK1 cDNA which remained WT after the mutagenesis procedure (mutagenesis efficiency about 70 %), was *in vitro* transcribed and injected in parallel with the deletion mutants. Injection of this WT mRNA always resulted in normal WT delayed rectifyer K currents. We are also convinced that the mutagenesis kit itself did not produce a systematic error in the cDNA, since we have made other RCK1 mutants in the P-region which yield functional currents (Tytgat & Carmeliet, in preparation). The fact that we have tried several independent clones for each of the constructs also persuades us that the lack of expression does not result from a spontaneous mutation in the cDNA.

In conclusion, although we have failed to functionally express our RCK1 deletion mutants, and, therefore, have not been able to study possible Na channel-like ion conduction properties conferred on K channels, our results show that the same mutations in highly homologous K channel proteins can produce different results, which point to unknown structural differences in the P-region of these channels. Since site-directed mutagenesis is often used as a tool to study structure-function relationships of different channel proteins, extrapolation of the structural and functional importance of residues should be done with caution, even when the channels belong to the same family.

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