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CREB regulates the expression of type 1 inositol 1,4,5-trisphosphate receptors

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MS TITLE: CREB regulates the expression of Type 1 Inositol 1,4,5-trisphosphate receptors

AUTHORS: Vikas Arige, Lara E Terry, Taylor R Knebel, Larry E Wagner, and David I. Yule
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

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Reviewer 1

Advance summary and potential significance to field

This paper by Arige et al. describes regulation of inositol 1,4,5-trisphospahe receptor (IP3R) type-1 by the CREB transcription factor in HEK293 cells. The authors show that prolonged stimulation of the cells with Forskolin (FK) increased the amount of type-1 but not type-2 or type-3 IP3Rs. Similar changes were elicited by expression of a constitutively active CREB variant (VP-16 CREB) and the effect of forskolin was inhibited by expression of a dominant negative version of CREB (KCREB) or by an inhibitor of PKA. In-silico analysis revealed the presence of CREB consensus binding elements in the putative promoter regions of all three IP3R genes and a mild positive correlation was found in data mining between the expression levels of CREB and all three IP3Rs. The authors also analyzed the Ca2+ responses to carbachol in cells transfected with the dominant negative version of CREB and also investigated the elementary Ca2+ events in response to uncaged IP3. These experiments showed a modest inhibition of the Ca2+ responses to carbachol and a more substantial inhibition of the number of elementary Ca2+ events in the KCREB-transfected cells.

These are potentially interesting and important results from a group that has contributed greatly to the understanding of IP3R biology. This study contributes to our understanding of the cross talk between the cAMP and IP3/Ca2+ signaling pathways by identifying the regulation of IP3R type-I expression at the transcriptional level by cAMP via the CREB transcription factor.

Comments for the author

My main criticism is the preliminary feel of the study and the relatively small changes that are found in the levels of the IP3R and, more importantly, in the Ca2+ responses of the cells after interfering with CREB functions. Even if one understands that using cell populations and transfections the changes would be mitigated by the contributions of non-transfected cells to the signals, the biological significance of the reported changes would require some further support.

Major points:
1. It is quite possible that the HEK293 cell is not the best model to demonstrate the importance of this regulation. Using another cell line, perhaps a neuronal one, to demonstrate the universality of this regulation would significantly strengthen the study.

2. Analysis of the promoter region of the various IP3Rs in a luciferase assay and its response to CREB would also strengthen the study. It would also confirm the specificity among the three IP3R isoforms. This is especially relevant as the in-silico analysis suggests the presence of CREB binding elements in the putative promoter region of all three IP3R, yet only IP3R1 is up-regulated in this study.

3. Related to the previous point, the amount of CREB may not be the best correlate with the regulatory importance of CREB.

4. The increase in IP3R expression after FK is much smaller in Fig. 4B than in Fig. 1A. Since all changes are evaluated from three relatively tight data points, such differences between the magnitudes of the responses between experiments is somewhat concerning.

5. The difference in the Ca2+ response in Fig. 5A is very tiny reaching significance only at one concentration even if one understands that many non-transfected cells also contribute to the response. In this respect, the analysis of the individual cells shown in Fig. 6 is more informative. However, these changes while do show a tendency to be smaller, still did not reach statistical significance except for a few columns. What would be the effect of overexpression of the active version of CREB in the Ca2+ responses to carbachol? Since the basal activity of the CREB on the IP3R is not known, it is possible that the changes in response to CREB activation would be larger than to its inhibition.

6. The most striking changes appear to be in the number of elementary events in response to the uncaged IP3, shown in Fig. 7. Again, would the expression of the active CREB elicit the opposite changes? Were these changes also specific for the cells expressing only the type-I IP3Rs?

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Minor:
Some of the Figures should be combined. They do not all justify occupying a separate Figure.
Fig. S4 is unnecessary.

Reviewer 2

Advance summary and potential significance to field

This paper provides a role for CREB in regulating expression and function of IP3 receptors in a isoform-selective manner. Whilst a role for acute regulation of IP3 receptors by cyclic AMP/PKA has been documented previously much less is known about chronic effects. Consequently the results presented are novel if not slightly over-egged.

Comments for the author

1. Fig. 2. Without further details of the predicted CREB elements in the promoters of ITPRs, this Table is uninformative. Indeed the predicted sites plus the correlation analysis for all three isoforms appears to contradict the ITPR1-specific effects of Forskolin effect reported in Fig. 1 (discussion noted).

2. Figure S3. The GAPDH blots look similar if not identical. Some clarification on how the IP3R2 and IP3R3 levels were determined is required.

3. Figure 4. 'The forskolin-induced endogenous IP3R1 protein level was markedly diminished upon blocking endogenous CREB (Fig. 4A, B).' This is overstating things. In these experiments, the forskolin induced increase in ITPR1 expression is reduced by KCREB. But KREB alone reduces expression. So the percentage increase in expression by forskolin in the absence and presence of KREB looks about the same to my eye. The statistics presumably refer to the control.

4. Figure 5. 'Agonist-induced Ca2+ release is markedly diminished..' Again this is overstating things. There is very little effect of KCREB expression here on agonist evoked signals (with only 1 point of statistical significance). Did the authors examine the effects of overexpressing CREB on Ca2+?

5. Figure 6. 'CCh-induced Ca2+ release is markedly attenuated ..'. Once again, this is overstating things (unless a single malt is to hand) as much of the data is not statistically significant.

6. Figure 8. This seems like a bit of an after-thought and is incomplete with a rationale apparently based on a 2011 paper. The in silico predictions in Table S2 are not so informative (see comments to Fig. 2A). It was not clear if forskolin regulates KRAP levels. And the relative role of KRAP v IP3R1 expression (or any other CREB target for that matter) on de-regulated Ca2+ signals mediated by CREB were unexplored. I am not sure this adds much to the study.

Minor comments
1. In the abstract, the results are described in the context of 'chronic activation of PKA' but there was little attempt to probe for PKA involvement directly. This should be toned down.
2. Fig. S1. Time course shows less than a 2 fold increase cf 3 fold increase from Fig.1.
3. Fig. S2. More details required for the phosphorylated IP3R blot. What is being measured here?
4. 'conclusively demonstrated' – let the readers decide that!
5. Check molecular weight markers are present in all blots (including those in Supplementary eg Fig. S2 and Fig. S3).
6. Fig. 3. Summary data presentation is inconsistent. 3B has no error bars for some conditions. Individual data points are aligned vertically in some instances but not others

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First revision

Author response to reviewers' comments

Reviewer 1

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Reviewer 1 Comments for the author

My main criticism is the preliminary feel of the study and the relatively small changes that are found in the levels of the IP3R and, more importantly, in the Ca2+ responses of the cells after interfering with CREB functions. Even if one understands that using cell populations and transfections the changes would be mitigated by the contributions of non-transfected cells to the signals, the biological significance of the reported changes would require some further support.

Response: We thank the reviewer for their time and constructive comments on our manuscript. We agree that the effects on IP3R1 protein levels and global Ca2+ signals are relatively small. While the effects on protein levels are significant, the data presented suggests that basal activity of the PKA/CREB axis contributes to the expression of IP3R1 (effect of PKA inhibitors and effect of dominant negative CREB in naïve cells) likely reducing the magnitude of further stimulation. We now report this finding in additional cell lines indicating this is a common phenomenon. We contend that the modest effect on global Ca2+ signals are likely because of non-uniform transfection of reagents, but also because of the amplification through calcium induced Ca2+ release and Ca2+ influx which is inherent property of the Ca2+ signaling machinery. In support of this idea when the fundamental Ca2+ release events (puffs) are isolated, a more impressive effect on IP3R1 activity is observed. We believe that the changes in IP3R1 together with its important binding partner KRAP could have a significant impact on the overall spatiotemporal Ca2+ signals. This idea is supported by a recent paper describing the important role of KRAP to influence the activity of IP3R. Following the reviewer’s suggestions, we have added substantive new data and made changes to the text of the manuscript to strengthen our claim regarding the biological significance of the data.

Major points:
1. It is quite possible that the HEK293 cell is not the best model to demonstrate the importance of this regulation. Using another cell line, perhaps a neuronal one, to demonstrate the universality of this regulation, would significantly strengthen the study.

Response: We thank the reviewer for their valuable suggestion. Accordingly, to address this, we inhibited PKA using H89 in SH-SY5Y and HeLa cells both of which predominantly express IP3R1 and found that both the endogenous IP3R1 and KRAP levels decreased in these cell lines as well. This

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effect is similar to PKA inhibition using PKI plasmid in HEK-293 cells. These results indicate a role for PKA-CREB axis in regulating IP3R1 and KRAP levels across various cell lines.
2. Analysis of the promoter region of the various IP3Rs in a luciferase assay and its response to CREB would also strengthen the study. It would also confirm the specificity among the three IP3R isoforms. This is especially relevant as the in-silico analysis suggests the presence of CREB binding elements in the putative promoter region of all three IP3R, yet only IP3R1 is up-regulated in this study.

Response: We thank the reviewer for their suggestion. As per their suggestion, we carried out luciferase assays using the proximal-promoter domain of IP3R1 or IP3R2 upon over-expressing CREB. We observed promoter activities of both IP3R1 and IP3R2 increased upon ectopic over-expression of CREB. The regulation of IP3R2 by CREB has been previously reported as stated in the manuscript. To further authenticate the role of CREB in regulating IP3R1 expression, we also co-transfected IP3R1 promoter construct with KCREB and observed an opposite trend to that of over-expressing CREB. In addition, PKA inhibition using H89 also suppressed the promoter activity. In total, these results indicate potential binding sites for CREB in the proximal promoter domain of IP3R1 similar to those already reported for IP3R2. While outside the scope of the current study, based on our in silico predictions, we plan to characterize the proximal promoter regions of all three IP3Rs sub-types along with KRAP in detail to gain molecular insights into differential/tissue-specific expression of these subtypes depending on the specific transcription factors expressed in those tissues.

3. Related to the previous point, the amount of CREB may not be the best correlate with the regulatory importance of CREB.

Response: Over-expression of constitutively active CREB caused a dose-dependent increase in the reporter activity and endogenous protein levels of IP3R1 in HEK-293 cells suggesting a correlation between the amount of CREB and IP3R1. Interestingly, blocking CREB or PKI had the opposite effect. Similarly, over-expression of constitutively active CREB caused a dose-dependent increase in the reporter activity of IP3R2 in HEK-293 cells. Data mining from GTEx portal suggests a correlation between the transcript levels of IP3Rs and CREB. Based on these observations, it is tempting to speculate that the amount of CREB or active CREB may correlate with the IP3R levels in most, if not, all tissues. However, we are aware that these speculations need to be further substantiated with experimental evidence.

4. The increase in IP3R expression after FK is much smaller in Fig. 4B than in Fig. 1A. Since all changes are evaluated from three relatively tight data points, such differences between the magnitudes of the responses between experiments is somewhat concerning.

Response: In Fig. 4, HEK-293 cells were transfected using lipofectamine-2000 with either pcDNA or KCREB prior to treatment with forskolin, however, the cells in Fig. 1A were not transfected before treatment with forskolin. We think the differences in magnitudes of responses between Fig. 4B and Fig. 1A may be due to these different experimental conditions. Moreover, we performed this experiment in the presence or absence of forskolin/KCREB multiple times and observed similar modest changes in IP3R1 levels each time supporting a role for CREB.

5. The difference in the Ca2+ response in Fig. 5A is very tiny reaching significance only at one concentration, even if one understands that many non-transfected cells also contribute to the response. In this respect, the analysis of the individual cells shown in Fig. 6 is more informative. However, these changes while do show a tendency to be smaller, still did not reach statistical significance except for a few columns. What would be the effect of overexpression of the active version of CREB in the Ca2+ responses to carbachol? Since the basal activity of the CREB on the IP3R is not known, it is possible that the changes in response to CREB activation would be larger than to its inhibition.

Response: We agree with the reviewer that the differences in Ca2+ signals are relatively small in our flex-station assays. We observed differences in CCh-stimulated global Ca2+ signals between the transfected cells at lower (threshold) concentrations of CCh which become less pronounced as the concentration of CCh increases. As stated above, Ca2+-induced Ca2+ release mediated by IP3Rs and the contribution of Ca2+ influx may further confound these assays at higher stimulus.

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We did not observe an increase in the global Ca2+ signals upon over-expression of CREB as compared to pcDNA. The data presented suggests that activity of the PKA/CREB axis contributes to the basal expression of IP3R1. Nevertheless, Ca2+ puffs are significantly enhanced upon over-expression of CREB in these cells.

6. The most striking changes appear to be in the number of elementary events in response to the uncaged IP3, shown in Fig. 7. Again, would the expression of the active CREB elicit the opposite changes? Were these changes also specific for the cells expressing only the type-I IP3Rs?

Response: We thank the reviewer for their suggestion. We now provide TIRF/puff data following over-expression of VP16-CREB. Notably, there was a significant increase in the number of puffs following over-expressing CREB in these cells compared to pcDNA. Our population based assays using hR3 endo cells and Western blots suggest that the effect of CREB is specific only to IP3R1 sub-type.

Minor:

Some of the Figures should be combined. They do not all justify occupying a separate Figure.

Response: We agree with the reviewer and according to their suggestion/journal guidelines, in our revised manuscript we combined figures where necessary.

Fig. S4 is unnecessary.

Response: We agree and have deleted this figure in the revised manuscript.

Reviewer 2

Advance summary and potential significance to field

This paper provides a role for CREB in regulating expression and function of IP3 receptors in a isoform-selective manner. Whilst a role for acute regulation of IP3 receptors by cyclic AMP/PKA has been documented previously much less is known about chronic effects. Consequently the results presented are novel if not slightly over-egged.

Response: We thank the reviewer for their time, critical comments and feedback on our manuscript. As per their suggestions, we have moderated our discussion of the magnitude of the changes, carried out additional experiments and made changes to the manuscript to support our findings.

Reviewer 2 Comments for the author

1. Fig. 2. Without further details of the predicted CREB elements in the promoters of ITPRs, this Table is uninformative. Indeed the predicted sites plus the correlation analysis for all three isoforms appears to contradict the ITPR1-specific effects of Forskolin effect reported in Fig. 1 (discussion noted).

Response: We agree with the reviewer and performed promoter-reporter assays to validate the potential role of CREB in regulating the expression of ITPR1 and ITPR2. Our in silico predictions, correlation assays, promoter-reporter assays support a key role for CREB in regulating the expression of ITPRs. Our results employing various molecular and pharmacological tools to modulate the levels of factors involved in PKA-CREB axis support a role of PKA-CREB axis in regulating the expression of ITPR1 in HEK-293. It should be noted that ITPR2 expression is regulated by CREB in hepatocytes as reported previously (mentioned in discussion section). We speculate that the difference in epigenetic modifications could result in differential expression of IP3R sub-types in various cell types/tissues.

2. Figure S3. The GAPDH blots look similar if not identical. Some clarification on how the IP3R2 and IP3R3 levels were determined is required.

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Response: We thank the reviewer for bringing this error to our attention. We have replaced this blot and paired it with the appropriate loading control.

3. Figure 4. 'The forskolin-induced endogenous IP3R1 protein level was markedly diminished upon blocking endogenous CREB (Fig. 4A, B).' This is overstating things. In these experiments, the forskolin induced increase in ITPR1 expression is reduced by KCREB. But KREB alone reduces expression. So the percentage increase in expression by forskolin in the absence and presence of KREB looks about the same to my eye. The statistics presumably refer to the control.

Response: We carried out this experiment multiple times and observe that the endogenous protein level of IP3R1 diminished upon blocking CREB compared to forskolin treated condition. Nevertheless the observation that KCREB or PKA inhibition reduces IP3R1 expression (or promotor activity) suggests that this pathway is active under basal conditions and likely contributes to the levels of IP3R1 expressed.

4. Figure 5. 'Agonist-induced Ca2+ release is markedly diminished.' Again, this is overstating things. There is very little effect of KCREB expression here on agonist evoked signals (with only 1 point of statistical significance). Did the authors examine the effects of overexpressing CREB on Ca2+?

Response: We did not observe an increase in the global Ca2+ signals upon over-expression of CREB as compared to pcDNA. Nevertheless, the Ca2+ puffs are significantly enhanced upon over-expressing CREB in these cells.

5. Figure 6. 'CCh-induced Ca2+ release is markedly attenuated.' Once again, this is overstating things (unless a single malt is to hand) as much of the data is not statistically significant.

Response: We modified this sentence in the revised manuscript.

6. Figure 8. This seems like a bit of an after-thought and is incomplete with a rationale apparently based on a 2011 paper. The in silico predictions in Table S2 are not so informative (see comments to Fig. 2A). It was not clear if forskolin regulates KRAP levels. And the relative role of KRAP v IP3R1 expression (or any other CREB target for that matter) on de-regulated Ca2+ signals mediated by CREB were unexplored. I am not sure this adds much to the study.

Response: We have expanded on the rationale for this experimental series and expand the experiments performed. We now provide new evidence that forskolin treatment causes an increase in endogenous protein level of KRAP. Moreover, inhibition of PKA using either PKI or H89 in various cell types resulted in diminished KRAP levels. These observations indicate involvement of PKA-CREB axis in regulating KRAP expression under basal and induced-conditions. KRAP is involved in immobilizing IP3Rs and recent evidence suggests that it plays substantial role in defining which IP3Rs are active or "licensed" to respond. We have cited this paper in our revised version and our findings in hR1 endo cells are in agreement with their observations in HeLa cells. While outside the scope of the current studies based on our in silico predictions, we plan to characterize proximal promoter regions of all the three IP3Rs along with KRAP in detail to gain molecular insights into differential/tissue-specific expression of these sub-types depending on the specific transcription factors expressed in those tissues.

Minor comments

1. In the abstract, the results are described in the context of 'chronic activation of PKA' but there was little attempt to probe for PKA involvement directly. This should be toned down.

Response: We provide evidence for involvement of PKA using forskolin which activates CREB via PKA. Furthermore, we demonstrate involvement of PKA in regulating the expression of IP3R1 and KRAP by blocking PKA using PKI or H89 across various cell lines. It should also be noted that the endogenous IP3R1 receptors remain phosphorylated even after 12 hours treatment with forskolin. Based on all these observations, in the abstract, we state ''PKA is chronically active''.

2. Fig. S1. Time course shows less than a 2 fold increase cf 3 fold increase from Fig.1.

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Response: We repeated this experiment and replaced this blot with a representative blot which shows nearly 3 fold increase in endogenous IP3R1 levels.

3. Fig. S2. More details required for the phosphorylated IP3R blot. What is being measured here?

Response: The blot is probed with a phospho-specific ab that recognized phosphorylated ser-1756. In the supplementary data, we now state, “HEK-293 cells were treated with 10 μM forskolin for 12 hours. Western blots depicting increase in the phosphorylation of the endogenous IP3R1 at the Ser-1756 residue and total IP3R1 protein levels upon treatment with forskolin.”

4. ‘conclusively demonstrated’ – let the readers decide that!

Response: We modified this in our revised manuscript.

5. Check molecular weight markers are present in all blots (including those in Supplementary eg Fig. S2 and Fig. S3).

Response: We thank the reviewer for noticing this. We incorporated molecular weight markers for all the blots.

6.Fig. 3. Summary data presentation is inconsistent. 3B has no error bars for some conditions. Individual data points are aligned vertically in some instances but not others.

Response: The scatter plots are now arranged consistently as scatter dot plots. 3B has error bars, probably not visible due to small variability and color that we used for representation.

Second decision letter

MS ID#: JOCES/2021/258875

MS TITLE: CREB regulates the expression of Type 1 Inositol 1,4,5-trisphosphate receptors

AUTHORS: Vikas Arige, Lara E Terry, Sundeep Malik, Taylor R Knebel, Larry E Wagner, and David I. Yule
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This is a revised version of the manuscript. The Authors have performed additional experiments and added important new data that significantly strengthened the study. They have addressed my concerns and the revised version is suitable for publication in JCR. This study advances our understanding of the cross talk between the cAMP and IP3/Ca2+ signaling pathways by identifying the regulation of IP3R type-I expression at the transcriptional level by cAMP via the CREB transcription factor.

Comments for the author

This is a revised version and the Authors have addressed my concerns to my satisfaction. The revisions made the manuscript significantly stronger.

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Reviewer 2

Advance summary and potential significance to field

This is a signficant study as outlined in my original review.

Comments for the author

I am happy with the revisions and recommend acceptance.

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