Drug-induced ion channel opening tuned by the voltage sensor charge profile

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Polyunsaturated fatty acids modulate the voltage dependence of several voltage-gated ion channels, thereby being potent modifiers of cellular excitability. Detailed knowledge of this molecular mechanism can be used in designing a new class of small-molecule compounds against hyperexcitability diseases. Here, we show that arginines on one side of the helical K-channel voltage sensor S4 increased the sensitivity to docosahexaenoic acid (DHA), whereas arginines on the opposing side decreased this sensitivity. Glutamates had opposite effects. In addition, a positively charged DHA-like molecule, arachidonyl amine, had opposite effects to the negatively charged DHA. This suggests that S4 rotates to open the channel and that DHA electrostatically affects this rotation. A channel with arginines in positions 356, 359, and 362 was extremely sensitive to DHA: 70 µM DHA at pH 9.0 increased the current >500 times at negative voltages compared with wild type (WT). The small-molecule compound pimaric acid, a novel Shaker channel opener, opened the WT channel. The 356R/359R/362R channel drastically increased this effect, suggesting it to be instrumental in future drug screening.

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are naturally occurring substances with important functions in normal physiology. As a component of the cell membrane, PUFAs and other fatty acids can directly affect the activity of membrane proteins like voltage-gated ion channels (Schmidt et al., 2006; Boland and Drzewiecki, 2008; Börjess and Elinder, 2008; Xu et al., 2008b). In addition, free PUFAs can affect different ion channels (Boland and Drzewiecki, 2008; Moreno et al., 2012), and their beneficial effects on heart arrhythmias and epilepsy have been known for a while (McLennan et al., 1985; Hock et al., 1990; Billman et al., 1994; Xiao et al., 1995; Vreugdenhil et al., 1996; Xiao and Li, 1999; Spector, 2001). PUFAs have been suggested to regulate neuronal excitability by closing sodium or calcium channels (Vreugdenhil et al., 1996; Tigerholm et al., 2012) and/or by opening potassium (K) channels (Börjess and Elinder, 2008, 2010; Xu et al., 2008a; Börjess and Elinder, 2011; Tigerholm et al., 2012).

A voltage-gated ion channel consists of a pore-forming unit surrounded by four voltage sensor domains (VSDs; Long et al., 2007; Börjess and Elinder, 2008). Each VSD is composed of four transmembrane helices (S1–S4), where S4 contains several regularly spaced positively charged amino acid residues (Fig. 1, A and B). These positive charges respond to alterations in the membrane voltage by sliding along negative countercharges in S1–S3, and this movement regulates whether the channel is open or closed (Papazian et al., 1995; Keynes and Elinder, 1998; Broomand and Elinder, 2008; DeCaen et al., 2008, 2009; Catterall, 2010; Henrion et al., 2012; Jensen et al., 2012). The VSD of voltage-gated K channels can be in at least four closed/resting configurations (C1 to C4) and one open/activated configuration (O; Delemotte et al., 2011; Henrion et al., 2012). PUFAs open the voltage-gated Shaker K channel by shifting the voltage dependence of the opening toward more negative voltages (Börjess and Elinder, 2008, 2010; Xu et al., 2008a; Börjess and Elinder, 2011). The PUFA molecule is suggested to be inserted into the lipid membrane, close to or in direct contact with the ion channel (Fig. 1 B). The negative charge of the fatty acid attracts the positively charged voltage sensor S4, primarily the last molecular conformational step (C1→O) that swings out the top charge (arginine R362 called R1) against the lipid bilayer (Fig. 1, C and D; Börjess and Elinder, 2011; Henrion et al., 2012). Thus, R1 is suggested to be a key player in determining the sensitivity to PUFA and PUFA-like molecules (Börjess and Elinder, 2011). From an earlier study, we know that the Shaker ILT mutant is more sensitive to docosahexaenoic acid (DHA) compared with the WT Shaker channel, supporting an effect by DHA on the last opening step (Börjess and Elinder, 2011).

In the present investigation, we aimed to construct a channel with increased sensitivity to PUFAs. Such a
channel would (a) gain us more insight into the biophysical mechanism of action of PUFA, (b) explain why some ion channels are more sensitive for PUFA than others (Boland and Drzewiecki, 2008), and (c) function as an important tool in the search for new substances with lipoelectric properties, acting as drugs against epilepsy, cardiac arrhythmias, and pain. In addition, we also report on electrostatic channel-opening effects of a small-molecule compound.

MATERIALS AND METHODS

Molecular biology and expression of ion channels

Experiments were performed on the Shaker H4 channel (Kamb et al., 1987), made incapable of fast inactivation by the Δ(6–46) deletion (Hoshi et al., 1990). Mutagenesis, eRNA synthesis, oocyte preparation, eRNA injection, and oocyte storage were performed according to the procedures described previously (Börjesson et al., 2010; Börjesson and Elinder, 2011). Animal experiments were approved by the local Animal Care and Use Committee at Linköping University.

Electrophysiology

Currents were measured with the two-electrode voltage-clamp technique (GeneClamp 500B amplifier, Digidata 1440A digitizer, and pClamp 10 software; Molecular Devices) 1–6 d after injection of RNA. The amplifier’s leak and capacitance compensation were used, and currents were low-pass filtered at 5 kHz. All experiments were performed at room temperature (20–23°C). The holding voltage was set to –80 mV (−120 mV for the L361R/R362Q mutant), and steady-state currents were achieved by stepping to voltages between −80 and 50 mV (adjusted for some of the mutants) for 80 ms in 5-mV increments. The control solution contained (mM): 88 NaCl, 1 KCl, 15 HEPES, 0.4 CaCl2, and 0.8 MgCl2. pH was adjusted to 7.4 with NaOH, yielding a final sodium concentration of ~100 mM. Pure control solution was added using a gravity-driven perfusion system. DHA was prepared, stored, and applied as previously described (Börjesson et al., 2008). Arachidonoyl amine (AA+) was provided by T. Parkkari (University of Eastern Finland, as previously described (Börjesson et al., 2008). Arachidonyl amine driven perfusion system. DHA was prepared, stored, and applied as previously described (Börjesson et al., 2010), to prevent COX-induced meta-

RESULTS

Place dependence of the top arginine for the effect of DHA

To explore the place dependence of the top arginine (R1) in S4 for DHA effects, we expressed the Shaker K channel in Xenopus laevis oocytes and measured ion currents with a two-electrode voltage-clamp technique. We studied eight channels in which either the region 356–362 was neutral or in which one charge at a time was introduced in each of the seven positions 356–362 (Fig. 1 E). All mutants expressed well, and the opening kinetics was essentially unaffected by the mutations (opening time constants were 0.5–2.0 times the opening time constant for the WT channel).
By shifting the positive charge along S4, the voltage dependence for the conductance versus voltage, \( G(V) \), curve also shifted along the voltage axis (Fig. 1 F); some mutated channels were opened at more positive voltages (green symbols) than the channel with a neutral 356–362 segment (open symbol), and some were opened at more negative voltages (red symbols; for \( G(V) \) curves, see Fig. S1). The two groups of residues are positioned on opposite sides of the S4 helix (Fig. 1 G). The simplest explanation for this orientation dependence is that a charged residue dislikes a hydrophobic environment and therefore destabilizes either the open state or closed state depending on in which state a particular residue faces the lipid bilayer (Yang et al., 2011). Side chain interactions within the VSD may also contribute to the voltage dependence of the arginine mutants: The right-shifted \( G(V) \) curve for M356R and A359R is probably supported by charge interactions with negative countercharges in S2 and S3 normally interacting with the gating charges in S4 (Yang et al., 2011; Henrion et al., 2012), whereas the open conformation for S357R, L358R, and L361R might be stabilized by interactions with negatively charged clusters in the S1–S2 loop or the S3–S4 loop (Henrion et al., 2012).

Shifting the top charge along S4 also altered the Shaker K channel sensitivity to DHA. Moving the arginine from position 362 to 361 abolished the effect of DHA (Fig. 2, A and B), whereas moving it two steps further clearly potentiated the effect (Fig. 2 C). A summary of DHA-induced shifts of the \( G(V) \) curves for all mutations (Fig. 2 D) shows that three (yellow bars) are not significantly different from the neutral mutant (open bar), two are significantly less affected (red bars), and two are significantly more affected (green bars), with A359R/R362Q as the most potent mutation, nearly doubling the shift from \(-6.1 \pm 0.6 \) (\( n = 9 \)) to \(-11.8 \pm 0.8 \) mV (\( n = 9 \); Fig. S1). When coloring the mutated residues in two suggested Kv channel structures (Henrion et al., 2012), C3 and O, based on their impact on DHA sensitivity, a clear pattern emerges (Fig. 2 E). The red residues point toward the lipid bilayer and the expected position of DHA (Börjesson and Elinder, 2011) in the closed C3 state, supporting the lack of channel-opening effects of DHA on these mutations. The green residues point toward

Figure 1. Charge distribution and voltage dependence of opening of WT and mutated Shaker K channels. (A and B) Gating charges R1–R4 in S4 denoted as blue sticks in a VSD structure (side view [A] and top view [B]) of an open K channel (the Kv1.2/2.1 chimera structure with Shaker side chains; Long et al., 2007; Henrion et al., 2012). The approximate interaction site for DHA is marked with the red encircled negative sign in B (Börjesson and Elinder, 2011). (C and D) R1 (R362) is highlighted in five superimposed structures of S4 (side view [C] from residue 356 and top view [D] from residue 361), for four closed (C1–C4) and one open (O) model states (Henrion et al., 2012). Backbones are color coded according to opening level, from light gray (C4) to black (O). In D, the approximate interaction site for DHA is marked with the red encircled negative sign (Börjesson and Elinder, 2011). The arrow in D denotes the movement of R1 when S4 moves from C1 to O, the step most sensitive to DHA (Börjesson and Elinder, 2011). (E) Amino acid sequences of the extracellular end of S4 (Shaker channel) from the eight single-residue mutations investigated. The positively charged arginines (R) are marked in blue. The mutated region is shown in gray. R1 = R362. (F) The voltage required to reach 50% of the maximum conductance \( (V_{1/2}) \) plotted against the residue number of the positive charge. The dotted line corresponds to \( V_{1/2} \) for the channel without charges in the studied region (R362Q). Green symbols denote positive midpoint voltages relative the neutral 356–362 segment (open symbol), and the red symbols denote negative midpoint voltages. (G) Residues 356–362 are denoted as sticks on the open Shaker VSD structure with same color coding as in F.
the lipid bilayer and the expected DHA position in the open state, thus supporting DHA-induced promotion of channel opening. The yellow residues are positioned toward the lipid bilayer in the open state, but at larger distances (vertically or horizontally) from the expected position of DHA in the open state, supporting a lack of electrostatic interaction. There is a significant correlation between the DHA-induced shifts of voltage for activation and the voltage required to reach 50% of the maximum conductance ($V_{1/2}$; Fig. 2F). This correlation suggests that, at least to some extent, the mechanisms for the induced shifts and the opening of the channels are common. A similar correlation has been found for metal ion effects on different WT as well as mutated K channels and suggested to depend on alterations in fixed surface charges (Elinder et al., 1996; Elinder and Århem, 2003; Broomand et al., 2007). In contrast, there is no correlation between the slope of the fitted Boltzmann curves of the arginine mutants and the DHA-induced shifts, suggesting that the altered sensitivity to DHA does not depend on altered energy barriers between the states with altered state occupancy as consequence (Fig. S2).

Rotation of S4 is required to open the channel:
**Sidedness of the charge effects**
To further test the hypothesis that there is an electrostatic interaction between charges in S4 and the DHA molecule, and not simply that additional charges in S4 dislike the lipid bilayer or hydrophobic pockets of the channel protein, we tested whether negatively charged glutamates at three consecutive positions, 359 to 361, had opposite effects to the arginines. A glutamate in position 359 significantly reduced the DHA-induced $G(V)$ shift from $-6.1 \pm 0.6$ to $-3.8 \pm 0.4$ mV ($n = 7$; Fig. 3A and B). This alteration is in the opposite direction of the positively charged arginine. The 359E mutant was further investigated by testing whether AA+, a positively charged PUFA analogue, shows the opposite effect compared with DHA. Although AA+ previously was shown to close the Shaker WT channel by shifting the $G(V)$ curve in a positive direction along the voltage axis (Börjesson et al., 2010), AA+ opened the 359E channel by shifting the $G(V)$ curve by $-8.9 \pm 1.7$ mV ($n = 4$; Fig. S3), which is significantly more than the shift induced by the negatively charged DHA on the same channel.

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**Figure 2.** DHA sensitivity of the single arginine mutants. (A–C) Representative current traces for voltages corresponding to 10% of $G_{\text{max}}$ in control solution at pH 7.4. Black traces indicate control, and red traces indicate 70 µM DHA. The increments in current amplitudes are R362R (WT), 2.2 times (A); for L361R/R362Q, 0.9 times (B); and A359R/R362Q, 2.7 times (C). (D) DHA-induced $G(V)$ shifts for the single-charge mutants (70 µM DHA at pH 7.4). The black line equals the DHA-induced shift for R362Q. Mean ± SEM ($n = 8, 8, 6, 9, 10, 14, 9, \text{and } 9$). DHA-induced shifts compared with R362Q (one-way ANOVA together with Dunnett’s multiple comparison test: **, $P < 0.01$; ***, $P < 0.001$). Green bars denote significantly larger DHA-induced shifts relative to the neutral 356–362 segment (open bar), the red bars denote significantly smaller shifts, and the yellow bars denote no significant differences. (E) Mutated residues are marked on one VSD of the Shaker K channel in states C3 and O. Same color coding as in D. (F) Correlations between the $V_{1/2}$ values and the DHA-induced shifts for the channels described in D. Slope is significantly different from zero (Pearson correlation test, and linear regression: $P < 0.01$ for both). The symbols denote mean ± SEM, the continuous line is the linear regression, and the dashed lines denote the 95% confidence interval.
Worley, 2001). Also in this system, the Kv2.1 look-a-like channel was sensitive to DHA, which displayed a relatively large DHA-induced shift of $-10.7 \pm 1.2$ mV ($n = 8$), but this effect was gained from 359R rather than 358R. The triple-R mutant M356R/A359R/R362R (hereafter referred to as 3R) turned out to be the most sensitive channel and was thus further explored.

In contrast to the experiments for 359 above, swapping the charge at position 361 from positive to negative had the opposite effect; the DHA-induced shift increased from $-0.8$ to $-7.0$ mV (Fig. 3, C and D), slightly more than for R362Q. The AA+ effect on 361E was not possible to evaluate because AA+ induced severe cellular toxicity for this mutant (see also Börjesson et al. [2010] for toxicity description) and caused substantial inactivation of 361E. For position 360, there was no difference between the glutamate and the arginine (Fig. 3 E). Calculations of the electrostatic effects are complicated because of several unknown parameters, like the exact positions of the amino acid charges, the dielectric constant, and uncertainty regarding the number of bound DHA molecules. However, the data are consistent with a simple model, locating one DHA molecule in the lipid bilayer just outside of S4 as indicated in Fig. 3 F. The last step in the channel opening, the transition from C1 to O (Henrion et al., 2012), is the step most sensitive to DHA (Börjesson and Elinder, 2011). During this step (Fig. 3 F, arrows), 359 approaches the negatively charged DHA molecule, 361 departs from the DHA molecule, and 360 keeps a relatively constant distance from the DHA molecule (Fig. 3 F). A positive charge at 359 or a negative charge at 361 promotes opening. A negative charge at 359 or a positive charge at 361 prevents opening. Notably, the negative glutamates have less of an effect than the positive arginines, compared with R362Q, probably because the negative charge of the glutamate can partly push away the negative DHA molecule from the channel or deprotonate the DHA molecule. These data strongly suggest that S4 is required to rotate in the last step to open the channel and that DHA electrostatically affects this rotation.

Combining arginine mutations to increase the DHA sensitivity

To search for a channel with even higher sensitivity to DHA than the single mutants, we explored the effect of DHA (70 µM at pH 7.4) on several combinations of positively charged residues in the positions 356, 358, 359, 360, and 362 (Fig. 4). The potentiating effect of A359R was independent (i.e., additive) of single-positive charges in positions 356, 358, 359, 360, and 362, and thus, A359R could easily be combined with these arginines to gain a larger sensitivity to DHA. In contrast, the potentiating effect of I360R was abolished in various combinations with A359R. In combination with A359R alone, I360R even abolished the potentiating effect of an arginine at position 359. Thus, I360R was not a good candidate for the construction of a highly DHA-sensitive channel. The L358R/A359R/R362Q mutant was generated to mimic the charge profile of the Kv2.1 channel because this channel is known to be sensitive to DHA (McKay and Worley, 2001).
The 3R mutant exhibits dramatically increased DHA sensitivity

There are no major differences between the activation kinetics of the 3R mutant and the control channel (i.e., a difference less than a factor of 2; Fig. 5, A and B). For the 3R mutant, 70 µM DHA at pH 7.4 increased the current 11-fold at 10 mV (Fig. 5 C) and shifted the voltage dependence by $-19.6 \pm 1.0$ mV ($n = 15$; Fig. 5 D), a shift more than three times larger than that for the R362Q and WT channels.

In a previous study, we showed that the DHA-induced $G(V)$ shift of the Shaker WT channel was pH dependent (Börjesson et al., 2008). The pH dependence is explained by incomplete deprotonation of DHA at pH 7.4, as the apparent pKa value of PUFAs in a lipid membrane is $\sim 7.5$ (Börjesson et al., 2008). Thus, the effect of DHA on the 3R channel was further investigated by altering the pH. At $-15$ mV and pH 9.0, a DHA concentration $\geq 3$ µM clearly increased the current; 7 µM caused a 10-fold increase (orange trace), and 70 µM caused a 40-fold increase (green trace; Fig. 6 A). Increasing concentrations of DHA at pH 9.0 gradually shifted the voltage dependence to more negative voltages (up to $-60$ mV), with minor effects on the maximal conductance (Fig. 6 B). However, at high concentrations, DHA also induced some inactivation, as previously reported for the WT Shaker channel (Börjesson et al., 2008). The DHA-induced shift caused by the highest DHA concentrations may therefore be the result of two separate mechanisms: lipoelectric opening of the channel and channel inactivation (possibly by DHA-induced conformational changes of the selectivity filter or block of the gate). As inactivation mainly occurs at the more positive voltages, we expect only minor effects of DHA-induced inactivation on the $G(V)$ shifts measured at the 10% level. DHA concentrations $>210$ µM were not tested because free fatty acids form micelles around 100 µM in physiological solutions (Richieri et al., 1992; Börjesson et al., 2008). The effect of DHA on the control channel versus the 3R channel was measured at different concentrations at pH 7.4 and 9.0 (Fig. 6 C). From these data, it is clear that the DHA sensitivity of the WT channel at pH 9.0 is similar to the DHA sensitivity of the 3R channel in pH 7.4. The increased DHA sensitivity of the 3R channel is likely explained by the additional three positive charges in the 3R channel that all point toward the lipid bilayer and therefore are able to electrostatically interact with DHA (Fig. 6 D).

Thus, although 70 µM DHA at pH 9.0 shifted the $G(V)$ of the WT channel by $-18.0 \pm 1.4$ mV ($n = 9$; Börjesson et al., 2008), it shifted the $G(V)$ of the 3R channel by $-47.9 \pm 4.2$ mV ($n = 4$). At low voltages these shifts can be converted to equivalent increases in current magnitude, $A = \exp(-\Delta V/4.7)$; Börjesson et al., 2010. The amplitude increase from WT to 3R is thus

$$A_{3R}/A_{WT} = \exp(-\Delta V/4.7).$$

Thus, introducing two extra positive charges at positions 356 and 359 increases the open probability of the Shaker K channel by a factor of 580 at 70 µM DHA at pH 9.0. This large potentiation simplifies the search for other compounds with similar properties.
Identifying a novel Shaker channel opener
DHA and other PUFAs display promising anti-excitatory effects on the Shaker K channel (Börjesson et al., 2008, 2010; Xu et al., 2008a; Börjesson and Elinder, 2011; Tigerholm et al., 2012). However, they are very promiscuous, a characteristic not appreciated in drug design. Therefore, in an attempt to find other candidate drugs, we have searched for small-molecule compounds with similar effects to DHA. PiMA, an amphipathic resin acid known to open Ca²⁺-activated BK channels (Imaizumi et al., 2002) has, in conformity with the fatty acids, a lipophilic domain and a carboxyl group supposed to be negatively charged at high pH (Fig. 7 A). Therefore, PiMA is also a possible candidate to open Kv channels. For the WT Shaker K channel, 70 µM PiMA at pH 7.4 shifted the $G(V)$ by $-4.6 \pm 0.7$ mV ($n = 15$; Fig. 7 B). For the 3R channel, the shift was almost doubled to $-8.4 \pm 1.2$ mV ($n = 4$; Fig. 7 B), indicating that PiMA also activates the Kv channel by electrostatically affecting the positive charges in the top of S4. Similar to the DHA effect, the PiMA effect was potentiated at increased pH (Fig. 7, B–F). Thus, we have found a small-molecule compound able to shift the $G(V)$ of the 3R channel at pH 9.0 by almost $-30$ mV.

**DISCUSSION**

In this study, we have investigated introduced charged amino acid residues in S4, and combinations of them, and how they affect the DHA-induced alteration of the Shaker K channel’s voltage dependence. We found that the single mutations A359R and I360R and combinations including A359R, in particular 3R, significantly increased the sensitivity to DHA. We have also identified a small molecule compound, PiMA, with similar effects as DHA on the channel’s voltage dependence. Furthermore, we found that residues on the opposite side of S4, S357R and L361R, significantly reduced channel sensitivity to DHA. The place dependence of arginines and glutamates for the DHA sensitivity supports a rotational S4 movement in the last opening step that is promoted by negatively charged lipophilic compounds, like PUFAs and PiMA. This model is supported by data for the positively charged PUFAs with similar effects as DHA on the channel’s voltage dependence. An understanding of the molecular details for the PUFAs–channel interactions is important for explaining and predicting differences in PUFAs sensitivity between channels. One of the channels constructed in this study mimics the S4 arginine profile of Kv2.1 (L358R/A359R/R362Q). The Kv2.1-mimicking mutant demonstrated increased DHA sensitivity, in line with experimental findings reporting clear shifts in Kv2.1 channel voltage dependence from low micromolar PUFAs concentrations (McKay and Worley, 2001). BK channels also display a favorable S4 charge profile for possible PUFAs effects by having a positive charge at the position equivalent to 359 in the Shaker K channel. BK channels are reported to be highly sensitive to PUFAs (Clarke et al., 2003; Sun et al., 2007; Hoshi et al., 2013a,b). However, these large effects on the BK channel could, at least partly, be explained by another mechanism because residues on the intracellular side of the channel are involved in the effect (Hoshi et al., 2013a). Other DHA-promoting or -preventing charge profiles are found in different pseudotetrameric sodium and calcium channels.
However, the physiological relevance is difficult to evaluate because only one out of four VSDs is expected to show increased or decreased PUFA sensitivity.

We have previously reported that part of S4 adapts a \(3_{10}\) helical structure and that the segment forming a \(3_{10}\) helix slides along the S4 helix during gating (Henrion et al., 2012). In our previous study, we proposed that the \(3_{10}\)-helical structure is downstream of R3 in the last opening step, the step most sensitive to DHA. Because the electric force varies inversely with the square of the distance between two charges, the electrostatic interaction between DHA and the gating charges in the \(3_{10}\)-helical segment during the opening step will be weak. Hence, the \(3_{10}\) helix structure and the sliding of that structure will likely be preserved in the presence of DHA.

The main finding in the present work is the 3R mutant, a constructed channel with an increased sensitivity to PUFAs with respect to the channel’s voltage sensitivity. 70 µM DHA increased the current of the 3R channel at pH 9.0 and negative voltages >500 times the WT channel. Thus, the 3R channel is a promising tool in the search for pharmacological compounds with beneficial effects against cellular hyperexcitability in diseases such as cardiac arrhythmia, epilepsy, and pain. As a first example of this, we here demonstrate 3R channel sensitivity to PiMA, a novel Shaker-channel opener.

The AA+ was generously provided by Teija Parkkari.

This work was supported by grants from the Swedish Research Council, the Swedish Heart-Lung Foundation, the Swedish Brain Foundation, the County Council of Östergötland, and King Gustaf V and Queen Victoria’s Freemasons Foundation.

The authors have no conflicting financial interests.

Kenton J. Swartz served as editor.

Submitted: 16 August 2013
Accepted: 19 December 2013

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Figure 7. Drug sensitivity is increased for 3R. (A) Structure of PiMA, here in the uncharged form with the carboxylic acid group protonated. (B) \(G(V)\) shifts induced by 70 µM PiMA at pH 7.4 and 9.0 for WT and 3R. Mean ± SEM (n = 12, 6, 4, and 6; one-way ANOVA together with Bonferroni’s pairwise comparison test: **, P < 0.01; ***, P < 0.001). (C) Representative current traces at pH 9.0 for WT in control solution (black) and in 70 µM PiMA (red) for a voltage corresponding to 10% of \(G_{\text{max}}\) in control solution (i.e., \(-35\) mV). The current amplitude is increased fourfold. (D) Representative \(G(V)\) curves. Same cell as in C (control, black symbols; PiMA, red symbols). \(G(V)_{\text{PiMA}} = -9.0\) mV in this example. (E) Representative current traces at pH 9.0 for 3R in control solution (black) and in 70 µM PiMA (red) for a voltage corresponding to 10% of \(G_{\text{max}}\) in control solution (i.e., 5 mV). The current amplitude is increased >10 times. (F) Representative \(G(V)\) curves. Same cell as in F (control, black symbols; PiMA, red symbols). \(G(V)_{\text{PiMA}} = -28.5\) mV in this example.


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Figure S1. Representative $G(V)$ curves for single arginine mutants. The continuous lines are fitted Boltzmann curves, $G(V) = A/(1 + \exp((V - V_{1/2})/s))$, where $A$ is the amplitude, $V_{1/2}$ is the midpoint in control, and $s$ is the slope forced to be equal in control and DHA. $V_{1/2}$ in DHA is set to $V_{1/2} + \Delta G(V)_{\text{DHA}}$. (A) R362Q: $V_{1/2} = 5.5$ mV, $\Delta G(V)_{\text{DHA}} = -5.7$ mV, $s = 13.3$. (B) M356R/R362Q: $V_{1/2} = 22.2$ mV, $\Delta G(V)_{\text{DHA}} = -5.8$ mV, $s = 9.1$. (C) S357R/R362Q: $V_{1/2} = 31.2$ mV, $\Delta G(V)_{\text{DHA}} = 0.1$ mV, $s = 8.7$. (D) L358R/R362Q: $V_{1/2} = 16.8$ mV, $\Delta G(V)_{\text{DHA}} = -6.5$ mV, $s = 14.9$. (E) A359R/R362Q: $V_{1/2} = 44.2$ mV, $\Delta G(V)_{\text{DHA}} = -10.3$ mV, $s = 12.7$. (F) I360R/R362Q: $V_{1/2} = 20.6$ mV, $\Delta G(V)_{\text{DHA}} = -7.9$ mV, $s = 9.4$. (G) L361R/R362Q: $V_{1/2} = -64.0$ mV, $\Delta G(V)_{\text{DHA}} = -0.6$mV, $s = 13.8$. (H) R362R (WT): $V_{1/2} = -26.9$ mV, $\Delta G(V)_{\text{DHA}} = -5.4$ mV, $s = 5.5$. 

SUPPLEMENTAL MATERIAL

Ottosson et al., http://www.jgp.org/cgi/content/full/jgp.201311087/DC1
**Figure S2.** Correlation between the slope ($s$ values) of the fitted Boltzmann curves and the DHA-induced shifts for arginine mutants. Slope of the linear regression is not significantly different from zero (Pearson correlation test and linear regression, $P = 0.23$). Mean ± SEM.

**Figure S3.** AA$^{+}$ sensitivity of A359E/R362Q. (A and B) Current families for A359E/R362Q in control solution (A) and after application of 70 μM AA$^{+}$ (B) when stepping the membrane voltage from −80 mV to voltages between −80 and 60 mV in 5-mV increments. Current traces for a voltage corresponding to 10% of $G_{\text{max}}$ in control solution (i.e., 0 mV) are highlighted with thick lines in black (A) or red (B). The current amplitude at 0 mV is increased 2.24-fold by AA$^{+}$ application. (C) $G(V)$ curves for the same cell as in A and B (control, black symbols; DHA, red symbols). $\Delta G(V)_{\text{AA}^{+}} = −7.2$ mV in this example.