Distinct $K_{\text{ATP}}$ Channels Mediate the Antihypertrophic Effects of Adenosine Receptor Activation in Neonatal Rat Ventricular Myocytes

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Running title: $K_{ATP}$ channels mediate antihypertrophic effects of adenosine

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Number of text pages: 26

Number of tables: 1

Number of figures: 9

Number of references: 20

Number of words in the Abstract: 209

Number of words in the Introduction: 401

Number of words in Discussion: 1254
JPET #110494

Abbreviations:

5-HD, 5-hydroxydecanoic acid;

Akt, serine/threonine kinase, protein kinase B

ANP, atrial natriuretic peptide.

AR, adenosine receptor;

CPA, $N^6$-cyclopentyladenosine;

CGS 21680, 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine;

HMR1098, 1-[[5-[2-(5-chloro-o-anisamido)ethyl]-2-methoxyphenyl]sulfonyl]-3-methylthiourea, sodium salt;

IB-MECA, $N^6$-(3-iodobenzyl)adenosine-5'-methyluronamide;

JC-1, 5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide, chloride;

mito$K_{ATP}$, mitochondrial ATP-sensitive potassium channel;

sarc$K_{ATP}$, sarcolemmal ATP-sensitive potassium channel;

PE, phenylephrine

Rhod-2, 1-[2-Amino-5-(3-dimethylamino-6-dimethylammonio-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid, chloride

Section options:

Cardiovascular
Abstract

Recent evidence suggests that both adenosine receptor (AR) and K<sub>ATP</sub> channel activation exert antihypertrophic effects in cardiac myocytes. We studied the relative contributions of mitochondrial K<sub>ATP</sub> (mitoK<sub>ATP</sub>) and sarcolemmal K<sub>ATP</sub> (sarcK<sub>ATP</sub>) to the antihypertrophic effects of ARs in primary cultures of neonatal rat ventricular myocytes exposed for 24 hours with the α1 adrenoceptor agonist phenylephrine (PE). The A<sub>1R</sub> agonist CPA, A<sub>2AR</sub> agonist CGS21680 and the A<sub>3R</sub> agonist IB-MECA all prevented PE-induced hypertrophy. Glibenclamide, a non-selective K<sub>ATP</sub> channel blocker reversed the antihypertrophic effect of all three AR agonists as determined by cell size and ANP expression and early c-fos upregulation. In contrast, the mitoK<sub>ATP</sub> blocker 5-HD selectively attenuated the effect of CGS21680 and IB-MECA whereas HMR1098, a specific blocker of sarcK<sub>ATP</sub>, only abolished the antihypertrophic effect of CPA. Moreover, both CGS and IB-MECA but not CPA decreased the mitochondrial membrane potential when PE was present similarly to that seen with diazoxide and both agents inhibited PE-stimulated elevation in mitochondrial Ca<sup>2+</sup>. All AR agonists diminish PE-induced phospho-Akt upregulation which was unaffected by any K<sub>ATP</sub> blocker. Our data suggest that AR-mediated antihypertrophic effects are mediated by distinct K<sub>ATP</sub> channels with sarcK<sub>ATP</sub> mediating the antihypertrophic effects of A<sub>1R</sub> activation whereas mitoK<sub>ATP</sub> activation mediates the antihypertrophic effects of both A<sub>2AR</sub> and A<sub>3R</sub> agonists.
Both opening of $K_{\text{ATP}}$ channels and activation of adenosine receptors exert cardioprotective effects against ischemic and reperfusion injury (Cohen et al., 2000). Early studies on $K_{\text{ATP}}$ channels suggested that the cardioprotective properties are mediated by sarcolemmal $K_{\text{ATP}}$ activation (Noma 1983; Auchampach et al., 1992). More recent studies have found that opening of mito$K_{\text{ATP}}$ also plays an important role in cardiac protection such as in ischemic preconditioning (Garlid et al., 1997). Cardiac hypertrophy has traditionally been considered as an adaptive response however prolonged hypertrophy is associated with increased risk of sudden death or progression to heart failure (Frey and Olson 2003). Emerging evidence suggests that in addition to their cardioprotective effects, both adenosine receptor and $K_{\text{ATP}}$ activation reduce the remodelling process and inhibit cardiac hypertrophy. For example, the mito$K_{\text{ATP}}$ opener nicorandil has been shown to reduce myocardial remodeling in rats treated with the NO synthase inhibitor L-NAME (Sanada et al., 2002) whereas the putative mito$K_{\text{ATP}}$ opener diazoxide inhibited PE induced cardiac hypertrophy in rat neonatal cardiomyocytes (Xia et al., 2004). Thus, these studies suggest a direct antihypertrophic effect of $K_{\text{ATP}}$ activation in the heart. Recent studies have also demonstrated an antihypertrophic effect of adenosine receptor activation in both cardiac myocytes as well as in a murine cardiac hypertrophy produced by aortic coarctation (Liao et al., 2003; Gan et al., 2005) although the nature of the receptors involved in this phenomenon is not known with certainty. For example, Liao et al. (Liao et al., 2003) showed that only $A_1$ receptor activation could attenuate cardiac hypertrophy although work from our laboratory has shown that multiple receptor subtypes ($A_1R$, $A_2AR$ and $A_3R$) activation share equal efficacy in their ability to attenuate PE-induced hypertrophy (Gan et al., 2005).
Previous studies have shown that $A_1$ receptor stimulation activates $K_{ATP}$ channels in rat cardiac cells (Kirsch et al., 1990). It has also been shown that activation of adenosine $A_1$ receptors induces myocardial preconditioning in the canine heart by opening $K_{ATP}$ channels (Auchampach and Gross, 1993). In view of this evidence and the finding that both diazoxide and adenosine receptor agonists attenuate hypertrophy, we hypothesized that the direct antihypertrophic effects of adenosine receptor stimulation could involve $K_{ATP}$ activation. Accordingly, the present study was designed to determine if $K_{ATP}$ channels mediate the antihypertrophic effect of adenosine receptors in neonatal rat ventricular myocytes and if so, to assess and identify the nature of $K_{ATP}$ involvement in mediating the antihypertrophic effect of adenosine receptor activation.

**Experimental procedures**

*Primary neonatal ventricular myocyte culture*

Myocytes were prepared from the ventricles of 4-day-old Sprague-Dawley rats as described in detail previously (Gan et al., 2003). In brief the ventricles were excised, washed and cut into small pieces in 15 ml Hanks’ Balanced Salt Solution (HBSS) (Invitrogen, Burlington, ON, Canada), then digested in 60 ml of HBSS containing 800 U collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA)/ventricle. The digestion was performed in a circulating water bath to maintain the reaction temperature at $37^\circ$C. The digestion was terminated by adding an identical volume of 20% Fetal Bovine Serum (FBS). The cells were sorted by a cell strainer to remove undigested particles and then centrifuged at 600 x g for 5 min at $4^\circ$C. The cell pellet was resuspended in a plating medium containing 10% FBS and 0.1 mM bromodeoxyuridine, and was preplated in tissue culture flasks for two times of 20 min to reduce
contaminating non-myocytes after which the cells were transferred into Primaria™ cell culture dishes (Becton Dickinson Labware, Mississauga, ON, Canada) and cultured for 48 hours. The medium was replaced with a serum-free maintenance medium and incubated for another 24 hours before being used for study. Approximately 95% of cells prepared by this method demonstrated sarcomeric myosin heavy chain staining, indicating relatively low nonmyocyte contamination (Rajapurohitam et al., 2003).

Drugs used and experimental design

In order to first determine if the activation of adenosine receptors inhibited PE (10 μM) induced cardiomyocytes hypertrophy via $K_{ATP}$ channels, we assessed the effect of $K_{ATP}$ inhibition with the following pharmacological agents which were added 30 minutes prior to adenosine receptor activation: the mito$K_{ATP}$ blocker 5–HD (100 μM), the sarc$K_{ATP}$ blocker HMR 1098 (100 μM), or the nonspecific $K_{ATP}$ blocker glibenclamide (50 μM). The following adenosine receptor agonists were then administered for a further 30 minutes after which PE was administered for 24 hours: the A₁R agonist CPA (1 μM), the A₂₅R agonist CGS21680 (100 nM) or the A₃R agonist IB-MECA (100 nM). For some experiments, the putative mito$K_{ATP}$ opener diazoxide (100 μM) was used as a positive control. All drugs were purchased from Sigma (Oakville, ON, Canada) except HMR 1098 which was a kind gift from Sanofi-Aventis, Frankfurt, Germany.

Cell surface area measurement

The cells were plated at a density of $1 \times 10^6$ cells/6 cm dish to obtain individually plated cells. At the end of the treatment period the cells were washed twice with PBS after which they were
viewed using a Leica DMIL inverted microscope equipped with a Polaroid digital camera. For each sample (n=1) eight random images were taken and at least 40 individual cell surface area measurements were made using Mocha software.

**Real-time PCR**

Myocytes were plated at a density of 6 x 10^6 cells/6 cm dish. After washing twice with PBS, RNA was isolated by adding 1 ml Trizol reagent (Invitrogen, Burlington, ON, Canada) to each dish. 5 μg of total RNA were applied for reverse transcription by Superscript II reverse transcriptase (Invitrogen). 1 μl from the 20 μl cDNA product was used for each PCR reaction. Real time PCR was performed with a DNA Engine Opticon Real Time System (MJ Research, Waltham, MA, USA) with SYBR Green JumpStart Taq ReadyMix kit (Sigma) according to the manufacturer’s instructions. Primer sequences for individual genes and PCR conditions are shown in Table 1.

**Fluorescence measurement of mitochondrial membrane potential (∆Ψ_m) and mitochondrial Ca^{2+} concentration**

Cells were plated at 1 x 10^6 cells/well in 24 well dishes. After the cells were treated with PE for 30 min, the mitochondrial membrane potential (∆Ψ_m) was measured by loading cells with 10 μg/ml of JC-1 (Molecular Probes, Oregon, USA) at 37°C for 15 min and the mitochondrial Ca^{2+} concentration ([Ca^{2+}]_m) was monitored with the Ca^{2+} fluorophore Rhod-2 (Molecular Probes, Oregon, USA). The cardiomyocytes were loaded with 10 μg/ml Rhod-2 for 120 minutes at 4°C and then incubated for 30 minutes at 37°C in the culture media. This 2-step cold loading/warm incubation protocol achieved exclusive loading of Rhod-2 into the mitochondria (Trollinger et
al., 2000). Myocytes loaded with JC-1 or Rhod-2 were washed with PBS and the fluorescence was measured by a TECAN multifunction microplate reader. JC-1 was excited at 488nm, the red emission fluorescence was detected at 595nm, and the green emission fluorescence was detected at 535nm. The $\Delta \Psi_m$ is presented as a ratio of f595 nm/535 nm compared to control cells. Rhod-2 was excited at 540 nm, with emission monitored at 605 nm.

**Confocal fluorescence imaging of $\Delta \Psi_m$**

Cells were plated at 3 x $10^6$ cells/ 3.5 cm glass bottom dish precoated with type I collagen. After the cells were treated with PE for 30 min, the cells were washed with PBS and JC-1 was added to each well to reach a final concentration as 10 $\mu$g/ml. The cells were incubated at 37°C for 30 min and then washed with PBS 3 times. Images were recorded on a Zeiss LSM 510 confocal microscope. JC-1 was excited at 488 nm by an argon ion laser with red and green emissions detected at 568 nm and 510 nm, respectively.

**Western blotting for phosphorylated and total Akt**

Cells were plated at a concentration of 6 x $10^6$ cells/ 6 cm dish. After washing twice with PBS the cells were scraped into 100 $\mu$l lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton-X 100, 10% Glycerol, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 200 $\mu$M Na$_3$VO$_4$, 10 mM Na$_4$P$_2$O$_7$, 40 mM b-glycerophosphate, 10 $\mu$g/ml leupeptin, 1 $\mu$M pepstatin A, 1 mM PMSF, and 1 $\mu$M colyculin A). The lysate was transferred to 1.5 ml Eppendorff tubes, sonicated and then centrifuged at 10000 x g for 5 min at 4°C. The supernatant was transferred to a fresh tube and the protein concentration was assayed by Bradford Protein Assay Kit (Bio-Rad, Mississauga, ON, Canada). 30 $\mu$g protein were loaded in 10% SDS-PAGE, and transferred to nylon
membranes (Amersham, Little Chalfont Buckinghamshire, UK). The membranes were blocked in 5% dry milk for 3 hours with primary antibody for 2 hours and secondary antibody for 1 hour, and then detected by ECL reagent (Amersham, Little Chalfont Buckinghamshire, UK). After detection by phospho-Akt (ser473) antibody, the same blot was stripped and reprobed by total Akt antibody to demonstrate equal sample loading. Antibodies against phospho-Akt (ser473) and total Akt were purchased from Cell Signaling (Beverly, MA, USA) and used at a 1:1000 dilution.

Statistical analysis

All values in the figures and text are presented as mean ± SEM. Sample size per experiment is indicated in the results. Data were analyzed by one-way ANOVA followed by a Tukey multiple comparison test using Prism (GraphPad Software) with P<0.05 considered to represent significant differences between groups.

Results

Influence of KATP blockers on antihypertrophic effect of adenosine receptor agonists

In order to identify the concentration of adenosine receptor agonists producing maximum inhibition of PE induced hypertrophy, increasing concentrations of these agents were used to perform the cell surface area experiment. As shown in Figure 1, 1 μM CPA, 100 nM CGS and 100 nM IB-MECA produced maximum inhibition of PE induced increase in cell surface area. Furthermore, our previous results have shown that the antihypertrophic effects of all three agonists can be completely reversed by their respective antagonists thus suggesting a receptor-specific effect of these agonists (Gan et al., 2005). We next determined if the inhibition of PE induced cardiomyocytes hypertrophy by adenosine receptor agonists is mediated by $K_{ATP}$ channels using primarily pharmacological approach. As shown in Figure 2, pharmacological
blockers of $K_{\text{ATP}}$ had diverse effects on the antihypertrophic effects of adenosine receptor agonists which, in general, reflected the nature of the adenosine agonist used. In the case of CPA, all three $K_{\text{ATP}}$ blockers reversed the inhibitory effect of CPA against PE-induced hypertrophy although the effect of 100 µM 5-HD was lower than that seen with either 100 µM HMR1098 or 50 µM glibenclamide. However, in the case of CGS21680 or IB-MECA, the antihypertrophic effect could be reversed by either 5-HD or glibenclamide whereas the sarc$K_{\text{ATP}}$ specific blocker HMR 1098 was without effect. Analysis of molecular markers of hypertrophy (ANP and $c$-fos mRNA expression) revealed generally identical responses to $K_{\text{ATP}}$ inhibitors as shown in Figures 3 and 4. Thus, inhibition of both ANP and $c$-fos upregulation by CPA was abrogated by both HMR 1098 and glibenclamide whereas 5-HD was without effect. With respect to either CGS 21680 or IB-MECA, both 5-HD and glibenclamide significantly reversed the antihypertrophic effects of the adenosine agonists vis a vis ANP and $c$-fos upregulation (Figures 3 and 4) akin to that seen with respect to cell surface area reported in Figure 2. Overall, these data suggest that the antihypertrophic effect of the A1R agonist CPA is dependent on sarc$K_{\text{ATP}}$ whereas mito$K_{\text{ATP}}$ mediates the antihypertrophic influence of $A_{2A}$R and $A_{3}$R activation.

The effect of adenosine receptor agonists on mitochondrial membrane potential in neonatal cardiomyocytes

To obtain direct evidence that CGS 21680 and IB-MECA inhibited PE induced hypertrophy through opening mito$K_{\text{ATP}}$ channels, the mitochondria membrane potential ($\Delta \Psi_{m}$) of these cells was measured. The cells were labeled with 10 µg/ml JC-1, and the fluorescence at 595nm and 535nm were measured by a fluorescence plate reader and imaged by confocal microscopy. Mitochondrial depolarization was indicated by a decrease of the ratio of f595/f535. Figure 5
shows the changes in ΔΨm when cells were treated with adenosine receptor agonists and diazoxide with or without PE. Neither diazoxide nor adenosine receptor agonists alone exerted any effect on ΔΨm compared to control cells (Figure 5A, white bars). However, in the presence of PE (Figure 5A, black bars) diazoxide significantly reduced ΔΨm by 23% in cells exposed to PE. Similarly, both CGS21680 and IB-MECA significantly reduced ΔΨm by 18% and 34%, respectively in cells exposed to PE. In contrast, CPA was without effect on ΔΨm.

The effect of diazoxide and adenosine receptor agonists on [Ca2+]m

We next examined treatments on [Ca2+]m in neonatal cardiomyocytes by Rhod-2 staining. As shown in Figure 6 panel A, PE significantly increased [Ca2+]m by 23% whereas this was completely abrogated by diazoxide as well as CGS 21680 and IB-MECA. In contrast, CPA was without effect. The data in panel B shows that the mitochondrial Ca2+ uniport inhibitor ruthenium red inhibited the Ca2+ overload induced by 100 µM H2O2 in mitochondria confirming that the Rhod-2 staining method is specific for mitochondrial Ca2+ measurement.

Effect of activation of adenosine receptors on Akt expression

In order to further address potential mechanisms underlying the antihypertrophic effects of adenosine receptor activation and the role of K_ATP we determine the potential contribution of Akt activation which has been implicated as a major contributor to the cardiac hypertrophic program. We first determined if PE or adenosine receptor agonists could activate Akt on their own. Interestingly, as shown in Figure 7, a significant increase in Akt phosphorylation was observed 30 min after PE administration whereas each of the adenosine receptors agonists increased Akt phosphorylation only 5 min after addition of the agent. As shown in Figure 8, although
adenosine receptor agonists had no effect on Akt phosphorylation at 30 min, all agents significantly prevented the effect of PE on Akt phosphorylation at this time point. However, this was unaffected by $K_{ATP}$ blockers. In view of these findings we next examined if the antihypertrophic effects of mito$K_{ATP}$ channels opening can also be dissociated from Akt activation. As shown in Figure 9, diazoxide had no effect on PE induced Akt activation in neonatal cardiomyocytes.

Discussion

Our study demonstrates for the first time that the antihypertrophic effect of adenosine receptor activation in rat neonatal cardiomyocytes, at least with respect to PE-induced hypertrophy, is dependent on $K_{ATP}$ activation and that distinct $K_{ATP}$ channels mediate the actions of specific adenosine receptors. Based on our results, we propose that sarc$K_{ATP}$ channels mediate the antihypertrophic effects of A$_1$R activation whereas mito$K_{ATP}$ channel activation mediates the antihypertrophic effects of both A$_2$AR and A$_3$R agonists. This reasoning is based on various lines of evidence. First, the overall role of $K_{ATP}$ in mediating the antihypertrophic effect of adenosine receptor activation was strongly suggested by the ability of pharmacological inhibitors of the channels to abrogate the effect of A$_1$R, A$_2$AR and A$_3$R activation. Although the non-specific $K_{ATP}$ blocker glibenclamide reversed the effect of all three AR agonists the sarc$K_{ATP}$ specific inhibitor HMR1098 was able to selectively prevent only the effects of the A$_1$R agonist CPA. In contrast, the mito$K_{ATP}$ specific blocker 5-HD had no effect on the antihypertrophic effect of CPA whereas it blocked the effects of both the A$_2$AR and A$_3$R agonists. These differences were particularly evident in terms of molecular hypertrophic markers although surprisingly 5-HD partially reversed
the antihypertrophic effect of CPA as determined by cell surface area although to a lesser degree than that seen with HMR1098. The basis for the diminished effect of 5-HD on cell surface area data compared to molecular indices of hypertrophy is uncertain although it may reflect a non-specific effect of 5-HD on cell size due to the drug’s fatty acid moiety.

Mitochondrial $K_{ATP}$ channel activation is associated with numerous effects including membrane depolarization and changes in $Ca^{2+}$ homeostasis (Holmuhamedov et al., 1998). Accordingly, to obtain further evidence that activation of $A_2A$R and $A_3$R opens mito$K_{ATP}$ channels, we assessed the changes in mitochondrial membrane potential and mitochondrial $Ca^{2+}$ uptake in intact myocytes using the fluorescence dyes JC-1 and Rhod-2, respectively. Diazoxide was used as a positive control although surprisingly, it was without effect on either mitochondrial membrane potential or mitochondrial $Ca^{2+}$ content on its own. However, diazoxide was able to depolarize the membrane and decrease $Ca^{2+}$ accumulation in the presence of PE suggesting that the mito$K_{ATP}$ channel does not play an important role in mitochondrial homeostasis under normal condition but its activation modulates the responses to cellular stimulation such as that in response to PE. Our finding with diazoxide is in agreement with a study by Ishida et al. (Ishida et al., 2001) who showed that diazoxide was without direct effects on mitochondrial membrane potential or $Ca^{2+}$ levels in ventricular myocytes but abrogated ouabain-induced mitochondrial $Ca^{2+}$ levels which was associated with decreased JC-1 fluorescence. Interestingly, in our study both the $A_2A$R agonist CGS21680 and $A_3$R agonist IB-MECA were also able to decrease the mitochondrial membrane potential as well as $Ca^{2+}$ accumulation in PE-treated myocytes similarly to that seen with diazoxide although the $A_1$R agonist CPA was without effect. It is important to note that mitochondrial $Ca^{2+}$ uptake is driven by the mitochondrial membrane potential (Gunter
and Pfeiffer 1990). It is therefore likely that A2AR and A3R activation opens mitochondrial KATP and results in the decreases in the mitochondria membrane potential, therefore reducing the driving force of Ca^{2+} influx and thus attenuating the mitochondria Ca^{2+} overload induced by PE. Thus, a reduction in “mitochondrial remodelling” may constitute an important contributor to the antihypertrophic effect of both A2AR and A3R activation. A brief depolarization of mitochondrial membrane may exert cardioprotection by preventing Ca^{2+} entry into the matrix. However, a prolonged reduction of mitochondrial membrane potential may indicate opening of the mitochondrial permeability transition pore. Indeed, we have demonstrated such a potential contribution of mitochondria to post-infarction responses in rats treated with a sodium-hydrogen exchange inhibitor (Javadov et al., 2005).

Although our data support the hypothesis that both A2AR and A3R activation inhibit PE induced cardiac hypertrophy through opening of mitoK_{ATP} channels in neonatal cardiomyocytes, the phenomenon is difficult to explain at present particularly because the structure and pharmacological profile of these two receptors are quite different. Although it could be suggested that rat cardiomyocytes do not express A3 receptor and IB-MECA induces unspecific effect through A2A receptor rather than A3 receptor we have found using Western blotting that the A1R, A2AR and A3R are all expressed in neonatal cardiomyocytes (data not shown). Thus, it is unlikely that the effects of the receptor agonists were due to nonspecific effects of these agents, although this cannot be ruled out with certainty at this time.

A question that remains concerns the molecular and cellular bases for the antihypertrophic effect of adenosine receptor activation and contributing roles of K_{ATP} channels. Mitogen-activated protein kinase kinase / extracellular signal-regulated kinase1 and 2 (ERK1/2) and
phosphatidylinositol 3-kinase (PI3-kinase)/Akt (also known as PKB) are important antiapoptotic signaling pathways which have recently been implicated in cardiac hypertrophy (Dorn and Force, 2005). However, we previously showed that ERK1/2 was not involved in the antihypertrophic effect of either diazoxide (Xia et al., 2004) or activation of adenosine receptors (Gan et al., 2005). In the current study, we focused on the potential contribution of Akt by determining the effects of treatments on Akt phosphorylation/activation. Indeed PE induced a potent Akt upregulation which reached maximum 30 min after PE addition whereas each of the three adenosine receptor agonists induced a much earlier (5 min) and transient Akt upregulation. This finding is generally similar to that reported by Germack et al. (2004) who demonstrated A1R and A3R but contrary to our findings, not A2AR-dependent Akt activation in neonatal cardiomyocytes with peak upregulation of Akt at 5 min with reversal to control by 30 minutes. Thus, adenosine receptor agonists appear to have complex effects on Akt activation, a direct early and transient direct activation and an ability to prevent subsequent PE-induced Akt phosphorylation. The basis for these diverse effects of adenosine receptor agonists is uncertain at present but the results suggest that prevention of Akt upregulation is associated with the antihypertrophic effects of adenosine receptor agonists. However, KATP blockers which reversed the antihypertrophic effects of adenosine receptor agonists failed to reverse the inhibitory effects of adenosine agonists on Akt phosphorylation. Moreover, diazoxide, which prevents PE-induced hypertrophy (Xia et al., 2004) failed to inhibit PE-induced Akt upregulation. These data suggest that KATP channel opening likely occurs downstream to Akt activation, which serves to explain why neither KATP blockers or diazoxide exerted any effect on inhibition of Akt activation by adenosine receptor agonists. When taken together, our results suggest a potential role of Akt activation in mediating the hypertrophic effect...
of PE. The ability of adenosine receptor agonists to prevent Akt activation are encouraging in terms of implicating Akt in the antihypertrophic effect of adenosine receptor activation. However, our results clearly indicate that the degree of Akt phosphorylation can also be dissociated from the hypertrophic response in view of the inability of K_ATP blockers to reverse Akt inhibition by adenosine receptor agonists despite their ability to reverse the antihypertrophic effect of these agents.

In conclusion, our study shows an important role for K_ATP in mediating the antihypertrophic effects of multiple adenosine receptor subtype activation. Based on our results we propose that the antihypertrophic effect of A_1R activation is dependent on sarcK_ATP channel activity whereas mitoK_ATP activity mediates the antihypertrophic effects of both A_2A and A_3 receptor activation. Although prevention of Akt phosphorylation was associated with the antihypertrophic effects of adenosine receptor activation, the precise role of this pathway needs to be studied further.
References


Footnotes

This work was supported by a grant from the Canadian Institutes of Health Research. M Karmazyn holds a Canada Research Chair in Experimental Cardiology.
Figure Legends

Figure 1
Dose-dependent effect of A1R agonist CPA (panel A), the A2AR agonist CGS21680 (panel B) and the A3R agonist IB-MECA (panel C) on inhibition of 10 µM phenylephrine (PE) induced hypertrophy as assessed by cell surface area. Values indicate mean ± SEM of N=4. *p<0.05 vs control values obtained in the absence of any treatment.

Figure 2
Effect of the KATP channel blockers 5-HD (100 µM), HMR 1098 (100 µM) and glibenclamide (50 µM) on the antihypertrophic effects of the A1R agonist CPA (1 µM), the A2AR agonist CGS21680 and the A3R agonist IB-MECA (100 nM) as assessed by cell surface area. Hypertrophy was produced by 24 hour exposure to 10 µM phenylephrine (PE). Values indicate mean ± SEM of N=6. ‡ ‡ p<0.01 vs control values obtained in the absence of any treatment, ##p<0.01 vs PE, *p<0.05, **p<0.01 vs PE+AR.

Figure 3
Effect of the KATP channel blockers 5-HD (100 µM), HMR 1098 (100 µM) and glibenclamide (50 µM) on the antihypertrophic effects of the A1R agonist CPA (1 µM), the A2AR agonist CGS21680 and the A3R agonist IB-MECA (100 nM) as assessed by ANP expression. Hypertrophy was produced by 24 hour exposure to 10 µM phenylephrine (PE). Values indicate mean ± SEM of N=6. ‡ p<0.05 vs control values obtained in the absence of any treatment, #p<0.05 vs PE, *p<0.05 vs PE+AR.

Figure 4
Effect of the $K_{ATP}$ channel blockers 5-HD (100 µM), HMR 1098 (100 µM) and glibenclamide (50 µM) on the antihypertrophic effects of the $A_1$R agonist CPA (1 µM), the $A_2A$R agonist CGS21680 and the $A_3$R agonist IB-MECA (100 nM) as assessed by upregulation of $c-fos$ expression. Hypertrophy was produced by 24 hour exposure to 10 µM phenylephrine (PE). Values indicate mean ± SEM of N=6, ‡ $p<0.05$ vs control values obtained in the absence of any treatment, # $p<0.05$ vs PE, * $p<0.05$ vs PE+AR.

**Figure 5**

The effect of diazoxide (100 µM) and adenosine receptor agonists on neonatal cardiomyocytes mitochondrial membrane potential in the presence or absence of 10 µM phenylephrine (PE). Panel A, quantitative JC-1 fluorescence values. The value of control was considered as 100%. Values indicate mean ± SEM of N=6 * $p<0.05$. Panel B, representative confocal images of JC-1 staining. The decrease of red/green ratio indicates the decrease of mitochondrial membrane potential. The bar in the images represents 50 µm.

**Figure 6**

The effect of diazoxide (100 µM) and adenosine receptor agonists on neonatal cardiomyocytes mitochondrial $Ca^{2+}$ uptake in the presence or absence of 10 µM phenylephrine (PE) measured by Rhod-2 staining (panel A), the value of control was considered as 100% . Values indicate mean ± SEM of N=6. * $p<0.05$. The effect of mitochondrial $Ca^{2+}$ uniport inhibitor ruthenium red on neonatal cardiomyocytes mitochondrial $Ca^{2+}$ uptake with or without 100 µM H$_2$O$_2$ treatment
measured by Rhod-2 staining (panel B). N=4, *p<0.05, #p<0.01. Ruthenium red inhibited the Ca^{2+} overload induced by H_{2}O_{2} in mitochondria indicated that this Rhod-2 staining method is specific for mitochondrial Ca^{2+} measurement.

**Figure 7**

Time-dependent effect of A_{1}R agonist CPA 1 \mu M (square), the A_{2A}R agonist CGS21680 10 nM (triangle), the A_{3}R agonist IB-MECA 10 nM (circle) and phenylephrine 10 \mu M (diamond) on Akt phosphorylation in neonatal cardiomyocytes. Representative western blots for phospho-Akt and Akt are shown above the chart. Values indicate mean ± SEM of N=3.

**Figure 8**

The effect of adenosine receptor activation on Akt phosphorylation induced by 30 minutes 10 \mu M PE treatment. Representative western blots for phospho-Akt and Akt are shown below the appropriate treatment groups. Values indicate mean ± SEM of N=3. *p<0.05 vs control values obtained in the absence of any treatment. #p<0.05 vs PE.

**Figure 9**

Lack of effect of 100 \mu M diazoxide (Dia) on phenylephrine (PE)-induced Akt phosphorylation. Values indicate mean ± SEM of N=3, *p<0.05 vs control.
Table 1A. Primer sequences for real time PCR analysis of various gene products

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Table 1B. Real-time PCR conditions

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<td>72°C 30 sec</td>
<td></td>
</tr>
<tr>
<td>c-fos</td>
<td>94°C 20 sec</td>
<td>34</td>
<td>58°C 25 sec</td>
<td>72°C 30 sec</td>
<td>72°C 5 min</td>
<td></td>
</tr>
<tr>
<td>18S</td>
<td>94°C 20 sec</td>
<td>34</td>
<td>55°C 20 sec</td>
<td>72°C 30 sec</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1

A. Relative Cell Surface Area (fold increase)

B. Relative Cell Surface Area (fold increase)

C. Relative Cell Surface Area (fold increase)
Figure 2

CPA

Relative Cell Surface Area (fold increase)

Phenylephrine - + + + + +
AR agonist                  - - + + + + +
5-HD                            - - - +            - -
HMR1098                     - ---+            -
Glibenclamide - - - - - +

CGS21680

Relative Cell Surface Area (fold increase)

IB-MECA

Relative Cell Surface Area (fold increase)

Phenylephrine - + + + + +
AR agonist                  - - + + + + +
5-HD                            - - - +            - -
HMR1098                     - ---+            -
Glibenclamide - - - - - +
Figure 3

Phenylephrine - +           +            +           +           +
AR agonist                - - +           +            +           +
5-HD                           - - - +            - -
HMR1098                   - - - - +           -
Glibenclamide - - - - - +

ANP Expression/18s (fold increase)

CPA

CGS21680

IB-MECA

*‡#
Figure 4

**Phenylephrine**

- AR agonist: +
- 5-HD: -
- HMR1098: -
- Glibenclamide: -

**AR agonist**

- +
- +
- +
- +
- +

**5-HD**

- -
- -
- +
- -
- -

**HMR1098**

- -
- -
- +
- -

**Glibenclamide**

- -
- -
- -
- -
- +

---

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Figure 5

A.

Mitochondrial Membrane Potential (% Control)

PE

Control  Diazoxide  CPA  CGS21680  IB-MECA

B.

Control  Diazoxide  CPA  CGS21680  IB-MECA

PE  Diazoxide+PE  CPA+PE  CGS+PE  IB-MECA+PE
Figure 6

A.

Mitochondrial Ca\(^{2+}\) Concentration (% Control)

PE
- + Control Diazoxide CPA CGS21680 IB-MECA

B.

Mitochondrial Ca\(^{2+}\) Concentration (% Control)

\(H_2O_2\)
- + Control Ruthenium Red
Figure 7

The figure shows Western blot analysis of pAkt expression in response to various treatments at different time points.

- **C PE CPA**: Samples at 5, 10, 15, 30, and 60 minutes.
  - 5 min: pAkt, Akt
  - 10 min: pAkt, Akt
  - 15 min: pAkt, Akt
  - 30 min: pAkt, Akt
  - 60 min: pAkt, Akt

- **C PE CGS**: Samples at 5, 10, 15, 30, and 60 minutes.
  - 5 min: pAkt, Akt
  - 10 min: pAkt, Akt
  - 15 min: pAkt, Akt
  - 30 min: pAkt, Akt
  - 60 min: pAkt, Akt

- **C PE IB-MECA**: Samples at 5, 10, 15, 30, and 60 minutes.
  - 5 min: pAkt, Akt
  - 10 min: pAkt, Akt
  - 15 min: pAkt, Akt
  - 30 min: pAkt, Akt
  - 60 min: pAkt, Akt

A graph below the blot images depicts the pAkt expression/Akt ratio over time for different treatments (PE, CPA, CGS, IB-MECA) with error bars indicating the fold increase.
Figure 8

Phenylephrine - + - - + + + +
AR agonist - - + + + + + +
5-HD - - - - + - - -
HMR1098 - - - - - + - -
Glibenclamide - - - - - - + +

* pAkt Expression/Akt (fold increase)
# pAkt Expression/Akt (fold increase)

CPA
CGS21680
IB-MECA