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RESEARCH PAPER

# $A_{2B}$ adenosine receptors inhibit superoxide production from mitochondrial complex I in rabbit cardiomyocytes via a mechanism sensitive to *Pertussis* toxin

Xiulan Yang[1], Wenkuan Xin[2], Xi-Ming Yang[1], Atsushi Kuno[1], Thomas C Rich[2], Michael V Cohen[1,3] and James M Downey[1]

*Departments of [1]Physiology, [2]Pharmacology, and [3]Medicine, University of South Alabama, College of Medicine, Mobile, AL, USA*

**Correspondence**
James M. Downey, Department of Physiology, MSB 3074, University of South Alabama, College of Medicine, Mobile, AL 36688, USA. E-mail: jdowney@usouthal.edu

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## BACKGROUND AND PURPOSE
$A_{2B}$ adenosine receptors protect against ischaemia/reperfusion injury by activating survival kinases including extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K). However, the underlying mechanism(s) and signalling pathway(s) remain undefined.

## EXPERIMENTAL APPROACH
HEK 293 cells stably transfected with human $A_{2B}$ adenosine receptors (HEK-$A_{2B}$) and isolated adult rabbit cardiomyocytes were used to assay phosphorylation of ERK by Western blot and cation flux through cAMP-gated channels by patch clamp methods. Generation of reactive oxygen species (ROS) by mitochondria was measured with a fluorescent dye.

## KEY RESULTS
In HEK-$A_{2B}$ cells, the selective $A_{2B}$ receptor agonist Bay 60-6583 (Bay 60) increased ERK phosphorylation and cAMP levels, detected by current through cAMP-gated ion channels. However, increased cAMP or its downstream target protein kinase A was not involved in ERK phosphorylation. *Pertussis* toxin (PTX) blocked ERK phosphorylation, suggesting receptor coupling to $G_i$ or $G_o$ proteins. Phosphorylation was also blocked by inhibition of PI3K (with wortmannin) or of ERK kinase (MEK1/2, with PD 98059) but not by inhibition of NO synthase (NOS). In cardiomyocytes, Bay 60 did not affect cAMP levels but did block the increased superoxide generation induced by rotenone, a mitochondrial complex I inhibitor. This effect of Bay 60 was inhibited by PD 98059, wortmannin or PTX. Inhibition of NOS blocked superoxide production because NOS is downstream of ERK.

## CONCLUSION AND IMPLICATIONS
Activation of $A_{2B}$ adenosine receptors reduced superoxide generation from mitochondrial complex I through $G_{i/o}$, ERK, PI3K, and NOS, all of which have been implicated in ischaemic preconditioning.

## Abbreviations
ADA, adenosine deaminase; Bay 60, Bay 60-6583 (2-[(3,4-dimethoxyphenyl)methyl]-7-[(1*R*)-1-hydroxyethyl]-4-phenylbutyl]-5-methyl-imidazo[5,1-f][1,2,4]triazin-4(1H)-one); CCPA, 2-chloro-$N^6$-cyclopentyladenosine; CNG, cyclic nucleotide–gated; CPT-cAMP, 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; HEK-$A_{2B}$, HEK cells transfected with $A_{2B}$ adenosine receptors; IBMX, 3-isobutyl-1-methylxanthine; IPC, ischaemic preconditioning; KHH, Krebs–Henseleit–HEPES; L-NAME, $N^{\omega}$-nitro-L-arginine methyl ester; MM-IBMX, 8-Methoxymethyl-IBMX; MPTP, mitochondrial permeability transition

pore; NECA, 5′-(N-ethylcarboxamido) adenosine; NO, nitric oxide; NOS, NO synthase; PDE, phosphodiesterase; pERK, phosphorylated ERK; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PKI, PKA inhibitor (myristolated PKI 14-22 amide peptide (GRTGRRNAI); PTX, *Pertussis* toxin; RISK, reperfusion injury salvage kinases; ROS, reactive oxygen species; Rp-8-Br-cAMPS, 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, Rp isomer

## Introduction

Adenosine receptors exist in four subtypes: $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ (nomenclature follows Alexander *et al.*, 2009). The $A_1$ and $A_3$ adenosine receptors are coupled to $G_i$ proteins, while the high-affinity $A_{2A}$ and low-affinity $A_{2B}$ adenosine receptors are coupled to $G_s$ proteins. The $A_{2B}$ adenosine receptors are involved in the regulation of diverse processes such as vascular tone (Rubino *et al.*, 1995), neurosecretion (Okada *et al.*, 1996) and mast cell degranulation (Feoktistov and Biaggioni, 1995). In ischaemic preconditioning (IPC), one or more short coronary occlusions followed by reperfusion cause hearts to become rapidly resistant to necrosis from a subsequent prolonged ischaemic insult. $A_1$ and $A_3$ adenosine receptors and bradykinin and opioid receptors participate in parallel prior to the ischaemic insult as physiological triggers of IPC (Yang *et al.*, 2010). The actual protection from IPC is thought to occur during reperfusion after the period of ischaemia has been terminated (mediator phase). Formation of mitochondrial permeability transition pore (MPTP) kills many cells in the first minutes of reperfusion, and this formation appears to be blocked in IPC hearts (Hausenloy *et al.*, 2004). We have proposed that $A_{2B}$ adenosine receptors play a critical role in suppressing MPTP formation in IPC (Kuno *et al.*, 2007).

Blocking $A_{2B}$ adenosine receptors early in the first hour of reperfusion prevents IPC from protecting rabbit hearts (Solenkova *et al.*, 2006) and giving an $A_{2B}$ adenosine receptor agonist at reperfusion protects rabbit hearts that do not have IPC (Kuno *et al.*, 2007). It was originally reported that *in situ* hearts of mice in which $A_{2B}$ adenosine receptors had been genetically deleted were not protected by IPC, while those lacking $A_1$, $A_{2A}$ or $A_3$ adenosine receptors were still protected (Eckle *et al.*, 2007). That suggested that the $A_{2B}$ adenosine receptor was the only receptor controlling MPTP formation. However, a recent study using isolated hearts with a different $A_{2B}$ adenosine receptor knock-out construct could still be protected by IPC (Maas *et al.*, 2010), suggesting other signalling pathways. In the latter study, Bay 60-6583 (Bay 60), a selective agonist of $A_{2B}$ adenosine receptors, still protected rat hearts.

In addition to coupling of $A_{2B}$ adenosine receptors to $G_s$ protein, these receptors also couple to phospholipase C and protein kinase C (PKC) through $G_q$ in some tissues such as mast cells (Feoktistov and Biaggioni, 1995) and cardiac fibroblasts (Feng *et al.*, 2010). Surprisingly, in rabbit hearts protected by IPC, we found $A_{2B}$ adenosine receptors to be downstream of PKC rather than upstream (Philipp *et al.*, 2006). We therefore proposed that PKC acts to lower the threshold for $A_{2B}$ adenosine receptor signalling such that endogenous adenosine can activate this normally low-affinity receptor at the onset of reperfusion (Kuno *et al.*, 2007). Protection by IPC in rabbit and rat hearts requires phosphorylation of the activation site on the extracellular-signal regulated kinase (ERK), which has two isoforms (p42 and p44). Blocking phosphorylation of ERK (pERK) at reperfusion prevented protection from either IPC (Hausenloy *et al.*, 2004) or an $A_{2B}$ adenosine receptor agonist (Yang *et al.*, 2004), and treatment of a non-preconditioned heart with an $A_{2B}$ adenosine receptor agonist increased ERK phosphorylation and protected the heart (Yang *et al.*, 2004). The present study investigated how activation of the $A_{2B}$ adenosine receptors could be protective and the signalling pathways involved. We first studied HEK 293 cells transfected with human $A_{2B}$ adenosine receptors and then tried to confirm our findings in isolated adult rabbit ventricular cardiomyocytes.

## Methods

### *Isolation and culture of rabbit ventricular cardiomyocytes*

All animal care and experimental procedures were in accordance with guidelines established by the National Institutes of Health (National Research Council, 1996) and were approved by our Institutional Animal Care and Use Committee. New Zealand White rabbits of either sex (between 2 and 3 kg) were used, and ventricular myocytes were isolated as described previously (Oldenburg *et al.*, 2002; Krieg *et al.*, 2004). Briefly, retrograde perfusion of a rabbit heart mounted on a Langendorff apparatus was carried out using Krebs–Henseleit–HEPES buffer (KHH buffer) containing 1.25 mM $CaCl_2$ and then switched to calcium-free KHH buffer, after which the heart was digested for about 40 min in KHH buffer containing collagenase type II. The softened heart was minced, and the digested heart tissues were passed through fine nylon mesh (about 310 µm diameter). Myocytes were made calcium-tolerant by stepwise restoration of calcium. Cells were plated in 60 × 15 mm plastic cell culture dishes in 3 mL of Medium 199 modified with Earle's salts containing L-glutamine and enriched with 24 mM $NaHCO_3$, 100 $U \cdot mL^{-1}$penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin, 5 mM creatinine, 2 mM L-carnitine and 5 mM taurine in a 37°C incubator. In selected experiments, Dulbecco's modified Eagle's medium (DMEM) was used in lieu of Medium 199. Viable myocytes rapidly attached to the bottom, while necrotic cells failed to adhere. The unattached cells in serum-free medium were removed after 1 h, and fresh Medium 199 was added. Cardiomyocytes were used within 4 days of isolation.

### *Measurement of superoxide generation*

Superoxide generation was measured in cultured cardiomyocytes with MitoSOX™. Cells were incubated with 2.5 µM

MitoSOX™ for 20 min in Tyrode's buffer containing (in mM) 145 NaCl, 4 KCl, 10 HEPES, 11 glucose, 2 $MgCl_2$, 1.25 $CaCl_2$ in a cell incubator at 37°C, washed three times with warm Tyrode's solution and observed under the microscope at 510 nm excitation.

Mitochondrial superoxide generation was analysed with InCytIm1 software from Intracellular Imaging (Cincinnati, OH, USA) after 20 min incubation with or without rotenone present. When Bay 60, a highly selective $A_{2B}$AR agonist, was used, it was added 10 min prior to rotenone, and when an inhibitor was used, it was added 10 min prior to Bay 60. In the *Pertussis* toxin (PTX) experiments, myocytes were pre-treated with 100 ng·mL$^{-1}$ PTX for 24 h prior to the study. In one protocol, endogenous adenosine generated by myocytes was removed by adding adenosine deaminase (ADA; 5 U·mL$^{-1}$) for 30 min before and during the experiments. For each group, at least three individual experiments were performed using separate myocyte isolations.

## *HEK-$A_{2B}$ cell culture*

HEK 293 cells stably transfected with human $A_{2B}$ adenosine receptors (HEK-$A_{2B}$) were a gift from Joel Linden (Linden *et al.*, 1999). HEK-$A_{2B}$ cells were grown at 37°C in plastic culture plates using DMEM containing 0.3 mg·mL$^{-1}$ G418, 50 U·mL$^{-1}$ penicillin, 50 μg·mL$^{-1}$ streptomycin, 2 mM L-glutamine, 10% fetal calf serum in 95% air/5% $CO_2$ (Liu *et al.*, 2010). All cultures were divided three times a week at a ratio of 1:5.

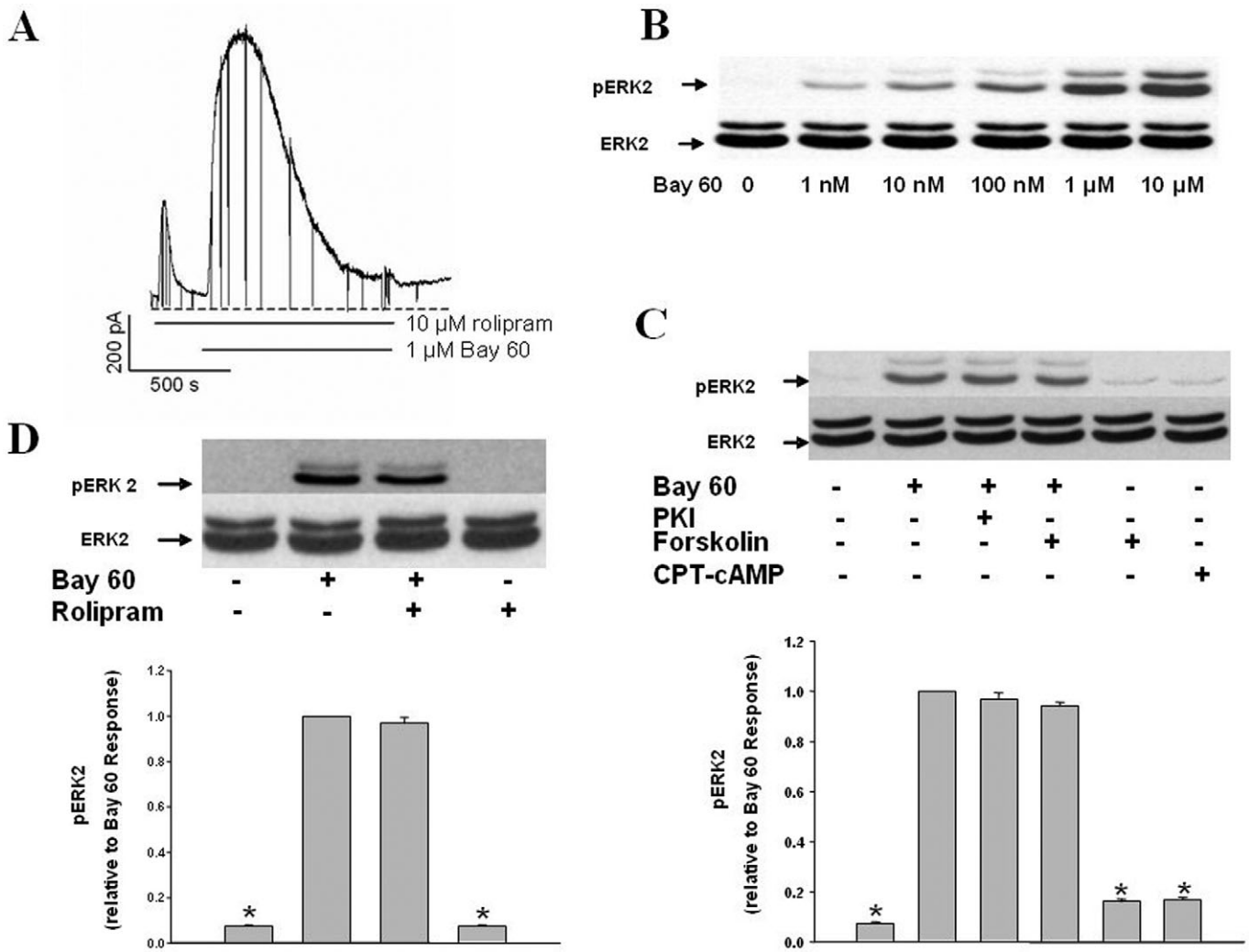
## *Protein phosphorylation and immunoblotting*

Protein phosphorylation of both isoforms of ERK was analysed by Western blotting with phosphospecific antibodies (Yang *et al.*, 2004). Prior to an experiment, HEK-$A_{2B}$ cells were serum-starved overnight to induce quiescence, washed twice with Tyrode's solution and stimulated with Bay 60 for 5 min. When protein kinase inhibitors were used (PKI, PD 98059), they were added 20 min before Bay 60. Either forskolin or the cell-permeable 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate (CPT-cAMP) was added to the medium alone or with Bay 60. PTX was applied 24 h prior to study. After two washes with ice-cold Tyrode's solution, cells were lysed in lysis buffer (70 mM β-glycerophosphate, 0.5% Triton X-100, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM NaF, 1 mM $Na_3VO_4$, 20 μg·mL$^{-1}$ aprotinin, 5 μg·mL$^{-1}$ leupeptin), and cellular debris were removed by centrifugation. Samples were denatured with Laemmli buffer, and protein content was determined by the Bradford assay. Proteins were separated by polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Equal loading of lanes was confirmed by density scanning of Ponceau S–stained membranes prior to immunoblotting. Membranes were blocked with 5% dry milk powder in phosphate buffered saline with Tween 20 and incubated with mouse monoclonal phospho-specific anti-phospho-Thr$^{202}$/Tyr$^{204}$ ERK overnight on a shaker at 4°C. After incubation with anti-mouse horseradish peroxidase–coupled secondary antibody, bands were visualized with Immobilon™ according to the manufacturer's instructions. To further test for equal loading, membranes were stripped and reprobed with non-phosphospecific anti-ERK antibody. Parallel changes occurred in both ERK 1 (44 kDa) and ERK 2 (42 kDa). All blots for each experiment were run on the same gel and any lane where total ERK differed from the rest was omitted.

## *Single cell cAMP study for HEK-$A_{2B}$ cells and cardiomyocytes*

HEK-$A_{2B}$ cells were plated at about 60% confluence in dishes for infection with an adenovirus encoding the cyclic nucleotide–gated channel (CNG) A2, with C460W and E583M mutations (Rich *et al.*, 2001) at a multiplicity of infection of about 10 pfu per cell. These channels have an apparent affinity for cAMP of 1.1 μM allowing the measurement of near-membrane free cAMP levels of 0.1 to 5 μM (Rich and Karpen, 2002). The binding of cAMP to CNG channels triggers a conformational change in the channel, allowing cation flux through the channels and resulting electric currents. The generated current reflects the concentration of near-membrane cAMP. Two hours following infection, hydroxyurea was added to the cell medium at a final concentration of 1 mM to inhibit viral replication. Twenty-four hours after infection, cells were detached with phosphate buffered saline containing 0.03% EDTA, resuspended in serum-containing medium and assayed within 12 h. All experiments were conducted at room temperature, 22–25°C. The whole-cell patch clamp technique was used to measure cAMP in single cells (Xin *et al.*, 2008). Recordings were made with a HEKA-EPC10 patch clamp amplifier system [EPC10 (HEKA Elektronik, Lambrecht/Pfalz, Germany), Nikon TE 2000-S microscope, MP-225 micromanipulator system (Sutter Instrument, Novato, CA, USA), SF-77B Perfusion Fast-Step system and VC-6 six-channel valve controller (Warner Instrument, Hamden, CT, USA)]. Pipette resistance was limited to 4 MΩ and averaged 2.2 ± 0.1 MΩ. Voltage offsets were zeroed with the pipette in the bath solution. After achieving whole-cell configuration, the preparation was allowed to equilibrate for at least 10 min to ensure sufficient time for dialysis of compounds from the patch pipette into the cell. Current records were typically sampled at 10 kHz, filtered at 2 kHz and stored on a PC. Currents were recorded during a 400-ms step to a membrane potential of –30 mV from a holding potential of 0 mV. The pipette solution contained (in mM) 140 KCl, 0.5 $MgCl_2$, 10 HEPES, 5 $Na_2GTP$ (pH 7.4); the bath solution contained (in mM) 140 NaCl, 4 KCl, 10 D-glucose, 10 HEPES and 0.1 $MgCl_2$ (pH 7.4). After establishment of a baseline, rolipram (10 μM), an inhibitor of phosphodiesterase (PDE) 4, was added to the cell bath. With rolipram still present, Bay 60 (1 μM) was added. Extracellular solutions were applied using a SF-77B solution switcher (Warner Instruments) with a mechanical switch time of 1–2 min. The experimental protocol is presented in Figure 1.

Almost the same procedure was used for detecting cAMP signals in cardiomyocytes. Specifically, after 24 h of transfection with the same adenovirus, a single rod-shaped cardiomyocyte was attached to a pipette and positioned in a chamber where it was superfused with buffer from one of three nozzles, each fed from a separate reservoir. Solenoid valves allowed rapid switching from one nozzle to another, thus permitting quick and effective change of the composition of the superfusate. A PDE inhibitor cocktail containing 100 μM 8-methoxymethyl-3-isobutyl-1-methylxanthine (MM-IBMX), 10 μM cilostamide and 10 μM rolipram, which inhibit PDE1, PDE3 and PDE4, respectively, was used to




## Figure 1

Effect of Bay 60-6583 (Bay 60) on HEK-$A_{2B}$ cells. (A) The $A_{2B}$ adenosine receptor agonist Bay 60 increased cAMP measured as cation flux in HEK-$A_{2B}$ cells (vertical axis). Downward spikes indicate times when CNG channels were momentarily blocked with $Mg^{2+}$ (current returns to baseline). (B) A dose–response to Bay 60 for ERK phosphorylation. The upper blot shows phosphorylated (activated) ERK, while the lower shows total ERK. (C) The effects of the PKA inhibitor PKI (1 μM), forskolin (10 μM) or CPT-cAMP (100 μM) on ERK phosphorylation induced by BAY 60. Each band was normalized to that recorded in response to Bay 60 alone. (D) The effect of the PDE inhibitor rolipram (10 μM) on Bay 60–mediated ERK phosphorylation. The number of repetitions for each set of experimental conditions was ≥4. *$P < 0.001$ versus Bay 60 alone.

maximize cAMP levels by blocking degradation. Isoprenaline, an agonist at β-adrenoceptors that are coupled to $G_s$ proteins, maximally increased cAMP production and increased current through CNG channels. The experimental protocols are presented in Figure 3. The effect of the $A_1$ adenosine receptor–selective agonist, 2-chloro-$N^6$-cyclopentyladenosine (CCPA, 200 nM), was also determined. Periodically 10 mM $Mg^{++}$, a permeant CNG channel blocker, was added to the superfusate to test whether other channels were contributing to membrane current. Under these experimental conditions, little or no agonist-induced currents were observed in myocytes that did not express CNG channels. All experiments were done at room temperature (22–25°C). Whole-cell patch recordings were made as described above. Data were analysed using customised scripts written in the MATLAB programming environment (MathWorks, v 7.4, Natick, MA, USA).

### *Data analysis*

All data are expressed as mean ± s.e.mean. One-way analysis of variance with Student–Newman–Keuls *post hoc* test was performed on baseline superoxide generation, superoxide levels produced under various conditions and pERK2 (ERK42) bands as quantified by Sigma Gel software (Aspire Software, Ashburn, VA, USA). $P < 0.05$ was considered significant. Quantification of immunoreactive bands was done by expressing the intensity of the band obtained with the $A_{2B}$ adenosine receptor agonist Bay 60 as 1, and the value of all other bands in control or drug-treated groups was normalized to the Bay 60 band using Sigma Gel. Changes in pERK1 and pERK2 were comparable for the various treatments. Because pERK1 bands were too light to see on many of the gels, only changes in pERK2 are presented.

## Materials

Cell culture medium, fetal calf serum, Rp-8-Br-cAMPS and MM-IBMX were from Sigma-Aldrich (St. Louis, MO, USA). Bay 60 was a gift from Thomas Krahn of Bayer Healthcare AB. PTX, rolipram, wortmannin, PD 98059, MRS 1754, the protein kinase A inhibitor PKI, forskolin, CCPA and rotenone were purchased from Tocris Bioscience (Ellisville, MO, USA); CPT-cAMP, isoprenaline and cilostamide from EMD Chemicals (Gibbstown, NJ, USA); and $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) from Enzo Life Sciences (Plymouth Meeting, PA, USA). Mouse monoclonal anti-phospho-$Thr^{202}$/$Tyr^{204}$ ERK, anti-ERK and anti-mouse horseradish peroxidase–coupled antibody were obtained from Cell Signaling Technology (Danvers, MA, USA), and Immobilon™ Western chemiluminescent HRP substrate from Millipore, (Billerica, MA, USA). MitoSOX™ was obtained from Cell Signaling Technology. Collagenase type II was purchased from Worthington Biochemical Corporation, (Lakewood, NJ, USA).

# Results

## HEK-$A_{2B}$ cells

*$A_{2B}$ adenosine receptor–induced cAMP changes.* We first studied HEK-$A_{2B}$ cells as they are a homogenous monoculture and can easily be grown in quantity, and Bay 60 is known to be highly selective for the human $A_{2B}$ adenosine receptors. We first confirmed that an $A_{2B}$ adenosine receptor agonist would increase cAMP levels close to the cell membrane (submembrane cAMP), in HEK-$A_{2B}$ cells transfected with CNG channels sensitive to cAMP. To increase sensitivity, we suppressed PDE activity with 10 μM rolipram, a PDE4 inhibitor. Rolipram caused a small inward current (Figure 1A), which subsided after several minutes presumably because of negative feedback suppression of adenylyl cyclase (Sobolewski *et al.*, 2004). Addition of 1 μM Bay 60 then induced a strong inward current, indicating a high level of cAMP in the submembrane space, thus confirming that membrane $A_{2B}$ adenosine receptors were functionally coupled to $G_s$ proteins in the HEK-$A_{2B}$ cells.

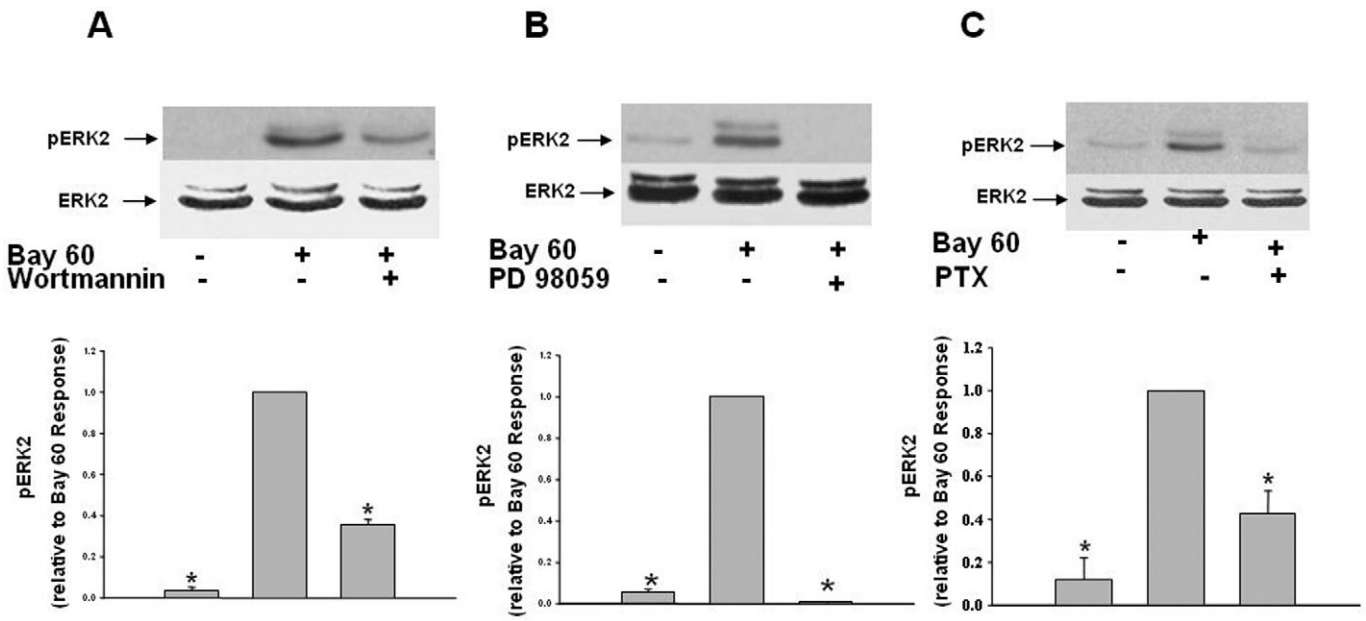
*ERK activation is independent of protein kinase A activity.* We had previously demonstrated that Bay 60 induced a profound increase of pERK in HEK-$A_{2B}$ cells (Liu *et al.*, 2010). Also, we found that $A_{2B}$ adenosine receptor-selective antagonists attenuated that increase, thus indicating that the changes in pERK were receptor-mediated. Figure 1B shows a dose–response curve for Bay 60 in HEK-$A_{2B}$ cells. Unless otherwise stated, all subsequent experiments used 1 μM Bay 60. We next tested whether increased cAMP might be contributing to Bay 60–induced ERK phosphorylation through protein kinase A (PKA). When forskolin was applied to cells in conjunction with 100 nM Bay 60 (we used a lower dose so that both increases and decreases in pERK could be observed), there was no additional effect on ERK phosphorylation and forskolin by itself only slightly increased pERK (Figure 1C). Furthermore, the PKA inhibitor PKI failed to attenuate ERK phosphorylation after Bay 60 (Figure 1C). Raising cAMP with rolipram with or without 100 nM Bay 60 (Figure 1D) or exposure of cells to the cell-permeant cAMP analogue CPT-cAMP (Figure 1C) had minimal effects on pERK. Thus, modification of cAMP levels or of PKA activity failed to mimic the effects of Bay 60 on ERK phosphorylation.

*Akt, pERK and NO.* Wortmannin, a potent inhibitor of phosphatidylinositol 3-kinase (PI3K) at 100 nM (Powis *et al.*, 1994), attenuated Bay 60–mediated ERK phosphorylation by ~60%, suggesting involvement of PI3K ($P < 0.05$) (Figure 2A). Pretreatment of cells with 20 μM PD 98059 (Dudley *et al.*, 1995), a MEK1/2 and therefore ERK1/2 antagonist, abolished the enhanced ERK phosphorylation triggered by Bay 60 (Figure 2B), confirming that the phosphorylation depended on MEK1/2 activation rather than phosphatase inhibition. Administration of L-NAME, 100 μM (Wunder *et al.*, 2007), did not affect the level of pERK-induced by Bay 60 (data not shown), indicating that nitric oxide synthase (NOS) was not involved. We used PD 98059, L-NAME and wortmannin because these inhibitors at these concentrations block protection from the $A_{2B}$ adenosine receptor agonist given at reperfusion in rabbit hearts (Yang *et al.*, 2004).

*$G_{i/o}$ coupling of A2B adenosine receptors.* We next investigated whether a $G_i$ coupling might be involved. Overnight pretreatment of isolated HEK-$A_{2B}$ cells with PTX causes ADP-ribosylation of the α subunit of both $G_i$ and $G_o$ proteins and thus prevents interaction of the protein heterotrimer with the receptor. PTX had no effect on basal pERK (data not shown). Bay 60 again significantly increased the pERK level and PTX attenuated this effect of Bay 60 by ~60% ($P < 0.05$) (Figure 2C). These results indicated that activation of the $A_{2B}$ adenosine receptors induced phosphorylation of ERK through a PTX-sensitive pathway implying $G_i$ (or $G_o$) protein coupling. These findings are consistent with our failure to show involvement of cAMP or PKA in the phosphorylation and activation of ERK.

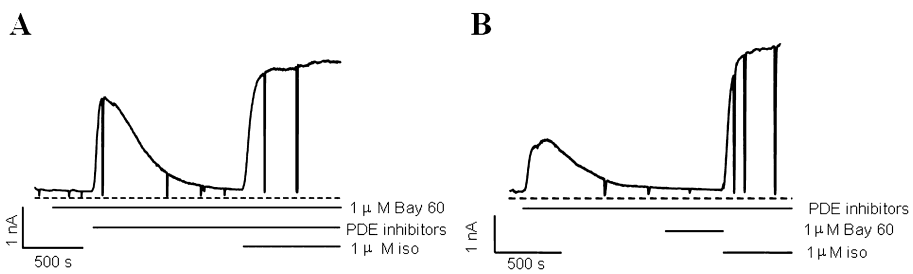
## Cardiomyocytes

*$A_{2B}$ adenosine receptor–induced cAMP changes.* We next attempted to see if cardiomyocytes would exhibit responses similar to those seen in HEK-$A_{2B}$ cells. We repeated our cAMP measurements on CNG channel–transfected cardiomyocytes. Surprisingly, Bay 60 at 1 μM did not change the current and, by implication, basal cAMP levels in cardiomyocytes (Figure 3A). Although the cells were not responsive to Bay 60, both the PDE inhibitor cocktail and isoprenaline produced significant currents (Figure 3A). The PDE inhibitor cocktail alone in single cardiomyocytes greatly increased the current, indicating a basal level of adenylyl cyclase activity even without receptor stimulation (Figure 3B). The increase in cAMP after PDE inhibition was transient, and the current slowly declined, presumably because of feedback inhibition of adenylyl cyclase and residual PDE activity (Beazely and Watts, 2006; Rich *et al.*, 2007; Xin *et al.*, 2008). To increase the sensitivity of our measurements, we altered the experimental procedure, as shown in Figure 3B. We gave the PDE inhibitor cocktail first and then waited for the current to decline before adding Bay 60. This was the same protocol used in Figure 1A, which resulted in a dramatic response to Bay 60 in HEK-$A_{2B}$ cells. With PDE inhibited, even a small




## Figure 2

Effect of various inhibitors on Bay 60-6583 (Bay 60)–induced ERK phosphorylation in HEK-$A_{2B}$ cells. (A) Pretreatment of cells with wortmannin (100 nM) for 20 min. (B) 20 μM PD 98059 pretreatment. (C) Overnight pretreatment of HEK-$A_{2B}$ cells with *Pertussis* toxin (PTX) (100 ng·mL$^{-1}$). $n \geq 4$ for all experimental conditions. All blots for each experiment were run on the same gel and any lane where total ERK differed from the rest was omitted. *$P < 0.001$ versus Bay 60 alone.

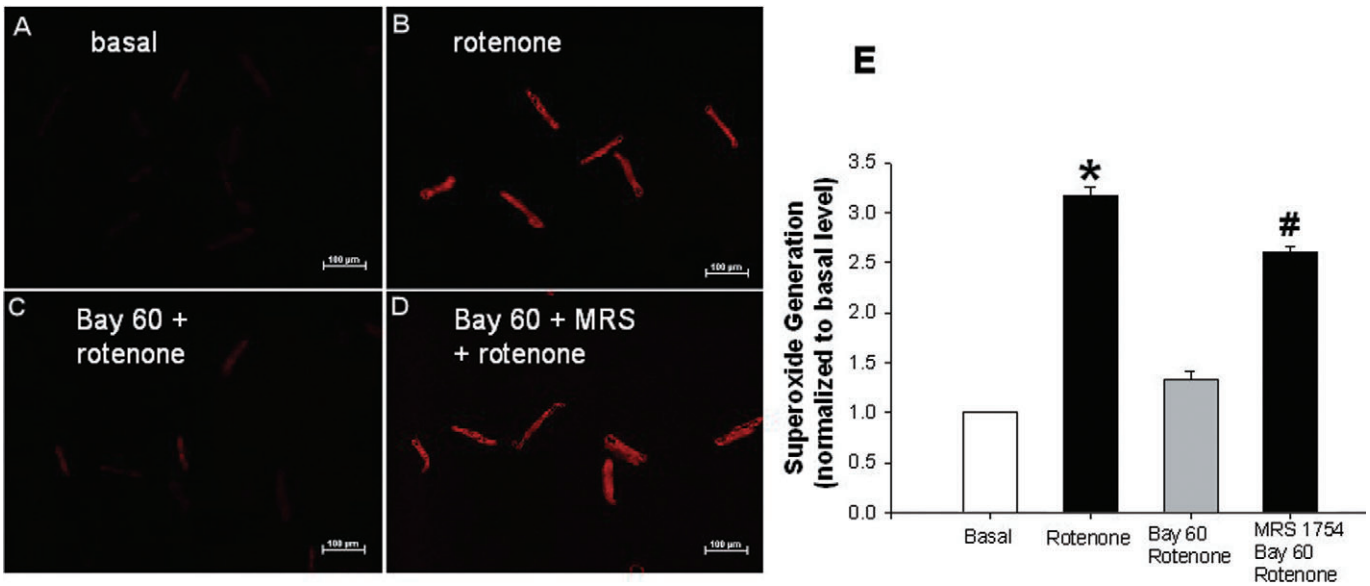



## Figure 3

cAMP detected as membrane current in rabbit cardiomyocytes transfected with CNG channels. (A) 1 μM Bay 60 did not change cAMP in single cardiomyocytes, although a phosphodiesterase (PDE) inhibitor cocktail and isoprenaline (iso) each induced a large increase ($n = 5$). (B) To increase sensitivity, Bay 60 was given after administration of a potent PDE inhibitor cocktail. Even with attenuated PDE activity, there was still no increase in cAMP in response to Bay 60.

increase in adenylyl cyclase activity should have been detected. However, Bay 60 still had no effect on the current. Thus, there was no sign of $G_s$-protein coupling of $A_{2B}$ adenosine receptors in these cells, indicating either that the sarcolemmal $A_{2B}$ adenosine receptor density was too low to detect, or that these receptors were not coupled to $G_s$ proteins.

As described in a preliminary report (Xin *et al.*, 2009), we previously tested if $A_{2B}$ adenosine receptors might instead be coupled to $G_i$ in these cells. $G_i$-coupled receptors can be detected by their ability to attenuate the response to a test dose of isoprenaline whose receptor couples to $G_s$. The increase in current in response to 50 nM isoprenaline was 56 ± 9% of the maximal current produced after treatment of cells with a PDE cocktail. In the presence of 200 nM CCPA (a $A_1$ adenosine receptor-selective agonist whose receptor couples to $G_i$), the response to isoprenaline was only 25 ± 11% ($P < 0.025$), showing antagonism by $G_i$ of the $G_s$-stimulated current. In the presence of Bay 60, the isoprenaline current (75 ± 11%) was not significantly changed from that for isoprenaline alone. Thus, we could not detect either an increase or decrease in cAMP in response to a receptor-saturating dose of an $A_{2B}$ adenosine receptor agonist, again suggesting few if any surface $A_{2B}$ adenosine receptors. Yet Bay 60 is highly protective in the ischaemic rabbit heart (Kuno *et al.*, 2007).



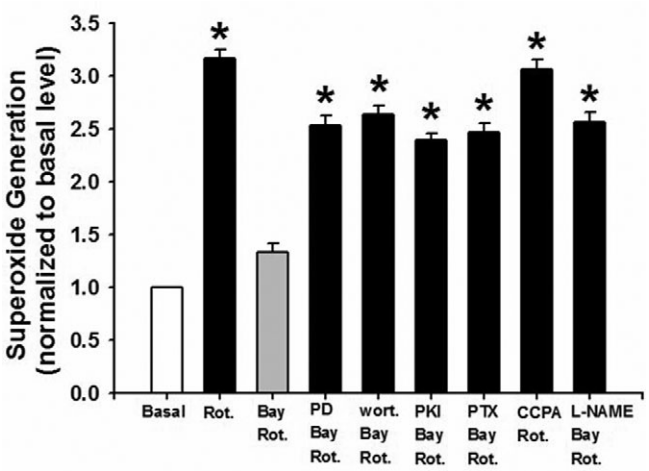

## Figure 4

Effect of Bay 60 on superoxide production detected by MitoSox™ in cardiomyocytes. (A) Basal superoxide generation in isolated rabbit cardiomyocytes. (B) 20 μM rotenone greatly increased superoxide production. (C) 1 μM Bay 60, an $A_{2B}$ adenosine receptor agonist, attenuated the effect of rotenone. (D) 20 nM MRS 1754, a highly selective $A_{2B}$ adenosine receptor antagonist, blocked the effects of Bay 60. (E) Summary of changes in superoxide generation in the four groups. * $P < 0.001$ between basal and rotenone-treated groups # $P < 0.001$ between Bay 60 + rotenone groups with and without MRS 1754.

*$A_{2B}$ adenosine receptors attenuate superoxide generation induced by rotenone in cardiomyocytes.* One possible explanation for these cAMP results might be that rabbit cardiomyocytes do not express $A_{2B}$ adenosine receptors, and that the protection emanates from receptors on some other cell type in the heart. Another possibility is that receptors are present, but their density is too low for our system to detect. A third possibility is that they may be present in the cardiomyocyte but are not located on the sarcolemma. We sought to demonstrate any $A_{2B}$ adenosine receptor–mediated effect in isolated cardiomyocytes. A biochemical measurement would not be definitive because the isolated myocyte culture is not a monoculture and is contaminated with other cell types including fibroblasts that do express $A_{2B}$ adenosine receptors (Feng *et al.*, 2010). We therefore looked for a response we could measure in a single cardiomyocyte. It has been proposed that reactive oxygen species (ROS) from complex I in mitochondria contribute to reperfusion injury (Ambrosio *et al.*, 1993; Tompkins *et al.*, 2006), so we measured superoxide production in cardiomyocytes. MitoSOX™ red is a novel fluorogenic dye that is highly selective for superoxide in mitochondria of live cells. Once taken up by mitochondria, MitoSOX™ is oxidized only by superoxide to make a red fluorescent product. We found a low, but detectable, superoxide generation in untreated isolated myocytes (Figure 4A and E). Administration of 20 μM rotenone, a mitochondrial complex I inhibitor, increased basal superoxide production by approximately threefold (Figure 4B and E). Pretreatment of myocytes with 1 μM Bay 60 blocked this effect of rotenone (Figure 4C and E). The effects of Bay 60 were blocked by 20 nM MRS 1754 (Ji *et al.*, 2001), a highly selective $A_{2B}$ adenosine receptor antagonist (Figure 4D and E), confirming that the effects of Bay 60 were mediated by $A_{2B}$ adenosine receptors.

Medium M199 in which most cells used in this investigation were incubated contains 1 $mg \cdot L^{-1}$ ATP that, after degradation, could produce up to 2 μM adenosine. This generated adenosine could theoretically activate other adenosine receptors and affect the response to Bay 60. To eliminate any effect of possible adenosine production in the medium, adenosine deaminase (ADA) was added 30 min prior to experiments. In the presence of ADA, Bay 60 still attenuated superoxide production induced by rotenone. In additional experiments, myocytes were instead cultured only in DMEM, which does not contain ATP; again the response to Bay 60 was not affected.

*pERK, PI3K, NOS and a PTX-sensitive G protein are in the pathway between $A_{2B}$ adenosine receptors and attenuated superoxide production.* These observations indicated that the cardiomyocyte does have functional $A_{2B}$ adenosine receptors, and that we could monitor the inhibition of rotenone-induced superoxide production as a reporter for $A_{2B}$ adenosine receptor activation in the cardiomyocyte. In hearts subjected to ischaemia/reperfusion *in vitro*, PD 98059 abolished protection by the $A_{2B}$ adenosine receptor agonist Bay 60 (Cohen *et al.*, 2010). Therefore, ERK phosphorylation might be involved in the superoxide production that we measured in rotenone-treated cells. Accordingly, we treated cultured cardiomyocytes with PD 98059 before exposure to rotenone and Bay 60 and found that this inhibitor (20 μM; Dudley *et al.*, 1995) attenuated the effect of Bay 60 on rotenone-induced super-




## Figure 5

The effect of various inhibitors on the ability of Bay 60-6583 (Bay) to attenuate superoxide production in rotenone (Rot.)–treated cardiomyocytes. PD 98059 (PD) (MEK1/2 inhibitor) attenuated the ability of Bay to suppress superoxide generation as did the PI3K inhibitor wortmannin (wort; 100 nM), the PKA inhibitor PKI (1 μM), 24 h of $G_i$ antagonist *Pertussis* toxin (PTX) treatment, and the NOS inhibitor $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME). The $A_1$ adenosine receptor–selective agonist, 2-chloro-$N^6$-cyclopentyladenosine (CCPA) had no effect on rotenone's ability to enhance superoxide generation. *$P < 0.001$ for comparison of any experimental group to either basal or Bay + rotenone group.

oxide production (Figure 5), indicating that pERK was involved in the processes between activation of $A_{2B}$ adenosine receptors and ROS production.

In the isolated rabbit heart model, the protective function of $A_{2B}$ adenosine receptors could be blocked by the PI3K inhibitor wortmannin (Cohen *et al.*, 2010), a potent, selective, cell-permeable and irreversible inhibitor of PI3K with an $IC_{50}$ of 2–4 nM. In our preparation of isolated cardiomyocytes, wortmannin also blocked the effect of Bay 60 on the rotenone-induced increase in superoxide generation (Figure 5).

Nitric oxide (NO) plays an essential role in the cardioprotective function of $A_{2B}$ adenosine receptors, and the NO step appears to be downstream of pERK, as the protective effects of NO could not be blocked by PD 98059 (Cohen *et al.*, 2010). Application of the NOS inhibitor L-NAME (100 μM) attenuated the effect of Bay 60 on rotenone's enhancement of superoxide generation (Figure 5), suggesting that there was also a NO-mediated step between the $A_{2B}$ adenosine receptors and ROS production, probably downstream of pERK. Such a pathway would explain why L-NAME did not block ERK activation in HEK-$A_{2B}$ cells.

*PKA is also involved in the effects of Bay 60 on rotenone-induced ROS generation.* In isolated cardiomyocytes, application of either 1 μM PKI, a cell-permeable protein kinase A (PKA)–selective inhibitor with an $IC_{50}$ = 36 nM (Glass *et al.*, 1989), or Rp-8-Br-cAMPS (data not shown), blocked the inhibition by Bay 60 of rotenone-induced superoxide production (Figure 5). These data suggested PKA involvement. This effect was clearly different from that seen in HEK-$A_{2B}$ cells in which blocking PKA had a very minor effect on ERK phosphorylation.

*$G_{i/o}$ coupling of $A_{2B}$ adenosine receptors.* These results with the PKA inhibitor prompted us to examine the effect of PTX on cardiomyocytes, to see if $A_{2B}$ adenosine receptors were coupled to $G_{i/o}$ proteins in these cells. After overnight incubation of cardiomyocytes in PTX, the effects of Bay 60 on rotenone-treated cells was almost completely blocked (Figure 5), confirming $G_{i/o}$ protein coupling in cardiomyocytes. Thus, in contrast to our results from HEK-$A_{2B}$ cells, in cardiomyocytes, we found evidence of both PKA- and PTX-sensitive components in the pathway by which $A_{2B}$ adenosine receptors suppressed superoxide production.

While Bay 60 is known to be highly selective for human $A_{2B}$ adenosine receptors, it has not been tested against rabbit receptors. To test the possibility that the effect of Bay 60 observed here might be because of non-specific $G_i$ protein–coupled $A_1$ adenosine receptor activation, myocytes were treated with the $A_1$ adenosine receptor–selective agonist CCPA before rotenone. Figure 5 shows that CCPA (100 nM) did not decrease rotenone-induced superoxide production.

## Discussion

The protection from ischaemia–reperfusion injury by the $A_{2B}$ adenosine receptor agonist Bay 60 given just before reperfusion is equivalent to that of IPC in the rabbit heart (Cohen *et al.*, 2010). ERK activation is essential for the protective effects of activated $A_{2B}$ adenosine receptors, as interference with ERK phosphorylation by PD 98059 abolished that protection (Hausenloy and Yellon, 2004; Cohen *et al.*, 2010). ERK inhibitors given at reperfusion also abolish protection from IPC in both rat (Hausenloy *et al.*, 2005) and rabbit (Solenkova *et al.*, 2006) hearts, as do $A_{2B}$ adenosine receptor antagonists (Solenkova *et al.*, 2006), suggesting that preconditioning protects by activating $A_{2B}$ adenosine receptors at reperfusion. The anti-infarct effect of IPC appears to involve suppression of the formation of the lethal MPTP through activation of the reperfusion injury salvage kinases (RISK; Hausenloy and Yellon, 2004), which include ERK and PI3K (Hausenloy *et al.*, 2009). MPTP are formed in the first minutes of reperfusion presumably as a result of ROS production. Suppression of ROS generation from mitochondrial complex I had been proposed to be protective to reperfused hearts (Ambrosio *et al.*, 1993; Tompkins *et al.*, 2006) and could be the mechanism by which the RISK pathway protects. We increased ROS production in isolated rabbit cardiomyocytes by blocking the passage of electrons from complex I to complex III with rotenone, and activation of $A_{2B}$ adenosine receptors clearly attenuated that ROS production. In our HEK-$A_{2B}$ cells, $A_{2B}$ adenosine receptor activation leads to phosphorylation of ERK, a member of the critical RISK group and, in our cardiomyocytes, the ability of the $A_{2B}$ adenosine receptor agonist to inhibit ROS depended on activation of ERK. Our results indicate that the $A_{2B}$ adenosine receptors are present in cardiomyocytes and that they couple to a pathway that includes ERK, a PTX-sensitive signalling element, and NOS and that the $A_{2B}$ adenosine

receptors may protect by suppressing ROS production from complex I in mitochondria of the reperfused heart.

## *Activation of $A_{2B}$ adenosine receptors may protect the heart by reducing ROS production from mitochondrial complex I during reperfusion*

After a prolonged period of ischaemia, reperfusion leads to significant tissue damage mainly because the surge of oxygen and metabolic substrates into cells leads to rapid re-establishment of respiration resulting in a burst of ROS generation that precipitates formation of MPTP. Mitochondrial complex I has been proposed to be the source of damaging ROS during reperfusion (Ambrosio *et al.*, 1993; Tompkins *et al.*, 2006). This complex (NADH ubiquinone oxidoreductase), composed of over 46 subunit enzymes, is a key site of electron entry into the respiratory chain in cardiac mitochondria. Rotenone blocks complex I at a distal locus, near or at the binding site for ubiquinone. When rotenone inhibits complex I, the proximal parts of the complex become fully reduced, thus favouring electron leak to oxygen. Mitochondria oxidizing NADH generate superoxide that is then released into the matrix of the mitochondria if the electrons cannot be passed to complex III, which is exactly what happens when rotenone is present or when the complex has been damaged by ischaemia (Barja, 1999; Muller *et al.*, 2004). In isolated rabbit cardiomyocytes, there is low but detectable superoxide generation under basal conditions. However, after application of rotenone, superoxide levels increased more than threefold. Pretreatment of cardiomyocytes with the $A_{2B}$ adenosine receptor agonist Bay 60 greatly decreased the superoxide burst induced by rotenone. $A_{2B}$ adenosine receptor activation critically affects mitochondria either directly at complex I or indirectly by affecting an upstream signaling step. This effect of Bay 60 could be blocked by the receptor blocker MRS 1754, which indicates that the effect was receptor-mediated. We do not yet know how activation of $A_{2B}$ adenosine receptors might bring about the inhibition of ROS production and whether such activation has other targets in or outside the mitochondria.

There are many sources of ROS in the cell, and many molecular species are generated. $G_i$-coupled receptors, such as those for bradykinin and the opioids, trigger entrance into a preconditioned state by activating PKC through a redox signaling mechanism (Pain *et al.*, 2000). Cardiomyocytes produce increased ROS when those receptors are occupied (Oldenburg *et al.*, 2004), and activation of $A_{2B}$ adenosine receptors acts to protect at the time of reperfusion, if the heart has been preconditioned (Kuno *et al.*, 2007). Our data suggest that $A_{2B}$ adenosine receptor signalling may protect by specifically suppressing superoxide formation from mitochondrial complex I.

## *PTX-sensitive $G_{i/o}$ protein coupling of $A_{2B}$ adenosine receptors*

In most cells, $A_{2B}$ adenosine receptors strongly couple to $G_s$ proteins (Gao *et al.*, 1999), and we confirmed that in HEK-$A_{2B}$ cells. Although $G_{q/11}$ protein coupling has also been reported for HEK-$A_{2B}$ cells (Gao *et al.*, 1999), Schulte and Fredholm (2003) could not detect evidence of that in CHO-$A_{2B}$ cells. Nor did they see any effects of the $G_{i/o}$ inhibitor PTX. Surprisingly, in cardiomyocytes, PTX pretreatment consistently caused dramatic attenuation of the rotenone-induced increase of superoxide generation and, in HEK-$A_{2B}$ cells, PTX attenuated ERK phosphorylation induced by the $A_{2B}$ adenosine receptor agonist Bay 60. The possibility of $A_1$ adenosine receptor activation in our cell models by Bay 60 application was excluded. This suggests that coupling depends both on the cell type and the pathway under study. To our knowledge, this is the first report indicating that $A_{2B}$ adenosine receptor signalling can be PTX-sensitive in both cardiac and non-cardiac cells.

Such a dual coupling is not unusual among G protein–coupled receptors. $\beta_2$-Adrenoceptors couple to both $G_s$ and $G_i$ proteins (Daaka *et al.*, 1997), and $\beta_2$-adrenoceptors also induce activation of ERK. That signalling results from a switch in receptor coupling from $G_s$ to $G_i$ proteins. Feedback phosphorylation by PKA of the $\beta_2$-adrenoceptor within the third intracellular loop and C-terminal tail attenuates receptor-$G_s$ coupling and facilitates coupling to $G_i$. ADP-ribosylation of the $G_i\alpha$ subunit with PTX inhibits $\beta_2$-adrenoceptor-mediated ERK activation. We now report a similar $G_i/G_s$ coupling for $A_{2B}$ adenosine receptors. However, in this case, PKA did not appear to be involved in HEK-$A_{2B}$ cells, although it may be in cardiomyocytes.

## *cAMP changes by Bay 60 are not detectable in cardiomyocytes, but PKA attenuates the effect of Bay 60 on rotenone-induced ROS generation*

In single cardiomyocytes, we were unable to detect Bay 60–induced changes in cAMP, although Bay 60 did modulate cAMP in HEK-$A_{2B}$ cells. Several explanations are possible. MRS 1754 blocked the effects of Bay 60 on superoxide generation, indicating that the effect was receptor-mediated. Thus, we can exclude the possibility that cardiomyocytes do not express $A_{2B}$ adenosine receptors. Another possibility is that $A_{2B}$ adenosine receptors may not be found in the myocyte sarcolemma, and we have recently proposed that these receptors are located near the mitochondria of cardiomyocytes (Grube *et al.*, 2011). Cytosolic adenosine receptors have also been reported in human skeletal muscle (Lynge and Hellsten, 2000). The second possibility is that $A_{2B}$ adenosine receptors are in the sarcolemma, but the density is too low to significantly affect adenylyl cyclase. A third possibility is that $A_{2B}$ adenosine receptors affect cAMP in an intracellular compartment distinct from that in which CNG channels detect cAMP. In rat cardiomyocytes, different PDEs catalyse the hydrolysis of cAMP depending on which membrane receptor generated the cyclic nucleotide (Mongillo *et al.*, 2004; Rochais *et al.*, 2006; Leroy *et al.*, 2008), suggesting that there are different receptor-specific compartments for cAMP within the cell. Similarly, previous work has demonstrated that CNG channels composed of CNGA2 subunits preferentially localize to non-caveolar lipid rafts (Brady *et al.*, 2004). Thus, $A_{2B}$ adenosine receptors in cardiomyocytes may affect cAMP in a compartment that does not communicate with the CNG channels in the sarcolemma. Also, if the receptors are located on an intracellular organelle, then modulation of subsarcolemmal cAMP would not be expected. It is interesting that PKA inhibition attenuated the effect of activated $A_{2B}$ adenosine

receptors on ROS production, and that would suggest that these receptors did influence cAMP in some compartment in the cell.

In CHO cells transfected with human $A_{2B}$ adenosine receptors, elevated cAMP plays a prominent role in the phosphorylation of ERK by agonists of $A_{2B}$ adenosine receptors (Schulte and Fredholm, 2003), but manipulation of cAMP caused only negligible effects on pERK in our HEK-$A_{2B}$ cells. In wild-type HEK 293 cells, forskolin also had no effect on phosphorylation of ERK induced by the $A_{2B}$ adenosine receptor agonist 5′-(N-ethylcarboxamido) adenosine (NECA) (Gao *et al.*, 1999). The most likely explanation is the difference of cell type. For example, cAMP can have either an inhibitory (in rat-1 or NIH3T3 cells) or stimulatory (in PC12 cells) effect on ERK activation (Gao *et al.*, 1999). As in Linden's study (Gao *et al.*, 1999), we noted that forskolin induced a slight increase in ERK phosphorylation, perhaps through B-Raf. But direct elevation of cAMP with either forskolin, CPT-cAMP or rolipram did not replicate the striking increases in pERK seen after Bay 60.

### *NO is in the signalling pathway from $A_{2B}$ adenosine receptors*

Both endogenous and exogenous NO protect the heart from ischaemia/reperfusion injury (Cohen *et al.*, 2006). NO participates in both the trigger (Cohen *et al.*, 2006) and mediator (Cohen *et al.*, 2010) pathways. In the trigger pathway, NO activates protein kinase G (PKG), leading to opening of the mitochondrial $K_{ATP}$ channel and ROS production (Oldenburg *et al.*, 2004). During the mediator phase, NO acts in a PKG-independent manner, perhaps by modifying sulfhydryl residues of proteins through S-nitrosylation (Sun and Murphy, 2010). Several studies have shown that S-nitrosylation of mitochondrial complex I can inhibit the activity of this complex and attenuate ROS generation during ischaemia/reperfusion (Burwell *et al.*, 2006; Shiva *et al.*, 2007; Sun *et al.*, 2007). Finally, NO can bind to superoxide to form peroxynitrite, which could act as either a protective or damaging molecule (Otani, 2009). In the present investigation, the blockade by Bay 60 of ROS generation by mitochondrial complex I of cardiomyocytes was effectively abolished by L-NAME, suggesting the involvement of NO. NOS is found in mitochondria (Ghafourifar and Richter, 1997; Giulivi *et al.*, 1998; Brookes *et al.*, 2000), so local NO production could be part of the protective signalling by activated $A_{2B}$ adenosine receptors. Further studies are needed to better define the role of NO.

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## Conflicts of interest

None.

## References

Alexander SPH, Mathie A, Peters JA (2009). Guide to Receptors and Channels (GRAC). 4th edn. Br J Pharmacol 158 (Suppl. 1): S1–S254.

Ambrosio G, Zweier JL, Duilio C, Kuppusamy P, Santoro G, Elia PP *et al.* (1993). Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radicals in intact rabbit hearts subjected to ischemia and reflow. J Biol Chem 268: 18532–18541.

Barja G (1999). Mitochondrial oxygen radical generation and leak: sites of production in states 4 and 3, organ specificity, and relation to aging and longevity. J Bioenerg Biomembr 31: 347–366.

Beazely MA, Watts VJ (2006). Regulatory properties of adenylate cyclases type 5 and 6: a progress report. Eur J Pharmacol 535: 1–12.

Brady JD, Rich TC, Le X, Stafford K, Fowler CJ, Lynch L *et al.* (2004). Functional role of lipid raft microdomains in cyclic nucleotide-gated channel activation. Mol Pharmacol 65: 503–511.

Brookes PS, Salinas EP, Darley-Usmar K, Eiserich JP, Freeman BA, Darley-Usmar VM *et al.* (2000). Concentration-dependent effects of nitric oxide on mitochondrial permeability transition and cytochrome c release. J Biol Chem 275: 20474–20479.

Burwell LS, Nadtochiy SM, Tompkins AJ, Young S, Brookes PS (2006). Direct evidence for S-nitrosation of mitochondrial complex I. Biochem J 394: 627–634.

Cohen MV, Yang X-M, Downey JM (2006). Nitric oxide is a preconditioning mimetic and cardioprotectant and is the basis of many available infarct-sparing strategies. Cardiovasc Res 70: 231–239.

Cohen MV, Yang X-M, Liu Y, Solenkova NV, Downey JM (2010). Cardioprotective PKG-independent NO signaling at reperfusion. Am J Physiol 299: H2028–H2036.

Daaka Y, Luttrell LM, Lefkowitz RJ (1997). Switching of the coupling of the β2-adrenergic receptor to different G proteins by protein kinase A. Nature 390: 88–91.

Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci USA 92: 7686–7689.

Eckle T, Krahn T, Grenz A, Köhler D, Mittelbronn M, Ledent C *et al.* (2007). Cardioprotection by ecto-5′-nucleotidase (CD73) and $A_{2B}$ adenosine receptors. Circulation 115: 1581–1590.

Feng W, Song Y, Chen C, Lu ZZ, Zhang Y (2010). Stimulation of adenosine $A_{2B}$ receptors induces interleukin-6 secretion in cardiac fibroblasts via the PKC-δ-p38 signalling pathway. Br J Pharmacol 159: 1598–1607.

Feoktistov I, Biaggioni I (1995). Adenosine $A_{2b}$ receptors evoke interleukin-8 secretion in human mast cells. An enprofylline-sensitive mechanism with implications for asthma. J Clin Invest 96: 1979–1986.

Gao Z, Chen T, Weber MJ, Linden J (1999). $A_{2B}$ adenosine and $P2Y_2$ receptors stimulate mitogen-activated protein kinase in human embryonic kidney-293 cells. Cross-talk between cyclic AMP and protein kinase C pathways. J Biol Chem 274: 5972–5980.

Ghafourifar P, Richter C (1997). Nitric oxide synthase activity in mitochondria. FEBS Lett 418: 291–296.

Giulivi C, Poderoso JJ, Boveris A (1998). Production of nitric oxide by mitochondria. J Biol Chem 273: 11038–11043.

Glass DB, Cheng H-C, Mende-Mueller L, Reed J, Walsh DA (1989). Primary structural determinants essential for potent inhibition of cAMP-dependent protein kinase by inhibitory peptides corresponding to the active portion of the heat-stable inhibitor protein. J Biol Chem 264: 8802–8810.

Grube K, Rüdebusch J, Xu Z, Böckenholt T, Methner C, Müller T *et al.* (2011). Evidence for an intracellular localization of the adenosine $A_{2B}$ receptor in rat cardiomyocytes. Basic Res Cardiol DOI: 10.1007/s00395-011-0151-6.

Hausenloy DJ, Ong S-B, Yellon DM (2009). The mitochondrial permeability transition pore as a target for preconditioning and postconditioning. Basic Res Cardiol 104: 189–202.

Hausenloy DJ, Tsang A, Mocanu MM, Yellon DM (2005). Ischemic preconditioning protects by activating prosurvival kinases at reperfusion. Am J Physiol 288: H971–H976.

Hausenloy DJ, Yellon DM (2004). New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. Cardiovasc Res 61: 448–460.

Hausenloy DJ, Yellon DM, Mani-Babu S, Duchen MR (2004). Preconditioning protects by inhibiting the mitochondrial permeability transition. Am J Physiol 287: H841–H849.

Ji X, Kim Y-C, Ahern DG, Linden J, Jacobson KA (2001). [$^3$H]MRS 1754, a selective antagonist radioligand for $A_{2B}$ adenosine receptors. Biochem Pharmacol 61: 657–663.

Krieg T, Cui L, Qin Q, Cohen MV, Downey JM (2004). Mitochondrial ROS generation following acetylcholine-induced EGF receptor transactivation requires metalloproteinase cleavage of proHB-EGF. J Mol Cell Cardiol 36: 435–443.

Kuno A, Critz SD, Cui L, Solodushko V, Yang X-M, Krahn T *et al.* (2007). Protein kinase C protects preconditioned rabbit hearts by increasing sensitivity of adenosine $A_{2b}$-dependent signaling during early reperfusion. J Mol Cell Cardiol 43: 262–271.

Leroy J, Abi-Gerges A, Nikolaev VO, Richter W, Lechêne P, Mazet J-L *et al.* (2008). Spatiotemporal dynamics of β-adrenergic cAMP signals and L-type $Ca^{2+}$ channel regulation in adult rat ventricular myocytes: role of phosphodiesterases. Circ Res 102: 1091–1100.

Linden J, Thai T, Figler H, Jin X, Robeva AS (1999). Characterization of human $A_{2B}$ adenosine receptors: radioligand binding, western blotting, and coupling to $G_q$ in human embryonic kidney 293 cells and HMC-1 mast cells. Mol Pharmacol 56: 705–713.

Liu Y, Yang X, Yang X-M, Walker S, Förster K, Cohen MV *et al.* (2010). AMP579 is revealed to be a potent $A_{2b}$-adenosine receptor agonist in human 293 cells and rabbit hearts. Basic Res Cardiol 105: 129–137.

Lynge J, Hellsten Y (2000). Distribution of adenosine $A_1$, $A_{2A}$ and $A_{2B}$ receptors in human skeletal muscle. Acta Physiol Scand 169: 283–290.

Maas JE, Wan TC, Figler RA, Gross GJ, Auchampach JA (2010). Evidence that the acute phase of ischemic preconditioning does not require signaling by the $A_{2B}$ adenosine receptor. J Mol Cell Cardiol 49: 886–893.

Mongillo M, McSorley T, Evellin S, Sood A, Lissandron V, Terrin A *et al.* (2004). Fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes reveals distinct functions of compartmentalized phosphodiesterases. Circ Res 95: 67–75.

Muller FL, Liu Y, Van Remmen H (2004). Complex III releases superoxide to both sides of the inner mitochondrial membrane. J Biol Chem 279: 49064–49073.

National Research Council (1996). Guide for the Care and Use of Laboratory Animals, 7th edn. National Academy Press: Washington, DC.

Okada M, Mizuno K, Kaneko S (1996). Adenosine A1 and A2 receptors modulate extracellular dopamine levels in rat striatum. Neurosci Lett 212: 53–56.

Oldenburg O, Qin Q, Krieg T, Yang X-M, Philipp S, Critz SD *et al.* (2004). Bradykinin induces mitochondrial ROS generation via NO, cGMP, PKG, and mito$K_{ATP}$ channel opening and leads to cardioprotection. Am J Physiol 286: H468–H476.

Oldenburg O, Qin Q, Sharma AR, Cohen MV, Downey JM, Benoit JN (2002). Acetylcholine leads to free radical production dependent on $K_{ATP}$ channels, $G_i$ proteins, phosphatidylinositol 3-kinase and tyrosine kinase. Cardiovasc Res 55: 544–552.

Otani H (2009). The role of nitric oxide in myocardial repair and remodeling. Antioxid Redox Signal 11: 1913–1928.

Pain T, Yang X-M, Critz SD, Yue Y, Nakano A, Liu GS *et al.* (2000). Opening of mitochondrial $K_{ATP}$ channels triggers the preconditioned state by generating free radicals. Circ Res 87: 460–466.

Philipp S, Yang X-M, Cui L, Davis AM, Downey JM, Cohen MV (2006). Postconditioning protects rabbit hearts through a protein kinase C-adenosine $A_{2b}$ receptor cascade. Cardiovasc Res 70: 308–314.

Powis G, Bonjouklian R, Berggren MM, Gallegos A, Abraham R, Ashendel C *et al.* (1994). Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. Cancer Res 54: 2419–2423.

Rich TC, Karpen JW (2002). Review article: cyclic AMP sensors in living cells: what signals can they actually measure? Ann Biomed Eng 30: 1088–1099.

Rich TC, Tse TE, Rohan JG, Schaack J, Karpen JW (2001). In vivo assessment of local phosphodiesterase activity using tailored cyclic nucleotide-gated channels as cAMP sensors. J Gen Physiol 118: 63–77.

Rich TC, Xin W, Mehats C, Hassell KA, Piggott LA, Le X *et al.* (2007). Cellular mechanisms underlying prostaglandin-induced transient cAMP signals near the plasma membrane of HEK-293 cells. Am J Physiol 292: C319–C331.

Rochais F, Abi-Gerges A, Horner K, Lefebvre F, Cooper DMF, Conti M *et al.* (2006). A specific pattern of phosphodiesterases controls the cAMP signals generated by different $G_s$-coupled receptors in adult rat ventricular myocytes. Circ Res 98: 1081–1088.

Rubino A, Ralevic V, Burnstock G (1995). Contribution of $P_1$-($A_{2b}$ subtype) and $P_2$-purinoceptors to the control of vascular tone in the rat isolated mesenteric arterial bed. Br J Pharmacol 115: 648–652.

Schulte G, Fredholm BB (2003). The $G_s$-coupled adenosine $A_{2B}$ receptor recruits divergent pathways to regulate ERK1/2 and p38. Exp Cell Res 290: 168–176.

Shiva S, Sack MN, Greer JJ, Duranski M, Ringwood LA, Burwell L *et al.* (2007). Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. J Exp Med 204: 2089–2102.

Sobolewski A, Jourdan KB, Upton PD, Long L, Morrell NW (2004). Mechanism of cicaprost-induced desensitization in rat pulmonary artery smooth muscle cells involves a PKA-mediated inhibition of adenylyl cyclase. Am J Physiol 287: L352–L359.

Solenkova NV, Solodushko V, Cohen MV, Downey JM (2006). Endogenous adenosine protects preconditioned heart during early minutes of reperfusion by activating Akt. Am J Physiol 290: H441–H449.

Sun J, Morgan M, Shen R-F, Steenbergen C, Murphy E (2007). Preconditioning results in S-nitrosylation of proteins involved in regulation of mitochondrial energetics and calcium transport. Circ Res 101: 1155–1163.

Sun J, Murphy E (2010). Protein S-nitrosylation and cardioprotection. Circ Res 106: 285–296.

Tompkins AJ, Burwell LS, Digerness SB, Zaragoza C, Holman WL, Brookes PS (2006). Mitochondrial dysfunction in cardiac ischemia-reperfusion injury: ROS from complex I, without inhibition. Biochim Biophys Acta 1762: 223–231.

Wunder F, Buehler G, Hüser J, Mundt S, Bechem M, Kalthof B (2007). A cell-based nitric oxide reporter assay useful for the identification and characterization of modulators of the nitric oxide/guanosine 3′,5′-cyclic monophosphate pathway. Anal Biochem 363: 219–227.

Xin W, Cohen MV, Rich TC, Downey JM (2009). Which preconditioning-associated G protein-coupled receptors are expressed on the sarcolemma? (Abstract). FASEB J 23: 793.25.

Xin W, Tran TM, Richter W, Clark RB, Rich TC (2008). Roles of GRK and PDE4 activities in the regulation of $\beta_2$ adrenergic signaling. J Gen Physiol 131: 349–364.

Yang X, Cohen MV, Downey JM (2010). Mechanism of cardioprotection by early ischemic preconditioning. Cardiovasc Drugs Ther 24: 225–234.

Yang X-M, Krieg T, Cui L, Downey JM, Cohen MV (2004). NECA and bradykinin at reperfusion reduce infarction in rabbit hearts by signaling through PI3K, ERK, and NO. J Mol Cell Cardiol 36: 411–421.