Tetraphenylporphyrin derivative specifically blocks members of the voltage-gated potassium channel subfamily Kv1

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Tetraphenylporphyrin derivatives represent a promising class of high-affinity ligands for voltage-gated potassium (Kv) channels. Herein, we investigated the mode of Kv channel block of one tetraphenylporphyrin derivative, por3, using electrophysiological methods, structure-based mutagenesis, and solid-state NMR spectroscopy. The combined data showed that por3 specifically blocks Kv1.x channels. Unexpectedly, 2 different por3 binding modes lead to Kv1.x channel block exerted through multiple por3 binding sites: first, por3 interacts in a highly cooperative and specific manner with the voltage sensor domain stabilizing closed Kv1 channel state(s). Therefore, stronger depolarization is needed to activate Kv1.x channels in the presence of por3. Second, por3 bind to a single site at the external pore entrance to block the ion conduction pathway of activated Kv1.x channels. This block is voltage-independent. Por3 appears to have equal affinities for voltage-sensor and pore. However, at negative voltage and low por3 concentration, por3 gating modifier properties prevail due to the high cooperativity of binding. By contrast, at positive voltages, when Kv1.x channels are fully activated, por3 pore blocking properties predominate.

Introduction

Voltage-gated potassium (Kv) channels constitute promising targets for the pharmaceutical industry to treat a large variety of diseases ranging from neurological, autoimmunological, cardiac, and diabetic disorders to cancer.1-3 However, the development of subtype-specific Kv channel drugs also presents a challenge to drug discovery because Kv channel antagonists and agonists often have undesirable side effects and lack specificity. A major challenge is to avoid blocking the cardiac hERG channel, which features a promiscuous drug binding pocket. HERG channel block potentially causes a devastating drug-induced cardiac arrhythmia. The characterization and investigation of venom peptides, specific antibodies and small molecules as potential antagonists of Kv channels has shown that the compounds mainly follow 2 types of targeting mechanism. The first mechanism involves a direct occlusion of the Kv channel pore by binding either to its outer or inner entrance. Alternatively, the drug may target a binding pocket near or at the conduction pathway to prevent the flow of ions through the Kv channel pore.4-6 The second mechanism leads to a modification of the Kv channel gating properties by interfering with voltage-sensor movements early or late in the activation pathway of the Kv channel. The drug effect on voltage-sensor movement may involve a direct targeting of the voltage-sensor or an indirect effect, due to an alteration of the lipid surrounding.7-10

Tetraphenylporphyrin derivatives have been developed as a new class of synthetic ligands for potassium channels.11 These water-soluble ligands have 4-fold symmetry and were designed to interact simultaneously with all 4 subunits of the tetrameric Kv channel to mimic the action of toxins that target the extracellular entrance of the Kv channel pore. Indeed, competitive binding assays with a pore-blocking radiolabelled scorpion toxin12 showed that tetraphenylporphyrin derivatives bind with nanomolar affinities to the Kv1.3 channel, which represents an interesting target for immunomodulation.2 The data suggested that tetraphenylporphyrin derivatives occlude the outer entrance of the Kv1.3 pore.

Solid-state NMR spectroscopy of a complex between tetraphenylporphyrin compound 3 (por3) and the bacterial potassium channel KcsA containing the external pore loop of the eukaryotic Kv1.3 channel (KcsA-Kv1.3) revealed that por3 did not interact simultaneously with all 4 subunits of the KcsA-Kv1.3 channel.12,13 Contrary to this expectation, the structural data indicated that por3 inserted one of its positively charged side chains into the K+ channel pore.12 Hence, por3 was not situated...
flat on the external surface of the channel pore, but apparently bound in a perpendicular fashion. Furthermore, it is worth noting that application of por3 to the Kv1.3 related Shaker channel did not completely block ionic current even at high concentration. The data seemed to contradict the observation that por3 binds to the Kv channel pore with high affinity.

In this study, we investigated these apparent inconsistencies in more detail and electrophysiologically characterized specificity and binding of por3 to Kv channels. The results showed that por3 specifically blocks members of the Kv1.x channel subfamily and preferentially binds to the closed Kv1 channel in a highly cooperative manner. Por3 block involves 2 distinct binding modes. One binding mode interferes with ion flow through the Kv channel pore, the other binding mode of por3 targets voltage-sensor movement. Thus, por3 represents an interesting template for developing either novel specific Kv1 channel gating modifiers or pore blockers.

**Results**

**Por3 specifically blocks members of the Kv1 subfamily**

To study the effect of por3 on Kv channels we used the *Xenopus laevis* oocyte expression system, which is convenient for expressing members of the Kv channel superfamily. However, it has the limitation that high concentrations of por3 (> 1 µM) in the bathing solution make the oocyte membrane leaky. Therefore, we limited our investigations to por3 concentrations of ≤ 1 µM.

First, we screened the sensitivity of members of the Kv1.x family (Kv1.1–Kv1.6) to por3 and recorded current amplitudes and voltage dependence of Kv1.1 to Kv1.6 channel activation in the absence and presence of 0.5 µM por3 (Fig. 1A–F). We made 2 observations in the data. First, Kv1.x channels displayed very distinct por3 sensitivities, and second, the por3 block was voltage sensitive, being more effective at negative than at positive voltages. Among the Kv1.x channels, Kv1.1 and Kv1.6 channels were the most sensitive ones since they were completely blocked at almost all test voltages. Kv1.4 and Kv1.5 channels, both of which have a positively charged amino acid side chain at their pore entrance, were the least sensitive. Next, we tested the influence of por3 on representative members of other Kv channel subfamilies, e.g., Kv2.1, Kv4.1, Kv7.1, and Kv11.1 (hERG). Note these Kv channels are expressed in cardiac cells. None of these channels was blocked to any significant extent (Fig. 1G; Fig. S1). Although we did not test every possible member of the Kv channel superfamily, we conclude that por3 specifically inhibits the activity of Kv1.x channels, in particular Kv1.1, Kv1.2, Kv1.3, and Kv1.6.

**Por3 interacts with Kv1.x channels in a highly cooperative manner**

Previous por3 data indicated that por3 binds to potassium channels from the outside and competes with scorpion toxins for a common or overlapping binding site. Plotting the fractional inhibition of Kv1.1 and Kv1.3 current, measured at +80 mV where Kv channels are fully activated, against por3 concentration, however, showed a very steep concentration dependence that was difficult to reconcile with a simple pore block with 1 binding site (Fig. 1H). By contrast, por3 concentration dependence of Kv1 block was quite well described by a Hill-equation assuming multiple cooperative por3 binding sites. For the por3 block of the Kv1.1 channel we obtained a Hill coefficient of 3.8 and an IC₅₀ of 0.15 µM, and for the 1 Kv1.3 channel a Hill coefficient of 2.2 and an IC₅₀ of 0.24 µM (Fig. 1H). In conclusion, the data indicated that por3 block of Kv1.1 and Kv1.3 channels is highly cooperative and should involve multiple binding sites.

![Figure 1. Specific inhibition of Kv1.x channels by por3.](image-url)
This conclusion was supported by investigating the kinetics of por3 current block and unblock. Application of 0.5 µM por3 to the bathing solution of outside-out patches caused a nearly complete (> 85%) and reversible Kv1.3 channel block at a test potential of −20 mV (Fig. 2A and B). The time course of por3 block was relatively slow and could be well described with 1 time constant \( \tau_{\text{on,por3}} = 13.5 \pm 0.8 \text{ s} \) (n = 4). Por3 washout, however, first showed a rapid phase of recovery, reaching approximately 70% of the original Kv1.3 current amplitude, followed by a very slow recovery phase (Fig. 2B). Hence, the time course of por3 washout at −20 mV was well described with 2 time constants, \( \tau_{\text{off,1}} = 25.8 \pm 4.5 \text{ s} \) and \( \tau_{\text{off,2}} = 295 \pm 16.5 \text{ s} \) (n = 4). The second \( \tau_{\text{off}} \) time constants differ by 1 order of magnitude and likely reflect por3 unbinding at 2 different Kv1 channel binding sites.

When we applied por3 to whole Xenopus laevis oocytes and followed the time course of Kv1.3 current inhibition and washout in 2-voltage electrode recordings at test potentials of −20 and +60 mV, we obtained comparable data (Fig. S2). Significantly, the kinetics of the por3 washout at −20 mV showed a delay (approximately 60 s) in the onset of recovery from inhibition (Fig. S2). The delay probably reflects a high degree of cooperativity in por3 binding. Importantly, after onset of recovery, biphasic washout kinetics were identical to those recorded at −20 mV. The combined binding data were difficult to reconcile with previous biochemical and structural data, which suggested a single por3-binding site at or near the K+ channel pore. A likely possibility to resolve the discrepancies between the data was the hypothesis that por3 has 2 binding modes. One mode targets the extracellular entrance to the pore as we previously suggested; the other mode may target the voltage sensors implying that por3 has gating modifier properties.

Por3 modifies Kv1.3 channel gating

First, we investigated how por3 affected Kv1.3 channel activation and deactivation. Under standard conditions the kinetics of activation and deactivation are too fast for a reliable analysis. Therefore, we slowed the kinetics by lowering the bath temperature to 16 °C and by replacing K+ with Rb+. The respective Kv1.3 current records are shown in Figure S3, scaled records in Figure 2C. Por3 significantly slowed the time rise to peak current in comparison to control experiments (at +60 mV 135 ± 16 ms vs. 65 ± 16 ms; n = 5) and altered the sigmoidicity of the Kv1.3 activation time course (Fig. 2C). In the tail current records, we observed that the continuous presence of por3 markedly accelerated the deactivation time course (\( \tau_{\text{deact}} = 59.2 \pm 9.8 \text{ ms} \) vs. \( \tau_{\text{deact}} = 37.4 \pm 2.3 \text{ ms} \) n = 5). It is worth noting that tail current kinetics reached a saturating value as por3 concentration.
The voltage-dependence of Kv1.3 channel block by por3 was further demonstrated when we measured Kv1.3 tail current-voltage relations in the presence of increasing por3 concentrations. Whereas at more depolarized test potentials, 0.5 µM por3 blocked approximately 70% of activated Kv1.3 channels (Fig. S5), 0.5 µM por3 completely blocked the Kv1.3 channel at test potentials of \( \geq -20 \text{ mV} \). In contrast to the por3 blocking data at +80 mV (Fig. 1H), the steep por3 concentration dependence of Kv1.3 block was very well described by a Hill-equation with an IC\(_{50}\) of 0.13 µM and a Hill coefficient of 3.9 at \(-20 \text{ mV}\) (Fig. 2F). The data indicated that por3 binding to the Kv1.3-channel is voltage-dependent, i.e., sensitive to the Kv1.3 channel state.

**Porphyрин-3 preferentially binds to the closed Kv1.3 channel**

Information about dependence of por3 block on Kv1.3 channel state is obtained by determining the ratio of Kv1.3 current amplitudes between onset and end of a depolarizing test pulse recorded in absence (\( I_{\text{u,control}} \)) and presence (\( I_{\text{u,por3}} \)) of por3 (Fig. 3B). Plots of the ratio \( I_{\text{u,control}}/I_{\text{u,por3}} \) vs. test-pulse time at voltages between 0 and +80 mV are shown in Figure 3C. At test pulse onset, the closed Kv1.3 channel was almost completely blocked showing a very low probability of unblock (\( U_{\text{C}} \)) (\( U_{\text{C}} = 0.06 \pm 0.01; n = 5 \)). The probability of opened unblocked channels (\( U_{\text{O}} \)) increased with increasing test pulse duration to values of 0.40 ± 0.04 (\( n = 5 \)) at 0 mV and 0.62 ± 0.05 (\( n = 5 \)) at +80 mV. The increase of \( I_{\text{u,control}}/I_{\text{u,por3}} \) over time was well described with 2 time constants \( \tau_{1,u} \) and \( \tau_{2,u} \) (Fig. 3C). They were sensitive to changes in membrane voltage and decreased exponentially (approximately 4-fold) between 0 and +80 mV (Fig. 3D). The data indicates that por3 blocks closed Kv1.3 channels with a higher affinity than opened Kv1.3 channels.

The \( \tau_{1,u} \) and \( \tau_{2,u} \) voltage relations provide information about the electrical distance through the plasma membrane \( \delta \), which por3 transverses to its binding site(s) on the Kv1.3 channel. To obtain this information, we used Equation 1, which resembles the Woodhull-equation describing voltage-dependence of pore block by channel inhibitor:

\[
\tau_{u}(V) = \tau_{u}(V_0) e^{-z\delta V/RT} \quad (\text{Eqn. 1}).
\]

\( \tau_{u}(V) \) is the time constant at a given test voltage, \( \tau_{u}(V_0) \) the time constant at 0 mV, \( z \) the charge of por3 (presumably +4), \( V \) the test voltage, \( F \), \( R \), and \( T \) have their usual meaning. The slope of the \( \tau_{u} \)-voltage relation in Figure 3D then corresponds to \( z\delta F/RT \). With \( z = 4 \) one obtains a value of \( \delta = 0.26 \) for both time constants demonstrating that por3 enters a significant distance into the electric field.

**Porphyрин effects gating charge movement**

Our observation that por3 has multiple binding sites, slows activation, accelerates deactivation and preferentially blocks the

\[\text{Figure 3. Dependence of por3 block on Kv channel state. (A) Exemplary Kv1.3 current traces (I_{\text{u,control}}) elicited from a holding potential of -80 mV to test potentials of } \geq 0 \text{ mV as indicated. Dotted line is base line recorded at -80 mV. Bars indicate current amplitude and test pulse duration. (B) Exemplary current traces as in (A), but recorded in the presence of 0.2 µM por3 (I_{\text{u,por3}}). (C) Plot of I_{\text{u,control}}/I_{\text{u,por3}} against test pulse duration to calculate } \tau_{u} \text{ at different test potentials. Dashed line indicates base line. (D) Plot of } \tau_{u} \text{ against voltage of test pulse. For further details see Results and Equation 1.} \]
closed channel suggested that por3 may bind to each Kv1.3 subunit and interfere with Kv1.3 gating charge movement. To investigate a gating modifying effect of por3, we mutated Kv1.3 Val 430 to Trp by analogy to the Shaker channel mutant V478W20,24 expecting that the mutation diminishes ionic Kv1.3 currents and, thereby, facilitates measurement of gating currents.21,22 Indeed, the mutant Kv1.3/V430W channel no longer mediated measurable ionic current. Under control conditions, depolarizing test potentials elicited Kv1.3 V430W on-gating currents (\(q_{on}\)) and, respectively, off-gating currents (\(q_{off}\)) after repolarization to the holding potential. Raw gating current traces are displayed in Figure 4A and Figure S6. The amount of charge (Q) displaced during a test pulse was obtained by integration of \(q_{on}\) and \(q_{off}\) over time. Since \(Q_{off}\) and \(Q_{on}\) were similar, movement of gating charges across the electric field was apparently reversible. The \(Q_{off} - V\) data were well described with a single Boltzmann function. Typically, the Kv1.3 V430W \(Q_{off} - V\) relation (\(V_{1/2, Qoff} = -22.1 \pm 1.1\) mV; \(n = 4\)) (Figure 4B) was shifted by approximately 14 mV to more negative potentials in comparison to the Kv1.3 G – V relation (\(V_{1/2} = -8.3 \pm 0.9\) mV; \(n = 5\)).

We observed 2 effects of por3 on Kv1.3 V430W gating currents. First, por3 shifted the Kv1.3 \(Q_{off} - V\) curve by about 12 mV to more depolarized potentials (\(V_{Qoff,1/2} = -10 \pm 0.5\); \(n = 4\)) (Figure 4B). This shift likely reflects a por3 effect on the energetics of the Kv1.3 voltage-sensor movement. Second, por3 block of Kv1.3 V430W gating charge movement was strongly voltage-dependent. At a test potential of –30 mV, 0.5 µM por3 blocked total gating charge transfer almost completely, at –20 mV by approximately 40–60%, and at 0 mV only by approximately 20% (Fig. 4B and C). The kinetics of the reversible block of Kv1.3 gating-charge movement (\(Q_{off}\)) was well described by one time constant for block (\(\tau_{on, Qoff} = 19.2 \pm 2.0\) s; \(n = 3\)) and one for unblock (\(\tau_{off, Qoff} = 24.6 \pm 3.7\) s; \(n = 3\)), respectively (Fig. 4C). Note both time constants were very similar, if not identical to those measured at a test potential of –20 mV for onset and recovery of Kv1.3 current inhibition by por3. This suggests that block of gating charge movement causes the observed por3 block of Kv1.3 current at negative potentials (≤ –20 mV).

Next, we investigated the por3 concentration dependence of \(Q_{off}\) block at –40 mV, where the Kv1.3 channel is closed (Fig. 4D). In order to provide a rational for the data, we argued that each of the 4 Kv1.3 subunits has an equivalent and independent binding site for por3. Since por3 interaction with the Kv1.3 channel was highly cooperative, it is likely that all por3 binding sites need to be occupied to achieve inhibition. Consistent with this view was also that onset of recovery of \(Q_{off}\) from por3 block started at –20 mV without significant delay (Fig. 4C). With these assumptions, the concentration dependence for fractional occupancy of the Kv1.3 V430W channel by por3 is then given by Equation 2, where \(\rho_4\) is the probability that all 4 por3 binding sites on the channel are occupied.

\[
\rho_4 \approx 1 - \left( \frac{\text{por3}}{\text{por3} + K_D} \right)^4 \quad \text{(Eqn. 2)}
\]

The por3 blocking data matched Equation 2 with an equilibrium dissociation constant (\(K_D\)) of 45 nM for each of...
the 4 cooperative porphyrin-binding sites (Fig. 4D, solid line). By contrast, an equation that is based on independent, non-cooperative binding site(s) for por3 significantly deviated from the data points (Fig. 4D, dashed line). The result suggests that por3 block of gating charge movement is effective when all 4 por3 binding sites are occupied. In summary, the data indicated that por3 preferentially interacts with the closed Kv1.3 channel at negative voltages and blocks gating charge movement.

Porphyrin blocks Kv1.3 channel pore

Since previous studies indicated por3 binding to the K+ channel pore,11,12 we hypothesized that por3, in addition to its effect on gating charge movement, acts as a pore blocker. To test this hypothesis, we generated a constitutively open Kv1.3 channel, where gate opening and voltage-sensor movement are uncoupled. Thus, we mutated residue Pro427 in the Kv1.3 pore domain to Asp (Kv1.3 P427D). The choice of this mutation originated from a report that the analogous mutation renders the Shaker channel constitutively open.23 Indeed, Kv1.3 P427D channels showed a comparable voltage-independent gating behavior (Fig. 5A). Kv1.3P427D currents recorded under symmetrical K+ conditions exhibited characteristics typically seen with inwardly rectifying K channels (Kir) that are devoid of a voltage-sensor (Fig. 5B). Application of 0.5µM por3 reduced the Kv1.3 current amplitude to approximately 50% in a voltage-independent manner (Fig. 5C). Por3 block of Kv1.3P427D was completely reversible (Fig. 5D). The time course of por3 block was well described with 1 time constant $\tau_{on} = 31.4 \pm 1.8$ s ($n = 4$), which was of similar magnitude to those obtained for por3 block of Kv1.3 gating ($Q_{off}$) and Kv1.3 ionic current, respectively (see Figs. 2B and 4C). Also, the time constant $\tau_{off} = 285 \pm 18.5$ s ($n = 4$) for washout of por3 was of similar magnitude as $\tau_{2off} = 295 \pm 16.5$ (n = 4), which described the slow phase of Kv1.3 current recovery from por3 block (Fig. 2B).

To further support the conclusion that por3 blocks Kv channel pores, we asked whether a pore mutation in the Kv1.5 channel, which is relatively por3 insensitive, could increase por3 sensitivity of the Kv1.5 channel. A positively charged amino acid chain near the extracellular pore entrance, like Kv1.5 Arg 487, hinders binding of pore

Figure 5. Por3 blocks constitutively opened Kv1.3P427D channel. (A) Exemplary Kv1.3 P427D currents elicited by depolarization to voltages between –80 mV and +80 mV. Bath solution contained 100 mM K+. Holding potential was at 0 mV. Control currents are in black, currents in presence of 0.5 µM por3 are shown in red. (B) Current-voltage relation of Kv1.3 P427D currents (n = 5) in absence (black dots) and presence of 0.5 µM por3 (red dots) as shown in (A). (C) Bar diagram of fractional inhibition of Kv1.3 P427D current obtained by 0.5 µM por3 application at different test voltages (n = 5). (D) Exemplary wash-in and washout kinetics of por3 block of Kv1.3 P427D. Red bar indicates duration of 0.5 µM por3 application by bath perfusion. Kv1.3 P427D current (open circles) was recorded every 20 s at +80 mV. Kinetics of por3 block were fit with $\tau_{on} = 31.3$ s and those of por3 unblock with $\tau_{off} = 285.0$ s (dashed curve). (E) Bar diagram of fractional inhibition of Kv1.5 and Kv1.5R487Y current in comparison to Kv1.1 and Kv1.3 block by por3 (n for each bar = 5). (F) Dependency of Kv1.1 (squares) and Kv1.3 channel block (circles) on por3 concentration. Fractional inhibition data were obtained measuring tail current amplitudes as in Figure 1. An equation assuming cooperative binding sites ($K_a$) and an additional independent single binding site ($K_s$) was fit to the data (solid curves) with a Hill coefficient of 4.5 and $K_a = 0.16$ µM and $K_s = 0.20$ µM in the case of Kv1.1 and with a Hill coefficient of 4.5 and $K_a = 0.18$ µM and $K_s = 0.23$ µM in the case of Kv1.3.
blocks to Kv1 channels, e.g., that of TEA and scorpion toxins.\textsuperscript{24} Hence, we replaced Arg 487 in Kv1.5 by tyrosine (Kv1.5R487Y), which occurs in an analogous location at the por3 sensitive Kv1.1 pore entrance. Por3 sensitivity of Kv1.5R487Y channels was investigated at +80 mV, where the channels are fully activated. The results clearly showed that Kv1.5R487Y channels are as por3 sensitive as Kv1.1 or Kv1.3 (Fig. 5E). Combining all binding data, we came to the conclusion that por3 has 2 binding modes. One binding mode interferes in a cooperative manner with gating charge movement and the other interferes with ion conduction. Both binding modes can be described by an equation, which combines a Hill equation for the cooperative binding to the Kv1.x voltage sensor ($K_{a}$) and one for por3 binding to an independent single site at the Kv1.x pore entrance ($K_{b}$). This equation well describes the puzzling por3 concentration dependence of Kv1.1 and Kv1.3 current block (Fig. 5F), which we measured at depolarized potentials (Fig. 1H). In conclusion, por3 binds to the closed Kv1.x channel and interferes with gating charge movement at low concentrations, whereas at higher concentrations por3 blocks the pore of the channel.

**Discussion**

Our data reveals that porphyrin derivatives like por3 are very specific inhibitors of voltage-gated Kv1.x channels and, therefore, may be useful tools for developing specific Kv1 channel antagonists in the future. The mode of action of por3, however, is quite complex. Por3 preferentially interacts with the closed Kv channel combining gating modifier and, in agreement with earlier data,\textsuperscript{11,12} pore blocking properties.

Previously, MacKinnon, Swartz and collaborators investigated how Hanatoxin (HaTx), a protein toxin from tarantula venom, modifies gating of the Kv2.1 channel.\textsuperscript{25} The HaTx data indicated that the toxin binds tightly at four equivalent sites, which correspond to the resting conformation of the voltage-sensor paddle in the Kv2.1 channel. Thereby, HaTx alters the energetics of Kv2.1 channel gating. Our por3 binding data shares many characteristics with those of HaTx, but also exhibits important differences. Both, HaTx and por3, alter the energetics of Kv channel gating. However, HaTx specifically interacts with Kv2 channels, whereas por3 preferentially binds to Kv1.x channels. Both, HaTx and por3 accelerate deactivation, and tail current decay kinetics reaches a saturating value as HaTx and por3 concentrations are increased. The results suggest that both HaTx and por3 stay bound to the Kv channel, while the channel is opened by depolarization. However, HaTx accelerates Kv2.1 channel deactivation much stronger than por3 Kv1.3 channel deactivation. The HaTx data fit to a model with 4 equivalent and independent HaTx binding sites on the Kv2.1 channel. The model implies that occupancy of one binding site already suffices to inhibit channel activation. Por3 also seems to have multiple binding sites, but the concentration dependence of fractional occupancy of the Kv1.3 channel by por3 suggests a different scenario. Por3 binding is highly cooperative and apparently inhibits channel activation only when all binding sites are occupied.

Both HaTx and por3 preferentially bind to the closed Kv channel and interfere with the movement of gating charge during activation. HaTx shifts the Q–V relation by about 40 mV, por3 by only 12 mV. Significantly, the apparent inhibition of gating charge movement by HaTx is overcome by strong depolarization and the maximal value of gating charge moved appears to be unaffected by HaTx.\textsuperscript{26} By contrast, por3 inhibition of gating charge movement is incompletely overcome by strong depolarization. Thus, an inhibition of gating charge movement of about 20% remains suggesting that the voltage sensors cannot undergo their full range of movement in the presence of por3. It is presently unclear if por3 interferes with a final concerted opening transition of the Kv channel, which accounts for approximately 10–20% of the total gating charge.\textsuperscript{17,26} This mode of action is consistent with the observed high cooperativity of por3 block and the complete inhibition of Kv1 channel activity that we observed at negative potentials. Note, however, distinct voltage-dependencies of por3 block for gating and ionic current, respectively. Also, we cannot exclude that por3 binds to the voltage-sensor in the ‘up’-position and thereby affects the final opening transition of the channel.
Tetraphenylporphyrin derivatives like por3 are amphiphilic in nature. Thus, the positively charged por3 may dip into the lipid bilayer and interact with negatively charged phospholipid head groups and thereby alter the surface charge of the external side of the plasma membrane. Note Kv1.3 channels expressed in the inside-out configuration of membrane patches are insensitive to por3 bath application (Fig. S7). The specificity of the por3 Kv channel block is difficult to reconcile with a general effect of por3 on surface charge. Nevertheless, we considered surface charge as a possibility to explain por3 block of gating charge movement. Hence, we prepared partially deuterated DMPC liposomes in absence and presence of por3 and then measured 2-dimensional $^1$H-$^1$H correlation spectra using solid-state NMR spectroscopy (Fig. 6A–C). The spectral data indicated cross correlations between por3 and methylene moieties of the lipid headgroup, in particular for those near both sides of the phosphate moiety (Fig. 6C, dashed boxes). Cross relaxation rates were deduced from NOESY experiments$^{27}$ for the protons of the lipid headgroup to the phenyl and amine protons of por3, which exchanged with water in the sample (Fig. 6D). In line with additional $^{31}$P 1D MAS spectra that indicate chemical shift changes in the $^{31}$P phosphate group upon addition of por3 (data not shown), the NMR data suggest an orientation of por3 with respect to the lipid molecules that is schematically illustrated in Figure 6E. The model proposes that por3 binds perpendicularly to the lipid bilayer and interacts with lipid head groups. It is likely that the interaction distorts the lipid bilayer. The notion is consistent with our observation that higher por3 concentrations produced leaky Xenopus laevis oocytes.

A close contact between negatively charged phospholipid headgroups and positively charged amino-acid side chains of the voltage sensors has been seen in the Kv1.2/Kv2.1 chimera crystal structure$^{28}$ and in molecular dynamics studies.$^{29}$ Negatively charged phospholipid head groups may serve as counter charges for the positively charged amino acid side chains of the voltage sensor.$^{30}$ Hence, binding of the positively charged por3 to phospholipid head groups potentially affects this interaction and thus affects voltage-dependence of Kv channels. Alternatively, negatively charged amphiphiles, for example, free polyunsaturated fatty acids (PUFAs) can potentially bind to the voltage sensor, modulating the voltage-dependence of Kv channel activation.$^{10,31}$ Furthermore, binding of por3 to phospholipid may be attenuated at positive potentials because of its positive charges. This property could well explain the voltage-dependence of Kv channel block by por3.

Por3 binds to both the voltage sensor and the external pore entrance of Kv1.x channels with comparable affinity ($K_a$ ~ 200 nM; see Fig. 5F). However, because of the high cooperativity in binding, the effect of por3 on gating charge movement saturates at lower concentrations than the occlusion of the conduction pathway, which implicates a single binding site. In fact, we were unable to reach a sufficiently high por3 concentration for complete pore block due to leakage problems occurring at por3 concentrations > 0.5 µM. Kv1.4 and Kv1.5 channels, which possess a positively charged amino acid (Lys 532 for Kv1.4, Arg 487 for Kv1.5) near the pore entrance, were less sensitive to por3 than Kv1.1, 1.2, 1.3, and 1.6. Replacing Arg487 in Kv1.5 by tyrosine (Kv1.5R487Y) significantly increased por3 sensitivity to that of Kv1.1. The combined data are consistent with the previous observation, that por3 blocks the K+ channel pore. Thus, por3 block of Kv1.x channels is associated with t2 modes of por3 action. One mode interferes with ion conduction and the other with gating charge movement. Apparently, Kv1.x channels are sensitive to both modes of por3 action, except Kv1.4 and Kv1.5 channels, where por3 may mainly affect voltage sensor movement.

During the preparation of this manuscript, Marzian et al. (ref. 32) reported on the subtype specific inhibition of Kv1.x channels by Psora-4, a cationophoric molecule. Comparison of the Psora-4 and por3 data are very informative as it illustrates 2 alternative modes of specific Kv1-channel inhibition by small molecules. Comparably to por3, Psora-4 inhibition of the Kv1.5 channel involves multiple binding sites and is highly cooperative, displaying a Hill coefficient of 2.81 ± 0.61. However, the mechanism of Psora-4 block is very distinct to the one reported here for por3. Psora-4 altered neither gating current kinetics nor G-V relationships. It preferentially blocked Kv1.5 channels in the opened and not in the closed state. Also in contrast to por3, Psora-4 apparently has to cross the plasma membrane to reach its binding sites located in the central cavity and in side pockets formed by the backside of S5 and S6 in conjunction with parts of S4 and the S4-S5 linker.

In summary, we showed that por3 blocks Kv channels by 2 different mechanisms: First, por3 interacts with the voltage sensor domain and stabilizes a closed Kv channel state(s). Therefore, stronger depolarization is needed to activate the Kv1.x channels. Por3 block of gating charge movement is strongly voltage-dependent. Second, por3 blocks the ion conduction pathway. By using a constitutively opened Kv channel (Kv1.3P427D), we showed that por3 pore block is voltage-independent. Future work in this area will focus on the synthesis and study of porphyrin derivatives that specifically act as gating modifier or pore blocker.

**Material and Methods**

**Porphyrin**

The porphyrin derivative, presented herein, consists of a tetraphenylporphyrin scaffold bearing 4 cationic side chains (tet-butyl N-(2-aminoethyl)carbamate).$^{11}$ One millimolar stock solutions of this compound were prepared in DMSO and were stored at ~80°C. For electrophysiological experiments, the final concentration of DMSO never exceeded 0.02%.

**Electrophysiology**

Oocytes from Xenopus laevis frogs were removed surgically and incubated with agitation for 2–3 h in a collagenase solution (2 mg/mL containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 5 mM HEPES at pH 7.5 with NaOH. Defolliculated oocytes (stage V–VI) were microinjected with cRNA as described.$^{14}$ Oocytes were kept at 17 °C in a solution containing 96 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 1.8 mM CaCl$_2$, 5 mM HEPES, 5 mM pyruvate and gentamycin (50 µg/mL, Sigma Aldrich) at pH 7.5 with NaOH, and were used for electrophysiological experiments 1 to 3 d after injection. During recordings oocytes
were perfused with a solution containing 20 mM KCl, 78 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES at pH 7.4 with NaOH. Data were filtered at 2 kHz and digitized at 10 kHz. Microelectrodes resistances were between 0.1 and 0.5 M when filled with 3 M KCl. Oocyte membrane voltage was controlled using a 2-electrode voltage technique (OC-725 oocyte clamp, Warner instruments). Voltage pulses were applied and data were acquired using PULSE software (HEKA).

Linear leak and capacitance currents were subtracted using P/4 subtraction paradigm. A Boltzmann function of the form 1/(1+(exp \[V_{1/2} - V]/k)) was fit to normalized conductance-voltage data.

On- and off-gating currents were recorded using the non-conducting Kv1.3-V430W channel. Test potential durations were of sufficient time to allow gating currents to return to baseline. Current traces were integrated to obtain an estimate of the respective gating charges (Q₁ and Q₂). Gating charge-voltage (Q-V) relations were determined by plotting normalized Q against voltage. A Boltzmann function was fit to the data. All data are given as means ± SEM.

Ion channel mutagenesis

Single base pair exchange was performed with a QuickChange site-directed mutagenesis kit (Stratagene).

NMR studies

To study tetraphenylporphyrin-lipid interactions, partially deuterated 1,2-dimyristoyl-D54-sn-glycero-3-phosphocholin (DMPC) was purchased from Avanti Polar Lipids, Inc. Liposomal samples were prepared with a tetraphenylporphyrin:DMPC ratio of 1:10. The sample contained approximately 60 mass % D₂O. NMR spectra were obtained after ultracentrifugation and packing into standard 4 mm Magic Angle Spinning (MAS) rotors. NOESY (ref. 27) experiments were conducted at 18.8 T employing 11 kHz MAS at about +10°C. The typical proton field strength for 90° pulses and SPINAL64 decoupling was 83 kHz. Two-dimensional experiments were acquired with 300 τ₁ experiments and 24 scans per τ₁ increment.34,35

In order to obtain cross-relaxation rates, integral intensities of diagonal peaks were determined for mixing times close to zero (5 ms) and 250 ms using TopSpin (Bruker Biospin). The relaxation rate matrix R can be calculated using matrix algebra according to the matrix equation: A(t) = exp(-Rt)A(0), where A(0) is the diagonal peak intensity matrix at zero mixing time.34,35 For N magnetically nonequivalent spins R is

\[
R = \begin{pmatrix}
\sigma_{11} & \sigma_{12} & \ldots & \sigma_{1N} \\
\sigma_{21} & \sigma_{22} & \ldots & \sigma_{2N} \\
\vdots & \vdots & \ddots & \vdots \\
\sigma_{N1} & \sigma_{N2} & \ldots & \rho_{NN}
\end{pmatrix}
\]

(Eqn. 3)

where \(\sigma_{ij}\) is by convention the cross-relaxation rate for magnetization transfer from spin j to i and \(\rho_{ij}\) is the effective relaxation rate of the diagonal peak due to spin i. In order to allow for relative comparability per proton transfer rates, \(\Gamma_i\) were computed by dividing the cross-relaxation rate \(\sigma_{ij}\) by the relative number of i spins N_i.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here:

http://www.landesbioscience.com/journals/channels/article/25848

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