molecular systems biology

Competing G protein-coupled receptor kinases balance G protein and β-arrestin signalling

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Review timeline:

Submission date:	22 August 2011
Editorial Decision:	04 October 2012
Revision received:	16 May 2012
Editorial Decision:	12 April 2012
Revision received:	18 May 2012
Accepted:	23 May 2012

Transaction Report:

(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 October 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest. They raise substantial concerns on your work, which, I am afraid to say, preclude its publication in its present form.

The reviewers acknowledged that this work presented a detailed modeling analysis of GRK signaling. Nonetheless, each reviewer provided very detailed reports that raised a series of concerns, some of which we feel are potentially fundamental.

Most importantly for the scope of Molecular Systems Biology, the reviewers had clear concerns regarding the depth and conclusiveness of the biological insights that are derived from this modeling analysis. The first reviewer was not convinced that this work had demonstrated the physiological relevance of the switch mechanism described here, writing "this is a primarily a modeling paper. Does this work really indicate that there is a physiological switch?" Similarly the second reviewer recognizes that this is a "carefully conducted and complete modeling study," but found it disappointing that the practical value of the model for uncovering new biology or developing better pharmacological agents had not been clearly demonstrated. We would like to emphasize that it will be to crucial to address these issues of physiological relevance and practical model value in any revised work. We recognize that conclusively addressing these concerns is likely to require additional analyses and experimental work.

In addition, the reviewers had a series of concerns regarding parameter selection and identifiability.

These concerns, particularly those raised by reviewer #3, seem especially important since the global parameter estimation method described here is a main point of methodological novelty in this work.

Finally, the reviewers made a series of suggestions to help improve to presentation of this work, especially with non-specialists in mind, which will be very important given the broad readership of Molecular Systems Biology.

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

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Sincerely,

Editor - Molecular Systems Biology
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Referee reports:

Reviewer #1 (Remarks to the Author):

In this paper Heitzler et al have modeled the angiotensin II type 1A (AT1A) receptor activation of ERK in HEK293 cells. The model is based on new data focused on quantitative measures of the kinetics of ERK1/2 and MEK1/2 phosphorylation and stoichiometry, coupled with manipulations of GRKs 2 and 5 levels by siRNA depletion, elimination of GRK phosphorylation of the AT1A by S/T to A mutations of the terminal 13 S/Ts (termed 13A), and inhibition of PKC by Ro-31-8425. Included in the model are previously derived data that had revealed augmentation and inhibition of ERK activation by knockdowns of GRK2/3 and GRK5/6 respectively. Additionally, while the necessary roles of Arr 1 and 2 had been previously demonstrated; i.e., depletion of Arr 2 impairs AT1A ERK activation, while depletion of Arr1 enhances ERK activity (Ahn et al, 2004A), new data is presented showing that Arr 2 depletion by siRNA clearly limited ERK activation in cells expressing either the WT or the 13A mutant. The model also pulls together previous data showing that there are unique pools of ERK that display differential kinetics, as well as the kinetics of DAG production. The model is an equilibrium ODE model with 54 parameters including 26 reactions and 18 variables. Of these, 16 parameters were either taken from the literature (7) or experimentally determined in house (9). The remaining 38 parameters were optimized by model training. Key findings were the phosphorylation-independent but Arr 2-dependent activation of ERK by the AT1A (Fig 3), the non-stoichiometric, enzymatically amplified activation of ERK/MEK, further indicators of the proposed unique roles of GRK2 versus 5, and the overall good fit of the data to simulations.

General Comments
This work is the first effort of this group to model a decade's worth of data on the G protein dependent and independent ERK activation by the AT1AR. The Lefkowitz group has led the way in characterizing roles of arrestin scaffolding in G protein-independent ERK activation, that distinguishes arrestin's role in activation versus uncoupling/desensitization of the receptor/G protein activation. While not the first model of GPCR activation of ERK (Chang, C., Neuropharmacology. 2009) I believe it is the first to attempt modeling of the GRK/arrestin role. The model parameters are clearly presented in stepwise fashion. The weaknesses are the rather large number of arbitrarily

assigned parameters (see list below), and the lack of data concerning specific sites of GRK phosphorylation on the AT1A. The first of these problems is to be expected with a first go at modeling a complex series of reactions, and the latter concerning the possibility of unique GRK sites on the AT1A (the barcode hypothesis), has been recently demonstrated in work with other receptors, most notably that from the Lefkowitz group (Zidar et al. and Nobles et al 2011).

Specific Comments:
This reviewer would like to see the major assumptions made in forming the model laid out together in a table that is in the main text together with the parameter table (not supplemental data). The major assumptions are all to be found in the main text, but one gets lost in wading through the detailed discussion of the model and tying it together with the parameter table and detailed model that are in the supplement.
I would like to suggests that you simulate, as a function of time after treatment, several more of the key parameters/components in the model - it would add a lot to a general understanding of the flow.

Given the recent work demonstrating with mass spectrometry some unique actions of GRK2 versus 5 on the 2AR it is tempting to ask for some further work or site-directed mutagenesis or mass spec showing that a similar mechanism works with the AT1A (mutagenesis/mass spec).

Parameter optimization is an important aspect of the modeling given the number of parameters for which no data is available. However, the data shown in S11 and S12 (% of variation) is not really discussed - that is, most of the discussion is concerned with how the sensitivity analysis was done, not a discussion of what it shows.

The title is not a good description of the paper. This is a primarily a modeling paper. Does this work really indicate that there is a physiological switch? This can be induced by various manipulations as shown by GRK and ARR depletion, but with physiological stimulation is there really a switch?
The Abstract and Discussion seem to be more about global interpretations; focus needs to be on the novel contributions of the new data and modeling in this work. In that regard, in the abstract "our study reveals that..."; I would delete points (i) and (ii) that had been previously determined by this group, and expand on those conclusions presented herein.

Further suggestions:

P6. A reader not too familiar with the G protein dependent vs. independent signaling might be a bit confused by the second paragraph (Desensitization and recycling of the receptor). Doesn't the ERK signaling also desensitize - it is confused with receptor/G protein desensitization? And a related question: P7. " assumption that the phosphorylated form of the AT1A (HRP1 was distinct from the desensitized form.." Is this really possible and necessary for the model? - and see notes on parameter 10 below.

Is there a good reason IP3/calcium signaling was ignored? Along the same lines, I would like to see some discussion of the paper from the Lefkowitz group (Xiao et al, 2010) that showed the pleiotropic reactions driven by AT1A stimulation (e.g., 38 protein kinases, 3 phosphatases). While one must simplify in modeling, it is important to put this model in the larger context of AT1A actions in the Discussion.

In places it seems that reviews, as opposed to the primary publications, are used. There also is a lot of redundancy in the repeated references to work showing unique GRK phosphorylations of other receptors. One gets the impression that the authors are repeating these references to support the lack of data on GRK phosphorylation sites on AT1AR.

P10 line 12. I was not aware that there was the possibility of "an unknown intermediate" nor is the evidence for eliminating that possibility clear in the subsequent discussion.

Page 9 "When expressed in HEK293 cells at similar levels, the two receptors behaved differently. In control conditions, the 13A triggered significantly more ERK phosphorylation than the WT, which possibly reflect the lack of GRK-mediated desensitization (Figures 3B and S4)"

If active structure of receptor is all that is required for arrestin recruitment and subsequent ERK

activation would increasing the 13A receptor levels lead to an increase in pERK levels that correlates with receptor overexpression? Do you have modeling studies to show the effect of increase in receptor number?

Page 10. "we compared WT and 13A AT1AR for their sensitivity to siRNA-mediated GRK depletion (Figure S5B, C, D)". Figure S5B, C and D do not show comparisons between WT and 13A AT1AR.

Page 11. "In order to limit data heterogeneity, we only considered data collected in a unique cellular model (i.e.: HEK293 cells). We used kinetic data on three variables of the model: previously published angiotensin-induced ERK phosphorylation data (Kim et al, 2005), DAG accumulation and PKC activity measured in real-time in AT1AR expressing HEK293 cells by using relevant FRET sensors (Violin et al, 2003)". The way the sentence is structured one assumes that previously published data on ERK phosphorylation, DAG accumulation and PKC activity was used. If that is correct then the assertion that experimental data was only from HEK293 is incorrect. Kim et al used HEK293 cells but the DAG and PKC measurements done by Violin et al per the reference provided was in HeLa and MDCK cells.

*There are a lot of typos; e.g., p13 top GRK23, 56; p14 heading. "inositol uptake" p 15 in ref to Fig 7?

Parameter Table Comments:

Given the units of the parameters what is the volume of the cell assumed in the simulations?

The readability of the table would be greatly enhanced if instead of using red and black text under the reference column if one of this was stated a) citation b) experimentally determined c) computationally optimized. Also considering that this is an electronic journal, space is not at a premium. The table would be more useful as part of the main text after the reaction figure.

Check units for all second order reactions
1. Initial Bound Receptor 8e-02 µmol.L-1 (Ahn 2004). The citation refers to a concentration range of 200-300 fmol/mg of protein. How did this translate into 8e-02 µmol.L-1 in the model? How was the cellular/membrane volume estimation done? Were all receptor species considered to be hormone bound? Given that the initial protein estimates were a range what was the concentration range that it converted into? Is the model behavior the same across that range? Such a commentary is required in the table for better understanding of the numbers used.

2. Initial G Protein 56.99 µmol.L-1 (computationally optimized). Do estimates of Gq/11 protein exist in other cell lines? If so, does the computationally optimized value compare well to the range of values observed?
3. Initial Activated G Protein 0 µmol.L-1 (experimentally determined). 9. Initial G Auto Activation 3.11e-04 sec-1 (computationally optimized) Given that estimates of G protein constitutive do exist in other cell lines how does the computationally optimized value compare to the range of values observed?
Considering that we are dealing with overexpressions wouldn't a change in receptor concentration actually increase basal activity? Since this was determined in house a reference to the methodology used to determine the concentration would be useful. Again a commentary in the table enhances the usefulness of the data presented.
4. Initial PIP2 9.97e-01 µmol.L-1 (computationally optimized). Do estimates of PIP2 exist in other cell lines? If yes, how does the computationally optimized value compare to the range of values observed?
5. Initial DAG 9e-03 µmol.L-1 (experimentally determined). How does this compare to estimates of DAG from other cell lines? Doesn't this imply that there is basal PLC activation? Also since this was experimentally determined the authors should have access to the range of concentrations obtained. Does the stated value fall at the upper or lower end of the DAG concentration? How was the cellular volume estimation done?
6. Initial PKC 8.84 µmol.L-1 (computationally optimized). Do estimates of PKC exist in other cell lines? If yes, how does the computationally optimized value compare to the range of values observed?

7. Initial Activated PKC 2e-03 µmol.L-1 (experimentally determined). Since this was experimentally determined the authors should have access to the range of concentrations obtained? Does the stated value fall at the upper or lower end of the activated PKC concentration?
8. Initial GpERK 1.5e-02 µmol.L-1 (Dupuy 2009)./ 35. Initial bpERK 1.5e-02 µmol.L-1 (Dupuy 2009). 45 Initial ERK 4.243 uM.L-1. Since the cited publication is from the same group the authors should have access to the range of concentrations obtained? Does the stated value fall at the upper or lower end of the pERK concentration? Also in the original citation pERK concentrations were given in ng/nL 0.8612{plus minus}0.022 (Dilution 1:2), 0.44{plus minus}0.014 (Dilution 1:4) and 0.2716{plus minus}0.009 (Dilution 1:8). How does this range of concentrations translate into a single unique concentration? In the light of the units of this parameter, again, how was cellular volume estimated? What fraction of ERK is converted to pERKs in simulations?
10. Activation of G by HRP1 1.8e-02 µmol-1.L.sec-1 (computationally optimized)/11. Activation of G by HR 7.6 µmol-1.L.sec-1 (computationally optimized). These values imply that desensitization of HR does not require Arr binding. Can an increase in one of the rates compensate a decrease in the other? How do these rates compare with experimentally determined rates of G protein activation by phosphorylated receptor vs. non- phos receptor in other receptor systems? With your rates what's the half-life of G protein activation?
12. Activation of DAG (should be PLC?) by active G protein 4.63 µmol-1.L.sec-1 (computationally optimized)./13. Activation of PKC by DAG 7.87e-02 µmol-1.L.sec-1 (computationally optimized). How do these rates compare with experimentally determined rates of DAG/PKC activation since PKC activation is 1/200 of PLC activation? With your rates what's the half-life of DAG/PKC activation? How do your rates compare to kinetic properties of PLC/ PKC as described in other studies?
14. Phosphorylation of ERK by PKC 2.65 µmol-1.L.sec-1 (computationally optimized). How do these rates compare with experimentally determined rates of ERK phosphorylation? With your rates what's the half-life of ERK phosphorylation?
15. Deactivation of G 5.10 sec-1 (computationally optimized). How do these rates compare with experimentally determined rates of G protein inactivation? How is this comparable to GTPase rates?
16. Deactivation of DAG (PLC?) 4.61e-01 sec-1 (computationally optimized)./ 17. Deactivation of PKC 1.77 sec-1 (computationally optimized)./ How do these rates compare with experimentally determined rates of inactivation?
18. Dephosphorylation of GpERK 3.04 sec-1 (computationally optimized)./ 44. Dephosphorylation of bpERK 7.62e-01 sec-1 (computationally optimized). How do these rates compare with experimentally determined rates of ERK dephosphorylation in other systems? C.f. comments for 29/30.
19. Initial GRK2/3 8.99e-01 µmol.L-1 (computationally optimized). The GRK2/3 levels are about 11 fold higher than RH levels in this model of a receptor overexpression system. How does this compare with reported 100 fold excess of GRKs2/5/6 in 2AR overexpression systems in HEK 293 cells (Tran TM et al 2007 Biochemistry) and by Nobles et al 2011?
20. Initial -Arrestin 1 8.58e-01 µmol.L-1 (Ahn 2004)./ 46. Initial -Arrestin 2 4.83e-01 µmol.L-1 (Ahn 2004)./ In Figure 1 of the cited paper -Arrestin1 and 2 levels are shown in blots and their relative levels are quantitated using densitometry. How do those measurements translate into concentrations given here? It is not clear from the blots how a 1.77 fold difference in -Arrestin 1 and 2 are calculated? In figure S5, blots are shown for -Arrestin1 and 2 levels in cells treated with control siRNA and they appear to be similar. Could this blot be used to estimate arrestin levels instead of an outside reference?
21. Initial HRP1barr1 0 µmol.L-1 (experimentally determined)./ 22. Initial HRP1barr2 0 µmol.L-1 (experimentally determined)./ 23. Initial HRP1 0 µmol.L-1 (experimentally determined)./ 33. Initial HRbarr2 0 µmol.L-1 (experimentally determined)./ 34. Initial HRP2barr2 0 µmol.L-1 (experimentally determined)./ 36. Initial HRP2 0 µmol.L-1 (experimentally determined). What antibodies were used to determine HRP1 vs. HRP2 phosphorylation? If that distinction cannot be made then in a footnote to the table it must be mentioned that total HRP was measured. It is interesting that overexpression has no effect on basal phosphorylation of the receptor.
24. GRK2/3 phosphorylation rate 2.27 µmol-1.L.sec-1 (computationally optimized)./ 37. GRK5/6 phosphorylation rate 5.90 µmol-1.L.sec-1 (computationally optimized)./ 31. HRP2 dephosphorylation 6.65e-02 sec-1 (computationally optimized). How do these rates compare with experimentally determined rates of receptor phosphorylation? With your rates what's the half-life of GRK phosphorylation of the receptor? Why does HRP1 not dephosphorylate in the model?
29.HRP1barr1 recycling rate 6.54e-05 sec-1 (computationally optimized)./ 30. HRP1barr1 recycling rate 7.23e-02 sec-1 (computationally optimized). These reactions are either incorrectly connected in

the figure or incorrectly labeled here. The way the reactions have been drawn HRP1barr1 and HRP2barr2 release arrestin and get dephosphorylated in the same step. Thus there is a hidden dephosphorylation of both HRP1 and 2. This is in addition to a separate dephosphorylation reaction for HRP2 (k17). Is the increase in GRK5/6 phosphorylation rate over GRK2/3 phosphorylation rate a mere compensatory mechanism to overcome this modeling artifact?
25/26/27/28/38/39/42/43. How do these rates compare to the half-life of arrestin recruitment to other GPCRs? Why can't HRP2 get phosphorylated by GRK2/3 to give a HRP1/2 state and vice versa for HRP1? Since you are knocking down arrestin2 what prevents HRP2 from being phosphorylated by GRK2/3? In the simulations to mimic arrestin 2 knockdowns you reduce arrestin2 levels by greater than 4000 fold. Is this consistent with about 80% KO? With such a huge reduction there should be accumulation of HRP2. An argument needs to be made as to why in the model HRP2 could not be phosphorylated by GRK2/3.
40/41. Would a 4000 fold reduction in barr2 levels not be required to simulate arrestin2 knockdown if there was not a 34000 fold difference in the two rates of phosphorylation?

Reviewer #2 (Remarks to the Author):

This work develops a new model for the activation (phosphorylation) of ERK in response to activation of 7-transmembrane receptors, a common class of drug targets. As the authors mention, models of this process have been suggested before. But the proposed model greatly extends the scope of previous efforts and is capable of addressing previously not analyzed questions on the role of beta-arrestins and GRKs. In particular, the authors introduce (and test) several details/assumptions needed for the model to work, such as (i) reversibility of reactions leading to beta-arrestin-dependent p-ERK, (ii) raising the possibility of enzymatic activity of the beta arrestin scaffold (not fully explained but supported by some experiments); and, experimentally supported GRK-independent beta-arrestin-mediated activation of ERK (page 9, figure 3). To some extent, the authors also assess the extent to which the model generalizes and makes predictions of a non-trivial nature. First, they seek to re-produce in a qualitative fashion the results from a set of previously published studies. Second, they experimentally verify a set of GRK2/3 and 5/6 knockdowns in the presence and absence of PKC inhibitors.

Overall I think this is a carefully conducted and complete modeling study with results that may be applicable for pharmacology and which give considerable new insight (at least, new strongly supported hypotheses) into possible mechanisms involving ERK activation by 7-TMRs. I think the authors strike a good balance between model complexity and available data, and I learned a lot from following their effort, e.g. choice of model scope, lumped variables, optimization techniques etc. I think that the ODE community and readers interested in pharmacological modeling would enjoy this article, as it constitutes a good starting point for further studies and an educational a clearly presented example to follow. (there are rooms for improvement in terms of presentation - below).

Being a bit more critical, I feel that this would be a much more exciting paper if the model was used for a purpose beyond showing the validity of the model itself, especially given the stated importance of these receptors to pharmacology. For instance, it would be spectacular if they can use the model to forsee ways of modulating the response to a 7-TMR in a medically meaningful way, or if the authors conducted a broader study of how their approach to modeling of ERK activation by 7-TMRs works across a range several pharmacologically relevant models. If the work was developed in such a way, I think it would further enhance general interest.

Scientific comments

I found it frustrating to get such a vague account of how the model connectivity was found. Did you try different alternatives manually? Or systematically? This seems to have been a key part of the modeling process, and it would be interesting and educational to hear more about it.

According to the text, several (9?) of the parameters are 'set arbitrarily', which I found totally confusing given the great care you give parameter estimation otherwise. Surely there must be some principle for choosing them?

Minor points and presentation

Figure 1, while being very SBML-consistent, could be much more informative. It would give readers a better overview and if the authors should point out which regulatory steps are new features of the model developed compared to previous work, perhaps with pointers to coming figures and the strength of evidence for the new interactions. This would also bring forward the strengths in the work in a better way. Note that diacylglycerol is annotated as a protein!

Figure 6 is not very easy to assess and is not well labeled (x,y,z). The statement is that it confirms results in two previous papers, but readers are not really given the chance to assess this claim without looking up the original references. It would be more convincing if the results were shown side-by-side (unless there is some sort of copyright violation).

While it is a strength that much detail is given, the manuscript is a rather heavy read and the tone is somewhat specialized (like assuming that readers know what Ro318425 is (a PKC inhibitor) and immediately understand why it is used (turn off signalling via G proteins)). I had to spend a lot of time cross-comparing the main text, figures and legends to understand what was really being done. Please keep general readers in mind if you revise the work. You could also add drugs and other perturbations to the schematics, which would make it easier to follow your experiments.

There seem to be several grammatical errors in the text.

Reviewer #3 (Remarks to the Author):

This manuscript describes the formulation of a mathematical model to analyze angiotensin receptor signaling in HEK293 cells. ERK activation is achieved by two distinct branches of the pathway: (i) via heterotrimeric G proteins leading to activation of PKCalpha and (ii) via beta-arrestins that trigger phosphorylation of the receptor as well as activation of the MAP-kinase cascade. Components of these two branches were selectively inhibited by siRNA or small molecules and the model parameters were estimated based on these data sets. Finally, model predictions were compared to previously published or newly obtained data.

Major comments:

1) The authors use a reverse phase protein array to analyze the ratio between phosphorylated ERK and MEK. They conclude that they find substantially more phosphorylated ERK then phosphorylated MEK and thus selected enzymatic catalysis rather than equimolar scaffolding to model ERK activation. Indeed, it has been previously suggested that ERK is activated to a higher degree than MEK (e.g. Fujikoka et al.,PMID:16418172). However, the authors calibrate their protein array measurement with commercially available recombinant proteins that have been phosphorylated in vitro. This analysis is only valid if both pERK2 and pMEK1 were double-phosphorylated to 100%. This is obviously not the case, see e.g. the analysis of recombinant ERK by Prabakaran et al (PMID:21487401). The phosphorylation degree of in vitro labeled MEK1 might even be much lower. Thus, the conclusions of Fig. 2 are only valid if the authors can demonstrate the phosphorylation degree of their recombinant standards. It might also be more appropriate to show the quantification of the standard curves (to confirm linearity) rather than pictures of the spots.

2) In the discussion, the authors comment on the problem of identifiability, e.g. the determination of unknown parameters based on the available amount of data. They claim to have solved this problem in a innovative fashion by combining global parameter estimation and qualitative constrains. However, while their best fit (Fig. 5) may represent the global optimum, probably many of the model parameters (Fig. S8) cannot be uniquely identified. The simulations (Fig. 6 and 7) and the sensitivity analyses (Fig. S11 and S12) directly depend on these parameter values. To demonstrate that these results are significant, the authors should either analyze the identifiability of the model as suggested e.g. in Raue et al. (cited in this manuscript) or obtain multiple parameter sets that are able to describe the data (e.g. Chen et al., PMID:19156131) and then perform the analyses with each parameter set.

Minor comments:

1) The simulations in Fig. 6D do not match with the experimental data of Ahn et al. (cited in this manuscript) as they correspond to different time scales. They need to be replaced by the correct

simulations (Fig. S10A). As the authors point out, the data set is not completely in line with the experimental data, which could be explained by different cell lysis conditions (cytoplasmic vs. whole cell lysis). It may be worthwhile to repeat this experiment with correct settings (30 min and whole cell lysis) to validate the model.

2) The colors in Fig. 3 should be consistent for clarity. All WT data could be depicted in blue and 13A data in red (corresponding to Fig 3A). CTL could be a solid line and the different siRNA treatments could be dashed. Along the same line, the figure legend of Fig. 6ABC could be more intuitive (e.g CTL, twofold GRK2/3, etc.).

3) The y-axis of Fig. 5 is incorrectly labeled. While the simulations may be in units of umol/L, the units of the axis are non-dimensional (pERK/ERK) or arbitrary units.

1st Revision - authors' response 16 May 2012

2nd Editorial Decision 12 April 2012

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who evaluated your revised study. As you will see, the referees felt that the revisions had improved this work substantially, and the last two reviewers are largely supportive of publication. The first reviewer, however, has raised some important issues, which we would ask you to carefully address in a final revision of this work.

Reviewer #1 has two key concerns:

Most crucially, this reviewer identifies an important technical issue with the VSMC 2AR experiments (H84 is known to antagonize 2AR), and felt that this issue complicates the interpretation of this experiment. In further discussion, this reviewer suggested that this issue may be directly addressable, writing "they can adjust the agonist concentration to surmount the receptor level inhibition by H89 (they should have used greater than or equal to 10 uM ISO)." The editor feels that this additional experimental work would be justified.

Second, this reviewer has a conceptual concern regarding the biological relevance of the "switch" analogy used in the manuscript, and particularly in the manuscript title. This issue will require due consideration and possibly some textual revisions.

In addition, the editor feels that this work could potentially benefit from a more active title. Also, we generally prefer to avoid titles with colons or abbreviations. Perhaps something like, "Competing G protein-coupled receptor kinases balance G protein and -arrestin signaling"? (100 characters max).

Thank you for submitting this paper to Molecular Systems Biology.

Sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Referee reports

Reviewer #1 (Remarks to the Author):

The authors have made substantial improvements to the manuscript, notably a much improved discussion of the iterations leading to the abstracted model, and an improved and detailed discussion of the model parameters - now clearly distinguishing experimentally determined parameters from the model-derived.

This reviewer had a problem with the title emphasizing a "switch" mechanism and that remains a problem in the title and in several places in the text. Interestingly, in Discussion section the authors refer repeatedly, and more rigorously, to the "balance" or "interplay" between GRK2/3 and 5/6. By any classical meaning of the word switch the implication is to an on or off mechanism, and the choice the authors made to use balance or interplay is more accurate.

In regard to the switch mechanism for the current work, the authors lean heavily on the recent paper by Nobles et al (2011) suggesting there is a switch mechanism for ISO-stimulated GRK2/3 versus GRK5/6. In fact the paper clearly shows that GRK5/6 and GRK2 both stimulate phosphorylation of residues 355/356 and 360/364 phosphorylation. With siRNA depletion for either sets of GRKs in this work, there remains substantial phosphorylation of these residues by the remaining GRKs. Further, several studies have clearly shown that internalization of the 2AR requires residues 355/356 and 360/364 (Seibold et al Mol Pharm, 2000,; Vaughan et al JBC, 2006, and Krasel et al JBC 2008). Overexpression of GRKs 2,5 and 6 leads to phosphorylation of residues 355/356 (Shenoy et al and Baameur et al). The statement made on p26 line 4 needs to be changed. Sites distal to the 355-364 do appear to play a role in internalization (Krasel et al), but it is not those sites that

are "primarily responsible" for internalization.

The authors, in response to Rev 2 have added work with VSMC cells that helps satisfy, for the most part, the criticism that physiological relevance should be demonstrated. However, there is a flaw in the 2AR study. It has been shown that H89 is an antagonist of the 2AR with a Kd nearly equivalent to ISO. Using ISO at 100 nM and H89 at 10 μM means that occupancy by ISO is reduced by a factor of 20-30. This has to be corrected.

Minor comments on parameters.
The authors note that determining GRK sites of phosphorylation for the AT1AR are too much to ask, and that is reasonable if as noted above the authors do not use the rationale based on the 2AR Nobles paper (see above comment) that HRP2 cannot be phosphorylated by GRK2/3 or vice versa for HRP1. This is an assumption for the AT1AR and can stand as such for modeling purposes.
On units for the AT1AR: the authors used a concentration of 6-9 X 10-8 M/L. Perhaps it should be noted the membrane concentration is unknown, and would certainly be about a 1000 times greater than that based on whole cell volume. Again, for purposes of modeling, this is not critical, but a note in the text or legend would clarify this point.

Reviewer #2 (Remarks to the Author):

The revised version of the paper is an interesting step forward in modeling the G-protein and Beta-arresting interplay downstream of the 7TM receptors.

The new experimental work, where the authors study the effect of additional ligands (substance P, FSH, vasopressin etc) and their corresponding receptors, as well as the study of angiotensin in cultured smooth muscle cells, provide strong support of the generality of the model.

I recommend publication of the paper as is.

Small comments on presentation:

The authors may want to add additional markup of figure 1 (presentation will be clearer if readers are helped in some way to locate the starting point of the process, i.e. a ligand binding to a 7TMR. This is contained in the node HR, but that may not be so clear from the figure and legend)

Similarly, it takes a bit of effort to parse the contents of figures 6 and 7, instead of using e.g. "V2R", "NK1R" etc as markup, you could put "stimulation of V2 vasopressin receptor with vasopressin", "stimulation of neurokinin 1 receptor with substance P" or similar information.

Reviewer #3 (Remarks to the Author):

The authors have sufficiently addressed the concerns raised. The revised manuscript has significantly improved.

2nd Revision - authors' response 18 May 2012

Acceptance letter 23 May 2012

Thank you again for sending us your revised manuscript. We are now satisfied with the modifications made and I am pleased to inform you that your paper has been accepted for publication.

Before we can send this work to production, we have some minor remaining format and content issues:

1. The quality of the Fig. 1 and 5 images remain a bit low (i.e. the text and line-art is noticeably blocky/blurry). Please provide higher resolution versions, and check to make sure that text/line-art remains clear even when zooming in. You may find that saving the images directly as EPS or PDF will better preserve the text resolution. If this does not help, you may need to remake the figures in a quality vector graphics program like Illustrator or the free opensource, alternative Inkscape. I would also recommend increasing the font sizes in Fig 5 by one or two pts, to improve readability.

2. Thank you for depositing your model at BioModels. We prefer to also archive biological models locally in our supplementary materials section. Please provide the SBML model file, and then add a line to the Supplementary Information pdf Table of Contents indicating that this model is available as a separate file.

3. If Fig. 1 was generated with a common software package, like CellDesigner, we would encourage you to send us the underlying machine readable file, which will be including as "figure source data" for Fig. 1.
Thank you very much for submitting your work to Molecular Systems Biology.

Sincerely,

Editor - Molecular Systems Biology
msb@embo.org

Reviewer #1 (Remarks to the Author):

The authors have addressed my problems adequately. I believe the manuscirpt is ready for publication.

