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Mechanism of De-energized Mitochondria Membrane Potential Formation in Permeabilized Cardiac Myocytes

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ABSTRACTS

The biophysical characteristics in de-energized mitochondria are not known very well. In this study, we carried out the investigation into the membrane potential formation at de-energized states of mitochondria. NADH, FAD and $\Delta\Psi_m$ were monitored simultaneously using permeabilized cardiac myocytes of the rat. We showed de-energized mitochondria in absence of mitochondrial substrates still have a membrane potential and it was about 56 ± 5 mV. To pursue the mechanism of that, firstly we tried the possibility of the involvement of mito-K_{ATP} channel. We tested effects of Pi /ATP, K_{ATP} channel blockers/opener, and K^+ replacement with meglumine on the de-energized mitochondria. The addition of Pi and ATP could dramatically hyperpolarized about 62 ± 7.7 mV. However, all agents blocking K_{ATP} channel did not show any significant effects. Interestingly, Diazoxide (DZX), K_{ATP} channel opener, and Oligomycin A (OligoA), F_1-F_0-ATPase inhibitor, itself could depolarize the Ψ_m in basal condition about 11 ± 1 mV and 46.5 ± 6 mV respectively, and the addition of Pi could reverse the effect. The ATP induced the hyperpolarization was occurred with DZX but was fully inhibited by OligoA. K^+ replacement with meglumine slowed down the speed of ATP induced hyperpolarization and made it transient. However, the change NMDG to K^+ could reverse and showed the full effect of ATP on Ψ_m. From these results, K_{ATP} channel and reverse mode of F_1-F_0-ATPase by the residual ATP hydrolysis might contribute the formation of the resting Ψ_m in de-energized mitochondria even though the mechanism of formation of the resting Ψ_m is still not clear. The K^+ environment seemed to be important for the activity of F_1-F_0-ATPase.

Key Words: Ψ_m ,mito-KATP channel, ATP, Diazoxide, Oligomycin A.

ABBREVIATION

EGTA,Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid;HEPES,N-2-hydroxylethypiperazineN'-2-ethanesulfonic acid; TMRE,Tetramethylrhodamine ethyl ester; NADH,Nicotinamide adenine dinucleotide FAD, Ψ_m, mitochondria membrane potential;ATP, Adenosine triphosphate;Pi, inorganic phosphate; K_{ATP} channel, ATP-sensitive potassium channel; 5-HD, 5-hydroxydecanoate; TEA,Tetraethylammonium; NMDG, N-methyl-D-glucamie chloride; ADP, Adenine diphosphate;AMP, Adenine monophosphate

I. INTRODUCTION

Mitochondria are organelles which play multiple critical functions in the cell, such as: regulate the cellular redox state, produce the cellular reactive oxygen species (ROS)[1)], buffer Ca^{2+}[2-4)], ATP production and etc. Among them the most prominent role of mitochondria is to generate the metabolic energy in cells[5)].They obtain this energy through the process called the oxidative phosphorylation. In 1961 *Peter Mitchell* proposed the chemiosmotic hypothesis to explain the mechanism of mitochondrial energy transduction. There are three processes; 1) Generating NADH, FAD by oxidizing the substrates of Krebs cycle, 2) Using NADH, FAD to generate Proton Motive Force (PMF) via Electron Transport Chain, 3) Using PMF to generate ATP via F_1F_0 ATPase complex. [6, 7)]

In addition, there were recent reports which showed that mitochondria involved with important processes such as apoptosis, ischemia-reperfusion injury, other diseases (diabetes, Huntington's disease, cancer) and etc. However, despite the fact that much research has been done on physiological mechanism of mitochondria, some of the bioenergetics backgrounds are still not clear including energy metabolism of de-energized mitochondria. De-energized states strongly related with I/R injury, heart transplantation and other mitochondrial disease. However, the functions of mitochondria are not known in de-energized states. As far as we know, the mitochondrial membrane potential (Ψ_m) is a key indicator of cellular viability; it results from redox transformations associated with activity of Krebs cycle and serves as an

intermediate form of energy storage which is used by ATP-synthase to make ATP. These transformations generate not only an electrical potential but also a proton gradient, and it forms the transmembrane potential (Ψ_m)[8]. The depolarization of $\Delta\Psi_m$ may induce unwanted loss of cell viability and be a cause of various pathologies[9-12)]

Accordingly, supplying substrates to Krebs cycle is critical to form the Ψ_m. Without energy substrates to mitochondria, Krebs cycle reactions will be stopped and Ψ_m may not be formed. For the first time, we carried out the investigation into the potential formation at de-energized states in this study. An ATP-sensitive K^+ channel is existed in mitochondria and the mitochondrial ATP-dependent K^+ channel (mitoK_{ATP}) is highly considered by many scientists to play a key protection role in I/R injury transplantation and other mitochondrial disease[13-16)].Therefore we tried to investigate the effects of Pi / ATP, pharmacological agents (K_{ATP} channel blockers/opener), and K^+ replacement with meglumin on the de-energized mitochondria.

II. MATERIAL AND METHOD:

1. Cell preparation:

SD (Sprague Dawley) Rat (8-10 week) was anesthetized with 0.1 cc Heparin and 0.9 cc Drug (Ketamine+Rompun, 4:1). The rat chest cavity was opened, the heart was excised and mounted on a Langendoff-type apparatus. Heart was perfused with Tyrode solution (NaCl 133.5; KCl 5.4; HEPES 5; Taurine 20; $MgCl_2$ 3; $CaCl_2$ 0.75; Glucose 5.5; pH adjusted to 7.4 at 37^oC with NAOH, (in mmol/L)) sufficiently to remove all blood from the heart. Subsequently, the heart was perfused Ca^{2+}-free Tyrode solution(NaCl 133.5; KCl 5.4; HEPES 5; Taurine 20; $MgCl_2$ 3; EGTA 0.5 Glucose 5.5; pH adjusted to 7.4 at 37 ^oC with NAOH, (in mmol/L)). And then digested by Tyrode solution (NaCl 133.5 ; KCl 5.4 ; HEPES 5; Taurine 20 ; $MgCl_2$ 3 ; $CaCl_2$ 0.05; Glucose 5.5 ; pH adjusted to 7.4 at 37 ^oC with NAOH, (in mmol/L)) containing enzyme Protease (2mg/100μl), enzyme Liberase (1mg/100μl), bovine serum albumin (83.5 mg/50ml). Cardiac myocytes were dispersed by gentle agitation of the digested heart in a high K^+ solution (K-Glut 75 ; KCl 40 ; HEPES 20 ; Taurine 20 ; EGTA 5 ; KH_2PO_4 20 ; $MgCl_2$ 1 ; Glucose 5.5 , pH adjusted to 7.1 at 37 ^oC with KOH (in mmol/L)). And finally the isolated cells were stored in the cell culture medium (Dulbecco's modified Eagle's medium) at room temperature (24-26^oC). Tetramethylrhodamine-ethyl ester (TMRE) was obtained from Invitrogen. The other chemicals were purchased from Sigma-Aldrich (Seoul, Korea).

2. Measurement of mitochondrial signals:

A microspectrofluorometric multi-parametric measurement system was used to measure NADH (excitation, 363nm; emission, 450 nm), FAD (excitation, 469 nm; emission, 500 nm) and TMRE (excitation, 530 nm; emission, 590 nm).A fast monochromator was used as an excitation light source. Near infrared from microscopic light was applied as a light source to visualize the myocytes during experiments using CCD camera and monitor. Cell area was calculated as pixel number in the captured image (0.178 μm^2/pixel). Four photomultiplier tubes (PMTs) were used to detect emission wavelengths NADH signal intensity was normalized with cell area[17)]

The *Nernst equation* was used to calculate Ψ_m with TMRE distribution. Therefore, TMRE signal was converted to Ψ_m following the equations:

$$\Psi_m\ (\mathrm{mV}) = 2.3026\frac{RT}{zF}log\frac{[TMRE]cyt}{[TMRE]mito}\ (1)$$

$[TMRE]_{mito}$, $[TMRE]_{cyt}$ are the signal of TMRE in mitochondria and cytosol, respectively. To obtain the pure $[TMRE]_{mito}$ or $[TMRE]_{cyt}$, the background signals were corrected. All data were displayed as mean ± S.E. Student t-test was used and significant level was setup at $p<0.05$.

III. RESULTS:

1. Membrane potential of de-energized mitochondria

The Ψ_m of mitochondria in the absence of substrates was tested. Cardiac myocytes were permeabilized by Saponin (0.1mg/ml) in 60s. The Fig.1 showed that, in the presence of 10nM TMRE, the signal intensity clearly increased in the presence of a cell. Without TMRE, there are intrinsic background signals from the cells because of the reflection of the leak light. All background signals were corrected before proceeding the experiment. Assuming TMRE concentration in the bath would be the same as in the cytosol, $[TMRE]_{cyt}$.

$$I_{bath} = k \times \text{field area} \times [TMRE]_{bath} \quad (2)$$

I_{bath} is the signal intensity of the free window with $[TMRE]_{bath}$ and k is a constant. The mitochondrial fraction is about 36%, therefore the mitochondria fraction was that cell size multiply with 0.36. When the cell was moved into the field, all signal changes was caused by the TMRE in mitochondria, the mitochondrial TMRE concentration was calculated as follows:

$$[TMRE]_{mito} = [I_T - I_{bath} / \text{field area} \times (\text{field area} - MA)]/(k \times MA) \quad (3)$$

I_T is a total TMRE signal intensity with the cell. MA is the mitochondria area calculated by cell area multiplying the mitochondrial fraction. in the cell. Based on the equation (1),(2) and (3), we calculated the Ψ_m in a resting condition without substrates was 56 mV ± 5 (n=42). Mitochondrial membrane potential was surprisingly generated even though energy substrates were not supplying.

2. *Effect of Pi/ATP on de-energized mitochondria Ψ_m*

To investigate the possibility of the involvement of mito-K_{ATP} channel, firstly, we tested the effects of Pi + ATP. The permeabilized cardiac myocytes were treated with Pi 1mM and followed by Pi + ATP (1mM). As shown in the Fig.2 & Fig.3 the addition of Pi could hyperpolarize further about 4 ± 4.8 mV (n=19). Interestingly, when we added both Pi and ATP, $\Delta\Psi_m$ was dramatically hyperpolarized about 62 ± 7.7 mV (n=19) and the removal of ATP returned Ψ_m to the initial value. In addition, FAD signals were slightly increased which reflected FADH oxidation, which means FADH is still existed in the mitochondria in the absence of substrate. Interestingly, Pi itself could decrease FAD signal, which means the decrease of FADH consumption**.** NADH signal change was changed but negligible.

Then, we checked the time course of ATP-dependent effect for 2 hours. The results showed that ATP-induced hyperpolarization was not maintained and continuously depolarize (Fig.4)

3. *Effect of K^+ channel blockers / opener on de-energized mitochondria Ψ_m and Pi/ATP induced hyperpolarization*

ATP-sensitive potassium (K_{ATP}) channels are composed of four pore-forming Kir6.2 subunits [18-20]. Binding of ATP to Kir6.2 leads to inhibition of channel activity [18, 21, 22].Since the ATP/ADP ratio determines K_{ATP} channel activity, the K_{ATP} channel is inactivated by

rising ATP and falling ADP [18, 23], not allowing K^+ flow in the matrix, thus may cause the hyperpolarization, we tested pharmacological agents to activate or to inhibit K_{ATP} channel and K^+ replacement. We investigated the effect of the pre-treatment of 4 different pharmacological agents such as K_{ATP} channel blocker (Glibenclamide (Glib.)(10 μM)) (Fig.5A), mK_{ATP} channel blocker (5-HD (100mM)) (Fig.5B), K^+ channel blocker (TEA (10mM)) (Fig.6), and K_{ATP} channel opener (Diazoxide (DZX) (100 μM)) (Fig.7). However, all blocking agents (Glib; 5-HD and TEA) did not show any significant effect on Ψ_m. Meanwhile DZX, K_{ATP} channel opener, could not block the ATP induced hyperpolarization, however, interestingly, itself could depolarize the Ψ_m in basal condition and $\Delta\Psi_m$ was about 11 ± 1 mV and the addition of Pi could return Ψ_m to basal level (Fig.7). It suggested that ion K^+ may participate to the resting membrane potential, therefore we replaced K^+ environment with NMDG. NMDG caused a small initial depolarization and slowed down the speed of ATP induced hyperpolarization and made it transient. However, the change NMDG to K^+ could reverse and showed the full effect of ATP on Ψ_m. (Fig.8)

We also investigated the effect of Glib., 5-HD and DZX on Pi/ATP induced hyperpolarization. The permeabilized cardiac myocytes were treated with Pi 1mM, and then, Pi + ATP (1mM), and followed by Pi+ATP+Glib (10 mM) or 5-HD (100 mM) during 2 hours (Fig.9) or DZX (100 μM) (Fig.10). Nevertheless, there was no significant effect on Pi/ATP induced hyperpolarization in these investigations

4. Effect of ADP or AMP on de-energized mitochondriaΨ_m

ATP might hydrolyze into ADP or AMP. These products could be candidates for ATP-induced hyperpolarization. Permeabilized cardiac myocytes were pre-treated with ADP (1mM) or AMP (1mM) (Fig.11). However, these agents could not make Ψ_m hyperpolarized, also there was no significant change on Ψ_m when treating ADP or AMP (Fig.11).

5. Effect of F_1F_0 ATPase inhibitor (Oligomycin A) on de-energized mitochondriaΨ_m and Pi/ATP induced hyperpolarization

ATP could be used for the substrate for F_1F_0-ATPase activity to pump out H^+ from the mitochondria. We tested Oligomycin A (OligoA) (5μg/ml), F_1F_0-ATPase inhibitor. Interestingly, OligoA, showed the similar effects as DZX, itself depolarizing Ψ_m and $\Delta\Psi_m$ was about 46.5 ± 6 mV and reversing by Pi (Fig.12A). However, ATP induced hyperpolarization was not affected by DZX but fully inhibited by OligoA (Fig.12). In addition, Oligo A could also completely block the increase of FAD induced by ATP. We suggested that the Pi/ATP induced change of Ψ_m might be related to the hydrolysis of ATP to ADP. This process makes proton H^+ pump out through F_1F_0 ATPase complex, might induce Ψ_m hyperpolarization when we treat Pi/ATP on permeabilized cardiac myocytes

I.V.DISCUSSION:

Mitochondrial studies have been carried out under the assumption that the mitochondria are in completely energized state. However, understanding the bioenergetics in de-energized status still has not been clear. Therefore, in this study, we investigated and showed the results related to the mechanism of Ψ_m formation in de-energized state.

The Ψ_m is formed by the transport of proton via ETC. The energy used for this process is the redox potential derived from reducing equivalents such as NADH and $FADH_2$ to oxygen. Therefore, in the Ψ_m formation, NADH and $FADH_2$ are crucial chemicals and should be replenish through reactions of TCA cycle which need the supply of substrates to be maintained. For this reason, if there is no supply of substrates to the mitochondria, the proton pumping via ETC should be stopped and Ψ_m may not be formed. However, this study showed 56 ± 5 mV of Ψ_m without substrates.

The reason why Ψ_m could be formed in the absence of substrates is puzzled. To pursue the mechanism of that, firstly we tried the possibility of the involvement of mito-K_{ATP} channel. We tested the effects of Pi /ATP, pharmacological agents (K_{ATP} channel blockers/opener**),** and K^+ replacement on the de-energized mitochondria. The addition of Pi could hyperpolarize further about 4 ± 4.8 mV in average (Fig2&3). Notably, when we apply both Pi and ATP, $\Delta\Psi_m$ was strongly hyperpolarized about 62 ± 7.7 mV (Fig2&3) and the removal of ATP returned Ψ_m to the initial value. The K_{ATP} channel is inactivated by rising ATP and falling ADP [18, 23] , therefore the replenish of ATP during experiments might leads to the

possibility of blocking K_{ATP} channel, prohibit influx K^+ to the matrix, thus may help the hyperpolarization. For that reason, we investigated pharmacological agents to activate or to inhibit K_{ATP} channel such as Glib., 5-HD, TEA and DZX and K^+ replacement. Nevertheless, the results showed the inhibition of K_{ATP} channel could not have the same hyperpolarization inducing with Pi/ATP effect (Fig.5&6). Meanwhile, DZX, K_{ATP} channel opener, could not block the ATP induced hyperpolarization (Fig.10). It is possible that ATP blocked K_{ATP} channels, and then lead to agents did not have effect on Ψ_m. We need to further investigate about residual ATP. Interestingly, DZX itself could depolarize the Ψ_m in basal condition about 11 ± 1 mV and the addition of Pi could return Ψ_m to basal level (Fig.7). There were some studies which also showed the similar. Isolated mitochondria from rat hearts were measured Ψ_m with tetraphenylphosphonium (TPP^+) and used K^+ channel openers to examine the effect of mitochondrial K_{ATP} channel opening on mitochondrial membrane potential. Measurements were made under continuous stirring of the mitochondrial suspension (1 mg protein/ml incubation medium). The standard incubation medium contained (in mM) 110 KCl, 5 K_2HPO_4, 5 succinate, 5 pyruvate, and 10 MOPS. The study showed that from a Ψ_m of −180 ± 15 mV, K^+ channel openers, pinacidil, cromakalim, and levcromakalim, induced membrane depolarization by 10 ± 7, 25 ± 9, and 24 ± 10 mV, respectively. This effect was abolished by removal of extramitochondrial K^+ or application of a K_{ATP} channel blocker [24]. We did not use aforementioned K^+ channel openers but used DZX to test its effect on Ψ_m. In our study, the resting Ψ_m is -56 ± 5 mV and DZX could induce resting Ψ_m depolarization

about 11 ± 1 mV. Our results showed similar effect with the reference[24]. In addition, on mitochondrial particles from the rat myometrium, by means of Rhodamine-123 fluorescence, the other study showed that activation of K_{ATP} channel in mitochondria caused partial depolarization of the Ψ_m[25]. This effect was completely blocked by glybenclamide[25]. In the presence of valinomycine (a cyclic peptide antibiotic which catalyses K^+ transport across mitochondria) and diazoxide together, depolarization also was detected, but in this case glybenclamide failed to restore mitochondrial potential. Thus, activation of mitoKATP from the rat myometrium causes partial depolarization of the inner membrane [25]. In our investigation, K^+ replacement with NMDG caused a small initial transient depolarization on the basal Ψ_m but slowed down the speed of ATP induced hyperpolarization and made it transient (Fig.8). It suggests that mitoK_{ATP} channel may not or negligibly participate on the formation of the resting Ψ_m of the de-energized mitochondria, and the opening of mitoK_{ATP} channel could depolarize it.

During experiments, ATP might be converted to ADP or AMP. These products could be candidates for ATP-induced hyperpolarization. But the addition of ADP or AMP did not have the significant effect on Ψ_m (Fig.11). Therefore, this possibility could be removed.

F_1F_o-ATPase can use the energy stored in ATP to drive the reverse process via hydrolysis of the nucleotide, i.e., it can induce the reverse rotation of the central stalk [26, 27]and use this to pump protons across the membrane [28-30]. Since ATP supply may activate the reverse mode of F_1F_0-ATPase activity, we investigated OligoA, F_1F_0-ATPase inhibitor. Interestingly,

OligoA showed the similar effects as DZX, depolarizing Ψ_m about 46.5 ± 6 mV and reversing by Pi (Fig.12A). It means there is a possibility that the residual ATP hydrolysis by the reverse mode of F_1-F_0-ATPase might contribute the resting Ψ_m. Studies from Rouslin's group showed that the nonselective F_1F_0-ATPase inhibitor oligomycin reduced the rate of ATP depletion in rats and dogs. A significant amount of ATP was conserved by oligomycin, with some variability in the actual degree of ATP conservation[31, 32]. From the dog heart, in the ischemic tissue that had not been exposed to oligomycin, ATP levels dropped off more or less linearly until ATP depletion was essentially complete at 80 min of autolysis[32]. Pretreatment with oligomycin resulted in a marked slowing of ATP depletion between -5 and 40 min of autolysis; however, after -60 min of autolysis there was an acceleration of ATP depletion in the oligomycin-treated hearts to a rate which paralleled that of the untreated hearts[32]. The reason for this acceleration is not known at this time, but it could be due to the activation of lysosomal hydrolases between 40 and 60 min of tissue autolysis[32]. From rat heart, ATP levels dropped off rapidly and ATP depletion was essentially complete after < 30 mins of autolysis[32]. Moreover, pretreatment of rat hearts with oligomycin resulted in the small but significant slowing of tissue ATP depletion evident only around 20 mins autolysis time point[32]. However, the production of ATP requires an electrochemical gradient, ADP and Pi, thus ATP synthesis activity was not performed in the absence of an electrochemical gradient, resulting from the absence of respiratory chain substrates. Therefore, we suggest ATP produced before permeabilization might be trapped or stored in mitochondria and it

may be used for generation of resting Ψ_m. OligoA may block F_1F_0-ATPase and caused the depolarization.

In addition, our results showed ATP induced hyperpolarization was fully inhibited by OligoA (Fig.12). It suggests that the Pi/ATP induced Ψ_m hyperpolarization might be related to the hydrolysis activity of ATP. On permeabilized cardiac myocytes, the replenish of ATP during experiments enhanced the ATP hydrolysis activity, made proton H^+ pump out through reverse mode of F_1-F_0 ATPase complex and might induce Ψ_m hyperpolarization. Danijel Pravdic et al investigated H^+-pumping activity by using 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence which is quenched when an H^+ gradient forms at membranes. Adding 1mM ATP and 1 μl ACMA on submitochondrial particles (SMPs), the result showed a representative trace of ACMA fluorescence quenching induced by ATP hydrolysis in SMPs. Oligomycin almost completely abolished the ATP-induced quenching[33] suggesting that ATP induced hydrolysis and Oigomycin effect can be work not only energized condition but also in de-energized condition. Moreover, there was a study on pancreatic acinar cells and found that the rate of loss of $\Delta\Psi_m$ was substantially larger with the addition of antimycin and oligomycin in combination, than with the addition of antimycin alone[34]. It suggests that oligomycin is a pharmacological inhibitor of both the forward and reverse mode of F_1F_0-ATP synthase, however in mitochondria with an inhibited ETC, oligomycin might only suppress the reverse mode [34]. Indeed, there could be a functional alteration in the F_1F_0-ATP synthase. The ATP synthase is able to work in two

directions: under normal conditions it harnesses the translocation of protons into the mitochondrial matrix in order to catalyze the synthesis of ATP, but under conditions of low $\Delta\Psi_m$ it acts in reverse, hydrolysis ATP in order to pump protons out of the mitochondria and thereby maintain a sufficiently high $\Delta\Psi_m$ to prevent the mitochondrial permeability transition [35]. That could support to our study when in the de-energized state, mitochondria were under condition of low $\Delta\Psi_m$, therefore it might induce the activation of reverse mode of F_1F_0-ATPase. And the addition of ATP could hyperpolarize the Ψ_m to about 118mV.

On NMDG experiment (Fig.8), the result showed that the change NMDG to K^+ could reverse and showed the full effect of ATP on Ψ_m. Hence the K^+ environment seemed to be important for the activity of F_1F_0-ATPase. When we treat ATP, FAD signal was slightly increased which reflected FADH oxidation (Fig.2), which means FADH is still existed in the mitochondria in the absence of substrate. Moreover, since Pi could decrease FAD signal (Fig.2), FADH may form and consume in the absence of the substrates. The addition of Pi (phosphoric acid group) might generate the NADPH from NADH[36-38]. Thus, in xenobiotics metabolism process, by enzyme flavin-containing monooxygenase (FMO), the FAD is reduced to $FADH_2$ by NADPH[39-41]. Therefore, it might explain why the addition of Pi leaded to the decrease of FAD signal. Subsequently, $FADH_2$ binds oxygen, producing peroxide. In interaction with a substrate (basic amines, sulfides, P-containing compounds…), the flavin peroxide is then transferred, and the FAD is restored via dehydration, releasing

$NADP^+$ [42-45]. It suggests that when we treated ATP, ADP production from ATP hydrolysis activity might be a substrate in the interaction with flavin peroxide and then restored FAD. It was also matched with our results (Fig.10), the inhibition of ATP hydrolysis activity by OligoA also declined FAD signal. In related manner, back to the change of Ψ_m by Pi application, the explanation might be that the reaction of Pi ($H_2PO_4^-$) and NADH may release proton and thus induce the slight hyperpolarization

In summary, from these results, the mechanism of formation of the resting Ψ_m is still not clear. We suggest that mitoK_{ATP} channel may not or negligibly participate on the formation of the resting Ψ_m of the de-energized mitochondria and Pi might inhibit the effect of DZX or OligoA on the resting Ψ_m. Besides, we also hypothesize that the reverse mode of F_1F_0-ATPase by the residual ATP hydrolysis might contribute resting Ψ_m. Additionally ATP-induced hyperpolarization may be induced by the reverse mode of F_1F_0-ATPase. And the K^+ environment seemed to be important for the activity of F_1F_0-ATPase. Pi might affect Ψ_m in more complex way however, the exact mechanisms are still not clear.

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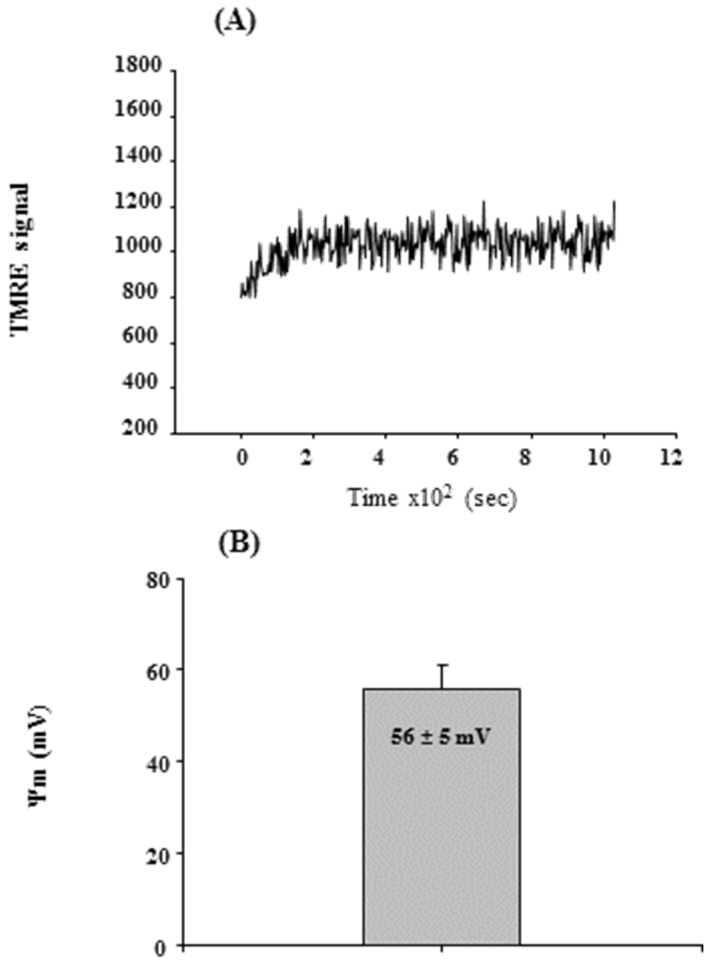

Fig.1 The Ψm of de-energized mitochondria. (A) In the presence of 10 nm TMRE, the signal intensity clearly increased in the presence of a cell. (B) The Ψm was 56 ± 5 mV (n=42)

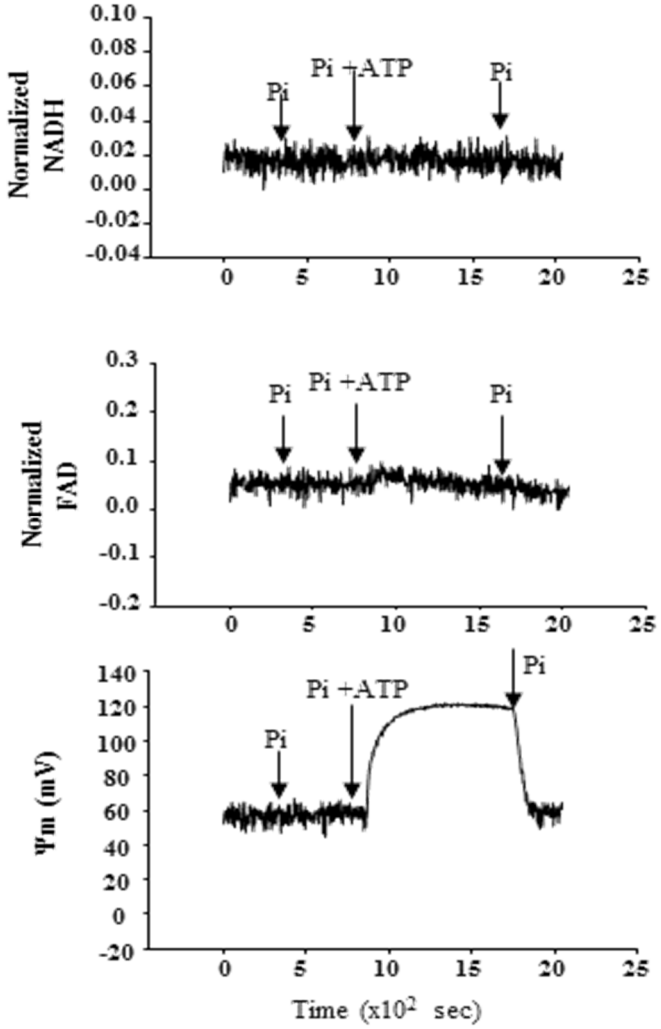

Fig.2 Pi/ATP effect on de-energized mitochondria. Permeabilized cardiac myocytes were treated with Pi 1mM and followed by Pi + ATP(1mM)

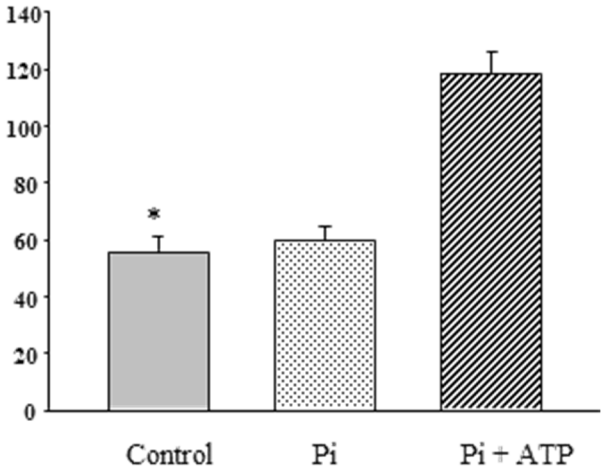

Fig.3 Summary of $\Delta\Psi_m$ induced by Pi /ATP. The Ψ_m at control was 56 ± 5 mV. Pi could hyperpolarize about 4 ± 4.8 mV and Pi/ATP could further hyperpolarize about 62 ± 7.7 mV. * $p< 0.005$ vs Pi.

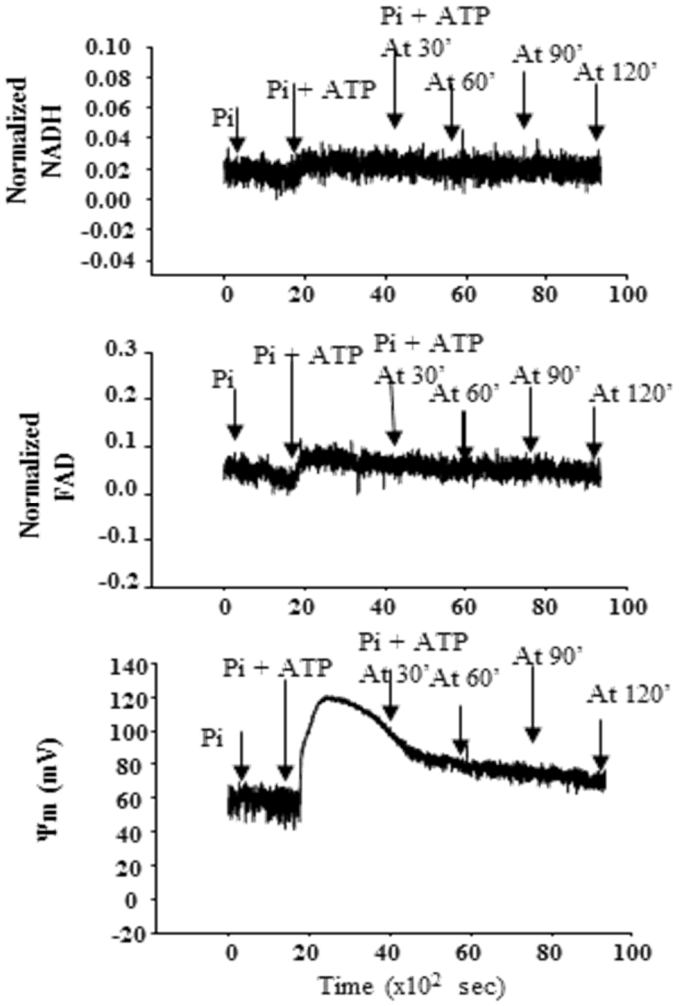

Fig.4 ***Effect of ATP on de-energized mitochondria Ψ_m during 2 hours***. Permeabilized cardiac myocytes were treated with Pi 1mM, followed by Pi + ATP (1mM) during 2 hours. ATP-induced hyperpolarization was not maintained and continuously depolarize

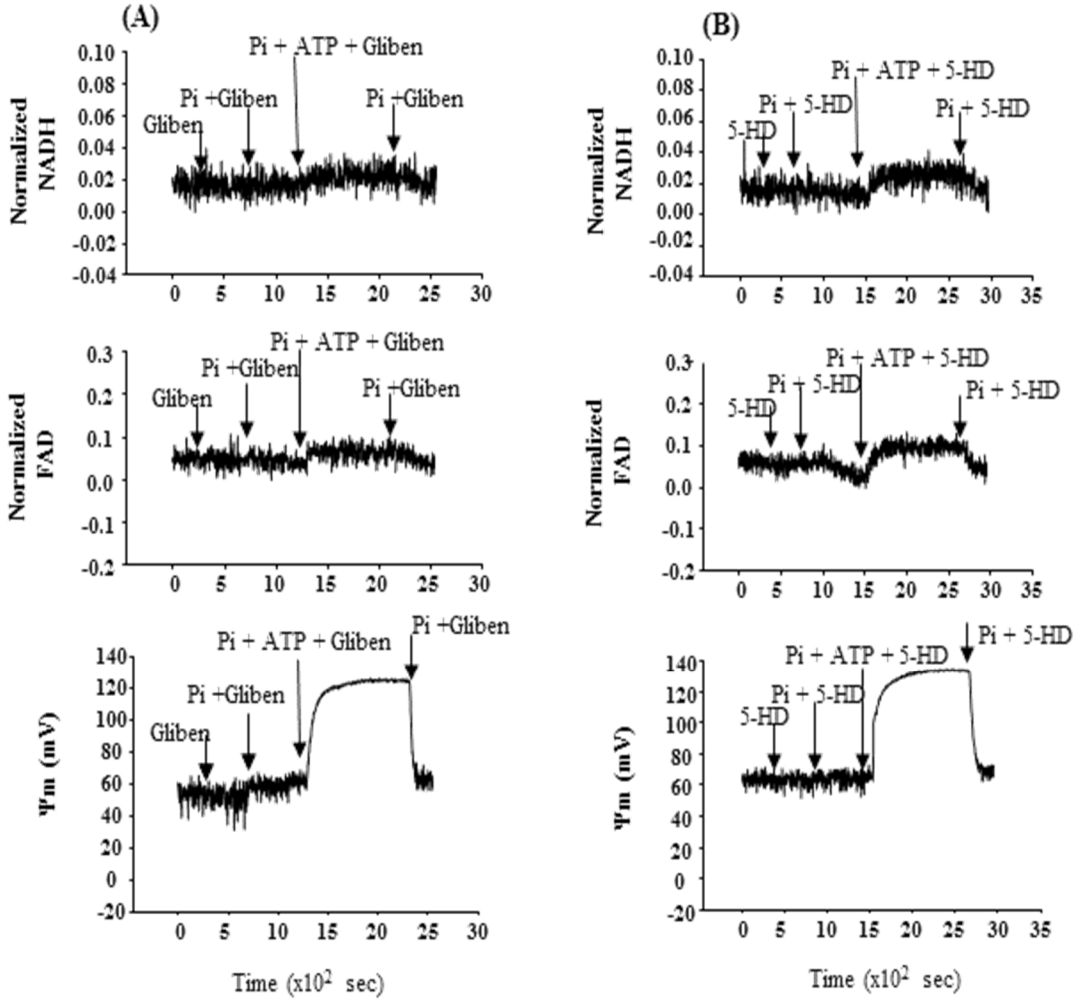

Fig.5 Effect of K_{ATP} channel blockers (Glib. and 5-HD) pre-treatment on de-energized mitochondria Ψ_m. (A)Permeabilized cardiac myocytes were pre-treated with Glib.(10 μM)**.** (B) Permeabilized cardiac myocytes were pre-treated with 5-HD (100 mM***)*** Glib.: K_{ATP} channel blocker;5-HD: mK_{ATP} channel blocker

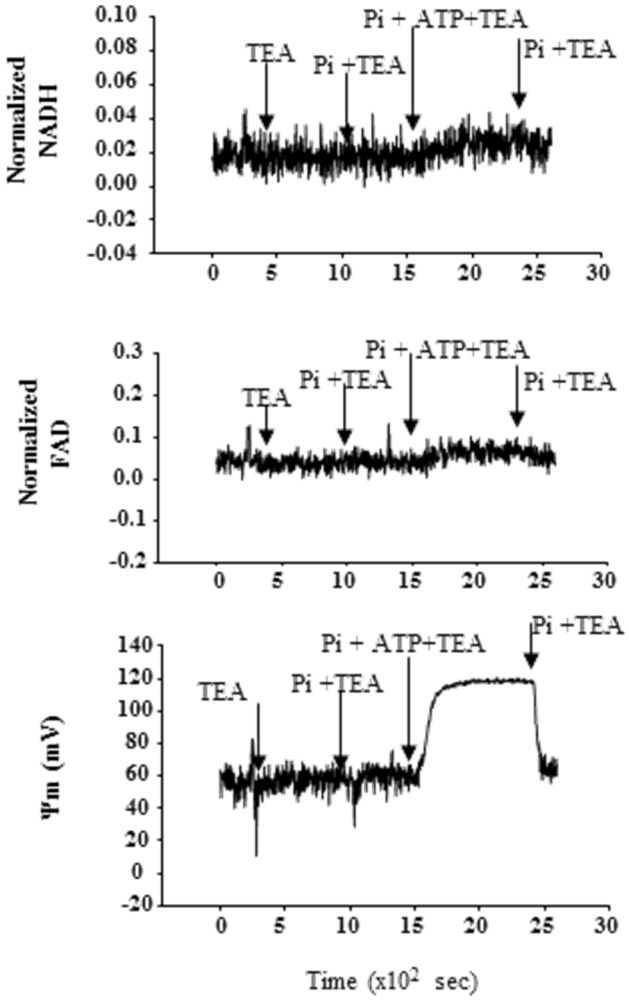

Fig.6 Effect of K^+ channel blocker (TEA) pre-treatment on de-energized mitochondria Ψ_m.

Permeabilized cardiac myocytes were pre-treated with TEA (10 mM).

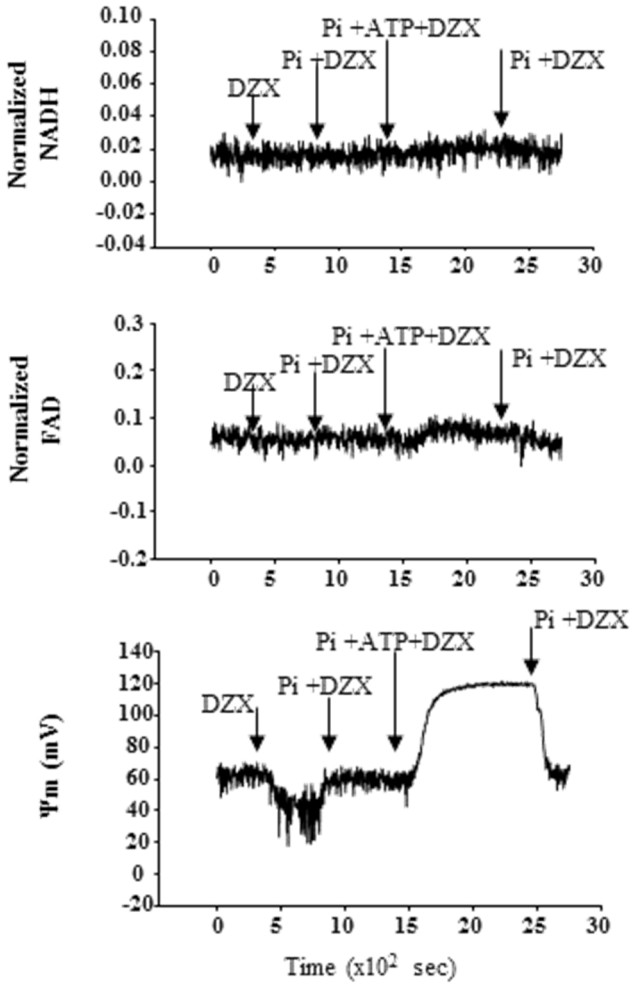

Fig. 7 Effect of K_{ATP} channel opener (DZX) on de-energized mitochondria Ψ_m. Permeabilized cardiac myocytes were pre-treated with DZX (100 μM).

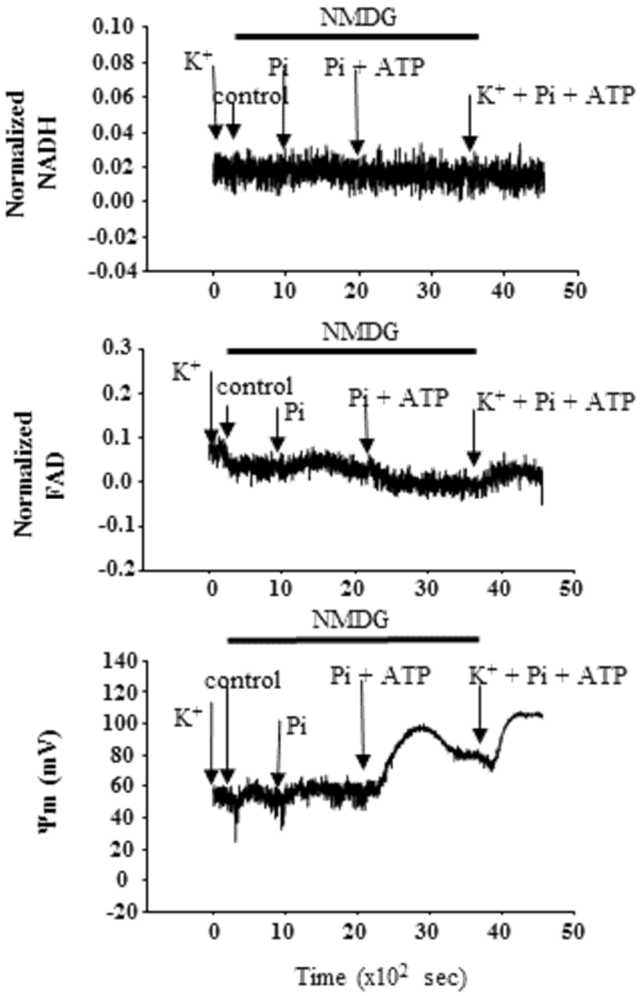

Fig.8 Effect of K^+ environment replacementwith NMDG on de-energized mitochondria Ψ_m.

Permeabilized cardiac myocytes were treated with Pi (1mM), Pi +ATP(1mM) in NMDG environment, then change from NMDG to K^+ environment at Pi +ATP condition

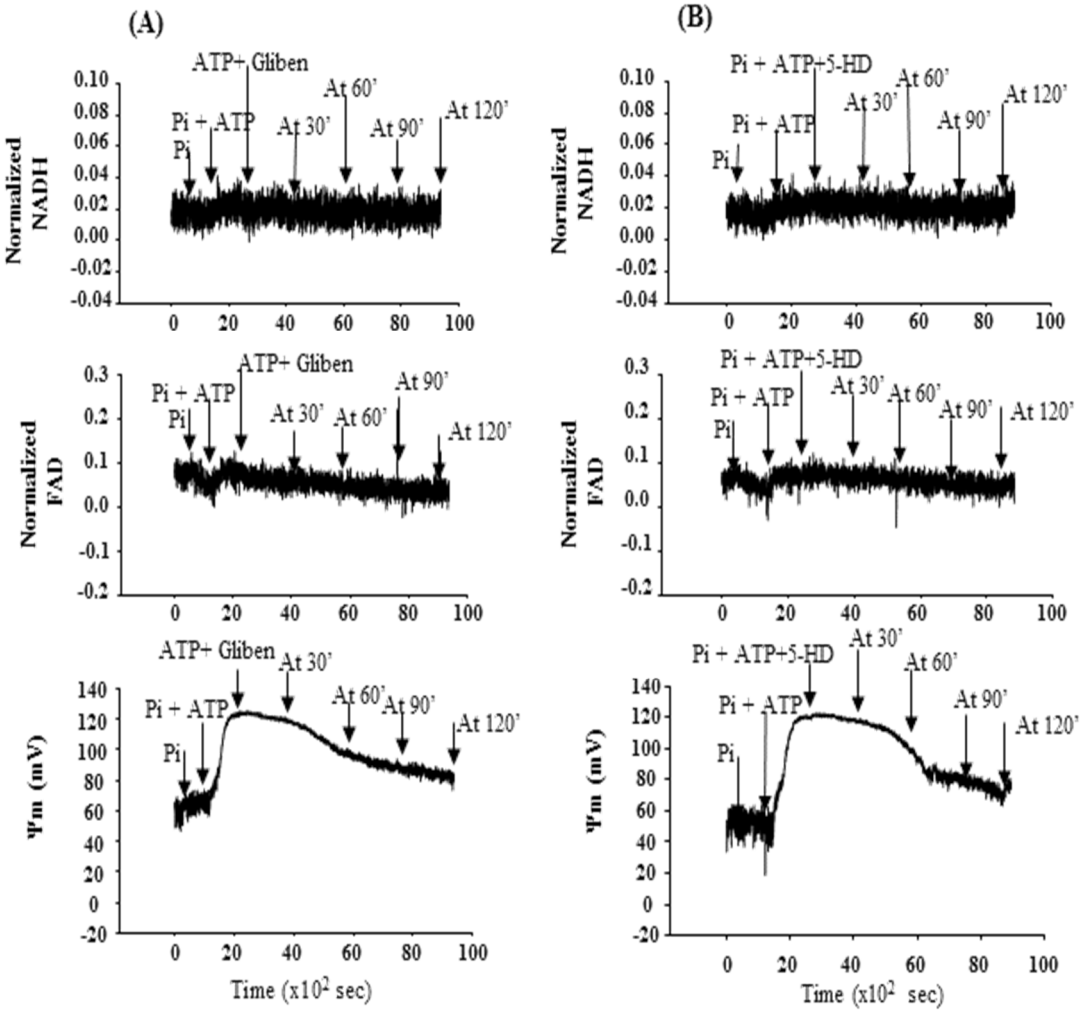

Fig.9Effect of K^+ channel blockers onPi/ATP induced hyperpolarization. Permeabilized ventricular myocytes were treated with Pi 1mM, Pi + ATP (1mM)

and followed by Pi+ATP+Glib (10 mM) **(A)** or 5-HD (100 mM) **(B)** during 2 hours

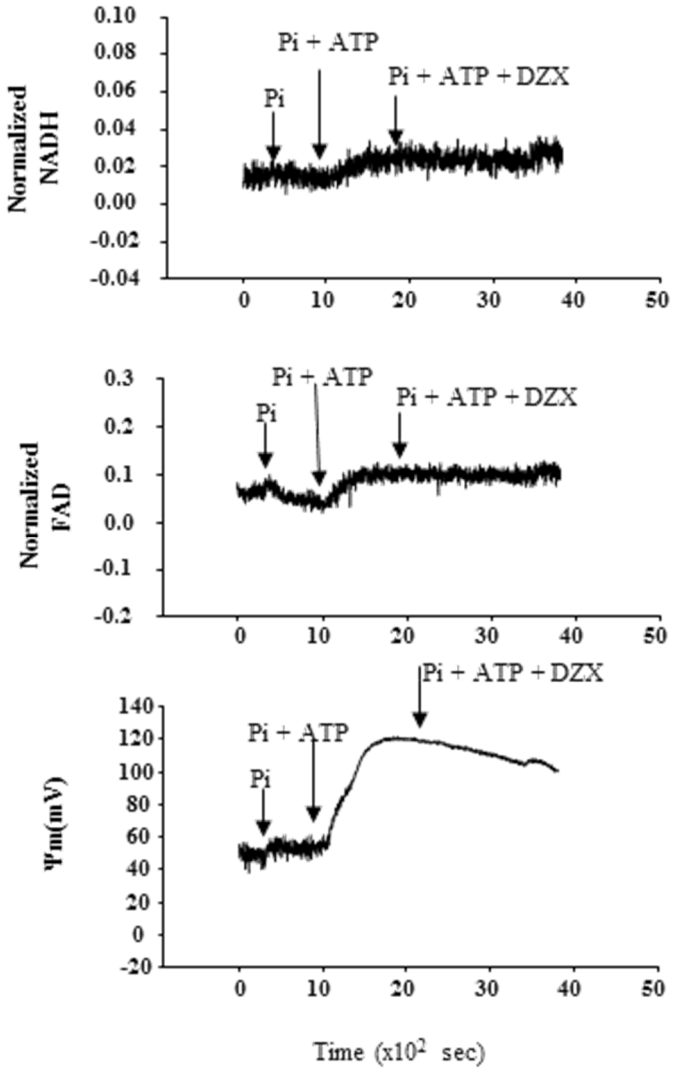

Fig.10 Effect of K^+ channel opener onPi/ATP induced hyperpolarization. Permeabilized ventricular myocytes were treated with Pi 1mM, Pi + ATP (1mM) and followed by Pi+ATP+DZX (100μM)

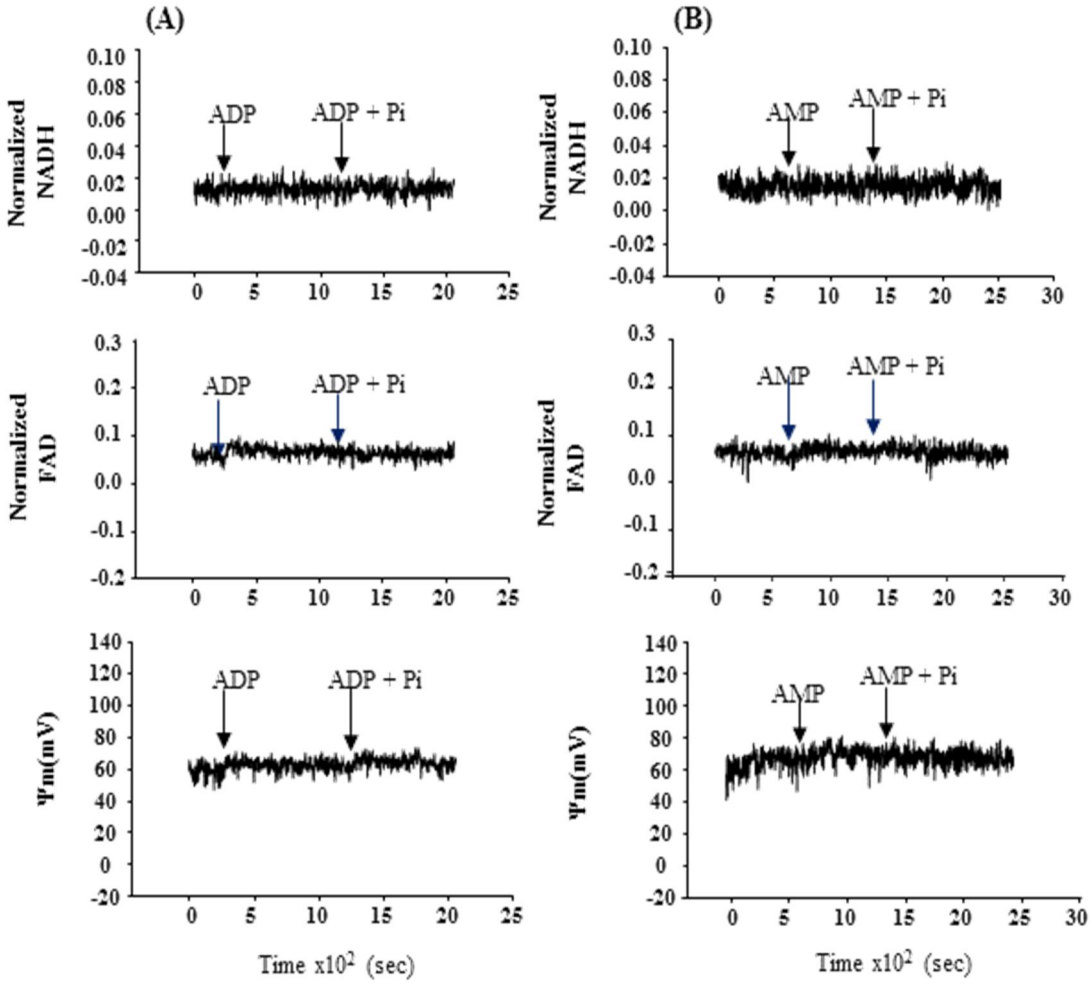

Fig.11 Effect of ADP and AMP on de-energized mitochondria. (A)Permeabilized cardiac myocytes were pre-treated with ADP (1mM). (B)Permeabilized cardiac myocytes were pre-treated with AMP (1mM)

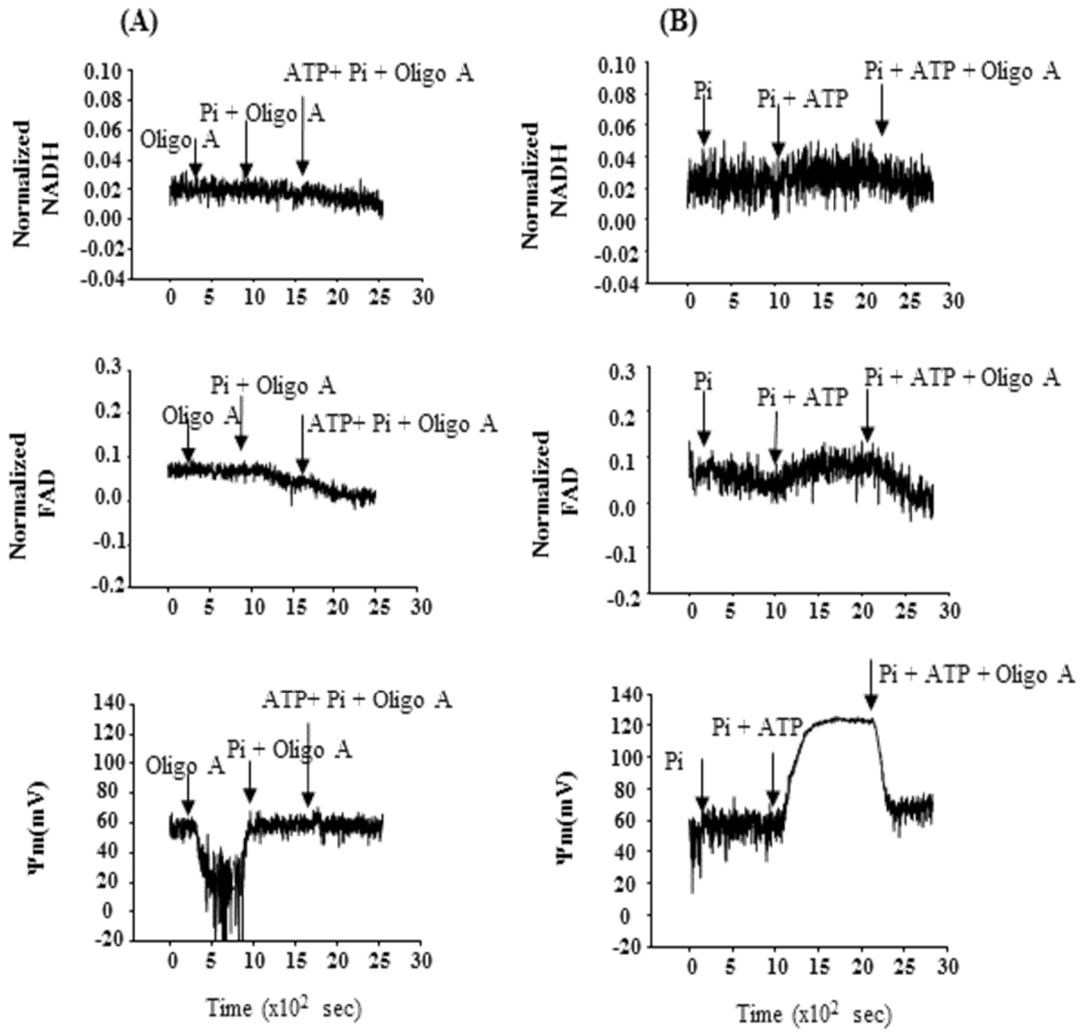

Fig.12 Effect of F_1F_0 ATPase inhibitor (Oligomycin A) on de-energized mitochondria and Pi/ATP induced hyperpolarization.(A) Permeabilized cardiac myocytes were pre-treated with OligoA(5μg/ml). (B)Permeabilized cardiac myocytes were treated with Pi (1mM), Pi+ATP(1mM)and follow by Pi+ATP+OligoA

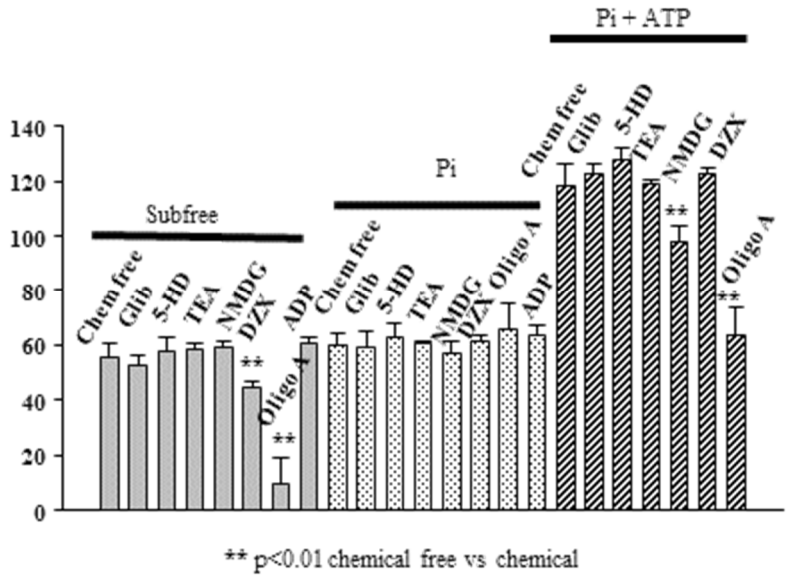

Fig.13 Summary of the effect of pharmacological agents on de-energized mitochondria.

** p<0.01 chemical free vs chemical

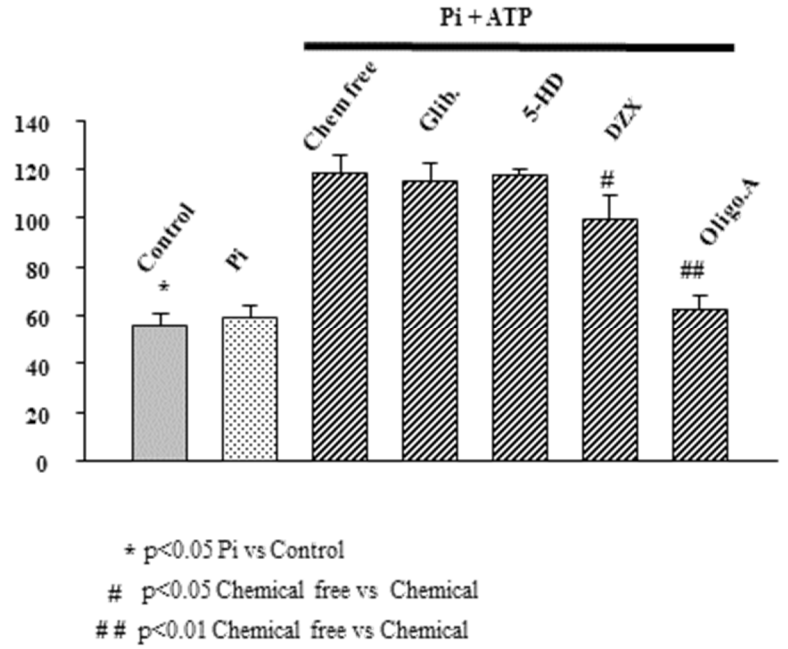

Fig.14Summary of the effect of pharmacological agents on Pi/ATP induced hyperpolarization.*p<0.05 Pi vs control; #p<0.05 chemical free vs chemical; ##p<0.01chemical free vs chemical.