Author's response to reviews

Title: Estrogen regulation of TRPM8 expression in breast cancer cells

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Author's response to reviews: see over

Reviewer 1: Luis PARDO

We wish to thank you for your helpful comments on our manuscript. Following are the detailed responses to your comments. The page and line numbers are for the revised version. Changes within the text of the revised manuscript are indicated using red font.

1. In accordance with the referee, we have now added in the new version, the effect of Icilin on the Ca^{2+} signal in cells treated with E_2 compared to starved cells. We observed that treatment of MCF-7 cells with E_2 (10 nM) for 24h did neither affect the number of cells responding to Icilin, nor the Icilin-induced Ca^{2+} increase. However, 48h treatment by E2 increased the number of cells responding to Icilin, without changing the amplitude of Icilin-induced Ca^{2+} entry (Results p11-12, Figure 3C). So, our results suggest that Estrogen controls the fraction of cells expressing TRPM8 channels rather than the amount of channels per cell (see discussion, p14).

2. According to Mr Diouf, biostatistician at the university hospital of Amiens, the Cox proportional hazard analysis is used for longitudinal study like survival that is not the case in our study. Indeed, the evaluation of TRPM8 expression was performed in all studied samples immediately after staining. The best statistic model to evaluate a correlation is the chi-square. Moreover, to determine if the combination of TRPM8 expression with ER status is more predictive than ER status or TRPM8 expression alone regarding the grade status, the logistic model seems to be the best. Statistical analysis performed by Mr Diouf revealed no correlation between tumour's grade and TRPM8, ER or TRPM8 and ER status (certainly due to the low number of samples, n=26). We just described at the end of the results the grade distribution in the TRPM8-expressing cancer tissues.

Minor revisions

2.   The best positive control for TRPM8 channel is of course prostate cancer (tissue or LNCaP cell line). We used the colon cancer tissue as positive control for

TRPM8 as described by the provider (ABCAM Chemistry) to assess the expression of TRPM8 channel. Moreover, we have used human colon cancer tissue because it was relatively easy for us to obtain some samples from the "Tumorothèque de Picardie" directed by Pr. H. Sevestre. We also added in the revised manuscript new data of western blot and immuno-precipitation confirming the expression of TRPM8 in MCF-7 cell line.

3. For the three experiments on the effect of ER siRNA, we added now the quantification of the ER mRNA decrease in Fig 4 B. In the 3 experiments we observed a reduction of the same magnitude of ER and TRPM8 mRNAs, but we have no explanation for that. Re.al-time PCR approach for TRPM8 mRNA analysis was difficult to perform because most of the published TRPM8 primers produced an amplicon with a size higher than 500 bp. We designed three couples of primers according to the Roche Lightcycler conditions (amplicon size less than 300 bp, no dimers, %GC between 50 and 60 …). But they did not pass the validation tests, and we investigated the variation of TRPM8 mRNA expression using semi-quantitative PCR using one of our couples of primers (described in material and methods, paragraph RT-PCR).

4. We corrected in the text: 17/26 cases correspond to 65.4%.

5. The siRNA concentration is now added in the material and methods.

6. The immunohistochemistry protocol is now more explicit (see material and methods).

7. The sentence has been rephrased accordingly.

9. The thickness in Fig.2 has been reduced. Moreover the Standard Error Means (S.E.M.) have been removed from the Ca^{2+}-imaging traces for clarity.

10. We have now removed the TRPM8-expressing cells, since we did not select cells expressing this channel.

11. The last sentences of the results section have been modified and we added a comment on the tumour grading system in material and methods. As we were interested in an association between TRPM8 expression and the differentiation status of the tumor tissues, we estimate the TRPM8 expression according to the tumour grade. Indeed, the Scarff, Bloom and Richardson (SBR) histopronostic grading system is based on cell differentiation, nuclear polymorphism and mitotic activity: Grade I (well-differentiated), Grade II (moderately-differentiated) and Grade III (poorly-differentiated). Our results suggest that TRPM8 is expressed in the early primitive breast cancers presenting a well-differentiated status (Grades I and II).

Reviewer 2: Karl Akerman

We wish to thank you for your helpful comments on our manuscript. Following are detailed responses to your comments. The page and line numbers are for the revised version. Changes within the text of the revised manuscript are indicated using red font.

1. We described the Icilin-activated current as a non-selective current because its reversal potential was close to 0 mV as it is described for the currents evoked by TRPM8 channels. We have added a new paragraph in the discussion to focus on this point. We have now modified the current traces of the figure 2 and it is easier to visualize the control current compared to the Icilin-activated one. We have also replaced "Icilin-sensitive current" by "Icilin-activated current" throughout the manuscript. Regarding the time course of current appearance, the current is activated within the 2-3 minutes following the perfusion of Icilin in the responding cells. However no « intermediate » state of activation was recorded: when the current was activated, its amplitude was maximal. Then, it is quite difficult to show a time course of the current activation. The timing of ramps (100 ms) is included in the Fig. 2 legend. The reversal potential was not significantly changed by Icilin and remained close to 0 mV indicating that the recorded current is a non-selective one. The sentence was modified accordingly in the revised manuscript.

2. We have studied the effect of Icilin on 199 cells from different passage numbers, and Icilin required about 3 min to increase $[Ca^{2+}]_c$. Previous studies on TRPM8 showed that Icilin effect on $[Ca^{2+}]_c$ is reversible. In our study, we have not washed the Icilin because we focused on the number of cancer cells responding to Icilin. Our aim was to determine if Icilin was able to induce a Ca influx in MCF-7 cells. Regarding the 340/380 ratio baseline, we have replaced the figure 2B by an averaged trace of 11 cells to homogenize with the figure 2C.

3. All the MCF-7 responded equally to thapsigargin which explain the low S.E.M.. Regarding the 340/380 ratio baseline, it is not very constant in MCF-7 cells

and could be modulated by the cell cycle. Our lab previously showed that $[Ca^{2+}]_i$ is low in early G1 phase and increases in end G1 and S phases (Ouadid-Ahidouch *et al.*, 2004). In this study, the cells were not synchronized in a particular phase of the cell cycle and this could explain the variability in the 340/380 ration baseline. Moreover, the ratio value could also change depending on time exposure for 340 nm and 380 nm.

4.   We improved the quality of the gels in Fig 3, particularly for the beta-actin, and we corrected the histograms Y axis according to the title in % of control. Moreover we included the definition of DCCFCS in material and methods and results, and the description of the analysis of the gels in figure legend.

5.   The quantitative data and statistics relative to the expression of TRPM8 in adenocarcinomas are described in Table 1. The Fig 5 was obtained using a Zeiss microscope equiped with an x 20 objective lens, and we included a scale bar on the images.

Reviewer 3: Greg Barritt.

We wish to thank you for your helpful comments on our manuscript. Following are detailed responses to your comments. The page and line numbers are for the revised version. Changes within the text of the revised manuscript are indicated using red font.

1.   We used the human breast cancer cell line MCF-7 as a cell model of ER+ adenocarcinoma. We have justified the cell line model in the last paragraph of the Introduction. Moreover, our results concerning TRPM8 expression are confirmed in human breast tissue samples as indicated in the Abstract as well as in the Discussion.

2.   Real-time PCR approach for TRPM8 mRNA analysis was difficult to perform because most of the published TRPM8 primers produced an amplicon with a size higher than 500 bp. We designed three couples of primers according to the Roche Lightcycler conditions (amplicon size less than 300 bp, no dimers, %GC between 50 and 60 …). But they did not pass the validation tests, and we investigated the variation of TRPM8 mRNA expression using semi-quantitative PCR using one of our couples of primers (described in material and methods).

3.   We added in Fig 1 the analysis by western blot of TRPM8 protein expression in MCF-7 cells and human tumoral colon tissue as positive control. As the amount of TRPM8 protein was very low, we confirmed this result by immunoprecipitation experiments.

4. A comment on the small fraction of responding cells has been added in the discussion. Our results suggest that TRPM8 channels are not widely functionally expressed at the plasma membrane of the MCF-7 cell line.

Minor essential revisions
5. Icilin is a synthetic agent which has been described as a possible activator of TRPM8 channels in plasma membrane as well as in ER membrane (Beck B. *et al.*,

2007). Then we suppose that Icilin is able to pass the lipid bilayer to mediate an intracellular effect. We have not found in the literature any comment regarding the permeability of the plasma membrane to icilin.

6. The sentence has been rephrased accordingly.

7. We added the recommended informations about number of experiments and cell cultures in Fig.1 and Fig.2 legends.

Reviewer 4: Roland Schoenherr

We wish to thank you for your helpful comments on our manuscript. Following are detailed responses to your comments. The page and line numbers are for the revised version. Changes within the text of the revised manuscript are indicated using red font.

Major compulsory revision

1. We corrected the histograms Y axis according to the title in % of control. Moreover real-time PCR approach for TRPM8 mRNA analysis was difficult to perform because most of the published TRPM8 primers produced an amplicon with a size higher than 500 bp. We designed three couples of primers according to the Roche Lightcycler conditions (amplicon size less than 300 bp, no dimers, %GC between 50 and 60 …). But they did not pass the validation tests, and we investigated the variation of TRPM8 mRNA expression using semi-quantitative PCR using one of our couples of primers (described in material and methods).

2. In accordance with the referee, we have added now, in the new version, the effect of Icilin on the Ca^{2+} signal in cells treated by E2 compared to starved cells. We observed that treatment of MCF-7 cells with E_2 (10 nM) for 24h neither affects the number of cells which respond to Icilin nor the increased of Ca^{2+} induced by Icilin (see results p11-12 and figure 3C). However, after 48h treatment by E2, we observed an increase of cells that respond to Icilin, without changing the Icilin-induced Ca^{2+} entry (see results p11-12 and figure 3C). Consequently, our results suggest that Estrogen control the fraction of cells expressing TRPM8 channels rather than the amount of channel per cell (see discussion, p14).

3. For PCR detection of TRPM8, we performed PCR amplification with 40 cycles because TRPM8 transcripts were present in low quantity in the cells, and the PCR signals were never saturated. Moreover the quantitative interpretation of PCR signals was performed by comparing the ratio TRPM8 on β-actin between the

different treatment-conditions of MCF-7 cells. For this study, it is not necessary to compare with another cell line. However, for the mRNA detection of TRPM8 in Fig 1, we used human colon cancer tissue as positive control. Indeed, this tissue is recommended by the provider (ABCAM Chemistry) to assess the expression of TRPM8 channel and it was relatively easy for us to obtain colon cancer samples, thanks to the "tumorothèque de Picardie" directed by Pr H. Sevestre.