Double gaps along Shaker S4 demonstrate omega currents at three different closed states

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The aim of the present study was to investigate in detail how the voltage sensor in the Shaker potassium channel moves during the gating process. After the publication of the open channel structure from the crystallized K\textsubscript{AP} channel in 2003, an alternative so-called "paddle" model was put forward in contrast to the existing helical screw model. The voltage sensor S4 contains 4 arginine residues relevant for gating, R1(362), R2(365), R3(368) and R4(371), each separated by 2 neutral residues. These charged residues coil as one of three threads on the S4-\(\alpha\)-helix. Based on a previous finding that the mutation R1S leads to the so-called omega leak current through a "gating-pore" in the closed state, we introduced gaps systematically along the arginine thread substituting long arginines by short serines. Mutations R2S or R3S did neither create transient nor steady leaks. The fact that the native residue A359, which is located three amino acids in front of R1, is a short one, motivated us to check its role. Mutation of A359 to arginine blocked the omega current in the R1S mutant indicating that the omega pore is occupied by A359 and R1. Introducing further double gaps (RR to ss) at sequential positions (0 + 1, 1 + 2, 2 + 3), produced clear leak currents which were remarkably stable over a wide voltage range. These leaks contradict that S4 would swing together with S3 in lipid according to the paddle hypothesis. Rather, our results show that during gating the S4 segment moves in 3 helical steps through a fixed pore formed by the channel protein.

Introduction

After the publication of the protein structure of voltage-gated potassium channels obtained from crystals of K\textsubscript{AP},\textsuperscript{1,2} a new proposal for the gating movement of the voltage sensor S4 was put forward. The observation that S4 appears to be in contact with the lipid phase led to the picture of a paddle consisting of the outer half of S3 and of S4 which together would swing loosely in the lipid. The same model of gating was also proposed for K\textsubscript{1.2} channels where the entire S3 segment, not only the outer half, together with S4 sensor forms the paddle.\textsuperscript{3,4} This was backed by extensive probing the depth of most residues in the outer paddle with tethers of different length.\textsuperscript{2,5} In contrast, the more traditional view sees the outer half of S4 to slide through a narrow hole formed by other segments of the channel protein, the so-called helical screw model.\textsuperscript{6,7} An important point in support of the screw model is the very conservative arrangement of the 4 arginines R1(362), R2(365), R3(368) and R4(371) in the outer half of S4 (Fig. 1A). Occurring at every third position, they form a helical thread on S4 in \(\alpha\)-helical conformation, and during the stepwise gating movement an arginine would jump to the position of the neighboring gating charge driven by the electric field across the membrane. Also, the following positively charged residues K5(374), R6(377), K7(380) and R8(383) lie on the same thread. Based on accessibility studies of the arginines from either the extracellular or the intracellular water space, the length of the voltage drop where the arginines are in the dielectric phase appears to be concentrated on a very short distance.\textsuperscript{8,9} Recently, the so-called omega current was demonstrated in Shaker K-channels, where a replacement of arginine R1(362) by a short residue led to a leak current at hyperpolarized potentials.\textsuperscript{10,11} This finding supported the previously proposed gating canal\textsuperscript{12} in the protein which would normally guide the thread of arginines during the gating movement of S4. The region of interest is depicted in the schematic of Figure 1B.

In the present study we searched for experimental evidence to decide whether the S4 slides through a fixed narrow pore according to the helical screw model, or, whether it would swing together with S3 as a paddle through a less well defined lipid phase. Our strategy was to systematically mutate arginine to serine one by one at the positions of R2 and R3 expecting to find transient leak currents when S4 would slide through the proposed narrow pore and the short serine would give way to a leak current while in the pore. The second expectation was to find an outward leak current in the mutant R4S if this serine would be the only residue occupying the omega pore at the final upward position of S4 in the depolarized state. The results were negative; we saw neither short transient leak currents in intermediate states nor steady state leak currents, e.g., in the final open state. So, either the short serines did not pass through or dwell in the gating-pore and S4
would move along a different path, or the present view about the pore was not correct. Comparing other voltage-gated channels, it was reported for sodium channels that in the resting state inward omega-leak current could be recorded when a longer gap was introduced by a double mutation of arginines to the shorter residue glutamine at the respective positions of R1 and R2 in domain 2, while for the double mutation of R2 and R3, an outward leak-current could be recorded in the activated state. As can be read in a review, it was almost a dogma that in Shaker potassium channels the omega pore had a length of one residue and in rat brain sodium channels (rBIIA) a length of two residues. In view of our negative findings, we then asked whether the hypothetical pore fitting around the arginine would be longer than just one residue as depicted in Figure 1B and perhaps also the next residue along the arginine thread would control the aperture of the pore. Three amino acids in front of R1 is a short residue, namely alanine 359 or, if counting along the arginine thread A0.

To probe the width of the pore (question mark in Fig. 1B), we introduced into the R1S omega mutant the additional mutation A0R. As the result, the omega current was blocked indicating that both residues at position 359 and 362, or 0 and 1 on the arginine thread, need to be short for the leakiness of the pore. This finding provided us a new strategy to reexamine how S4 moves during gating by applying double gaps along S4.

### Results

Blocking of the omega current by mutation A0R. Table 1 introduces the shorthand notation used in this paper for the various combinations of the mutations within the stretch of alanine and the first four arginines of S4. Accordingly, wild-type S4 would be aRMMM and, asRMMM corresponds to the classical omega construct used by us (serine reportedly produced in Shaker the largest omega current); in addition, to visualize short vs. long residues, small vs. capital letters are used. All constructs were made in the inactivation preserved Shaker potassium channel with the alpha-pore closed by mutation W434F such that the gating currents and omega currents could well be detected. Further, the C-terminal was tagged with eGFP in order to monitor the expression and implementation of the channels into the oocyte membrane. The channels were expressed in Xenopus oocytes and the omega- and gating-currents were measured using the two electrode voltage clamp technique. Figures 2A and 2B show the results for the classical omega construct asRMMM compared to RsRMM. The currents obtained from different cells with varying expression were normalized to the total gating charge measured from the same oocytes (Fig. 2D). More than 95% of the omega current measured at -200 mV was blocked in the case of RsRMM compared to that measured at the same voltage from asRMMM channels. This indicates that the classical omega-mutation R362S in the asRMMM channel produces omega current as a
was restored (Fig. 2C). The normalization of the measured omega current to gating charge did not seem appropriate when two important positive gating charges were missing in one construct, and in consequence the charge per channel might be altered too much for a comparison of the different constructs. Therefore, the measured omega current was normalized to the relative channel number as obtained from the total fluorescence of the eGFP marked channels. The vertical scale is normalized to the current in asRRR at -200 mV. The current-to-voltage plots are linear-leak subtracted. Error bars denote s.e.m.

Figure 2. Omega current in asRRR mutant becomes blocked in RsRRR mutant and is restored in RssRR mutant. (A–C) Measured currents for pulses from -200 to +80 in steps of 10 mV in lower part and proposed schematic models in upper part for the 3 mutant constructs. (D) Current vs. voltage for asRRR (n = 6) in comparison to RsRRR (n = 11) normalized to the gating charge used as counter for the channel number. (E) New batch of experiments comparing asRRR (n = 5), RsRRR (n = 11) and RssRRR (n = 11), now with the currents normalized to the relative channel number as obtained from the total fluorescence of the eGFP marked channels. The vertical scale is normalized to the current in asRRR at -200 mV. The current-to-voltage plots are linear-leak subtracted. Error bars denote s.e.m.

Double gap strategy. The finding that two successive short residues are needed to conduct omega current offered a fresh possibility to test whether S4 slides through a narrow pore and consequently also double gaps at other positions of S4 would create leak currents. To check on this, the second arginine in the blocking construct RsRRR was mutated to serine to obtain the next double gap construct RssRR. As a result, the omega current was restored (Fig. 2C). The normalization of the measured omega current to gating charge did not seem appropriate when two important positive gating charges were missing in one construct, and in consequence the charge per channel might be altered too much for a comparison of the different constructs. Therefore, the measured omega current was normalized to the quantified fluorescence emitted from the eGFP tagged ion channels; this technique was shown previously to reliably reflect the number of channels in the membrane. The vertical scale is normalized to the current in asRRR at -200 mV. The current-to-voltage plots are linear-leak subtracted. Error bars denote s.e.m.
the quantified fluorescence. The results showed again that the long arginine at position 0(359) in the RssRR channel blocked more than 95% of the omega current measured at -200 mV in the classical omega construct asRRR. On the other hand, the channel construct RsRR restored only 70–80% of the omega current. The reason for this smaller leak could be the presence of the long residue A359R at the entrance of the omega pore as compared to the wider entrance in asRRR with methionine at position 356 (on the extended arginine thread).

Single vs. double gap mutations. Figure 3 shows the results obtained for further single and double gaps along the thread of the positively charged residues of S4. The single gap constructs aRsRR and aRRsR produced only small almost linear leak currents which are comparable to the leak of non-injected control cells or wild-type aRRRR; the single gap mutation at position 371, aRRRs, did not express channels in our hands. In contrast, the double gap construct aRssR showed a pronounced omega leak current. It can be seen, that here the omega pores start to open at a less hyperpolarized potential of about -30 mV compared to about -50 for RssRR and about -70 mV for aRssR.

while, remarkably, all stay open down to -200 mV. This suggests that S4 in aRssR needs a less negative pulling force from the inside to bring the gap into the leaking position. Preliminary trials with the double gap construct aRRss did not seem to produce expression of channels, as was observed for the construct aRRRs which also includes serine at position 371.

As can be seen in the I/V curves (Figs. 2 and 3), the three gaps along the arginine thread each cause a leak current over an astonishingly wide voltage range down to the tested potential of -200 mV. This indicates that for each of the three mutated S4 segments the leaking position seems to be relatively stable. In fact, with these double gap mutants we seem to have found a means to “freeze” the S4 voltage sensor in three different states; normally during the gating process these two positions are probably only short lived in contrast to the closed or the open positions. An explanation may be that in these leaky positions no voltage sensing arginine is in the pore and ions of the leak current short circuit the force normally acting upon the S4 helix via a charged residue. For the classical omega construct asRRR the stable position most likely corresponds to the closed resting state. Then, S4 cannot move any deeper since even at -200 mV arginine R1 in wild-type still keeps the pore closed while in

Figure 3. Leak currents of different single and double gap constructs compared to control non-injected oocytes. (A and B) Typical linear leak currents of single gap mutations aRsRR and aRRsR for pulses from -200 to +100 in steps of 20 mV. (C) Double gap construct aRssR shows non-linear leak current (omega current). (D) I/V-plots of leak currents from recordings in (A–C) and from non-injected control cells vs. voltage; note that in the I/V plots the linear leak has been subtracted by fitting a line at positive potentials. The double gap construct aRssR shows a large omega-current in comparison to the linear leak of the single gap constructs aRsRR and aRRsR. Also note that the omega-current starts at about -30 mV for aRssR in contrast to about -50 mV for RssRR (Fig. 2E) and around -50 to -70 mV for asRRR (Fig. 2D and E).
omega-conductance decreased again indicating a closure by R1 stepping opposite to E283 (unpublished results).

**Discussion**

S4 passes a fixed pore in three steps during gating. Figure 4 summarizes in schematic form what we conclude from our data. We propose 4 different positions I to IV for S4 during gating. Position IV reflects the structure found for the crystallized K\_1.2 channel and corresponds most likely to the open state and thus for the most outward state of S4.\(^3,4\) On the other hand, position I reflects the most inward state of S4. Here we find the omega pore blocked for wild-type aRRRR (position IA), but open in the case of the classical omega mutation asRRR (position IB) and again closed for the “blocked omega” mutation RsRRR (position IC). Note that these conditions are true over the wide voltage range of -70 to -200 mV; if this position I would not be the most inward state, one would expect that the wild-type S4 would leak if it could move one step more inward. From these results obtained in position I it is already clear that the omega pore must be longer than just one residue, and appears to be controlled by either one of two sequential arginines (359 or 362). Position II and III each reflect the states where S4 with either one of the other double gap mutations RssRR or aRssR is situated such that its gap lies in the pore. This confirms firstly that the pore length must span two arginine residues and secondly that S4 slides sequentially through this narrow pore. Each of the three mutants, asRRR, RssRR and aRssR, creates a leaky opening between the external and the internal aqueous space due to the pair of shortened residues. The space normally filled by the longer arginines in wild-type is left open by the serine double gap, and the body of the helix does not fill this opening and is not pressed into it. It also appears very unlikely that the lipid phase of the membrane has contact with the omega pore since lipid would most likely obstruct the pore lumen. Since the same leak occurs for all three mutants, it appears impossible that the outer half of S4 swings in lipid as the
paddle model argued. What we see, corresponds to three different positions of S4 relative to a fixed pore, and during gating these three positions are passed by S4 in a stepwise sliding movement towards a further open state. Other evidence for an independent movement of S4 relative to S3 was recently reported. The fixed pore would also provide a solid handle for S4 relative to the channel protein such that it can exert force to open the channel at the inside. Compared with the results obtained from skeletal muscle sodium channel NaV 1.4, it has been found there that mutation of only one of the two outermost gating charges in the S4 voltage sensor in domain II induces omega currents at hyperpolarized potentials. On the other hand, mutation of the third arginine in the same domain produced an outward omega current at depolarized potentials. This would indicate that S4 in domain II of NaV 1.4 moves only two steps during gating and the omega pore spans only the size of one arginine.

**Implication for the length of the omega pore.** With respect to the length of the omega pore, our results give an estimate in inter-arginine distances as shown in Figure 5. Since we never observed any leak current in the case of a single gap with one short residue between two long ones, but always a pronounced leak current in the case of a double gap, we argue that the length of the pore should span minimally about 1.5 and maximally about 2.5 inter-residue distances in order to block the single gap but allow a leak through the double gap. What this means, expressed in Angstrom units, depends on several aspects. We argue that one should really consider the positions of the positively charged ends of the arginines which seem to control the passage through the omega pore; often the distance of the α- C-atoms along the helical axis is addressed. Between the positively charged ends of two arginines as seen on the helix of Figure 1A, we measured a distance of about 9 to 10 Å; thus, the canal comprising the ends might have a length of around 14 Å. Also, as the representation of the S4-helix in Figure 1A visualizes, it makes a difference whether the distance orthogonal to the membrane, or along the helical axis is taken. Such aspects may be relevant when we want to compare our results with other work dealing with the length of the confined electric field or narrow pore. In effect, different lengths for the omega pore have been proposed.

![Figure 5. Estimation of the length of the omega pore according to our data. Minimal length will allow only minute or no leak current in the case of a single gap (dotted yellow line; one short serine in red); maximal length should still allow leak current in the case of a double gap (thick yellow line; two short serines in red). Consequently, the length of the pore is expected to be about 1.5 to 2.5 inter-arginine distances; note, however, these distances would be at about the ends of the arginines as one can visualize e.g., from the arginine thread of the 3-D Figure 1A.](image)

![Figure 6. Comparison of our data with a published hypothetical structure for the closed state of Shaker. Based on experimental data which proposes that in the closed state R1(Arg362) would be opposite to E1(Glu283) in S2, a structure was obtained using the ROSETTA program. We used the coordinates as published in a follow paper to show in top-view the region of the omega-pore and surrounding segments S1 to S4 (image constructed using the Program PyMOL, Delano Scientific LLC). S1 to S4 of one domain are shown; S5 and S6 on the left are cut. It can well be seen how the omega pore is surrounded by a proteinaceous wall provided by S1 to S4. (A) Wild-type where R1 closes the pore and is opposite to E1. (B) Mutation R1S leaving the omega-pore open. (C) Our mutation A0R(RsRRR) which as we have shown closes the pore. However, in this structure the arginine at position 0(359) does not block the proposed omega pore. In contrast, we propose that the position of A0 or A0R is opposite to E1 as shown in Figure 4, and R1 would oppose I287; i.e., the blue thread seen on S4 (Fig. 1A) steps for the closed state one ratchet deeper such that A0 and R1 fill the longer omega-pore.](image)
In an earlier perspective\textsuperscript{23} the “gating canal” was estimated to be occupied by 2 to 3 residues spanning 13.5 Å. The recent conclusion from the same lab for a short pore of one residue R1 only\textsuperscript{10,11} was based on the omega leak due to the R1 mutation and should now be adjusted to include A0. It has to be noted here that in the rBIIa Na\textsubscript{+} 1.2a channel the length of the omega pore seems also to span two residues.\textsuperscript{13} Through a series of measurements of the electric field strength profile in the Shaker K\textsuperscript{+} channel an upper limit of 10 Å for the hydrophobic constriction was estimated.\textsuperscript{21} Other experiments using tethers of different lengths suggest a focused electric field over a stretch of about 4–8 Å.\textsuperscript{22}

Which amino acids would form the omega pore? Many studies addressed the question, which amino acids would form the omega pore? Tombola et al.\textsuperscript{11} reported that mutation of glutamic acid 283 to aspartic acid doubles the amplitude of the classical R362S omega current. Campos et al.\textsuperscript{23} showed that I283 in segment S2 is in atomic proximity to R1 in the closed state by using cysteine scanning experiments. On the other hand, some models assume that R1 interacts with E283 in the closed state.\textsuperscript{10} Taking these results together, and in view of our new data, we suggest that R362 and A359 point towards I287 and E283 respectively, which then form the narrow part of the gating pore as shown in Figure 4, position IA. An elaborate structure for the closed state of the Shaker channel has recently been obtained.\textsuperscript{24} As important constraint for this structure, the above cited proposal that R1 would face E283 was taken; and then, the ROSETTA program was used to optimize the 3-D structure coming from the open state of K\textsubscript{1,2}. To test this structure against our new results, we used the coordinates published in a later paper\textsuperscript{25} to build the top-views of the proposed omega pore region for the wild-type, the open omega pore and our new “blocked omega” mutant RsRRR as shown in Figure 6A–C. While R1 blocks and R1S leaves the omega pore open—the constraints for the modeling by ROSETTA—it can clearly be seen that the arginine in the mutation A0R does not close the omega pore which is in contrast to our experimental result. This implies that the proposed structure for the closed state\textsuperscript{25} needs a readjustment to explain our new data as proposed schematically in our Figure 4 position IC. In our view, the closed state structure should be adjusted with S4 one step deeper in inward direction and a possibly adapted canal for the omega current. This might have the further effect to increase the gating charge per domain which should be around three elementary charges.

Part of the presented data have been communicated in short form elsewhere.\textsuperscript{26}

\section*{Materials and Methods}

Point mutations on Shaker \textit{H4} cDNA were carried out using the QuickChange method (Stratagene) and verified by sequencing. The coding DNA was in a high expression vector, and the plasmid was injected into oocytes using a micropipette. Measurements were carried out in Modified Barth’s Solution (in mM: 88 NaCl, 1 KCl, 2.4 NaHCO\textsubscript{3}, 0.82 MgSO\textsubscript{4}, 0.33 Ca(NO\textsubscript{3})\textsubscript{2}, 0.41 CaCl\textsubscript{2}, 10 HEPES, titrated with TrisBase to pH 7.3) at 15°C. For gating current measurements, the omega leak current in the voltage region, where normally the subtraction pulses are applied, gave a problem. This was minimized in two ways: (i) the pulses for subtraction of the linear capacitance were obtained not in the usual voltage region below -100 mV but at positive voltages where no omega and no gating current would flow. (ii) Full transients including capacitance and gating current were recorded from a holding potential of -100 mV pulses in the range from +60 to -200 mV without subtraction pulses. Then, the total currents were integrated after subtraction of the baseline as obtained after the end of the gating current (containing the normal and the omega leak); the displacement charge vs. voltage was then obtained from the integrated full transients as described.\textsuperscript{27,28} Quantification of the number of expressed ion channels with fluorescence was carried out as described elsewhere.\textsuperscript{17}

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