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THESIS APPROVAL

The abstract and thesis of Venkat Raghav Aachi for the Master of Science in Biology were presented March 13, 2009, and accepted by the thesis committee and the department.

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ABSTRACT

An abstract of the thesis of Venkat Raghav Aachi for the Master of Science in Biology presented March 13, 2009.

Title: Preliminary characterization of Mitochondrial ATP-sensitive potassium channel (MitoKATP) activity in mouse heart mitochondria.

Myocardial ischemia, infarction, heart failure and arrhythmias are the manifestations of coronary artery disease. Reduction of ischemic damage is a major concern of cardiovascular biology research. As per recent studies, the mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}) opening is believed to play a key role in the physiology of cardioprotection, protection against ischemia-reperfusion injury or apoptosis. However, the structural information of mitoK_{ATP} is not precisely known. Elucidating the structural integrity and functioning of the mitoK_{ATP} is therefore a major goal of cardiovascular biology research. The known structure and function of the cell ATP-sensitive potassium channel (cellK_{ATP}) is functional in interpreting the structural and functional properties of mitoK_{ATP}.

The primary goal of my research was to characterize the activity of mitoK_{ATP} in the isolated mitochondria from the control mouse heart. The mitoK_{ATP} activity, if preliminarily characterized in the control strains through the light scattering technique, then the structure of the channel could possibly be established and analyzed by means

of the transgenic model and with the help of immunological techniques such as western blotting and immunoflorescence.

With this experimental model it was possible to demonstrate that the mitoK_{ATP} activity in control mouse heart mitochondria is activated by potassium channel openers (KCOs) such as diazoxide and cromakalim and activators of mitoK_{ATP} such as PMA (phorbol 12 myristate-13-acetate), and inhibited by K_{ATP} inhibitors such as glibenclamide and 5-hydroxydecanoate (5 HD).

It was evident that the mitoK_{ATP} activity in mouse heart mitochondria was comparable to that exhibited by the rat heart mitochondria. The various selective and non-selective activators and inhibitors of the channel elicited their activity at a similar concentration used for the rat heart mitochondria. The results were reproducible in five independent experiments for each combination, further reinforcing the significance of existing channel activity in the mouse heart mitochondria.

PRELIMINARY CHARACTERIZATION OF MITOCHONDRIAL ATP-SENSITIVE POTASSIUM CHANNEL (MITOKATP) ACTIVITY IN MOUSE HEART MITOCHONDRIA

by

VENKAT RAGHAV AACHI

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGY

Portland State University
2009

DEDICATION

My work is dedicated to my parents, my brother and to all my friends

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I would like to thank my advisor, Dr. Keith D. Garlid, for being truly supportive of my work throughout my tenure as a graduate student. I am thoroughly indebted to Dr. Garlid for having introduced me to the world of clinical research, which I am sure, has helped me grow as a clinician in specific, and a person in general.

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Finally, I would want to thank my family and friends, without whom this journey would not have been this worthwhile.

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ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
APD	Action potential duration
ATP_i	Intracellular ATP
BLM	bilayer lipid membrane
BSA	bovine serum albumin
$CellK_{ATP}$	plasma membrane K_{ATP} channel
CoA	coenzyme A
CRM	cromakalim
DAG	diacylglycerol
DEAE-cellulose	diethylaminoethyl-cellulose
DZX	diazoxide
DMSO	Dimethyl Sulfoxide
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
eNOS	endothelial nitric oxide synthase
FA	fatty acid
$FADH_2$	reduced flavin adenine dinucleotide
FL-glyburide	BODIPY FL glibenclamide
GDP	guanosine diphosphate

GTP	guanosine triphosphate
GLI	glibenclamide
5-HD	5-hydroxydecanoate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IP_3	inositol 1, 3, 4 triphosphate
KCO	potassium channel opener
Kir	inward rectifying potassium channel
$MitoK_{ATP}$	mitochondrial K_{ATP} channel
MPT	mitochondrial permeability transition
NADH	α-nicotinamide-adenine dinucleotide
NBF	nucleotide binding fold
PAGE	polyacrylamide gel electrophoresis
PKC	protein kinase C
PMA	phorbol 12 myristate-13-acetate
PKG	protein kinase G
Pi	phosphate
PLC	phospholipase C
PIP_2	phosphatidyl inositol 4, 5 bisphosphate
SDS	sodium dodecyl sulfate
SUR	sulfonylurea receptor
TEA	tetraethylammonium cation
TPP^+	tetraphenylphosphonium ion

W_A	Walker A motif
W_B	Walker B motif

CHAPTER 1

INTRODUCTION

Cardiovascular disease can manifest as acute myocardial infarction, myocardial ischemia or arrhythmias [72, 75, 84]. Myocardial ischemia, as defined, is a reduction in the blood supply to the heart muscle owing to the constriction or obstruction of the coronary arteries. The lack of blood supply to the heart leads to a reduction in the oxygen supply to the contractile heart muscle and eventual accumulation of toxic metabolites, leading to a permanent damage to the heart tissue. The irreversible necrosis of the heart tissue owing to the compromised blood supply manifests as infarction. The infarcted heart tissue loses the power to regenerate and subsequently ends up with a compromised ventricular function. [72, 75, 84]

Reducing the morbidity and improving the clinical outcome in patients with coronary artery disease is a major concern of cardiovascular biology research. The mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}) is thought to have a significant effect in the physiology of ischemic signaling pathway, protecting the myocardium against the ischemia-reperfusion injury (considering the reduction in the size of infarct when subjected to mitoK_{ATP} specific potassium channel openers during ischemic preconditioning; an effect reversed in the presence of potassium channel blockers) [46, 47]. It is therefore important to address questions regarding the

structural and functional properties of mitoK$_{ATP}$ to further delineate its role in health and disease.

1.1 Rationale for mouse as the study model:

Mice are probably the only mammalian species that are subjected to routine genetic manipulations. Also, the mouse genome is one of the most extensively studied genomes. The structural subunit assembly of mitoK$_{ATP}$ is quite unclear. It is still a matter of ambiguity what channel subunits constitute the mitoK$_{ATP}$.

Through my study I intended to preliminarily characterize the mitoK$_{ATP}$ activity in control mouse heart mitochondria. I believe that the mitoK$_{ATP}$ activity if successfully characterized in the control mouse strains would eventually facilitate in understanding the structural constitution and assembly of the channel subunits through the study model of transgenic mice and various immunological techniques. Also, I intended to test the hypothesis that the mitoK$_{ATP}$ activity exhibited in control mouse heart mitochondria is similar and comparable to that exhibited in rat heart mitochondria.

The structure of mitoK$_{ATP}$ could be understood in much detail by pursuing the model of genetically modified mice (transgenic mice) with their cellK$_{ATP}$ channel subunits over-expressed or knocked-out. Performing light scattering experiments on the isolated heart mitochondria from the transgenic mouse model, and by subjecting the isolated mitochondria to various selective and non selective KCOs and blockers,

would eventually facilitate in characterization of the mitoK_{ATP} activity in these study models. The structural subunits constituting the mitoK_{ATP} could be analyzed through various immunological techniques such as western blotting and immunoflorescence.

CHAPTER 2

ATP- SENSITIVE POTASSIUM CHANNELS (K_{ATP})

There are two types of K_{ATP} channels constituting the cardiac myocyte. These include, the cellK_{ATP} channel / sarcolemmalK_{ATP} channel in the plasma membrane, and the mitochondrial K_{ATP} (mitoK_{ATP}) in the inner membrane of the mitochondria [1, 44, 77, 82].

2.1 Plasma Membrane / Sarcolemmal K_{ATP} Channels (CellK_{ATP})

The potassium channels form the prime component of plasma membrane of cells of various tissues [1-6]. The potassium channels function in setting up a resting membrane potential, regulating the electrical activity and ion transport across the membrane. The ATP-sensitive potassium channels (K_{ATP}) were first identified in the plasma membrane of cardiac myocyte by Noma through his experiments using single channel patch clamp techniques [1]. CellK_{ATP} are expressed in diverse tissues of the body that include pancreatic β cells, skeletal muscle, smooth muscle and neurons of the brain parenchyma, in addition to the cardiac cells. [2, 3, 4, 5, 6]

The cell ATP-sensitive potassium channels are involved in a variety of regulatory functions that include shortening of the action potential duration in cardiac myofibrils, loss of K^+ ions during functional inhibition in heart, regulation of the skeletal muscle

excitation, pancreatic β-cell release of insulin, relaxation of smooth muscle and the release of various neurotransmitters from the neurons of brain parenchyma [7, 8, 9].

2.2 Physiological roles of CellK_{ATP}

The opening of the cellK_{ATP} leads to hyperpolarization of the cell plasma membrane. Hyperpolarization of the pancreatic β-cells inhibits glucose-stimulated insulin secretion by preventing the increase in intracellular Ca^{+2}. Depolarizing the plasma membrane of the pancreatic β-cells leads to opening of voltage gated Ca^{+2} channels, elevation of intracellular Ca^{+2} and eventual exocytosis of insulin granules [10]. In the myocardium, excess efflux of K^{+} leads to a reduced influx of Ca^{+2} by decreasing the action potential duration. This reduced intracellular Ca^{+2} results in a reduction in the contractility of the heart and possible rhythm disturbances during ischemia [11, 12]. The cellK_{ATP} opening in the arteriolar smooth muscles results in a reduction in blood pressure, primarily mediated through a fall in the arteriolar resistance [13].

2.3 Pharmacological directive of cellK_{ATP}

Intracellular ATP (ATP_i) and ADP primarily regulate the activity of the cellK_{ATP} [4]. ATP_i is functional in closing the channel and maintains the channel activity in the presence of Mg^{+2} [14-17]. Under normal physiological concentrations of ATP_i, the probability of cellK_{ATP} to be in an open state is minimal. The ATP_i is viewed to be bound to the cellK_{ATP}, for the channel to remain in the closed state. Hydrolysis of ATP

in the presence of Mg^{+2} emphasizes the pivotal role of protein phosphorylation in maintaining the channel activity [7].

The ATP inhibition of K_{ATP} channels is reversed by a class of drugs called potassium channel openers (KCOs) that include cromakalim, diazoxide, minoxidil, pinacidil and nicorandil. The properties of K_{ATP} channels vary among tissues. For example pinacidil activates cardiac cellK_{ATP} but diazoxide does not; the pancreatic β-cellK_{ATP} activity is weak in the presence of pinacidil, but shows significant activity with diazoxide. Both diazoxide and pinacidil effectively activate smooth muscle cellK_{ATP} resulting in vasodilatation [7, 21, 22]. The differential activities exhibited by cellK_{ATP} lead to the assertion that there are receptor subtypes among cellK_{ATP}. [21, 22]

Glyburide and glibenclamide, which belong to the sulfonylurea class of anti-diabetic drugs, have also been found to block the activity of K_{ATP}. Inhibition of K_{ATP} channel in the pancreatic β-cells leads to a series of events that eventually result in the release of insulin, and hence sometimes referred to as insulin secretagogues considering their pro-insulin action [3, 24, 25]. Their action on the cardiac and smooth muscle cells requires higher concentrations than in the pancreatic β-cells [23]. Glyburide and glibenclamide are not really indicated in patients susceptible to ischemia, as they have been found to reverse the cardioprotective effects of the KCOs and result in rhythm disturbances [13, 19]. The inhibition of K_{ATP} by ATP is independent of phosphorylation and the sensitivity of K_{ATP} channel to ATP is reduced in the presence

of ADP [70]. Glyburide and Glibenclamide are classified as non-specific K_{ATP} channel antagonists, considering their inhibitory action on the cellK_{ATP} and the mitoK_{ATP}. (Table1)

2.4 Structure of cellK_{ATP}

The complex of two different protein subunits namely, the inward-rectifying potassium channels (Kir) and the sulfonylurea receptor (SUR), form the cellK_{ATP} [28]. The Kir can exist either as Kir6.1 or Kir6.2 [18, 27] and the SUR can exist as SUR1, SUR2A or SUR2B [29-31]. The regulatory SUR belongs to the ATP-binding cassette transporter family (ABC) [32, 33, 72, 77]. The Kir subunit acts as a potassium conducting pore. The SUR subunit primarily regulates cellK_{ATP} activity [29, 30, 72, 77].

The complementarity-determining DNA (cDNA) that encoded for Kir6.1 was initially isolated from the pancreatic islet cDNA library of the rat [25]. The composition of rat Kir6.1 primarily included two transmembrane domains with a 424-amino acid residue protein (M_r = 47,960). The Kir6.2 fragment was isolated by means of the Kir6.1 as the probe for screening the human genomic library [34]. The Kir6.1 was almost 70% identical to the Kir6.2 as revealed by the amino acid sequences [34, 35]. The truncated form of Kir6.2 (with the last 26 or 36 amino acids deleted), when expressed in the absence of SUR, is found to be insensate to sulfonylureas, diazoxide, cromakalim and Mg-ADP [18, 36].

SUR1 was recognized by means of radiolabeled analogs of glyburide. They were isolated in pancreatic β-cells as a 140 kD protein on SDS-Polyacrylamide gel electrophoresis [26, 37]. Molecular cloning of SUR1 exposed that it encoded proteins of 1582 amino acids with 13 transmembrane domains and two nucleotide-binding folds (NBF) [31].

The cellK_{ATP} from the pancreatic β-cells co-express SUR1 and Kir6.2 [34]. Mutations in NBF1 and NBF2 are viewed as functional in the pathophysiology of familial hyperinsulinemic hypoglycemia of infancy [38, 39]. Walker A (W_A) motifs of NBF1 and NBF2 are thought to be prospective sites of Mg-ADP activation of cellK_{ATP} [40]. The activation of K_{ATP} channels by diazoxide required the W_A motifs of NBF1 and not NBF2 [40].

The diverse isoforms of SUR, when co-expressed in various combinations with Kir subunits, demonstrates different sensitivities [29, 36]. For example the SUR2A / Kir6.2 complex regulates the activity of cellK_{ATP} in cardiac and skeletal muscle. This combination is more sensitive to pinacidil than to diazoxide [29]. The SUR2B / Kir6.2 complex regulates the cellK_{ATP} activity of smooth muscle and is sensitive to both pinacidil and diazoxide [30]. Thus, the sensitivity of cellK_{ATP} to sulfonylureas, diazoxide and cromakalim requires the presence of SUR and the activation of the cellK_{ATP} by Mg-ADP also requires the presence of SUR [36].

The verity that SUR1 contains two NBF whereas Kir6.2 has none, favors the assumption that inhibition of the channel by ATP requires binding of ATP to SUR [29, 31]. The sensitivity of truncated Kir6.2 to ATP is enhanced by SUR1 as is evident from the shifting of K_i from approximately 100 μM to 10 μM [36].

As per recent studies it has been demonstrated that the Kir and the SUR subunits are associated with 1:1 stoichiometry [41, 42, 43, 77]. The data also indicate that the channel pore of cellK_{ATP} is lined by four Kir subunits and four SUR regulatory subunits surrounding it, eventually functioning as a hetero-octameric structure [72, 77].

The mitoK_{ATP} is thought to be qualitatively similar to the cellK_{ATP}, comprising the potassium conducting pore forming Kir subunit and the regulatory SUR subunit surrounding it. [51, 65, 90] [Fig 2]

2.5 Mitochondrial K_{ATP} Channels (MitoK_{ATP})

Inoue et al., in 1991, through their patch clamp studies on fused giant mitoplasts (mitochondria stripped of their outer membrane) from rat liver mitochondria, first reported the existence of ATP sensitive K^+ channels in the inner membrane of the mitochondria [44]. The principal properties of mitoK_{ATP} include its improved sensitivity to K^+ ions in comparison to Na^+ and TEA^+, high affinity inhibition with ATP and long chain acyl-CoA esters [44, 45], KCOs activation of the ATP inhibited

channels [46, 47], and inhibition of the channel by glyburide, glibenclamide and 5-hydroxydecanoate (5HD) [45, 47, 48]. All these physiological and pharmacological actions exhibited by mitoK_{ATP} are thus similar to cellK_{ATP}, although with a much lower conductance (10 pS in 100 mmol/L cytosolic K+ and 33 mmol/L matrix K^+) [44].

2.6 Regulation of matrix volume by MitoK_{ATP}

The opening of mitoK_{ATP} has been proposed to cause an increase in steady state matrix volume, respiratory stimulation (uncoupling), and alkalization of the mitochondrial matrix [72, 77]. The electrophoretic K^+ uptake across the inner mitochondrial membrane is mediated through the process of diffusion and mitoK_{ATP}, as demonstrated by the mitochondrial potassium cycle [Fig. 1]. This influx of K^+ under steady state is balanced by the electrophoretic H^+ efflux through the electron transport chain. The mitochondrial matrix volume is determined by the net flux of K^+ across the inner membrane along with the flux of anions like phosphate and osmotically regulated water [49, 70, 72, 77]. Opening of the mitoK_{ATP} leads to the influx of K^+ across the inner mitochondrial membrane and an eventual swelling of the mitochondrial matrix, with a minimal effect on the matrix K^+ concentration. This net influx of K^+ activates the electroneutral K^+ / H^+ antiporter that provide a compensatory K^+ efflux so as to maintain a new steady state for a higher matrix volume [49-51]. The proton motive force derived from the electron transport chain provides the required energy to cause the movement of ions across the membrane. [Fig 1]

2.7 Oxidative phosphorylation

The electrons carried by NADH and $FADH_2$ get combined with molecular oxygen through the electron transport chain. Peter Mitchell proposed the chemiosmotic hypothesis in order to understand the synthesis of ATP through the coupling of substrate oxidation [52]. The energy required to pump the H^+ across the inner mitochondrial membrane from the matrix to the intermembrane space, is derived from the movement of electrons from the hydrogens on NADH and $FADH_2$, as they pass along the respiratory chain. The synthesis of ATP by ATP synthase is mediated through the electrochemical gradient created by the movement of electrons. Oxidative phosphorylation is eventually concluded by the catalytic translation of ADP and Pi to ATP [52].

2.8 Myocardial Ischemia and cardioprotection

Myocardial ischemia is a phenomenon of reduced blood supply to heart muscle due to the constriction or obstruction of the coronary arteries, resulting in an imbalance between the supply and demand for coronary arterial blood supply. As a result of this imbalance there is an accumulation of potentially toxic metabolites such as protons, carbon dioxide and lactic acid. Also there is a dearth of oxygen, substrates and energy in the tissues. The energy for myocardial contractility is derived from mitochondrial oxidative phosphorylation which subsequently influences the myosin ATPase activity.

The functions of the mitochondria are thus of prime importance in the molecular events that lead to compromised myocardial function. [75]

Subjecting the myocardium to repetitive alternating periods of ischemia followed by reperfusion, eventually followed by a prolonged ischemia-reperfusion, reflects ischemic-preconditioning (IPC) [75, 82]. Following ischemia, there is ATP depletion and subsequent reperfusion washes out the ischemic metabolites [53]. KCOs when used during ischemic preconditioning are cardioprotective, as evidenced by the size of the heart muscle infarct [20, 54, 75, 82]. This protective effect of KCOs is blocked by glyburide, glibenclamide and 5-hydroxydecanoate (5HD) [19, 20, 55, 75, 82].

KCOs through their action on the cardiac cellK_{ATP} are considered to cause a shortening of the action potential duration (APD) and hence protect the ischemic myocardium [56]. However, a lack of correlation between monophasic APD and cardioprotection by KCOs has resulted in contradicting this hypothesis, thus suggesting that the site of action of KCOs in cardiac muscle cells is discrete from cellK_{ATP} [57, 58]. This discrete site of action of cardioprotective effects of various KCOs is believed to be the mitoK_{ATP}. [65, 72]

2.9 MitoK_{ATP} as a receptor for KCOs and their inhibitors

MitoK_{ATP}, a central intracellular pharmacological receptor is inhibited by ATP, and this inhibited mitoK_{ATP} is potentially activated by KCOs [46, 47, 48, 59, 60]. Cromakalim and Diazoxide, the prime activators of mitoK_{ATP}, act in the low

micromolar range [46]. The distinct nature of the two cardiac K_{ATP} channels i.e., cellK_{ATP} and mitoK_{ATP} could probably be established through diazoxide which is more potent in activating the cardiac mitoK_{ATP} (K ½, 0.4 μmol/L), roughly 2000 times more potent than for activating cardiac cellK_{ATP} (855 μmol/L) [46]. Glyburide, glibenclamide and 5HD, when studied in intact mitochondria, have been shown to act as inhibitors of mitoK_{ATP} in the presence of ATP, $Mg^{+2}$and a pharmacological opener such as diazoxide, cromakalim, or a physiological opener such as GTP [48].

Cardiac mitoK_{ATP}, but not cardiac cellK_{ATP}, is susceptible to the action of 5 HD and diazoxide and the cardioprotective effects of diazoxide are blocked by 5 HD [47]. It was eventually hypothesized by Garlid et al. that the phenomena of cardioprotection by KCOs in cardiac muscle cells was mediated through mitoK_{ATP} [47]. Liu et al. through their experiments on intact ventricular myocytes, showed that diazoxide was mitoK_{ATP} specific and not cellK_{ATP} specific [59]. Protein kinase C (PKC) mediated activation of mitoK_{ATP} is a key element in the phenomenon of ischemic-preconditioning (IPC), as emphasized by Sato et al. [60-64].

It was recommended by Inoue et al. [44] and Halestrap [83] that the regulatory sites of the mitoK_{ATP} nucleotides faced the mitochondrial matrix. Subsequently Garlid et al. through their studies on mitoK_{ATP} reconstituted into proteoliposomes and lipid bilayer membranes (BLM), concluded that the mitoK_{ATP} was unidirectional with respect to

nucleotide access and that the nucleotide binding sites of mitoK$_{ATP}$ faced the cytosol [70].

2.10 Sulfonylurea receptor of mitoK$_{ATP}$:

Garlid et al. viewed that the mitoK$_{ATP}$ comprised an inward-rectifying K^+ channel (mitoKir) and a regulatory sulfonylurea receptor (mitoSUR), as was thought of in cellK$_{ATP}$ [51, 65, 90]. Paucek et al. through their studies using BODIPY-FL glyburide to photolabel inner mitochondrial membrane vesicles identified a protein that was precise and migrated at 63kD on SDS-Polyacrylamide gel electrophoresis [66, 67]. The fraction containing the 63kD protein (identified on partial purification on DEAE-cellulose medium), when reconstituted in lipid vesicles, exhibited mitoK$_{ATP}$ properties. It was henceforth hypothesized to be the regulatory sulfonylurea receptor of mitoK$_{ATP}$ [67].

Szewczyk et al. through photoaffinity labeling of sub-mitochondrial particles with [I^{125}]-glibenclamide, recognized a 28kD inner mitochondrial membrane protein. This identified membrane protein demonstrated the activity of low-affinity sulfonylurea-binding protein with an apparent K_D of 360 nM and B_{MAX} of 48 pmol per mg of inner membrane protein [68].

2.11 Kir subunit of mitoK$_{ATP}$.

Mironova et al., on reconstituting an ATP-dependent K^+ channel in the lipid bilayer using rat liver mitochondria, identified an activity with 55kD protein, that was inhibited with low affinity by ATP (K1/2 approximately 550 μM). They eventually suggested it to be the Kir subunit [69, 90]. MitoK$_{ATP}$ activity was witnessed in liposomes that were reconstituted with partially purified fraction using rat liver and beef heart mitochondria by Paucek et al. [45]. The identified protein contained a major protein band at 54kD [45], with a saturating conductance of 30 pS (1 mol/L KCl). Further studies are awaited that can help identify and sequence the alleged Kir subunit of mitoK$_{ATP}$.

The mitoK$_{ATP}$ activity characterized in the control mouse model would eventually facilitate in understanding further the subunit assembly of mitoK$_{ATP}$ through the medium of genetically modified/transgenic mice and various immunological techniques.

CHAPTER 3

MATERIALS AND METHODS

3.1 Mitochondrial isolation:

Inbred strain mice of type C57BL/6J from the Jackson lab were used for all the experiments. Three mice were used for each experiment in order to isolate significant amount of mitochondrial protein. The mice were anesthetized with CO_2 for one minute, incised through the abdomen, sternotomy performed and the hearts were excised. It was our observation that the time interval between the hearts excision and completion of homogenization influenced the channel activity (brief period of 2 minutes); the lesser the time interval, the better the channel activity. The hearts were washed in ice cold Buffer A (250mM sucrose, 10mM HEPES, 5mM EGTA and pH 7.2). The hearts were pounded with protease 1mg/ml (type XXIV Sigma). The suspension was diluted up to two to threefold with buffer A supplemented with 0.5% bovine serum albumin (BSA). The suspension was then poured into a motorized Teflon pestle so as to get it homogenized. The homogenized suspension was then centrifuged in a Sorvall centrifuge for 3 minutes at 1500 g. The supernatant obtained was then centrifuged at 9000 g for 5 minutes. The consequential pellet was resuspended in Buffer A without BSA and centrifuged for 3 minutes at 2300 g. The ensuing supernatant was centrifuged for 5 minutes at 9000 g. The resulting final pellet was resuspended in Buffer A without BSA so as to have the final concentration as 35-

40 mg/ml protein and was placed on ice. This technique of mitochondrial isolation is in agreement with that described by Jaburek et al. [48, 72, 77, 84] (Appendix A). The mitochondrial protein concentration was projected by means of the Biuret reaction [71]. Mitochondrial protein was assayed within two hours of isolation. The isolated mitochondria were placed on ice and were continuously stirred with the help of a magnetic stirrer to ensure free oxygen access during the experiments. This practice is in agreement with the American Physiological Society's "Guiding principles in the Care and Use of Animals", and was permitted by the Institutional Animal Care and Use Committee (IACUC) at Portland State University, Oregon. [72, 77, 84]

3.2 Assay Medium:

The mitochondria were suspended in an assay medium consisting of potassium chloride (KCl) (120mM); EGTA (0.1mM) (chelator of calcium ions); succinate (10mM) (provides electrons to electron transport system); phosphoric acid (5mM); 0.5 mM $MgCl_2$ and pH 7.2. The osmolality range of the assay medium, as measured by the osmometer, was between 275-280 mosmol/kg H_2O. All the runs were supplemented with 5 μM Rotenone and 0.67 μM Oligomycin. The experiments were performed at 30.5 ^{0}C [72, 77, 84].

Rotenone is functional in inhibiting the reverse electron transfer from complex II to complex I of the electron transport chain. Oligomycin inhibits F_0F_1-ATP synthase of

the electron transport chain. The synthesis of ATP is henceforth interrupted and the membrane potential is subsequently decreased. [72, 77, 84]

The volume of the assay medium used for experiments involving rat heart mitochondria was 3 ml in my lab. I tried to reduce the volume and work with the 1.5 ml assay medium in order to perform significant number of light scattering runs to validate the mitoK_{ATP} activity.

3.3 Measurement of mitochondrial volume:

K^+ uptake is assayed following the mitochondrial matrix swelling. The light scattering technique is functional in measuring the changes in mitochondrial volume that escort the net salt transport into the mitochondria. The light scattering technique is based on the principle that the reciprocal absorbance (A^{-1}) at 520 nm and 0.1 mg/ml of the mitochondrial suspension at 30.5 ^{0}C, is linearly related to matrix volume, as described by Beavis et al. [71, 72]. β is a dimensionless factor that normalizes A^{-1} (inverse absorbance) for mitochondrial protein concentration, P (mg/ml)

$$\beta = P\,(A^{-1} - \alpha) / P_s$$

α is a machine constant (0.25 for our apparatus) and P_s equals 1 mg/ml. [72, 77, 84]

An increase in matrix volume, owing to the osmotic swelling of mitochondria (due to uptake of salts and water), is accompanied by a decrease in light intensity. The light scattering technique measures this change in matrix volume of the mitochondria. The

light scattering technique is primarily functional in investigating the mitoK_{ATP} activity in intact isolated mitochondria. The light scattering technique is beneficial in comparison to the reconstitution studies, considering that the mitochondrial ion channels and the bioenergetic apparatus are intact and also the various regulatory agents could possibly be applied directly to the mitochondria. Limitations include the unphysiological ion gradient's usage, substrate or nucleotide concentrations, and a possible influence of neighboring organelle membranes [48]. The data measured by a Brinkmann PC 700 light probe is converted to digital information (A^{-1}) using a "Lab view" program designed exclusively for use in our laboratory. [72, 84]

Diazoxide, and other hydrophobic compounds soluble in dimethyl sulfoxide (DMSO), are always added 2-3 seconds after the mitochondrial suspension to guarantee equal distribution. Also, diazoxide is not effective in the absence of ATP, since it cannot activate a channel that is previously in its open state. Similarly, 5 hydroxydecanoate (5 HD), a specific mitoK_{ATP} antagonist is ineffective in the absence of ATP and the KCO diazoxide. This is because 5 HD prevents the diazoxide mediated mitoK_{ATP} activation, but by itself does not inhibit the channel activity. The same observations hold good for cromakalim, PMA and glibenclamide as well. Light scattering traces were initiated on adding the mitochondrial suspension with the first 5-7 seconds omitted for precision. The channel activity was followed over a period of 180 seconds. [72, 77, 84]

The final concentration of the stock mitochondria added to the assay medium was 0.1 mg protein/ml. The volume of the assay medium was 1.5 ml for each run. Data analysis followed each individual experiment. Rate of swelling was determined by the slope of the traces on addition of the various KCOs, activators of mitoK_{ATP} and their blockers. The analysis program used was "ORIGIN", a data-analysis program by Microcal (Northampton, MA, USA). [72, 84]

Statistical analysis: Data are presented as mean +/- SD. Data were analyzed using unpaired Student's t-test of the means using Microcal Origin software (Northampton, MA). A value of $P < 0.05$ is considered statistically significant.

3.4 Chemicals

All the chemicals used for the experiments were from Sigma Chemical Co. (St. Louis, MO, USA). Mice were ordered from the Jackson laboratory (Maine, USA).

CHAPTER 4

K_{ATP} CHANNEL OPENERS AND BLOCKERS:

One of the most credible means to establish and identify the functions of cardiac sarcolemmal and mitochondrial K_{ATP} channels is through the medium of K_{ATP} channel openers and blockers. The pharmacological agents could probably be classified as selective and non-selective based on their putative sites of action in heart, the former acting exclusively on the mitoK_{ATP} and the latter acting either on sarcK_{ATP} / mitoK_{ATP} as shown in Table 1 and represented structurally in figures 15 and 16.

4.1 Non-selective K_{ATP} agonists:

Cromakalim, synthesized in 1980 represents the archetype of the benzopyrans class of drugs. Used as a cardioprotective drug in experimental routines, it lacks specificity in its site of action (mitoK_{ATP} vs cellK_{ATP}). It functions in shortening the action potential duration (APD) and causes vasodilatation [80, 82].

Nicorandil, belongs to the pyridyl nitrate class of drugs and causes significant vasodilatation due to its NO (nitric oxide) donor properties. It has a non-specific site of action in the cardiac myocyte [80, 82].

P 1075 is a widely studied potent cyanoguanidine. It lacks selectivity versus the mitoK_{ATP} as was shown in ischemia-reperfusion studies on rat and rabbit cardiac myocytes [82].

4.2 Selective mitoK_{ATP} agonists:

Diazoxide, a benzothiazidine class of drug is a selective mitoK_{ATP} agonist under normal conditions. It also causes activation of smooth muscle K_{ATP} and endothelial K_{ATP} isoforms. Diazoxide is probably the only K_{ATP} opener that binds equivocally to SUR 1 and SUR 2B subunits. It is routinely used in low doses to establish the importance of mitoK_{ATP} in preconditioning. A long term use of diazoxide is related to the development of hyperglycemic state owing to its role in insulin inhibition [46, 82].

BMS-191095 ((3*R*)-*trans*- 4-((4-chlorophenyl)-*N*-(1*H*-imidazol-2-ylmethyl)dimethyl-2*H*-1-benzopyran-6-carbonitril monohydrochloride) belongs to the benzopyrans class of drugs with a higher cardiac selectivity and anti-ischemic potency. It was shown to improve the post- ischemic cardiac function and a reduction in the LDH (lactate dehydrogenase) release. Grover et al. through their studies using reconstituted mitoK_{ATP} observed that BMS191095 activated the mitoK_{ATP} and the activation was inhibited by both 5 HD and glibenclamide [79, 82].

4.3 K_{ATP} channel blockers:

5 HD (5 hydroxydecanoate), regarded as a specific inhibitor of mitoK_{ATP}, is used in most studies to assess the activity of the mitoK_{ATP} channel. It has been observed that the diazoxide activated mitoK_{ATP} influx of K^+ ions is reversed in the presence of 5 HD in isolated rat heart mitochondria [48].

Glibenclamide, an anti-diabetic drug belonging to the sulfonylurea class, is implemental in blocking the activity of the K_{ATP} channel. It shows marked variability in its actions with different affinity grades, exhibiting SUR1 blocking properties involving pancreatic K_{ATP} and the SUR2A/2B subunits expressed on the cardiac and the vascular cells [80, 82].

HMR 1098 belongs to the sulfonylthiourea class and is considered as a selective cellK_{ATP} blocker. It is generally used along with a non-specific K_{ATP} blocker to examine the physiology of IPC [81].

CHAPTER 5

RESULTS:

The effects of ATP, KCOs (diazoxide, cromakalim), mitoK$_{ATP}$ activators (such as PMA) and inhibitors (5 HD and glibenclamide) on mitochondrial matrix swelling are potassium-specific.

The mitochondrial potassium cycle is an important constituent in maintaining the mitochondrial volume homeostasis [Fig 1]. The swelling of the mitochondrial matrix is witnessed as an increase in light scattering and is proportional to the net salt influx [72, 77, 84]. The light scattering technique is functional in investigating the mitoK$_{ATP}$ activity in intact isolated mitochondria. The typical light scattering traces from control mouse heart mitochondria in a respiring K^+ rich medium are shown in figures 4 – 9.

Figure 4 demonstrates light scattering traces from mouse heart mitochondria respiring in succinate supplemented K^+ medium at 30.5 °C. The light scattering parameter β, an index of matrix volume, is plotted against time. In the absence of ATP (open/control), the mitochondrial matrix attained a higher steady state volume. On addition of 200 μM ATP there is an apparent lower rate of swelling and ultimately a lower steady state volume (ATP). The ATP inhibition of the channel was reversed by 30 μM diazoxide (ATP + DZX), and the opening effect of diazoxide was blocked by 300 μM 5 HD (ATP + DZX + 5 HD). [Fig. 4]

Figure 5 demonstrates light scattering traces from mouse heart mitochondria respiring in the same succinate supplemented K^+ medium at 30.5 °C. In the absence of ATP, the mitochondrial matrix scaled to a higher steady state volume. Addition of 200 μM ATP lowered the rate of swelling. The inhibition of the channel by ATP was reversed by 30 μM diazoxide, and the activator effect of diazoxide was inhibited by 10 μM glibenclamide. [Fig. 5]

Figures 6 and 7 demonstrate light scattering traces initiated from mouse heart mitochondria respiring in the same K^+ medium supplemented with succinate. It was evident that the mitochondria had swollen to a new steady state volume in the absence of ATP (open) and the addition of 200 μM ATP lowered the swelling. The channel was re-activated by cromakalim (50 μM) and this activator effect was inhibited by both 5 HD (300 μM) and glibenclamide (10 μM). [Fig. 6 & 7]

Figures 8 and 9 illustrate the light scattering traces demonstrated by isolated mouse heart mitochondria respiring on succinate supplemented K^+ medium. The mitochondria, owing to the matrix swelling, attained a new steady state volume in the absence of ATP (open/control trace), an effect inhibited in the presence of 200 μM ATP. On addition of a PKCε activator PMA (0.2 μM), the mitoK_{ATP} got reactivated and almost superimposed on the open trace. But this activated mitoK_{ATP} was inhibited by both 5 HD (300 μM) and glibenclamide (10 μM). [Fig. 8 & 9]

Figures 10, 11 and 12 graphically illustrate the summary of the percentage mitoK_{ATP} activity exhibited by isolated mouse heart mitochondria in the presence of activators diazoxide, cromakalim, PMA, and inhibitors 5 HD, glibenclamide. The data establish the capacity of ATP to inhibit the channel, the openable effects of various KCOs and the inhibitory actions of K^+ channel blockers. P value was less than 0.05 and hence statistically significant. The data also validates the monitoring capacity of the assay medium. The results summarized represent five independent experiments for each combination. Data are presented as mean +/- SD. Data were analyzed using unpaired Student's t-test of the means using Microcal Origin software. $P < 0.05$ is considered statistically significant.

Figure 13 represents typical light scattering traces from rat heart mitochondria respiring in succinate supplemented K^+ medium (120mM) at 30.5 °C. It can be noted that the mitochondrial matrix had swollen to a higher steady state in the absence of ATP (open/control). When 200 μM ATP was added it resulted in a lower steady state volume. ATP inhibition was overcome by 30 μM diazoxide and the diazoxide activated channel was inhibited by 300 μM 5 HD [Fig. 13]. The light scattering traces look similar to those in the mouse heart mitochondria. The results obtained with the combination of diazoxide and 5 HD were reproducible by combining diazoxide + glibenclamide (10 μM), cromakalim (50 μM) + 5 HD or cromakalim + glibenclamide, and also PMA (0.2 μM) + 5 HD or PMA + glibenclamide. (data not shown)

Figure 14 demonstrates bar graphs representing the comparison between percentage mitoK$_{ATP}$ activity exhibited by rat and mouse heart mitochondria under the same experimental conditions. Rates in the absence of ATP were set as 100% and in the presence of ATP as 0%. The effects of various KCOs and blockers are assessed in relation to the set controls. It can be seen that the percentage mitoK$_{ATP}$ activity exhibited by mouse heart mitochondria is comparable to that exhibited by rat heart mitochondria when subjected to various potassium channel openers and their blockers. Data are presented as mean +/- SD. Data were analyzed using unpaired Student's t-test of the means using Microcal Origin software.

5.1 Mechanism of activation of mitoK$_{ATP}$ by PMA:

PMA is a protein kinase C epsilon (PKCε) activator which eventually activates the mitoK$_{ATP}$. The activation of PKCε phosphorylates the mitoK$_{ATP}$ and ultimately opens it. PKCε has been shown to be bound to the inner membrane of the mitochondria and associated in a functional complex with mitoK$_{ATP}$. This was demonstrated using the techniques of detergent extraction and chromatographic isolation of mitochondrial membrane proteins. The PKCε activated mitoK$_{ATP}$ results in an influx of potassium, an effect negated in the presence of phosphatases. The K^+ influx causes the matrix alkalinization, retarding the reduction of Q to QH_2 at Complex 1 of the electron transport chain and eventually resulting in the H_2O_2 production by reducing the molecular oxygen. MitoK$_{ATP}$ activated K^+ influx and eventual ROS (reactive oxygen

species) production is mediated through the KCOs and also mitoK_{ATP} associated PKCε activation [75, 78]. [Fig 3]

When used in the 0.2 μM concentration, the PKCε mediated activation of mitoK_{ATP} by PMA was inhibited by both 5HD and glibenclamide. [Fig 8, 9, 12]

Table 1:

Pharmacological agents currently under investigation to validate the K_{ATP} channel activity:

Pharmacological agent	Putative site of action in heart	Agonist/Antagonist
Cromakalim	$CellK_{ATP}/MitoK_{ATP}$	Agonist
Nicorandil	$CellK_{ATP}/MitoK_{ATP}$	Agonist
Diazoxide	$MitoK_{ATP}$	Agonist
P-1075	$CellK_{ATP}/MitoK_{ATP}$	Agonist
BMS191095	$MitoK_{ATP}$	Agonist
5-hydroxydecanoate	$MitoK_{ATP}$	Antagonist
Glyburide/Glibenclamide	$MitoK_{ATP}$ /$CellK_{ATP}$	Antagonist
HMR-1098	$CellK_{ATP}$	Antagonist
TPP	$MitoK_{ATP}$ /$CellK_{ATP}$	Antagonist

Table 1 demonstrates the various pharmacological agents currently under investigation worldwide to assess the ATP-sensitive potassium channel (K_{ATP}) activity along with their putative sites of action in heart and their mode of action (agonist vs. antagonist). Cromakalim, Nicorandil and P-1075 are considered as non-selective agonists of K_{ATP} since they are believed to act on the $cellK_{ATP}$ and the $mitoK_{ATP}$. Diazoxide and BMS191095 are considered to be $mitoK_{ATP}$ specific agonists. 5-hydroxydecanoate (5HD) is a $mitoK_{ATP}$ specific antagonist and HMR-1098 is a $cellK_{ATP}$ specific antagonist. Glyburide, Glibenclamide and TPP are non-selective K_{ATP} channel antagonists.

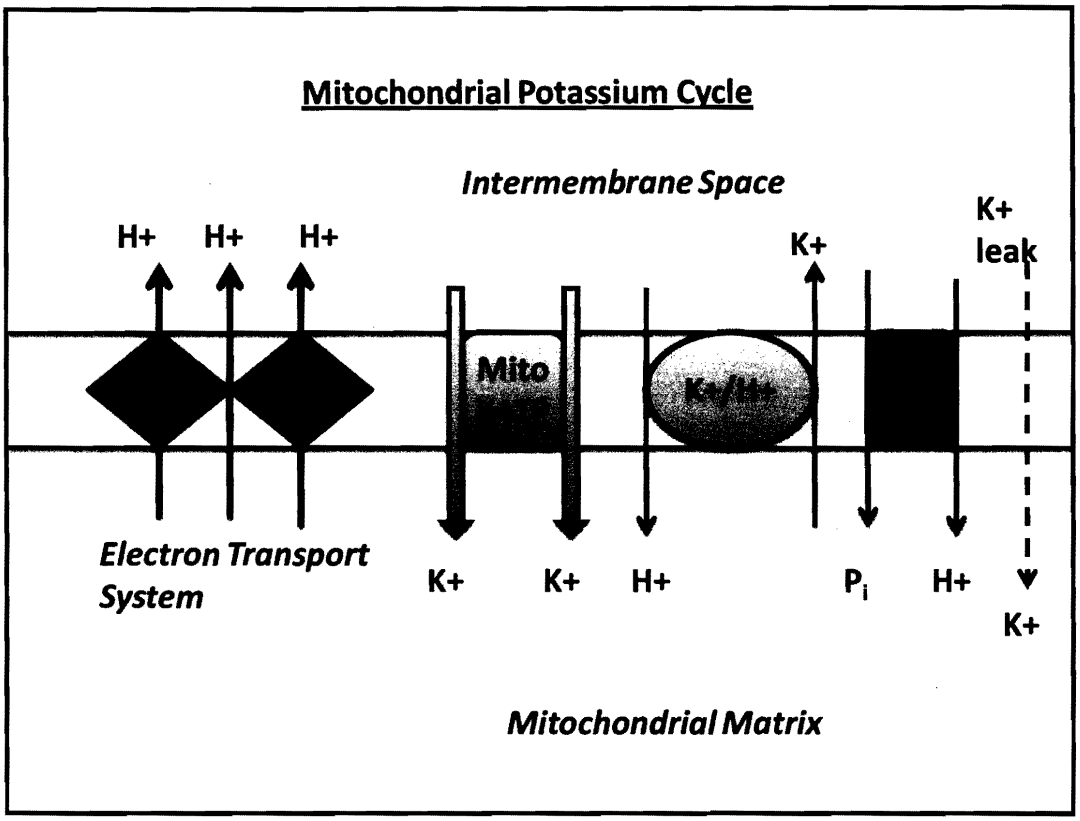


Fig 1: **Mitochondrial Potassium cycle.** The proton motive force that is generated by the electron transport system creates a membrane potential that drives K^+ influx via diffusion (K^+ leak) and mitoK_{ATP} (located on the inner membrane). The electroneutral Pi/OH exchanger drives phosphate inside so that the net K^+ influx is accompanied by anions and osmotically obligated water. The electroneutral K^+/ H^+ antiporter regulated on the matrix side by volume changes, Mg^{+2} and H^+, provides an efflux of excess K^+ accompanied by Pi and water, thereby preventing excessive matrix swelling. [49, 50, 51, 77]

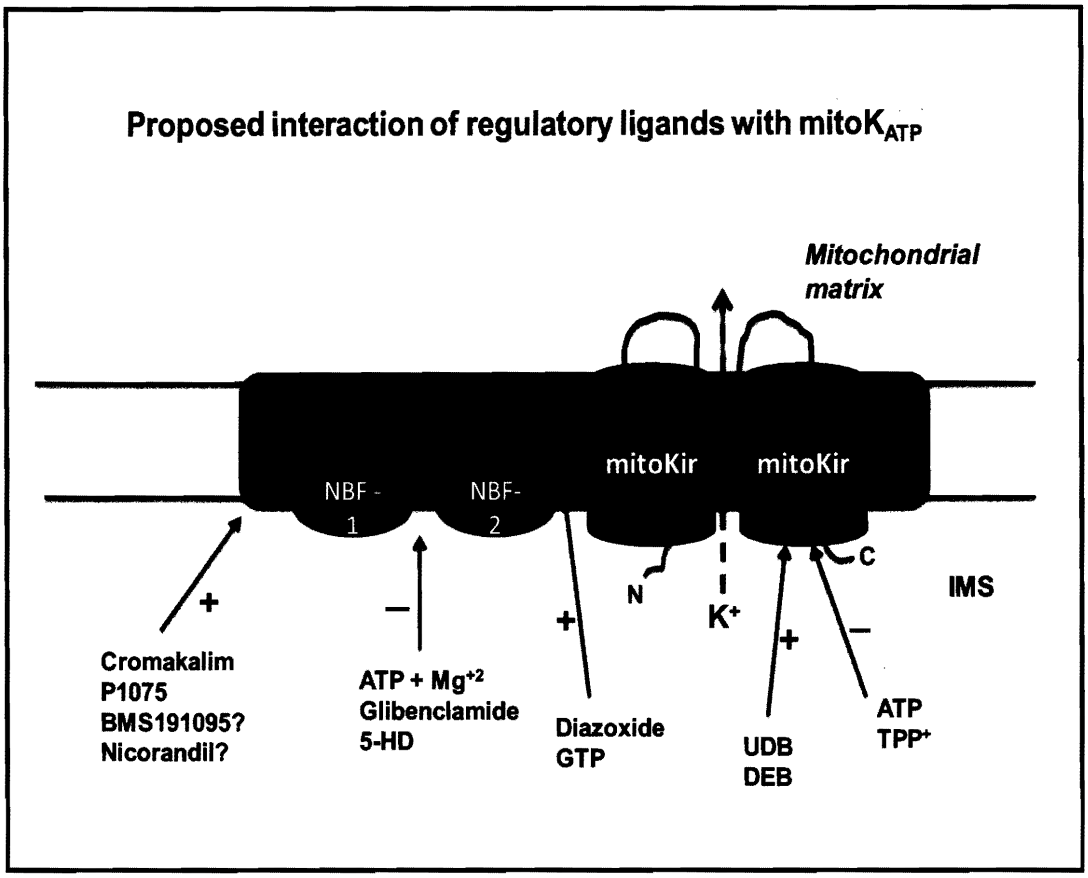


Fig 2: A proposed illustration of the mitoK$_{ATP}$ channel subunits with the possible interaction sites of the regulatory ligands (illustration modified from reference [90]). MitoK$_{ATP}$ is thought to be represented by four 55-kDa Kir subunits and four 63-kDa SUR subunits, functioning as a hetero-octamer. MitoKir is viewed as a conduction pore of the channel and is surrounded by the regulatory SUR subunits that contain one or more nucleotide binding folds (NBF). The Kir subunits regulate the movement of K^+ across the membrane. The Kir subunits are thought to mediate the inhibitory effects of ATP (in the absence of Mg^{+2}) and TPP^+, and activation effects of UDP and DEB. The SUR subunits, belonging to the ABC family, demonstrate the sites of possible inhibition by ATP + Mg^{+2}, Glibenclamide and 5-HD. The activation of the channel by diazoxide, GTP, cromakalim, P1075 and possibly BMS191095 and nicorandil, is thought to be mediated through the SUR subunits. [90]

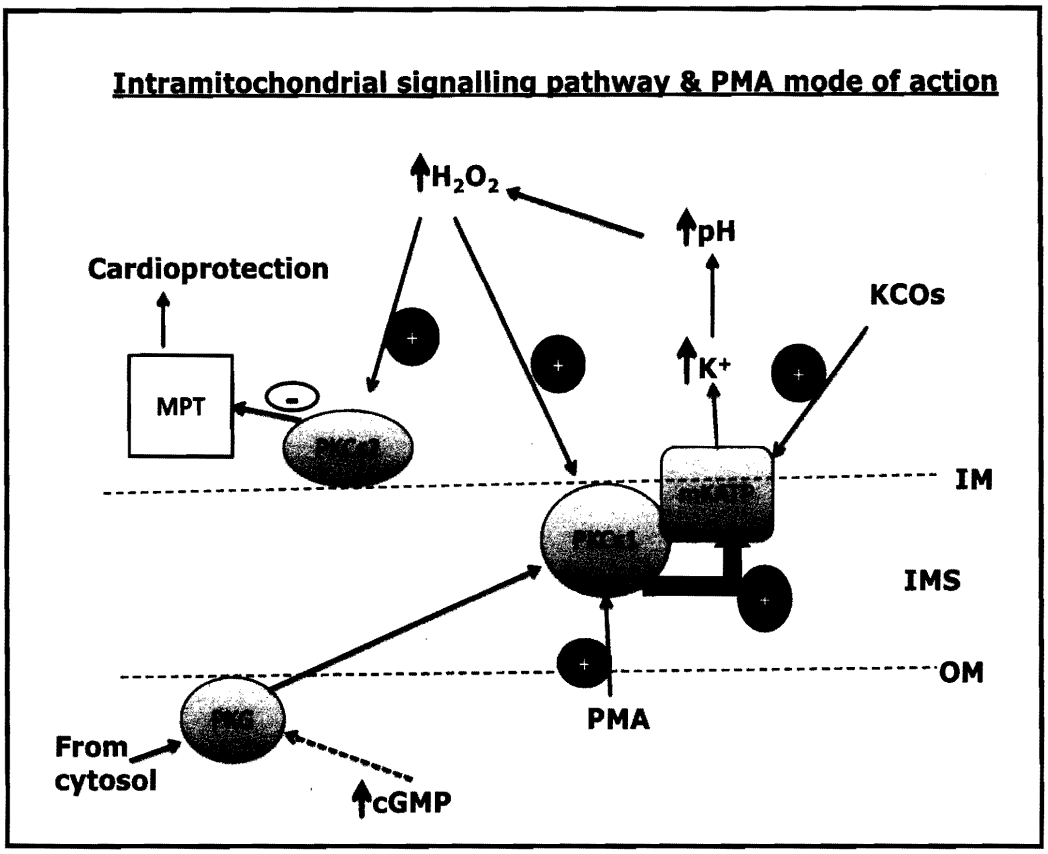


Fig 3: Intramitochondrial signalling pathway triggered by cGMP activated mitoK$_{ATP}$ (illustration modified from reference [78]). PKG delivers the signals to the mitochondria that arise from the G_i- coupled receptors. The signal is transmitted to PKCε1 located on the inner mitochondrial membrane (IM) from PKG phosphorylation of an unknown mitochondrial outer membrane (OM) protein. Activated PKCε1 causes phosphorylation and opening of the mitoK$_{ATP}$. MitoK$_{ATP}$ opening via PKCε1 activation by PMA or by K$_{ATP}$ channel openers such as diazoxide or cromakalim results in K^+ influx, increase in matrix pH and increased H_2O_2 (reactive oxygen species) production. The H_2O_2 produced inturn activates PKCε1 and PKCε2. The activated PKCε2 inhibits the mitochondrial permeability transition (MPT) which eventually decreases cell necrosis, infarct size and inhibits apoptosis. [75, 78, 82]

Abbreviations used: cGMP, cyclic Guanosine monophosphate; PKG, protein kinase G; PMA, Phorbol 12-myristate-13-acetate; OM, outer membrane; IM, inner membrane; IMS, inter membranous space; PKC ε, protein kinase C epsilon; H_2O_2, hydrogen peroxide; MPT, mitochondrial permeability transition; mkATP, mitochondrial K$_{ATP}$; "+" sign indicates activation; "-" sign indicates inhibition

Light scattering traces illustrating mitoK$_{ATP}$ activity in mouse heart mitochondria:

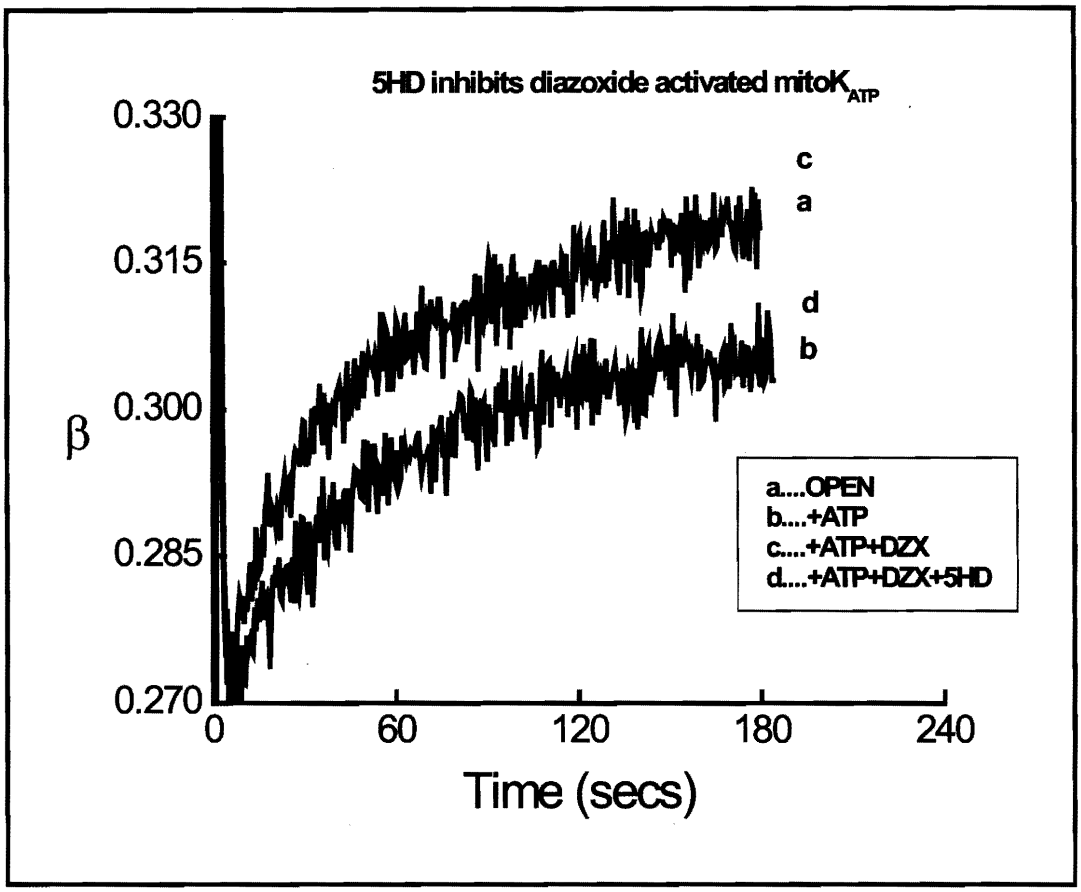


Fig 4: 5-hydroxydecanoate (5 HD) inhibits the pharmacological opening of mitoK$_{ATP}$ by diazoxide. Light scattering traces from mouse heart mitochondria respiring on succinate in K$^+$ medium (120 mM) at 30.5 ^{0}C. The light scattering parameter β, an index of matrix volume, is plotted against time.

Trace a: K$^+$ influx in the absence of ATP (open/control)

Trace b: K$^+$ influx in the presence of 0.2 mM ATP

Trace c: Reversal of ATP inhibition by 30 μM Diazoxide (the trace almost superimposes on the open trace)

Trace d: Reinhibition by 300 μM 5-hydroxydecanoate in the presence of 30 μM diazoxide and 0.2 mM ATP (the trace almost superimposes on trace 'b')

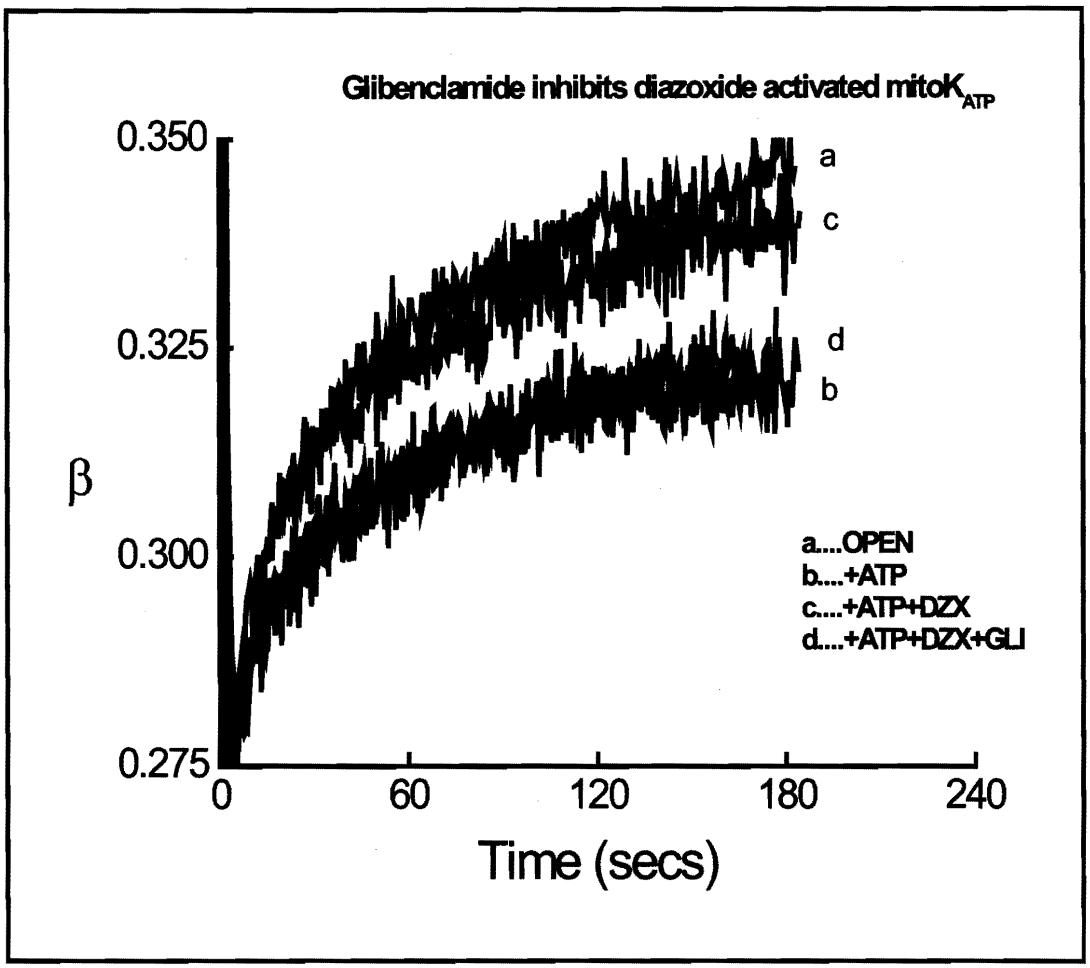


Fig 5: Glibenclamide inhibits the pharmacological opening of mitoK$_{ATP}$ by diazoxide. Light scattering traces of mouse heart mitochondria respiring on succinate rich K^+ medium (120 mM) at 30.5 ^{0}C. The light scattering parameter β, an index of matrix volume, is plotted against time.

Trace a: Resting mitoK$_{ATP}$ channel in open state (control)

Trace b: Inhibition of mitoK$_{ATP}$ by ATP (0.2 mM)

Trace c: Activation of mitoK$_{ATP}$ by diazoxide (30 μM) in the presence of ATP (0.2 mM) (the trace almost superimposes on the open trace)

Trace d: Inhibition of diazoxide activated mitoK$_{ATP}$ by glibenclamide (10 μM) in the presence of ATP (the trace almost superimposes on trace 'b')

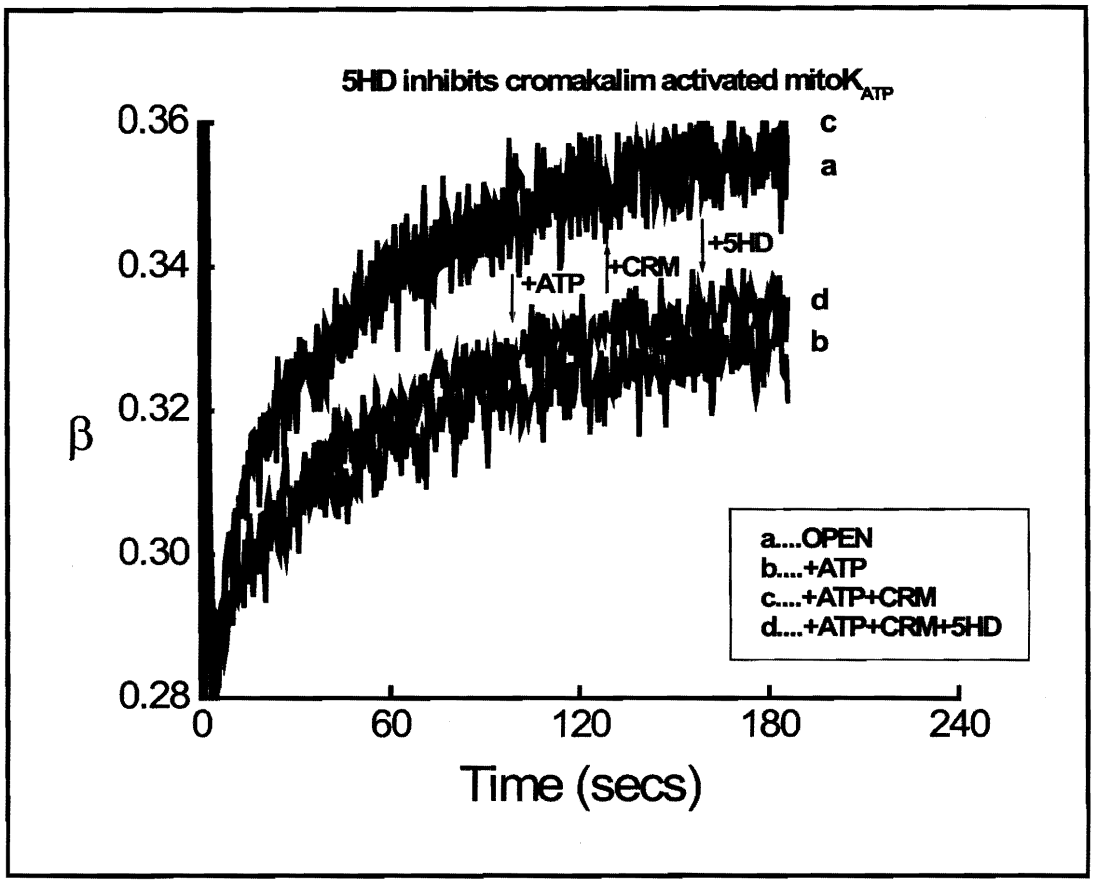


Fig 6: 5-hydroxydecanoate (5 HD) inhibits cromakalim (CRM) activated mitoK$_{ATP}$. Light scattering traces showing the activity of mitoK$_{ATP}$ in mouse heart mitochondria respiring on succinate supplemented K^+ medium (120 mM) at 30.5 ^{0}C. The light scattering parameter β, an index of matrix volume, is plotted against time.

Trace a: Resting mitoK$_{ATP}$ channel in open state (control)

Trace b: Inhibition of mitoK$_{ATP}$ by ATP (0.2 mM)

Trace c: Activation of mitoK$_{ATP}$ by cromakalim (50 μM) in the presence of ATP (0.2 mM) (the trace almost superimposes on control trace)

Trace d: Inhibition of cromakalim activated mitoK$_{ATP}$ by 5HD (300 μM) in the presence of ATP (the trace almost superimposes on trace 'b')

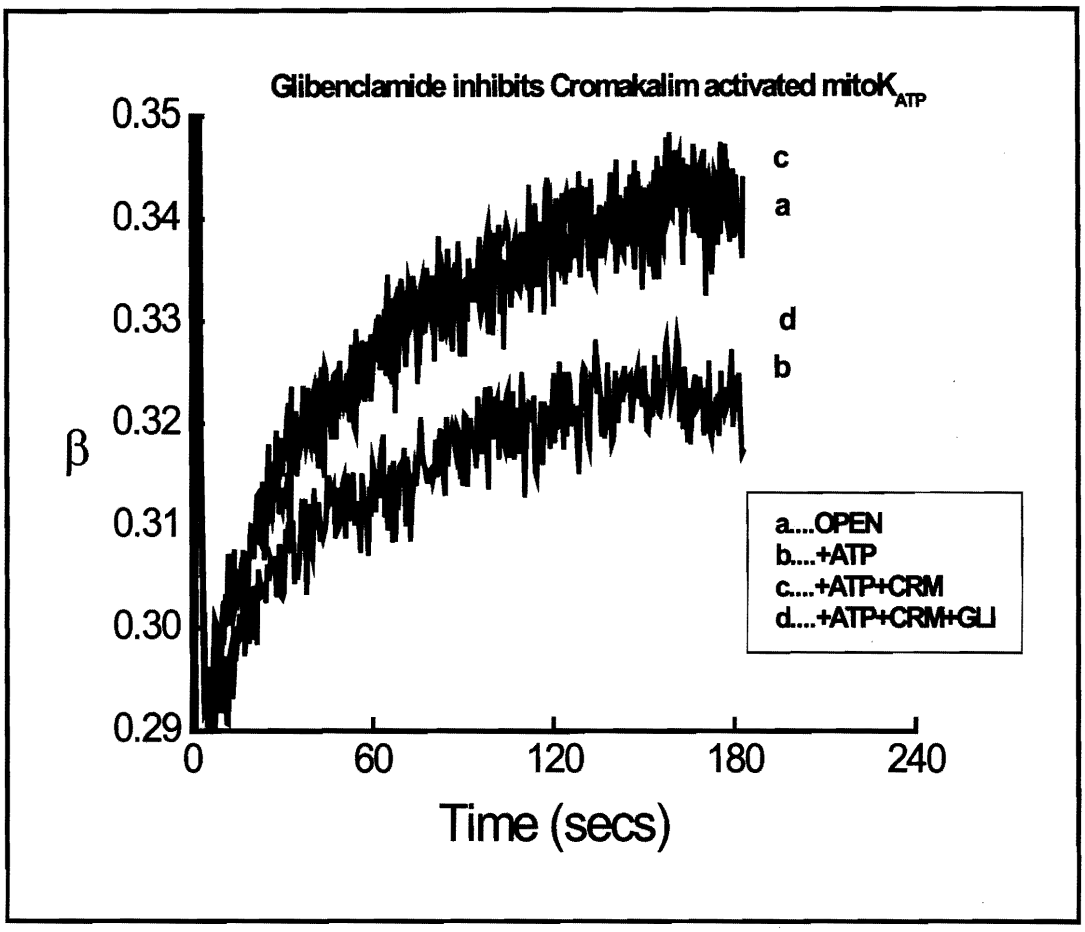


Fig 7: Glibenclamide inhibits the cromakalim activated mitoK$_{ATP}$. Light scattering traces showing the activity of mitoK$_{ATP}$ in mouse heart mitochondria respiring on succinate supplemented K^+ medium (120 mM) at 30.5 ^{0}C. The light scattering parameter β, an index of matrix volume, is plotted against time.

Trace a: Resting mitoK$_{ATP}$ channel in open state (control)

Trace b: Inhibition of mitoK$_{ATP}$ by ATP (0.2 mM)

Trace c: Activation of mitoK$_{ATP}$ by cromakalim (50 μM) in the presence of ATP (0.2 mM) (trace almost superimposing on trace 'a')

Trace d: Inhibition of cromakalim activated mitoK$_{ATP}$ by glibenclamide (10 μM) in the presence of ATP (the trace almost superimposes on trace 'b')

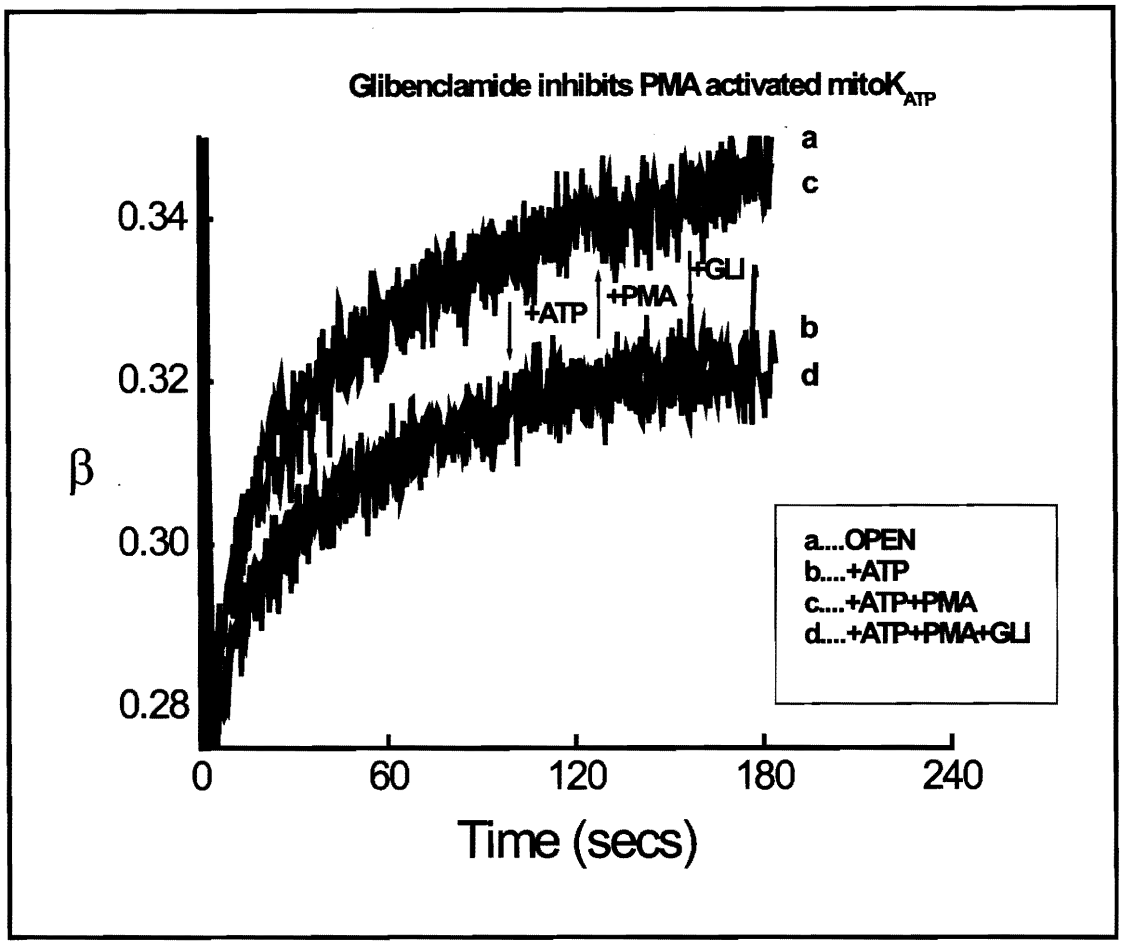


Fig 8: Glibenclamide inhibits the PMA activated mitoK$_{ATP}$. Light scattering traces showing the activity of mitoK$_{ATP}$ in mouse heart mitochondria respiring on succinate supplemented K^+ medium (120 mM) at 30.5 ^{0}C. The light scattering parameter β, an index of matrix volume, is plotted against time.

Trace a: Resting mitoK$_{ATP}$ channel in open state (control)

Trace b: Inhibition of mitoK$_{ATP}$ by ATP (0.2 mM)

Trace c: Activation of mitoK$_{ATP}$ by PMA (0.2 μM) in the presence of ATP (0.2 mM) (trace almost superimposing on trace 'a')

Trace d: Inhibition of PMA activated mitoK$_{ATP}$ by glibenclamide (10 μM) in the presence of ATP (the trace almost superimposes on trace 'b')

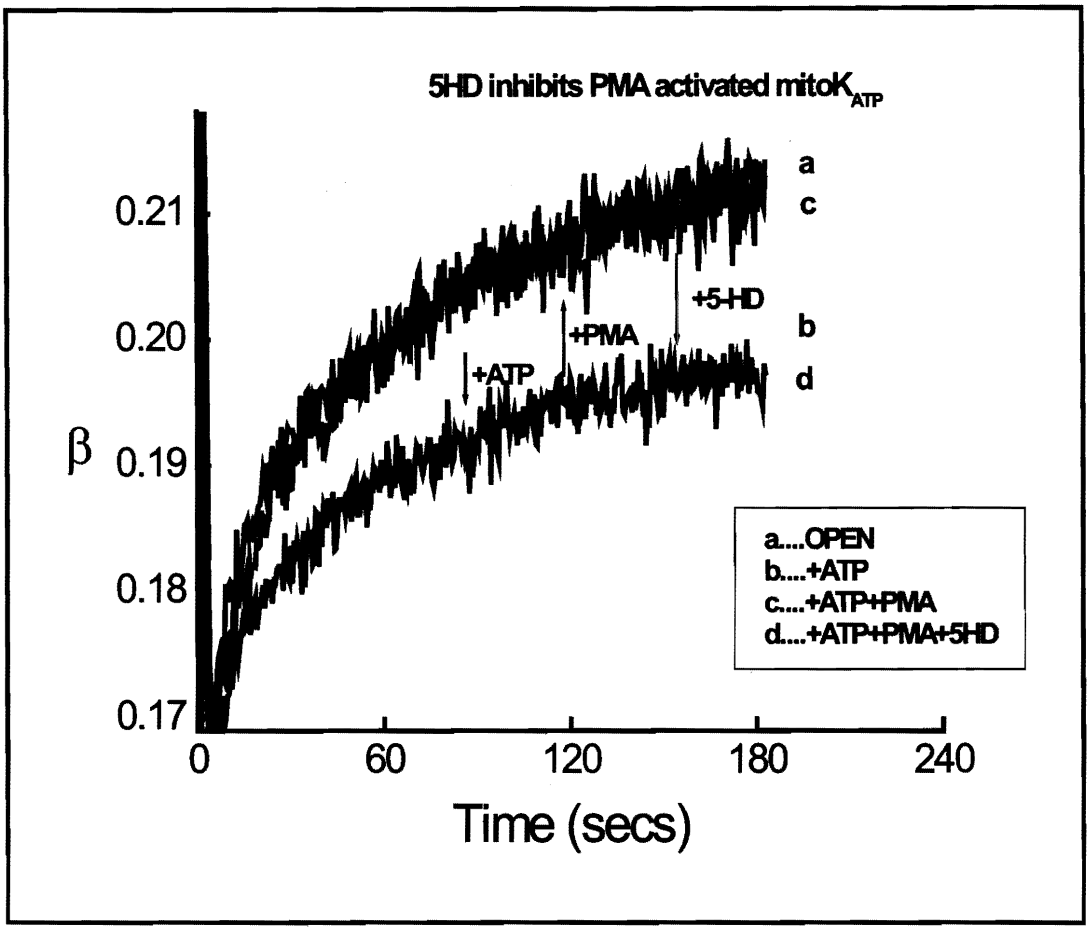


Fig 9: 5 HD inhibits PMA activated mitoK$_{ATP}$. Light scattering traces showing the activity of mitoK$_{ATP}$ in mouse heart mitochondria respiring on succinate supplemented K$^+$ medium (120 mM) at 30.5 ^{0}C. The light scattering parameter β, an index of matrix volume, is plotted against time.

Trace a: Resting mitoK$_{ATP}$ channel in open state

Trace b: Inhibition of mitoK$_{ATP}$ by ATP (0.2 mM)

Trace c: Activation of mitoK$_{ATP}$ by PMA (0.2 μM) in the presence of ATP (0.2 mM) (trace seen almost superimposing trace 'a')

Trace d: Inhibition of PMA activated mitoK$_{ATP}$ by 5 HD (300 μM) in the presence of ATP (the trace almost superimposes on trace 'b')

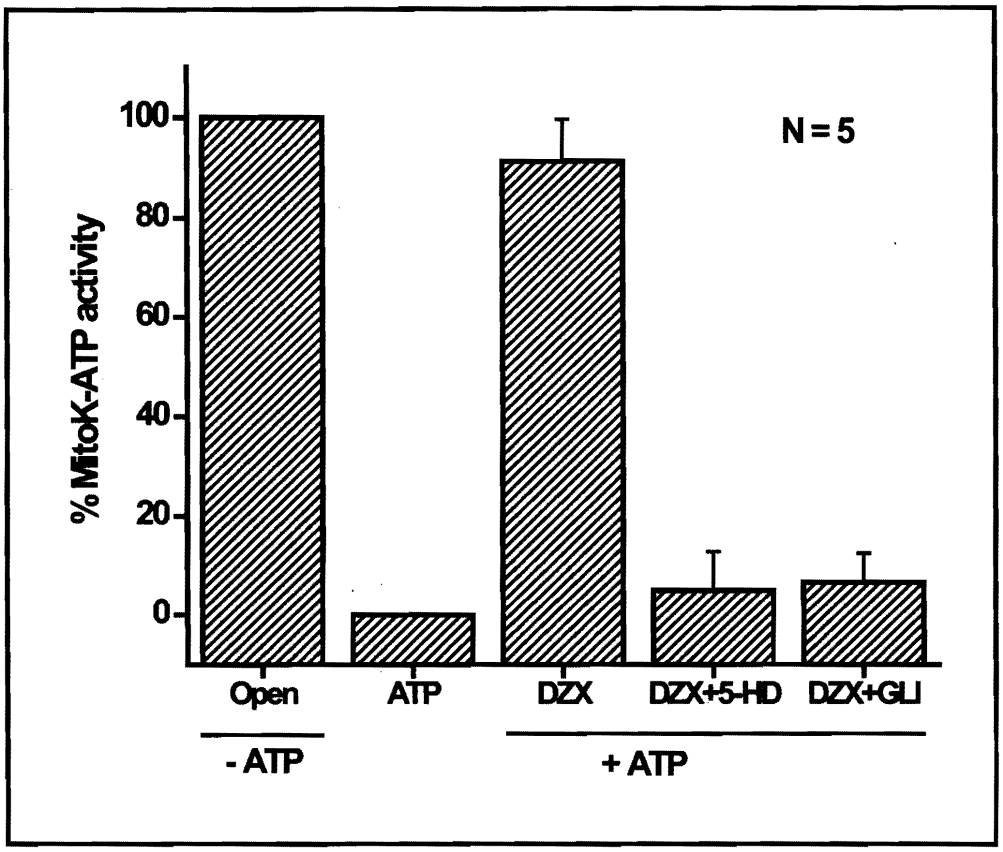


Fig 10: Summarized data for the percentage mitoK$_{ATP}$ activity exhibited by isolated mouse heart mitochondria when subjected to KCO diazoxide and blockers 5 hydroxydecanoate (5 HD) and glibenclamide. The results summarized are for five independent experiments for each combination. Error bars represent SDs from the average of five individual experiments.

Open: Control traces with no ATP (100% mitoK$_{ATP}$ activity)

ATP: % mitoK$_{ATP}$ activity in the presence of 0.2 mM ATP (0%)

DZX: % mitoK$_{ATP}$ activity in the presence of 30 μM diazoxide + 0.2 mM ATP

DZX+5-HD: % mitoK$_{ATP}$ activity with 30 μM DZX+ 300 μM 5-HD+ 0.2 mM ATP

DZX+GLI: % mitoK$_{ATP}$ activity with 30 μM DZX+ 10μM glibenclamide + 0.2 mM ATP

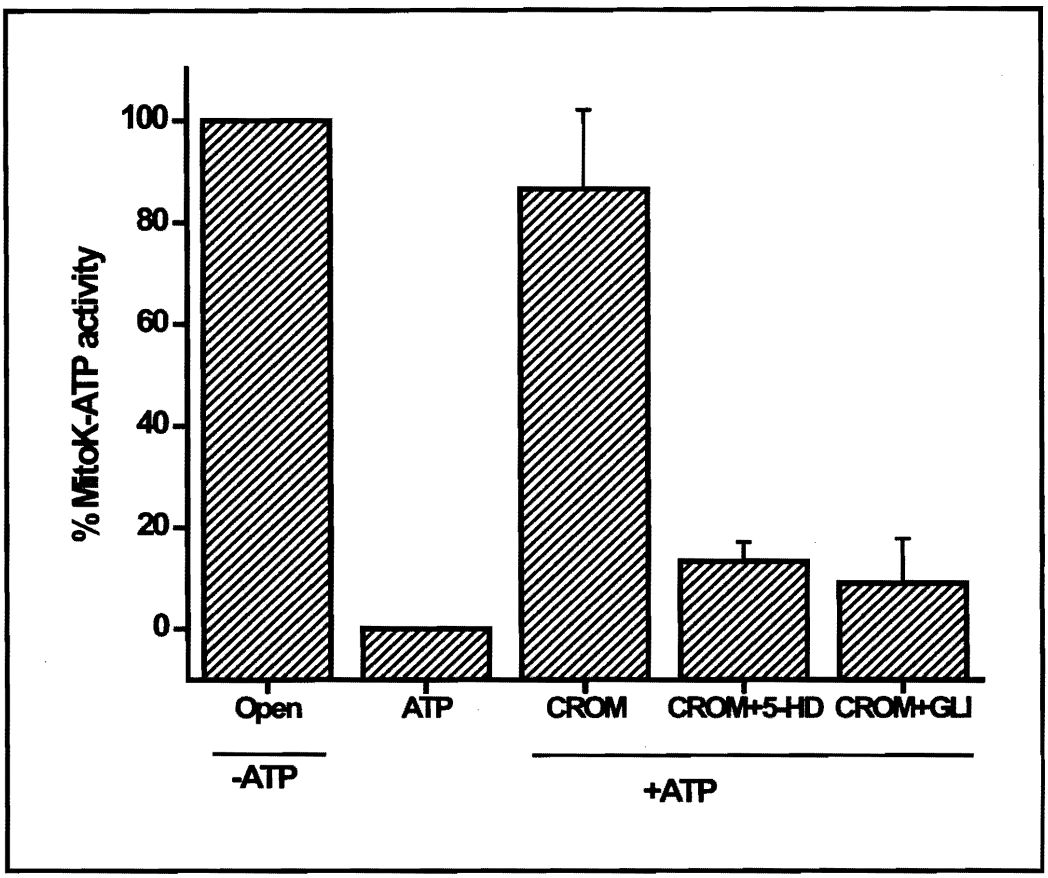


Fig 11: Summarizing the % mitoK$_{ATP}$ activity exhibited by isolated mouse heart mitochondria when subjected to cromakalim alone and in combination with 5-HD and glibenclamide. The results summarized are for N=5 for each combination. Error bars represent SDs from the average of five individual experiments.

Open: Control traces with no ATP (100% mitoK$_{ATP}$ activity)

ATP: % mitoK$_{ATP}$ activity in the presence of 0.2 mM ATP (0%)

CROM: % mitoK$_{ATP}$ activity in the presence of 50 μM cromakalim + 0.2 mM ATP

CROM+5-HD: % mitoK$_{ATP}$ activity with 50 μM cromakalim+ 300 μM 5-HD+ 0.2 mM ATP

CROM+GLI: % mitoK$_{ATP}$ activity with 50 μM cromakalim+ 10μM glibenclamide + 0.2 mM ATP

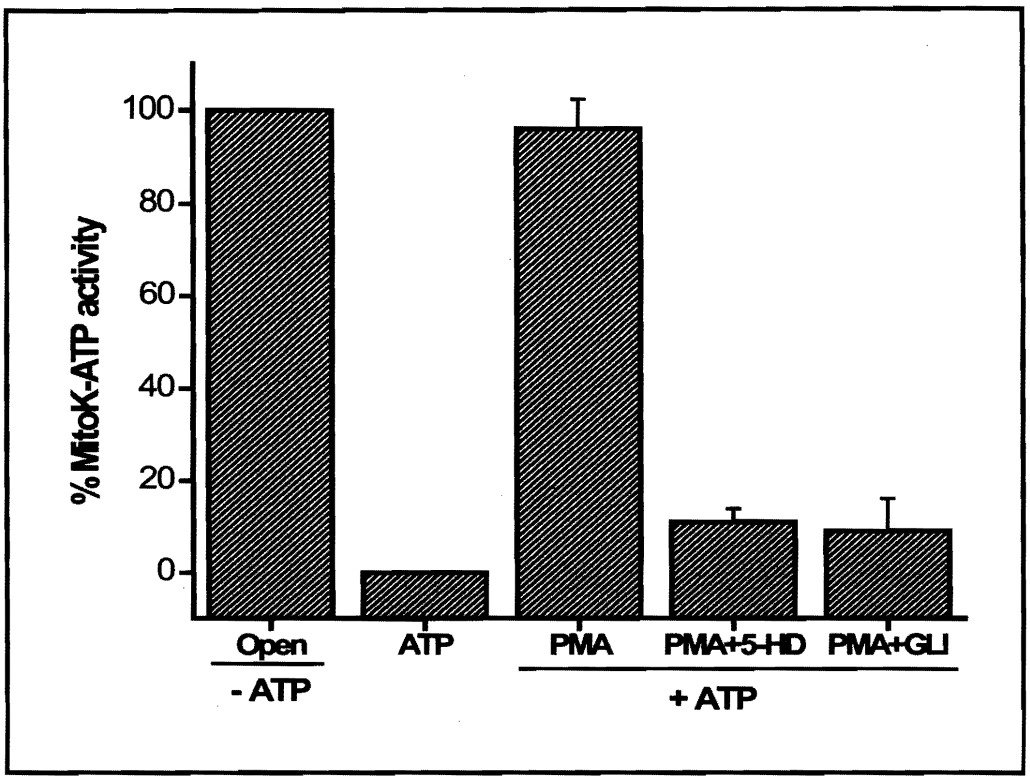


Fig 12: Summarizing the % mitoK$_{ATP}$ activity exhibited by isolated mouse heart mitochondria when subjected to PMA alone and in combination with 5-HD and glibenclamide. The results summarized are for N=5 for each combination. Error bars represent SDs from the average of five individual experiments.

Open: Control traces with no ATP (100% mitoK$_{ATP}$ activity)

ATP: % mitoK$_{ATP}$ activity in the presence of 0.2 mM ATP (0%)

PMA: % mitoK$_{ATP}$ activity in the presence of 0.2 μM PMA + 0.2 mM ATP

PMA+5-HD: % mitoK$_{ATP}$ activity with 0.2 μM PMA+ 300 μM 5-HD+ 0.2 mM ATP

PMA+GLI: % mitoK$_{ATP}$ activity with 0.2 μM PMA+ 10μM glibenclamide + 0.2 mM ATP

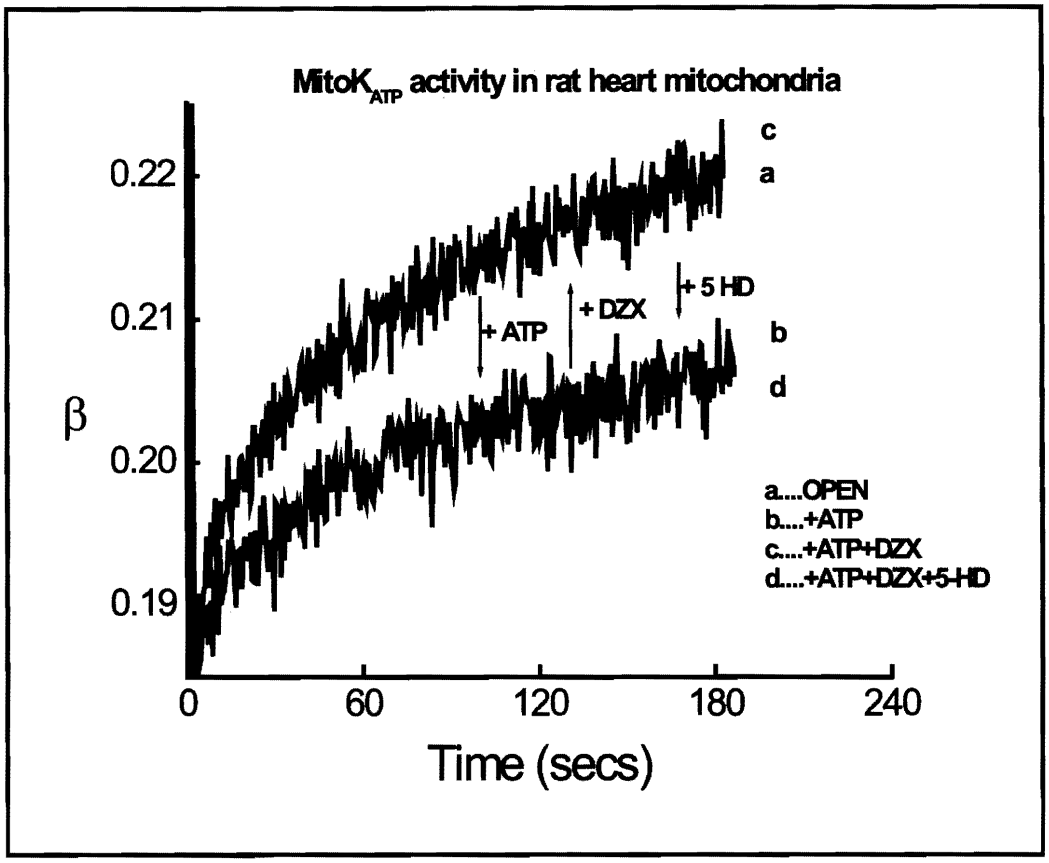


Fig 13: A typical light scattering trace showing the activity of mitoK$_{ATP}$ in rat heart mitochondria under the same experimental conditions i.e., respiring on succinate supplemented K^+ medium (120 mM) at 30.5 ^{0}C. The light scattering parameter β, an index of matrix volume, is plotted against time.

Trace a: Resting mitoK$_{ATP}$ channel in open state (control/open)

Trace b: Inhibition of mitoK$_{ATP}$ by ATP (0.2 mM) (+ ATP)

Trace c: Activation of mitoK$_{ATP}$ by KCO diazoxide (30 μM) in the presence of ATP

Trace d: Inhibition of activated mitoK$_{ATP}$ by 5 HD (300 μM) in the presence of ATP and diazoxide

Note: The results obtained with the combination of diazoxide and 5 HD were reproducible by combining diazoxide + glibenclamide (10 μM), cromakalim (50 μM) + 5 HD or cromakalim + glibenclamide, and also PMA (0.2 μM) + 5 HD or PMA + glibenclamide. (data not shown)

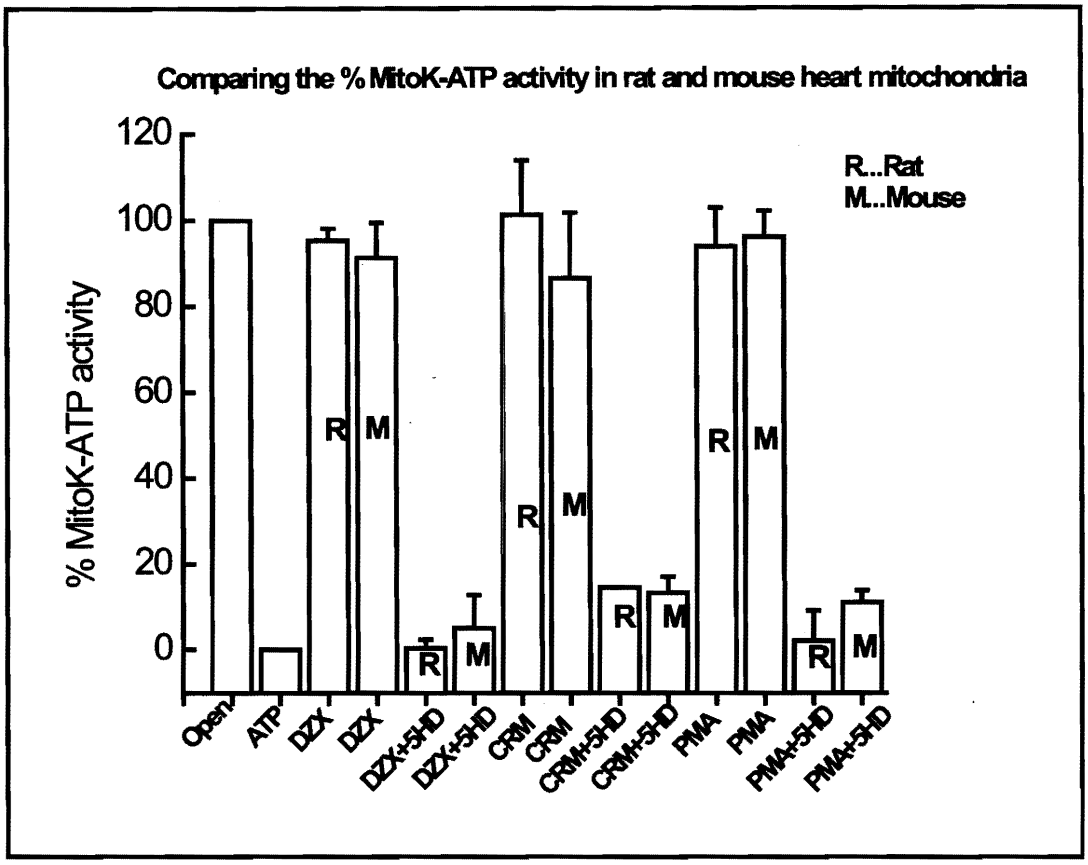
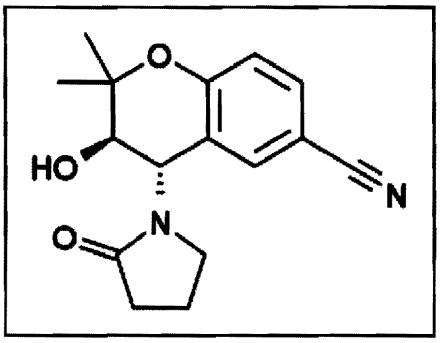


Fig 14: The following figure compares the % mitoK$_{ATP}$ activity activity exhibited by both rat and mouse heart mitochondria under the same experimental conditions and in the presence of various potassium channel openers and blockers. Error bars represent SDs from the average of five individual experiments. The rates in the absence of ATP (open) were set as 100% and in the presence of ATP as 0%. The various openers and blockers are analyzed in relation to these set controls.

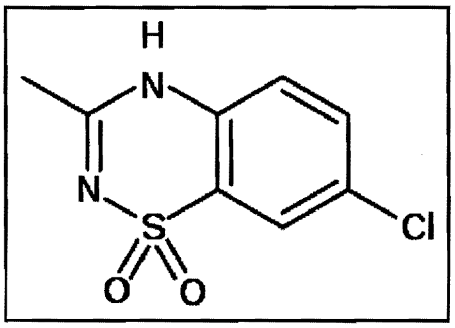
- Diazoxide (DZX) has caused a significant reactivation of the mitoK$_{ATP}$ in both rat and mouse heart mitochondria. This effect is significantly inhibited by 5HD in both study models.
- The openable effects of cromakalim (CRM) on mitoK$_{ATP}$ are significantly inhibited by 5HD in both rat and mouse heart mitochondria.
- The activation of mitoK$_{ATP}$ by PMA is significantly inhibited in the presence of 5HD in both the study models.

Fig 15: Chemical structures of K_{ATP} channel activators

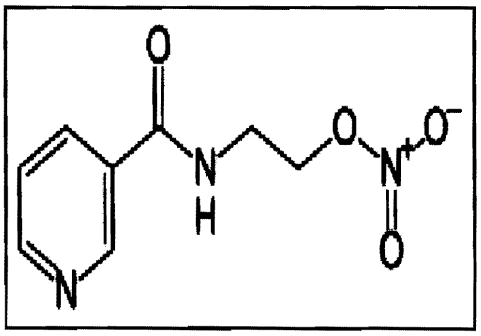
Cromakalim



Diazoxide



Nicorandil



PMA

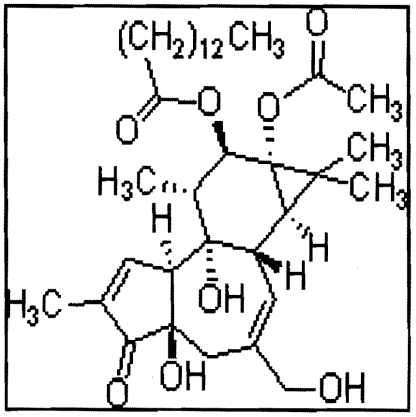
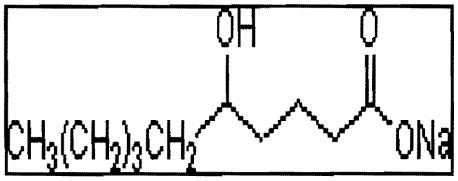
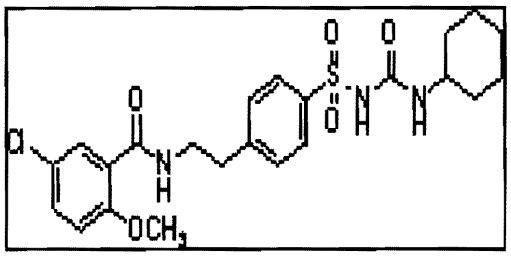


Fig 16: Chemical structures of K_{ATP} blockers

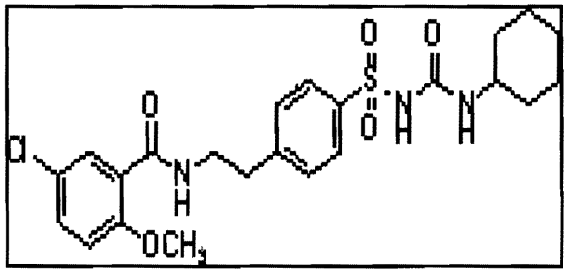
5 hydroxydecanoate (5 HD)



Glibenclamide



Glyburide



CHAPTER 6

DISCUSSION:

Mitochondrial ATP sensitive potassium channels (mitoK_{ATP}) are believed to be integral in the physiology of cardioprotection and cardiac ischemia related manifestations [46, 47, 72, 77]. The various cardioprotective drugs such as diazoxide are thought to act through the mitoK_{ATP} and hence involved in cardioprotection, as evidenced by the infarct size post ischemic-preconditioning and ischemia-reperfusion injury [46, 47, 51, 75, 78, 82].

ATP-sensitive potassium channels (K_{ATP}) are found in a variety of tissues that include pancreatic β-cells, neurons of brain parenchyma, heart, skeletal and smooth muscle cells [1]. Cardiac cells contain two divergent K_{ATP} channels, the classical one in the sarcolemma (cellK_{ATP}) [1] and the other in the inner membrane of the mitochondria (mitoK_{ATP}) [44, 73]. Garlid et al. observed that the mitoK_{ATP} exhibited several properties comparable to those of the cellK_{ATP}. [45, 69, 77, 90]

CellK_{ATP} are implicated in a variety of roles and are implemental in transitioning the metabolic status of a cell into a physiological effect [21, 86, 87, 88, 89]. Kir and SUR subunits, that are heteromultimers, constitute the cellK_{ATP} [29, 31, 69, 90]. Kir is believed to assemble with SUR as a 4:4 complex and function as an octameric channel [29, 31, 41, 42, 69, 90]. The SURs are members of the ATP-binding cassette (ABC) family [29, 31, 69, 77]. The genes that encode SUR1 and SUR2 are mostly

homologous, while SUR2A (cardiac) and 2B (smooth muscle) are splice variants [29, 77, 90]. SUR1 is thought to regulate the pancreatic β channel activity, SUR2A the cardiac channel, and SUR 2B the smooth muscle channel [29, 30, 31, 77]. Kir channels are tetramers and have two transmembrane domains linked by a pore loop. [29, 30, 31, 37, 77]

The subunits that configure the mitoK_{ATP} are thought to be qualitatively equivalent to the cellK_{ATP} [77, 90]. The activity of mitoK_{ATP} is modulated by various biochemical and pharmacological agents, some reacting with mitoSUR (mitochondrial sulfonylurea receptor), and others with mitoKir (mitochondrial inward rectifying potassium channel receptor) [Fig 2] [77, 78, 90]. Garlid et al. showed that mitoK_{ATP} is a high affinity receptor for K^+ channel openers (KCOs), including cromakalim, diazoxide and PMA (a PKCε activator), and this activation is inhibited in the presence of 5 HD and glibenclamide. [46, 47, 48, 72, 75, 90]

Through my experiments I have tried to characterize the activity of mitoK_{ATP} in the mitochondria of control mouse by testing the activity of this channel in the presence of various specific and non-specific potassium channel openers and their blockers. It was evident from my results that the channel was significantly active in the mouse heart mitochondria. The light scattering traces initiated in the presence of various KCOs and their inhibitors showed results that were comparable to those in the rat heart mitochondria [Fig 13 & 14]. The various selective and non-selective activators and

inhibitors of the channel exhibited their activity at a concentration that was identical to that used for the rat heart mitochondria. Diazoxide, a selective KCO activated the channel in 30 μM range, a concentration at which a significant channel activity was witnessed in rat heart. This activator effect of diazoxide was inhibited by 5 HD (300 μM) and glibenclamide (10 μM). Cromakalim, a non-selective KCO demonstrated significant channel opening when added as a 50 μM final concentration. The channel activity was inhibited by both 5 HD and glibenclamide. Phorbol 12-myristate-13-acetate (PMA), which is a PKCε (Protein Kinase C epsilon) activator, caused a significant activation of the mitoK_{ATP} in the 0.2 μM range; an effect that was inhibited by 5-hydroxydecanoate (5 HD). Channel activity was evident considering and comparing the rates of various light scattering traces initiated.

The mitoK_{ATP} activity, having been characterized in control mouse heart mitochondria, would eventually be an ideal platform to explore further the structural components of the mitoK_{ATP}. The model of transgenic mouse/genetically modified mouse, powered with the light scattering technique and immunological tools such as western blots and immunoflorescence techniques, would be one of the most ideal and logistic extensions in identifying and establishing the subunit assembly of the mitoK_{ATP}. Also, from the data analysis it seems evident that the mitoK_{ATP} activity exhibited by control mouse heart mitochondria is similar and comparable to that exhibited by the rat heart mitochondria considering the channel activity exhibited in the presence of various potassium channel openers and their blockers. The data

validates the use of the mouse as a comprehensive study model for the analysis of mitoK_{ATP} activity in the heart mitochondria.

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APPENDIX A: Mouse heart mitochondria isolation protocol

Solutions

Buffer A		*Buffer B*	
Sucrose	250 mM	Sucrose	250 mM
HEPES pH 7.2	10 mM	HEPES pH 7.2	10 mM
EGTA	5 mM	EGTA	5 mM
BSA	5 mg/mL	[i.e. Buffer A without BSA]	

Procedure

1. Anesthetize 2-3 mice with carbon dioxide *(~30 sec)*

2. Quickly remove heart(s) and place in a 20 mL beaker, on ice, filled with ice-cold Buffer B.

3. Using a squeezing action, manually remove as much blood as possible. Decant off the bloody buffer. *(~15 sec)*

4. Add 10-15 mL of Buffer B supplemented with 1mg/mL protease (Nagarse – protease Sigma Type XXIV) and cut into very small pieces with a sharp scissor. *(30-60 sec)*

5. Pour the diced suspension into a 50 mL, ice-jacketed Potter-Elvegen tissue grinder with Teflon pestle, add 30-40 mL ice-cold Buffer A and homogenize at moderate speed (75% of max). Usually 5-6 strokes are enough for a good homogenization. NOTE: Additional strokes will not necessarily increase protein yield, but certainly will increase the time of isolation and thus mitochondria exposure to protease, although Buffer A contains 0.5% BSA. *(30-60 sec)*

6. Pour the homogenate into two (2) pre-cooled 50 mL centrifuge tubes and centrifuge 3 minutes x 1500g.

7. Decant the supernatant into two, clean, pre-chilled 50 mL centrifuge tubes and centrifuge 5 minutes x 10,000g.

8. Discard the supernatants and resuspend each of the pellets in ~0.5 mL of Buffer B using a pipetor to gently "wash" the pellets into homogeneous suspension. Combine all suspensions into one (1) 50 mL centrifuge tube, fill with Buffer B, and centrifuge 3 minutes x 2300g.

9. Carefully decant the supernatant (containing the mitochondrial fraction) into a clean, pre-chilled 50 mL centrifuge tube. Discard the "fluffy" pellet that results from this low-speed spin.

10. Centrifuge the supernatant for 5 minutes x 10,000 *g*.

11. Pour off (discard) the supernatant, and resuspend the pellet in ~150 to 200 μL of Buffer B (see Step 8, above).

12. Alternatively, the centrifugation times can be cut to 3-5-3-5 min, respectively