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All preconditioning-related G protein-coupled receptors can be demonstrated in the rabbit cardiomyocyte

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Abstract

G protein-coupled receptors for adenosine (A₁, A₃, A_{2A} and A_{2B}), bradykinin (B₁) and opioids (δ) are all involved in the mechanism of ischemic preconditioning. Although the heart is comprised of many tissue types it has been assumed that preconditioning's protective signaling occurs in the cardiomyocyte. We critically tested that hypothesis by testing for the presence of each of these receptors in isolated adult rabbit ventricular myocytes that had been transfected with cyclic nucleotide-gated (CNG) ion channels. Because subsarcolemmal cAMP opens the CNG channels we could monitor cAMP levels within a single cardiomyocyte by measuring channel current with a patch pipette. The presence of a receptor would be confirmed if we could alter cAMP in the cell with a selective agonist to the receptor being studied. Superfusion with the β-adrenergic G_s-coupled receptor agonist isoproterenol (50 nM) transiently increased cAMP levels, and, therefore, channel current. Pretreatment with selective agonists to A₁ or A₃ adenosine receptors (AR) that are G_i-coupled markedly attenuated the response to isoproterenol indicating inhibition of adenylyl cyclase by increased G_i activity. Agonists to bradykinin or δ-opioid receptors also attenuated isoproterenol's response. A_{2A}AR and A_{2B}AR are G_s-coupled. The A_{2A}AR-selective agonist CGS21680 increased current through CNG channels but only in the presence of phosphodiesterase (PDE) inhibitors indicating low surface receptor activity and high intracellular PDE activity. As we previously reported, BAY 60–6583, an A_{2B}AR-selective agonist which mimics preconditioning's protection in rabbit heart, neither increased nor decreased membrane current in transfected cardiomyocytes, suggesting absence or a markedly limited number of A_{2B}AR in the sarcolemma. However, RT-PCR of purified cardiomyocytes yielded an A_{2B}AR band implying that rabbit cardiomyocytes do indeed express A_{2B}AR. These data reveal that all receptors reported to be involved in ischemic preconditioning do exist on or within the cardiomyocyte.

Keywords

adenosine A_{2B} receptor; cAMP signaling; cardiomyocytes; G protein-coupled receptors

Introduction

Preconditioning the heart with a brief period of ischemia (ischemic preconditioning or IPC) increases the heart's resistance to infarction from a subsequent prolonged period of ischemia.¹ IPC is triggered in part by endogenous adenosine which is quickly produced in the ischemic heart and which binds to its G_i-coupled receptors (G_iPCR), the A₁² and A₃^{3,4} adenosine receptor (AR) subtypes. Bradykinin^{5,6} and opioids⁷⁻⁹ are also released by ischemic myocardium and they also contribute to IPC's protection by binding to their respective G_iPCR as well. All four of these G_iPCR act in parallel to trigger entrance into the preconditioned state. Actual protection is thought to occur early in reperfusion when signaling prevents opening of lethal permeability transition pores in mitochondria.¹⁰ We have found evidence that activation of G_s-coupled A_{2B}AR early in reperfusion is required for preconditioning's protection¹¹⁻¹³ and a highly ₁₃ selective A_{2B}AR agonist, BAY 60-6583, given at reperfusion mimics IPC's anti-infarct effect. Finally, activation of the G_s-coupled A_{2A}AR along with A_{2B}AR has been proposed to also be necessary during reperfusion in the IPC heart.¹⁴

This study critically tests the hypothesis that preconditioning's signaling pathway occurs in the cardiomyocyte. Although the heart's ventricle is composed of many cell types (vascular, neural, fibroblasts, cardiac muscle, etc.) it has been assumed that the above mentioned receptors that are involved in preconditioning reside on the sarcolemma of the ventricular cardiomyocytes, but not all of these receptors have actually been demonstrated in the myocytes. Although many investigators have tried to mimic ischemic or pharmacological preconditioning in isolated cardiomyocytes, the dynamics of ischemic injury and cardioprotection in those models are quite different from those in an intact heart. It is possible that the protective signaling actually occurs in another cell type in the heart and then protects the cardiomyocyte through a diffusible autocrine such as nitric oxide. The cardiomyocyte hypothesis of preconditioning would be disproved if it could be shown that one or more of the receptors used by IPC's signaling is not expressed by the cardiomyocyte. Adenosine A₁AR stimulation markedly inhibits isoproterenol (iso)-induced increase in contractility¹⁵ as does bradykinin¹⁶ indicating that both of those receptors co-exist with adrenergic receptors on cardiomyocytes. It has been more difficult to demonstrate the presence of the remaining receptors. An A_{2A}AR agonist reportedly modestly enhances cardiac contractility.¹⁷ However, that could have been an artifact resulting from A_{2A}AR-mediated dilation of the coronary vasculature.¹⁸ There is even less evidence that A₃AR or opioid receptors are present on the cardiomyocyte.

We have recently presented histological evidence that A_{2B}AR are expressed by the rat cardiomyocyte but reside on or near the mitochondria and likely not in the cell's sarcolemma.¹⁹ We subsequently tested for the functional presence of A_{2B}AR in single rabbit cardiomyocytes with a technique which monitors intracellular cAMP levels.²⁰ Isolated ventricular myocytes were transfected with cyclic nucleotide-gated (CNG) channels. Increasing the concentration of cAMP near the sarcolemma opens the channel which in turn causes increased membrane current that can be measured with a whole-cell patch clamp technique. Cationic current through CNG channels has been shown to be proportional to cAMP concentration.²¹ Activation of a G_s-protein coupled receptor (G_sPCR) stimulates adenylyl cyclase activity and raises cAMP, while activation of a G_iPCR inhibits adenylyl cyclase activity and lowers cAMP. These changes in cAMP are then reflected in the magnitude of the membrane current measured with a patch pipette. This method has been shown to have high resolution for subsarcolemmal cAMP.^{22,23} Consistent with our immunohistochemistry findings that A_{2B}AR are on or near mitochondria,¹⁹ we failed to alter membrane current with an A_{2B}AR-selective agonist confirming that rabbit cardiomyocytes do not have A_{2B}AR in their sarcolemma.²⁰ In the present study we used this

same model to test whether any of the remaining receptors associated with IPC could be found in the rabbit cardiomyocyte.

Materials and Methods

Isolation and culture of rabbit ventricular myocytes

New Zealand White rabbits of either gender were used. The Institutional Animal Care and Use Committee of the University of South Alabama College of Medicine approved these protocols. All animals were cared for and handled in accordance with guidelines established by the National Institutes of Health.²⁴ Rabbit ventricular myocytes were isolated as described previously.²⁵ Briefly, a rabbit heart was mounted on a Langendorff apparatus and retrogradely perfused with calcium-free Krebs–Henseleit–HEPES buffer containing collagenase. The softened heart was minced and extruded through fine nylon mesh. Myocytes were made calcium-tolerant by stepwise restoration of calcium. Cells were plated on laminin-coated glass cover slips in 2 ml Medium-199 modified with Earle's salts containing L-glutamine and enriched with 24 mmol/L NaHCO₃, 0.2% BSA, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 mmol/L creatinine, 2 mmol/L L-carnitine, and 5 mmol/L taurine and placed in an incubator at 37°C. Four hours later the medium was replaced and cells were not disturbed thereafter. Myocytes were used within 4 days of isolation.

Measurement of cAMP signals in single myocytes

Cyclic AMP signals near the plasma membrane were measured using genetically modified CNG channels as described.^{21, 26, 27} Myocytes were transfected using adenovirus encoding CNGA2 subunits with the C460W and E583M mutations. 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 10 µM.²⁸ Binding of cAMP to CNG channels triggers a conformational change in the channel allowing cation flux through the channels thus producing electric currents. Currents were measured with the whole-cell patch clamp technique. Patch pipettes were filled with buffer containing (in mmol/L) 140 KCl, 10 HEPES, 5 Na₂ATP, 0.5 Na₂GTP, and 0.5 MgCl₂ at pH 7.4. The perfusion buffer contained (in mmol/L) 145 NaCl, 4 KCl, 10 HEPES, 10 D-glucose and 0.1 MgCl₂ at pH 7.4. Pipette resistance, access resistance, and cell capacitance averaged 2.5±0.1 MΩ, 5.1±0.2 MΩ, and 98±4 pF (n=54), respectively. Inward currents were recorded during repeated 400 ms steps to a membrane potential of -30 mV from a holding potential of 0 mV. Changes in cAMP-mediated currents were calculated as the average current during each 400 ms step. Baseline current was taken as the pre-drug current. 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. Isoproterenol (iso), a β-adrenergic G_sPCR agonist, increased cAMP production and increased current through CNG channels. Maximal iso-induced currents (*i*_{max}) were achieved by subsequent addition of a phosphodiesterase (PDE) inhibitor cocktail (100 µmol/L 8-methoxymethyl-IBMX, 10 µmol/L cilostamide and 10 µmol/L rolipram which inhibit PDE1, 3 and 4, respectively). The ratio of peak iso-triggered current (*i*_p) to maximal current (*i*_{max}) recorded in the presence of PDE inhibitor cocktail (*i*_p/*i*_{max}) was measured. Periodically the nozzle was moved to bathe the cell in 10 mmol/L Mg⁺⁺, a reversible CNG channel blocker. If current rapidly returned to zero, then it was assumed that no other channels were contributing to the membrane current. Also, under these experimental conditions, little or no agonist-induced currents were observed in myocytes not expressing CNG channels. All experiments were done at room temperature (23–25°C). Whole-cell patch recordings were made using a HEKA EPC 10 amplifier system (HEKA Elektronik,

Germany), Nikon TE 2000-S microscope, MP-225 micromanipulator system (Sutter Instrument), SF-77B Perfusion Fast-Step system and VC-6 six-channel valve controller (Warner Instrument).

Determination of the presence of GPCRs in myocytes

Protocol 1 (Fig. 1) was used for each receptor agonist to determine if G_i - or G_s -coupled receptors were present. The cell was bathed for three minutes in buffer to establish a baseline. Addition of an agonist of one of the heart's G_s PCRs would be expected to activate adenylyl cyclase and increase membrane current. If there were no response to the agonist alone, then 50 nmol/L iso was infused in addition to the agonist to raise cAMP and test whether the cell was viable and peak current (i_p) was noted. If the agonist instead activated G_i -coupled receptors, then it should reduce cAMP and membrane current. Resting rabbit cardiomyocytes have high PDE activity causing cAMP to be below the threshold for channel opening. Hence further lowering of cAMP would not be detected. We, therefore, tested whether the agonist could attenuate the response to 50 nM iso. At the end of the experiment PDEs were inhibited in the presence of iso to establish the maximal iso-induced current (i_{max}). If the agonist significantly decreased i_p/i_{max} compared to that seen with iso alone, then we concluded the agonist had activated G_i PCR.

The cardiomyocyte's high basal PDE activity could keep cAMP from rising in response to a G_s PCR agonist if the receptor density were very low.²⁹ Therefore, if protocol 1 did not reveal receptors to be either G_s - or G_i -coupled, we tested for PDE masking with protocol 2 (Fig. 1). The PDE inhibitor cocktail causes cAMP to rise revealing a high background adenylyl cyclase activity. After several minutes the cAMP level begins to subside, presumably because of feedback inhibition of adenylyl cyclase activity as well as residual PDE activity.^{26, 27, 30} The G_s PCR agonist is then added during this declining phase. With the inhibition of PDE activity even a small increase in adenylyl cyclase activity should be observable. Concentrations of agonists were approximately 10-fold their published K_d s.

RT-PCR of cardiomyocytes for adenosine A_{2B} receptor message

RNA was extracted from cardiomyocytes using the RNeasy fibrous tissue kit from QIAGEN. In brief, the myocytes were digested with Proteinase K, and the supernatant was passed through the RNeasy Mini spin column. After RNase-free DNase digestion, the RNA pellets were washed off the column in nuclease-free water by centrifugation and stored at -80°C . RNA was reverse transcribed using the iScript cDNA Synthesis Kit from Bio-Rad. The resulting cDNA was amplified by PCR with primers designed to span one intron region and electrophoresed through 3% agarose gels. The primers for amplification of the 300 bp rabbit GAPDH were: forward primer = GAT GGT GAA GGT CGG AGT G, reverse primer = AAG ACG CCA GTG GAT TCC AC. The primers for amplification of the 273 bp rabbit adenosine A_{2B} AR were: forward primer = CTG CTT CGT GCT TGT GCT, reverse primer = AAC AGA CAC TTG ACG AGG CA. The primers for amplification of the 235 bp A_1 AR were: forward primer = GCC ACC TTC TGC TTC ATC G, reverse primer = GCG TCA CCA CTG CCT TGT A. All primers were designed using Primer 5.0 software.

Three groups of cardiomyocytes were studied. First we used the crude cardiomyocyte preparation described above which is obviously contaminated with other cell types. The second group was myocytes labeled with tetramethylrhodamine methyl and ethyl esters (TMRE). To purify the myocytes cells with high fluorescence were sorted with a Becton-Dickinson FACS Vantage SE flow cytometer. The third group consisted of several hundred myocytes which were individually picked up with a manually guided micropipette under a microscope.

Statistics

All values are reported as mean $\pm$ SEM. Differences between groups were determined by one-way analysis of variance and Student-Newman-Keuls post hoc test. $P < 0.05$ was considered significant.

Results

Iso-triggered cAMP signals in single myocytes

In rabbit cardiomyocytes transfected with CNG channels, 50 and 100 nmol/L iso caused dose-dependent transient increases of cAMP monitored as whole-cell current (Figs. 2A and B). Adding a PDE inhibitor cocktail increased current to a plateau which we considered to be maximal current ($i_{\max}$). The ratio of iso-triggered peak current (i_p) to maximal current ($i_{\max}$), i.e., $i_p/i_{\max}$, averaged 0.56 ± 0.09 ($n = 5$) for 50 nmol/L iso and 0.72 ± 0.10 ($n = 5$) for 100 nmol/L iso (Fig. 2C). Because 50 nmol/L iso caused a sub-maximal response we chose that dose for the remaining protocols. Using our protocol iso caused a negligible change in current in cardiomyocytes not transfected with CNG channels ($n=8$) (data not shown).

G_iPCR agonists attenuate iso-triggered cAMP signals

A G_sPCR agonist such as iso stimulates adenylyl cyclase activity which increases synthesis of cAMP. Simultaneous activation of G_iPCRs would compete with that activation and reduce cAMP production. We determined expression of G_iPCRs like A₁AR on cardiomyocytes by documenting their ability to attenuate the response to isoproterenol. Myocytes were pretreated with the highly-selective A₁AR agonist 2-chloro-N⁶-cyclopentyladenosine (CCPA, 200 nmol/L)³¹ using Protocol 1 (Fig. 1). CCPA had no effect by itself but clearly attenuated the iso-induced rise in cAMP (Fig. 3A). Washout of CCPA was always accompanied by a transient increase of cAMP as G_i-mediated inhibition was removed (Fig 3A). CCPA significantly decreased the average $i_p/i_{\max}$ for iso ($p < 0.025$) (Fig. 4).

A₃AR is another G_iPCR associated with ischemic preconditioning.⁴ The A₃AR-selective agonist N⁶-(3-iodobenzyl)-adenosine-5'-N-methylcarboxamide (IB-MECA, 200 nmol/L)³² also significantly attenuated the iso-triggered cAMP signal and wash-out of IB-MECA caused cAMP to rise again (Fig. 3B). The average $i_p/i_{\max}$ for iso was significantly decreased as well ($p < 0.02$) (Fig. 4).

Bradykinin B₂ and δ -opioid receptors are G_i-coupled and both, like A₁AR and A₃AR, trigger a preconditioned state.^{5, 6, 9, 33} We sought to determine whether their receptors could be detected on rabbit cardiomyocytes. Pretreatment with 500 nmol/L bradykinin either completely abolished or greatly attenuated iso's effect on cAMP (Fig 3C). The average decrease was highly significant ($p < 0.005$) (Fig. 4). The δ -opioid-selective agonist [D-Ala², D-Leu⁵]-enkephalin (DADLE) (20 nmol/L)⁷ performed similarly (Fig. 3D). The average decrease was again very significant ($p < 0.005$) (Fig. 4).

A_{2A}AR and A_{2B}AR – G_sPCRs

It is controversial whether functional A_{2A}AR reside in or on cardiomyocytes. A_{2A}AR reportedly couple to G_s.³⁴ The highly A_{2A}-selective agonist CGS 21680 (300 nmol/L) alone did not change the basal cAMP level and no current was detected. Addition of PDE inhibitor cocktail in the presence of CGS 21680 caused a large increase in measured current indicating that adenylyl cyclase activity had been masked by the PDEs (Fig. 5A). However, this protocol could not determine whether A_{2A}AR had contributed to that activity. According to protocol 2 (Fig. 1) the cell was first treated with the PDE inhibitor cocktail which increased the current and revealed a high adenylyl cyclase activity even without

receptor stimulation. That increase in cAMP was transient, however, and the current slowly declined, presumably because of feedback inhibition of adenylyl cyclase and increased PDE activity.^{26, 27, 30} Adding CGS 21680 during that decline caused a clear increase in current (Fig. 5B). Therefore, a small but detectable population of functional A_{2A}AR on cardiomyocytes could be uncovered but only when PDE activity was inhibited.

A_{2B}AR are also reported to be G_s-coupled.³⁴ We recently reported that BAY 60–6583, a highly A_{2B}-selective agonist, did not change membrane current in the transfected myocytes even in the presence of PDE inhibitors.²⁰ Representative data from those experiments appear in Figs. 5C and 5D. To test if these receptors might possibly be G_i-coupled, we investigated whether pretreatment with 1 μmol/L BAY 60–6583 would attenuate iso-triggered cAMP signals. It did not (Fig. 4). Thus there was no sign of either G_s or G_i action of BAY 60–6583 indicating the A_{2B}AR density is too low to influence subsarcolemmal cAMP levels in rabbit cardiomyocytes. When BAY 60–6583 was given to HEK 293 cells transfected with human A_{2B}AR and the CNG channels, it caused increased membrane current in a dose-dependent manner.²⁰

RT-PCR

Single-cell PCR revealed that rat cardiomyocytes express A_{2B}AR,¹⁹ but it is unknown if rabbit hearts express them. Figure 6 shows the results of the RT-PCR studies. The crude myocyte preparation showed bands for both A₁AR and A_{2B}AR. However, we know that this preparation is contaminated with other cell types including fibroblasts and coronary vascular cells, and hence we could not unequivocally attribute the receptor bands to the cardiac myocytes. We tried to purify the cardiomyocytes by sorting them for TMRE fluorescence with our flow cytometer. The culture was labeled with TMRE which concentrates in healthy mitochondria which are polarized. Because cardiomyocytes are both large and rich in mitochondria they were easy to identify by their bright fluorescence. Although the A_{2B}AR message was reduced, it was still detectable. We still were not satisfied that our culture was 100% cardiomyocytes so we hand-selected approximately two hundred myocytes from the FACS-sorted cells using a micropipette and a microscope. The A_{2B}AR band could still be seen in the hand-sorted cells. Therefore, our results indicate that cardiomyocytes do express A_{2B}AR, but, like the case in rat cardiomyocytes,¹⁹ they do not appear to be in the sarcolemma.

Discussion

We found clear evidence for the functional expression of five of the six receptors associated with ischemic preconditioning's protective pathway in the cardiomyocyte's sarcolemma. We have shown that A₃AR are involved in preconditioning in the rabbit heart.⁴ The A₃-selective agonist CI-IB-MECA was able to induce protection against hypoxia in a neonatal rat cardiomyocyte model,³⁵ but there are little available data confirming whether A₃AR are actually expressed in adult cardiac myocytes. Zhang et al.³⁶ found that A₁AR-selective agonists inhibited high dihydro-ouabain sensitive Na⁺-K⁺ current in guinea pig ventricular myocytes, whereas CI-IB-MECA had no effect causing them to conclude that adult cardiomyocytes do not express A₃AR, at least in that species. The present results, however, clearly reveal A₃AR in the sarcolemma of adult rabbit cardiomyocytes. It is well known that cardiomyocytes express A₁AR since activation of A₁AR has a marked anti-adrenergic effect on the rat heart,³⁷ which is consistent with our observation that A₁AR are in the sarcolemma. Bradykinin also competes with adrenergic stimulation of the heart.¹⁶

Our rabbit cardiomyocytes have surprisingly high basal adenylyl cyclase activities resulting in a futile cycling of cAMP synthesis and breakdown in these quiescent cells. This basal adenylyl cyclase activity is reflected in the rise in cAMP when the major PDEs were

inhibited with the PDE inhibitor cocktail. As a result we had to inhibit PDEs before we could demonstrate the presence of A_{2A}AR. We cannot determine whether the high PDE activity is representative of cells in the intact heart or if it is induced by our cell culture conditions because it takes 48 hours in culture to express CNG channels. Our results are consistent with previous observations in which inhibition of PDE activity alone increased cAMP levels in neonatal rat cardiac myocytes³⁸. PDE inhibition alone also potentiated PKA-mediated L-type Ca²⁺ channel activity³⁹ and total cAMP content⁴⁰ in adult rat cardiomyocytes. But unlike the present study, inhibition of PDE activity alone induced little or no increase in CNG channel activity in adult rat cardiomyocytes, possibly due to buffering of free cAMP by PKA or PKA-mediated effects on PDE and adenylyl cyclase activities⁴¹. Yet PDE inhibition still greatly potentiated iso-induced cAMP signals. These observations suggest that basal PDE and adenylyl cyclase activities may vary substantially between species.

A_{2A}AR-selective agonists have a modest positive inotropic effect in rat⁴² and chick⁴³ hearts suggesting their presence on cardiomyocytes, but that could have been an artifact from coronary dilation by the agonist. Our findings confirm A_{2A}AR activity in isolated rabbit heart muscle cells. Unlike the case with iso, PDE inhibition was required to observe an A_{2A}AR agonist-induced increase in cAMP in cardiac myocytes. Yet the concentration of CGS21680 that we used should have occupied nearly 100% of A_{2A}ARs. The difference was likely due to different densities of β-adrenergic and A_{2A}ARs on the sarcolemma, but could also reflect some compartmentalization of cAMP.

We^{13, 44} and others⁴⁵ have found the A_{2B}AR to be an important part of IPC's protective pathway. It has been established that A_{2B}ARs are present in coronary vessels where they cause dilation.⁴⁶ They are also present in cardiac fibroblasts.^{47, 48} As a result cardiac biopsies yield message for A_{2B}AR, but that does not prove that they are expressed in cardiomyocytes. RT-PCR revealed message for A_{2B}AR in isolated rat cardiomyocytes¹⁹ and in the present study we were able to show message for A_{2B}AR in highly purified rabbit cardiomyocytes. We were unable to show any effect on cAMP when the myocytes were treated with BAY 60–6583, an A_{2B}AR-selective agonist, and that was assumed to be related to their apparent intracellular location.¹⁹ Receptors on or near the mitochondria would likely not affect the subsarcolemmal cAMP pool that controls the CNG channels. The intracellular receptors do appear to be functional in that we could inhibit rotenone-induced superoxide production in isolated rabbit cardiomyocytes with BAY 60–6583 and that could be blocked by an A_{2B}AR-selective antagonist MRS 1754.²⁰

In summary, we were able to demonstrate receptor-mediated changes in cAMP for A₁AR, A_{2A}AR, and A₃AR as well as bradykinin and δ-opioid receptors in single rabbit ventricular cardiomyocytes and genetic evidence of A_{2B}AR in isolated ventricular myocytes thus supporting the hypothesis that preconditioning's signaling occurs wholly within the heart's cardiomyocytes.

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References

1. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation*. 1986; 74(5):1124–1136. [PubMed: 3769170]

2. Liu GS, Thornton J, Van Winkle DM, Stanley AWH, Olsson RA, Downey JM. Protection against infarction afforded by preconditioning is mediated by A₁ adenosine receptors in rabbit heart. *Circulation*. 1991; 84(1):350–356. [PubMed: 2060105]
3. Maddock HL, Mocanu MM, Yellon DM. Adenosine A₃ receptor activation protects the myocardium from reperfusion/reoxygenation injury. *Am J Physiol*. 2002; 283(4):H1307–H1313.
4. Liu GS, Richards SC, Olsson RA, Mullane K, Walsh RS, Downey JM. Evidence that the adenosine A₃ receptor may mediate the protection afforded by preconditioning in the isolated rabbit heart. *Cardiovasc Res*. 1994; 28(7):1057–1061. [PubMed: 7954592]
5. Goto M, Liu Y, Yang X-M, Ardell JL, Cohen MV, Downey JM. Role of bradykinin in protection of ischemic preconditioning in rabbit hearts. *Circ Res*. 1995; 77(3):611–621. [PubMed: 7641331]
6. Wall TM, Sheehy R, Hartman JC. Role of bradykinin in myocardial preconditioning. *J Pharmacol Exp Ther*. 1994; 270(2):681–689. [PubMed: 8071859]
7. Bell SP, Sack MN, Patel A, Opie LH, Yellon DM. Delta opioid receptor stimulation mimics ischemic preconditioning in human heart muscle. *J Am Coll Cardiol*. 2000; 36(7):2296–2302. [PubMed: 11127476]
8. Schultz, Jel-J.; Hsu, AK.; Nagase, H.; Gross, GJ. TAN-67, a δ_1 -opioid receptor agonist, reduces infarct size via activation of G_{i/o} proteins and K_{ATP} channels. *Am J Physiol*. 1998; 274(3):H909–H914. [PubMed: 9530203]
9. Schultz JE, Rose E, Yao Z, Gross GJ. Evidence for involvement of opioid receptors in ischemic preconditioning in rat hearts. *Am J Physiol*. 1995; 268(5):H2157–H2161. [PubMed: 7771566]
10. Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. *Cardiovasc Res*. 2004; 61(3):448–460. [PubMed: 14962476]
11. Solenkova NV, Solodushko V, Cohen MV, Downey JM. Endogenous adenosine protects preconditioned heart during early minutes of reperfusion by activating Akt. *Am J Physiol*. 2006; 290(1):H441–H449.
12. Philipp S, Yang X-M, Cui L, Davis AM, Downey JM, Cohen MV. Postconditioning protects rabbit hearts through a protein kinase C-adenosine A_{2b} receptor cascade. *Cardiovasc Res*. 2006; 70(2):308–314. [PubMed: 16545350]
13. Kuno A, Critz SD, Cui L, et al. Protein kinase C protects preconditioned rabbit hearts by increasing sensitivity of adenosine A_{2b}-dependent signaling during early reperfusion. *J Mol Cell Cardiol*. 2007; 43(3):262–271. [PubMed: 17632123]
14. Xi J, McIntosh R, Shen X, et al. Adenosine A_{2A} and A_{2B} receptors work in concert to induce a strong protection against reperfusion injury in rat hearts. *J Mol Cell Cardiol*. 2009; 47(5):684–690. [PubMed: 19695259]
15. Dobson JG Jr, Fenton RA, Sawmiller DR. The contractile response of the ventricular myocardium to adenosine A₁ and A₂ receptor stimulation. *Ann N Y Acad Sci*. 1996; 793:64–73. [PubMed: 8906156]
16. Arvola L, Bertelsen G, Ytrehus K. Bradykinin induced sustained anti-adrenergic actions in guinea pig heart muscle involves PKC. *Scand Cardiovasc J*. 2007; 41(2):89–94. [PubMed: 17454833]
17. Monahan TS, Sawmiller DR, Fenton RA, Dobson JG Jr. Adenosine A_{2a}-receptor activation increases contractility in isolated perfused hearts. *Am J Physiol*. 2000; 279(4):H1472–H1481.
18. Gregg DE. Effect of coronary perfusion pressure or coronary flow on oxygen usage of the myocardium. *Circ Res*. 1963; 13:497–500. [PubMed: 14120967]
19. Grube K, Rudebusch J, Xu Z, et al. Evidence for an intracellular localization of the adenosine A_{2B} receptor in rat cardiomyocytes. *Basic Res Cardiol*. 2011; 106:385–396. [PubMed: 21246204]
20. Yang X, Xin W, Yang X-M, et al. A_{2B} adenosine receptors inhibit superoxide production from mitochondrial complex I in rabbit cardiomyocytes via a mechanism sensitive to Pertussis toxin. *Br J Pharmacol*. 2011; 163(5):995–1006. [PubMed: 21366548]
21. Rich TC, Fagan KA, Nakata H, Schaack J, Cooper DMF, Karpen JW. Cyclic nucleotide-gated channels colocalize with adenylyl cyclase in regions of restricted cAMP diffusion. *J Gen Physiol*. 2000; 116(2):147–161. [PubMed: 10919863]

22. Rich TC, Fagan KA, Tse TE, Schaack J, Cooper DMF, Karpen JW. A uniform extracellular stimulus triggers distinct cAMP signals in different compartments of a simple cell. *Proc Natl Acad Sci USA*. 2001; 98(23):13049–13054. [PubMed: 11606735]
23. Rich TC, Tse TE, Rohan JG, Schaack J, Karpen JW. In vivo assessment of local phosphodiesterase activity using tailored cyclic nucleotide-gated channels as cAMP sensors. *J Gen Physiol*. 2001; 118(1):63–78. [PubMed: 11429444]
24. National Research Council. Guide for the Care and Use of Laboratory Animals. 7. Washington, DC: National Academy Press; 1996.
25. Armstrong S, Downey JM, Ganote CE. Preconditioning of isolated rabbit cardiomyocytes: induction by metabolic stress and blockade by the adenosine antagonist SPT and calphostin C, a protein kinase C inhibitor. *Cardiovasc Res*. 1994; 28(1):72–77. [PubMed: 7509260]
26. Rich TC, Xin W, Mehats C, et al. Cellular mechanisms underlying prostaglandin-induced transient cAMP signals near the plasma membrane of HEK-293 cells. *Am J Physiol*. 2007; 292(1):C319–C331.
27. Xin W, Tran TM, Richter W, Clark RB, Rich TC. Roles of GRK and PDE4 activities in the regulation of β_2 adrenergic signaling. *J Gen Physiol*. 2008; 131(4):349–364. [PubMed: 18347080]
28. Rich TC, Karpen JW. Review article: cyclic AMP sensors in living cells: what signals can they actually measure? *Ann Biomed Eng*. 2002; 30(8):1088–1099. [PubMed: 12449769]
29. Jurevičius J, Fischmeister R. cAMP compartmentation is responsible for a local activation of cardiac Ca^{2+} channels by β -adrenergic agonists. *Proc Natl Acad Sci USA*. 1996; 93(1):295–299. [PubMed: 8552625]
30. Beazely MA, Watts VJ. Regulatory properties of adenylate cyclases type 5 and 6: a progress report. *Eur J Pharmacol*. 2006; 535(1–3):1–12. [PubMed: 16527269]
31. Lohse MJ, Klotz K-N, Schwabe U, Cristalli G, Vittori S, Grifantini M. 2-Chloro- N^6 -cyclopentyladenosine: a highly selective agonist at A_1 adenosine receptors. *Naunyn Schmiedeberg Arch Pharmacol*. 1988; 337(6):687–689. [PubMed: 3216901]
32. Gallo-Rodriguez C, Ji X-D, Melman N, et al. Structure-activity relationships of N^6 -benzyladenosine-5'-uronamides as A_3 -selective adenosine agonists. *J Med Chem*. 1994; 37(5):636–646. [PubMed: 8126704]
33. Schultz JEJ, Hsu AK, Gross GJ. Ischemic preconditioning in the intact rat heart is mediated by δ_1 - but not μ - or κ -opioid receptors. *Circulation*. 1998; 97(13):1282–1289. [PubMed: 9570199]
34. Fredholm BB, Ijzerman AP, Jacobson KA, Klotz K-N, Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev*. 2001; 53(4):527–552. [PubMed: 11734617]
35. Germack R, Dickenson JM. Adenosine triggers preconditioning through MEK/ERK1/2 signalling pathway during hypoxia/reoxygenation in neonatal rat cardiomyocytes. *J Mol Cell Cardiol*. 2005; 39(3):429–442. [PubMed: 16005018]
36. Zhang Z, Guo H-C, Zhang L-N, Wang Y-L. Isoform-specific regulation of the Na^+ - K^+ pump by adenosine in guinea pig ventricular myocytes. *Acta Pharmacol Sin*. 2009; 30(4):404–412. [PubMed: 19305421]
37. Romano FD, Naimi TS, Dobson JG Jr. Adenosine attenuation of catecholamine-enhanced contractility of rat heart in vivo. *Am J Physiol*. 1991; 260(5):H1635–H1639. [PubMed: 2035682]
38. Mongillo M, McSorley T, Evellin S, Sood A, Lissandron V, Terrin A, Huston E, Hannawacker A, Lohse MJ, Pozzan T, Houslay MD, Zaccolo M. Fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes reveals distinct functions of compartmentalized phosphodiesterases. *Circ Res*. 2004; 95(1):67–75. [PubMed: 15178638]
39. Verde I, Vandecasteele G, Lezoualc'h F, Fischmeister R. Characterization of the cyclic nucleotide phosphodiesterase subtypes involved in the regulation of the L-type Ca^{2+} current in rat ventricular myocytes. *Br J Pharmacol*. 1999; 127(1):65–74. [PubMed: 10369457]
40. Rochais F, Abi-Gerges A, Horner K, Lefebvre F, Cooper DM, Conti M, Fischmeister R, Vandecasteele G. A specific pattern of phosphodiesterases controls the cAMP signals generated by different Gs-coupled receptors in adult rat ventricular myocytes. *Circ Res*. 2006; 98(8):1081–8. [PubMed: 16556871]

41. Rochais F, Vandecasteele G, Lefebvre F, Lugnier C, Lum H, Mazet J-L, Cooper DMF, Fischmeister R. Negative feedback exerted by cAMP-dependent protein kinase and cAMP phosphodiesterase on subsarcolemmal cAMP signals in intact cardiac myocytes: an *in vivo* study using adenovirus-mediated expression of CNG channels. *J Biol Chem*. 2004; 279(50):52095–52105. [PubMed: 15466415]
42. Dobson JG Jr, Fenton RA. Adenosine A₂ receptor function in rat ventricular myocytes. *Cardiovasc Res*. 1997; 34(2):337–347. [PubMed: 9205548]
43. Liang BT, Haltiwanger B. Adenosine A_{2a} and A_{2b} receptors in cultured fetal chick heart cells. High- and low-affinity coupling to stimulation of myocyte contractility and cAMP accumulation. *Circ Res*. 1995; 76(2):242–251. [PubMed: 7834835]
44. Yang X-M, Krieg T, Cui L, Downey JM, Cohen MV. NECA and bradykinin at reperfusion reduce infarction in rabbit hearts by signaling through PI3K, ERK, and NO. *J Mol Cell Cardiol*. 2004; 36(3):411–421. [PubMed: 15010280]
45. Eckle T, Krahn T, Grenz A, et al. Cardioprotection by ecto-5'-nucleotidase (CD73) and A_{2B} adenosine receptors. *Circulation*. 2007; 115(12):1581–1590. [PubMed: 17353435]
46. Olanrewaju HA, Qin W, Feoktistov I, Scemama J-L, Mustafa SJ. Adenosine A_{2A} and A_{2B} receptors in cultured human and porcine coronary artery endothelial cells. *Am J Physiol*. 2000; 279(2):H650–H656.
47. Epperson SA, Brunton LL, Ramirez-Sanchez I, Villarreal F. Adenosine receptors and second messenger signaling pathways in rat cardiac fibroblasts. *Am J Physiol*. 2009; 296(5):C1171–C1177.
48. Feng W, Song Y, Chen C, Lu ZZ, Zhang Y. Stimulation of adenosine A_{2B} receptors induces interleukin-6 secretion in cardiac fibroblasts via the PKC-δ-p38 signalling pathway. *Br J Pharmacol*. 2010; 159(8):1598–1607. [PubMed: 20050850]

Figure 1.

Experimental protocols. The horizontal axis is time. Protocol 1 is used to test for the functional presence of a G_s -coupled receptor with a highly selective agonist for that receptor. The subsequent addition of isoproterenol as a positive control tests whether the cell is viable and expressing the CNG channels. Finally, the PDE inhibitors reveal the level of PDE activity in the cell. If activity is not seen in protocol 1, protocol 2 is then used. By starting the PDE inhibitor prior to the agonist we can test whether high PDE activity had masked the increase in cAMP expected following occupation of the receptor in protocol 1. Abbreviations: EC = extracellular solution PDE = phosphodiesterase

Figure 2.

Typical isoproterenol (iso)-triggered cAMP signals in rabbit ventricular myocytes measured as currents through cyclic-nucleotide gated (CNG) channels. 50 (A) and 100 (B) nmol/L iso triggered transient increases in cAMP levels. Subsequent addition of a phosphodiesterase (PDE) inhibitor cocktail increased currents to a plateau which is the iso-induced maximal current (i_{max}). Average ratio of iso-triggered peak current (i_p) to i_{max} in the presence of the PDE inhibitor cocktail for both groups is presented in panel C. Numbers in parentheses indicate numbers of cells studied and the bars represent SEM.

Figure 3.

Activation of adenosine A₁ (A), adenosine A₃ (B), bradykinin (C), and δ -opioid (D) receptors inhibit isoproterenol (iso)-triggered cAMP signals in rabbit cardiomyocytes. In each case washout of the agonist revealed a second increase of cAMP as the inhibitory effect of G_i activity on adenylyl cyclase was removed. Abbreviations: CCPA = 2-chloro-N⁶-cyclopentyladenosine; DADLE = [D-Ala², D-Leu⁵] enkephalin; IB-MECA = N⁶-(3-iodobenzyl)-adenosine-5'-N-methylcarboxamide; PDE = phosphodiesterase

Figure 4.

Ratio of peak isoproterenol (iso) current to the maximum current observed after inhibition of phosphodiesterases (i_p/i_{max}) is plotted for 50 nmol/L iso alone and iso plus each of the 5 agonists tested with protocol 1. Numbers of cells tested are indicated in parentheses. The first four agonists caused a significant decrease of the iso response, but BAY 60 did not. Abbreviations: BAY 60 = BAY 60-6583; CCPA = 2-chloro- N^6 -cyclopentyladenosine; DADLE = [D-Ala², D-Leu⁵] enkephalin; IB-MECA = N^6 -(3-iodobenzyl)-adenosine-5'- N -methylcarboxamide. Statistical significance of difference between experimental group and iso: * $p < 0.025$

Figure 5.

Adenosine A_2 receptor-mediated cAMP signals in rabbit cardiomyocytes. (A) The A_{2A} -selective agonist CGS 21680 did not change the basal cAMP level before application of phosphodiesterase (PDE) inhibitors ($n=3$). (B) PDE inhibitor cocktail transiently increased cAMP and addition of the A_{2A} agonist while cAMP level was declining, presumably because of feedback inhibition of adenylyl cyclase, could then increase adenylyl cyclase activity in face of the effective PDE blockade and elevate cAMP ($n=5$). Thus a small but measurable expression of $A_{2A}AR$ was found. However, the $A_{2B}AR$ -selective agonist BAY 60–6583 (Bay 60) did not have any effect on cAMP whether introduced before (C) ($n=5$) or after (D) ($n=4$) the PDE inhibitor cocktail. Abbreviations: iso = isoproterenol

Figure 6. RT-PCR of message for adenosine GAPDH, and A₁ and A_{2B} adenosine receptors from crude and purified rabbit cardiomyocyte preparations. See text for details.