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Coordinated signal integration at the M-type potassium channel upon muscarinic stimulation

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1st Editorial Decision 14 February 2012

Thank you for submitting your manuscript to the EMBO Journal. I am very sorry for the delay in getting back to you with a decision, but I have now received the comments from the three referees.

I think that you pleased to see that all three referees appreciate your work and find it suitable for publication in the EMBO Journal. They raise a number of different concerns that shouldn't involve too much additional work to address. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript that addresses the raise concerns.

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, 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

Potassium M-channels (usually composed of Kv7.2 and Kv7.3 subunits) play a major role in regulating the excitability of nerve cells. The channels themselves are regulated by several biochemical messengers, including phosphatidylinositol-4,5-bisphosphate (PIP2), protein kinase C, calmodulin (CaM) and calcium (via CaM). These are known to participate, to varying extents, in the closure of the channels produced by different neurotransmitters and other extracellular messengers - a process of wide physiological significance. The question arises how do these regulators interact? Since previous work (some cited in this paper) has identified overlapping targets for the regulators in the C-terminal of the subunit proteins, one possibility (explicitly suggested in a previous review (Delmas & Brown 2005 - cited) and alluded to in other papers) is that they might all interact in such a way as to modify the sensitivity of the channels to PIP2. However, the veracity of this suggestion, and the details of how this interaction might occur, are not yet clear.
In the present paper, the authors focus on the role of PKC-mediated phosphorylation and its interaction with calmodulin. In previous papers the authors showed that PKC mediated phosphorylation at (primarily) serine 541 in Kv7.2 plays a role in mediating the closure of the channels by stimulating muscarinic acetylcholine receptors (mAChRs), and that this requires binding of PKC to the scaffolding protein AKAP79/150 as an auxillary protein (Hoshi et al, 2003,2005 - cited). They now report two important additional discoveries. First, they show that this does indeed appear to reduce the 'affinity' of the channel to PIP2, since a pseudo-phosphorylated (S541D) Kv7.2 channels, or one exposed to phorbol dibutyrate, shows a greater sensitivity than the normal channel to the intracellular PIP2-scavenger neomycin. Second, they show that such phosphorylation (by PKC or a muscarinic agonist) or pseudophosporylation (S341D) leads to the dissociation of CaM from the channel protein as measured by in vitro binding or in situ by FRET. From these two observations they suggest the sequential scheme:

mAChR stimulation → PKC phosphorylation → CaM dissociation → reduced PIP2 activation → channel closure

ASSESSMENT
This paper contains interesting new information that provides an important advance in knowledge regarding the regulation of M-channels, which may be relevant to signalling mechanisms in general. The experiments are carefully performed and with some reservations) clearly described, and some experiments (e.g., using neomycin and a PIP2 analog) are quite ingenious. It is therefore potentially suitable for publication in EMBO J. However, there are some aspects of the paper (both experimental and interpretational) that need attention, either by discussion or perhaps by additional information or experimentation.

MAJOR POINTS.

1. The authors do not DIRECTLY show that it is the loss of CaM that reduces the 'affinity' for PIP2, as opposed to the alternative possibility that the conformational change inducing such loss is itself responsible for the change in PIP2 affinity (i.e., two parallel events, not sequential events). The nearest they get to that is the effect of the mutant R353G, which reduces CaM binding but not phosphorylation. This implies that phosphorylation itself is not necessary, but the R353G mutant might well produce a similar conformational change to that produced by phosphorylation. Further, Exteberria et al (cited) say that this mutant affects trafficking, so presumably the currents were reduced (the authors do not state the current amplitudes, and only show normalized currents in Fig.4). Indeed, the shift of the neomycin curve produced by this mutation (Fig.4E) is quite different from that in Fig 4D, which suggests that the two may not be caused by the same mechanism. One way of testing whether it really is the loss of CaM that shifts PIP2 affinity might be to see if the effects of the mutations are reversed by over-expressing CaM (assuming here that the reduced CaM binding by the S541D and R353G mutants reflect a change in CaM-binding affinity - it would be useful to have this quantitated).

2. The Discussion of previous reports concerning the role of CaM in regulating M-channels in paragraph 1 of page 6 requires some amendment: it is not that the Shapiro group contend that CaM itself suppresses M-channel function, it is that low-affinity calcium-bound CaM (Gamper et al., 2005: Mol.Biol.Cell, 16, 3538) is required for calcium-mediated inhibition. Indeed, that group is

largely in agreement with the authors in showing that wild-type CaM competes for AKAP binding to M-channels (Bal et al., 2010: J. Neurosci, 30, 2011). Since the present authors have shown that AKAP is the carrier for PKC, this is essentially complementary to their current observations, and should be referred to.

3. In the context of (2) above, can the authors clarify how far their observations refer to Ca-free CaM or to Ca-bound CaM (or to both)? For example, was Ca present in the in vitro solutions used in Fig.2? Would phosphorylation / pseudophosphorylation cause dissociation of a CaM that is unable to bind Ca? What was the free Ca2+ concentration in their pipette solutions? (In sympathetic neurons cytoplasmic free [Ca2+] is around 100 nM, rising to around 200 nM during Ca-mediated M-channel inhibition by bradykinin: Hernandez et al., 1998, PNAS, 95, 7151.)

4. The authors' sequential scheme is at variance with the dynamics of the system as described by Suh & Hille (2006: Pflug. Arch., 453,293), in which the initial event is the rapid hydrolysis and depletion of PIP2, with DAG formation (and hence PKC activation) occurring many seconds later. Further, these authors and others have provided strong evidence that mAChR stimulation does indeed cause a profound loss of PIP2 and that M/Kv7 channels closure can be well-explained by that. The authors' scheme has no role for this primary event. Presumably the justification for this is the experiment in Fig.6, in which inhibition of wild-type or native (presumably PKC-phosphorylated?) channel is not affected by 'substituting' non-hydrolysable ms-PIP2 for normal PIP2. However, the authors give no reference or background information about ms-PIP2 with which to judge the significance of this observation. Further, it is difficult to understand why, in Fig.6C, the effect of ms-PIP2 should differ from that of diC4-PIP2 since, at 500 µM, no appreciable amount of the latter could possibly be hydrolysed by PLC: the equivalent cytoplasmic concentration of endogenous membrane PIP2 is around 10 µM (McLaughlin et al, 2002: Ann. Rev. Biophys, 31,151), and PLC is essentially saturated at normal PIP2 levels (Jensen et al, 2009: J.Gen.Physiol., 133, 347). These experiments are sufficiently important, and the interpretation sufficiently at odds with previous work showing that elevation of endogenous PIP2 greatly reduces muscarinic inhibition of M-current (e.g., Winks et al.,2005; Suh et al, 2006 - both cited), as to require careful discussion.

[I have raised these points, not to be excessively critical, but because the paper is important and novel enough as to warrant a more thorough critique by the authors.]

POINTS OF DETAIL.

These relate primarily to methodological and descriptive details (not all of which are clear) and to some of the references and their usage. Going through in order:

1. P.3, para 2, line 3. Evidence for a role of M-channels in the slow after-hyperpolarization (Tsigounis & Nicoll, 2008) is not very convincing. Indeed, there is ample evidence for a role in regulating firing frequency independent of the sAHP (e.g., Peters et al., 2005: Nat Neurosci 8: 51-60).

2. P.3, para 2, line 11. In reference to the axon initial segment, authors might note a functional follow-up to Pan et al (2006) in Shah et al., 2008: PNAS, 105, 7869.

3. P.4, para 1, line 13. The more recent work of Hernandez et al., 2008 (J.Gen.Physiol., 132, 361) implying a rather different PIP2 binding site should be referred to.

4. Page 4, last para. "....reduced ion conducting properties" Meaning reduced open probability? (Ionic conductance of the channel is unchanged.)

5. Page 5, line 6. BIS IV. What does IV mean? (There are no chemicals listed in Methods.) How was this applied and for how long?

6. Page 5, line 9. Phosphorylation (spelling).

7. Page 6, para 2, line 1". ....increased functional expression" - misleading, expression is unchanged - increased activity?

8. Page 6, para 2. Was calmodulin Ca-bound? Were immunos done in presence or absence of calcium? (see major Point 3)

9. Page 7, line 10. CaM-V5. What is this? (No description in Methods).

10. Page 8, para 2, line 3. "...marginal change ...with KCNQ2(S541A)" - not really - looks about half that with wt.

11. Page 8, para 2, line 5. "these results infer that..." Not infer, imply. (The experiment implies, the observer infers.)

12. Page 9, first sentence. See Detail point (3) above and re-think.

13. Page 9, line 5. Infers again - should be implies (see 11)

14. Page 9, line 5 "calmodulin binding affects" - might affect?

15. Page 9, line 7. Utilized, not utilize.

16. Page 14, para 1, last line. "Figs 2 ...and 3 ...show that CaM dissociation from the channel complex triggers suppression of the M-current". They don't, since no currents are shown - "is associated with" perhaps.

17. Fig.1. B,D. How are currents normalized?
F. What are the two bands at 100 and 75 kD?

18. Fig.3. What is meant by n=100? 100 cells? 100 experiments?

19. Fig. 4. Again explain normalization procedure. Also, please give mean current densities for each construct in the legend.
Were the IC50s in Figs D (S541D) and E(R353G) significantly different from wt? Please give full details of parameters and significance levels in text or legend.

20. Fig 5. No effect of PDBu in absence of neomycin: this presumably means that PIP2 is normally too high for an affinity change to have much effect - then much of the effect of Oxo-M in Fig 1 must be due to PIP2 depletion, not affinity change (see Major Point 3).

21. Fig.6. Explain precisely what panels C and F show - currents in Oxo-M? what does that mean?

22. It is not clear which experiments were done using perforated patches and which with whole-cell patching. Please state clearly in the legends.

23. The title seems a bit vague, and doesn't give much indication of exactly what the paper is about. Better perhaps: "[Co-ordinated] signal integration by protein kinase C, calmodulin and PIP2 at the M-type potassium channel following muscarinic stimulation."

Referee #2

This is an interesting, well thought-out and executed study by Kosenko and colleagues from Naoto Hoshi's lab in collaboration with John Scott's group. The authors show that block of PKC effects, either by the PKC inhibitor BIS or by an Ala mutation of a specific phosphorylation site in the KCNQ2 channel (S541), attenuate the oxo-M induced inhibition of this current. In contrast, the phosphomimetic mutant (S541D) accentuates the oxo-M induced inhibition. KCNQ2 has been shown to interact with calmodulin (CaM) altering channel function, although the direction of its effect differs among published studies. By tagging KCNQ2 and CaM with variants of YFP and CFP, respectively, the authors show that compared to its absence the presence of oxo-M reduces the CaM/KCNQ2 interactions more so in the S541 mutants than the wild-type KCNQ2. Through direct interactions of tagged CaM and KCNQ wt and mutant channels, the authors demonstrate that PKC-dependent phosphorylation at S541 causes dissociation of CaM and KCNQ2, a result that is also

shown for stimulation by oxo-M. These data are consistent with a model in which the presence of CaM stabilizes somehow currents through KCNQ2 and oxo-M acting through Gq-coupled receptors stimulates PKC to phosphorylate KCNQ2 involving S541, an event that leads to reduced interactions of CaM and the channel and thus destabilizes the open channel state and reduces activity. I feel this part of the study does not need to be changed in any way to enhance its message. The authors proceed to investigate how does this PKC modulated interaction of CaM with KCNQ2 and "M" channels control activity. They perform whole-cell experiments and present data that CaM enhances channel-PIP2 affinity. The experiment to suggest this interpretation is a comparison of dose-response curves of neomycin included in the patch pipette, where the KCNQ2(S541D), the phosphomimetic mutant that greatly attenuated CaM association with the channel, showed increased block by neomycin, suggesting a decreased affinity of the channel for PIP2. Interestingly, direct PKC activation by PDBu did not cause a decrease in KCNQ2 current by itself, but it did in the presence of 100 μM neomycin, a low enough concentration that normally did not inhibit channel activity. This effect of neomycin was occluded by the S541A mutant. These results are consistent with the interpretation that PKC phosphorylation at S541 by PDBu weakened channel-PIP2 affinity enough so that 100 μM neomycin was sufficient to cause current inhibition. Thus, the notion of a decreased affinity for PIP2 by PKC-mediated phosphorylation that prevents CaM association with the channel is further strengthened by these experiments. The final experiments are performed with oxo-M again to show that with inhibition of PKC phosphorylation, either with the KCNQ2(S541A) or with BIS treatment of SCG M-currents, not only increased KCNQ currents but the oxo-M effects were greatly attenuated. I found the inclusion of 500 μM diC4-PIP2 or ms-PIP2 not terribly convincing. I am not aware of studies showing directly (in inside-out patches) effects on channel activity of soluble PIP2 that has acyl chains less than 8-carbon long (diC8). I have a couple suggestions to improve this second part of the paper dealing with the effects of PKC phosphorylation on channel-PIP2 affinity.

1) The study would be greatly strengthened by showing direct effects of PKC phosphorylation on apparent affinity for PIP2. A dose-response of KCNQ2 (or Q2/Q3) activation by diC8-PIP2 could be shown for wt, and the S541A and S541D mutants to assess whether the lack of phosphorylation or the phosphomimetic mutations, respectively, alter channel-PIP2 affinity.
2) This study on KCNQ inhibition by PIP2 hydrolysis and PKC activation resembles a study in Kir3 channels by Keselman et al., 2007 Channels1:113-123. The authors may benefit by considering the results of this study.

Referee #3

This is a very interesting paper. But it also suffers from some shortcomings.
(1) The handling and interpretations of the FRET experiments is a bit superficial, mainly with some missing control experiments. This can be improved by some changes in text, adding some data that is probably already exist from the existed experiments, and the addition of a small additional experiment of FRET.
(2) I am a little bit concerned about the level of certainty of the displayed model based on the presented experiments.
Major issues:
1. It should be checked if FRET differences between the groups don't reflect differences in flexibility and/or orientations of the xFP-tagged proteins and not by interaction levels. One way to cope with this issue is by using anisotropy measurements. The second and much easier experiment might be to repeat the experiments shown in Figure3 in the presence of BIS-IV vs. PDBu. Thus, without a potential change in the protein structure, the change in kinase activity should be reflected by FRET readouts.
2. One cannot state that (p. 8, bottom) "Collectively, these results further infer that muscarinic stimulation induces dissociation of Calmodulin from channel complex through...". There might be several other reasons for the author's observations that FRET decreases such as conformational changes between the molecules without their dissociation or orientation changes between fluorescent proteins that effect their dipole-dipole orientation.
3. In Fig. 1F normalized traces for KCNQ2 S541A mutants are shown while raw FRET efficiency data is shown only for KCNQ2 S541D. In order to assess the dynamic range of the FRET measurements between negligible- to constitutively- phosphorylated in the context of FRET readout between CaM and the M-Channel, and to strengthen the measurement reliability the missing row

data for 551A mutant should be added to figure 3A.
4. According to the model FRET should decrease upon channel phosphorylation, in Figure 3 however, the decrease in FRET signal in the presence of OXO is not completely abolished; can the authors address the possibility that CaM may be associated with the channel while phosphorylated?
Minor:
1. Page 6 - The reference the fig. 1E and 1F contain wrong n-numbers. (n=4 instead of n=42 as shown in the Figure legend.

2. I don't understand the significant discrepancy between the Ns in the calculation of current densities, showing 30-50 cells for each group and the normalized traces showing ~10- cells per group.

1st Revision - authors' response 18 March 2012

Referee #1

MAJOR POINTS.

1. The authors do not DIRECTLY show that it is the loss of CaM that reduces the 'affinity' for PIP2, as opposed to the alternative possibility that the conformational change inducing such loss is itself responsible for the change in PIP2 affinity (i.e., two parallel events, not sequential events). The nearest they get to that is the effect of the mutant R353G, which reduces CaM binding but not phosphorylation. This implies that phosphorylation itself is not necessary, but the R353G mutant might well produce a similar conformational change to that produced by phosphorylation. Further, Exteberria et al (cited) say that this mutant affects trafficking, so presumably the currents were reduced (the authors do not state the current amplitudes, and only show normalized currents in Fig.4). Indeed, the shift of the neomycin curve produced by this mutation (Fig.4E) is quite different from that in Fig 4D, which suggests that the two may not be caused by the same mechanism. One way of testing whether it really is the loss of CaM that shifts PIP2 affinity might be to see if the effects of the mutations are reversed by over- expressing CaM (assuming here that the reduced CaM binding by the S541D and R353G mutants reflect a change in CaM-binding affinity - it would be useful to have this quantitated).
Responses: 1) Direct evidence for loss of CaM reduced affinity for PIP2. We agree that mutant analyses can only represent stationary conditions. Demonstration of the dynamic change in PIP2 affinity by dissociation of calmodulin would be ideal. However, we do not yet know adequate stimulation to dissociate calmodulin from KCNQ2 channel (besides oxo-M). Rescue experiments by over expression of calmodulin as the reviewer suggested could be valuable supportive evidence. However, we would like to point out that calmodulin is an abundant protein, which is reported to be 160 ng/10^6 cells in CHO-K1 cells corresponding to 0.1% of total protein (Cell 36,73-81, 1984). In some cells such as neurons, it is known to be 2% of total protein (Klee Annual rev. Biochem 1980). Thus, it is extremely difficult to manipulate cellular concentration of this protein. Indeed, we did not detect overall increase in calmodulin in our transient expression experiments when assessed by Western blotting using anti-calmodulin antibody, even though transfection efficiency exceeded 50%. In addition, we did not detect major changes in electrophysiological data when coexpressing KCNQ2 and calmodulin in our condition.

Whether loss of CaM and reduction in PIP2 affinity are two parallel or sequential events is a very profound question. To address this point, we have added scatter plots showing close correlations between calmodulin deficient mutants and PIP2 related parameters in a new supplementary figure 1. Data from mutants of a single amino acid residue may lead to false interpretation that links unrelated events as a concern raised by the reviewer. To avoid this possibility, we added analyses of KCNQ2(S534D), a secondary PKC phosphorylation mutant with a milder phenotype in addition to KCNQ2(R353G). All parameters from these mutants showed strong correlations between calmodulin binding, neomycin IC50 and various PIP2 related parameters. Thus, we think that it is more likely that these two events are closely related rather than coincidental. In addition, our immunoprecipitation indicates that CaM binding does not require PIP2 since it is washed out during the procedure. Since PIP2 binding does not govern CaM binding, it is most likely that CaM binding controls PIP2 affinity. **These new data are included in the new supplementary figure 1 and in the text (page 11-12). In addition we added discussion regarding this issue in page 17, third paragraph.**

2) **R353G mutant.** As the reviewer predicted, current density of R353G is lower than that of the wildtype. As per the reviewer's suggestion, we have quantified the levels of KCNQ2(R353G) bound calmodulin. **All these new data are included in the scatter plots in the new supplementary figure.** In addition, we agree with the reviewer that phosphorylation itself is not the only mechanism to dissociate calmodulin. Rather, what we think is important is that the R353G mutant, which shows disturbed calmodulin binding by another mechanism, has similar phenotype regarding PIP2 affinity. We think that this is a critical piece of supportive evidence that these two events are related. **This statement is added to page 11, line 20-23.** As mentioned in the text, KCNQ2(R353G) is known to have disturbed trafficking. However, we would like to point out that patch clamp data represent functional channels at the cell surface. In this regard, trafficking may affect current density but should not affect neomycin sensitivity per se. To examine PIP2 sensitivity in this channel directly, we tested whether addition of diC8-PIP2 in the patch pipette augments KCNQ2(R353G) shown in supplementary figure 1 (as suggested by reviewer 2).

2. The Discussion of previous reports concerning the role of CaM in regulating M-channels in paragraph 1 of page 6 requires some amendment: it is not that the Shapiro group contend that CaM itself suppresses M-channel function, it is that low-affinity calcium-bound CaM (Gamper et al., 2005: Mol.Biol.Cell, 16, 3538) is required for calcium-mediated inhibition. Indeed, that group is largely in agreement with the authors in showing that wild-type CaM competes for AKAP binding to M-channels (Bal et al., 2010: J. Neurosci, 30, 2011). Since the present authors have shown that AKAP is the carrier for PKC, this is essentially complementary to their current observations, and should be referred to.

Responses: We thank the reviewer for this constructive suggestion. The original sentence was confusing, therefore in accordance with the reviewer 1 suggestion we have amended it to state, "conformational change of constitutively bound calmodulin regulates channel activity" (page 16, line 2-3). We cited a paper by Bal et al. (J. Physiol. 2008), instead of the one suggested.

3. In the context of (2) above, can the authors clarify how far their observations refer to Ca-free CaM or to Ca-bound CaM (or to both)? For example, was Ca present in the in vitro solutions used in Fig.2? Would phosphorylation / pseudophosphorylation cause dissociation of a CaM that is unable to bind Ca? What was the free Ca2+ concentration in their pipette solutions? (In sympathetic neurons cytoplasmic free [Ca2+] is around 100 nM, rising to around 200 nM during Ca-mediated M-channel inhibition by bradykinin: Hernandez et al., 1998, PNAS, 95, 7151.)

Responses: We performed binding assay in Ca-free conditions with the lysis buffer containing both 5 mM EDTA and 5 mM EGTA. This condition was described in the methods, but is now clearly stated in the text (page 7, line 4). In addition, free calcium concentration (90 nM) in the pipette solution is stated in the methods. In our hands, apo-CaM is the KCNQ2 binding partner; Ca-bound CaM did not bind KCNQ2. This is briefly mentioned in the methods, but we did not include it in this manuscript since it would require a full article worth of data set to characterize this phenomenon. We will focus on this issue in another manuscript as soon as possible.

4. The authors' sequential scheme is at variance with the dynamics of the system as described by Suh & Hille (2006: Pflug. Arch., 453,293), in which the initial event is the rapid hydrolysis and depletion of PIP2, with DAG formation (and hence PKC activation) occurring many seconds later. Further, these authors and others have provided strong evidence that mAChR stimulation does indeed cause a profound loss of PIP2 and that M/Kv7 channels closure can be well-explained by that. The authors' scheme has no role for this primary event. Presumably the justification for this is the experiment in Fig.6, in which inhibition of wild-type or native (presumably PKC-phosphorylated?) channel is not affected by 'substituting' non-hydrolysable ms-PIP2 for normal PIP2. However, the authors give no reference or background information about ms-PIP2 with which to judge the significance of this observation. Further, it is difficult to understand why, in Fig.6C, the effect of ms-PIP2 should differ from that of diC4-PIP2 since, at 500 μM, no appreciable amount of the latter could possibly be hydrolysed by PLC: the equivalent cytoplasmic concentration of endogenous membrane PIP2 is around 10 μM (McLaughlin et al, 2002: Ann. Rev. Biophys, 31,151), and PLC is essentially saturated at normal PIP2 levels (Jensen et al, 2009: J.Gen.Physiol., 133, 347). These experiments are sufficiently important, and the interpretation sufficiently at odds with previous work showing that elevation of endogenous PIP2 greatly reduces muscarinic inhibition of M-current (e.g., Winks et al.,2005; Suh et al, 2006 - both cited), as to require careful discussion.

Responses: Thank you or providing this basis for deeper discussion.

1) Discrepancy from cellular dynamics of signaling molecules. With all due respect, there is no variance with the dynamics described by Suh and Hille even though their main claim is very different from ours. They described equimolar production of DAG and IP3 accompanied by rapid fall of PIP2. Thus, there should be no delay in DAG production. What they described was that application of DAG alone did not induce suppression of KCNQ2 channel activity, which is also confirmed in our condition. Regarding the dynamics of PKC phosphorylation and channel modulation, our previous paper analyzed cellular PKC phosphorylation and KCNQ2 channel activity (Mol. Cell 2010). In that paper, we demonstrated that the time course of oxo-M responses for both KCNQ2 current and AKAP79-mediated PKC phosphorylation were almost identical. Thus we think that sequential events proposed in this manuscript are consistent with previous observations. **We included the above discussion in the revised manuscript (page 16, second paragraph) and reconcile with newly cited paper by Suh and Hille.**

In the older version of our manuscript, we did not emphasize depletion of PIP2 since we took it as an established fact. However, as the reviewer correctly indicated, it would be helpful for general readers to provide background information (page 16, second paragraph). We think that PIP2 depletion and reduction of PIP2 affinity are equally important (page 18, line5-8). In addition, as mentioned in the text, these two mechanisms represent a fail-safe mechanism. We think that this fail safe mechanism is our key claim since most of PIP2 depletion experiments were done with supra maximal concentration of oxo-M (10 uM). Considering that acetylcholine is released at synapses, the receptor activation in physiological conditions should be local and weaker than activating all receptors on the cells. We think that reduction in PIP2 affinity is critical in this regard since it amplifies weak signals. Furthermore, we found a publication just came out from Brown lab showing that KCNQ2 channel binds not only to PIP2 but also to a broad spectrum of negatively charged lipids (Telezhkin et al. JBC e-pub ahead 2012, doi:10.1074/jbc.M111.322552). This raises a serious question as to why other negatively charged lipids do not mask the PIP2 depletion pathway; we think that reduction of PIP2 affinity should also reduce bindings of other negative lipids. We added this discussion in the revised manuscript (page16, line 20-22).

2) Difference between ms-PIP2 and diC4-PIP2. As the reviewer anticipated, we first used diC4-PIP2 to prevent depletion of PIP2. However, diC4-PIP2 did not prevent oxo-M response of KCNQ2 currents. Thus we switched to ms-PIP2. We realized that the estimate of cellular concentration of PIP2 as 10 uM assumes that lipid PIP2 diffused into cytosol makes it 10 uM, thus membrane PIP2 concentration is much higher. We agree that we should consider the difference between 3D diffusion through cytoplasm versus 2D diffusion within membrane which was not discussed in the earlier version of the manuscript. Indeed, McLaughlin described membrane concentration of PIP2 to be ~1000-fold higher than if it was diffusing throughout the cytoplasm of a cell, (from cheap trick 1 box from Ann Rev. Biophys above). These differences would explain why membrane PIP5 kinase could counteract PLC, while diC4-PIP2 through pipette could not. In addition, our data indicate that augmentation by diC4-PIP2 required around two minutes to reach peak amplitude. This latency may attribute to slow diffusion of PIP2 in our experimental condition. <u>This slow diffusion of PIP2 would be why PIP2 could not counteract PLC in our condition.</u> As discussed above, we are confident that our data is solid. However, as the reviewer indicated, diC4-PIP2 experiments in Fig. 6c seem to cause confusion rather than help to understand the experiment, thus we deleted PIP2 columns from Fig. 6c.

POINTS OF DETAIL.

1. P.3, para 2, line 3. Evidence for a role of M-channels in the slow after-hyperpolarization (Tsigounis & Nicoll, 2008) is not very convincing. Indeed, there is ample evidence for a role in regulating firing frequency independent of the sAHP (e.g., Peters et al., 2005: Nat Neurosci 8: 51-60).

Responses: As per suggestion, we deleted "slow after-hyperpolarization". We also updated the reference accordingly in page 3, line 10.

2. P.3, para 2, line 11. In reference to the axon initial segment, authors might note a functional follow-up to Pan et al (2006) in Shah et al., 2008: PNAS, 105, 7869.

Responses: Thank you very much for reminding us of the significant paper. This literature as well as other references are added and updated in the relevant paragraph in introduction (page 3, lin17-19).

3. P.4, para 1, line 13. The more recent work of Hernandez et al., 2008 (J.Gen.Physiol., 132, 361) implying a rather different PIP2 binding site should be referred to.
Responses: We appreciate the detailed analysis of PIP2 binding by Hernandez et al. (2008). One caveat of this paper is that they used a wrong PIP2 binding domain of Kir2.2 for their modeling. Recently, McKinnon group demonstrated that different domains of Kir2.2 interact with PIP2 by co-crystallizing these molecules (Nature 477,495-498, 2011). However, the mutation analyses conducted by Hernandez et al. are valid and were recently reproduced by another group (Telezhkin et al. JBC e-pub ahead 2012). Thus, these recent findings have been incorporated into the text (page 4, line 14-15; page 9, line 17-18).

4. Page 4, last para. "....reduced ion conducting properties" Meaning reduced open probability? (Ionic conductance of the channel is unchanged.)
Responses: Thank you for the correction. It is now stated “reduced channel activity” (page 4, line 23).

5. Page 5, line 6. BIS IV. What does IV mean? (There are no chemicals listed in Methods.) How was this applied and for how long?
Responses: Materials and reagents section is added to the methods as well as chemical name. IV stands for four. It is seems to be common to call bisindolylmaleimide compounds with roman numbers to identify analogues. BIS IV is treated at 10 micromolar for more than 5 min. This is stated in the methods (page 22, line 10-12).

6. Page 5, line 9. Phosphorylation (spelling).
Responses: Corrected (page 5, line 12).

7. Page 6, para 2, line 1". ....increased functional expression" - misleading, expression is unchanged - increased activity?
Responses: Changed to “current densities were increased in cells expressing…” (page 6, line 5)

8. Page 6, para 2. Was calmodulin Ca-bound? Were immunos done in presence or absence of calcium? (see major Point 3)
Responses: Calcium-free condition is stated (page 7, lane 4).

9. Page 7, line 10. CaM-V5. What is this? (No description in Methods).
Responses: We added a statement “V5 epitope tagged calmodulin” in the text (page 7, line 3) as well as in Methods (page 19, line 2)

10. Page 8, para 2, line 3. "...marginal change ...with KCNQ2(S541A)" - not really - looks about half that with wt.
Responses: We agree it looks half that of wt. However the differences are not statistically significant from no oxo-M control even with n=78 in the original manuscript. Thus we avoided describing it as “FRET signal is reduced”. Regarding KCNQ(S541A), reviewer 3 raised a concern with comparing FRET signals between mutants since it may have different anisotropies. Thus we added FRET experiments using BIS IV treated cells, which showed essentially identical responses to that of KCNQ2(S541A). Therefore, instead of mentioning “marginal change”, we stated “Oxo-M induced FRET responses were suppressed to a similar degree” in either KCNQ2(S541A) and BIS IV treated cells in the revised manuscript (page 9, line 10-11).

11. Page 8, para 2, line 5. "these results infer that..." Not infer, imply. (The experiment implies, the observer infers.)
Responses: Corrected (page 9, line 14).

12. Page 9, first sentence. See Detail point (3) above and re-think.
Responses: We revised the text as suggested (page 9, line 17,18).

13. Page 9, line 5. Infers again - should be implies (see 11)
Responses: Corrected (page 9, line 23).

14. Page 9, line 5 "calmodulin binding affects" - might affect?
Responses: Corrected according to the suggestion (page9, line 23).

15. Page 9, line 7. Utilized, not utilize.
Responses: Corrected according to the suggestion (page 10, line 1).

16. Page 14, para 1, last line. "Figs 2 ...and 3 ...show that CaM dissociation from the channel complex triggers suppression of the M-current". They don't, since no currents are shown - "is associated with" perhaps.
Responses: Corrected according to the suggestion (page 16, line7).

17. Fig.1. B,D. How are currents normalized?
Responses: The M-current and KCNQ2 currents are normalized to the current at t=0. This is included in the figure legends.

F. What are the two bands at 100 and 75 kD?
Responses: The immunoblots of KCNQ2 usually give these two bands; a sharp and faint 75kDa band and a fuzzy 100kDa band. Our previous study (Nat. Neurosci 2003) indicated that only 100kDa is phosphorylated, which suggests that 75kDa is unglycosylated immature KCNQ2 and 100kDa is glycosylated KCNQ2. This is mentioned in the text (page 6, line 13).

18. Fig.3. What is meant by n=100? 100 cells? 100 experiments?
Responses: n=100 stands for 100 cells. This is stated in the text (page 8, line 17). Please note new values since it is from new experiments.

19. Fig. 4. Again explain normalization procedure. Also, please give mean current densities for each construct in the legend.
Were the IC50s in Figs D (S541D) and E(R353G) significantly different from wt?
Please give full details of parameters and significance levels in text or legend.
Responses: All normalization referred to is the current amplitude at t=0. This is now stated in the figure legends. Fit parameters are now shown in the supplementary figure 1a.

20. Fig 5. No effect of PDBu in absence of neomycin: this presumably means that PIP2 is normally too high for an affinity change to have much effect - then much of the effect of Oxo-M in Fig 1 must be due to PIP2 depletion, not affinity change (see Major Point 3).
Responses: We would like to remind that this is supraphysiological ATP concentration (4 mM), which we believe that it leads to an increase in endogenous PIP2 (see initial increase in KCNQ2 current in Fig. Xa, which is equivalent to diC4-PIP2 effect). In addition, it would be better to emphasize that phosphorylated KCNQ2 channel is mediated by AKAP79 anchored PKC. The PKC-AKAP79 interaction is through the pseudosubstrate domain, thus PDBu is not a potent agonist. In addition, we demonstrated in the previous paper that application of PDBu induced only around 50 % cellular PKC activity compared to application of 3 uM oxo-M (Plos One 2011). Thus, unlike oxo-M stimulation, KCNQ2 would be partially phosphorylated. We believe that this is one reason that PDBu did not suppress KCNQ2 currents. From KCNQ2(S541D) mutant analysis (Fig. 4), however, we expect that endogenous PIP2 for phosphorylated KCNQ2 is not sufficient for full activity. Regarding PIP2 depletion, we do agree it is an important part of M-channel regulation. We think that PIP2 depletion and PKC phosphorylation are like two wheels of a cart, both parts are equally important. This discussion is added as new paragraphs in the discussion (page16 second paragraph to page 18).

21. Fig.6. Explain precisely what panels C and F show - currents in Oxo-M? what does that mean?
Responses: These panels show the amplitudes of current relative to current amplitude at t=0. In other words, normalized currents at t=1 shown in panel a and b are extracted as a histogram to depict differences among conditions. The titles of y-axis are corrected and this is stated in the figure legend (page 32, Fig. 6 legend).

22. It is not clear which experiments were done using perforated patches and which with whole-cell patching. Please state clearly in the legends.
Responses: M-current measurement from SCG in figure 1 used perforated patch and rest of experiments used whole-cell patch technique. The statements for patch clamp mode are moved to the beginning of the sentences in the figure legends to avoid confusion. We also added more information in Methods to clarify which experiments used which technique (page 18).

23. The title seems a bit vague, and doesn't give much indication of exactly what the paper is about. Better perhaps: "[Co-ordinated] signal integration by protein kinase C, calmodulin and PIP2 at the M-type potassium channel following muscarinic stimulation."
Responses: We wish to follow this suggestion, but the title is already close to the journal title limit of 100 characters (the original title is 90 characters). Thus we cannot put more information.

Referee #2

These data are consistent with a model in which the presence of CaM stabilizes somehow currents through KCNQ2 and oxo-M acting through Gq-coupled receptors stimulates PKC to phosphorylate KCNQ2 involving S541, an event that leads to reduced interactions of CaM and the channel and thus destabilizes the open channel state and reduces activity. I feel this part of the study does not need to be changed in any way to enhance its message.
We appreciate reviewer's strong assent.

I found the inclusion of 500 μMdiC4-PIP2 or ms-PIP2 not terribly convincing. I am not aware of studies showing directly (in inside-out patches) effects on channel activity of soluble PIP2 that has acyl chains less than 8-carbon long (diC8).
Responses: We agree that diC4-PIP2 is not very common and has some limitations. We newly added experiment using diC8-PIP2 and compared with results obtained with diC4-PIP2, which were revealed to be equally effective for KCNQ2 channel. This result is consistent with the recent findings that KCNQ channel family tolerates various acyl groups including diC4 since 1) diC4-PIP4 was successfully used to facilitate KCNQ1 channel in inside-out patches (Proc. Nat. Acad. Sci. USA 108,9095-100 (2011)), 2) various negatively charged lipids with other acyl chain moieties can facilitate KCNQ2 channel (Telezhkin et al. JBC e-pub ahead 2012, doi:10.1074/jbc.M111.322552). This information is added to the text (page 10, line 11-17).

1) The study would be greatly strengthened by showing direct effects of PKC phosphorylation on apparent affinity for PIP2. A dose-response of KCNQ2 (or Q2/Q3) activation by diC8-PIP2 could be shown for wt, and the S541A and S541D mutants to assess whether the lack of phosphorylation or the phosphomimetic mutations, respectively, alter channel-PIP2 affinity.
Responses: To address this key question of the direct effects of PKC phosphorylation on apparent affinity for PIP2, we examined change in mutant channel activities after rupturing membrane with 500uM diC8-PIP2. The results suggest that endogenous PIP2 level is near the saturating concentration for wild type and S541A but is not sufficient for S541D, which supports our view that S541D has lower PIP2 affinity. Furthermore, new scatter plots show close correlation (r=0.91) between diC8-PIP2 induced augmentation and IC50 of neomycin. These results are included in new Figure 4F and summarized in the new Supplementary figure 1. We appreciate reviewer's suggestion.

2) This study on KCNQ inhibition by PIP2 hydrolysis and PKC activation resembles a study in Kir3 channels by Keselman et al., 2007 Channels1:113-123. The authors may benefit by considering the results of this study.
Responses: Thank you for informing us of this interesting literature. This is cited and used in the discussion (page 17, line7).

Referee #3

Major issues:
1. It should be checked if FRET differences between the groups don't reflect differences in flexibility and/or orientations of the xFP-tagged proteins and not by interaction levels. One way to cope with this issue is by using anisotropy measurements. The second and much easier experiment might be to repeat the experiments shown in Figure3 in the presence of BIS-IV vs. PDBu. Thus, without a potential change in the protein structure, the change in kinase activity should be reflected by FRET readouts.
Responses: We agree that there are potential problems with using various mutants. As per suggestion, we performed FRET experiments in the presence of BIS IV, which blocked KCNQ2 phosphorylation. BIS IV treatment showed almost identical response to that of KCNQ2(S541A),

which suggest that PKC phosphorylation of S541 is key for this response. In light of the reviewers concerns new data has been incorporated into Figure 3F.

2. One cannot state that (p. 8, bottom) "Collectively, these results further infer that muscarinic stimulation induces dissociation of Calmodulin from channel complex through...". There might be several other reasons for the author's observations that FRET decreases such as conformational changes between the molecules without their dissociation or orientation changes between fluorescent proteins that effect their dipole-dipole orientation.
Responses: We understand reviewer's concern regarding anisotropy rather than dissociation. However, we would like to respectfully point out that Fig. 2c demonstrates dissociation of calmodulin using immunoprecipitation, which is induced by oxo-M. This biochemical experiment suggests that decrease in FRET signal is due to dissociation rather than change in dipole orientation. This rationale is stated in the text (page 9, line 4-8). In the original manuscript, we used the word "collectively" to sum up previous figures and other sections of the text. However, we agree that this wording is misleading and thus corrected (page 9 line 14).
In addition, we agree "infer" is not a correct wording as the reviewer 1 indicated (page 9 line 14).

3. In Fig. 1F normalized traces for KCNQ2 S541A mutants are shown while raw FRET efficiency data is shown only for KCNQ2 S541D. In order to assess the dynamic range of the FRET measurements between negligible- to constitutively- phosphorylated in the context of FRET readout between CaM and the M-Channel, and to strengthen the measurement reliability the missing row data for 551A mutant should be added to figure 3A.
Responses: As per reviewer3's suggestion, we have redone side-by-side comparison among wt, S541D and S541A and added to the new Fig. 3a. KCNQ2(S541A) showed equivalent FRET efficiency to that of wild type. In addition the new data showed reduced FRET efficiency (68.2 +/- 9.8%) for KCNQ2(S541D), which were very similar to the value in the original determination (71.6 +/- 9.8%) (page8, line 21).

4. According to the model FRET should decrease upon channel phosphorylation, in Figure 3 however, the decrease in FRET signal in the presence of OXO is not completely abolished; can the authors address the possibility that CaM may be associated with the channel while phosphorylated?
Responses: It is an interesting possibility that phosphorylated CaM stays on the channel while reducing FRET signal. As mentioned above, our immunoprecipitation suggests reduction of bound calmodulin. Nonetheless, one possibility is that calmodulin would shuttle between AKAP79 and KCNQ2, which would be in the vicinity of KCNQ2 channel. However, analyzing this would be beyond the scope of this paper. In addition, we believe that oxo-M induced calmodulin dissociation is not an all-or-none response. As shown in Fig. 2c, oxo-M reduces about 50% of calmodulin binding. Since KCNQ2 channel is a tetramer, 50% reduction would roughly correspond to the loss of two calmodulin per channel. This is a rough estimate, but we would like to point out that this could be sufficient to affect all channels. In addition, as shown in a new reference suggested by reviewer 2 (Channels1:113-123 (2007)), maximal FRET responses are usually around 10-20% at most (another example, Nature 437, 569 (2002) for a typical FRET probe; AKAR). One thing we should keep in mind is that, as mentioned in reviewer1 question#1, CaM is an abundant protein. Thus most of KCNQ2-YFP is bound to endogenous CaM. Hence initial FRET signal is diluted, which may deteriorate the dynamic range. We hope that we can improve the dynamic range of FRET in the future.

Minor:
1. Page 6 - The reference the fig. 1E and 1F contain wrong n-numbers. (n=4 instead of n=42 as shown in the Figure legend.
Responses: Thank you for identifying an error in Fig. 1E. The original manuscript was confusing since not all n numbers are listed. We corrected this point as well as N number for 1E). To avoid confusion, N value for 1F is now included in the figure legend to avoid confusion. (page 6, line 6-12)

2. I don't understand the significant discrepancy between the Ns in the calculation of current densities, showing 30-50 cells for each group and the normalized traces showing ~10- cells per group.
Responses: Not all cells that established patch clamp were used in a single experiment. After initial evaluation of current densities, cells were used in different experiments, such as different oxo-M

concentrations, different time course, and other experiments that were not included in a single panel or in the manuscript. This is why the n of current density is higher than that in other panels. On the other hand, N of below 10 cells is widely accepted for electrophysiological studies.

2nd Editorial Decision 17 April 2012

Thank you for submitting your revised manuscript to the EMBO Journal. Your revision has now been seen by the three referees and their comments are provided below.

As you can see, the referees appreciate the introduced changes and support publication in the EMBO Journal. Referee #1 raises a few minor issues to be addressed before publication here. Once we receive the revised version, we will proceed with its acceptance here.

Thank you for submitting your interesting study to the EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The authors have dealt with my comments satisfactorily, with the following exceptions which require minor revision:

Major point #2. Bal et al (2010) is better than the authors' choice of Bal et al (2008) since the former directly addresses the interaction of AKAP with calmodulin binding, and hence is most relevant to the present paper.

Minor point 5. Add BIS IV as an abbreviation in para 1, p 19 (Methods).

Minor point 19. The statistical significance of the IC50 differences in Fig 4E (and now supplemental table 1) is still not given.

Referee #2

The data added as Figure 4F and also summarized in the new Supplementary Figure 1 satisfied the concern I had raised in my prior review. I feel that the authors have been very responsive to the critiques and that the manuscript will be a valuable addition to the literature.

Referee #3

The authors made major efforts to revise the manuscript to comply with the reviewers concerns. As to my judgement I have no further comments on the manuscript.

2nd Revision - authors' response 19 April 2012

Referee #1

1) Bal et al (2010) is better than the authors' choice of Bal et al (2008) since the former directly addresses the interaction of AKAP with calmodulin binding, and hence is most relevant to the present paper.

Response: Bal (2010) has been cited and the reference list is updated accordingly.

2) Minor point 5. Add BIS IV as an abbreviation in para 1, p 19 (Methods).
Response: Abbreviation BIS IV is added to para1, p19.

3) Minor point 19. The statistical significance of the IC50 differences in Fig 4E (and now supplemental table 1) is still not given.
Response: P values are indicated in the new supplementary table 1.

We also updated the reference information for Telezhkin et al. JBC (2012) since publication information became available.

