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Block of Kv1.7 potassium currents increases glucose-stimulated insulin secretion

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1st Editorial Decision 11 March 2011

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

In particular, reviewer #1 highlights that the in vivo data should be developed further to convincingly address the translational implications of the study. Importantly, this reviewer also points out that it is crucial to investigate the response of the different Kv channels to Conk-S1 in actual islet cells and makes specific suggestions how the data suggesting specificity of Conk-S1 for Kv1.7 could be strengthened. In addition, reviewer #3 notes concerns about the quality of the islets under study, which should be investigated.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the space and time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged differently with the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

In this study, the authors have investigated the effects of a cone snail toxin peptide, Conk-S1, on regulation of ion channel activity and insulin secretion. They demonstrate that Conk-S1 has preferential inhibitory activity against the voltage-gated potassium (Kv) channel Kv1.7 relative to several other Kv1 or Kv2 channels when expressed in oocytes. Conk-S1 is shown to have modest effects of delayed rectifier currents and Rb efflux in rodent islet cells, with some evidence of specific effects on beta-cells. Administration of Conk-S1 to animals causes a very modest enhancement of insulin secretion and glucose clearance during glucose tolerance tests or glucose clamp experiments. The authors conclude that agents that target Kv1.7 may be safe new therapeutic agents for enhancing insulin secretion in type 2 diabetes.

Whereas the experiments appear to be technically sound and carefully performed, the study is quite preliminary in its current form and does not go far enough to prove that Kv1.7 is in fact the molecular entity that is targeted in normal pancreatic islets by Conk-S1. Moreover, there are some apparent inconsistencies in the data, and the effects of Conk-S1 in vivo are vanishingly small, such that any conclusions about potential diabetes therapies are completely unjustified at this early stage. Key major points are as follows:

1) A major concern is that the key experiment attempting to establish specificity of Conk-S1 for Kv1.7 is the comparison of IC50 values relative to those of a panel of other Kv proteins expressed in Xenopus oocytes. As the authors are well aware, Kv proteins expressed in oocytes may not engage in the same heteromeric complexes as found in normal islet cells, and this could have major effects on the activities and responses of the different Kv channels to Conk-S1 in actual islet cells. Experiments demonstrating that knock-out or knock-down of Kv1.7 expression in islets eliminates the effects of Conk-S1 on delayed rectifier currents and insulin secretion are absolutely needed in order for the authors to support their current conclusions.

2) The authors show potentiation of GSIS by Conk-S1 at 4 mM glucose in Figure 2B, but fail to demonstrate key electrophysiologic effects of Conk-S1 such as integrated depolarization, spike frequency, or Fluo-4 Ca2+ fluorescence at 5 mM glucose. How do the authors reconcile these seemingly disparate results?

3) The effects of Conk-S1 on insulin secretion and glucose levels during the glucose tolerance test are miniscule, and occur only at 1 time point for glucose and possibly never attain significance with regard to insulin (not clear about this from the labeling of the right panel of Figure 4A). The authors suggest that these data support the idea that Kv1.7 inhibition can be considered a therapeutic target for diabetes. Again, the data are far too preliminary to support such a claim, and must be further developed with studies in diabetic models and possibility in knock-out mice to fully address the translational implications.

4. The claim of beta-cell specificity of Conk-S1 regulation of Kv1.7 activity should be further tested by measurement of glucagon and somatostatin release from islets treated with and without Conk-S1.

Referee #2 (Comments on Novelty/Model System):

I have followed this topic for years and think this manuscript will add a lot to the field. This work introduces a new player in beta cell physiology (Kv1.7) and the conotoxin specificity and data presented suggest we have a new diabetes treatment on the horizon.

Referee #2 (Other Remarks):

This manuscript presents an interesting and timely story with direct clinical relevance. It adds much to the field of beta cell membrane potential control over insulin release. However, the following concerns need to be addressed.

Major points.

I am concerned with the analysis of the Fig. 2B data. Could the significance at 4- to 10 mM glucose simply be due to the decreased insulin release at 0.3 M Conk-S1? It seems that a one way ANOVA is not the best way to analyze these data. Means with the SEM are shown and the N varies form 3-11, making the reader wonder about the relationship between the error and N. In addition, exactly where are differences plotted in Fig. 2C from? This is an important figure and it needs to be better presented and discussed.

Minor points.

1. Why does Fig. 1 C not show the use of a more saturating Conk-S1 concentration?

2. The abstract should state the species in which the in vivo work was done.

3. In the Rb+ flux experiments, is Kv1.7 blocked by the 10 mM TEA?

4. In the Fig. 2A legend, it is not really an "inset". The figure legend explanation of the Rb+ flux could be better presented.

5. With respect to Fig1C and Fig. S1B, all 10 cells clamps had the single cell RT-PCR done?

6. Why not make Fig. S3 similar to Fig. 3. But put all the 15 mM glucose data in Fig. 3 and the 5 mM glucose data in the supplement.

7. Why not also show cell images in Fig. S4B? Also in this figure, are the intensities from the entire field, all the cells or a selected ROI?

8. In Fig. 3C, why does N=5 if there are 8 traces in Fig. 2B?

9. A discussion of peptide access to the beta cells following IV delivery would be nice as would a discussion of the Conk-S1 concentrations used relative the Kv1.7 IC50.

Referee #3 (Comments on Novelty/Model System):

Overall, there are some very nice things about the manuscript and I appreciate the novelty and potential importance of the focus on Kv 1.7 channels for the beta cell field. However, I am concerned about some technical aspects with regards to the quality of the islets under study: they appear active to too great an extent in 5 mM glucose, which should be subthreshold, and they do not secrete as much as I would expect for healthy rat islets to a glucose challenge. I do not think an alternate model would be better.

Referee #3 (Other Remarks):

This is an interesting paper that describes studies of the role of Kv1.7 type delayed rectifier K channels in beta cell function. The authors used a combined approach to show that the the conopeptide Conk-S1 selectively blocks a component of delayed rectifier K currents in rat beta cells and that this leads to an increase in spike frequency and spike duration on the electrical level. Conk-S1 also dose dependently potentiates GSIS from rat islets and improves glucose tolerance when injected into the whole animal without attendance hypoglycemia.

It is an impressive range of studies and the results seem quite consistent. However, I do have some concerns as addressed below:

1. The study rests importantly on results obtained from rat islets but there is a concern about the functional competence of these since they only appear to secrete 2x more insulin in 16 vs. 1 mM glucose alone (Fig. 2B), which is on the low side. In addition, this could reflect unusually high basal

secretion, as the islets were apparently active in 5 mM glucose, usually considered to be subthreshold for GSIS in rodents, and there was a trend towards increased secretion in 5 mM glucose as Conk-S1 concentration was raised (also 2B). Moreover, the electrical recordings shown in Fig. 3 indicate spiking in 5 mM glucose which is odd; normal beta cells are quiescent in the range of 2.8-5 mM glucose. Also, another possible indication of compromised health is the lack of functional bursting seen in Fig. 3A even though the recordings were to the best of my knowledge from only partly dispersed islets. Were islets completely quiet and not secreting in 2.8 mM glucose or less?

2. The effects of Conk-S1 on spiking were reasonable looking but this is perhaps surprising given the rather modest effect of the toxin on the K current tracings shown in Fig. 1C. Perusal of the IV curves shown in S1B indicates no difference in Kv current at -20 mV even though this is the peak voltage of the voltage spikes recorded from rat beta cells (3B). Thus, there are some disconnects in the story that might reflect either unaccounted for voltage offsets or off target effects on other channels (do we know Conk-S1 is without effect on Ca channels for example?), albeit these would seem unlikely. This certainly needs to be discussed at least.

I was also concerned that the tests were done using 10 μM Conk-S1 while the OC50 obtained for Kv1.7 was 439 nM. This is >20x the IC50 of the toxin.

3. The data shown in Fig. 4A suggests that including Conk-S1 increases insulin at 10 min. by 100% yet this only decreases glucose levels in the tolerance test (at 30 min.) by 20% or so. Can the authors explain what is happening here?

4. Glucose is also regulated by CNS mechanisms and it would seem possible that in vivo effects of the toxin could be mediated by hypothalamic neurons. Do the authors know whether or not the Conk-S1 can cross the blood brain barrier?

5. There are some complex things happening in Fig. 2B. Increasing the dose of the toxin seems to dose dependently increase 'basal' secretion although this was not statistically significant, and at higher glucose, adding toxin seemed to decrease secretion. This should at least be commented upon.

1st Revision - Authors' Response 20 December 2011

Here, we outline our approach to the revision, and the changes incorporated into the revised manuscript.

New Experiments
In our revised manuscript, we report new experiments showing that Conk-S1 inhibits heteromeric constructs containing Kv1.7 alpha-subunits, with an affinity comparable to that for homotetrameric Kv1.7 used in our broad screening of Conk-S1 targeting. A new, moderate throughput screen showed some dependence of release of glucagon and pancreatic polypeptide on glucose concentration, no glucose dependence of somatostatin release, and as expected, no detectable release of leptin. In contrast to our extensive studies on insulin release and islet cell excitability, these new data revealed no systematic enhancement by Conk-S1 of release of any of these four additional peptides.

New Analysis
In the revision, we have also expanded our statistical analyses using more versatile software. Notably, the islet insulin release data of Fig. 2 show highly significant dependencies on both [ConkS1] ($P=0.0009$) and [glucose] ($P<0.0001$) with 2-way ANOVA. A similar high level of significance for Conk-S1 dependence is also indicated by a 1-way ANOVA, which is an alternate test suggested by the software documentation (GraphPad Prism for Mac, v5.0d). Post-hoc pairwise comparisons show increasing significance of the glucose-associated change with higher Conk-S1 concentrations.

Limitations
In the time available, we have not had the resources to establish molecular knock down/out studies. Adding a rigorous examination on either a knockout (ideally a conditional knock-out or knock down), or a disease model, would require repetition of most of the functional studies already performed and reasonably would occupy at least one additional full-length paper. We have, in fact, done preliminary experiments in both these areas, but we argue such complete studies would go beyond the reasonable scope of the present submission, and are precluded at present by lack of suitable animals and certain supporting reagents, including specific high-quality antibodies.

To recapitulate, we provide:
1. Molecular expression studies showing Conk-S1 preferential inhibition of both homomeric and heteromeric Kv channels containing Kv1.7.
2. Reductionist analysis of Conk-S1's action by voltage-current clamp of single cells in expression systems.
3. Confirmation of changes of electrical activity in partially dissociated islet tissue stimulated by current clamp, which offers a far more sensitive test of Conk-S1'a action than voltage clamp alone, because of the regenerative nature of islet cell electrical bursting.
4. Two types of whole animal study indicating subtle but significant effects on insulin release and control of blood glucose levels.
Results obtained at all of these levels of organization are self-consistent and statistically significant.

We agree that many more detailed experiments could be done, and we hope that our work will stimulate such studies, but we argue that the breadth and rigor of our present data and analysis are equal to many papers in elite, general interest journals. We are confident that the current draft meets the goal of providing a rigorous, multi-faceted account of a hitherto unrecognized contributor to the control of insulin release.

We identify the key outcome aspect of our work as the insight into the role of Kv1.7 as a fundamental, specific element in the control of insulin release. While the data reveal clear potential for translational application, we note that elaborating the details of such an application goes well beyond the reasonable and practical scope of our present paper.

Changes to Figures & Tables:
Fig. 2 – revised, showing new analysis, Fig. 2C deleted (0.3uM data removed from figure – see response to Reviewer #2)
Fig. 5 – new, demonstrates preferential Conk-S1 block of Kv1.7-containing heteromers
Fig. S3 – new, shows lack of effect of Conk-S1 on 4 metabolic hormones
Fig. S5 – revised (cell images added)
Tables S2, S3 – new, present statistical analysis details

We appreciate the probing comments of the reviewers, which we believe have helped us to substantially strengthen the manuscript. We hope that you find the revised manuscript suitable for publication in EMBO Molecular Medicine, and we look forward to hearing from you.

Specific responses to comments of the individual reviewers are described below. Each response is indented below the relevant quoted remarks of the Reviewer.

Reviewer #1

1. "A major concern is --- the key experiment attempting to establish specificity of Conk-S1 for Kv1.7 --- in Xenopus oocytes. As the authors are well aware, Kv proteins expressed in oocytes may not engage in the same heteromeric complexes as found in normal islet cells, and this could have major effects on the activities and responses of the different Kv channels to Conk-S1 in actual islet cells."

> We agree, and for this reason we have systematically addressed this issue in experiments on dimer-of-dimers constructs of the form Kv1.2/Kv1.x or Kv1.x/Kv1.2. In these heteromeric constructs, Kv1.7 dominated the interaction with Conk-S1, increasing its affinity by more

than 10-fold above that shown by homomeric Kv1.2 channels (Fig. 5, revised ms). In the context of our broad screen of Conk-S1's inhibitory action against homotetrameric Kv channels, this new evidence argues strongly that Kv1.7 provides the basis of Conk-S1's action in islet cells.

"Experiments demonstrating that knock-out or knock-down of Kv1.7 expression in islets eliminates the effects of Conk-S1 on delayed rectifier currents and insulin secretion are absolutely needed --- "

We agree that these experiments should provide a further important test, but also consider that the dimer-of-dimers experiments clearly and directly demonstrate the ability of Kv1.7 to direct Conk-S1 targeting. In contrast, Kv1.7 knock-down would not, per se, identify interactions with heteromeric channels.

Further on this issue, please see the "Limitations" paragraph in the Cover Letter.

2. "The authors show potentiation of GSIS by Conk-S1 at 4 mM glucose in Figure 2B, but fail to demonstrate key electrophysiologic effects of Conk-S1 such as integrated depolarization, spike frequency, or Fluo-4 Ca2+ fluorescence at 5 mM glucose. How do the authors reconcile these seemingly disparate results?"

We do not consider these results to be "disparate", given that these experiments involve measuring very different parameters from various preparations through the use of diverse techniques. Given the nonlinearity of the different steps along the signaling pathway that leads to insulin release, it would be surprising if different measured parameters showed identical dose dependencies. Nevertheless, small effects on action potential waveform (Fig. 3B), depolarization and spike frequency are clearly visible, even though there are not statistically significant changes in the integrated depolarization or firing frequency at 5 mM glucose, near the lower end of the activating range for insulin secretion (now shown in Fig. S4B, Supplementary Information).

3. "The effects of Conk-S1 on insulin secretion and glucose levels during the glucose tolerance test are miniscule, and occur only at 1 time point for glucose and possibly never attain significance with regard to insulin (not clear about this from the labeling of the right panel of Figure 4A). The authors suggest that these data support the idea that Kv1.7 inhibition can be considered a therapeutic target for diabetes. Again, the data are far too preliminary to support such a claim, and must be further developed with studies in diabetic models and possibility in knock-out mice to fully address the translational implications."

Please see also the comments in the cover letter to the Editor. We did not intend to imply that inhibiting Kv1.7 is established as a therapeutic tactic, but we do consider that demonstrating Kv1.7's modulatory role, at levels from the molecular to the whole animal, does justify our conservative reference to it as a potential therapeutic target, and we are open to other suggestions as to specific wording.

Regarding the observed Conk-S1 effect in the whole animal studies presented in the current ms, the Conk-S1 data for glucose and insulin levels in the glucose tolerance test, and the insulin levels during the transient in the glucose clamp are significantly different from the respective control values and are entirely self-consistent with each other. Furthermore, given that Kv1.7 is quantitatively a small component of the total Kv currents in beta cells, only limited effects are expected (see also below).

In the revised Fig. 4, we have moved the asterisks to reduce the possibility of associating it with the wrong comparison. Our apologies for any confusion caused by this, but we note that the comparison highlighted by the asterisk was and is correctly identified in the figure legend.

4. "The claim of beta-cell specificity of Conk-S1 regulation --- should be further tested by measurement of glucagon and somatostatin release from islets treated with and without Conk-S1"

New data have been added to the Supplemental Information which support the conclusion that the Conk-S1 enhancement of peptide secretion is specific to insulin (Fig. S3) in a screen of a panel of 4 additional hormonal peptides.

Overall, we underline the point that the subtlety and selective targeting of the effects of Conk-S1 (both molecular and functional), which result from Kv1.7's limited expression, are in fact important to its potential pharmacological interest.

Referee #2 (Comments on Novelty/Model System):

"This manuscript presents an interesting and timely story with direct clinical relevance. It adds much to the field of beta cell membrane potential control over insulin release. However, the following concerns need to be addressed."

Major points.
"I am concerned with the analysis of the Fig. 2B data. Could the significance at 4- to 10 mM glucose simply be due to the decreased insulin release at 0.3 μM Conk-S1? It seems that a one way ANOVA is not the best way to analyze these data. Means with the SEM are shown and the N varies form 3-11, making the reader wonder about the relationship between the error and N. In addition, exactly where are differences plotted in Fig. 2C from? This is an important figure and it needs to be better presented and discussed."

These data have been re-analyzed using more flexible software.
Fig. 3C seems to have been more a source of confusion than a clarification in the presentation and thus it has been deleted. Equivalent information is provided by the 2-way ANOVA, with Bonferroni pairwise comparisons, for Fig. 2B. See also Supplemental Information. It is open to debate as to which statistical test provides the most direct mechanistic insight, but the statistical significance of the dependence of insulin secretion on [Conk-S1] and [glucose] is unambiguous.

A special note is pertinent with regard to the reviewer's comments on the perceived "decreased" insulin secretion at 0.3 μM Conk-S1. First, this appeared only to be significant at a single [Glucose] (7mM) in the previous submission. The simplest implementation of 2-way ANOVA utilizes values of Mean, SEM, and N under each condition. This is the calculation which is presented in the revised ms. With this approach, empty cells for particular combinations of [glucose] and [Conk-S1] could not be used, and for this reason, the data in the Revised Fig 2 do not include the data for 0.3 μM Conk-S1. For this reason, we performed the following additional analyses to address the reviewer's concerns. Both 2-way and 1-way ANOVAs were carried out on the data for all [Conk-S1], including 0.3 μM Conk-S1, but with [glucose] = 4-16 mM. Also, a 2-way ANOVA was employed on the full data set entered as individual values, rather than means, SEMs, and N values. This allows an analysis working around missing data points. All of these analyses yielded significant dependence of insulin secretion on Conk-S1 at a level of $p<0.0001$.

We conclude that there is a highly significant dependence of insulin release on Conk-S1.

Minor points.
1. "Why does Fig. 1 C not show the use of a more saturating Conk-S1 concentration?"

The figure serves the purpose of illustrating clear, reversible Conk-S1 inhibition at a concentration near the IC_{50} for block of homotetrameric Kv1.7 in mammalian cells. The best measurements for this are determination of the relatively small difference currents for control, and Conk-S1 solutions, with subsequent washout of the peptide. It was not practical for us to expend the requisite amount of Conk-S1 to determine a complete dose-response curve using this experimental protocol. However, higher Conk-S1 concentrations in various other protocols gave no evidence of non-specific effects of the peptide.

2. "The abstract should state the species in which the in vivo work was done."
Added (rat).

3. "In the Rb+ flux experiments, is Kv1.7 blocked by the 10 mM TEA?"
Based on mKv1.7 screening experiments, this concentration would be expected to block at least 80% of homomeric Kv1.7 channels.

4. "In the Fig. 2A legend, it is not really an "inset". The figure legend explanation of the Rb^+ flux could be better presented."
The "inset" is relabeled as follows (A, right panel) Expanded presentation of Kv channel fluxes.

5. "With respect to Fig1C and Fig. S1B, all 10 cells clamped had the single cell RT-PCR done?"
Yes. This is now noted in the Methods of the main manuscript file.

6. "Why not make Fig. S3 similar to Fig. 3. But put all the 15 mM glucose data in Fig. 3 and the 5 mM glucose data in the supplement."
Done.

7. "Why not also show cell images in Fig. S4B? Also in this figure, are the intensities from the entire field, all the cells or a selected ROI?"
Images have been added to the figure as suggested.
Intensity traces were taken from a single ROI, which enclosed the chosen cell(s), with minimal background area, for the whole period of the recording.

8. In Fig. 3C, why does N=5 if there are 8 traces in Fig. 2B?
The example traces in 3B are from a single experiment; for 3C, 5 experiments were analyzed and summarized.

9. A discussion of peptide access to the beta cells following IV delivery would be nice as would a discussion of the Conk-S1 concentrations used relative the Kv1.7 IC_{50}.
Clearly, there are likely to be additional diffusion barriers for Conk-S1 to access secreting cells in islets and in intact animals than for isolated cultured cells in the voltage clamp experiments. However, given that Conk-S1 blocks from the extracellular surface, and that islets are highly vascular, access of the hydrophilic Conk-S1 presumably would follow pathways, taken in the reverse direction, similar to those traversed by pancreatic hormones (e.g. insulin) after secretion. As mentioned in the detailed methods in the Supplementary Information, Conk-S1 was administered 130 min prior to the glucose challenge, to give an estimated plasma concentration of 1-2 μM during the experimental measurements, which is about 2-4x IC_{50}.

<u>Referee #3</u> (Comments on Novelty/Model System):
"Overall, there are some very nice things about the manuscript and I appreciate the novelty and potential importance of the focus on Kv 1.7 channels for the beta cell field. However, I am concerned about some technical aspects with regards to the quality of the islets under study: they appear active to too great an extent in 5 mM glucose, which should be subthreshold, and they do not secrete as much as I would expect for healthy rat islets to a glucose challenge. I do not think an alternate model would be better."

We thank the reviewer for the positive comments, and will address the specific issues below. In our view, a major strength of the work is that the qualitative conclusions from the variety of experimental models used converge to a common interpretation of the action of Conk-S1 and role of Kv1.7.
Precise quantitative comparison of certain parameters is unrealistic in some cases because of the different variables measured and the complexity of their inter-relationships. There are differences in conditions imposed by technical considerations (e.g. islet bursting electrical activity is more easily observed at higher temperatures – closer to body temperature – than the room temperature used for voltage clamp screening experiments).

Referee #3 (Other Remarks):
" --- It is an impressive range of studies and the results seem quite consistent. However, I do have some concerns as addressed below:"

1. "The study rests importantly on results obtained from rat islets but there is a concern about the functional competence of these since they only appear to secrete ≈2x more insulin in 16 vs. 1 mM glucose alone (Fig. 2B), which is on the low side. In addition, this could reflect unusually high basal secretion, as the islets were apparently active in 5 mM glucose, usually considered to be subthreshold for GSIS in rodents, and there was a trend towards increased secretion in 5 mM glucose as Conk-S1 concentration was raised (also 2B). Moreover, the electrical recordings shown in Fig. 3 indicate spiking in 5 mM glucose, which is odd; normal beta cells are quiescent in the range of 2.8-5 mM glucose. Also, another possible indication of compromised health is the lack of functional bursting seen in Fig. 3A even though the recordings were to the best of my knowledge from only partly dispersed islets. Were islets completely quiet and not secreting in 2.8 mM glucose or less?"

> As shown in Fig. 2B, we detected small amounts insulin release at 0 and 1 mM glucose. During current clamp protocols, there was considerable "cell to cell", or "clump to clump" variation in burst behaviour, with a substantial number of recordings showing no AP activity at 5mM glucose. This could result from a variety of causes, including variable cell-to-cell coupling following the partial dissociation of the pancreas requited to provide access to the islets. For practical reasons, electrical, flux, and secretion measurements were necessarily done on separate islet preparations

2." The effects of Conk-S1 on spiking were reasonable looking but this is perhaps surprising given the rather modest effect of the toxin on the K current tracings shown in Fig. 1C. Perusal of the IV curves shown in S1B indicates no difference in Kv current at -20 mV even though this is the peak voltage of the voltage spikes recorded from rat beta cells (3B). Thus, there are some disconnects in the story that might reflect either unaccounted for voltage offsets or off target effects on other channels (do we know Conk-S1 is without effect on Ca channels for example?), albeit these would seem unlikely. This certainly needs to be discussed at least."

> Effects on Cav channels at synapses, or in the cardiovascular system would be expected to lead to systemic side effects in the whole animal experiments. Such outcomes were not observed.

"I was also concerned that the tests were done using 10 μM Conk-S1 while the IC50 obtained for Kv1.7 was 439 nM. This is >20x the IC50 of the toxin."

> As noted in the response to Referee #2, higher Conk-S1 concentrations were deliberately chosen to be relatively high in order to increase the likelihood of adequate access to the islets, and to ensure block of the majority of Kv1.7-containing channels. No detectable side effects were observed.

3. "The data shown in Fig. 4A suggests that including Conk-S1 increases insulin at 10 min. by 100% yet this only decreases glucose levels in the tolerance test (at 30 min.) by 20% or so. Can the authors explain what is happening here?"

> In the Conk-S1 animals, the insulin drops rapidly after 20' to values below or at control levels. This may blunt the Conk-S1 action to reduce the glucose compared to the more dramatic effect of glibenclamide, which caused a more profound and sustained drop in [glucose]. It also may part of the reason that Conk-S1 modulation of bursting, via inhibition of Kv1.7, appears to act most strongly on the transient response to a glucose challenge.

4. "Glucose is also regulated by CNS mechanisms and it would seem possible that in vivo effects of the toxin could be mediated by hypothalamic neurons. Do the authors know whether or not the Conk-S1 can cross the blood brain barrier?"

> To our knowledge, the only Kunitz-related peptides reported to cross the blood-brain barrier are BPTI fragments, specifically chosen and/or engineered to do so. Conk-S1 is large and

highly hydrophilic, and thus unlikely to do so. The glucose clamp experiment using pithed rats (Fig. 4B) rules out the possibility of direct central nervous input being necessary for the Conk-S1 effect, and the lack of side effects in the glucose tolerance test (Fig. 4A and Fig. S5) makes a CNS site of action for Conk-S1 highly unlikely, probably because it does not cross the blood-brain barrier.

5. "There are some complex things happening in Fig. 2B. Increasing the dose of the toxin seems to dose dependently increase 'basal' secretion although this was not statistically significant, and at higher glucose, adding toxin seemed to decrease secretion. This should at least be commented upon."

The reviewer's sharp eye appears to have focused on a single point (0.3 μM Conk-S1, 7 mM glucose) where there seemed to be a decrease in insulin secretion. This point did seem to be significantly lower than the corresponding control (0 μM Conk-S1, 7 mM glucose point), it was the only Conk-S1 data point for which that was seen, and we suggest that this is not a systematic effect.
On the other hand, overall association of higher insulin secretion with increasing [Conk-S1] is robust, and is verified by several analyses with and without including the 0.3 μM Conk-S1 data (please see also the response to Referee #2 regarding Fig. 2B).

2nd Editorial Decision 14 December 2011

Thank you for the submission of your revised manuscript "Block of Kv1.7 potassium currents increases glucose-stimulated insulin secretion" to EMBO Molecular Medicine and please accept my apologies for the delayed reply. We have now received the reports from the reviewers who were asked to re-review your manuscript.

You will be glad to see that they are now globally supportive and we can proceed with official acceptance of your manuscript pending the minor changes detailed below.

Please see below for information regarding EMBO Molecular Medicine guidelines for statistical analysis of data. Please mention the actual p value in each case it was calculated.

Statistical analysis:
The description of all reported data that includes statistical testing must state the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the actual P value for each test (not merely 'significant' or 'P < 0.05').

I look forward to seeing a revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #2 (Comments on Novelty/Model System):

I believe additional ways to modulate insulin release can only be of potential medical benefit. This story is well done and describes a new drug target not unlike how Ca2+ channel blocking conotoxins led to new pain treatments. I liked this manuscript the first time around and now it is better. The request by one reviewer for Kv1.7 knock-out or knock experiments in islets is, in my opinion, unreasonable.

Referee #2 (Other Remarks):

My limited concerns have been addressed.

Referee #3 (Comments on Novelty/Model System):

The paper reports results from a variety of techniques which is good, but the data are of average quality, especially the secretory data. As for medical impact, one would have to say that is limited for now, as there is only potential medical impact as for many basic science studies of new approaches.

Referee #3 (Other Remarks):

I think the authors have done a good job of responding to the reviews overall. There would have been some benefit to adding gene silencing experiments and perhaps refining the insulin secretion experiments but overall the new added data is supportive.

2nd Revision - Authors' Response 12 January 2012

Here, we outline the changes made in response to your request for additional minor revisions to our manuscript.

The names of all statistical tests and the numbers of independent experiments are provided. An account of probabilities provided by the different statistical tests has been added where needed. Where available from the software, individual p values are stated. In some cases, this information was available only in the form of graded inequalities, generally spaced no more than 10-fold apart (e.g. <0.05, <0.01, <0.001, etc.) thus giving a clear ranking of the relative certainty that can be assigned to inferences drawn from different results. This detailed statistical information has been provided throughout, either on the Figure Legends or in the Supplementary Information, as follows.

Figure legends
Where possible without making the text unwieldy, individual values for numbers of independent experiments and p values were included directly in the figure legends of the main article, as follows:
Fig. 3 and Fig. 5.
Fig. S1, Fig. S4B, and Fig. S6

Supplementary Information
In some cases, we judged that it was more appropriate to tabulate larger collections of p values in the online Supplementary Information, rather than disrupt the narrative with lengthy tables of important, but negative results. However, when these details were relegated to the online data, the ranges and trends were summarized in the main article in sufficient detail to give a clear indication of reliability of the conclusions, along with a citation of the more complete tabulations in the online Supplementary information. Cases documented in this manner were:
Fig. 2A – Table S2, Fig. 2B – Table S3.
Fig. 4A – Table S5, Fig. 4B – Table S6.
Fig. S3 - Table S4, Data on metabolic hormones other than insulin (Table S3 in the previous submission)

Figures were checked and revised as needed to conform as closely as possible to the journal's specifications.

As indicated below, there were no new revisions requested by the Referees.

***** Reviewer's Comments *****

Referee #2 (Comments on Novelty/Model System):
I believe additional ways to modulate insulin release can only be of potential medical benefit. This story is well done and describes a new drug target not unlike how Ca2+ channel blocking conotoxins led to new pain treatments. I liked this manuscript the first time around and now it is better. The request by one reviewer for Kv1.7 knock-out or knock experiments in islets is, in my opinion, unreasonable.
Referee #2 (Other Remarks):
My limited concerns have been addressed.

Referee #3 (Comments on Novelty/Model System):
The paper reports results from a variety of techniques which is good, but the data are of average quality, especially the secretory data. As for medical impact, one would have to say that is limited for now, as there is only potential medical impact as for many basic science studies of new approaches.
Referee #3 (Other Remarks):
I think the authors have done a good job of responding to the reviews overall. There would have been some benefit to adding gene silencing experiments and perhaps refining the insulin secretion experiments but overall the new added data is supportive.

