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## RESEARCH ARTICLE

# THE EFFECTS OF ADENOSINE AND ADENOSINE MONOPHOSPHATE ON CALCIUM INDUCED MITOCHONDRIAL SWELLING

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**ABSTRACT**

The aim of this research is to primarily determine the effect of calcium on isolated rat liver mitochondria and to discover the possible effects of adenosine and adenosine monophosphate on calcium induced mitochondrial swelling. About 100 male albino rats weighing between 180-260g were recruited into the study. The male albino rats were from animal house of Faculty of Biological Sciences, University of Nigeria, Nsukka. The effects of calcium, adenosine and adenosine monophosphate on isolated normal male rat liver mitochondria were studied and also the effects of adenosine and adenosine monophosphate on calcium-induced mitochondria were also studied by use of standard biochemical techniques and read at 520nm in spectrophotometer. From the study, we could infer that calcium at lower concentration will induce swelling of the mitochondria and invariably rapid swelling at higher concentration of calcium. It was also inferred that the magnitude of the effect of concentration of adenosine or adenosine monophosphate was invariably related to calcium concentration, hence prevented swelling in the presence of high concentration of calcium.



## INTRODUCTION

Mitochondria have a central role in energy metabolism and calcium ion haemostasis in cells (**Brookes *et al*. (2004), Passarella *et al*. (2003) and Gunter *et al*. (2004**). Adenosine is a purine nucleoside composed of a molecule of adenine attached to a ribose sugar molecule (ribofuranose) moiety of β-$N_9$- glycosidic bond. Adenosine plays important role in biochemical processes, such as energy transfer as adenosine triphosphate (ATP) and adenosine diphosphate (ADP) as well as in signal transduction as cyclic adenosine monophosphate (cAMP). It is also a neuro-modulator believed to play a role in promoting sleep and suppressing arousal. Adenosine also plays a role in regulation of blood flow to the various organs through vasodilation (**Sato *et al*., 2005; Costa and Biaggoni, 1998 and Morgan *et al*., 1991**). Several triggers of mitochondrial permeability transition (MPT) results in cell apoptopic cell death (**Sparagna *et al*., 2000; Sakurai *et al*., 2001a and Sakurai *et al*., 2001b**). MPT is associated with an increase in the permeability of the mitochondrial inner membrane, allowing the transmission of a solute with the molecular mass of up to approximately 1.5KDa. MPT is also associated with mitochondrial proteins including Cytochrome C (**Tsujimoto *et al*., 2006**). Cyt. C with molecular weight of approximately 12KDa is loosely bound to phospholipids of the outer surface of the inner mitochondrial membrane, primarily cardiolipin and functions to transmit electrons from complex III to complex IV of the electron transport chain in mitochondria (**Ott *et al*., 2002 and Petrosillo *et al*., 2003**). Mitochondria from different organs (liver, heart, brain) have different sensitivity to permeability inducers (**Sabine *et al*., 2011**) and hence different swelling behavior. $Ca^{2+}$ is transferred via the calcium uniporter into the mitochondria and induces mitochondrial swelling via the binding to an opening-inducing $Ca^{2+}$ binding site on the matrix side of mitochondrion (**Saris, 2005**). Enzymes found in the mitochondrion catalyze the oxidation of organic cell nutrients by molecular oxygen to yield carbondioxide and water, much chemical is released during these oxidations which are used to generate ATP, the major energy-carrying molecule of cells. ATP formed by mitochondria diffuses to all parts of the cell, where it is used to carry out cellular functions. The normal state of mitochondria which is dependent on the active transport of certain materials is in most cases not maintained due to the presence of some agents that cause swelling of mitochondria *invitro*. These agents include calcium of inorganic phosphate, thyroxine, reduced glutathione. (GSSH), arsenate (**Lehininger, 1982**).

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## MATERIALS AND METHODS

### Chemicals and reagents

All chemicals and reagents used were of analytical standard.

**Samples**

Calcium chloride solution, adenosine and adenosine monophosphate solution were prepared.

**Experimental Animals**

Male albino rats (180 – 260)g were purchased from the animal house of the Faculty of Biological Science, University of Nigeria, Nsukka.

**Isolation of Rat Liver Mitochondria**

This was carried out according to the methods of Guerra, 1971. The rate was weighed, stunned and decapitated. It was quickly dissected open and the liver excised and washed in air ice-cold isolation medium, (0.34M sucrose, and 25mM Tris HCl pH 7.4). The liver was then weighed, chopped into pieces with scissors and then homogenized in the isolation medium (1:4 w/v) for 5 minutes. The homogenate, after filtering to remove unwanted debris, was centrifuged at 3200 rpm (1200xg) for 10minutes in the IEC B-20A at -4$^0$C with A192 rotor head. The precipitate containing cell nuclei and debris was discarded. The supernatant was centrifuged again at 8000 rpm, (2700 x g) for 10 minutes at the same temperature. The fluffy supernatant containing the fraction was discarded. The precipitate was gently swirled in the medium to aspirate contents and recentrigued at 8000 rpm, (2700 x g) for 10minutes at -4$^0$C. The mitochondria were used within five hours from the time of isolation.

**Extinction Measurements**

The mitochondrial swelling of the normal male isolated rate liver was assayed using a Coleman spectrophotometer. The swelling was induced by adding the mitochondria to a reaction mixture containing the isolation medium and various other additives, as specified below, so that a final volume of 3ml was obtained in each case. Mitochondrial swelling was followed by measuring the extinction at 520nm over a range of time at a two-minute interval.

**Effect of calcium**

Mitochondrial suspension (0.03ml) was added into the reaction cuvette containing the isolation medium (2.77ml) followed by calcium (0.20ml), 1mM. This was repeated for each of the following concentrations of calcium solution, 5mM, 10mM and 15mM. In each case the final volume of the reaction mixture in the cuvette was 3ml. Extinction was measured and recorded as described above.

**Effect of Adenosine**

This was carried out to know the effect of adenosine on mitochondria and on calcium-induced swelling of the mitochondria. This was done by adding the mitochondrial suspension (0.03ml) to the isolation medium (2.77ml) in the reaction cuvette. This was followed by the addition of adenosine (0.20ml of 3ng/ml, 7ng/ml, 13ng/ml and 27ng/ml respectively), to the reaction cuvette, making a total volume of 3ml in the reaction cuvette. Extinction was recorded as usual.

**Effect of AMP**

This was performed to know the effect of AMP on the mitochondria and on calcium-induced swelling of the mitochondria. Mitochondrial suspension (0.03ml) was added to a cuvette containing 2.77ml of the isolation medium. AMP (0.20ml of 3ng/ml, 7ng/ml, 13ng/ml and 27ng/ml) was added to the reaction cuvette making same total volume of 3ml in the reaction cuvette. Extinction was then taken as before.

**Effect of Adenosine on Calcium-induced Mitochondrial Swelling**

Mitochondrial suspension (0.03ml) was added into a cuvette containing 2.57ml of the isolation medium. Adenosine (0.20ml of 3ng/ml) was added and finally 10mM $Ca^{2+}$ solution (0.20ml was then added making a final volume of 3ml).Extinction was read as usual. The experiment was repeated using 7ng/ml, 13ng/ml and 27ng/ml respectively.

**Effect of AMP on Calcium-induced Mitochondrial Swelling**

In a cuvette containing 2.5ml of the isolation medium, a mitochondrial suspension (0.03ml) was added. Next, 0.20ml AMP (3ng/ml, 7ng/ml, 13ng/ml and 27ng/ml respectively) was added. 0.20ml of 10mM $Ca^{2+}$ solution was then added making a final volume of 3ml.Extinction was recorded as before over a range of time.

## RESULTS

The following results were obtained and each indicated the mean of various values obtained for several experiments carried out. (Table 1-5).

**Effect of Calcium on the Extinction of Isolated Mitochondria**

A suspension of mitochondria was added to the isolation medium and its extinction recorded every two minutes interval for sixteen minutes. When $Ca^{2+}$ was included in the buffered medium, there was a concentration depending drop in the extinction of the suspension with time (See Table 1).

**Effect of AMP on Mitochondrial Swelling**

When AMP was included in the mitochondrial suspension in the buffered medium, there was a drop in the extinction of the suspension showing swelling, (Table 2). It was interesting to observe that the drop had an inverse relationship with AMP concentration; at higher concentration (27ng/ml) no swelling was observed.

**Effect of Adenosine on Mitochondrial Swelling**

The inclusion of a graded concentration of adenosine (3ng/ml to 27ng/ml) to mitochondrial suspension produced the following effects, lowering of OD at lower concentrations but

**Table 1. The effect of varying concentrations of calcium chloride on mitochondrial swelling**

| Time (Mins) | Extinction (520)nm | | | |
|---|---|---|---|---|
| | 1mM | 5mM | 10mM | 15mM |
| 2 | $0.095\pm2.0\times10^{-3}$ | $0.090\pm2.0\times10^{-3}$ | $0.085\pm5.0\times10^{-4}$ | $0.078\pm0.5\times10^{-3}$ |
| 4 | $0.092\pm0.8\times10^{-3}$ | $0.085\pm1.0\times10^{-3}$ | $0.081\pm1.0\times10^{-3}$ | $0.075\pm2.0\times10^{-3}$ |
| 6 | $0.090\pm2.0\times10^{-3}$ | $0.083\pm2.0\times10^{-3}$ | $0.080\pm2.0\times10^{-3}$ | $0.072\pm0.5\times10^{-3}$ |
| 8 | $0.090\pm2.0\times10^{-3}$ | $0.083\pm2.0\times10^{-3}$ | $0.078\pm2.5\times10^{-3}$ | $0.072\pm0.5\times10^{-3}$ |
| 10 | $0.085\pm1.0\times10^{-3}$ | $0.080\pm0.5\times10^{-3}$ | $0.074\pm3.5\times10^{-3}$ | $0.070\pm0.5\times10^{-3}$ |
| 12 | $0.085\pm1.0\times10^{-3}$ | $0.079\pm0.8\times10^{-3}$ | $0.073\pm4.0\times10^{-3}$ | $0.065\pm0.5\times10^{-3}$ |
| 14 | $0.085\pm1.0\times10^{-3}$ | $0.078\pm0.5\times10^{-3}$ | $0.072\pm3.0\times10^{-3}$ | $0.065\pm0.5\times10^{-3}$ |
| 16 | $0.082\pm1.0\times10^{-3}$ | $0.076\pm0.5\times10^{-3}$ | $0.076\pm2.5\times10^{-3}$ | $0.063\pm1.0\times10^{-3}$ |

These were the optical densities at 520nm of isolated mitochondria alone and of mitochondrial suspension containing a graded concentration of $CaCl_{2(aq)}$. There was a concentration dependent drop in the extinction of the mitochondrial suspension with time.

**Table 2. The effect of AMP at varying concentrations on mitochondrial swelling**

| Time (Mins) | Extinction (520)nm | | | |
|---|---|---|---|---|
| | 3ng/ml | 7ng/ml | 13ng/ml | 27ng/ml |
| 2 | $0.030\pm3.0\times10^{-3}$ | $0.070\pm0.5\times10^{-3}$ | $0.085\pm2.0\times10^{-3}$ | $0.104\pm2.0\times10^{-3}$ |
| 4 | $0.055\pm2.0\times10^{-3}$ | $0.065\pm2.0\times10^{-3}$ | $0.080\pm0.5\times10^{-3}$ | $0.102\pm2.0\times10^{-3}$ |
| 6 | $0.055\pm2.0\times10^{-3}$ | $0.066\pm0.5\times10^{-3}$ | $0.080\pm0.5\times10^{-3}$ | $0.100\pm4.5\times10^{-3}$ |
| 8 | $0.055\pm2.0\times10^{-3}$ | $0.064\pm2.0\times10^{-3}$ | $0.080\pm0.5\times10^{-3}$ | $0.099\pm3.5\times10^{-3}$ |
| 10 | $0.045\pm0.5\times10^{-3}$ | $0.064\pm2.0\times10^{-3}$ | $0.075\pm0.8\times10^{-3}$ | $0.098\pm5.5\times10^{-3}$ |
| 12 | $0.045\pm0.5\times10^{-3}$ | $0.066\pm0.5\times10^{-3}$ | $0.075\pm0.8\times10^{-3}$ | $0.095\pm5.5\times10^{-3}$ |
| 14 | $0.040\pm2.0\times10^{-3}$ | $0.062\pm0.5\times10^{-3}$ | $0.077\pm0.5\times10^{-3}$ | $0.094\pm3.5\times10^{-3}$ |
| 16 | $0.039\pm0.5\times10^{-3}$ | $0.060\pm2.0\times10^{-3}$ | $0.070\pm0.5\times10^{-3}$ | $0.086\pm7.5\times10^{-3}$ |

(3ng/ml to 13ng/ml) AMP lowered the OD (520nm) of mitochondrial suspension in an inverse manner but a higher concentration produced very little effect i.e. lowered OD to value close to those of mitochondria only.

**Table 3. The effect of adenosine at varying concentrations on mitochondrial swelling**

| Time (Mins) | Extinction (520)nm | | | |
|---|---|---|---|---|
| | 3ng/ml | 7ng/nl | 13ng/ml | 27ng/ml |
| 2 | $0.095\pm0.8\times10^{-3}$ | $0.105\pm0.8\times10^{-3}$ | $0.112\pm8.5\times10^{-3}$ | $0.125\pm0.5\times10^{-3}$ |
| 4 | $0.090\pm5.5\times10^{-3}$ | $0.101\pm0.5\times10^{-3}$ | $0.108\pm7.5\times10^{-3}$ | $0.123\pm2.0\times10^{-3}$ |
| 6 | $0.080\pm2.0\times10^{-3}$ | $0.095\pm0.8\times10^{-3}$ | $0.104\pm6.5\times10^{-3}$ | $0.120\pm1.0\times10^{-3}$ |
| 8 | $0.080\pm2.0\times10^{-3}$ | $0.094\pm0.5\times10^{-3}$ | $0.102\pm5.5\times10^{-3}$ | $0.115\pm0.8\times10^{-3}$ |
| 10 | $0.070\pm0.5\times10^{-3}$ | $0.094\pm0.5\times10^{-3}$ | $0.099\pm6.0\times10^{-3}$ | $0.115\pm0.8\times10^{-3}$ |
| 12 | $0.067\pm2.0\times10^{-3}$ | $0.090\pm0.5\times10^{-3}$ | $0.098\pm7.0\times10^{-3}$ | $0.110\pm0.5\times10^{-3}$ |
| 14 | $0.065\pm0.5\times10^{-3}$ | $0.085\pm0.8\times10^{-3}$ | $0.095\pm5.0\times10^{-3}$ | $0.105\pm0.5\times10^{-3}$ |
| 16 | $0.065\pm0.5\times10^{-3}$ | $0.081\pm0.5\times10^{-3}$ | $0.092\pm5.0\times10^{-3}$ | $0.102\pm0.5\times10^{-3}$ |

The inclusion of graded concentration of adenosine (3ng/ml to 27ng/ml) to mitochondrial suspension produced the following effects: lowering of OD at lower concentration but at higher concentrations the values are close to those of mitochondria only.

**Table 4. The effect of adenosine on $CaCl_2$-induced mitochondrial swelling**

| Time (Mins) | Extinction (520)nm | | |
|---|---|---|---|
| | 10mM $Cacl_{2(aq)}$ | 13ng/ml adenosine | 13ng/ml adenosine + 10mM $Cacl_{2(aq)}$ |
| 2 | $0.085\pm5.0\times10^{-4}$ | $0.112\pm8.5\times10^{-3}$ | $0.089\pm0.5\times10^{-3}$ |
| 4 | $0.081\pm1.0\times10^{-3}$ | $0.108\pm7.5\times10^{-3}$ | $0.087\pm0.8\times10^{-3}$ |
| 6 | $0.080\pm2.0\times10^{-3}$ | $0.104\pm6.5\times10^{-3}$ | $0.089\pm0.5\times10^{-3}$ |
| 8 | $0.078\pm2.5\times10^{-3}$ | $0.102\pm5.5\times10^{-3}$ | $0.089\pm0.5\times10^{-3}$ |
| 10 | $0.074\pm3.5\times10^{-3}$ | $0.099\pm6.0\times10^{-3}$ | $0.088\pm2.0\times10^{-3}$ |
| 12 | $0.073\pm4.0\times10^{-3}$ | $0.098\pm7.0\times10^{-3}$ | $0.085\pm2.0\times10^{-3}$ |
| 14 | $0.072\pm3.0\times10^{-3}$ | $0.095\pm5.0\times10^{-3}$ | $0.086\pm0.5\times10^{-3}$ |
| 16 | $0.068\pm2.5\times10^{-3}$ | $0.092\pm5.0\times10^{-3}$ | $0.085\pm2.0\times10^{-3}$ |

In the presence of adenosine (13ng/ml), the effect of $Cacl_2$ on the fall of the OD of mitochondria was mitigated i.e. it decreases the ability of $Cacl_2$ to induce swelling.

**Table 5. The effect of $CaCl_{2(aq)}$ on mitochondrial suspension containing AMP**

| Time (Mins) | Extinction (520)nm | | |
|---|---|---|---|
| | 10mM $Cacl_2$ | 27ng/ml AMP | 27ng/ml AMP + 10mM $Cacl_{2(aq)}$ |
| 2 | $0.085\pm5.0\times10^{-4}$ | $0.104\pm2.0\times10^{-3}$ | $0.093\pm0.5\times10^{-3}$ |
| 4 | $0.081\pm1.0\times10^{-3}$ | $0.102\pm2.0\times10^{-3}$ | $0.093\pm0.5\times10^{-3}$ |
| 6 | $0.080\pm2.0\times10^{-3}$ | $0.100\pm4.5\times10^{-3}$ | $0.093\pm0.5\times10^{-3}$ |
| 8 | $0.078\pm2.5\times10^{-3}$ | $0.099\pm3.5\times10^{-3}$ | $0.093\pm0.5\times10^{-3}$ |
| 10 | $0.074\pm3.5\times10^{-3}$ | $0.098\pm5.5\times10^{-3}$ | $0.091\pm0.8\times10^{-3}$ |
| 12 | $0.073\pm4.0\times10^{-3}$ | $0.095\pm5.0\times10^{-3}$ | $0.092\pm0.5\times10^{-3}$ |
| 14 | $0.072\pm3.0\times10^{-3}$ | $0.094\pm4.5\times10^{-3}$ | $0.090\pm0.5\times10^{-3}$ |
| 16 | $0.068\pm2.5\times10^{-3}$ | $0.086\pm7.5\times10^{-3}$ | $0.090\pm0.5\times10^{-3}$ |

Again, in the presence of AMP (27ng/ml), the effect of $Cacl_2$ on the fall of the OD of mitochondria was again mitigated, i.e. It decreased the ability of $Cacl_2$ to induce swelling.

At higher concentrations the values were close to those of mitochondria only (Table 3).

**Effect of Adenosine on Ca- induced Mitochondrial Swelling**

In the presence of adenosine (13ng/ml), the effect of Ca on the fall of the OD of mitochondria was mitigated i.e. it decreased the ability of Ca to induce swelling, (Table 4).

**Effect of Ca on Mitochondrial Suspension Containing AMP**

In the presence of AMP (27ng/ml), the effect of Ca on the fall of the OD of mitochondria was again mitigated, i.e. it decreased the ability of Ca to induce swelling, (Table 5).

## DISCUSSION

The results obtained during this experiment showed that mitochondria from the normal rat liver were very tightly coupled. This explained why no swelling occurred until calcium was added. From the results presented, it was evident that calcium induced swelling of the mitochondria. **Saris, (2005)** reported that $Ca^{2+}$ was transferred into the mitochondria and induced mitochondrial swelling via the binding to an opening inducing $Ca^{2+}$ binding site on the matrix side of mitochondrion. Thus, the swelling of mitochondria induced by calcium during the first 5 minutes might be caused by hydrolysis of phospholipids at a key locale, thus resulting in changes in the nature of mitochondrial membrane. Therefore, inhibition of swelling of mitochondria induced by calcium during the early period of incubation might owe to inhibition of phospholipids an activity, **(Naoko Harada, 1976).** A calcium induced non-specific increase in the permeability of the mitochondrial inner membrane of isolated beef heart mitochondria, which they termed mitochondrial permeability transition (MPT) was described by **Hunter *et al*. (1976).** Green and **Kromer, (2004)** also reported that such permeability transition was lethal because it resulted in the release of death inducing molecules from and/or in metabolic failure of mitochondria.

The inhibitory effects of adenosine and adenosine monophosphate were studied in the course of this research work and it was found that the effect of the concentration of adenosine and adenosine monophosphate was inversely related to calcium concentrations. The adenosine monophosphate had its phosphate ionized in physiologic solution, the transport or passage of this ion would be difficult to cross the mitochondrial membrane. Once this shrinkage occurs, the calcium ion could be removed as an insoluble sulphate, **Harris (1972),** later **Berman *et al.* (2000)** reported a mathematical model that was also capable of explaining the shrinking preceding the actual swelling of mitochondria. These enzymes found in the mitochondrion could catalyze the oxidation of organic cell nutrients by molecular oxygen yielding carbon-dioxide and water, and thus ATP was generated. Much chemical was released during these oxidations. ATP formed by mitochondria diffused to all parts of the cell, where ATP was used to carry out cellular functions. That the normal state of mitochondria which was dependent on the active transport of certain materials, was in most cases not maintained, thus some agents such as calcium of inorganic phosphate, thyroxine, reduced glutathione. (GSSH), arsenate could cause swelling of mitochondria *in-vitro* as reported by **Lehninger, (1982).**

**Conclusion**

This investigation revealed that calcium at low concentrations induced regular swelling of the isolated rat mitochondria but showed rapid swelling at higher concentrations. Moreover, the magnitude of the effect of any given concentration of adenosine or adenosine monophosphate was inversely related to calcium concentration. Both do prevent swelling, even in the presence of increasing concentrations of calcium.

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