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Creation of a Reactive Oxygen Species-Insensitive Kcv Channel

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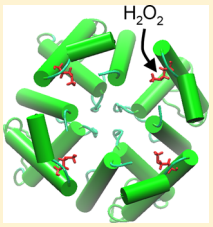
ABSTRACT: The current of the minimal viral K^+ channel Kcv_{PCBV-1} heterologously expressed in *Xenopus* oocytes is strongly inhibited by reactive oxygen species (ROS) like H_2O_2. Possible targets for the ROS effect are two cysteines (C53 and C79) and four methionines (M1, M15, M23, and M26). The C53A/C79A and M23L/M26L double mutations maintained the same ROS sensitivity as the wild type. However, M15L as a single mutant or in combination with C53A/C79A and/or M23L/M26L caused a complete loss of sensitivity to H_2O_2. These results indicate a prominent role of M15 at the cytosolic end of the outer transmembrane helix for gating of Kcv_{PCBV-1}. Furthermore, even though the channel lacks a canonical voltage sensor, it exhibits a weak voltage sensitivity, resulting in a slight activation in the millisecond range after a voltage step to negative potentials. The M15L mutation inverts this kinetics into an inactivation, underlining the critical role of this residue for gating. The negative slope of the $I-V$ curves of M15L is the same as in the wild type, indicating that the selectivity filter is not involved.



Bioengineering of ion channels is an emerging field. It is expected to provide new approaches for two major issues: (1) testing models of structure−function relationships and (2) monitoring and influencing functions inside living organisms. Some approaches start from β-barrels like staphylococcal α-hemolysin and introduce selective binding sites for a desired analyte.[1] Monitoring the changes of open and closed times then allows the distinction of different analytes. Others use chimeras of cellular receptor molecules and ion channels. Some examples are a G-protein receptor coupled to Kir6.1[2] and a prokaryotic cyclic nucleotide binding domain fused to a KcsA-derived K^+ channel.[3]

We have chosen the viral K^+ channel Kcv_{PBCV-1} as a launching pad for protein engineering. For instance, voltage sensitivity could be introduced into this simple channel by connecting the voltage sensor of *Ciona intestinalis* phosphatase to the pore unit of Kcv.[4] The viral protein Kcv_{PBCV-1} is a miniature potassium channel that is used by the virus PBCV-1 to depolarize the plasma membrane of its host *Chlorella* NC64A during infection.[5] The functional channel is tetrameric with two transmembrane segments in each pore-lining subunit, 94 amino acids long. The protein lacks any cytosolic domains, which normally harbor sensors or regulatory elements.[6] This lack of inherent sensors provides a good basis for serving as a pore element in the bioengineering of synthetic sensor channels.[4] However, in spite of its minimal design, Kcv_{PBCV-1} is not completely oblivious to the environment. The channel can be blocked by standard potassium channel blockers like Ba^{2+} and TEA;[7−9] it even has a small voltage sensitivity.[10] Here, we show that it is also sensitive to reactive oxygen species (ROS). This ROS sensitivity may restrain the use of Kcv_{PBCV-1} for bioengineering. Cellular functions and in particular malfunctions like ischemia[11] or aging and neurodegenerative diseases[12] often generate ROS.[13] Hence, any specific signal of a Kcv-based biosensor could be negatively influenced by an unwanted ROS sensitivity. A way to overcome this problem is the engineering of a ROS-insensitive Kcv mutant.

Redox-sensitive sites in proteins are also interesting from another point of view, because they can be used as a tool for the structural analysis of proteins. The study of structure−function correlates often draws structural information from intrinsic ROS-sensitive residues. For instance, in the thermosensitive TRP channel, the C617P and C622S mutations revealed the role of the cysteines in the turret for sensing temperature and capsaicin.[14] Wang and colleagues[15] concluded that the activation mechanism of TRPA1 (transient receptor potential ankyrin 1) may involve N-terminal conformational changes and disulfide bonding between critical cysteine residues. Another aspect of structural research is based on the introduction of cysteines at specific locations. For instance, disulfide bridges between engineered cysteines of *Shaker* mutant E418C/V451C or E418C/G452C can be opened and closed by redox agents. This sheds light on the function of C inactivation as normally caused by the interaction of S4 movement and the tryptophan ring of the selectivity filter.[16] Engineered disulfides further served for the study of gating in anthrax toxin channels.[17] A basic requirement for the application of this technique is again the engineering of a channel that has no inherent ROS sensitivity.

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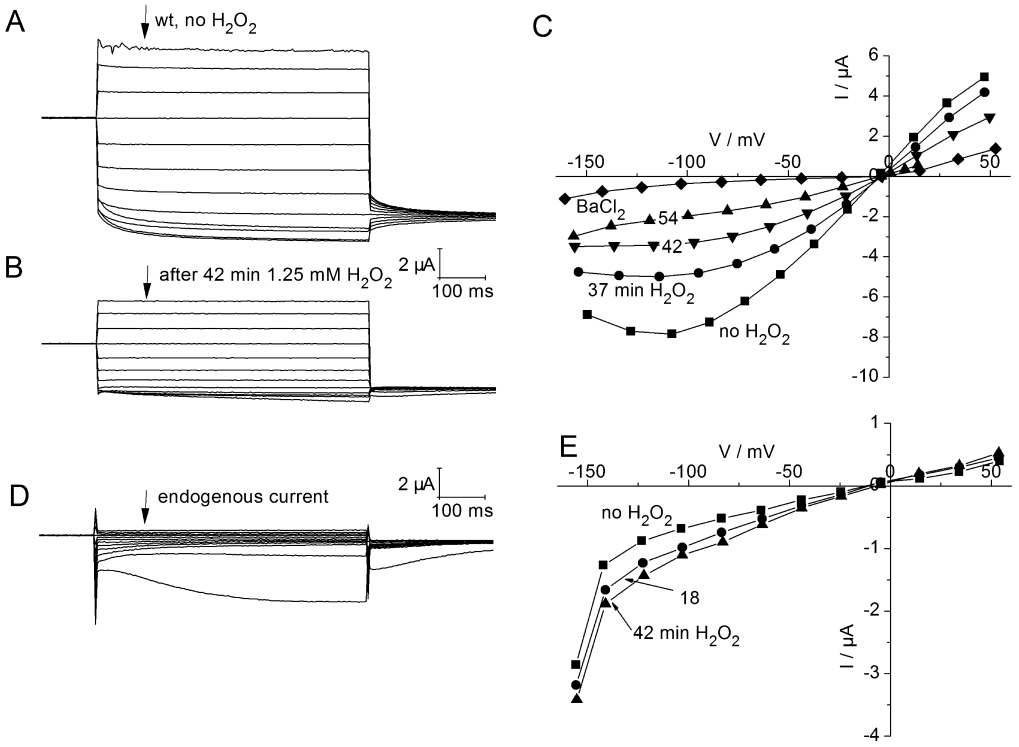

Figure 1. ROS gradually inhibits the wt Kcv_{PBCV-1} current but has no effect on endogenous oocyte currents. Current traces of an oocyte expressing wt Kcv_{PBCV-1} obtained in a TEVC experiment (A) before and (B) 42 min after addition of 1.25 mM H_2O_2. The holding potential was −20 mV; the voltage steps started at 60 mV and decreased, in steps of −20 mV, to −160 mV. Tail currents were recorded at −80 mV. Currents for panel C were measured at the arrows. (C) Steady-state current−voltage relationships ($I-V$ curves) for the same experiment as in panels A and B: (■) control before addition of H_2O_2, (●, ▼ and ▲) currents 37, 42, and 54 min after addition of 1.25 mM H_2O_2, and (◆) currents recorded after addition of 1 mM $BaCl_2$ to the bath medium to block Kcv_{PBCV-1} inward currents.[8,37,38] (D) Current traces from a control oocyte with no RNA injected in the control solution following the same voltage protocol that was used for panels A and B. (E) Effect of H_2O_2 on the $I-V$ curves obtained from the same oocyte as in panel D: (■) control experiment (data taken at the arrows in panel D) and currents (●) 18 min and (▲) 42 min after addition of 2.5 mM H_2O_2.

These considerations motivated us to search for the ROS-sensitive residues in Kcv and for mutants eliminating such a sensitivity. The Kcv_{PBCV-1} channel harbors only six residues, which are potential targets for ROS modification: two cysteines and four methionines (Figure 3 in the Results). Both types of amino acids are known to make ion channels ROS-sensitive. Examples are cysteines in Kv7 channels,[18] in KCNH1,[19] in hERG1 K^+ channels,[20] in CLIC1,[21] in capsaicin receptor TRPV1[22] or in SKOR,[23] and in $I_{K,out}$[24] in plant cells. ROS sensitivity caused by methionine was found in BK channels,[25] *Shaker*,[26,27] HERG channels,[28] or skeletal muscle sodium channel $Na_V1.4$.[29]

Redox-sensitive sites in proteins are informative because oxidizing and reducing conditions can form or disrupt disulfide bridges between pairs of cysteines. In this way, the interaction and distance between individual amino acids can be mapped. In contrast, methionines do not form disulfide bridges. Oxidation leads to the formation of methionine sulfoxide, which has a more rigid and polar side chain than methionine. With strongly oxidizing agents, higher oxidation levels are also possible.[30] The reduction of methionine sulfoxide to methionine requires an enzyme (MsrA, methionine sulfoxide reductase),[30−32] whereas cysteines can easily be reduced by reducing agents like DTT (dithiothreitol).[18,23]

To localize the ROS sensitivity of native Kcv_{PBCV-1}, we studied here the effects of mutating the cysteines and methionines into redox-insensitive amino acids. This led to the identification of M15 in the outer transmembrane domain as the key residue for ROS sensitivity in wt Kcv_{PBCV-1}. The functional properties of the M15L mutant also reveal that this site affects channel gating and hence sheds some light on the still unsettled question of the function of an inner gate in Kcv_{PBCV-1}. Even though a pattern of salt bridges at the intracellular entrance of the channel has been identified as being crucial for the channel's function,[33] a full understanding of the inner gate has not yet been achieved.

■ MATERIALS AND METHODS

Wild-type Kcv_{PBCV-1}[8] and its mutants were expressed in *Xenopus laevis* oocytes. The cDNA was cloned into the pSGEM vector (a modified version of pGEM-HE, provided by M. Hollmann, Max Planck Institute for Experimental Medicine, Göttingen, Germany). We employed the QuikChange method (Stratagene) to create point mutations and confirmed them by sequencing the DNA insert. cRNA was prepared by T7 polymerase transcription. *X. laevis* oocytes were prepared according to standard methods, and 18 or 36 ng of RNA per oocyte was injected. Measurements were performed 2−4 days after injection.

A two-electrode voltage clamp amplifier (Turbo TEC-03X, npi electronic GmbH, Tamm, Germany) was used to record K^+ currents from oocytes with the following protocol: holding potential of −20 mV for 200 ms, steps of voltage ranging from 60 to −160 mV in steps of −20 mV lasting for 600 ms, and tail currents measured at −80 mV for 300 ms. The micropipets were made from filament glass tubes (GB 150-85, Science

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products, Hofheim, Germany) using a PP-830 microelectrode puller (Narishige, Tokyo, Japan) and were filled with 3 M KCl. They had a resistance of 0.4−0.8 MΩ in 100 mM KCl.

The bathing medium in the experiments was a standard bath solution containing 100 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES (pH adjusted to 7.4 with KOH). The redox agents or blockers were taken from a stock solution, and an adequate amount was added to the standard solution to give the concentrations mentioned in the Results. All experiments were conducted at room temperature (18−24 °C).

RESULTS

Wild-Type Kcv_{PBCV-1} Is Inhibited by H_2O_2. Figure 1 illustrates the effects of H_2O_2 on the currents obtained from oocytes expressing Kcv_{PBCV-1}. They are compared with the effects on water-injected and noninjected oocytes. The current traces obtained from oocytes expressing Kcv_{PBCV-1} in the absence of H_2O_2 (Figure 1A) display the familiar behavior of this channel. Voltage steps from the holding potential to negative or positive voltages elicit currents with two phases. The first is an instantaneous current, which is superimposed at negative voltages by a slow activating component. At positive voltages, the deactivation of the latter becomes apparent. Control oocytes not treated with Kcv_{PBCV-1} (Figure 1D) showed a different behavior. The currents were much smaller, and the voltage-induced transients were deactivating at negative potentials. At very hyperpolarizing voltages, a large and slowly activating chloride current[34,35] became evident.

Upon addition of H_2O_2, the Kcv_{PBCV-1}-generated currents decreased gradually (Figure 1B,C). After 42 min in H_2O_2, the activation of the remaining currents (Figure 1B) at negative potential was much slower than in the control; this suggests that the remaining currents are mainly carried by endogenous inward currents (Figure 1D).

Figure 1C presents the $I-V$ curves generated from the current values measured ∼100 ms into the voltage step. This early time point was chosen to reduce the level of interference by the slowly activating chloride currents (Figure 1D). In the relevant voltage window, Kcv_{PBCV-1} is activated with a time constant between ∼3 and 30 ms.[10] Hence, 100 ms is sufficient for Kcv to reach the steady state. The control curve in the absence of H_2O_2 shows a reduction in the current at high negative voltages (negative slope), typical for Kcv.[8,10,36] The $I-V$ curves in the presence of H_2O_2 demonstrate the progressive decrease of the Kcv current after 37, 42, and 54 min. $BaCl_2$ (1 mM) added after a long exposure to H_2O_2 blocked the remaining inward current. Ba^{2+} is a known voltage-dependent blocker of Kcv currents.[8,37−39]

While the Kcv_{PBCV-1} currents gradually decreased in the presence of H_2O_2, the endogenous currents were apparently inert. The exemplary $I-V$ relation measured in a control oocyte not expressing Kcv_{PBCV-1} showed a small gradual current increase, which started with the impalement. An analysis of the progressive increase in the current following impalement revealed that the background conductance increased at a reference voltage of −60 mV exponentially with an amplitude of −0.54 ± 0.31 μA and a time constant of 84 ± 47 min ($N = 6$ oocytes). This spontaneous increase in conductance was not affected by the addition of H_2O_2.

Figure 2A shows the temporal development of the block of the wt Kcv_{PBCV-1} channel with 2 mM H_2O_2 at three different voltages (60, −60, and −160 mV) obtained from an experiment similar to that described in the legend of Figure 1. All three

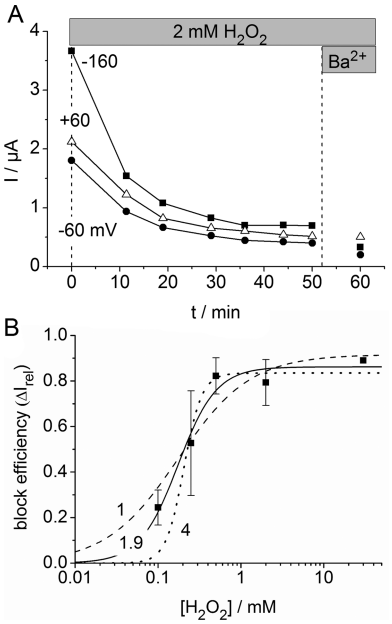

Figure 2. (A) ROS-induced decrease in the absolute values of current in wt Kcv_{PBCV-1} measured at −160 (■), −60 (●), and 60 mV (△). For the sake of comparison, currents at negative potentials are inverted. Time course of the steady-state currents measured after addition of 2 mM H_2O_2. (B) Dose−effect curve of relative current reduction (eq 1) by H_2O_2 in wt Kcv_{PBCV-1} measured at −60 mV at the steady level 30−60 min after addition of H_2O_2. The lines are obtained from the fit with the Hill equation (eq 2) with $n = 1$ (---), $n = 1.9$ (—, best fit), and $n = 4$ (···).

curves in this experiment showed a clear exponential time course. However, in many experiments, the data obtained at −160 mV suffered from the interference of endogenous currents, which increased with time (Figure 1E). To separate the remaining Kcv current in H_2O_2 from the background current, we blocked the remaining Kcv_{PBCV-1} current with Ba^{2+} at the end of the experiment (Figure 2A). The fact that this blocker inhibited the Kcv inward current while barely affecting the outward currents provided an additional means of identifying Kcv_{PBCV-1} currents (60 mV trace in Figure 2A). The time constant of the blocking activity of 2 mM H_2O_2 was obtained from nine different oocytes and resulted in a τ value of 17.7 ± 4.9 min at −60 mV and a τ value of 17.3 ± 7.5 min at 60 mV. The apparent voltage independence seems to be in contrast to the voltage-dependent H_2O_2 block of SKOR in *Arabidopsis*.[23] However, the protocol was different. Here, Kcv was subject to the test voltage for only 600 ms approximately every 3−5 min. In contrast, SKOR was exposed most of the time to the test voltage. However, the difference in voltage dependence was also expected from structural differences: The ROS-sensitive residue C168 in SKOR is on helix S3α, and the exposure of this locus to the agent is controlled by helix S4 of the voltage sensor;[23] this can explain a voltage dependency of H_2O_2 action. Kcv_{PBCV-1} is very weakly voltage-dependent. M15 in Kcv_{PBCV-1} (identified as the ROS sensor in Figure 4) is accessible at all potentials.

From the experiments with wt Kcv, the dose−effect curve of the action of H_2O_2 at −60 mV was generated (Figure 2B). For this purpose, the relative decrease in the Kcv current, ΔI_{rel}, was calculated as follows

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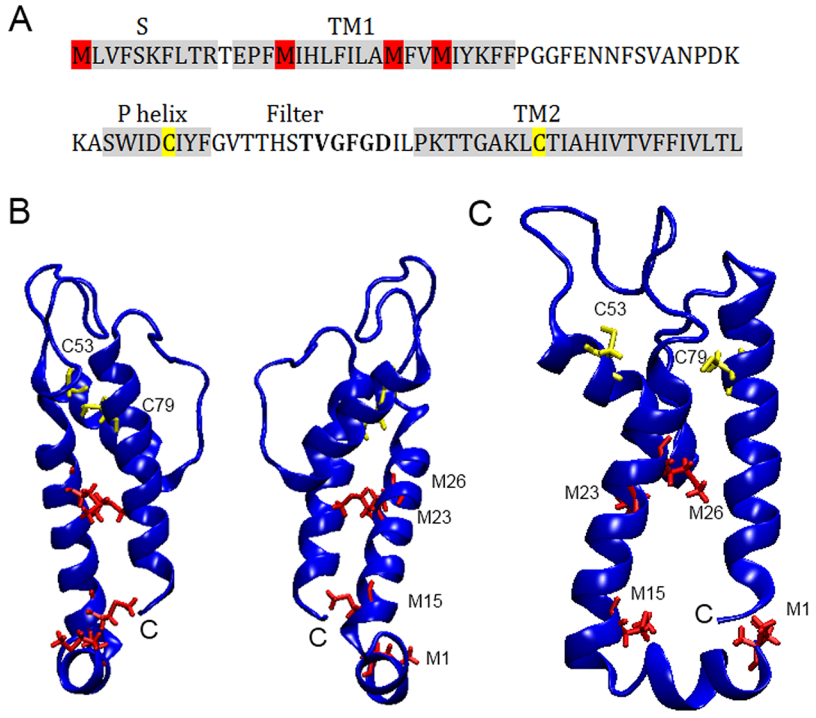

Figure 3. Locations of cysteines C53 and C79 (yellow) and methionines M1, M15, M23, and M26 (red) on Kcv_{PBCV-1}. (A) Amino acid sequence.[8] α-Helices are highlighted in gray (S, slide helix; P helix, pore helix; TM1 and TM2, transmembrane helices). The potassium channel signature sequence is printed in bold (adapted from ref 51). (B) Side view of two opposing monomers. (C) A single monomer, rotated by 90° in relation to that in panel B and slightly tilted backward to improve visibility. The structure used in panels B and C was developed by Tayefeh and co-workers.[41] The Protein Data Bank file was kindly provided by S. Kast (Technical University of Dortmund, Dortmund, Germany).

$$\Delta I_{rel} = \frac{I_0 - I_{min}}{I_0 - I_{Ba}} \quad (1)$$

where I_0 is the current at −60 mV immediately before the addition of H_2O_2 and I_{min} is the lowest current value obtained after the addition of H_2O_2 at the same voltage; this was usually reached after 30−60 min in H_2O_2. I_{Ba} is the remaining endogenous current in the presence of 1 mM $BaCl_2$. The Kcv blocker Ba^{2+} was used to estimate the contribution of the endogenous currents (Figure 2A).

Fitting the dose effect curve in Figure 2B by means of a Hill equation

$$\Delta I_{rel} = \frac{\Delta I_{rel,max}[H_2O_2]^n}{K + [H_2O_2]^n} \quad (2)$$

resulted in a $\Delta I_{rel,max}$ of 0.86 ± 0.05, a half-maximal concentration (K) of 0.17 ± 0.03 mM H_2O_2, and a Hill coefficient (n) of 1.9 ± 0.6. In Figure 2B, the fit with an n of 1.9 is compared with fits with n values of 1 (Michaelis−Menten) and 4 (number of subunits). It is obvious that Michaelis−Menten and n = 4 values do not fit the data. An n value of >1 is related to the fact that the channel is a tetramer. However, the complexity of the theory of the Hill coefficient[40] makes us hesitate to conclude that an n of 2 implies that the cooperativity of exactly two subunits is involved.

Candidate Amino Acids for Conferring ROS Sensitivity. Sensitivity to reactive oxygen species is usually caused by the sulfur-containing amino acids cysteine and methionine.[12,30] The homotetrameric Kcv_{PBCV-1} contains two cysteines and four methionines (including Met1) per monomer. These amino acids are marked on the sequence (Figure 3A) and on a realistic structure[41] of the channel (Figure 3B,C). The cysteines are located on the pore helix (C53) and on inner helix TM2 (C79). With the exception of Met1, all methionines are on outer helix TM1. Here, we mutate all cysteines and methionines with the exception of Met1 and test the mutants for sensitivity to H_2O_2.

Cysteines Are Not the Cause of ROS Sensitivity in Kcv_{PBCV-1}. We first tested whether one of the two cysteines in Kcv is responsible for H_2O_2 sensitivity. We started with the C79A single mutant. The bar in Figure 4 shows that sensitivity

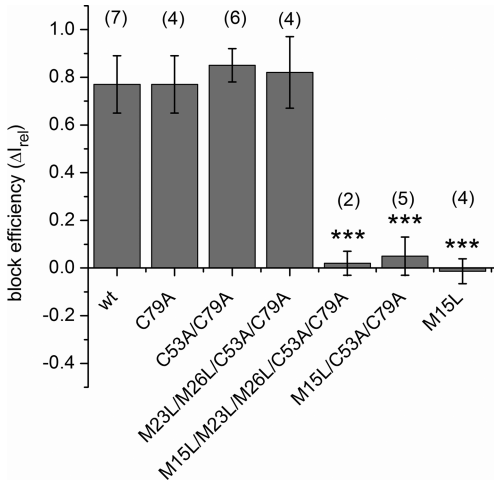

Figure 4. Bar graph presenting the relative changes in current (ΔI_{rel}, eq 1) induced by 2 mM H_2O_2 in different mutants of Kcv_{PBCV-1} and measured at −60 mV. The error bars give the standard deviations. Negative values result from drift. The numbers of experiments are shown in parentheses. Statistical significance was determined by the two-tailed Student's t test (***$p < 0.001$).

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to ROS is not different from that of the wild type. Also, the C53A/C79A double mutant shows no appreciable loss of sensitivity to H_2O_2 (Figures 4 and 5). The results of these experiments imply that the ROS sensitivity is not mediated by the cysteines.

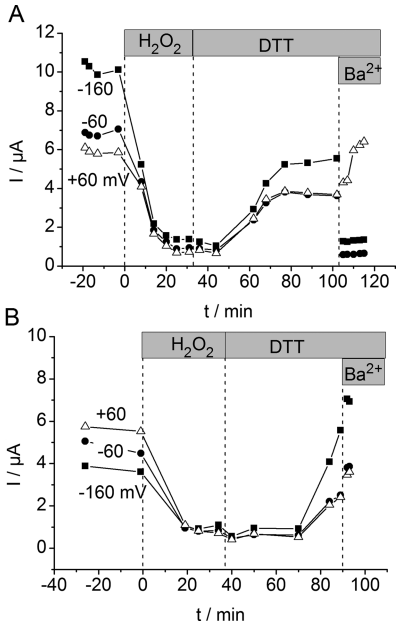

Figure 5. Variability of DTT-induced recovery from inactivation by 2 mM H_2O_2 in the C53A/C79A Kcv mutant. Absolute current values are measured at −160 (■), −60 (●), and 60 mV (△). For the sake of comparison, the sign is inverted for negative potentials as in Figure 2. (A) Current partially recovered after addition of 10 mM DTT. This current is assigned to the C53A/C79A mutant, because it could be blocked by 1 mM Ba^{2+}. (B) In another oocyte from the same batch, the Ba^{2+} test revealed no recovery of the current through the C53A/C79A mutant. The increase in current in DTT is not a recovery but caused by an increase in endogenous currents.

Met15 Is the ROS-Sensitive Site. Among the four methionines in Kcv_{PBCV-1} (Figure 3), Met1 cannot be mutated. The other methionines were replaced with leucine, which preserves the approximate size and hydrophobicity of methionine. As a first methionine mutant, we tested M15L/M23L/M26L on the background of C53A/C79A. The data show that the inhibitory effect of 2 mM H_2O_2 is absent in this mutant (Figure 4). In the next step, we created different mutants to identify the responsible methionine residue. It occurs that the M23L/M26L/C53A/C79A quadruple mutant is ROS-sensitive like the wild-type channel and the cysteine mutants (Figure 4). The result of these experiments draws the attention to M15. Figure 4 shows that all three mutants that include the M15L mutation have lost ROS sensitivity.

Reversibility. The reducing compound DTT has often been used to test the reversibility of ROS-induced effects.[18] This worked fine when cysteines mediated ROS sensitivity,[23] but the picture was less clear in the case of methionines.[26,31] This was also obvious in our results with Kcv_{PBCV-1}. The current generated by the C53A/C79A mutant only partially recovered from the H_2O_2-induced inhibition after addition of 10 mM DTT to the bath medium (Figure 5A). The fact that the recovered current could be blocked by Ba^{2+} proved that this current was indeed conducted by this Kcv_{PBCV-1} mutant.

In Figure 5B, the same experiment was conducted with another oocyte from the same batch and on the same day. There was also a recovery after addition of DTT, but this current was not sensitive to Ba^{2+}. Instead, there must have been a simultaneous increase in endogenous currents, which mimicked a recovery of Kcv_{PBCV-1} current. Two messages resulted from these experiments. (a) The Ba^{2+} test was necessary to separate changes in Kcv_{PBCV-1} current and spontaneous changes in endogenous currents. (b) There was a great variability in the effect of DTT.

This variability of DTT action is known from experiments in *Xenopus* oocytes expressing the ShC/B potassium channels. This has been explained by the facts that the reduction of methionine sulfoxide by DTT[26,42] has to be mediated by the enzyme MsrA (methionine sulfoxide reductase A)[30,32] and the levels of endogenous MsrA are extremely variable in oocytes.[26,31]

Reduction of methionine by MsrA requires a 1.82 Å hydrogen bond between the oxidized methionine and E53 of MsrA.[32] Hence, in a channel protein only Met, which can be accessed by MsrA, can recover from oxidation. In *Shaker*, this holds for M3,[26,31] but not for M440.[27] With these constraints, it may be questioned whether MsrA can reach the oxidized methionine in Kcv. Figure 3 shows that M15 in Kcv is located on the cytosolic side at the edge between TM1 and slide helix. If we look at the van der Waals representation of this structure, M15 is no longer visible from the cytosolic side. However, the mean structure of Figure 3 does not reveal the full flexibility of the proteins, which is particularly high in this region of the protein.[43] Flexibility is crucial for protein−protein interactions[44,45] or ligand binding.[46,47] Whether the high flexibility of the region around M15 and the replacement of the nonpolar methionine with the more hydrophilic methionine sulfoxide are sufficient to allow the docking of MsrA remains to be elucidated. Nevertheless, the variability of the DTT-mediated recovery is a further indication that a methionine is the culprit.

Functional Significance of Met15. The M15L mutation is not only eliminating the ROS sensitivity of Kcv but also altering some of the gating characteristics of the channel. Typical for the wt Kcv channel is some inward rectification with a slow activation at negative voltages and an inactivation at positive voltages. Figure 6 shows that the M15L single mutant and also the M15L/M23L/M26L/C53A/C79A quintuple mutant invert this voltage sensitivity. The mutants exhibit a slow inactivation at negative voltages and activation at positive voltages. The conductance with an inverted voltage dependency is still a Kcv-type current. The same phenotype with an inversion of the voltage dependency has been observed for Kcv orthologs.[48] In previous work, it was also found that a single mutation at position 19 (F19L), e.g., an amino acid, which is on the α-helix facing the same direction as M15, can generate the same switch in voltage dependency.[49] As expected for a Kcv_{PBCV-1} channel, the current is still blocked by Ba^{2+} (Figure 6C).

Important to note is the fact that the negative slopes of the related $I-V$ curves are maintained in the mutants. They are inherent features of the Kcv pore.[8,10,36,37,48,49] The same is true for the aforementioned mutants, which also invert the voltage dependency of the channel. Abenavoli and co-workers[36] have explained the occurrence of the negative slope in terms of the "depletion theory".[50] In the framework of this theory, the negative slope results from averaging over fast gating in the microsecond range as caused by an instability of the selectivity

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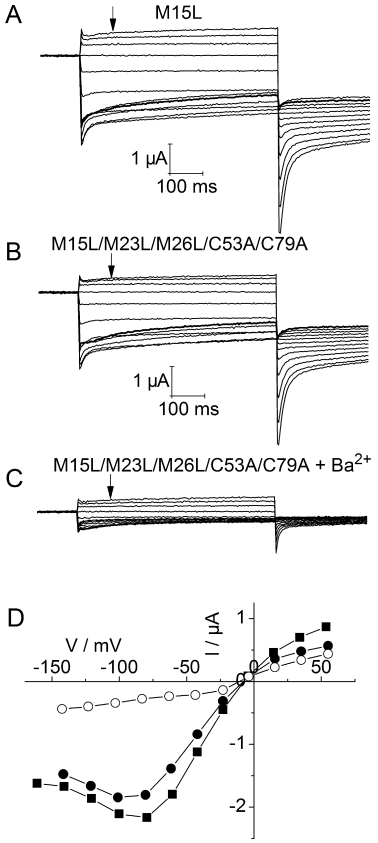

Figure 6. Characteristics of ROS-insensitive mutants. (A) Current traces obtained from the M15L mutant during a TEVC protocol with steps in membrane potential ranging from 60 to −160 mV. (B) The M15L/M23L/M26L/C53A/C79A mutant was measured with the same voltage protocol. (C) The same oocyte as in panel B after addition of 1 mM $BaCl_2$. (D) *I*–*V* curves taken 100 ms into the voltage step (arrows in panels A–C) from M15L (■) and M15L/M23L/M26L/C53A/C79A before (●) and after (○) barium block.

filter. This instability is presumably induced by ion depletion in the filter at high absolute membrane potentials. The fact that this negative slope remains unchanged in M15L (Figure 6D) is an indication that the selectivity filter is not modified by the M15L mutation.

■ DISCUSSION

The data show that the miniature viral K^+ channel Kcv_{PBCV-1} exhibits redox sensitivity in spite of its small size. By systematically screening the mutants of potential redox-sensitive amino acids, we identified a single methionine near the cytosolic end of the outer transmembrane domain as the only site that confers this sensitivity. The channel protein tolerates a mutation of the relevant methionine 15 to leucine, the effect being that the channel is still active but becomes fully inert against H_2O_2.

These data further underscore the relevance of this domain. The oxidation of Met15 in this position causes a closing of the Kcv channel, suggesting that the increased stiffness and/or polar nature of methionine sulfoxide affects the gating of the channel. Furthermore, the exchange of hydrophobic amino acid methionine with a similar hydrophobic amino acid, leucine, in this position has a dramatic effect on the kinetics of the channel. This might imply that the size and geometry of the side chain in this position are more important that its (non-) polarity. A similar inversion of gating in the opposite direction has been observed with a Kcv channel from virus MA-1D in response to the V19F mutation.[49] Further experiments will show how this part of the channel interferes with the inner gate of Kcv_{PBCV-1}. Kcv_{PBCV-1} gating relies on salt bridges that are formed between the COOH group of the terminal residue (L94) and positive residues (K6 and R10) on the slide helix. M15 is located at the beginning of TM1 (Figure 3). Mutations in the first half of TM1, which is the flexible part of this TM domain, frequently result in altered kinetics and/or gating[51,52] because TM1 is connected to the slide helix, and the slide helix plays a direct role in Kcv_{PBCV-1} gating.

These data show that the M23L and M26L mutations have no influence on whole-cell Kcv_{PBCV-1} current under oxidizing conditions. Hence, the two Met residues are not accessible to the oxidants (unlikely for H_2O_2), or their modification has no structural relevance with respect to Kcv_{PBCV-1} function.

It was a surprise to find that none of the two cysteines in Kcv_{PBCV-1} exhibited any ROS sensitivity even though cysteines are the most frequent targets in ROS-mediated control of channel activity. When two cysteines are separated by a few amino acids, disulfide bridges can be formed under oxidizing conditions, which in turn modify the fold of a protein. In the case of the TRP5 receptors, for example, two cysteines on the turret can form disulfide bridges when the external reducing protein TRX approaches the channel and thus opens it.[53] In Kcv_{PBCV-1}, the smallest distance between cysteines is 12 Å, namely between the Cα atoms of C79 and C53 on the same subunit. The shortest distances between the Cα atoms of cysteines on different subunits are those between neighboring C53 and C79, which are 15–17.5 Å apart. The optimal distances between cysteines for disulfide bridges are 2.9–4.6 Å for Cβ[54] and 8 Å for Cα. By backbone movement, this can be extended to a distance constraint of 13 Å.[55] The rigidity of the Kcv channel in the respective parts of the protein does not imply large backbone movements.[43] Still, intrasubunit formation of disulfide bridges between C53 and C79 cannot be fully excluded. However, if disulfide bonds were formed by ROS, it has to be concluded that they do not influence the function of the Kcv channel. This resembles the situation found in SKOR of *Arabidopsis*, where mutations of the cysteines on the S5 pore helix, C228 and C234, had no significant effect on the sensitivity to H_2O_2.[23]

In conclusion, we have created a redox-insensitive Kcv_{PBCV-1} channel. The redox-insensitive M15L mutant can now be used as a launching pad for the engineering of biosensors, which do not have any unwanted inherent ROS sensitivity. Furthermore, the ROS-insensitive mutant provides a good platform for a systematic cysteine scanning mutagenesis. This method offers detailed information about the relative position of amino acids in a functional protein as mentioned in the introductory section.

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Notes

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■ ABBREVIATIONS

DTT, dithiothreitol; *I*−*V* curve, current−voltage relationship; MsrA, methionine sulfoxide reductase A; ROS, reactive oxygen species; wt, wild type.

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