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

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Abbreviations:

5-HD,	5-hydroxydecanoic acid;
Akt,	serine/threonine kinase, protein kinase B
ANP,	atrial natriuretic peptide.
AR,	adenonosine 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;
mitoK_{ATP},	mitochondrial ATP-sensitive potassium channel;
sarcK_{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

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Abstract

Recent evidence suggests that both adenosine receptor (AR) and K_{ATP} channel activation exert antihypertrophic effects in cardiac myocytes. We studied the relative contributions of mitochondrial K_{ATP} (mitoK_{ATP}) and sarcolemmal K_{ATP} (sarcK_{ATP}) 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_1R agonist CPA, $A_{2A}R$ agonist CGS21680 and the A_3R agonist IB-MECA all prevented PE-induced hypertrophy. Glibenclamide, a non-selective K_{ATP} 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_{ATP} blocker 5-HD selectively attenuated the effect of CGS21680 and IB-MECA whereas HMR1098, a specific blocker of sarcK_{ATP}, 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^{2+}. All AR agonists diminish PE-induced phospho-Akt upregulation which was unaffected by any K_{ATP} blocker. Our data suggest that AR-mediated antihypertrophic effects are mediated by distinct K_{ATP} channels with sarcK_{ATP} mediating the antihypertrophic effects of A_1R activation whereas mitoK_{ATP} activation mediates the antihypertrophic effects of both $A_{2A}R$ and A_3R agonists.

Both opening of K_{ATP} channels and activation of adenosine receptors exert cardioprotective effects against ischemic and reperfusion injury (Cohen et al., 2000). Early studies on K_{ATP} channels suggested that the cardioprotective properties are mediated by sarcolemmal K_{ATP} activation (Noma 1983; Auchampach et al., 1992). More recent studies have found that opening of mitoK_{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_{ATP} activation reduce the remodelling process and inhibit cardiac hypertrophy. For example, the mitoK_{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 mitoK_{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_{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, $A_{2A}R$ 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 37^0C. 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 4^0C. 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^{TM} 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 mitoK_{ATP} blocker 5–HD (100 μM), the sarcK_{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 A_1R agonist CPA (1 μM), the A_{2A}R agonist CGS21680 (100 nM) or the A_3R agonist IB-MECA (100 nM). For some experiments, the putative mitoK_{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 x 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 ($\Delta\Psi_m$) 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 ($\Delta\Psi_m$) was measured by loading cells with 10 μg/ml of JC-1 (Molecular Probes, Oregon, USA) at 37^0C 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 4^0C and then incubated for 30 minutes at 37^0C 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 μg/ml. The cells were incubated at 37^0C 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 μl lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton-X 100, 10% Glycerol, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 200 μM Na_3VO_4, 10 mM $Na_4P_2O_7$, 40 mM b-glycerophosphate, 10 μg/ml leupeptin, 1 μM pepstatin A, 1 mM PMSF, and 1 μM colyculin A). The lysate was transferred to 1.5 ml Eppendorff tubes, sonicated and then centrifuged at 10000 x g for 5 min at 4^0C. 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 μg 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_{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_{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 sarcK_{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_{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 A_1R agonist CPA is dependent on sarcK_{ATP} whereas mitoK_{ATP} mediates the antihypertrophic influence of $A_{2A}R$ and A_3R 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 mitoK_{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 $\Delta\Psi_m$ 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 $\Delta\Psi_m$ compared to control cells (Figure 5A, white bars). However, in the presence of PE (Figure 5A, black bars) diazoxide significantly reduced $\Delta\Psi_m$ by 23% in cells exposed to PE. Similarly, both CGS21680 and IB-MECA significantly reduced $\Delta\Psi_m$ by 18% and 34%, respectively in cells exposed to PE. In contrast, CPA was without effect on $\Delta\Psi_m$.

The effect of diazoxide and adenosine receptor agonists on $[Ca^{2+}]_m$

We next examined treatments on $[Ca^{2+}]_m$ in neonatal cardiomyocytes by Rhod-2 staining. As shown in Figure 6 panel A, PE significantly increased $[Ca^{2+}]$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 Ca^{2+} uniport inhibitor ruthenium red inhibited the Ca^{2+} overload induced by 100 μM H_2O_2 in mitochondria confirming that the Rhod-2 staining method is specific for mitochondrial Ca^{2+} 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 mitoK_{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 sarcK_{ATP} channels mediate the antihypertrophic effects of A_1R activation whereas mitoK_{ATP} channel activation mediates the antihypertrophic effects of both $A_{2A}R$ and A_3R 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_1R, $A_{2A}R$ and A_3R activation. Although the non-specific K_{ATP} blocker glibenclamide reversed the effect of all three AR agonists the sarcK_{ATP} specific inhibitor HMR1098 was able to selectively prevent only the effects of the A_1R agonist CPA. In contrast, the mitoK_{ATP} specific blocker 5-HD had no effect on the antihypertrophic effect of CPA whereas it blocked the effects of both the $A_{2A}R$ and A_3R 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_3R opens mitoK_{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 mitoK_{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_3R 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_1R 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 $A_{2A}R$ and A_3R activation opens mitochondrial K_{ATP} 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 $A_{2A}R$ and A_3R 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 $A_{2A}R$ and A_3R 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 A_3 receptor and IB-MECA induces unspecific effect through A_{2A} receptor rather than A_3 receptor we have found using Western blotting that the A_1R, $A_{2A}R$ and A_3R 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 antihypertrphic 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 A_1R and A_3R but contrary to our findings, not $A_{2A}R$-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, K_{ATP} 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 K_{ATP} channel opening likely occurs downstream to Akt activation, which serves to explain why neither K_{ATP} 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 parthway needs to be studied further.

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Footnotes

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Figure Legends

Figure 1

Dose-dependent effect of A_1R agonist CPA (panel A), the A_{2A}R agonist CGS21680 (panel B) and the A_3R 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 K_{ATP} channel blockers 5-HD (100 μM), HMR 1098 (100 μM) and glibenclamide (50 μM) on the antihypertrophic effects of the A_1R agonist CPA (1μM), the A_{2A}R agonist CGS21680 and the A_3R 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 K_{ATP} channel blockers 5-HD (100 μM), HMR 1098 (100 μM) and glibenclamide (50 μM) on the antihypertrophic effects of the A_1R agonist CPA (1μM), the A_{2A}R agonist CGS21680 and the A_3R 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<0.05vs 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_1R agonist CPA (1μM), the $A_{2A}R$ agonist CGS21680 and the A_3R 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 rutherium red on neonatal cardiomyocytes mitochondrial Ca^{2+} uptake with or without 100 μM H_2O_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_2O_2 in mitochondria indicated that this Rhod-2 staining method is specific for mitochondrial Ca^{2+} measurement.

Figure 7

Time-dependent effect of A_1R agonist CPA 1 μM (square), the A_{2A}R agonist CGS21680 10 nM (triangle), the A_3R agonist IB-MECA 10 nM (circle) and phenylephrine 10 μM (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 μM 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 μM 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

Gene	**Product Length**	**Primers Sequence**
ANP	320	Sense 5'-CTGCTAGACCACCTGGAGGA-3' Antisense 5'-AAGCTGTTGCAGCCTAGTCC-3'
c-*fos*	435	Sense 5'-GTCTGCGTTGCAGACCGAGATTGC-3' Antisense 5'-CTCCAGCTCTGTGACCATGGG-3'
18S	150	Sense 5'- GTAACCCGTTGAACCCCATT-3' Antisense 5'- CCATCCAATCGGTAGTAGCG-3'

Table 1B. Real-time PCR conditions

Gene	**Initial Denaturation**	**Cycles**	**Denaturation**	**Annealing**	**Extension**	**Prolonged Extension**
ANP	94^0C 15 min	34	94^0C 20 sec	59^0C20sec	72^0C30sec	72^0C5min
c-*fos*		34		58^0C25sec	72^0C30sec	
18S		34		55^0C20sec	72^0C30sec	

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Figure 1

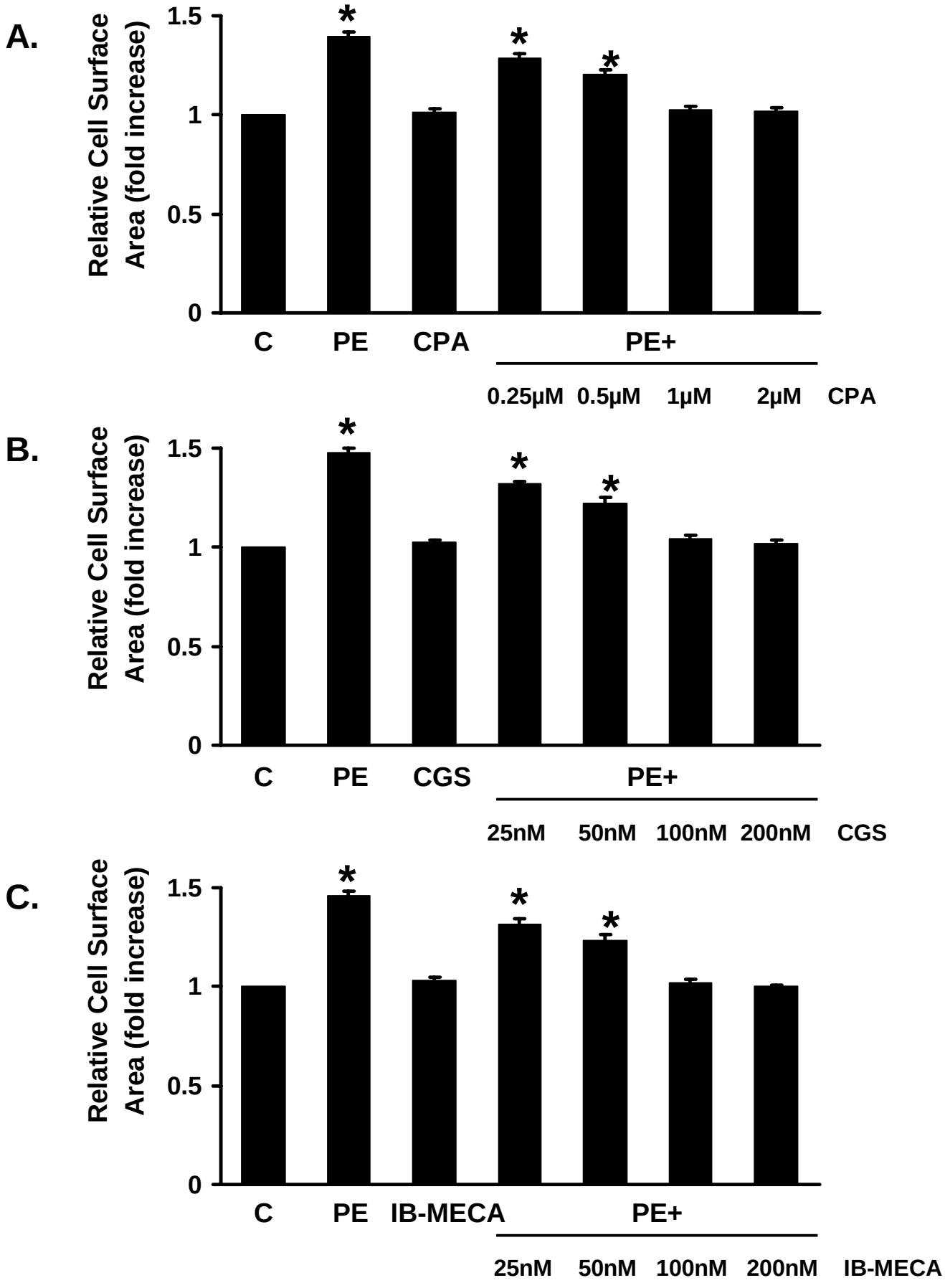

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Figure 2

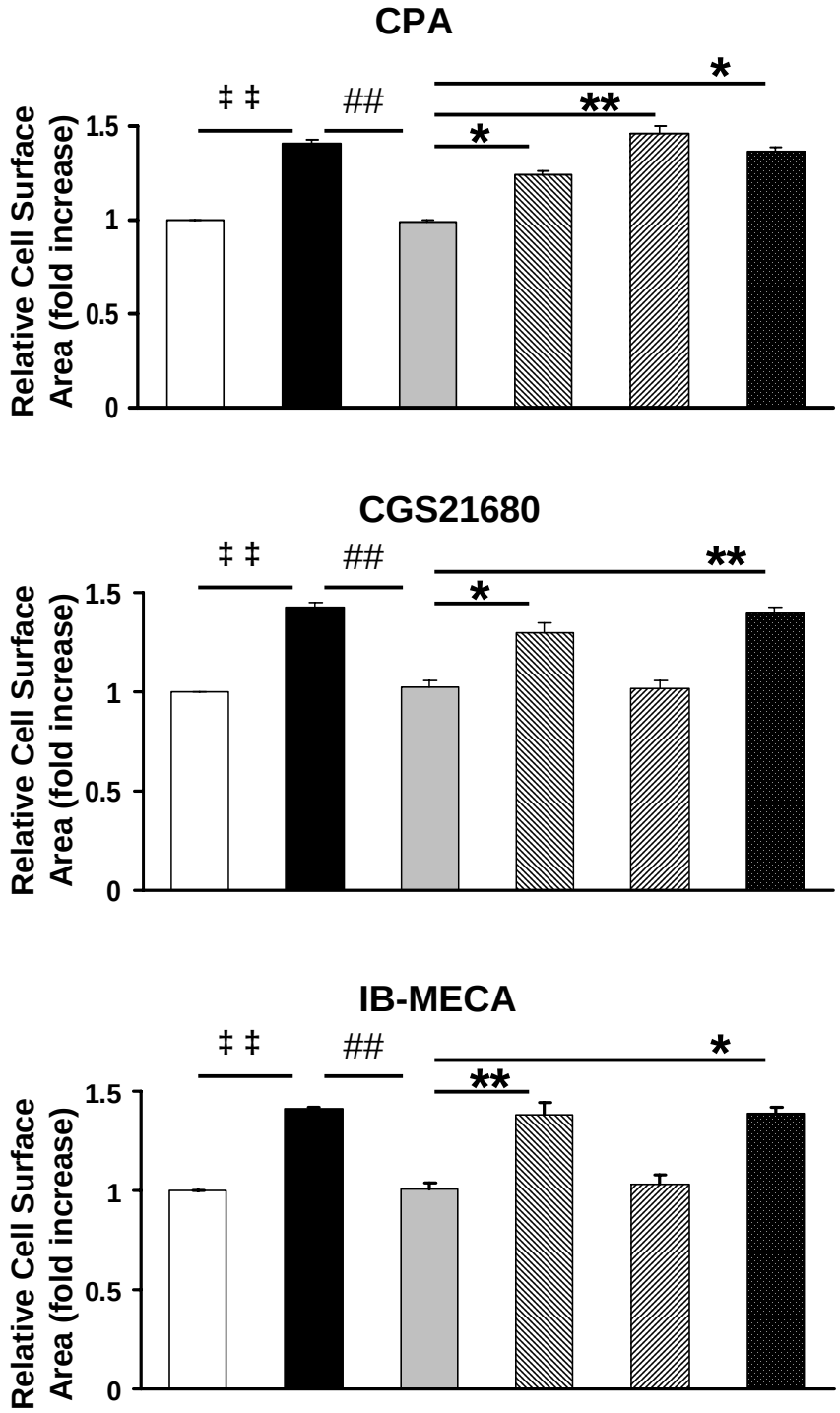

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Figure 3

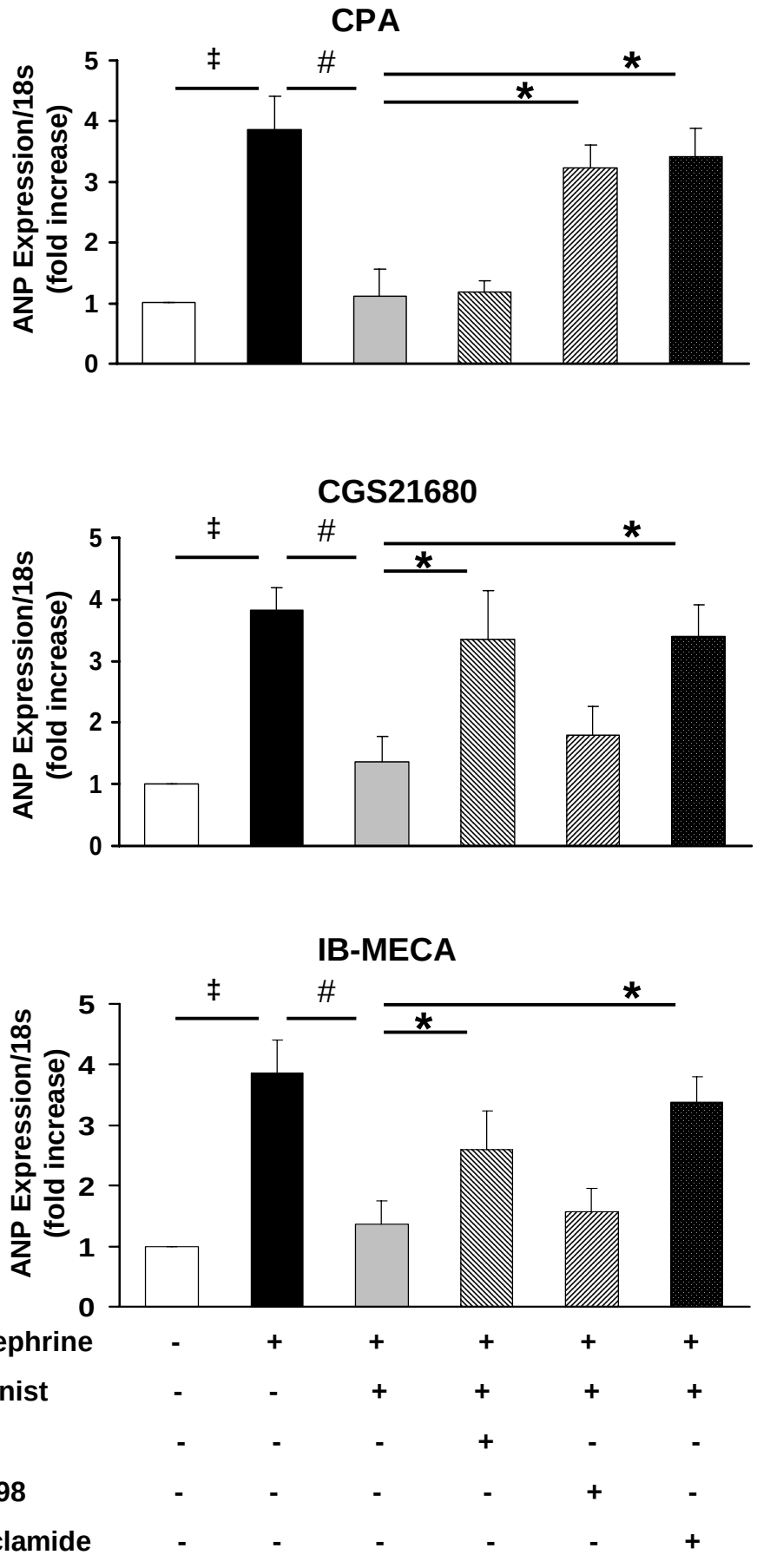

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Figure 4

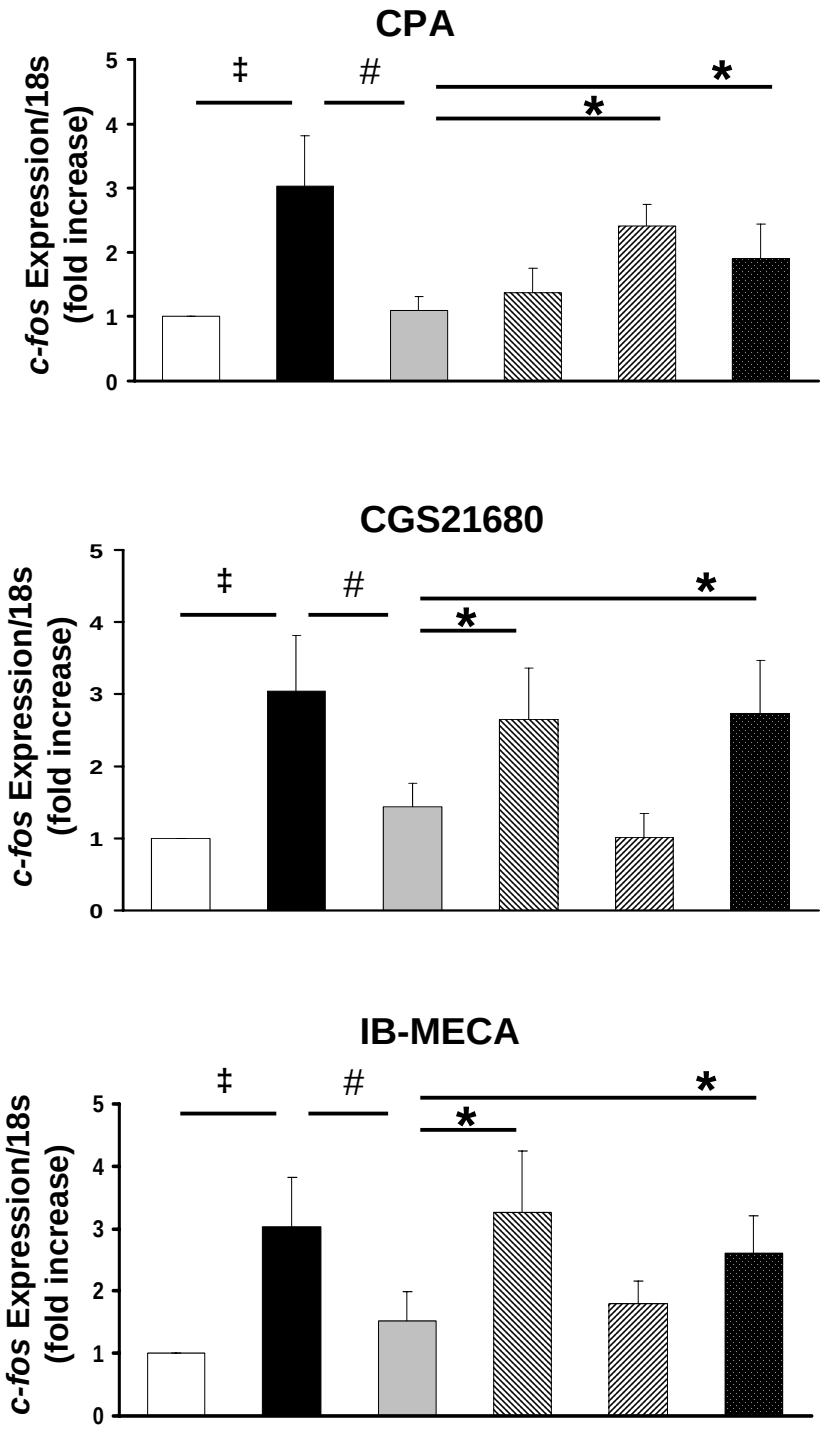

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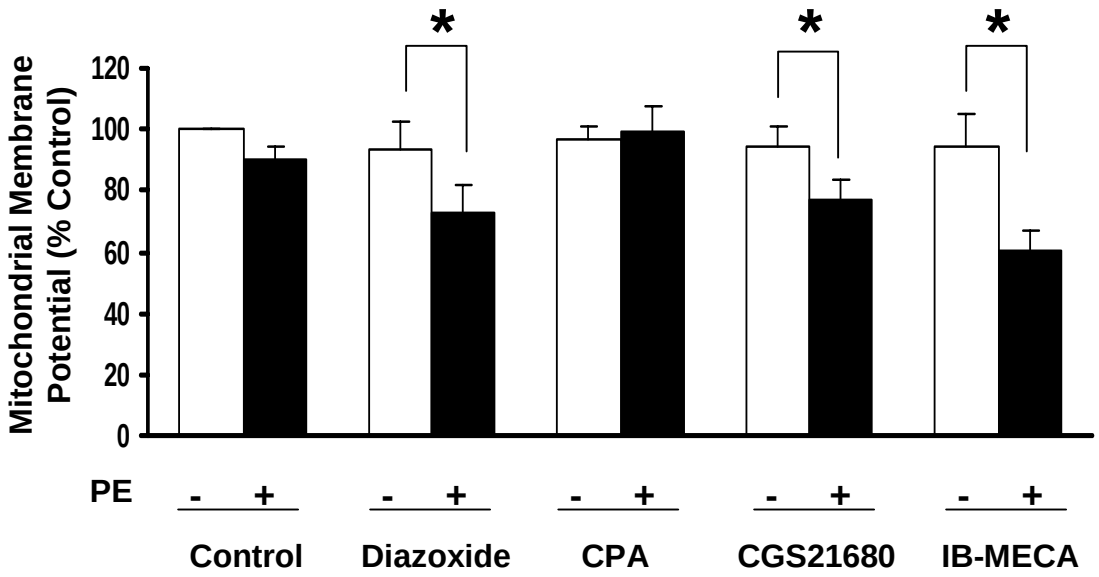
Figure 5

A.



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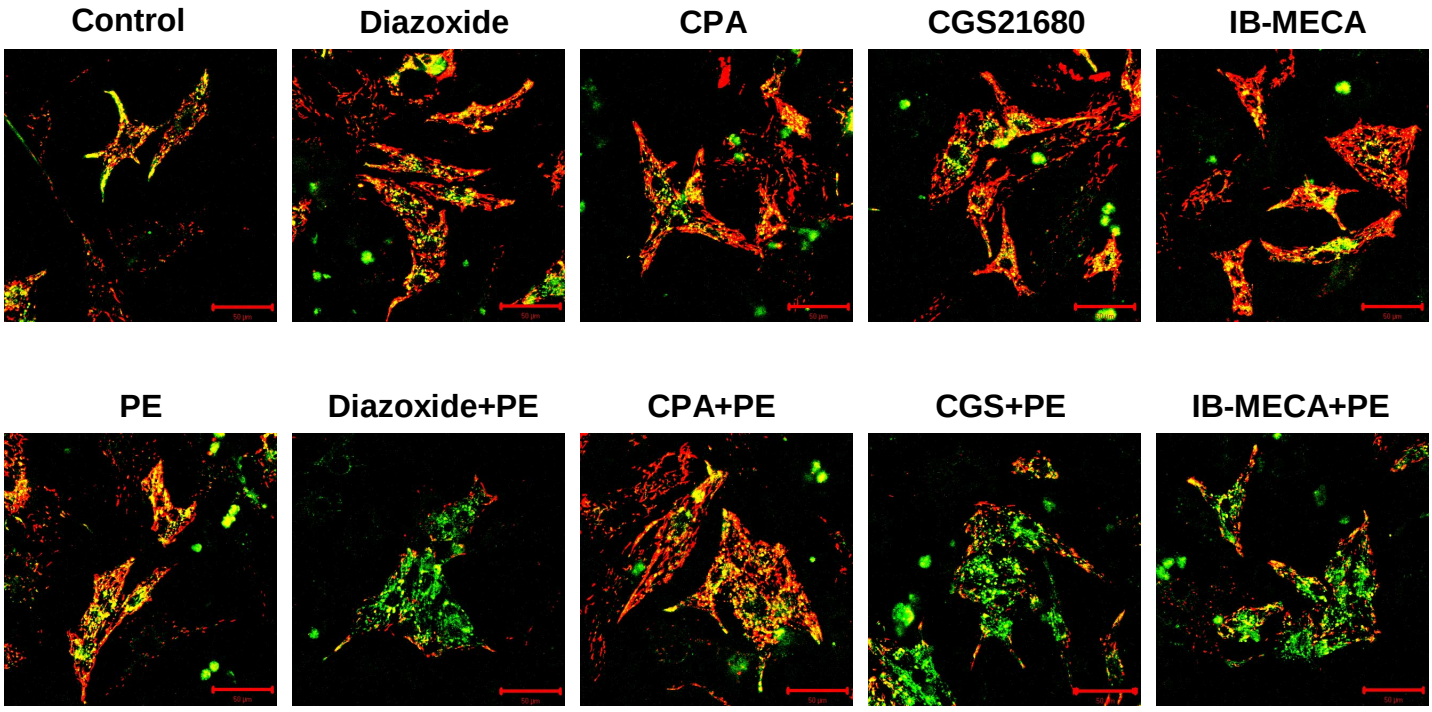

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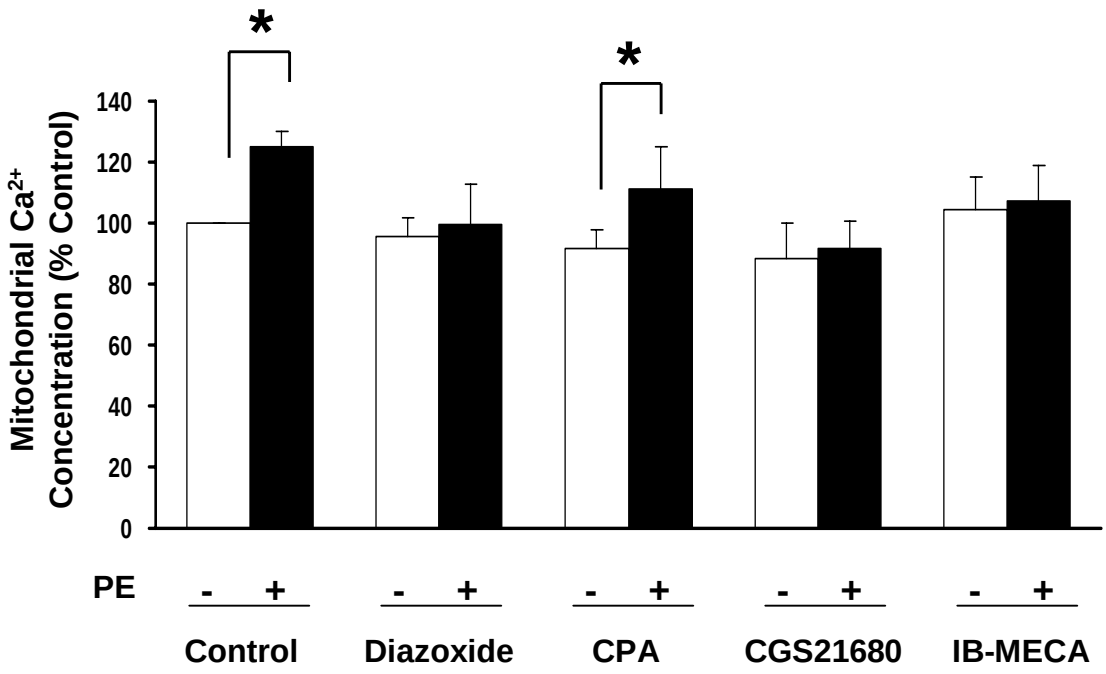
Figure 6

A.



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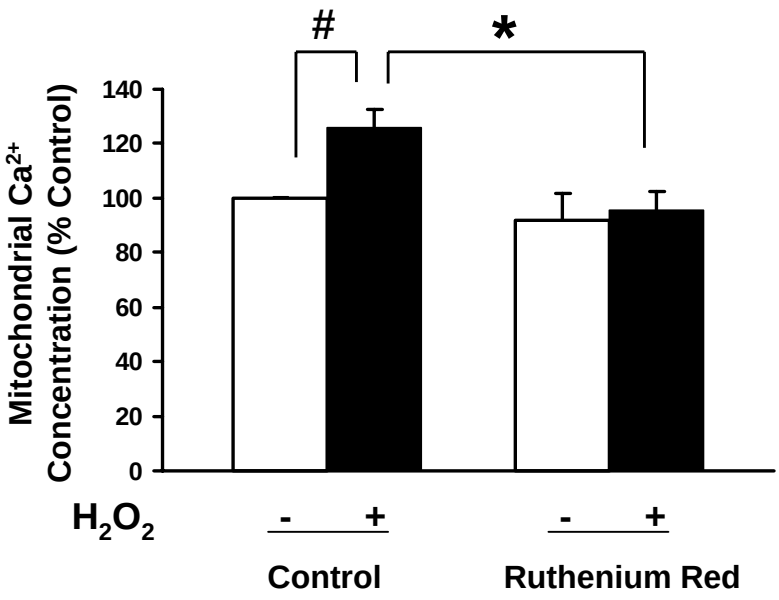

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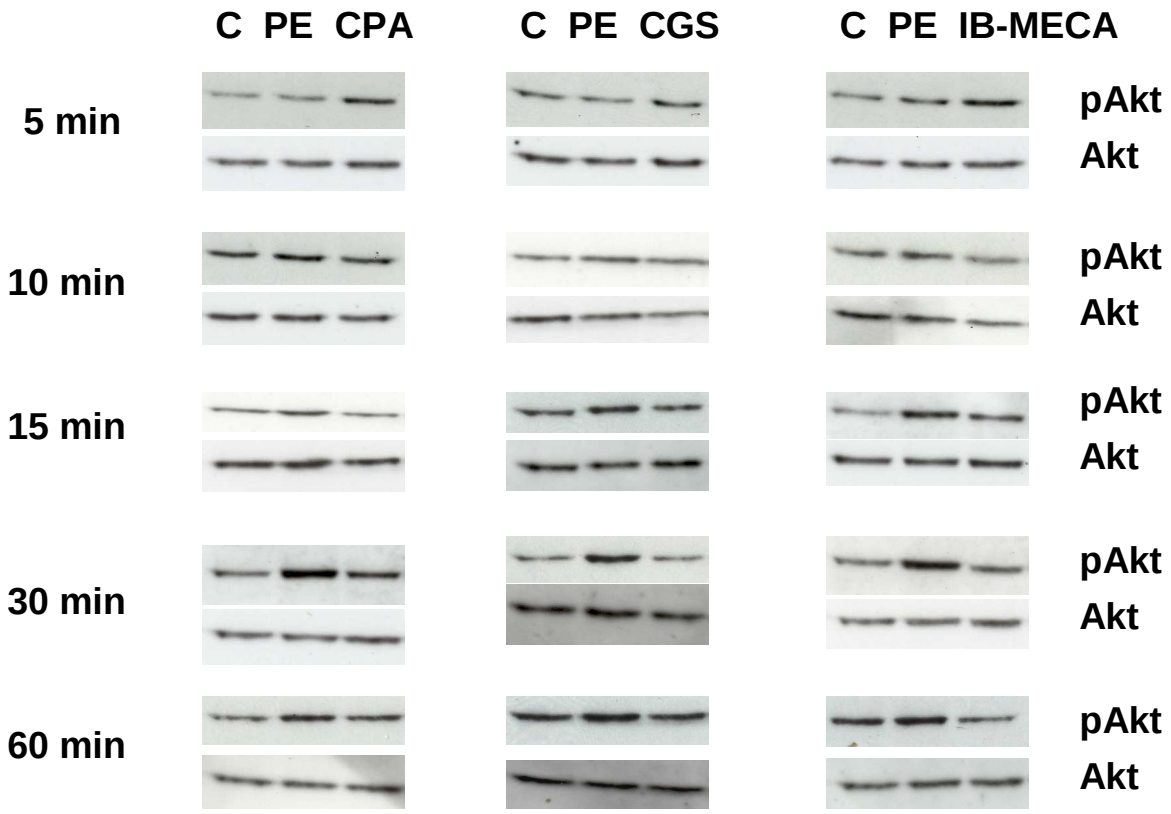
Figure 7



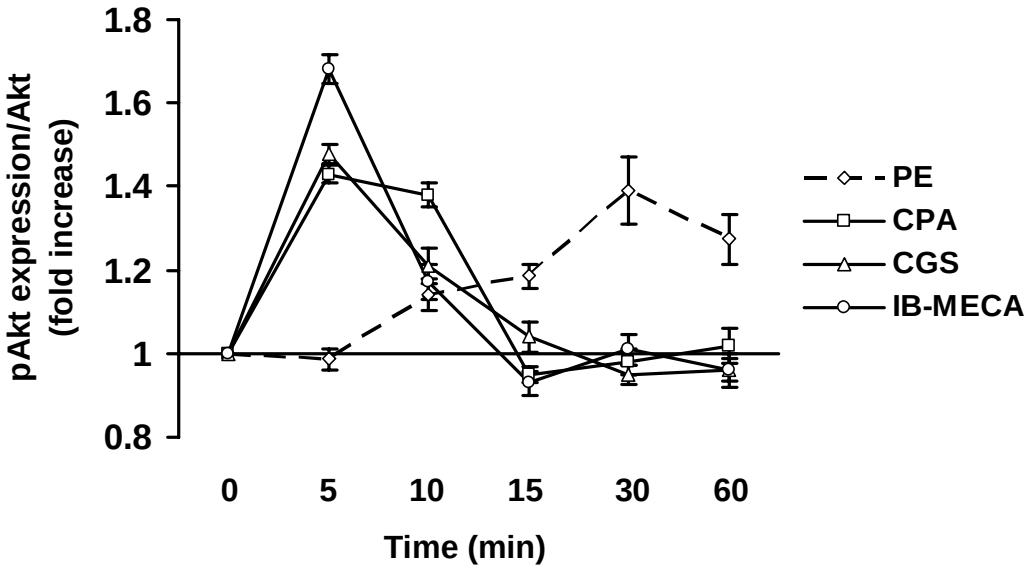


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Figure 8

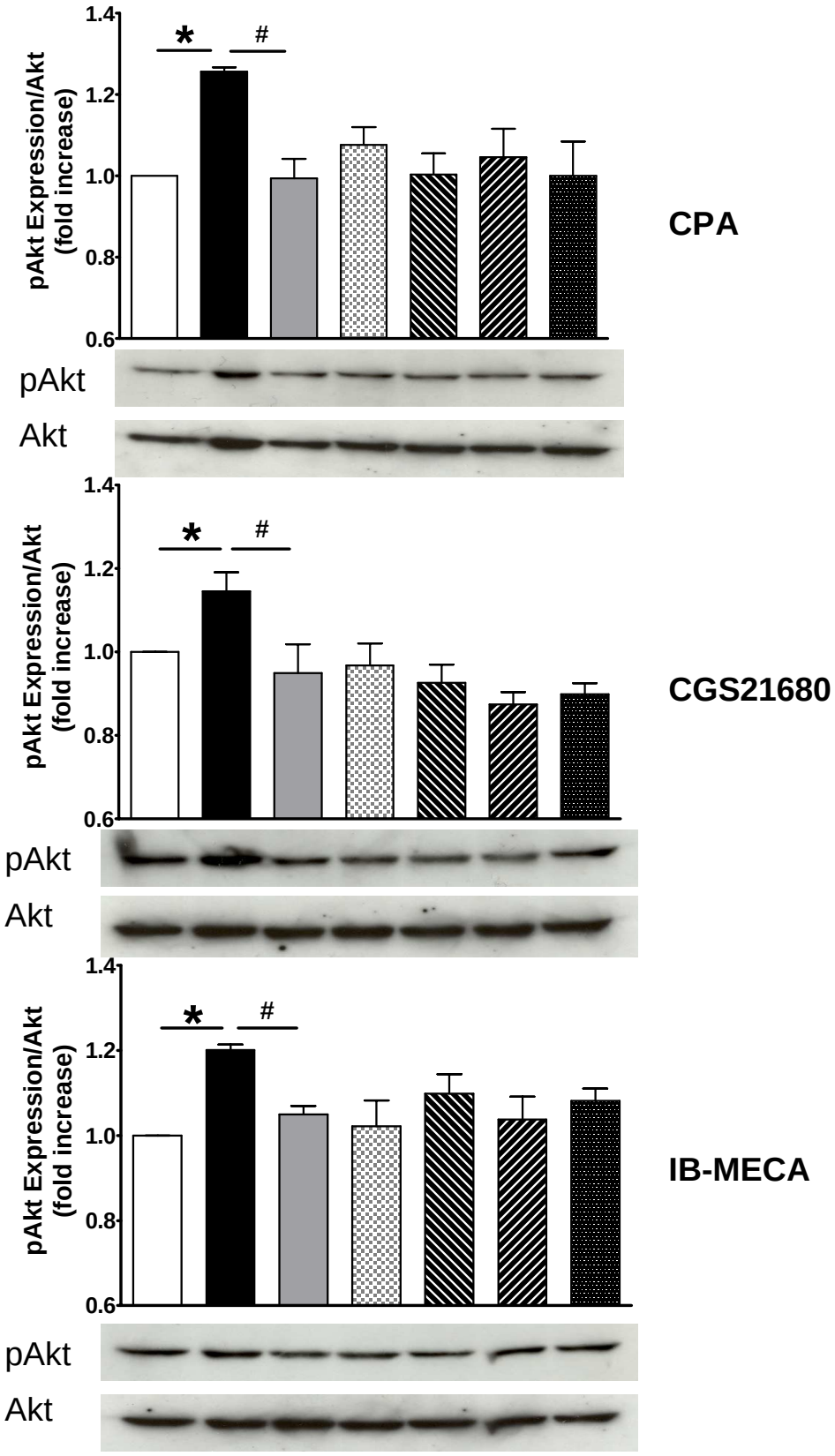

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Figure 9