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Magnesium Green for fluorometric measurement of ATP production does not interfere with mitochondrial respiration

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Reviewer 2

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Reviewer 2

The present technical communication describes the viability of an improved method for the determination of a classical bioenergetic parameter. The results are clear and concise, and will be of great use for the further development of the Magnesium Green assay as a universal method for adequate kinetic determination of the phosphorylating capacity. The question has been addressed with the appropriate approach.

I have one major point to address which is either a small mistake or a significant methodological error. In addition, I would like to recommend –some lesser and some greater- improvements which in my opinion would elevate the quality of the present communication, but the decision is left up to the Author and Editor. However, beyond these points, the manuscript is of very high quality and no further modifications are required.

Major point:

Either the EGTA concentration unit of the respiratory solution is a typo or is a serious concern. Considering pKa1 = 9.5, pKa2 = 8.8, pKa3 = 2.7, lgK = 5.2, [Mg]t = 1 mM, [EGTA]t = 0.5 mM (Data for Biochemical Research, 3rd ed., Dawson, R.M.C., et al., Oxford University Press (New York, NY: 1986), pp. 404-405.) the calculated [EGTA-Mg] concentration at pH = 7.1 is approximately 0.437 mM which is in a comparable range with the [Mg]free, therefore it has to be accounted for in all cases.

In [Mg]free calibration the standard complex formation equation may be used. I am surprised there was a reasonable fluorescence difference at lower (<0.5 mM) Mg concentrations despite such high sequestration.

The apparent Kd determination of ADP and ATP becomes a cubic equation instead of quadratic one.

The ATP calculation gets an additional component of
-{[EGTAt/(Mg+KdEGTA*α)]/[1/(Mg+KdATP)-1/(Mg+KdADP)]}
which ends up being fairly significant at this concentration.

Also because of the decreased free Mg there is expected to be an increase in the determination error (Christos Chinopoulos, Szilvia Vajda, László Csanády, Miklós Mándi, Katalin Mathe, Vera Adam-Vizi; A Novel Kinetic Assay of Mitochondrial ATP-ADP Exchange Rate Mediated by the ANT; Biophysical Journal, Volume 96, Issue 6, (2009) 2490-2504) which is important for the evaluation of the data quality and reliability. The error of the method has also not been described yet, such as recovery of added known amounts of nucleotide in known ratios.

Authors

We added new experiments, determining the K_d' for ATP^{4-}-Mg^{2+} and ADP^{3-}-Mg^{2+} with 500 µM (the concentration in MiR05 and used in the present work) and 5 µM (as used by Chinopoulos et al. 2009 and 2014). The new experiments are described in the new Figure 2 and in pages 6–7. In summary: "50 µM EGTA was needed (Figure 2c and d), with similar results at 500 µM EGTA (Figure 2e and f)". The use of EGTA in the medium is important to chelate free Ca^{2+}, which, besides the possibility of affecting mitochondrial respiration, can bind MgG with higher affinity than Mg^{2+} thus affecting the fluorometric measurements. The advantages and disadvantages of using different concentrations of Mg^{2+} and ADP are explored and well described by Chinopoulos et al, 2009. We included a sentence: "As shown by Chinopoulos et al (2009), too low concentrations of free Mg^{2+} decrease the signal-to-noise ratio" (page 7).

Reviewer 2

Greater recommendations: I have not seen it explicitly mentioned in the description, but has the initial fluorescence been taken into account in the calculation of [Mg]free? There was quite a bit of difference between individual measurements and it has been addressed in (Christos Chinopoulos, Gergely Kiss, Hibiki Kawamata, Anatoly A. Starkov; Measurement of ADP–ATP Exchange in Relation to Mitochondrial Transmembrane Potential and Oxygen Consumption, Methods in Enzymology, Academic Press, Volume 542, (2014) 333-348) that such corrections may need to be done. If they were done it would be important to mention them in the description along the other boundary values (Lines 144-147) of ATP calculation for the data manipulation to be reproducible.

Authors

We added to the text: "After calibration of the fluorescence signal in terms of free [Mg^{2+}], ATP concentration in the medium is calculated…" and "converted from current to voltage U (free [Mg^{2+}] = (a × U^2) + (b × U) + c) (Chinopoulos et al 2014)" (section 2.5, page 5).

Besides this, we included in the Results and discussion section: "it is advisable to perform a calibration with each sample tested under the same experimental conditions (same batches of media, chemicals and MgG, in the same instrumental chamber used)".

Reviewer 2

While it has been elaborated in (Erich Gnaiger, Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply, Respiration Physiology, Volume 128, Issue 3, (2001) 277-297) why it is important to use saturating ADP concentrations. In this particular case however, the results would benefit from using limiting amounts of ADP (200-400 uM) in the absence of exogenous ATP, because under these conditions ADP/O gives a good approximation of P/O and it would serve as an internal quality control, since the theoretical near complete (STUCKI, J.W. (1980), The Optimal Efficiency and the Economic Degrees of Coupling of Oxidative Phosphorylation. European Journal of Biochemistry, 109: 269-283.) (U. Küster, R. Bohnensack, W. Kunz, Control of oxidative phosphorylation by the extramitochondrial ATP/ADP ratio, Biochimica et Biophysica Acta (BBA) - Bioenergetics, Volume 440, Issue 2, (1976) 391-402) and calculated recovery can be compared after entry into state 4 respiration. This ADP/O can also be used as a reference point when saturating conditions are investigated. This evaluation would drastically increase the credibility of the present communication.

While the lack of statistical difference has been shown in the present manuscript, in 5 out of 6 cases there has been a less than significant but consistent decrease in leak and active respiration. The noxious effect of MgG was only investigated at the concentration of 1.1 uM while higher concentrations have been used in the literature (2.1 and 5 uM) with mixed effect (both decreasing and increasing) on mitochondrial respiratory parameters when the MgG assay is compared with other respiratory assays in the same paper. (Napa et al. 2017, Pham et al. 2014, Devaux et al. 2019). It is beyond the scope of the present communication, but rather than trying to show the absence of an effect, it is more convincing to show the detrimental effect at a higher concentration, characterise it, establish a concentration dependence and then extrapolate to a "no effect level" as in standard toxicology.

Authors

Such experiments would be important under the scope of a different investigation, aiming at analysing P»/O_2 ratios under saturating ADP concentration versus non-saturating concentration. In the current work, the P»/O_2 ratios are presented to illustrate what is possible to achieve with the technique, but its comparison was not among our aims. We included changes in the text, see answers to reviewer 1 doi:10.26124/bec:2021-0001.r1.

Reviewer 2

Minor points:

Line 50 – I would suggest removing the word "slightly" as the ADP addition causes a decrease of approximately 15-20% of all fluorescence and 90-95% of the working range.

Lines 76-80 – The word diluted may be replaced with the verbs dissolved or mixed depending on the state (solid, liquid, stock solution) of the substance in question.

Lines 82-89 – The quality of the description would increase by including the relative humidity (RH%) of the environment (usually 45-65% for rodents) and the microbiological category of the animal facility section the animals were kept in (conventional or SPF etc.).

Line 101 – "on" should be replaced with "in" since the homogeniser is a container.

Line 127 – The 2 in "MgCl2" is a subscript.

Line 127 - The comma in "EGTA 0,5 mM" should be a decimal dot to keep it consistent with the notation used in the rest of the paper.

Line 150 – "amperometric" is missing a t letter.

Figure 2 - The y-axis of graph b renamed as Mg fluorescence or something similar to make it easier to read.

Figure 2 – The x-axis of the c graph would be easier to align in the reader's head if it followed the same divisions as graph a and b. Cutting out the empty intervals could make space for a graph representing the calculated [Mg]free concentration with respect to time.

Figure 3 – Same modifications as figure 2 are recommended.

Line 229 – The word "used" is unnecessary in context of the sentence.

Authors

The points above have been corrected in the text. The Figures 2 and 3 from the original manuscript are now Figures 3 and 4 in the resubmitted manuscript and incorporate changes suggested by the reviewer.

Reviewer 2

Lines 166-168 – For a clearer description it would be advised to mention which inhibitor and uncoupler was used in which protocol. Also why were different one the particular choice or as later elaborated, why they are comparable still.

Authors

Addition to Section 2.6: "Both inhibitors have the same function in the context of the present experiments, to induce LEAK respiration by inhibition of the phosphorylation system. This was followed by stepwise titration of uncouplers up to the optimum concentration, when the maximum O_2 flux was achieved as a measure of ET capacity - CCCP (0.5 µM steps) or SF 6847 (25–50 nM steps) were used. Both are protonophores and have the same function". Prior to these experiments only oligomycin and CCCP were in use in our laboratories, with the acquisition of carboxyatractyloside and SF6847, these chemicals were also tested and used in the experiments here described.

As emphasized previously in the Results and Discussion section, "Respiration in these two LEAK states was similar, but slightly lower in *L*(Omy) with the N-protocol (Figure 3a, Table 1). *L*(n) stabilized quickly, whereas for *L*(Omy) it took a long time to fully inhibit respiration by the low concentration of 7.5–10.0 nM oligomycin". In contrast, "Inhibition by carboxyatractyloside (0.3–0.4 μM) was immediate, and *L*(Cat) tended to be slightly lower than *L*(n) (Table 1)".

Reviewer 2

In 5 out of 6 cases there was an increase in MgG fluorescence upon energisation of mitochondria, the 6th having comparatively rapid additions. I think it would increase the value of the communication to at least take note of such observation with or without hypothesis.

Authors

New Figure 6 shows that the increase in fluorescence upon addition of fuel substrates (particularly pyruvate and succinate) happened even in the absence of mitochondria. Page 13, 1st paragraph: "Addition of succinate and pyruvate in the absence of sample resulted in a chemical background effect (Figure 6a and b)…". Due to this chemical background effect, the K_d' of ADP and ATP to Mg^{2+} has to be determined in the presence of substrates used in the experiment, as emphasized by Chinopoulos et al (2014).