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The adenosine transporter, mENT1, is a target for adenosine receptor signaling and PKC in hypoxic and pharmacological preconditioning in the mouse cardiomyocyte cell line, HL-1.

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Abbreviations

Ado-adenosine; **BIM**-Bisindolymaleimide; **CGS15943**-5-amino-9-chloro-2-(2-furyl)-[1,2,4]-triazolo[1,5-c]quinazoline; **CHA**-N^6-cyclohexyladenosine; **Chloro-IB-MECA**-2-chloro-N^6-(3-iodobenzyl)adenosine-5`-N-nethyluronamid; **DIPY**-dipyridamole; **DPCPX**-1,3-dipropyl-8-cyclopentylxanthine; **EHNA**- erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride; **IO** – 5-Iodotubercidin; **mENT1** mouse equilibrative nucleoside transporter 1; **MRS1220**-9-chloro-2-(2-furanyl)-5-([[phenylacetyl] amino)(1,2,4)-triazolo[1,5-c]quinazoline; **NTs**-nucleoside transporters; **NBTI**- nitrobenzylthioinosine; **NECA**- 5`-N-ethyl-carboxamidoadenosine; **PC**-preconditioning; **PMA**-phorbol-12-myristate-13-acetate

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Abstract

Brief exposure of the heart to hypoxia results in less cellular damage after subsequent hypoxia, an effect known as preconditioning (PC). PC has been widely studied but is still not fully understood. Adenosine (Ado), adenosine receptors and protein kinase C (PKC), have been implicated as integral components of PC. Adenosine (nucleoside) transporters (NTs) facilitate flux of Ado across cell membranes but their role in PC is unknown. Therefore, we used the murine cardiomyocyte cell line, HL-1, and asked if there was feedback regulation of NTs by Ado, Ado receptors and PKC following either hypoxic or pharmacological PC.

Activation (by specific agonists) of A_1 or A_3 Ado receptors or PKC resulted in PC in HL-1. The A_1 (but not A_3) receptor is coupled to PKC and activation of PKC (by specific peptide agonist) resulted in PC. Moreover, PKC stimulates Ado uptake via the predominant NT in HL-1, mENT1. Studies in primary neonatal mouse cardiomyocytes confirmed our observations in HL-1 cells. Hypoxic challenge led to a rapid increase in, and efflux of, intracellular Ado from cells, which was blocked by NT inhibitors (DIPY/NBTI). Moreover, NT inhibition during hypoxia or PC was highly protective suggesting that Ado loss contributes to decreased cell viability. Our data suggest that hypoxic challenge causes an efflux of Ado via ENTs, activation of A_1 and/or A_3 receptors, signaling through PKC and activation of ENT1. Since Ado is required for ATP synthesis on reperfusion, this feedback regulation of mENT1 would promote re-uptake of Ado.

Introduction

Ischemia (reduced blood flow) and hypoxia (reduced oxygen) are serious problems in cardiology, from prenatal to geriatric settings and we need a better understanding of the causes and consequences of ischemia/hypoxia in order to develop strategies to combat resultant damage. Although cardiomyocytes cannot rapidly proliferate to replace damaged tissues, they can respond to, and resist, the cellular stress caused by ischemia/hypoxia. Thus, cardiomyocytes are less damaged by ischemia/hypoxia, if they have previously been briefly exposed to ischemia/hypoxia, a phenomenon known as ischemic or hypoxic preconditioning (PC) (e.g. Nakano et al., 2000; Mubagwa and Flameng; 2001).

PC has been widely studied and involves the purine nucleoside, adenosine (Ado) and protein kinase C epsilon (PKCε) (e.g. Nakano et al., 2000; Schulz et al., 2001) in addition to other unidentified components. However, the interactions between Ado, PKCε and other components involved in PC are still not well understood. An increased understanding of PC will improve therapeutic strategies aimed at activating or enhancing cellular processes that reduce cell damage and promote cell survival during situations where ischemia/hypoxia occur (e.g. heart attack or surgery).

Ado is one of the major effectors of PC. Following hypoxic challenge, Ado is produced by cardiomyocytes and acts as a paracrine and autocrine anti-stress hormone and cardioprotectant. Clinically, Ado has been in use since 1989, when it was introduced as an anti-arrhythmia drug (Pelleg et al., 2002). Moreover, Ado analogs and PC are used, or have been proposed for clinical use, to treat heart disease (e.g. de Jong et al., 2000; Pouzet et al., 2002). Therefore, we need to understand more precisely how Ado exerts its cardioprotective effects.

Ado is not lipophilic. Movement of Ado across the cell membrane is mediated by nucleoside transporters (NTs, Baldwin et al., 2004, Pastor-Anglada et al. 2004). Cardiomyocytes possess predominantly or exclusively equilibrative NTs (ENTs), which facilitate diffusion of Ado down its concentration gradient (Chaudary et al., 2002). Ado concentrations in the heart fluctuate enormously depending on cellular metabolic demands and activity of enzymes involved in Ado metabolism (Deussen 2000). In neural cells, ENTs are involved in modulating extracellular adenosine levels and appear to be subject to regulation by complex signal transduction pathways (Cunha et al. 2000). However, the influence of ENTs on Ado flux within cardiomyocytes is unknown. When metabolic demands for ATP exceed oxygen supply (as in ischemia/hypoxia), Ado levels rise. Extracellular Ado interacts with Ado receptors (A_1, A_{2A}, A_{2B} and/or A_3), which have varying affinities for Ado, and which are coupled to different signaling pathways. Receptor activation (an integral part of PC) triggers the cell to respond to the cellular assault. Indeed, various clinically important cardiovasodilatory and cardioprotectant pharmacological agents such as dipyridamole (DIPY) inhibit ENTs and their use is based on the premise that preventing re-uptake of Ado will potentiate receptor activation.

The nature of the relationship between Ado receptors and ENTs in heart is unclear and the role of ENTs in Ado-dependent processes is poorly understood. However, recent studies suggests a physiologically significant correlation in distribution of certain Ado receptors and ENTs in human neural tissues (Jennings et al., 2001; Bailey et al., 2002). Moreover, ENTs play a role in Ado-receptor dependent regulation of K^+ channels in epithelial cells (Szkotak et al., 2001). Given the very high expression of ENTs in heart (Pennycooke et al., 2001), and their role in mediating Ado flux across the cell membrane, we believe that ENTs are likely to be an integral, but over-looked, component of Ado physiology in cardiomyocytes. Moreover, since PKC□is

integral to PC, and PKC□ and/or PKC□ regulate ENT1 in mammalian cells (Coe et al., 2002), we hypothesized that ENTs would be regulated by Ado-receptor dependent mechanisms in cardiomyocytes such that Ado flux across the cell membrane is homeostatically controlled. Given the clinical significance of Ado-related physiology in cardiomyocytes, an enhanced understanding of the role of ENTs in these cells is needed. Therefore, we used the mouse cardiomyocyte cell line HL-1, which we have previously established as a viable model for studies on Ado physiology (Chaudary et al., 2002), in combination with primary cultures from neonatal mice, to investigate the relationship between ENTs, Ado receptors, and PKC□ and PC.

Methods

Materials. Claycomb media was purchased from JRH Biosciences Inc. (Kansas, USA). FBS, CHA, DPCPX, NECA, CGS 15943, NBTI, DIPY, Ara-C and BrdU were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). MRS 1220 and 2-Cl-IB-MECA were purchased from Tocris Cookson Inc. (Ellisville, MO. USA). PMA and IO was purchased from Calbiochem/ EMD Biosciences Inc. (San Diego, CA, USA). BIM was purchased from Bioshop Canada, Inc., (Burlington, Ontario, Canada). PKC peptides were initially provided as a kind gift from Dr. Daria Mochly-Rosen (Stanford University, CA, USA) and were used for all the viability assays. The PKC peptide agonist was V1-7 and the PKC antagonist peptide was V1-2. An additional negative control was a scrambled V1-2 peptide. All peptides are cross-linked via N-terminal Cys-Cys bond to *Drosophila* Antennapedia homeodomain-derived carrier peptide (Tat). They have been described in more detail elsewhere (Schechtman and Mochly-Rosen 2002). Identical peptides were subsequently purchased from Calbiochem. Plexiglass GasPak container, Fyrite oxygen analyzer and oxygen electrode were purchased from VWR Canlab (Mississauga, ON, Canada).

Cell Culture. The HL-1 cells are a cardiac muscle cell line, derived from the AT-1 mouse, atrial cardiomyocyte tumor lineage and were maintained as previously described (Chaudary et al., 2002). Briefly, HL-1 cells were grown in Claycomb medium supplemented with 10% (v/v) fetal bovine serum, 100 mM norepinephrine, 4 mM L-glutamine, 1 x antibiotic/antimycotic solution. Culture flasks and plates were precoated with 1mg/cm^2 fibronectin/0.02% (w/v) gelatin solution.

PC and cell viability. For viability assays, HL-1 cells were seeded in 60mm dishes and experiments were conducted when cells reached 75-80% cell confluency. The preconditioning

protocol used for HL-1 cells consisted of 90 min hypoxia and 60 min normoxia for one cycle. Following this preconditioning cycle, cells were exposed to 20 h of hypoxia and cell viability was determined. Non-preconditioned cells were exposed to 150 min normoxia and then 20 h hypoxia. Control cells were exposed to normoxia for equivalent times. For hypoxia, HL-1 cells were transferred to a vented Plexiglas GasPak container and maintained at 37°C with a humidified hypoxic atmosphere of 2% O_2, 5% CO_2, 93% N_2. Controls were maintained at 5% CO_2 and 95% air at 37°C. For hypoxic challenge, degassed medium was used. Oxygen concentrations in the chamber were measured using a FYRITE Oxygen Analyzer. Oxygen concentrations in the media were measured using an oxygen electrode. Following PC and/or hypoxia, cell viability was determined by a standard trypan blue exclusion assay following the various treatment conditions as described below. Counting was done by a "blinded" observer who was unaware of the treatment condition of each sample being counted. Eight fields of view were counted for each sample and values shown as % dead cells (mean ± S.E.).

Adenosine receptors. To determine if Ado receptor activation was involved in PC in HL-1, cells were treated with general Ado receptor agonist, NECA (1 M, 10min) prior to 20 hours of hypoxia and the general Ado receptor antagonist, CGS 15943 (4nM, 20 min) prior to initiating the PC cycle followed by 20 h hypoxia. To discriminate between Ado receptors, cells were treated with specific A_1 Ado receptor agonist, CHA (300nM, 20min) or A_3 Ado receptor agonist, MECA (100nM, 20min), prior to 20 h hypoxia (Jacobsen 1998). Recent reports suggest that a number of pharmacological agents used in signaling studies also act as Ado receptor antagonists (Schulte and Fredholm 2002) or Ado transport inhibitors (Huang et al., 2003). Therefore, we used pharmacological agents at concentrations which ensured maximal activation or blockade of the respective receptors without interfering with ENT-dependent transport (Sinclair et al., 2000)

by pre-testing them in Ado transport assays. All the pharmacological agents were tested on cells under normoxic conditions, and showed no effect on cell viability compared to control (untreated). To confirm the role of Ado receptors in PC, cells were treated with the A_1 Ado receptor antagonist, DPCPX (1 μM, 20min) and A_3 Ado receptor antagonist, MRS 1220 (200nM, 20min) to block Ado receptor activation during PC. Control cells were treated with vehicle, DMSO.

PKC. To determine if PKC was involved in PC in HL-1, cells were treated with PMA (500nM, 10min) in the presence or absence of the PKC inhibitor, BIM (10μM, 15min) prior to long-term hypoxia (20 h). Control cells were treated with BIM alone. To determine the role of PKCε specifically, cells were treated with a PKCε peptide agonist (0.25μM, 30 min) prior to 20 hr hypoxia and PKCε peptide antagonist (0.5μM, 30 min) prior to PC.

NT inhibitors. To determine the role of NTs, the NT inhibitor DIPY (15μM) was added to cells prior to and during 20 h hypoxia. To determine if inhibition of NT during PC would affect cell viability, cells were treated with both NBTI (100 nM) and DIPY (15 μM) to inhibit efflux of Ado via both ENTs.

Ethanol. To determine the effect of alcohol, a cardioprotectant that works, in part, by activating PKCε and/or inhibiting adenosine reuptake via ENT1 (Nagy et al., 1990; Miyamae et al., 1998), HL-1 cells were treated with varying doses of ethanol (50mM or 200mM) for 4 min prior to 20 hr hypoxia.

Adenosine uptake. Adenosine uptake (at 10 secs) was measured according to previously described methods (Chaudary et al., 2002). To determine the effect of Ado receptor activation on adenosine uptake, HL-1 cells were seeded in 12-well plates and serum-starved for 18 hours. Cells were treated with CHA (300nM, 20min) and/or MECA (100nM, 20min) prior to measuring

adenosine uptake. For comparison, transport was also measured in the presence of serum with similar treatment conditions. To determine the effects of activation of PKC on transport, cells were treated with PMA (250nM, 20min) prior to transport assays. Cells were treated with the following conditions prior to conducting transport: NBTI (100nM, 15min) in the absence and presence of PKC agonist (0.25 M, 30 min). In addition, cells were treated with PKC antagonist (0.5 M, 30min) alone and together with PKC agonist. As controls, the classical PKC agonist peptide (0.25 M, 30min) and scrambled (nonsense) peptide (0.25 M, 30min) were also used. To determine how long the activation lasted, cells were preconditioned and then uptake measured immediately (0 min) and at 90 and 120 min post-PC.

Western analysis. Protein (membrane and cytosol) samples were analyzed by SDS-PAGE and immunoblotting as previously described (Chaudary et al., 2002). Translocation of PKC to the membrane fraction was used as an assay for activation as previously described (e.g. Gray et al., 1997). Identical treatment conditions were used to those described in adenosine uptake assays.

Primary culture of neonatal mouse cardiomyocytes. Neonatal mouse cardiomyocytes were isolated using a Neonatal Cardiomyocyte Isolation System Kit (Worthington Biochemical Corporation, New Jersey, USA), according to manufacturer's instructions with minor modifications for mouse hearts. Healthy neonatal mouse pups were decapitated in accordance with the York University Animal Use and Care Protocol, approved by the Animal Care Committee. Briefly, hearts were aseptically isolated from 6-7 day old neonatal CD1 mice and rinsed with ice-cold calcium and magnesium-free Hank's Balanced Salt Solution (CMF-HBSS). Hearts were transferred to a petri dish and minced using small scissors. Tissue was digested overnight with 50 g/ml trypsin at 4^{o}C. The next day tissue was transferred to a 50ml sterile centrifuge tube on ice using a wide-mouth pipet. Trypsin inhibitor (0.2mg/ml) was added to the

tissue. The tube was warmed in a 37°C water bath for 15 min and 80units/ml of collagenase was added and warmed for additional 40 min at 37°C on a shaker. Cells were dispersed and released by gentle trituration 10-15 times and filtered through a cell strainer and left undisturbed for 20 min at room temperature. Cells were spun for 3 minutes at low speed and then suspended in 10ml complete Claycomb medium (10% FBS) and pre-plated for 2hours at 37°C to allow for non-cardiomyocytes to attach. Non-attached cells were transferred to complete Claycomb medium with 0.1mM BrdU and 25□M Ara-C and placed at 37°C incubator undisturbed for 24 hours. The medium was then changed to regular Claycomb medium with only 0.1mM BrdU and left for 3 days at 37°C prior to experimental use. A small proportion of cells were used to determine cell viability and cell number. Mouse myocyte yield was approximately $0.7x10^6$ per heart (n=27 pups/isolation).

HPLC analysis of adenosine release. Adenosine was measured as described for PC-12 cells (Kobayshi et al., 2000). Briefly, HL-1 cells were plated in 35-mm dishes and grown to approximately 80% confluency. Cells were rinsed twice with 0.5 ml of a Krebs solution (135 mM NaCl, 1.5 mM NaH_2PO_4, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 15 mM HEPES and 10□M EHNA). Prewarmed Krebs solution (0.5ml) was added to each plate and cells were incubated under normoxic conditions for 30 minutes. In one plate, cells were treated with NBTI (500 nM) and DIPY (30□M). The cells were transferred to hypoxic conditions for 5min, 15 min or 30 min. Following hypoxic and normoxic treatment the Krebs solution was collected and the number of cells in each plate were counted. Measurement of adenosine released was performed by HPLC as previously described (Gayden et al., 1991). C18 column was used with UV detection at 259 nm to separate compounds in collected extracellular fluid. The mobile phase contained 125 mM potassium dihydrogenphosphate, 1.5% (v/v) acetonitrile, 20 mM

triethylamine and 1.0 mM tetrabutylammonium hydrogen sulfate (TBAHS) (pH 6.5) and adjusted to pH 6.5 with 8 M potassium hydroxide. The flow rate was 3 ml/min and the assays were performed at room temperature. Peaks were identified on the basis of retention times and comparison with adenosine standards.

Data Analysis. Statistical analyses were done using either paired Student's t-test (comparison of two variables), or analysis of variance test (one-way ANOVA), followed by post-hoc Student-Newman-Keuls test. All statistical analyses were done using Graphpad Prism 3.0a for Macintosh (Graphpad Software, San Diego, Calif., USA). A *P* value of less than 0.05 was considered to be statistically significant. Data are presented as mean ± S.E. or S.D.

Results

Preconditioning in HL-1 cells. We have already established that HL-1 cells are typical of murine cardiomyocytes in terms of their adenosine transporter, glucose transporter, adenosine receptor, and PKC isoform profiles, and hypoxic response (Chaudary et al., 2002, Chaudary et al., 2004). However, HL-1 has not been used previously as a model for the study of PC. Therefore our initial aim was to establish a protocol for hypoxic preconditioning that was equivalent to that previously described for primary cultures or whole hearts (Gray et al., 1997). As shown in figure 1A, a preconditioning (PC) protocol of 90 min hypoxia followed by 60 min normoxia, resulted in reduced cell death in HL-1 cells following subsequent prolonged hypoxic challenge (Hypoxia), which was consistently slightly higher but not significantly different from that found in cells that had not been exposed to long-term hypoxia (Normoxia). We found that additional cycles of hypoxia/normoxia did not lead to enhanced cell viability, but were in fact as damaging as hypoxia alone. Thus, we have clearly demonstrated a protective effect, typical of hypoxic PC, in this cell model.

We then investigated the various components that were responsible for PC in HL-1. We found that activation of Ado receptors, using the non-specific Ado receptor agonist NECA, was equivalent to PC in reducing cell death following long-term hypoxia. These data suggest that Ado receptor dependent pathways are involved in PC in HL-1. This supposition was confirmed by the observation that inhibition of Ado receptors during PC, using the non-specific antagonist CGS, eliminates the protective effects of PC.

The Ado receptors that have been primarily implicated in PC in cardiomyocytes are A_1 and A_3. Therefore, we used specific agonists and antagonists to differentiate between these receptors and found that activation of either the A_1 or A_3 Ado receptor provided equivalent protection

compared to hypoxic PC. Moreover, one receptor did not appear to be more effective in mediating protection than the other. Inhibition of either the A_1 or A_3 Ado receptor during PC resulted in a complete loss of the protective effects of PC.

The signaling pathways that are coupled to Ado receptors are not fully described but one component that has been strongly implicated in PC is PKC, therefore we determined whether activation of PKC by PMA could mimic hypoxic PC in HL-1. We found that this was indeed the case and that this effect could be blocked by the PKC inhibitor BIM. To determine if PKC was involved in this response we used a cell-permeable PKC specific agonist peptide and found that this was equally effective in protecting cells from long-term hypoxia to PC. This effect was completely blocked in the presence of the PKC antagonist peptide.

Ethanol has long been known to be a cardioprotectant, in part because it mimics the effects of PC (Miyamae et al., 1997). Ethanol has a number of effects including the activation of PKC (Miyamae et al., 1998) and the inhibition of adenosine re-uptake via ENT1 (Nagy et al., 1990) suggesting that it influences Ado-receptor dependent signaling at two levels. Indeed, we found that short-term exposure of HL-1 cells to ethanol was highly effective in protecting cells, to a level that was equivalent to that seen with hypoxic PC. Higher concentrations of ethanol appeared to be slightly more effective at enhancing cell viability than lower concentrations, although the difference was not statistically significant.

Since Ado flux across cardiomyocyte cell membranes occurs via ENTs, we determined if inhibition of this flux during hypoxia would influence cell viability. Dipyridamole (DIPY) has been widely used as a NT inhibitor on the premise that it blocks Ado reuptake and therefore potentiates Ado-receptor activation. Indeed, we found that cells were strongly protected from hypoxia if DIPY was present prior to and during long term hypoxia. These data suggest that loss

of Ado from cells contributes to increased cell death. Paradoxically, we saw no significant difference in cell viability compared to normoxic or PC cells, if DIPY and/or NBTI were present during PC, which would not be expected if Ado efflux and activation of Ado-receptor dependent compensatory pathways is required for enhanced cell viability. However, given the strongly protective effect of DIPY alone during hypoxia, these data suggest that prevention of Ado loss is as effective as activation of Ado-receptor dependent pathways linked to compensatory mechanisms in protecting cells from hypoxic damage. These data also suggest that DIPY may act by protecting cellular stores of Ado as well as by potentiation of Ado receptor activation.

These findings are summarized in figure 1B, which shows the outcome of hypoxic or pharmacological preconditioning (enhanced cell viability) and the effects of inhibition of either Ado receptors or PKC during PC (loss of protection from long-term hypoxia). These data suggest that the underlying mechanisms of PC involve A_1 and/or A_3 Ado-receptor dependent activation of signaling pathways including PKC and that loss of Ado via ENTs contributes to the increase in cell death seen after long-term hypoxia.

Source of adenosine. The data described above suggest that Ado dependent processes are involved in PC. However, we have not determined the source of the Ado involved in this process in HL-1 cells. Literature reports suggest that metabolic stress results in an increase in intracellular levels of Ado as ATP hydolysis exceeds ATP synthesis (Deussen 2000). This would lead to an increase in intracellular Ado, which would efflux from cells via ENTs once the intracellular concentration exceeds the extracellular concentration. To determine if this was the case in HL-1 cells, we measured extracellular Ado levels following hypoxic challenge and found a rapid and significant increase (fig. 2). This increase was completely blocked by the DIPY and NBTI suggesting that the source of the Ado is intracellular and flux is outwards. Thus, hypoxic

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stress causes a build up in intracellular levels of Ado, which is transported down its concentration gradient by ENTs to the extracellular space.

Adenosine receptor coupled signaling in HL-1. Since activation of A_1 or A_3 Ado receptors or PKC results in pharmacological PC, we determined if either receptor was coupled to PKC as indicated by translocation of the kinase to the membrane fraction. PMA is a potent activator of all classic PKC isoforms and causes complete activation of PKC (fig. 3A) in both HL-1 and neonatal cardiomyoytes. The specific A_1 Ado receptor agonist CHA causes a similar activation (fig. 3B) in HL-1. However, we found that the A_3 Ado receptor agonist MECA had no effect on PKC translocation even at concentrations higher than those used in pharmacological PC (data not shown). These data suggest that A_1 Ado receptors are coupled to PKC and mediate PC via this pathway in HL-1 cells but that A_3 Ado receptors are coupled to PKC-independent pathways, which can also promote PC.

Adenosine receptor and PKC regulation of adenosine transport in HL-1. Since we have previously shown that PKC regulates ENT1 in other cells, we investigated whether activation of A_1, A_3, PKC or PKC regulated adenosine uptake in HL-1 cells. We found that acute PMA treatment of HL-1 cells resulted in a modest but significant increase in adenosine uptake suggesting that PKC was involved in regulation of the ENTs. We then investigated individual components in more detail and found that activation of A_1 or A_3 Ado receptors significantly enhance Ado uptake, with the effect of A_1 Ado receptor activation being stronger than the other two treatments. Activation of both receptors together was not additive in terms of enhancing adenosine uptake but did significantly increase uptake further. To determine if A_1 could be regulating adenosine uptake via PKC used the cell-permeable peptides and found that the non-specific agonist peptide (which activates all classic forms of PKC), and the PKC specific

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agonist, both increased adenosine uptake significantly to the equivalent level seen with A_1 receptor activation (fig. 4). This effect was completely blocked by the specific PKCε antagonist and there was no effect of a scrambled ε peptide. To confirm that the increase in uptake was due to activation of mENT1, we treated cells with both PKCε agonist peptide and NBTI, which specifically inhibits ENT1 but not ENT2. Although there appeared to be a slight increase in uptake via ENT2 (NBTI insensitive transport) in the presence of the PKCε agonist peptide this was not statistically significant. To determine if the increase in uptake was due to enhanced phosphorylation of intracellular adenosine, we measured adenosine kinase following PKCε activation (using the same conditions as for uptake assays) and found no increase (data not shown). Similarly, inhibition of adenosine kinase by IO (500 nM) did not abrogate the increased adenosine uptake observed following PKCε activation (data not shown) suggesting that changes in adenosine kinase activity were not responsible for our observations. Taken together, these data suggest that A_1 receptors, coupled to PKCε, can regulate mENT1-dependent Ado uptake in HL-1 cells.

PKCε regulation of mENT-dependent Ado transport in neonatal mouse cardiomyocytes. The data described herein are the first (to our knowledge) to describe Ado receptor-PKC regulation of a NT. To confirm physiological relevance of these findings, we used mouse neonatal cardiomyocyte cultures to investigate PKCε regulation of mENT1. Mouse neonatal cardiomyocytes are technically challenging to produce (compared to the more widely used rat model), more resistant to hypoxia (and therefore not appropriate for studies on hypoxic preconditioning) and less robust than immortalized cells over extended periods. However, we found that PKCε activation, either by PMA or agonist peptide, using identical approaches to those described above, resulted in a significant increase in adenosine uptake in mouse neonatal

cardiomyocytes (fig. 5). This effect could be blocked by the specific PKC antagonist peptide. These data suggest that mouse cardiomyocytes, like HL-1 cells, possess ENT proteins that are regulated by PKC.

mENT1 activation is short-lived in HL-1. If ENT1 activation by Ado-receptor coupled PKC signaling is a rapid cellular response to acute elevated extracellular Ado levels, then we would expect the effect to be transient. To test this, we measured Ado-uptake at various time points post-PC (fig. 6). Ado-uptake was approximately 25% higher than control (non-PC) levels immediately post-PC, but then gradually returned to control levels by 2 hours. These data suggest that ENT1 activation is an acute effect and part of the rapid compensatory response to hypoxic challenge.

Taken together, these data suggest hypoxic challenge causes the release of Ado, which activates A_1 Ado receptors resulting in the activation and translocation of PKC, which, in turn, stimulates mENT1-dependent adenosine transport in HL-1 cells. A_3 Ado receptors are also activated and involved in mENT1 regulation but the intracellular signaling pathways involved are not clear. Identical regulatory pathways coupling PKC and mENT1 exist in mouse neonatal cardiomyocytes. In addition, loss of Ado is a significant factor in promoting cell death due to hypoxia, demonstrating the importance to cardiomyocytes of coordinated regulation of Ado flux via ENTs in order to maintain intracellular pools of Ado.

Discussion

The novel findings presented in this study include the use of the murine cardiomyocyte immortalized cell line, HL-1, as a model for detailed studies on hypoxic and pharmacological PC and in determining the relationships between Ado receptors, signaling pathways and Ado transporters. Our findings support earlier data demonstrating a role for A_1 and A_3 Ado receptors plus PKC in PC in cardiomyocytes (e.g. Mubagwa and Flameng 2001). Other novel findings include the observation that the adenosine transporter, mENT1, is a downstream target of the A_1 Ado receptor and PKC To our knowledge, these are the first data to show that a NT is regulated by a specific PKC isoform. In addition, we propose that prevention of Ado efflux by the cardiovascular drug, DIPY, contributes to its cardioprotective behaviour.

The PC protocol we used in this study is similar to other reports (e.g. Gray et al., 1997) although we found that a single cycle of hypoxia/normoxia was sufficient to elicit a preconditioned effect in HL-1 and repeated cycles were no more effective. We[1] and others (Ostadal et al., 1999) have found rodent neonatal cultures to be considerably more resistant to hypoxia than adult cardiomyocytes necessitating the use of an alternative model such as HL-1 cells, which show hypoxic responses that are typical of adult cells (Nguyen et al., 1999; Chaudary et al. in press). Therefore, we believe the findings presented here support the contention that HL-1 represents a valid model for the study of Ado-dependent physiology in cardiomyocytes.

Our data suggest that the sequence of events involved in PC in murine cardiomyocytes involves a build up of intracellular Ado as a consequence of hypoxic challenge followed by efflux via ENTs, activation of A_1 and/or A_3 Ado receptors, signaling via PCK and other as yet unidentified factors, and regulation of target proteins including the Ado transporter, mENT1.

The source of Ado produced during hypoxic challenge has been the subject of some controversy, with reports of 5'-ectonucleotidases and endothelial cells being responsible for the increase in extracellular Ado concentration in the heart (Deussen 2000, Mubagwa and Flameng 2001). ENTs would then be responsible for reuptake of Ado into cells and would contribute to termination of Ado receptor activation. Inhibition of ENTs by drugs such as DIPY would potentiate receptor activation (Figueredo et al., 1999). However, ENTs are bi-directional and will facilitate diffusion of Ado in either direction depending on prevailing Ado concentrations inside and outside cells. Moreover, the half-life of extracellular adenosine is very short (Deussen 2000) questioning the significance of re-uptake to end receptor activation. Our data suggest that hypoxic challenge leads to an increase in intracellular Ado in cardiomyocytes as previously reported (Deussen 2000). It has been proposed that this increase is due to increased cytosolic 5' NT activity or decreased Ado kinase activity (Decking et al., 1997), possibly as a consequence of A_1 Ado receptor activation (Sinclair et al., 2000). However, we found no change in the activity of either enzyme following hypoxic challenge (data not shown) and our data suggest that DIPY acts to prevent loss of Ado, an essential metabolite required for the post-ischemic repletion of AMP, ADP and ATP in cardiomyocytes.

Since loss of Ado is potentially highly damaging to cells, it is plausible that cells would respond with mechanisms aimed at reducing Ado loss, possibly by rapid recovery of extracellular Ado. This response would require coordinated signaling between Ado receptors, which "sense" extracellular Ado and Ado transporters responsible for flux of Ado across the cell membrane. Thus, a novel finding in this study is the feedback regulation between A_1 Ado receptors, PKC and the Ado transporter, mENT1 in murine cardiomyocytes. These data are consistent with, and build on previous findings in mouse heart, which demonstrated a role for extracellular Ado and

A_1 Ado receptor activation in cardioprotection (Peart et al., 2001). Moreover, this study suggested that cardioprotection may be enhanced by activation of other adenosine receptor subtypes (e,.g. A_3 Ado receptors) and that an adenosine salvage pathway was likely to be essential for the full effects of adenosine cardioprotection to be realized. The A_1 Ado receptors and PKC are now well established to be involved in PC in a number of models and evidence for a contribution by A_3 also exists (e.g. de Jong et al., 2000). However, the relative roles, coupled signaling pathways and downstream effectors remain unclear. Here we show that specific activation of A_1 Ado receptors or A_3 Ado receptors will elicit PC and provide evidence that the A_1 Ado receptor activates PKC This is consistent with reports linking A_1 Ado receptors to G_i/G_o and pathways involving phospholipase C (PLC), phospholipase D, and PKC (Mubagwa and Flameng 2001; Klinger et al., 2002). Moreover, A_1 Ado receptor coupling to PKC has been described in PC in coronary smooth muscle cells (Nayeem et al., 2002) and cardiomyocytes (Lester et al., 2000). The A_3 receptor has been implicated in PC, but its role is not as well understood as for A_1.

The physiological relevance of use of more than one Ado receptor isoform in PC is not immediately clear. However, since Ado receptors generally have different affinities for substrate (A_1 is high affinity, A_3 is low affinity) and are linked to different G-protein coupled signaling pathways, a cell possessing various receptor subtypes has the ability to respond differentially to a range of concentrations of extracellular Ado. Moreover, recent data from chick cardiomyocytes suggest that the temporal profile of PC is related to differential A_1/A_3 activation since A_1-dependent effects can be short-lived while A_3 effects can be long-lasting (Mozzicato et al. 2004).

We did not investigate the role of the A_2 Ado receptors and cannot rule out a contribution or modifying effect of these receptors in mediating PC in HL-1 since they have been implicated in PC in other systems (Lasley et al., 2001) and have been shown to be present in HL-1 cells (Chaudary et al. 2002). However, the data for a role of A_2 Ado receptors in PC in cardiomyocyte systems are less compelling than those for A_1 and A_3 and we found that A_1 or A_3 are sufficient and necessary for Ado-receptor dependent PC in HL-1 cells.

Our observation that treatment of HL-1 cells with ethanol mimics PC is expected given that ethanol is known to be cardioprotective, due, at least in part, to its activation of PKC (Miyamae et al., 1998). Moreover, these data support the contention that PKC is an integral component of PC. In addition, the use of cell-permeable isoform specific peptides (Schechtman, and Mochly-Rosen 2002) confirmed the importance of PKC in PC as previously described (Gray et al., 1997). The same peptide activated Ado uptake via mENT1 in both HL-1 and neonatal murine cardiomyocytes suggesting that this transporter is a target of the kinase.

The downstream targets of PKC in PC are not well defined but may include mitochondrial ATP-sensitive K^+ (K_{ATP}) channels (Mubagwa and Flameng 2001). Here we show for the first time that the Ado transporter, mENT1, is a target of PKC, in cardiomyocytes such that PKC activation of mENT1 leads to enhanced adenosine uptake. We have previously found that PKC and/or were involved in regulation of ENT1 in human cells (Coe et al., 2002). Our current work suggest that PKC dependent stimulation of mENT1 activity represents a homeostatic attempt to recover extracellular adenosine, which has been lost as a consequence of hypoxic challenge. Adenosine is an essential metabolite within energetically active cells, and salvage pathways for maintenance of intracellular pools are likely to be more energetically favorable compared to *de novo* synthesis (Deussen 2000). Therefore, the rapid efflux will quickly lead to

increased extracellular concentrations, which will eventually favour inward movement of Ado down its concentration gradient via ENTs. A mechanism which enhances reuptake could be of considerable benefit to the cell.

While underlying mechanisms of regulation of any ENTs are unknown, our data are suggestive of post-translational modification. The mENT1 protein possesses consensus sites for PKC and other kinases suggesting that activity could be regulated by direct phosphorylation as described for other transporters (e.g. Vaughan et al., 1997). However, it is also possible that PKC regulates other proteins (kinases/phosphatases), which, in turn, are responsible for phosphorylation of mENT1. Increased availability of research tools and improved analytical techniques for the study of membrane proteins will allow us to dissect regulation of mENT1 in more detail in the future.

In summary, we have developed a novel model for the study of the relationships between Ado receptors, signaling pathways and Ado transporters in both hypoxic and pharmacological PC. We have identified feedback regulation, which involves components of PC that have been previously identified (A_1/A_3 Ado receptors, PKC) but not clearly linked, and also demonstrated the role of this signaling pathway in regulation of a novel target, the Ado transporter, mENT1. Loss of Ado via the ENTs is detrimental to cells and a significant action of clinically used NT inhibitors such as DIPY is likely to be retention of Ado pools, in addition to their more well-known action of potentiating receptor activation. The precise nature of mENT1 regulation by PKC is unclear, but is likely to involve post-translational rather than transcriptional modification. Given the importance of ENTs in modulating flux of Ado across the plasma membrane, we propose that these proteins be considered an integral part of the PC response in cardiomocytes.

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Footnotes

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[1] Coe and Chaudary, unpublished observations

Figure Legends

Figure 1. Hypoxic or pharmacological PC enhances HL-1 cell viability after chronic hypoxia.

A. Cell viability was determined (trypan blue exclusion assay) after the following treatments of HL-1 cells: **Normoxia**- cells maintained in normoxia (5% CO_2 and 95% air, 20 hr); **Hypoxia** – cells exposed to hypoxia (2% O_2, degassed media, 20 hr); **PC** – cells hypoxically preconditioned (90 min hypoxia, 60 min normoxia, 20 hr hypoxia). Pharmacological PC was induced by treatment of HL-1 cells with Ado receptors agonists or PKC activators as follows: **NECA** – general Ado receptor agonist, 1 M, 10 min; **CHA** – specific A1 Ado receptor agonist, 300 nM, 20 min; **MECA** – specific A3 Ado receptor agonist, 100 nM, 20 min; **PMA** – activates conventional PKC isoforms, 500 nM, 10 min; **PKC agonist peptide** – PKC specific activator, 0.25 M, 30 min; **Ethanol** – PKC specific activator, 50 or 200 mM, 4 min. Following pharmacological PC, cells were exposed to 20hr of hypoxia. Specificity of action of PMA and PKC agonist peptides was confirmed in the presence of antagonists, **BIM** – general PKC inhibitor, 15 M, 15 min, and **PKC peptide antagonist**, 0.5 M, 30 min. The role of Ado receptors in hypoxic PC was investigated using Ado receptor antagonists prior to PC as follows; **CGS 15943** – nonspecific Ado receptor antagonist, 4 nM, 20 min; **DPCPX** – specific A_1 Ado receptor antagonist, 1 M, 20 min; **MRS 1220** – specific A_3 Ado receptor antagonist, 200 nM, 20 min. Following Ado receptor antagonism and hypoxic PC, cells were exposed to 20 hr hypoxia. The role of Ado transporters was investigated by treating HL-1 cells with 15 M **DIPY** during 20 hr hypoxia or with 100 nM **NBTI**+15 M **DIPY** during PC followed by 20 hr hypoxia. Pooled data are shown, mean ±S.E., n 3, * $P < 0.05$ compared to PMA; ** $P < 0.001$

compared to normoxia, # $P < 0.01$ compared to NECA; ^ $P < 0.01$ compared to MECA; † $P < 0.05$ compared to CHA; § $P < 0.01$ compared to PKC agonist).

B. A schematic representation summarizing the treatments, PC time course and outcome of experiments described above.

Figure 2. Hypoxic challenge leads to an efflux of Ado via ENTs in HL-1 cells

Extracellular levels of Ado (in the presence and absence of ENT inhibitors, 500 nM NBTI+30 M DIPY) were determined by HPLC as described in Methods. Representative experiment shown (mean ± S.D.), each condition conducted in duplicate. Experiments repeated at least twice with similar results.

Fig. 3. PKC is activated by PMA and CHA in HL-1 and mouse cardiomyocytes.

HL-1 cells and primary neonatal mouse cardiomyocytes were treated **A)** with PMA (500nM, 15min) or **B)** CHA (300nM, 20min). Western blot analysis of cytosolic fraction (C) and membrane fraction (M) show activation of PKC (translocation to the membrane fraction). Representative experiment shown, repeated at least twice with identical results.

Figure 4. Activation of A_1 or A_3 Ado receptors, or PKC, stimulates adenosine uptake in HL-1 cells.

Activation of A_1 and/or A_3 receptors (using identical treatment to those described in figure 1) result in a significant increase in adenosine uptake in HL-1 cells. (Pooled data, mean ± S.E or S.D., n 2, each condition conducted in triplicate or sextuplicate, * = $P < 0.05$ versus control, ** = $P < 0.001$ compared to control, # = $P < 0.01$ compared to CHA or MECA alone). There was no

statistically significant difference between non-NBTI inhibited adenosine uptake in the presence or absence of PKC agonist.

Figure 5. Activation of PKC stimulates Ado uptake in mouse cardiomyocytes

Activation of PKC (by PMA, 500 nM, 20 min or peptide agonist 0.25 M, 30 min) stimulates adenosine uptake in primary cultures of mouse neonatal cardiomyocytes. Similar treatment conditions were used as described in figure 4. (Pooled data, mean ± S.E., n=3, each condition conducted in triplicate, * P <0.05 compared to control).

Figure 6. Ado uptake returns to basal levels following PC in HL-1 cells.

HL-1 cells were subjected to hypoxic PC (as described in figure 1) and Ado uptake measured following 0, 90 and 120min of post-PC normoxia. Uptake is significantly increased immediately following preconditioning (0 min post PC, ** = P <0.001 compared to control) but gradually returns to basal levels (90 min post PC, * = P <0.05 compared to control; 120 min post PC, no significant difference compared to control). Representative experiment shown as percent adenosine uptake relative to control (mean ± S.E.), repeated 3 times with similar results, each condition conducted in triplicate.

Figure 1A

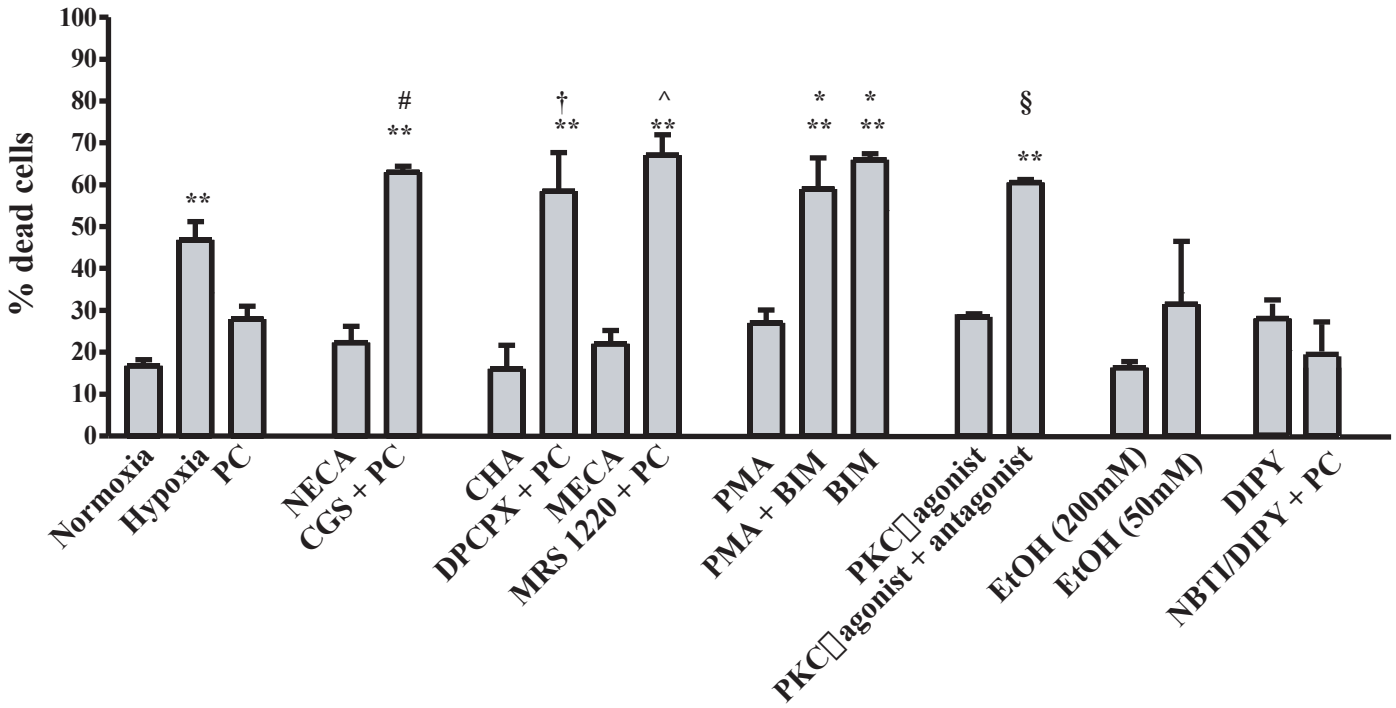


Figure 1B

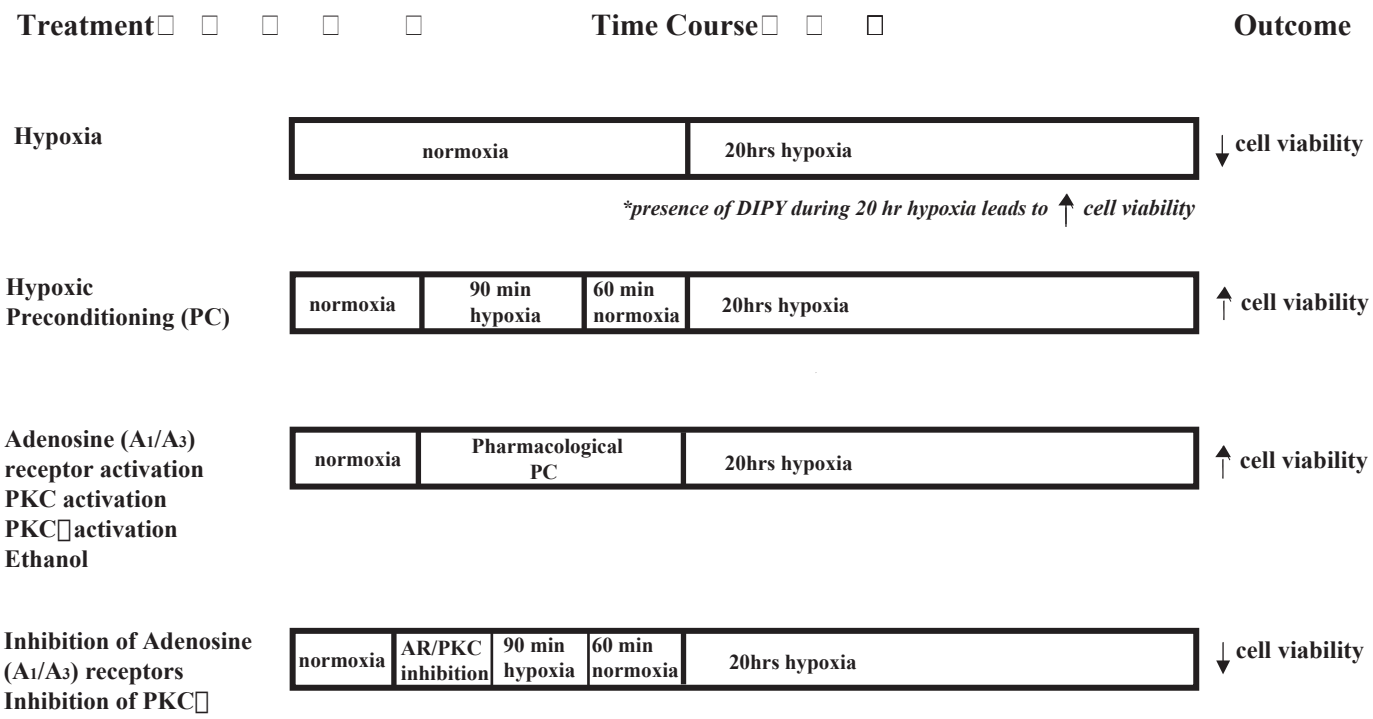
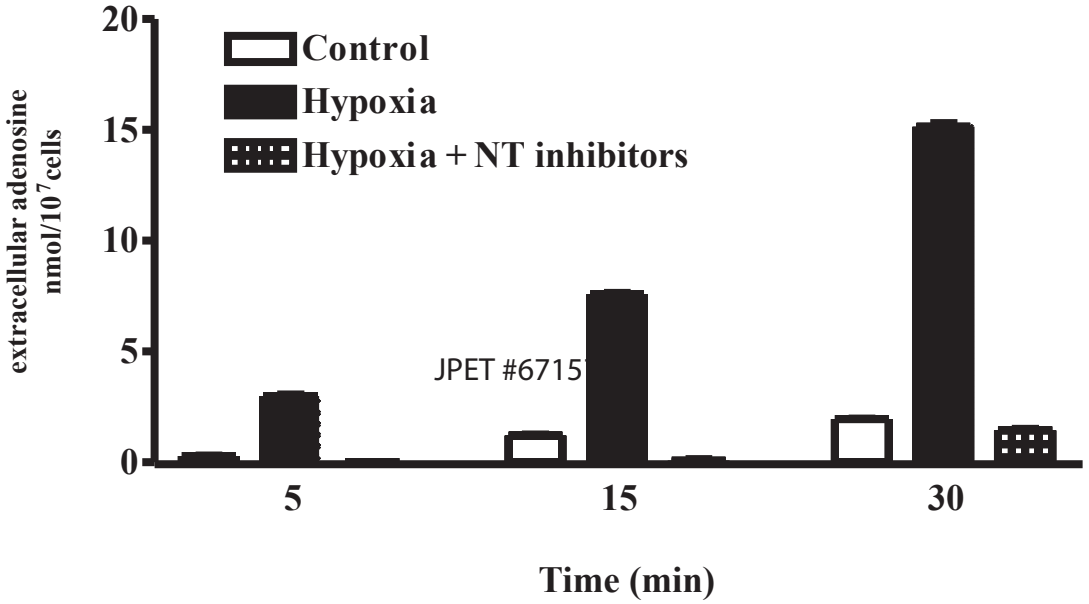


Figure 2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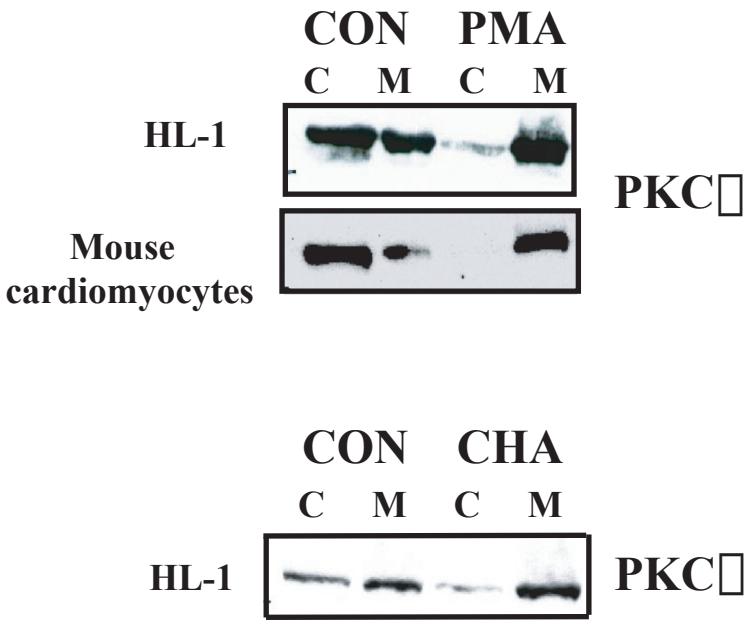
30
Time (min)

Figure 3


A
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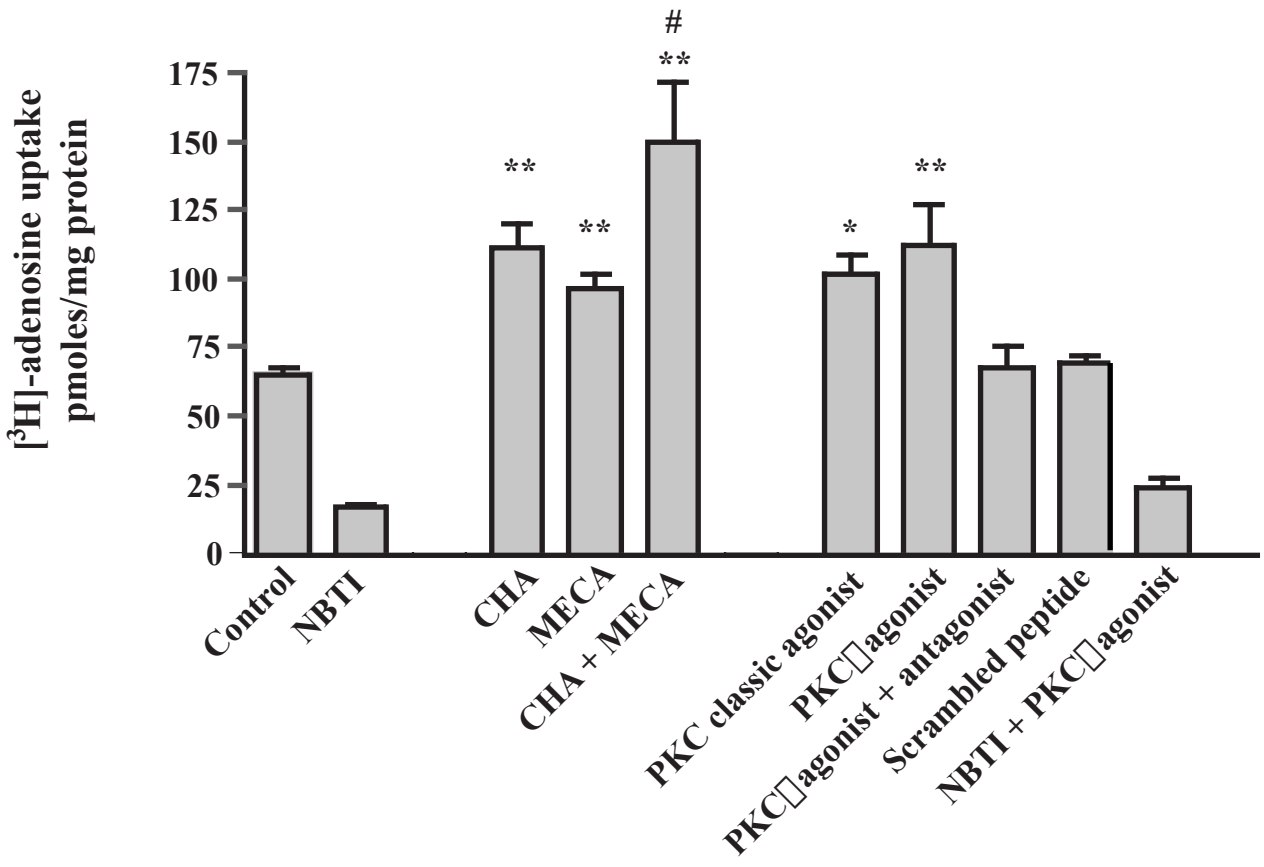
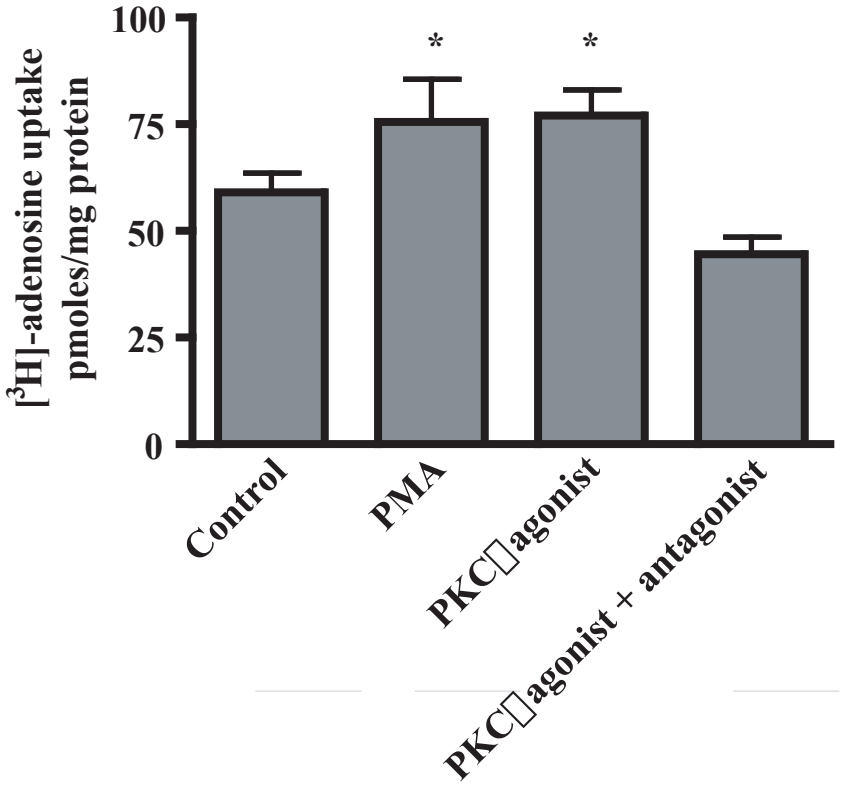


Figure 4

Figure 5


100
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[3H]-adenosine uptake
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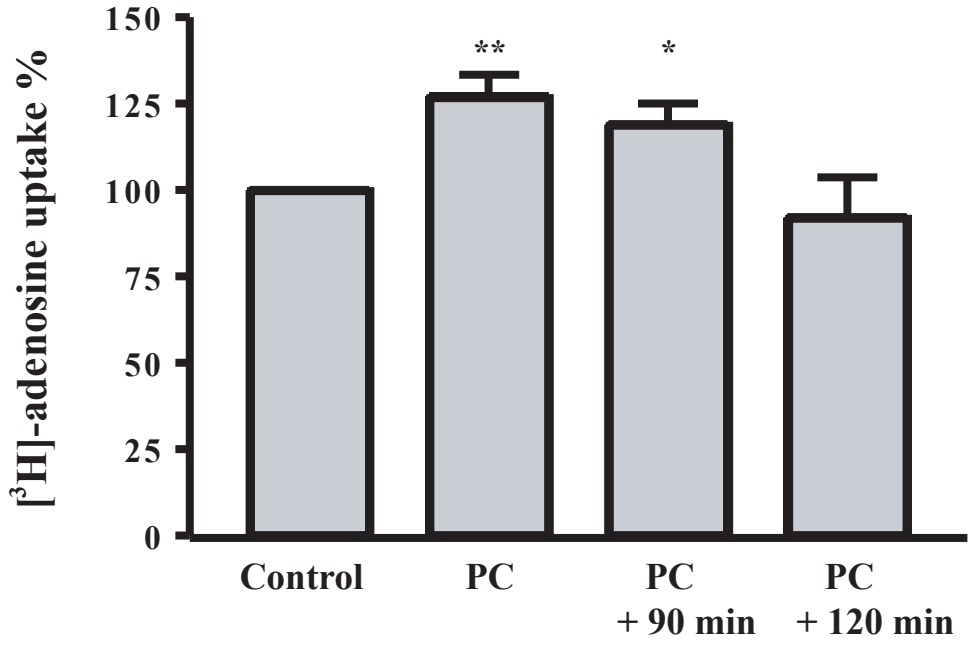
*
Control
PMA
PKC agonist
PKC agonist + antagonist

Figure 6


[3H]-adenosine uptake %
150
125
100
75
50
25
0
**
*
Control
PC
PC
+ 90 min
PC
+ 120 min