Compensatory Endocytosis in Bladder Umbrella Cells Occurs through an Integrin-Regulated and RhoA- and Dynamin-Dependent Pathway

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Review timeline:

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submission date</td>
<td>05 November 2009</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>23 December 2009</td>
</tr>
<tr>
<td>Revision received</td>
<td>12 March 2010</td>
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<tr>
<td>Editorial Decision</td>
<td>13 April 2010</td>
</tr>
<tr>
<td>Revision received</td>
<td>16 April 2010</td>
</tr>
<tr>
<td>Accepted</td>
<td>20 April 2010</td>
</tr>
</tbody>
</table>

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 23 December 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the exceptionally long delay in getting back to you with a decision. However, the referees were not able to return their reports as quickly as initially expected.

Your manuscript has now finally been seen by three referees whose comments to the authors are shown below. As you will see while referee 1 is very positive referee 2 and to some extent referee 3 feel that while they consider the study as interesting in principle considerably stronger causal data are needed before the study is sufficiently convincing. In fact, referee 2 feels strongly that a rather substantial amount of additional work would be required and he/she is therefore not in favour of publication of the study here. Given the interest expressed by the referees in principle and given the support provided by referees 1 and 3 we have come to the conclusion that we could give you the chance to address the referees' concerns by revision. However, you would need to address the concerns of referees 2 and 3 in an adequate manner and to their satisfaction. I should add that the revised manuscript would need to be seen again by the referees and that a final decision on acceptance or rejection can only be made at this stage.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees.

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 initiative, 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 COMMENTS

Referee #1 (Remarks to the Author):

This paper characterizes the mechanism of a highly specialized form of endocytosis, compensatory endocytosis in bladder cells. The authors integrate reagents and approaches from the wider field of clathrin-independent endocytosis.

The paper is clearly written and the data is convincing. The results do not amount to an actual description of the mechanism for compensatory endocytosis, but by applying inhibitors and other reagents from the wider field in a careful and well controlled way to this specific case the authors have made a valuable contribution.

Referee #2 (Remarks to the Author):

In this manuscript Khandelwal et al., discuss the role of RhoA-dependent clathrin-independent endocytosis involved in a physiological context, namely compensatory endocytosis (CE), triggered during the voiding phase of bladder function. Their data are consistent with their interpretation that a ROCK-activated RhoA-dependent endocytic process that contributes to the restoration of membrane surface area during the voiding phase, post exocytosis of DFVs in the filling process.

A general comment is that there is a lack of rigorous quantification in almost all experiments, and where there is an attempt at quantification, it is unclear how the authors have accomplished this. Furthermore, the authors appear to have done each experiment once with a maximum of three bladder tissue samples. (see Experimental Procedures, pages 18, 20). If this is true, the authors must repeat their findings at least once if not more times to report a verifiable finding. Nonetheless, the findings are potentially interesting, but they display a lack rigour, that leaves many an alternative explanation possible.

Some specific instances are documented below:

1) The claim that endocytosed PJAEs are distinct from recycling DFVs is based on the fact that ferritin-labeling is observed in irregular endosomes in EM sections. A single section with a single labeled irregular vesicle with no label in DFVs is the only evidence or this. Given that there is almost 30% of membrane being re-internalized (from the cacitance results), the authors need to account for the this aspect quantitatively and then show that none of this labeled membrane is associated with DFVs. The colocalisation of endocytosed WGA with Rab 11 is compared to the colocalization observed with actin (Fig 2), which is not a reasonable comparison. Endocytic structures do exhibit a higher colocalisation with Rab 11 than with EEA1. Considering that Rab11 is a marker used to define DFVs as well (see Khandelwal et al 2008), these studies are not adequate to call the CE-derived endosomes, distinct compartments.

2) Additionally, dextran/WGA filled endosomes are sort of diffuse and the figures do not show the high co-localisation with ZO-1 that the graphs exhibit (Fig. 2B). Especially at later time points, the fact that these vesicles are enriched near the junctions (and hence PJAEs) is not obvious.

3) The lack of any role of caveolin, has been shown by the absence of staining using one antibody
(see Fig 3). This level of evidence is not convincing, since several investigators [for example see del Pozo et al, Science, (2005)] have implicated a role for caveolin and dynamin in integrin-based signal-induced endocytosis especially during de-adherence. A rigorous role for this would be to show by a PCR method that no caveolin is expressed in umbrella cells and/or that caveolin-null umbrella cells exhibit a similar endocytic process. Ditto, for the claim of an absence of an effect for flotillin/clathrin. Especially for clathrin, the authors have access to a host of dominant negative inhibitors for accessory proteins [see review Benmerah and Lamaze, Traffic (2007)]

4) Similarly, in the absence of any quantification of a change in cholesterol levels, the authors cannot claim that filipin treatment (Fig 3B) does or does not inhibit this process.

5) Although, the inhibition of CE (or not) by electrophysiology is quite clear for many of the pharmacological inhibitors, where possible it is necessary to look at molecular perturbations. The dependence (or its lack) of the pathways is based solely on inhibitors, except for the use of Dn constructs of dynamin and RhoA. These experiments are almost anecdotal, being buttressed by evidence from a single cell. A rigorous quantification is necessary to document a convincing effect. And several control DN constructs (siRNA) of other members of the Rho family GTPases should be tried to provide convincing evidence for this effect.

6) The link with integrin signaling is extremely interesting. Here again, the authors ignore that they appear to have triggered a huge increase in filling-induced exocytosis (see Fig 7A-c left panels), and it is possible that this additional increased exocytic membrane is not being correctly processed during the voiding process. Nothing in the data rule this out. The effect of the FAK/PI3K inhibitors may all be explained by an overall suppressed activation of ROCK activity.

The effect of voiding and filling would be important to demonstrate on overall RhoA activation status; the effect of inhibitors of this process should be documented at both stages of this process. In the graph shown in Fig 7D, this reviewer is unclear what is being measured. A detailed description of the normalization and numbers reflecting the arguments noted above need to be provided.

Other:
1) It is completely unclear why the authors show a graph of Total intensity/um2 of tissue area, and all the control values appear as 50 units...for all the graphs shown quantifying internalized WGA or Dextran levels- note first bar of panels in 2D and 4D. A detailing of actual values - the signal and noise in each measurement must be documented.

2) Actin staining looks inconsistent, and changes with every figure.

Referee #3 (Remarks to the Author):

Article EMBOJ-2009-73072 by Khandelwal and colleagues on compensatory endocytosis in bladder umbrella cells.

During the cycle of bladder filling and voiding, subapical discoidal and fusiform vesicles (DFV) undergo a cycle of regulated exocytosis and endocytosis. In this manuscript, the authors have analyzed the endocytic process in further detail. Clathrin, caveolin or flotillin are not required, as opposed to dynamin and actin. Furthermore, the requirement for RhoA indicates similarities to the IL2 receptor pathway. After endocytosis, internalized apical membrane is found in ZO1-positive vesicles, and is then targeted to late endosomes/lysosomes for degradation. The process appears to be regulated by integrin-dependent signaling pathways.

The processes by which molecules are removed from the plasma membrane by endocytosis are currently under intense scrutiny, notably also the clathrin-independent pathways, and studying compensatory endocytosis in a system that mimics the physiological process of bladder voiding is quite important. A number of points need to be addressed, however, to render the study more convincing.

Page 6, middle: The authors should show the data on WGA and dextran uptake prior to voiding. This data would document the constitutive pathways (including the high capacity GEEC pathway) and allow getting a better idea on the relative importance of endocytosis events that are induced
during voiding.

A key point is found in the middle of page 9 and 10. The authors report that flipin and methyl-beta-cyclodextrin (data should be shown) have no effect on the compensatory endocytosis pathway that the authors are analyzing. This finding is highly surprising and unexpected as most if not all clathrin-independent and actin-dependent endocytosis processes are cholesterol sensitive. This notably also includes the IL2 receptor pathway. The authors must include a positive control in their flipin and methyl-beta-cyclodextrin experiments (experiments should be shown), such as to document that both treatments are effective. The authors should also use the conventional detergent extraction/fractionation method in the cold to determine to what extent their markers of apical compensatory endocytosis are detergent insoluble in control or cholesterol perturbation conditions. In the context of the current raft debate, this data would be of quite some interest.

Page 9 bottom and page 10 top: The concentrations of dynasore that are used in this study are astonishingly high, but the authors' hypothesis on reduced accessibility in bladder tissue is reasonable. Yet, the additional control by K44A expression is quite important here. What is the percentage of cells that were transfected by the K44A mutant? Is this figure high enough so that capacitance measurements could be performed? If not, it should at least be shown how many of the transfected cells have the mutant phenotype concerning the uptake of FITC-WGA.

Page 10 bottom and page 11 top: I could not find details in results or methods on how toxin B and C3 were introduced into cells. This must be detailed. It should also be described where these materials came from and how they were prepared.

The model on mechano-signaling that the authors propose at the end of the discussion section is quite exciting. Of course, at this stage many key aspects remain to be analyzed. Among these, the question of how apical membranes are bent in a clathrin and caveolin-independent manner in response to the basolateral beta1 integrin-stimulated signaling is of particular importance. While the authors may not have a definite response at this stage, they should speculate on the possible mechanisms that are likely to apply here.

1st Revision - authors' response 12 March 2010

Reviewer #1:

The reviewer had few comments and felt that our studies made a valuable contribution to our understanding of compensatory endocytosis.

Reviewer #2:

This reviewer also found our results potentially interesting, but expressed a general concern about the lack of rigorous quantitation, the small number of replicates, and inadequate description of methods in our studies. In the revised manuscript we are careful to quantify all data and perform statistical analyses, wherever possible. We also note that all experiments were performed multiple times, on different dates, using bladder preparations from multiple animals. In the revised manuscript we provide an indication of the number of replicates and the number of preparations that were used for each experimental approach. In the revised Materials and Methods we are also careful to better describe the quantitative methods we have employed in our studies.

Specific concerns:

1. The reviewer expressed reservations about whether our analysis adequately demonstrated that the endocytic structures, which we termed PIAEs, are distinct from DFV. To address this issue we have measured the degree of colocalization between endocytosed Alexa647-WGA and DFV labeled with virally expressed human growth hormone (hGH), which others and we have shown is efficiently packaged into DFV (Kerr et al., 1998; Khandelwal et al., 2008). In these experiments we observe that there is minimal colocalization between these markers (colocalization coefficient of ~ 0.1; Fig.
S3), which is consistent with our previous conclusion that DFV are not endocytic in nature (Khandelwal et al., 2008). The reviewer also questioned the utility of our EM analysis; however, we retained the electron micrograph showing cationized ferritin-positive PJAEs (Fig. 1H), and included additional micrographs of PJAEs, as we believe that these images speak to the morphology of PJAEs. We also note that in the examination of the EM sections from these experiments, the large majority of morphologically identifiable DFV are not labeled with cationized ferritin and this is consistent with the other studies that have explored endocytosis in umbrella cells (Alenghat et al., 2004; Amano et al., 1991; Khandelwal et al., 2008; Truschel et al., 2002).

The reviewer was also concerned that we compared endocytosed WGA to rab11a, however, we previously showed that DFV are Rab11a positive and that Rab11a regulates DFV exocytosis (Khandelwal et al., 2008). Although we show some colocalization between WGA and Rab11a, (Mander colocalization coefficient of ~ 0.2), the vast majority of WGA-labeled PJAEs are distinct from Rab11a-positive structures such as DFV. This is apparent not only in revised Fig. 2A and 2C, but also in new figures that we have added to the supplementary figures (revised Fig. S2), as well as a movie that better shows the distribution of the two markers (movie S4). The reviewer also commented that in our studies we appeared to compare the degree of WGA colocalization with Rab11a with that of actin. However, Rab11a and actin are different markers and any comparisons made in the original manuscript were unintentional.

Finally, the reviewer asked that we quantify the amount of internalized membrane and compare this to the 30% decrease we observed in our capacitance studies. We have performed this analysis and the results are reported on page 6 of the revised manuscript. Our analysis depends on two assumptions, both of which we feel are reasonable. Our first assumption is that the majority of endocytosed membrane is derived from the apical membrane. Second, we assume that the apical membrane is fairly flat in our samples (which is generally the case when we examined the umbrella cells by electron microscopy). Using either endocytic tracer we observe that ~ 25% of the apical membrane is internalized during voiding, which we feel is in reasonable agreement with the ~30% decrease in apical membrane measured by our capacitance studies.

2. The reviewer suggested that the WGA/dextran staining is diffuse, and that the colocalization between these endocytic tracers and ZO-1 is not clear. To our eyes the staining for the endocytic markers is clearly punctate and this is best visualized in the included movies. As for the issue with endocytic tracers and ZO-1 we have included a movie that shows the distribution of FITC-WGA and ZO-1 (revised movie S5). Furthermore, we have included a new figure in the supplementary materials (Fig. S2), which shows the individual markers in different combinations that we believe make the colocalization easier to discern. In addition, we have included PDM images in Fig S2, which show voxels that are positively correlated (i.e. colocalized).

3. The reviewer wanted more rigorous evidence about the lack of caveolin, flotillin, and clathrin expression at the apical membrane of the umbrella cells. In this regard, we have performed a quantitative analysis of clathrin-coated pits and flask-shaped invaginations (the morphology of both caveolae and flotillin-positive carriers) at the apical surface of the umbrella cells. This analysis was performed in tissue prior to voiding and just after voiding. In no condition could we identify a single clathrin-coated pit or flask-shaped invagination at the apical surface of the umbrella cells. I also note that in a previous analysis we found no evidence that DFV were connected to the apical cell surface or that they were part of network (Truschel et al., 2002); therefore, it is unlikely we would misidentify DFV undergoing fusion as being endocytic structures. However, we did identify an occasional clathrin-coated pit along the membranes of the intermediate/basal cells, and the subjacent myofibroblasts had clathrin-coated pits and a relatively large number of flask-shaped invaginations. As further evidence for a lack of caveolin expression in the uroepithelium we have used a number of additional antibodies and see a similar pattern of expression – that is, caveolin expression in endothelial cells, myofibroblasts, and smooth muscle cells, but no expression in the uroepithelium. This is consistent with previous published studies (Baressi et al., 2006; Fong et al., 2003). Furthermore, at the reviewer’s suggestion we have performed RT-PCR and cannot find any expression of caveolin-1 or -2 in rabbit or rat uroepithelium (but do observe it in other bladder tissues). The reviewer suggested we may look at caveolin knockout mice, however, at our institution we have a significant problem importing mice because of previous MPV outbreaks. As a result, all mice that enter the institution must be re-derived, a process that takes several months. The reviewer further suggests that we try other approaches to rule out roles for clathrin- and flotillin-dependent...
processes; however, the flotillin pathway is not well studied and we are unaware of selective cargoes for this pathway. We have not yet developed techniques to express shRNA in bladder tissue, although this is our goal for the future. As for use of dominant-negative clathrin constructs, we have had significant issues generating adenoviruses expressing these proteins and could not find other investigators that had already prepared viruses encoding these constructs. Nonetheless, the lack of clathrin-coated pits, clathrin, or AP-2 at the apical plasma membrane of umbrella cells, and the insensitivity of apical CE to chlorpromazine and K⁺ depletion all point to a clathrin-independent process.

4. The reviewer indicated that we could not rule out a role for filipin in the absence of a measured change in cholesterol. Based on these comments and those of the third reviewer we have used Triton X-100 treatment and sucrose gradients to show that UP3a-positive apical membranes float (and are Triton insoluble), but treatment with 10 mM MbCD results in the complete solubilization of these membranes (revised Fig. 5C). Furthermore, we show that MbCD causes a significant inhibition of WGA and dextran uptake (revised Fig. 5E). We also used up to 10 µg/ml of filipin in these studies, but could find no effect of this drug on membrane solubilization. As 10 µg/ml is at the high end of concentrations employed by other investigators we decided to remove these data and focus on the MbCD data, which yielded positive results.

5. The reviewer asked that we buttress our pharmacological experiments with additional quantitative studies using dominant-negative constructs. In response, we have performed a quantitative analysis of WGA uptake in transduced cells expressing DN-dynaminK44A, Cdc42N17, RhoAN19, Rac1N17, and Arf6T27N (revised Fig. 4F and 6A). This analysis was repeated multiple times and at least 70 cells were examined per condition. We find that dynamin K44A and RhoA had significant effects on WGA uptake, but the other constructs did not.

6. The reviewer had issues with the original studies in which we explored the role of integrins and associated signaling pathways in CE. The reviewer noted that there was a large impact on exocytosis that may affect the subsequent voiding response. In our original studies we added inhibitors and other reagents at the onset of experimental filling, which would impact both filling-induced and voiding-induced CE. Inhibition of the former would lead to a significant increase in exocytosis. Because our studies are focused on voiding-induced CE we have removed the original experiments and substituted them with ones in which inhibitors were added after the initial period of filling (revised Fig. 7A-C). Under these conditions, we observe no significant effects on exocytosis, but we still observe effects on voiding-induced CE. The reviewer also had questions about how we did the RhoA activation experiments and how the data were quantified. We have included both additional text in the supplementary methods and results to better explain what was done. Furthermore, we have added additional experiments in which we examine the impact of these treatments on inward bowing (revised Fig. 7E-F), and they show a similar activation of RhoA and dependence on PI3K and FAK.

Minor comments:

1. The reviewer wanted additional information about how the image analysis was performed. This information is now contained in the revised materials and methods.

2. The reviewer felt that the actin staining looked different from panel to panel. These differences are likely the result of several factors. First, the uroepithelium is sometimes referred to as the transitional epithelium, in part, because the umbrella cells (and other cell layers) can undergo dramatic changes in morphology depending on the filling status of the bladder. Second, in some of the panels the umbrella cells are fairly flat and when we collect the Z-stacks one can see the cortical actin associated with the underlying intermediate cells. We think this is best visualized in the movies, where the patches of actin that appear at the center of the umbrella cells in the stationary images are actually at the base of these cells and contributed by the underlying cell layer.

Reviewer #3:

1. The reviewer asked that we report the amount of uptake prior to voiding. There was a small amount of uptake, but less than 3% of that observed during voiding (mean intensity of ~ 1.2 prior to
voiding, versus ~ 43.7 afterwards). These data are reported on page 6 of the revised manuscript.

2. The reviewer asked that we show experiments that prove the effectiveness of the MbCD and filipin treatments. As described in our response to reviewer #2 (point #4) we have used Triton X-100 treatment and sucrose gradients to show that 10 mM MbCD solubilizes the umbrella cell apical membrane and causes a significant inhibition of WGA and dextran uptake (revised Fig. 4D). However, as described above we were unable to show a positive effect of filipin and have removed these experiments from the revised manuscript.

3. The reviewer expressed concern about the DN-dynaminK44A experiments. As noted in our response to reviewer #2 (point #5) we have now included experiments that quantify the effectiveness of this mutant (revised Fig. 4F).

4. The reviewer asked how the toxins were introduced into the cell and where they were sourced. A cell-permeable variant of C3 transferase was used in our studies and was purchased from Cytoskeleton. Toxin-B is inherently membrane permeable and can be added directly to most cell types in the absence of any permeabilizing agent. We have added comments in the Results and Materials and Methods to clarify these facts.

5. The reviewer asked that we comment on possible mechanisms by which apical membrane is internalized. In the last paragraph of the Discussion we include discussion of some possible molecules (e.g. actin and associated motors and GRAF1-like proteins) that may play a role in this process.

2nd Editorial Decision

13 April 2010

Thank you for sending us your revised manuscript. Our original referees 2 and 3 have now seen it again. In general, both referees are now positive about publication of your paper. However, referee 2 feels that there are a few issues that still need to be addressed (see below) before we can ultimately accept your manuscript. Now, I can see that according to your point-by-point response there are technical issues with expressing shRNA in bladder tissue, which will make a dynamin 2 siRNA experiment difficult to perform. Furthermore you point to problems with dominant-negative clathrin expressing adenoviruses. Also, an analysis of a role of GRAF1 would certainly go beyond the scope of this study. I would therefore like to ask you to address the remaining issues raised by the referee in an adequate manner and to at least respond to the more problematic ones.

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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Please let us have a suitably amended manuscript as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

REFeree COMMENTS

Referee #2 (Remarks to the Author):

In their revised manuscript Khandelwal et al., have clarified most questions that were raised earlier, and their manuscript is much improved on all counts. The quantification is more detailed in terms of giving number of samples, colocalization and amount of membrane endocytosed. They have done additional experiments with dominant negative constructs of Rho-A, Dynamin, CDC42, Rac and
Arf6. They also have redone experiments regarding the integrin signaling such that the exocytosis is not affected by using the inhibitors after the voiding step. The images are also much improved in this version.

However, I still have some concerns regarding the use of the dominant negative constructs of several of the reagents in the absence of independent corroborations. This is in particular for the Dynamin2 DN constructs which could affect the actin-polymerization step necessary for the RhoA-dependent process. Is it then appropriate to claim that dynamin is involved as a component, or rather its DN version acts as an an inhibitor. In their discussion the authors do discuss this issue, but then a simple experiment using a dynamin2 siRNA containing adenovirus construct could resolve their convoluted argument. The siRNA induced knockdown will not generate a dynamin isoform that interferes with the actin machinery. The demonstration of a role for GRAF would also certainly add mechanistic value to understanding the pathway involved in CE, and would provide a novel player in the RhoA-pathway in general. The claim that this pathway resembles the ILR2 pathway is not very likely since the IL2R pathway appears to be constitutive; a role for integrin-induction has also not been explored in this system.

Other suggestions:
Another issue is the causal connection with integrin-induced CE and RhoA activation. A simple experiment to test this idea would be to express virus expressing activated RhoA constructs and determine if this could rescue the effect of integrin-induced activation.

Minor:
1. To show that clathrin-dependent endocytosis does not occur, the authors have counted the number of CCPs (there being none) and have only shown that chlorpromazine and K+ depletion data has no effect on endocytosis. The authors claim that they had problems with generating adenoviruses expressing these proteins for dominant negative inhibitors. But they have done the other DN negative constructs (Rho-A, CDC42 etc).

2. The minor issues about giving details of actual value and the signal to noise in each measurement are not documented. They have also explained how they quantify the intensity/um2 (using object area) and then finally multiplying by calibrated area. But they do not mention what these areas are qualitatively and why they have to do the calculation this way.

Referee #3 (Remarks to the Author):
Revised version of EMBOJ-2009-73072 by Khandelwal and colleagues on compensatory endocytosis in bladder umbrella cells.

The authors have responded in a satisfactory manner to the comments I had on the initial version of the manuscript.

Reviewer #2:
We are pleased that this reviewer felt that our revised manuscript was much improved. However, the reviewer had a few additional concerns, which are addressed below.

Specific concerns:
1. The reviewer is concerned about the mode of action of dynamin in our system. There are at least two possibilities: one is that dynamin is acting to promote endocytic vesicle scission and the other is
that dynamin is acting to regulate the actin polymerization step. Of course, dynamin could function in both contexts. In our revised Discussion (page 17, second paragraph, first several lines) we have edited the text to be clear that either of these possibilities may explain dynamin function in CE. The reviewer further suggests that by down regulating dynamin expression (using siRNA) that we can ensure that DN-dynamin does not alter the actin machinery. However, we are unclear whether this experiment would be revealing, because if we observe an inhibition of CE we will not know if this is due to dynamin-dependent scission and/or regulation of actin dynamics. We also have a technical problem, one that your noted in your recent letter, and that is we have not yet developed techniques to express shRNA in bladder tissue, although this is our goal for the future.

2. The reviewer further suggests that we explore GRAF1 function and that this would add novel insight into the RhoA pathway. However, we agree with your own determination and believe that this is beyond the scope of the current paper and that our finding of the integrin dependence of RhoA-regulated CE is itself highly novel and revealing.

3. The reviewer does not like the similarities we have drawn between RhoA-regulated CE and the RhoA-regulated constitutive pathway that was previously described for the IL2 receptor. In the revised manuscript we are more careful to describe what is similar and what is different between the two pathways (page 16, second paragraph, first 5 lines).

4. The reviewer wanted us to demonstrate a causal connection between integrin-induced CE and RhoA activation. We have added a new experiment, found in revised Figure 7E-F, that shows that a b1-blocking antibody significantly impairs both integrin-triggered endocytosis and RhoA activation. These new data are included in the revised Results section (page 14, first paragraph, last 7 lines).

The reviewer suggested that a causal link could be established by determining whether an activated §RhoA construct could rescue the effect of integrin-induced activation. We assume that the reviewer meant for us to show that activated RhoA would stimulate endocytosis even when the integrin pathway was inhibited. However, these experiments are technically challenging in our tissue model as we cannot effectively strip rat bladder tissue and thus do not have access to the serosal surface of the tissue. Furthermore, performing such experiments in rabbit bladders is experimentally challenging because of the large volume capacity of the rabbit bladder (100-150 ml) and the necessity to obtain an animal protocol to perform such experiments on a non-exempt vertebrate species, a process that can take several months at our institution.

Minor concerns:

1. The reviewer previously asked for us to use dominant-negative clathrin constructs; however we previously noted that we are having significant issues generating adenoviruses expressing these proteins and can not find other investigators that have already prepared viruses encoding these constructs. As noted previously, the lack of clathrin-coated pits, clathrin, or AP-2 at the apical plasma membrane of umbrella cells, and the insensitivity of apical CE to chlorpromazine and K⁺ depletion all point to a clathrin-independent process. The reviewer also noted that we added experiments using various RhoA constructs; however, viruses expressing these constructs were prepared by and kindly gifted to us by Drs. Bamburg and Casanova.

2. The reviewer wanted additional information about how the image analysis was performed. In the revised Methods section we have added information about the threshold values, the values of the signals, the signal-to-noise values, and values for the calibrated areas. We multiplied the intensity values by the image area because in rabbit umbrella cells it is difficult to assign individual PJAEs to one cell or the other. However, in the rat tissue this was not necessary because the endocytic structures are somewhat more dispersed and the cells are very flat.