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RNA editing modulates the binding of drugs and highly unsaturated fatty acids to the open pore of Kv potassium channels

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 04 January 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three express significant interest in your work and are broadly in favour of publication. However, referees 1 and 3 raise a number of concerns that would need to be addressed experimentally before we can consider publication of your manuscript. The reports are explicit, and therefore I will not go into detail here, but I would in particular point out the comments of referee 1 regarding the importance of determining the stoichiometry of the lipid-channel interaction. In addition, both referees 1 and 3 raise concerns regarding the results of the experiments conducted in neurons that would need to be resolved.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Decher and colleagues describe a very interesting correlation between Kv1.1 mRNA editing and sensitivity of the respective Kv1.1 channel to block by lipids. Editing of Kv1.1 mRNA leads to an altered amino acid residue (I400V) within the inner pore of the channel. Kv1.1 mRNA editing is spatially restricted in the central nervous system being most prominent in spinal cord, thalamus, and medulla. The I400V mutation partially disrupts the interaction between the inactivating domain of Kvβ1.1 and the pore. Decher et al. now show that the I400V mutation also affects inactivation of the Kv1.1 channel by lipids. The authors propose that RNA editing may be used to induce lipid resistance to the Kv1.1 channel. This work is very interesting and of sufficient general interest to warrant publication in the EMBO journal.

Carefully executed experiments suggest that unsaturated lipids like arachidonic acid (AA) and anandamide modulate Kv1.1 channel gating by binding to the open pore of the channel. The conclusions are based on single channel analyses, competition experiments with TEA, analysis of channel deactivation, and scan mutagenesis of pore lining residues. Despite this set of experiments, important information concerning the lipid interaction with the Kv1.1 channel is missing and should be provided in a revised version of the manuscript.

i) It is important to determine in inside-out patches the stoichiometry of lipid binding to the Kv1.1 channel. Given that 2 µM AA induce an almost complete inhibition, this should be possible. The results will provide important information on the binding mode of the lipid and may also help to explain the dominant negative mutational effects on binding.

ii) The differences in block between Fig. 1 E and 1 I suggest that both wild type and mutant Kv1.1 channel are similarly blocked by 10 µM AA, whereas the mutant channel is almost insensitive at 2 µM. This is difficult to reconcile with a simple pore block.

iii) Quantitative data on the block by anandamide (concentration dependence) should be provided.

iv) Previous work (see Bhalla and colleagues) has shown that editing effects may differ between Shaker and Kv1.1 channels. This may also hold for Kv1.1 and Kv1.5. To my knowledge, Kv1.5 mRNA is not edited. Therefore, one would like to have information on Kv1.1 (and Shaker) with respect to editing-related mutagenesis.

v) Given the dramatic difference in Kv1.1 lipid sensitivity seen between two-electrode voltage-clamp and the inside-out patch clamp experiments, it is important to measure lipid sensitivity of Kv1.1 mutants in the inside-out patch-clamp configuration.

vi) The AA block of Kv1.1 seems voltage-independent, the AA block of Kv1.1 + Kvβ1.1 voltage-dependent (Fig. 3 C). There are several alternatives to explain these data. Additional experiments should be carried out to clarify. A control experiment with Kv1.1 + Kvβ2 may be helpful.

vii) The docking experiments illustrated in Fig. 3 D are crude. A model illustrating the pore lining residues suffices.

viii) Measurements of the relative distribution of edited Kv1.1 mRNA in the central nervous system have been done previously. The authors should refer to those (Hoopengardner et al., 2003) and omit Fig. 7 A.

ix) The interpretation of the electrophysiological data in Fig. 7 is very speculative. It is recommendable to include knock-down experiments and to carry out pharmacological experiments on neurons, which specifically express Kv1.x outward current while other Kv channels are blocked. A control with neurons expressing solely unedited Kv1.1 mRNA should be included.

x) Minor point: The introduction contains twice the same para (on p.3 and p.4). Single channel conductance and single-channel bursting behaviour of the Kv1.1 channel was first reported by Stühmer et al. in 1988.

Referee #2 (Remarks to the Author):

In this manuscript, the authors address previous observations of the effects of highly unsaturated fatty acids (e.g. AA) on the functioning of Kv3.1 voltage gated K+ channels. Given a variety of proposed models (since the mechanism of action is unknown) the authors proceed from an initial observation that AA also effectively causes wild type Kv1.1 channels expressed in Xenopus oocytes to undergo what appears be fast inactivation, while a mutated version (I400V) is resistant to this effect. This is particularly important when considering that the same amino acid conversion occurs in Kv1.1 channel transcripts through the natural process of RNA editing. The effect is not specific to AA, but to a class of HUFA and the effect is markedly suppressed by the effect of editing. Another compelling observation made is that in Kv3.1 (the non-inactivating channel used in the initial report of HUFA conversion to inactivating properties) becomes resistant to AA when one converts the equivalent isoleucine to valine by mutation. For both Kv1.1 which is edited and Kv3.1 which is not, the effect of the I-to-V change appears to most affect the off-rate of inactivation while the on rate is nearly the same. The authors also show that the effect of AA is very rapid if applied to inside-out macro patches in the bath solution. These observations are most consistent with a model of open channel block (rather than membrane access, etc). Further classic tests of the open channel pathway (competition of AA binding by TEA and a beta subunit) are consistent with open channel block, as well as the "foot in the door" effect of crossing over seen in deactivation curves in the presence/absence of AA. Single channel parameters were also determined and the addition of AA is consistent with the action of an open channel blocker (decreased burst duration and summed open probability).

Mutagenic studies (á la Zhou et al 2001) were performed and determined that, like for the natural inactivation particle, alteration affecting block occur in a helical nature and are channel lining residues. Interestingly, residues deeper into the channel pore than the crucial I400V seem to have large affects as well on AA's effect (V505, I502). The authors then show that only Kv1.1 channels undergo editing, and do so in a region specific manner consistent with previous reports. The effects of open channel blocking drugs are also tested and in all cases, including 4-AP, the edited version of the channel has a higher IC50 than the unedited version. Since Kv1.1 channels form tetramers, the authors then determine the effects of edited versus unedited channel stoichiometries. While these experiments do not follow the predictions of binomial distribution for Psora-4, they are quite consistent with the suggestion that for AA, the presence of a single edited subunit can confer resistance to the inactivating properties of HUFA and drugs. In addition, because Kv1.1 channels can co-assemble with other Kv1.x family members, the authors perform excellent mixing experiments with Kv2-6 subunits. All of the other Kv1.x family members are inhibited by Psora-4 and AA, and all of them are also rendered significantly resistant to these compounds by expression of I400V, in some cases strikingly so (Kv1.3 and 1.5), implying that even in heterotetramers, fractionally I400V-incorporated channels are resistant to HUFA and open channel blockers. The last set of experiments are performed in native neurons and show that only a portion of the Hg-Tx blocked channels (total Kv1.x) are blocked by Psora-4. This implies that there is a significant fraction of Psora-4 resistant Kv1.x channels (Kv1.1 edited subunit incorporated) in native neurons and is an excellent set of end experiments for the article. It would be nice to see some electrophysiology on a neuronal subtype which has reduced levels of editing- low levels of editing should correlate with less resistance. However, without knowing the expression levels in a given cell

type of the other Kv1.x family members, the results from such studies could be quite difficult to interpret. Thus, the example demonstrated in LGN neurons is certainly a great beginning, in that the effect was seen, even without knowing the relative contributions of Kv1.x genes.

The manuscript is extremely well written and tightly argued, and provides a striking example of the "subtlety" of molecular regulation of channel function by post-transcriptional mechanisms. In fact, the authors would not be naïve in highlighting the fact that this regulatory mechanism changes the chemical structure of the channel by a single methyl group- in a channel with just one edited subunit, only one methyl group in the tetramer! This is a fact that is sure to be lost in the shuffle with readers who are not familiar with the chemical structure of amino acid side chains, but nevertheless, is a point which the reviewer thinks is one of the most amazing observations in the paper. There is beauty in simplicity. The manuscript is sure to be of interest to molecular biologists, geneticists, neurobiologists, ion channel jocks, and even with the appropriate mentions (see below), evolutionary biologists.

Comments

1. Similar mutagenesis results (Fig.3) were done by MacKinnon's group (Zhou et al, 2001) and these authors should correlate/comment on how well their data fit with the residues identified in that study on effectiveness of inactivation by mutants. Residues lining the inner cavity should be of similar effect when mutated and the inactivation is from a hydrophobic peptide accessing the cavity, in the open state. The authors mention the overlapping binding site idea, but don't mention how well their data mesh with MacKinnon's.

2. In the mutagenic studies, there is an issue that should be addressed. The position equivalent to Kv1.1's I400 is also an I-to-V editing site in fly Shab (Kv2) and squid sqKv2 channels, a clear case of convergent evolution (Bhalla et al, 2004, Ryan et al 2008, Patton et al 1997). Both Ryan, 2009 and Patton, 1997 present data on the functional consequences for editing at this paralogous position to the isoleucine present in mammalian Kv1.1. But of course, HUFA treatments were not conducted in these studies. Nevertheless, the authors provide strong evidence for the modulation of Kv.X channels by HUFA and open channel blocking compounds, and the portability to non-editors (the Kv3.1 I-to-V experiment). So, this aspect of channel biology should be discussed and the authors should assess whether the functional studies in these papers could intersect with their own.

3. Another issue with the mutagenic studies are the positions of the residues mutated in Kv1.5 that are further (V505, I502) into the pore structure than the I400 edited position. In particular, I502A. This position seems to have as much of an effect on resistance to AA as I508A (which is presumably comparable in effect as I400V in Kv1.1). How does this position confer such an affect given its location in the structure? This deserves some attention, if only because seems to be an unexpected result. In addition, this position is ALSO the location of a natural site of modification by RNA editing in Drosophila Shaker channel (Hoopengardner et al. 2003) and has been studied at the level of functional properties (Ingelsby et al, 2009). Since this site was shown in Inglesby, 2009 to have a substantial effect on deactivation properties, it may have implications for AA's effects on the Kv1.5 I502A mutant.

Referee #3 (Remarks to the Author):

In this work, the authors studied the mechanisms of action and the binding site of highly unsaturated lipids such as arachidonic acid, docosahexaenoic acid and anandamide, which produce an apparent fast inactivation in Kv1 voltage-gated K+ channels. They found that these lipids interact with hydrophobic residues lining the inner cavity of the pore at the middle of the S6 segment and more specifically with the residue isoleucine 400 of Kv1.1 that lines the inner cavity of the pore. These unsaturated lipids were shown to compete with intracellular tetraethylammonium and Kvβ subunits, suggesting that the inactivation they produce probably reflects occlusion of the permeation pathway, similar to drugs that produce an open-channel block. Performing docking experiments with anandamide, the authors showed that the pore is wide enough to accommodate the anandamide molecule and that its binding will significantly narrow the pore width so that its remaining diameter is too small to allow a hydrated potassium ion to permeate the channel. In addition, they found that the open-channel block by arachidonic acid, docosahexaenoic acid and anandamide is substantially

decreased in 'edited' Kv1.1 channels, when residue I400 is edited to V400. Further, they showed that RNA editing in neurons of the lateral geniculate nucleus of the thalamus markedly alters the sensitivity of Kv1 channels to pharmacological inhibition.

This manuscript is interesting and reflects an extensive study that is technically well done and clearly described. The work describes an important topic since it addresses the issue of the effect of physiologically relevant unsaturated lipids on voltage-gated Kv1 potassium channels and its impact on their edited version in neurons. However, I have some concerns with specific issues that should be clarified.

1-The authors provide some lines of evidence that the unsaturated lipids work on WT Kv1.1 channels by docking to the open channel pore cavity, thereby occluding the permeation pathway. This mechanism implies that K+ influx will expel the lipid molecule from its binding site and will result in an acceleration of recovery from "inactivation-block" in high external K+ as was demonstrated for the inactivation particle of Shaker K+ channels by Demo and Yellen (Neuron 1991). To test directly reopening of Kv1.1 channels during recovery from "inactivation-block", the authors should examine the kinetics of tail currents in the presence of high external K+ (120-140 mMK+).

2-It is not clear to the reviewer how the authors checked the editing of Kv1.1 channels in the specific neurons they record, since the quantification they provide represents a an average value. How other K+ conductances that are not sensitive to the unsaturated lipids, independently of the editing process, are taken into account? The authors should also provide a set of recording from neurons that express the unedited version (I400) of Kv1.1 as a positive control.

3-In the introduction, an identical paragraph, page 4, line 15-23 "amphiphilic substances..."appears twice in the introduction page 3.

1st Revision - Authors' Response 25 March 2010

Referee #1 (Remarks to the Author):

Decher and colleagues describe a very interesting correlation between Kv1.1 mRNA editing and sensitivity of the respective Kv1.1 channel to block by lipids. Editing of Kv1.1 mRNA leads to an altered amino acid residue (I400V) within the inner pore of the channel. Kv1.1 mRNA editing is spatially restricted in the central nervous system being most prominent in spinal cord, thalamus, and medulla. The I400V mutation partially disrupts the interaction between the inactivating domain of Kvß1.1 and the pore. Decher et al. now show that the I400V mutation also affects inactivation of the Kv1.1 channel by lipids. The authors propose that RNA editing may be used to induce lipid resistance to the Kv1.1 channel. This work is very interesting and of sufficient general interest to warrant publication in the EMBO journal.

Carefully executed experiments suggest that unsaturated lipids like arachidonic acid (AA) and anandamide modulate Kv1.1 channel gating by binding to the open pore of the channel. The conclusions are based on single channel analyses, competition experiments with TEA, analysis of channel deactivation, and scan mutagenesis of pore lining residues. Despite this set of experiments, important information concerning the lipid interaction with the Kv1.1 channel is missing and should be provided in a revised version of the manuscript.

i) It is important to determine in inside-out patches the stoichiometry of lipid binding to the Kv1.1 channel. Given that 2 µM AA induce an almost complete inhibition, this should be possible. The results will provide important information on the binding mode of the lipid and may also help to explain the dominant negative mutational effects on binding.

In the revised manuscript we have included current traces of Kv1.1 and Kv3.1 with different concentrations of the lipids (Figures 1E-G). In addition, we have provided dose-response curves and the IC_{50} values of Kv1.1 and Kv1.1^{I400V} for AA and AEA (Figures 1E-F and Results page 6, first paragraph). In fact, block of Kv1.1 by AEA and AA has a steep dose-response

curve which is reflected in the Hill coefficient (H = close to 2). However, AA and AEA are very hydrophobic molecules and are known to be absorbed by e.g. the tubing of the application system. Therefore, is it possible that especially for the lower concentrations the free concentration is lower than the calculated concentration and this could produce an artificial steep dose-response curve. Thus, we would like to put not too much weight on the interpretation of the Hill coefficient in respect to stoichiometry. However, if we assume the Hill coefficient is correct, then it would indicate positive co-operativity which can be caused by a binding of multiple molecules or by a complex binding mode of a single molecule. It is not possible from the Hill coefficient to extrapolate how many molecules bind to the central cavity. From the size of the inner cavity however, it appears possible that two molecules can enter, when they are tightly packed within the central cavity. The $Kv1.1^{I400V}$ *channels have a slightly shallower dose-response curve for AA and AEA (H = close to 1.6), however this does not necessarily indicate an altered mechanism of block. From these data however it is hard to speculate why the I400V exchange acts in a dominant-negative manner.*

ii) The differences in block between Fig. 1 E and 1 I suggest that both wild type and mutant Kv1.1 channel are similarly blocked by 10 µM AA, whereas the mutant channel is almost insensitive at 2 µM. This is difficult to reconcile with a simple pore block.

The differences in block of Kv1.1 and $Kv1.1^{I400V}$ *are still present, however less pronounced when higher concentrations of the lipids (10 µM) are used. As mentioned under point i), we have included patch clamp traces into the revised manuscript that show the effects of different AA and AEA concentrations on Kv1.1 (Figures 1E-F). Due to the steep dose-response curves the about 4-fold shift in* IC_{50} *by the I400V exchange is enough to explain the observation that at 2 µM* $Kv1.1^{I400V}$ *is insensitive while at 10 µM a substantial amount of the current is blocked.*

iii) Quantitative data on the block by anandamide (concentration dependence) should be provided.

We have included these data in the revised manuscript, see also point i)

iv) Previous work (see Bhalla and colleagues) has shown that editing effects may differ between *Shaker* and Kv1.1 channels. This may also hold for Kv1.1 and Kv1.5. To my knowledge, Kv1.5 mRNA is not edited. Therefore, one would like to have information on Kv1.1 (and Shaker) with respect to editing-related mutagenesis.

In the first version of the manuscript, we show that Kv1.5 does not undergo an I-to-V editing in the S6 segment (Fig. 4A). To exclude differences between Kv1.1 and Shaker, we also recorded the effects of the I400V exchange on AA and AEA sensitivity in Shaker channels (which are also edited). We have included a sentence in the Results section (page 5, end of the first paragraph) which states that the I400V exchange has a similar effect in Kv1.1, Shaker and Shab channels. The data is also discussed at page 17, second last paragraph. See also comment 2 of referee 2.

v) Given the dramatic difference in Kv1.1 lipid sensitivity seen between two-electrode voltage-clamp and the inside-out patch clamp experiments, it is important to measure lipid sensitivity of Kv1.1 mutants in the inside-out patch-clamp configuration.

We have included measurements of the lipid sensitivity of Kv1.1 and $Kv1.1^{I400V}$ *in the revised manuscript, using inside-out patch-clamp experiments, see also point i).*

vi) The AA block of Kv1.1 seems voltage-independent, the AA block of Kv1.1 + Kvß1.1 voltage-dependent (Fig. 3 C). There are several alternatives to explain these data. Additional experiments should be carried out to clarify. A control experiment with Kv1.1 + Kvß2 may be helpful.

Block of Kv1.1 by AA, as well as block of Kv1.1 + Kvß1.1 is not voltage dependent, only a weak tendency is observed which is not statistically significant. Using Student's t-Test no significant

voltage dependence of block was observed even when the values of 0 mV were compared to the values at +70 mV. We have now included a similar statement in the Figure Legend of Fig. 3C.

vii) The docking experiments illustrated in Fig. 3D are crude. A model illustrating the pore lining residues suffices.

We have included a new Fig. 3D which illustrates the residues we have identified by our Ala-scan. The stereo-view picture clearly shows that the identified residues are primarily pore facing. A figure of the docking experiments can be found in the Supplementary Information of the revised manuscript (Supplementary Figure 4), as these give still a good impression of the size of an AEA molecule in the inner cavity.

viii) Measurements of the relative distribution of edited Kv1.1 mRNA in the central nervous system have been done previously. The authors should refer to those (Hoopengardner et al., 2003) and omit Fig. 7 A.

We suppose that you were referring to Fig. 4A (not Fig. 7A), as this panel shows the relative distribution of Kv editing. We were aware that for Kv1.1 a relative distribution has been previously reported and we quoted the work by Hoopengardner et al. in the Results section. We did not intend to claim that the Kv1.1 data are novel. The Kv1.1 editing rates given in Fig. 4A are only control experiments illustrating that we can confirm the high editing rates of Kv1.1 in these tissues while at the same time other Kv1.x channels, as well as Kv2.1, Kv3.1 and Kv4.3, are not edited. Nevertheless, we have removed the sequencing data of the Kv1.1 samples from Fig. 4A to de-emphasize the control data of Kv1.1 editing. Tissues in which Kv1.1 editing was not previously examined were also analyzed, but these data are shown in the separate Fig. 4B.

ix) The interpretation of the electrophysiological data in Fig. 7 is very speculative. It is recommendable to include knock-down experiments and to carry out pharmacological experiments on neurons, which specifically express Kv1.x outward current while other Kv channels are blocked. A control with neurons expressing solely unedited Kv1.1 mRNA should be included.

Thank you for this suggestion. We have performed control experiments in neurons that have much less Kv1.1 editing, as there are no neurons known to have a complete lack of Kv1.1 I-to-V editing. We have analyzed cells from the hippocampus, as these express Kv1 channels and have reportedly little Kv1.1 editing. We have quantified the Kv1.1^{I400V} editing ratio in the CA1 to CA3 region of the rat and found that only 7 % of the mRNA transcripts are I-to-V edited. Consistently, we found in patch clamp experiments of rat CA1 neurons that only 15 % of the Kv1.x current is Psora-4 resistant, in contrast to the 63 % in LGN neurons. These data show that there is a strong correlation of I400V editing and Psora-4 drug-resistance. The additional data is incorporated in the Results section (page 14), Figures 7J-L and the Supplementary Figure 5.

The suggested experiment to record Kv1.x currents while other Kv channels are blocked is currently not feasible, as there are no specific blockers known to completely inhibit Kv2.x, Kv3.x and Kv4.x channels without side effects. Therefore, the use of the Kv1.x specific Psora-4 and HgTx1 is currently the "cleanest" pharmacological way to isolate Kv1.x currents and to describe drug-resistance with the highly selective HgTx1.

Another way to analyze changes in pharmacology, would be the study of ADAR2 knock-out mice which should have no editing-related drug-resistance. We have already started to analyze the pharmacological changes in different regions of the brain using the ADAR2 knock-out mouse. However, these extensive studies are beyond the scope of the current manuscript.

A similar comment/criticism was made by another referee, see also response to referee 3, comment 2.

x) Minor point: The introduction contains twice the same para (on p.3 and p.4). Single channel conductance and single-channel bursting behaviour of the Kv1.1 channel was first reported by Stühmer et al. in 1988.

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Thank you. We have removed the paragraph that appeared twice. In addition, we have included the appropriate citation of Stühmer et al. in the Results section and in the Supplementary Information file where also single channel data appear.

Referee #2 (Remarks to the Author):

In this manuscript, the authors address previous observations of the effects of highly unsaturated fatty acids (e.g. AA) on the functioning of Kv3.1 voltage gated K^+ channels. Given a variety of proposed models (since the mechanism of action is unknown) the authors proceed from an initial observation that AA also effectively causes wild type Kv1.1 channels expressed in *Xenopus* oocytes to undergo what appears be fast inactivation, while a mutated version (I400V) is resistant to this effect. This is particularly important when considering that the same amino acid conversion occurs in Kv1.1 channel transcripts through the natural process of RNA editing. The effect is not specific to AA, but to a class of HUFA and the effect is markedly suppressed by the effect of editing. Another compelling observation made is that in Kv3.1 (the non-inactivating channel used in the initial report of HUFA conversion to inactivating properties) becomes resistant to AA when one converts the equivalent isoleucine to valine by mutation. For both Kv1.1 which is edited and Kv3.1 which is not, the effect of the I-to-V change appears to most affect the off-rate of inactivation while the on rate is nearly the same. The authors also show that the effect of AA is very rapid if applied to inside-out macro patches in the bath solution. These observations are most consistent with a model of open channel block (rather than membrane access, etc). Further classic tests of the open channel pathway (competition of AA binding by TEA and a beta subunit) are consistent with open channel block, as well as the "foot in the door" effect of crossing over seen in deactivation curves in the presence/absence of AA. Single channel parameters were also determined and the addition of AA is consistent with the action of an open channel blocker (decreased burst duration and summed open probability).

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The manuscript is extremely well written and tightly argued, and provides a striking example of the "subtlety" of molecular regulation of channel function by post-transcriptional

mechanisms. In fact, the authors would not be naive in highlighting the fact that this regulatory mechanism changes the chemical structure of the channel by a single methyl group- in a channel with just one edited subunit, only one methyl group in the tetramer! This is a fact that is sure to be lost in the shuffle with readers who are not familiar with the chemical structure of amino acid side chains, but nevertheless, is a point which the reviewer thinks is one of the most amazing observations in the paper. There is beauty in simplicity. The manuscript is sure to be of interest to molecular biologists, geneticists, neurobiologists, ion channel jocks, and even with the appropriate mentions (see below), evolutionary biologists.

Comments

1. Similar mutagenesis results (Fig. 3) were done by MacKinnon's group (Zhou et al, 2001) and these authors should correlate/comment on how well their data fit with the residues identified in that study on effectiveness of inactivation by mutants. Residues lining the inner cavity should be of similar effect when mutated and the inactivation is from a hydrophobic peptide accessing the cavity, in the open state. The authors mention the overlapping binding site idea, but don't mention how well their data mesh with MacKinnon's.

Thank you for the suggestion to include a more precise analysis/discussion of the overlapping binding sites between Kvβ1 and AA. In fact the AA data mesh very well with the Kvβ binding site reported by MacKinnon´s group. Zhou et al. found that Kvβ1.1 binds to residues of Kv1.4 which are homologous to V505, I508, V512 and V516 of Kv1.5 (V551 of Kv1.4 corresponds to V505, I554 of Kv1.4 corresponds to I508, V558 of Kv1.4 corresponds to V512, V562 of Kv1.4 corresponds to V516). These residues line the inner cavity and consistently they interact with both Kvβ1.1 and AA. In addition, block by AA was perturbed by I502 and P513. These residues were previously not identified for Kvβ1.1 and they are not perfectly facing into the pore. However, as the alkyl chains of AA and AEA are highly flexible an additional interaction with these residues is possible. We have included a brief discussion of the overlapping binding sites identified for Kvβ1.1 and AA in the second paragraph of the Discussion on page 16.

2. In the mutagenic studies, there is an issue that should be addressed. The position equivalent to Kv1.1's I400 is also an I-to-V editing site in fly Shab (Kv2) and squid sqKv2 channels, a clear case of convergent evolution (Bhalla et al, 2004, Ryan et al 2008, Patton et al 1997). Both Ryan, 2009 and Patton, 1997 present data on the functional consequences for editing at this paralogous position to the isoleucine present in mammalian Kv1.1. But of course, HUFA treatments were not conducted in these studies. Nevertheless, the authors provide strong evidence for the modulation of Kv.X channels by HUFA and open channel blocking compounds, and the portability to non-editors (the Kv3.1 I-to-V experiment). So, this aspect of channel biology should be discussed and the authors should assess whether the functional studies in these papers could intersect with their own.

This is an excellent point. In the revised manuscript we have included data for Shaker and Shab channels introducing the Kv1.1^{I400V} equivalent exchange. In fact, the HUFA sensitivity was also altered for Shaker and Shab channels. We have included the novel data at the end of the first paragraph of the Results section (page 5) and have included a brief discussion, as you suggested, on page 17, second last paragraph of the Discussion section. See also comment iv) of referee 1.

3. Another issue with the mutagenic studies are the positions of the residues mutated in Kv1.5 that are further (V505, I502) into the pore structure than the I400 edited position. In particular, I502A. This position seems to have as much of an effect on resistance to AA as I508A (which is presumably comparable in effect as I400V in Kv1.1). How does this position confer such an affect given its location in the structure? This deserves some attention, if only because seems to be an unexpected result. In addition, this position is ALSO the location of a natural site of modification by RNA editing in Drosophila *Shaker* channel (Hoopengardner et al. 2003) and has been studied at the level of functional properties (Ingelsby et al, 2009). Since

this site was shown in Inglesby, 2009 to have a substantial effect on deactivation properties, it may have implications for AA's effects on the Kv1.5 I502A mutant.

An interaction with V505 makes perfectly sense and most Kv open-channel blockers, as well as Kvß1.3 interact with this residue (Decher et al, 2008; Decher et al, 2004; Strutz-Seebohm et al, 2007).

As stated in the response to comment 1, the residue I502 is not facing directly into the central cavity, but the side chains of the highly flexible fatty acid could easily interact with this residue. Most importantly however, I502 has been previously identified as a binding site for Kv open-channel blockers like disopyramide and AVE0118 (Arechiga et al, 2008; Decher et al, 2006). Given the non perfect orientation of the I502 side chain, the role of I502 in drug-binding and AA-binding is still not clear. It is also possible that the drug and AA effects are caused by allosteric mechanisms, as the I502A mutant has a poor expression and altered inactivation/deactivation properties.

As I502 is an editing site in Drosophila Shaker channels, we also examined whether Kv1.x channels of the thalamus, spinal cord and medulla have a homologous editing site in humans (data not shown). However, the only S6 segment editing we have observed was the I400V of Kv1.1. In future studies we aim to analyze the role of I502 in lipid binding which will include the study of the Shaker I464V editing exchange.

Arechiga et al. (2008) Kv1.5 open channel block by the antiarrhythmic drug disopyramide: molecular determinants of block. J Pharmacol Sci 108: 49-55

Decher et al. (2004) Molecular basis for Kv1.5 channel block: conservation of drug binding sites among voltage-gated K^+ channels. J Biol Chem 279: 394-400

Decher et al. (2006) Binding site of a novel Kv1.5 blocker: a "foot in the door" against atrial fibrillation. Mol Pharmacol 70: 1204-1211

Decher et al. (2008) Structural determinants of Kvbeta1.3-induced channel inactivation: a hairpin modulated by PIP_2. Embo J 27: 3164-3174

Strutz-Seebohm et al. (2007) Comparison of potent Kv1.5 potassium channel inhibitors reveals the molecular basis for blocking kinetics and binding mode. Cell Physiol Biochem 20: 791-800

Referee #3 (Remarks to the Author):

In this work, the authors studied the mechanisms of action and the binding site of highly unsaturated lipids such as arachidonic acid, docosahexaenoic acid and anandamide, which produce an apparent fast inactivation in Kv1 voltage-gated K^+ channels. They found that these lipids interact with hydrophobic residues lining the inner cavity of the pore at the middle of the S6 segment and more specifically with the residue isoleucine 400 of Kv1.1 that lines the inner cavity of the pore. These unsaturated lipids were shown to compete with intracellular tetraethylammonium and Kvß subunits, suggesting that the inactivation they produce probably reflects occlusion of the permeation pathway, similar to drugs that produce an open-channel block. Performing docking experiments with anandamide, the authors showed that the pore is wide enough to accommodate the anandamide molecule and that its binding will significantly narrow the pore width so that its remaining diameter is too small to allow a hydrated potassium ion to permeate the channel. In addition, they found that the open-channel block by arachidonic acid, docosahexaenoic acid and anandamide is substantially decreased in 'edited' Kv1.1 channels, when residue I400 is edited to V400. Further, they showed that RNA editing in neurons of the lateral geniculate nucleus of the thalamus markedly alters the sensitivity of Kv1 channels to pharmacological inhibition.

This manuscript is interesting and reflects an extensive study that is technically well done and clearly described. The work describes an important topic since it addresses the issue of the effect of physiologically relevant unsaturated lipids on voltage-gated Kv1 potassium channels and its impact on their edited version in neurons. However, I have some concerns with specific issues that should be clarified.

1-The authors provide some lines of evidence that the unsaturated lipids work on WT Kv1.1 channels by docking to the open channel pore cavity, thereby occluding the permeation pathway. This mechanism implies that K^+ influx will expel the lipid molecule from its binding site and will result in an acceleration of recovery from "inactivation-block" in high external K^+ as was demonstrated for the inactivation particle of Shaker K^+ channels by Demo and Yellen (Neuron 1991). To test directly reopening of Kv1.1 channels during recovery from "inactivation-block", the authors should examine the kinetics of tail currents in the presence of high external K^+ (120-140 mM K^+).

We have performed the experiments reported by Demo and Yellen (Neuron 1991) and we did not observe an acceleration in the recovery from "inactivation-block" by AA in high extracellular K^+ (tau = 98 ± 9 ms (n = 5) in 120 mM K^+) compared to low K^+ (tau = 90 ± 7 ms (n = 5) in 4 mM K^+). However, in all of the instances of knock-off reported for K^+ channels, the open-channel blocker has a positively charged moiety (e.g. the highly charged ball peptide, TEA^+, TBA^+ or Charybdotoxin) that appears to interact with specific sites near the internal or external mouth of the pore. Further, the knock-off phenomenon appears to involve interactions of the blocker with a K^+ binding site (Park and Miller, 1992; Thompson and Begenisich, 2003; Boccaccio et al., 2004). AEA and AA are uncharged or negatively charged and are not likely to interact with a K^+ binding site directly or reduce K^+ binding via electrostatic repulsion and, thus, it is quite reasonable that knock-off is not seen. It is also noteworthy, that other primarily uncharged open-channel blockers with an established binding site in the pore cavity in Kv1.5 lack an effect of extracellular K^+ (Strutz-Seebohm et al, 2007).

Boccaccio et al. (2004) Binding of kappa-conotoxin PVIIA to Shaker K+ channels reveals different K+ and Rb+ occupancies within the ion channel pore. J Gen Physiol 124: 71-81

Park and Miller (1992) Interaction of charybdotoxin with permeant ions inside the pore of a K+ channel. Neuron 9: 307-313

Strutz-Seebohm et al. (2007) Comparison of potent Kv1.5 potassium channel inhibitors reveals the molecular basis for blocking kinetics and binding mode. Cell Physiol Biochem 20: 791-800

Thompson and Begenisich (2003) Functional identification of ion binding sites at the internal end of the pore in Shaker K+ channels. J Physiol 549: 107-120

2-It is not clear to the reviewer how the authors checked the editing of Kv1.1 channels in the specific neurons they record, since the quantification they provide represents a an average value. How other K^+ conductances that are not sensitive to the unsaturated lipids, independently of the editing process, are taken into account? The authors should also provide a set of recording from neurons that express the unedited version (I400) of Kv1.1 as a positive control.

This is an excellent point, as the quantification of RNA editing in the LGN tissue might include a set of different cell populations. Therefore, the cleanest way would be single-cell RT-PCRs of the cells that were patched. Although, we did not perform single-cell RT-PCRs the level of RNA editing and the level of drug-resistance from a set of cells in this particular region seem to correlate very well. This is strengthened by the additional control experiments we have performed in CA1 hippocampal neurons (see below).

Other K^+ conductances that are not sensitive to unsaturated lipids were not pre-blocked as it would demand a pharmacological cocktail containing a large diversity of blockers that leave Kv1.x channel unaffected. To our knowledge this is currently not possible, due to the lack of specific compounds. Therefore, the use of the Kv1.x specific Psora-4 and HgTx1 seems currently the "cleanest" pharmacological way to isolate Kv1.x currents and to describe drug-resistance with the highly selective HgTx1.

As suggested, we have performed control experiments in neurons that have less Kv1.1 editing. Unfortunately, there are no neurons known to have a complete lack of Kv1.1 I-to-V editing. We have analyzed cells from the hippocampus, as these express Kv1 channels and have reportedly little Kv1.1 editing. We have quantified the $Kv1.1^{I400V}$ editing ratio in the CA1 to CA3 region of the rat and found that only 7 % of the mRNA transcripts are I-to-V edited.

Consistently, we found in patch clamp experiments of rat CA1 neurons that only 15 % of the Kv1.x current is Psora-4 resistant, in contrast to the 63 % in LGN neurons. These data show that there is a strong correlation of I400V editing and the observed Psora-4 drug-resistance in native tissue. The additional data is incorporated in the Results section (page 14), Figures 7J-L and the Supplementary Figure 5.

A similar comment/criticism was made by another referee, see also response to referee 1, point ix).

3-In the introduction, an identical paragraph, page 4, line 15-23 "amphiphilic substances..."appears twice in the introduction page 3.

Thank you. We have removed the paragraph that appeared twice.

2nd Editorial Decision 14 April 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2009- 73316R. It has now been seen again by all three referees, whose (brief) comments are enclosed below. As you will see, all three reviewers are happy with the revision. Therefore, I am pleased to be able to tell you that we can accept your manuscript for publication in the EMBO Journal without further revision. You should receive the formal acceptance message shortly.

Many thanks for choosing the EMBO Journal for publication of this very nice piece of work!

Best wishes,

Editor

EMBO Journal

Referee 1:

The authors have satisfactorily answered the reviewers' queries. The revised ms is now acceptable for publication.

Referee 2:

I think that the authors have made heroic efforts to answer all reasonable reviewer comments and the paper is definitely better for it, and should be accepted without delay.

Referee 3:

The authors adequately addressed all concerns raised in the previous version.

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