45 years of cGMP research in Dictyostelium: Understanding the regulation and function of the cGMP pathway for cell movement and chemotaxis

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RE: Manuscript #E21-04-0171
TITLE: 45 years of cGMP research in Dictyostelium: Understanding the regulation and function of the cGMP pathway during cell movement and chemotaxis

Dear Dr. Van Haastert:

First, apologies for the delay in reviewing the paper. We had to spend some time chasing one of the reviewers. Both reviewers had positive remarks, but both had some issues with the nature of the paper (part review, part data, part model), and specific details. Overall, the impression is that more clarity is desirable about what is already established versus open questions.

One reviewer made extensive suggestions. Please implement as many as possible and indicate in your cover letter any that you disagreed with (and why).

From my own reading of the paper, its readability and usefulness as a solid review of the field could be improved by adding more synthesis of the overall "lay of the land", and a more directed comparison of methods/results/conclusions, rather than just listing them. At the moment, the many technical names, genes, proteins, and various details (all clearly important) also make it hard to read comfortably and to extract the overall message. This is easily fixed by adding one or two directed sentences here and there, indicating that message.

Sincerely,

Leah Edelstein-Keshet
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Van Haastert,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made, as described in the Monitoring Editor's decision letter above and the reviewer comments below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you haveopted out of publishing the review history.

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In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised manuscript, and figures, use this link: Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager

MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

In this study, van Haastert et al. investigated the cGMP pathway in Dictyostelium chemotaxis and migration. In the past few years, the authors and other groups in the field made tremendous progress on understanding on cGMP production, function and regulation during cell migration by using Dictyostelium cell as a model. They have summarized some of the results in their previous review published in 2002. Here, they discussed and summarized some new findings in perspective of biochemical and functional data on cGMP in Dictyostelium. The quality of the data is overall good. I have a few suggestions for improving this manuscript:

1. Various mutant cell lines were used to compare cGMP production. Have sGC or GCA level been measured and compared among strains?

2. It is well known that both sGC and GCA are responsible for cGMP production, especially at vegetative stage. The cGMP production measurement in Table 1 (folate) assumed that sGC is the only source for cGMP generation. I feel that GCA contribution should not be ignored. Therefore the authors may need to either explain it more or add the experiment in GCA null background cell.

3. In Figure 5, the authors claimed the sequential activation upon cAMP stimulation. cGMP production is measured in cell suspension; while Ras activation sGC translocation and F actin formation were imaged when cell was attached onto the surface. It is not convincing that the kinetics generated from different condition are comparable, especially when you try to distinguish millisecond time scale. Based on the methods, the images were recorded at 1 frame/sec. I am not convinced the accuracy of their deduction and conclusion for sequential activation (Ras 0.4s; sGC 0.8s; F actin 0.9s).

4. In Figure 3 reconstitution assay, the lysate was from WT cell. GTPrS only increase about 50% compared with control. A lysate from rasC/G null would decrease the background dramatically and should give more clear comparison.

5. Figure 9B, are data shown the means of replicates?

6. I suggest the authors provide a model Figure to summarize the major findings, which will help readers understand better.

Reviewer #2 (Remarks to the Author):

I am sorry for the delay in my response. However, the ms is not a simple research paper and required much thought to give the best guidance to review.

The ms is a model/review paper to explain the cGMP synthesis/degradation pathway in response to chemoattractant activation. This combines a mixture of published and new data to expand what is known about cG accumulation. The title emphasizes the function of cGMP, but there is little there apart from that published. Also quite little of the data involves actual chemotaxis, again its in the title, and eventually related in the discussion.

As an overview, I think this is a timely and valued ms. However, it is requires work by the reader to see what is fundamentally new and what are new data. This needs to be made far more evident at every stage. I am not saying they do not add references, it is just not so easy to see. Also, I think more clarity to the system is required. Workers outside of Dictyostelium, may not understand the differences between chemotaxis during growth and cell aggregation (development). Neither, are they likely to be aware of the complexity to cGMP signaling, in the context of activation/accumulation and de-activation/degradation (perhaps an early figure). These need to be far better explained at the outset. Axes labelings are not always consistent, so it not easy to understand activation level differences. Controls are not always present. Some additional experimental combinations are required. The data may already exist as published, with data in figs for actual visual comparisons. I will try to provide appropriate guidance.

Primarily, this is about cGMP signaling during development. Nearly all assays are on staved (early developed) cells where the chemoattractant is cAMP. Chemotaxis during growth uses a different chemoattractant, it may not be assumed that all rules during development equally apply for growth. I would modify the title here too. I am not even certain if the GCs used are precisely the same during both stages.

The initial path for cGMP synthesis by cAMP activation requires the cAMP receptor, its coupled G protein and Ras C and G

proteins. In fig 1 We see a 20x-cGMP level difference between WT cells and cells lacking any of these factors. They also express a DN Ras under control of TET - but 1) this is not labeled as such in the fig, 2) they use +/- DOX (not TET) without explanation, and 3) in the text the writing is confusing as it talks about "these cells" without indication that it is the DOX-induced cells that do not activate RAS.

One major issue regards the different cGMP synthases. There are 2 GCs, sGC (soluble) and GCA (membrane). sGC represents 85% activity in development (not growth), and so GCA is dismissed without further discussion. All data beyond assumes regulations all depend on sGC, but no GCA-null and few sGC-null controls are ever presented. I suggest adding them. data exist.

Next (fig2) they look to GC stimulation in cell lysates. Here they do direct activation of G proteins with GTPgS of WT and null cells. Here heterotrimertic Gs are not required for GC, but Ras is. Relative activity is, as with fig1, 20x. Again sGC, GCA null controls are missing.

These data argue that Ras and not Gs are direct activators of GC, but Gs activate Ras.

They then did this in a different way adding activated Ga2 or activated RasG directly to lysates (fig 3). G+ as no effect, while RasG+ activates GC. BUT induced activity differences are not 20x, but only 1.5x. No explanation. I presume this relates to differences in the assay. But why is it not done exactly like in fig 2 so experiments can be compared? This does not make sense. They argue that Gbeta and not Ga2 must activate Ras.

The next section relates to sGC association with the cytoskelton (F-actin). But the entire concept is poorly introduced. First, sGC is termed the soluble GC. I have no issue with old terminology, but later it is clear there is significant basal membrane association and that membrane association and not actin association are important for activity. Neither is this at all clear in the abstract. F-actin associations would seem minimally important.

A reason to consider surface v cytosol sGC binding relates to how Ras must activate sGC. IN response to cAMP, Ras jumps to membranes where it is activated. Presumably, sGC must also be t the membrane if it is to be activated by Ras. It is previously published that, like Ras, sGC, in response to cAMP, jumps to membranes. It may be expected, but not so stated, that jumping then relates to activity. Effectively they measure ras, sGC jumping kinetics, GC activation kinetics, etc. and conclude that full GC activation precedes any sGC jumping. Still the arguments to Ras-GFP delay and jump kinetics and the plot in 5B v GC activity are confusing. Also Ras activity time kinetics can be measured, separate to GFP. What are they? Do they match GC, regardless of GFP jump timings? More GC-del that does not jump is activated normally as is GC in cells treated with LAT that block F-actin formation.

Also they draw an aside conclusion that sGC association with the cortex is inhibitory and include this as part of their model. No data apart from time association supports this, and clearly other players are inhibitory. Does the cortex have any effect?

They could do a time course for cGMP in WT, Lat, GC-Ndel cells. In WT, cGMP would increase and then decrease. If sGC association to actin had effect, esp. inhibitory, the kinetics for cGMP declining would be very delayed in cells where sGC has no actin associations. What are the data?

Later they then address the issue of sGC association to membrane apart from F-actin. Where there is constitutive low level (20% basal) association of sGC with membrane. This should all be moved up, the GbpC data should not separate the actin/membrane association experiments.

For the membrane data, they look to GTPgS activation of GC activity in pellets/membranes and cytosol fractions of cells expressing full length sGC, sGC+ lat (to inhibit F-actin), and sGC-del. That does not bind f actin. If membranes have only sites enough to very low % of total GC activity, especially in GC over expressing cells, should be in membranes in Mn assays. But pellet activity levels are >25% of total activity. GFP % localizations should be shown by westerns. WT cells and sGC cells should be shown for pellet and sup assays. These are important controls as they would give us a better sense of binding site saturation on membranes, etc.

Basal pellets can be activated. Basal sups can not be. Actin associations are not required. Ideally pellet/sup mixes with various other cell types (eg rasC-nulls), would push the model further, for membrane GC activation by Ras-GTP.

GC turn off - kinetics indicate that cAMP activates GC to produce cGMP, but after ~10 sec, accumulation stops and cGMP is degraded, this implies that GC activity is turned off after short time.

mM Ca2+ inhibits GC activity. And cAMP induces Ca2+ accumulation, but wth with kinetics slower than GC activation. They argue that Ca is an inhibitory factor, testing pellets and Sups and mixes prepared with Ca or EGTA, etc. + GTPgS.

In various mixes they show that Ca treated pellets can not be activated, even with added EGTA to chelate Ca. But adding untreated Sup to the pellet allows activation.

They argue that Ca dissociates sGC, but the pellet released of sGC is able to re-recruot new sGC from the cytosol. This could be tested with western to prove actual sGC loss. Even with sGC-GFP.

If Ca plays a big role in GC de-activation one should see effects in cGMP kinetics in vivo. While they argue yes, to me the inhibitory time course role of Ca is minimal.

Permiabilized cells are given mM Ca, EGTA, or nothing and activated by cAMP.

Ca should inhibit and EGTA should by hyper active. If Ca were the major inhibitor, there should be continuous accumulation of cGMP. There is not, plus degradation kinetics of EGTA v controls are similar. Ca treated cells have lower activation and seemingly faster turn off rates than with EGTA, but I am not convinced one can exactly compare where there is a >3x cGMP max level difference between assays. Cells exist with low level Ca ipl-null. How what are cG kinetics in these cells?

The gbpC data should, be placed after Ca.

The discussion does a nice summary of their model. A figure is really required to follow. I have an additional problem with aspects of their inhibitory model. They say Ras-GTP is required to activate sGC. Once activated does GC stay active in the absence of Ras-GTP? This is testable, but ignored in my readings of the ms. Ras-GTP activated by cAMP is only transient. A simple model places GefA as a rapid activator of RasC, with slower activation of ras-GAP that deactivates RasC. There is fast time in vivo course for ras-GDP to rasGTP back to rasGDP. In response to cAMP. If sGC is inactived in the absence of ras-GTP, Ca, cortex and gbpC become less significant to sGC inactivation, as the major pathway could be by ras-deactivation.

Reply to the reviewers.

We thank the reviewers for their detailed and instructive comments. We realize that the manuscript is an unusual combination of reviewing 45 years of published experiments that is extended with new experiments to address specific questions.

The main changes are

- At each experiment information is added indicating the status of the field on that subject (review), and the main question(s) that is addressed in the new experiment.
- The role of the two guanylyl cyclases GCA and sGC is addressed in detail in the new table 1, indicating that sGC provides the major contribution to enzyme activity and cGMP response.
- A schematic figure is added that summarizes the major findings.

All questions are answered in detail below; reviewers text is in black, our answers are in red.

Reviewer #1 (Remarks to the Author):

In this study, van Haastert et al. investigated the cGMP pathway in Dictyostelium chemotaxis and migration. In the past few years, the authors and other groups in the field made tremendous progress on understanding on cGMP production, function and regulation during cell migration by using Dictyostelium cell as a model. They have summarized some of the results in their previous review published in 2002. Here, they discussed and summarized some new findings in perspective of biochemical and functional data on cGMP in Dictyostelium. The quality of the data is overall good. I have a few suggestions for improving this manuscript:

1. Various mutant cell lines were used to compare cGMP production. Have sGC or GCA level been measured and compared among strains?

Measuring separate sGC and GCA activity is very difficult, because the enzymes have similar (inhibition by Ca2+, Mg2+-dependence) or overlapping properties (GCA is fully and sGC partly membrane localized). The measurement of mRNA expression levels may give some indirect information, but has not been performed in the many mutants discussed in the paper. The role of GCA and sGC is discussed on page 5 of the revised paper (see also question 2 below, and a question of reviewer 2).

2. It is well known that both sGC and GCA are responsible for cGMP production, especially at vegetative stage. The cGMP production measurement in Table 1 (folate) assumed that sGC is the only source for cGMP generation. I feel that GCA contribution should not be ignored. Therefore the authors may need to either explain it more or add the experiment in GCA null background cell.

The reviewer is correct that in vegetative cells both GCA and sGC are responsible for the observed folate-induced cGMP response. The role of sGC and GCA is addressed in the revised manuscript in the new table 1. The experiment presented in table 1 (table 2 in revised manuscript) has the aim to show that folate in ga4-null cells induces good Ras activation but no cGMP response, indicating that Ras activation is not sufficient to activate guanylyl cyclase (GCA and sGC).

3. In Figure 5, the authors claimed the sequential activation upon cAMP stimulation. cGMP production is measured in cell suspension; while Ras activation sGC translocation and F actin formation were imaged when cell was attached onto the surface. It is not convincing that the kinetics generated from different condition are comparable, especially when you try to distinguish millisecond time scale. Based on the methods, the images were recorded at 1 frame/sec. I am not convinced the accuracy of their deduction and conclusion for sequential activation (Ras 0.4s; sGC 0.8s; F actin 0.9s).
The reviewer is correct that the lag times of activation for Ras, sGC and F-actin are similar. However, the aim of the analysis was to show that sGC translocation to the cortex is much later and occurs well after activation of sGC (and Ras and F-actin). In the revised manuscript on pages 9 and 10, the kinetics is described in two paragraphs: it is explained first that that activation of Ras, sGC and Factin are fast, and that sGC translocation to the cortex occurs well after activation of sGC, and secondly that the deduced lag times give the most likely sequence of events (Ras 0.4s; sGC 0.8s; F actin 0.9s).

4. In Figure 3 reconstitution assay, the lysate was from WT cell. GTPrS only increase about 50% compared with control. A lysate from rasC/G null would decrease the background dramatically and should give more clear comparison.
These in vitro reconstitution experiments are experimentally quite challenging, because the purified proteins and especially sGC in the lysate are not very stable. Therefore the lysate must be prepared fresh and used within 3 minutes for each reconstitution. Each freshly prepared lysate was assayed simultaneously with three incubations: a control and two activators. We have considered, as the reviewer suggested, to use the appropriate mutant for testing the effect of a potential activator, so e.g. GTPgS in Ras-null, Ga2-GppNHp in ga2-null lysate etc. We have decided not to use different lysates, because this would make the experiment extremely complicated and comparison of the data more complicated. Instead we use one strain for all lysates and accept a higher background.

5. Figure 9B, are data shown the means of replicates?
The replicates of the data shown is indicated in the legend of the revised manuscript.

6. I suggest the authors provide a model Figure to summarize the major findings, which will help readers understand better.
A model is shown in figure 10 of the revised manuscript.

Reviewer #2 (Remarks to the Author):

I am sorry for the delay in my response. However, the ms is not a simple research paper and required much thought to give the best guidance to review.

The ms is a model/review paper to explain the cGMP synthesis/degradation pathway in response to chemoattractant activation. This combines a mixture of published and new data to expand what is known about cG accumulation. The title emphasizes the function of cGMP, but there is little there apart from that published. Also quite little of the data involves actual chemotaxis, again its in the title, and

eventually related in the discussion.
The manuscript concentrates on the properties and regulation of the cGMP pathway in Dictyostelium. It is known that this pathway is involved in chemotaxis and cell movement. The interpretation of the properties of the pathway are in the context of its function. In the revised manuscript this is better explained in the title (changed from "Understanding the regulation and function of the cGMP pathway during cell movement and chemotaxis" to "Understanding the regulation and function of the cGMP pathway for cell movement and chemotaxis"), and at the end of the introduction on page 4.

As an overview, I think this is a timely and valued ms. However, it is requires work by the reader to see what is fundamentally new and what are new data. This needs to be made far more evident at every stage. I am not saying they do not add references, it is just not so easy to see. Also, I think more clarity to the system is required. Workers outside of Dictyostelium, may not understand the differences between chemotaxis during growth and cell aggregation (development). Neither, are they likely to be aware of the complexity to cGMP signaling, in the context of activation/accumulation and de-activation/degradation (perhaps an early figure). These need to be far better explained at the outset. Axes labelings are not always consistent, so it not easy to understand activation level differences. Controls are not always present. Some additional experimental combinations are required. The data may already exist as published, with data in figs for actual visual comparisons. I will try to provide appropriate guidance.
Thank you for the guidance.

Primarily, this is about cGMP signaling during development. Nearly all assays are on staved (early developed) cells where the chemoattractant is cAMP. Chemotaxis during growth uses a different chemoattractant, it may not be assumed that all rules during development equally apply for growth. I would modify the title here too. I am not even certain if the GCs used are precisely the same during both stages.
The reviewer is correct that the two GCs may have different contributions during development, with sGC prominent during aggregation while GCA may also contribute during growth and late development. The manuscript contains very little information on development; it also has no aim to inform the reader on the role of cGMP during different stages of development. The focus on understanding the cGMP pathway during aggregation is better explained in the introduction on page 4 of the revised manuscript.

The initial path for cGMP synthesis by cAMP activation requires the cAMP receptor, its coupled G protein and Ras C and G proteins. In fig 1 We see a 20x-cGMP level difference between WT cells and cells lacking any of these factors. They also express a DN Ras under control of TET - but 1) this is not labeled as such in the fig, 2) they use +/- DOX (not TET) without explanation, and 3) in the text the writing is confusing as it talks about "these cells" without indication that it is the DOX-induced cells that do not activate RAS.
The DN Ras is expressed under the control of a tetracyclin-inducible promotor that was activated by doxycyclin, not tetracyclin. This is now explained in the text on page 6 of the revised manuscript.
Figure 2 is now labelled with "+dox"
"these cells" is replaced by "these cells expressing the dominant negative RasG-S17N"

One major issue regards the different cGMP synthases. There are 2 GCs, sGC (soluble) and GCA (membrane). sGC represents 85% activity in development (not growth), and so GCA is dismissed without further discussion. All data beyond assumes regulations all depend on sGC, but no GCA-null and few sGC-null controls are ever presented. I suggest adding them. data exist.

This is an important point. We have added a new table 1 describing the activity and activation of GCA and sGC by folate and cAMP, showing that sGC always provides the major contribution.

Next (fig2) they look to GC stimulation in cell lysates. Here they do direct activation of G proteins with GTPgS of WT and null cells. Here heterotrimertic Gs are not required for GC, but Ras is. Relative activity is, as with fig1, 20x. Again sGC, GCA null controls are missing.

These data argue that Ras and not Gs are direct activators of GC, but Gs activate Ras.

Measurements on GCA and sGC are now described in the new table 1.

They then did this in a different way adding activated Ga2 or activated RasG directly to lysates (fig 3). G+ as no effect, while RasG+ activates GC. BUT induced activity differences are not 20x, but only 1.5x. No explanation. I presume this relates to differences in the assay. But why is it not done exactly like in fig 2 so experiments can be compared? This does not make sense. They argue that Gbeta and not Ga2 must activate Ras.

The experiment is done exactly as was performed in figure 2, but the expression of activity is different. In figure 3 we express GC activity in the presence of activators relative to the control without activators; then GTPgS induces a 55% increase of activity. In figure 2 the increase of activity of the parental strains by GTPgS varied between 0.5 and 2-fold. Since each mutant has a different parental strain, we expressed the increase of activity by GTPgS in the mutant relative to the increase of activity by GTPgS in its parental strain, which is now defined in the method section on page 22 of the revised manuscript.

The next section relates to sGC association with the cytoskelton (F-actin). But the entire concept is poorly introduced. First, sGC is termed the soluble GC. I have no issue with old terminology, but later it is clear there is significant basal membrane association and that membrane association and not actin association are important for activity. Neither is this at all clear in the abstract. F-actin associations would seem minimally important.

The association of sGC to the and membrane and cytoskeleton is reversible. GCA is always membrane bound. In the introduction of the revised manuscript we mention that GCA has membrane spanning regions and sGC has not and therefore named soluble GC. This is mentioned on page ¾ of the revised manuscript.

A reason to consider surface v cytosol sGC binding relates to how Ras must activate sGC. IN response to cAMP, Ras jumps to membranes where it is activated. Presumably, sGC must also be t the membrane if it is to be activated by Ras. It is previously published that, like Ras, sGC, in response to cAMP, jumps to membranes. It may be expected, but not so stated, that jumping then relates to activity. Effectively they measure ras, sGC jumping kinetics, GC activation kinetics, etc. and conclude that full GC activation precedes any sGC jumping. Still the arguments to Ras-GFP delay and jump kinetics and the plot in 5B v

GC activity are confusing. Also Ras activity time kinetics can be measured, separate to GFP. What are they? Do they match GC, regardless of GFP jump timings? More GC-del that does not jump is activated normally as is GC in cells treated with LAT that block F-actin formation.
The view on Ras activation is slightly different. The Ras protein is always at the membrane, approximately in a uniform distribution (as detected with Ras-GFP). cAMP does not change this distribution but activates Ras by converting Ras-GDP to Ras-GTP. This activation can be uniform or localized, depending on how cAMP is added. This activation is detected with the sensor RBD-Raf-GFP that bonds to Ras-GTP and therefore translocates from the cytoplasm to the boundary of the cell. This is now described on page 7 of the revised manuscript.
The reviewer is correct that sGC probably has be to present at or near the membrane to be activated by Ras-GTP (or by Ga2-GTP). Indeed we observe that significant sGC moves to the boundary of the cell. It was proposed that this translocation of sGC to the boundary is related to its activation. In the revised manuscript we have introduced this question, and then investigate this issue in detail: First, kinetics show that translocation occurs after sGC activation (fig 5). Furthermore, after adding LatA all boundary-localized sGC disappears, indicating that most boundary localized sGC is associated with the F-actin cortex and not with the plasma membrane where Ras-GTP is localized. Finally, sGC in LatA or deltN-sGC, that both do not localize to the boundary, are activated well by cAMP, indicating that localization to the F-actin cortex is not essential for activation of sGC.
The reviewer asks on the kinetics of Ras activation separate to GFP sensors. This has been down previously using pull-down experiment with a resolution of 6 s. However to compare it with GFP sensor pull down experiments should be done with a resolution of 2 seconds, which is not possible for pull-down experiments.
In the revised manuscript on page 7 we better introduce Ras localization and activation, on page 11 introduce the possible connection between translocation of sGC to the cortex for its activation, on page 10 make a statistical statement that Ras activation, sGC activation and F-actin are all fast, and sGC translocation is significantly slower.

Also they draw an aside conclusion that sGC association with the cortex is inhibitory and include this as part of their model. No data apart from time association supports this, and clearly other players are inhibitory. Does the cortex have any effect?
They could do a time course for cGMP in WT, Lat, GC-Ndel cells. In WT, cGMP would increase and then decrease. If sGC association to actin had effect, esp. inhibitory, the kinetics for cGMP declining would be very delayed in cells where sGC has no actin associations. What are the data?
The conclusion that sGC association with the cortex is inhibitory comes from the extended activation in vivo of sGC in LatA or of deltaN-sGC (figure 6).

Later they then address the issue of sGC association to membrane apart from F-actin. Where there is constitutive low level (20% basal) association of sGC with membrane. This should all be moved up, the GbpC data should not separate the actin/membrane association experiments.
GbpC data are placed at the end of the result section of the revised manuscript.

For the membrane data, they look to GTPgS activation of GC activity in pellets/membranes and cytosol fractions of cells expressing full length sGC, sGC+ lat (to inhibit F-actin), and sGC-del. That does not bind f actin. If membranes have only sites enough to very low % of total GC activity, especially in GC over expressing cells, should be in membranes in Mn assays. But pellet activity levels are >25% of total activity. GFP % localizations should be shown by westerns. WT cells and sGC cells should be shown for pellet and sup assays. These are important controls as they would give us a better sense of binding site saturation on membranes, etc.

The reviewer suggests to quantify sGC-GFP in sups and pellets using Western blots. This would be a very good experiment, but unfortunately Western blots of this very large 300 kDa protein is difficult for sups and pellets. First, the protein is not very stable in lysates and the time required to collect sample of sup and pellet. Second, the amount in the pellet is the most important part of the experiment, but extraction of this very large proteins from the pellet is not easily quantitative. And third, the amount in the pellet is the minor component (~20%), which further complicates quantitative determinations of sGC in pellets by Western blots.

Basal pellets can be activated. Basal sups can not be. Actin associations are not required. Ideally pellet/sup mixes with various other cell types (eg rasC-nulls), would push the model further, for membrane GC activation by Ras-GTP.

This is a very interesting experiment that we have considered during writing of an early version of the manuscript. However, the protocol is experimentally not simple. The entire experiments including the controls must be completed within 4 minutes after cell lysis because sGC is not stable. For instance to investigate the role of RasC requires the following steps that are performed in the cold room: 1) simultaneously lyse rasC-null cells and wild-type cells in Ca2+. 2) immediately centrifuge the lysates for 1 min, take supernatants and redissolve pellets. 3) within a 30 s time-window make four reconstitutions in EGTA (to allow reassociation): a) WT pellet + wt sup (positive control), b) rasC-null pellet with rasC-null sup (negative control), c) wt pellet with rasC-null sup (does sGC from rasC-null reassociate to wt-membranes?; expectation: yes) d) rasC-null pellet with wt-sup (the interesting experiment, does sGC from wt reassociate to rasC-null membranes, i.e. is RasC required for membrane association?). 4) the reconstitutions are immediately transferred to a water bath and incubated for 1 min at 22 oC. 5) within a 30 s time-window start eight incubations with substrate +/- GTPgS ; at 30 s and 60s after the start of each incubation withdraw a sample and transfer to tubes with PCA. It is clear that such experiments require at least two very skilled and tightly coordinating persons. We have done such type of experiments, but they are not simple. We could start with the most interesting reconstitution d), but it is our experience that -whatever the outcome- conclusions require the other reconstitutions.

GC turn off - kinetics indicate that cAMP activates GC to produce cGMP, but after ~10 sec, accumulation stops and cGMP is degraded, this implies that GC activity is turned off after short time.

mM Ca2+ inhibits GC activity. And cAMP induces Ca2+ accumulation, but wth with kinetics slower than GC activation. They argue that Ca is an inhibitory factor, testing pellets and Sups and mixes prepared with Ca or EGTA, etc. + GTPgS.

In various mixes they show that Ca treated pellets can not be activated, even with added EGTA to chelate Ca. But adding untreated Sup to the pellet allows activation.

They argue that Ca dissociates sGC, but the pellet released of sGC is able to re-recruot new sGC from the cytosol. This could be tested with western to prove actual sGC loss. Even with sGC-GFP.
As mentioned above, quantitative Western blots of pellets and sups are not possible.

If Ca plays a big role in GC de-activation one should see effects in cGMP kinetics in vivo. While they argue yes, to me the inhibitory time course role of Ca is minimal.

Permiabilized cells are given mM Ca, EGTA, or nothing and activated by cAMP.

Ca should inhibit and EGTA should by hyper active. If Ca were the major inhibitor, there should be continuous accumulation of cGMP. There is not, plus degradation kinetics of EGTA v controls are similar. Ca treated cells have lower activation and seemingly faster turn off rates than with EGTA, but I am not convinced one can exactly compare where there is a >3x cGMP max level difference between assays. Cells exist with low level Ca ipl-null. How what are cG kinetics in these cells?

Inhibition of sGC by Ca2+ ions is one of multiple inhibitory reactions that lead to a transient cGMP response as indicated in the manuscript. These processes also including adaptation between receptor and sGC, cGMP-stimulation of PDE5, and cGMP-binding to GbpC and subsequent inhibition. Therefore, the effects of increasing or decreasing Ca2+ are large or small, depending on its contribution relative to the other regulators.
In unstimulated cells, addition of 1 mM Ca2+ to permeabilized cells lead to a 70 % reduction of basal cGMP levels with a half-time of about 30 s (Fig. 8). Considering that cGMP degradation in vivo has a half-time of about 20 seconds, the effect of Ca2+ on basal sGC activity is fast and relatively large. Addition of EGTA to permeabilized cells has a moderate effect (25% increase), suggesting that in unstimulated non-permeabilized cells basal sGC activity is only slightly inhibited by basal Ca2+ levels. This is consistent with the measured 50 nM basal Ca2+ concentration in cells and the observed inhibition of sGC in vitro by 50 nM Ca2+.
Upon cAMP-stimulation, permeabilized cells in EGTA exhibit a larger response and permeabilized cells in Ca2+ have a smaller response to cAMP, compared to non-permeabilized cells. The magnitude of the effect of different Ca2+ concentrations on the cAMP-stimulated cGMP response is similar to the magnitude of Ca2+ on basal cGMP levels. So this is all consistent. The cGMP response in permeabilized cells in EGTA is still transient, indicating that inhibition by Ca2+ is not a major regulator of sGC activity. Additional information is given in the revised manuscript on page 18 to indicate that regulation by Ca2+ ions is one of the multiple components that regulate sGC activity and cGMP degradation to provide the transient response observed.

The gbpC data should, be placed after Ca.
GbpC data are placed at the end of the result section of the revised manuscript.

The discussion does a nice summary of their model. A figure is really required to follow. I have an additional problem with aspects of their inhibitory model. They say Ras-GTP is required to activate sGC. Once activated does GC stay active in the absence of Ras-GTP? This is testable, but ignored in my readings of the ms. Ras-GTP activated by cAMP is only transient. A simple model places GefA as a rapid activator of RasC, with slower activation of ras-GAP that deactivates RasC. There is fast time in vivo course for ras-GDP to rasGTP back to rasGDP. In response to cAMP. If sGC is inactived in the absence of ras-GTP, Ca, cortex and gbpC become less significant to sGC inactivation, as the major pathway could be by ras-deactivation.

In the revised manuscript a model on the regulation pof the cGMP pathway is presented in figure 10. In the strain expressing dominant active Ras basal cGMP levels and the cGMP response are not different from wild type cells as was indicated the original manuscript. This suggests that active Ras alone cannot activate sGC (sGC needs input from cAMP-stimulated Ga2), and that in the presence of active Ras the cGMP response is still transient, suggesting that the transient cGMP response in wild-type is not only due to the transient Ras response.

RE: Manuscript #E21-04-0171R
TITLE: "45 years of cGMP research in Dictyostelium: Understanding the regulation and function of the cGMP pathway for cell movement and chemotaxis"

Dear Dr. Van Haastert:

The second reviewer has a number of additional specific suggestions. Please consider these and try to address as many as you can, possibly with minor edits of the manuscript. Please summarize how you responded. I plan to accept the paper once this is done.

Sincerely,
Leah Edelstein-Keshet
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Van Haastert,

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript requires minor revisions before it can be published in Molecular Biology of the Cell, as described in the Monitoring Editor's decision letter above and the reviewer comments (if any) below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision include a rebuttal letter that details, point-by-point, how the Monitoring Editor's and reviewers' comments have been addressed. (The file type for this letter must be "rebuttal letter"; do not include your response to the Monitoring Editor and reviewers in a "cover letter.") Please bear in mind that your rebuttal letter will be published with your paper if it is accepted, unless you have opted out of publishing the review history.

Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

In preparing your revised manuscript, please follow the instruction in the Information for Authors (www.molbiolcell.org/info-for-authors). In particular, to prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision as described.

To submit the rebuttal letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Authors of Articles and Brief Communications whose manuscripts have returned for minor revision ("revise only") are encouraged to create a short video abstract to accompany their article when it is published. These video abstracts, known as Science Sketches, are up to 2 minutes long and will be published on YouTube and then embedded in the article abstract. Science Sketch Editors on the MBoC Editorial Board will provide guidance as you prepare your video. Information about how to prepare and submit a video abstract is available at www.molbiolcell.org/science-sketches. Please contact mboc@ascb.org if you are interested in creating a Science Sketch.

Thank you for submitting your manuscript to Molecular Biology of the Cell. Please do not hesitate to contact this office if you have any questions.

Sincerely,

Eric Baker
Journal Production Manager
MBoC Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

The revised manuscript answers the major concerns. The conclusions are well supported by the data and the experimental work is well done.

Reviewer #2 (Remarks to the Author):

I appreciate the many changes and responses to my previous review. Apparently, many of the suggested experiment were not technically feasible. However, several things are still not clear and as detailed I am not certain to some conclusions. Mostly, these center to the role Ga2 and rasG/C and on cGMP adaptation.

During aggregation (early developmental), cells deficient in Ga2 or both rasG/C are clearly very deficient in cGMP activation by cAMP (Fig 1). It would seem that rasG plays the larger role, contributing 3x the regulatory activity, as rasC. In vitro studies show that direct activation of ras by GTPgS is able to bypass the requirement of Ga2. The simple conclusion is that cAMP activates receptors which activate Ga2bg, which activate rasG/C, which active sGC to make cGMP. If so cells deficient in Ga2 would not be able to activate RasG at all. But this is not true; cells deficient in Ga2 active rasG to 50%, but not GC. They conclude that Ga2 has a separate required input to sGC than ras, and which is also independent of Gb. But without knowing the activations of rasC and other Ras proteins in Ga2 nulls or seeing a full time course to rasG regulation, conclusions are not so simple .To test this further they compare cGMP in cells with and without constitutively activated RasG and find no significant difference. These data are in the text and should be in a table. Also as best I understand, they express constitutively activated RasG in WT cells. They should express in Ga2-nulls and rasG/C-nulls.
If they are correct, Ga2-nulls expressing constitutively activated RasG should still not activate cGMP, but rasG/C-nulls would be rescued. Regardless a full time course for rasG activation in Ga2 nulls compared to WT is needed.

I also still have concern to the role ras plays in cGMP adaptation. After activation by cAMP, there is a rapid accumulation of cGMP and then decline as cGMP is degraded and sGC is turned off. They had invoked three main players Ca+2 accumulation, actin association, and GbpC in sGC inhibition. I had suggested that conversion of ras-GTP (or perhaps also Ga2-GTP) to GDP bound forms might be the more significant player. They dismissed my postulate without showing any data.
Nor did they test further their model. GbpCp-nulls cells, treated with latA and EDTA (with all various single, double control combinations), might show almost no adaptation. They should also show a full time course for cGMP using constitutively activated RasG as above to show it does not regulate adaptation. They should also show a full time course for cGMP using constitutively activated RasG as above to show it does not regulate adaptation.
They also need to run a full time course of activation, to if constitutively activated RasG has an effect on cGMP inhibition (see below).

Specific points:
1. They need to add an explanation of DIX to the figure legend of figure 1.
2. Figure 10 model, the feedback inhibition arrows from Ca, GbpC, etc need to go directly sGC.
3. Also in Fig 10, they need to add ras-GAP as a cAMP-induced inhibitor of ras.
4. I still do not understand why the data in fig 3 are expressed differently than the data in other figs, when the authors state clearly that the experiments were done the same way. In other figs measurement differences are 10-times, but here differences are only 50%, because the measurement terms used are not the same.
5. Nearly every experiment uses aggregation competent (early developed) cells and thus response to the developmental chemoattractant cAMP. Yet, in figure 10 they add an entire arm for regulation of sGC by folate during growth. I think this arm should be clearly stated to be a modeled extension of what they propose from studies during early development.
6. Fig 10, their model is only partially explained in the legend. Each component should be explained and their relative contribution to each pathway commented.

Reply to reviewer #2 (comments reviewer in black, reply in bold-red)

Reviewer #2 (Remarks to the Author):

I appreciate the many changes and responses to my previous review. Apparently, many of the suggested experiment were not technically feasible. However, several things are still not clear and as detailed I am not certain to some conclusions. Mostly, these center to the role Ga2 and rasG/C and on cGMP adaptation.

During aggregation (early developmental), cells deficient in Ga2 or both rasG/C are clearly very deficient in cGMP activation by cAMP (Fig 1). It would seem that rasG plays the larger role, contributing 3x the regulatory activity, as rasC. In vitro studies show that direct activation of ras by GTPgS is able to bypass the requirement of Ga2. The simple conclusion is that cAMP activates receptors which activate Ga2bg, which activate rasG/C, which active sGC to make cGMP. If so cells deficient in Ga2 would not be able to activate RasG at all. But this is not true; cells deficient in Ga2 active rasG to 50%, but not GC. They conclude that Ga2 has a separate required input to sGC than ras, and which is also independent of Gb. But without knowing the activations of rasC and other Ras proteins in Ga2 nulls or seeing a full time course to rasG regulation, conclusions are not so simple .To test this further they compare cGMP in cells with and without constitutively activated RasG and find no significant difference. These data are in the text and should be in a table. Also as best I understand, they express constitutively activated RasG in WT cells. They should express in Ga2-nulls and rasG/C-nulls.
If they are correct, Ga2-nulls expressing constitutively activated RasG should still not activate cGMP, but rasG/C-nulls would be rescued. Regardless a full time course for rasG activation in Ga2 nulls compared to WT is needed.
A full time course of Ras activation by cAMP in ga2-null cells has been presented in Figure 6B of reference 38, and reveals that the response is transient with slightly faster kinetics compared to the response in WT cells. This is now described on page 7 of the re-revised manuscript. The experiment with dominant active Ras was in WT as assumed by the reviewer; this is now mentioned on page 8 of the re-revised manuscript. The data on the dominant Ras are only four values; we prefer not to use a separate table with only four entries, but mention them in the text. The proposed experiment with dominant active Ras expressed in ga2-null and rasC/G null cells is likely to be only confirmative, as the reviewer already predicts the only logic outcome.

I also still have concern to the role ras plays in cGMP adaptation. After activation by cAMP, there is a rapid accumulation of cGMP and then decline as cGMP is degraded and sGC is turned off. They had invoked three main players Ca+2 accumulation, actin association, and GbpC in sGC inhibition. I had suggested that conversion of ras-GTP (or perhaps also Ga2-GTP) to GDP bound forms might be the more significant player. They dismissed my postulate without showing any data.
Nor did they test further their model. GbpCp-nulls cells, treated with latA and EDTA (with all various single, double control combinations), might show almost no adaptation. They should also show a full time course for cGMP using constitutively activated RasG as above to show it does not regulate

adaptation. They should also show a full time course for cGMP using constitutively activated RasG as above to show it does not regulate adaptation.
They also need to run a full time course of activation, to if constitutively activated RasG has an effect on cGMP inhibition (see below).
Activation and adaptation of the cGMP response is very fast as was shown in 1987 (reference 57): Guanylyl cyclase is half-maximally activated at 0.85 seconds after stimulation and half-maximally adapted at 2.4 seconds after stimulation. The time-course of cAMP-stimulated RBD-Raf-GFP translocation to the boundary of the cell, detecting Ras-GTP, suggests that Ras is maximally activated after about 8 seconds and recovers half-maximally to prestimulus levels at about 15 seconds after stimulation. This clearly shows that the adaptation of sGC is completed before Ras-GTP declines. Therefore we consider that the conversion of Ras-GTP does not play a dominant role in the decline of cGMP levels.

Specific points:
1. They need to add an explanation of DIX to the figure legend of figure 1.
Dox (not Dix) is explained in the legend of the re-revised manuscript

2. Figure 10 model, the feedback inhibition arrows from Ca, GbpC, etc need to go directly sGC.
The regulators Ca2+, GbpC and F-actin have multiple effects on sGC, but all have an effect on cGMP production. Some regulators change the localization of sGC by which sGC can be activated by Ga2, some regulators have a direct effect on the catalytic activity, while the working mechanisms of still other regulators is unknown. In figure 10A we show that they all effect the production of cGMP, while in figure 10B we present details for some regulators on the localization of sGC are presented in 10B.

3. Also in Fig 10, they need to add ras-GAP as a cAMP-induced inhibitor of ras.
Figure 10 explains all the details on the cGMP pathway. We prefer not to go in too much detail on how RasC/G are activated, because this would include several GEFs and GAPs.

4. I still do not understand why the data in fig 3 are expressed differently than the data in other figs, when the authors state clearly that the experiments were done the same way. In other figs measurement differences are 10-times, but here differences are only 50%, because the measurement terms used are not the same.
The stimulation of sGC activity by GTPgS in figure 3 is 1.55 relative to the control without GTPgS. This experiment is a wild-type. If these data were obtained in figure 2 for the wild-type, then this 0.55 fold increase of activity is set at 100% and the increase of activity of the mutant run in parallel was presented relative to the increase of this wild-type. The label at the Y-axis is changed in the re-revised to (relative to control) .

5. Nearly every experiment uses aggregation competent (early developed) cells and thus response to the developmental chemoattractant cAMP. Yet, in figure 10 they add an entire arm for regulation of sGC by folate during growth. I think this arm should be clearly stated to be a modeled extension of what they propose from studies during early development.

The unexpected finding that sGC activation requires both a Ga protein and Ras is very clear for folate, and therefore an essential component of the figure. In the re-revised manuscript the legend indicates that information for cAMP is derived from starved cells and for folate from vegetative cells.

6. Fig 10, their model is only partially explained in the legend. Each component should be explained and their relative contribution to each pathway commented.

We added additional information in the legend to the figure of the re-revised manuscript, but are careful not to recapitulate the discussion in the main text.

RE: Manuscript #E21-04-0171RR
TITLE: "45 years of cGMP research in Dictyostelium: Understanding the regulation and function of the cGMP pathway for cell movement and chemotaxis"

Dear Dr. Van Haastert:

I am pleased to accept your manuscript for publication in Molecular Biology of the Cell.

Congratulations! Your paper is accepted.

Sincerely,
Leah Edelstein-Keshet
Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Van Haastert:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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