Published in final edited form as:
Basic Res Cardiol. 2010 January ; 105(1): 129–137. doi:10.1007/s00395-009-0056-9.

AMP579 is revealed to be a potent A_{2b}-adenosine receptor agonist in human 293 cells and rabbit hearts

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Abstract

The mixed A_1/A_{2a}-adenosine agonist AMP579 given at reperfusion is protective in animal models of myocardial infarction. Receptor-blocking studies have indicated that the protection came from an adenosine receptor (AR), but neither A_1- nor A_{2a}-selective agonists could duplicate its protection. We recently found that A_{2b}-selective agonists given at reperfusion are protective, and, therefore, tested whether AMP579 might also be an A_{2b} agonist. We used human embryonic kidney cells overexpressing human A_{2b} receptors as an assay system. In these cells, A_{2b} receptor occupancy causes phosphorylation of ERK. AMP579 induced ERK phosphorylation with an EC_{50} of 250 nM and this phosphorylation could be blocked by MRS1754 or PSB1115, two highly selective blockers of human A_{2b} receptors. We attempted to confirm our A_{2b} hypothesis in a rabbit heart model of ischemia–reperfusion. AMP579 (500 nM) for 1 h starting at reperfusion reduced infarct size in isolated rabbit hearts exposed to 30 min of regional ischemia and 2 h of reperfusion (12.9 ± 2.2% infarction of risk zone vs. 32.0 ± 1.9% in untreated hearts). PSB1115 (500 nM) given for the first 15 min of reperfusion blocked AMP579's protection (32.2 ± 3.1% infarction) which is consistent with an A_{2b} mechanism. We conclude that AMP579 is a non-selective, but potent A_{2b}-AR agonist, and that its protection against infarction is through that receptor.

Keywords

Adenosine; A_{2b} receptor; AMP579; Cardioprotection; Myocardial infarction

Introduction

Adenosine is a purine nucleoside that is ubiquitous in the body. Its concentration may increase to 100-fold during periods of oxygen depletion and ischemia when there is net dephosphorylation of ATP. There are four known adenosine receptor (AR) subtypes: A1AR and A_3AR are G_i-coupled while the high-affinity A_{2a}AR and low-affinity A_{2b}AR are G_s-coupled. ARs are involved in the signaling by which ischemic preconditioning (IPC) makes the ischemic heart resistant to infarction. IPC involves a trigger phase prior to ischemia during

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which transient activation of the trigger pathway puts the heart into a protected state that persists even after the trigger stimulus has been withdrawn. We have shown that the A_1AR [18] and A_3AR [17] participate in triggering of the protected state of IPC, but A_1-selective agonists offer no protection from infarction when given just prior to reperfusion [1]. Although these ARs are physiological triggers of IPC, opioid, and bradykinin receptors also participate in parallel. Because of this redundancy, loss of the ability of one or two receptors to bind agonists does not block the ability of ischemia to precondition the heart. Rather, their absence only raises the threshold of ischemia required to trigger protection [6]. Accordingly knocking out either A_1, A_{2a}, or A_3 AR in mice failed to block the ability of IPC to protect their hearts [5]. The A_{2b}AR knockout mice, however, seemed to be incapable of being protected by IPC indicating that the A_{2b} receptor plays a critical and unique role in this protection.

The mediator phase of IPC's protection is initiated after the ischemic heart is reperfused when a series of signal transduction events acts to inhibit permeability transition pore formation in the mitochondria [8]. Transition pores form in the first few minutes of reperfusion and kill cells by destroying mitochondria. IPC's signaling pathways make the heart resistant to transition pore formation, but the signal transduction pathways must be activated in the critical first seconds of reperfusion. We have recently shown that the A_{2b}AR plays a key role in this protective pathway [23]. The A_{2b}AR normally has a low affinity for adenosine. IPC through PKC activation sensitizes the A_{2b}AR such that endogenous adenosine released by the ischemic heart can activate the protective pathway early in the reperfusion period [14,15].

The adenosine A_1/A_{2a}-receptor agonist AMP579 has also been reported to protect the ischemic heart in animal models of myocardial infarction when it was administered at reperfusion [2, 19,24,30]. Protection from AMP579 requires activation of an AR [30]. However, neither adenosine nor A_1- or A_{2a}-selective agonists infused at reperfusion can duplicate preconditioning's protection [1,2,7,12,26–28]. This was indeed a puzzling observation. Because little was known about the A_{2b}AR at the time AMP579 was being investigated, the drug was not tested for A_{2b}AR binding. Furthermore, because the A_{2b}AR has such a low affinity for adenosine (EC_{50}~5 μM) [20,22], it was largely dismissed as being unimportant because adenosine concentration in the heart seldom approaches that value even in deep ischemia.

Because protection in IPC hearts seems to depend on a change in the A_{2b}AR's sensitivity to adenosine, administration of an A_{2b} agonist at a dose high enough to occupy the low-affinity receptors should protect even a non-preconditioned heart. We indeed confirmed that prediction using the highly selective A_{2b}AR agonist BAY 60-6583 [14]. 5′-(*N*-ethylcarboxamido) adenosine (NECA) is a potent, although not selective, A_{2b}AR agonist with an EC_{50} for raising cAMP in cells expressing A_{2b}AR of ~3.1 μM [4]. NECA at reperfusion is as protective as AMP579, and the protection is dependent on the involvement of A_{2b} receptors [23]. Moreover, the structure of AMP579 is very similar to that of NECA. Therefore, we hypothesized that AMP579 is also an A_{2b} agonist and that it protects, like NECA, by binding to the A_{2b}AR to mimic IPC's signaling. To test that hypothesis, we used both HEK293 cells that had been transfected with human A_{2b}AR to overexpress them as an assay system for A_{2b} activity and an isolated rabbit heart model of myocardial infarction.

Materials and methods

Cell culture and biochemical studies

We used human embryonic kidney (HEK) 293 cells that had been stably transfected with human adenosine A_{2b} receptors by Anna Robeva at the University of Virginia who generously shared them with us [16]. In brief, a plasmid for recombinant A_{2b} receptors was introduced into HEK 293 cells by lipofectin. The construct included a gentamycin-resistant gene so that successfully

transfected cells could be selected by growing them in a medium containing G418, which resulted in a stable cell line that overexpresses A_{2b}AR. The stably transfected cells were grown in laminin-coated dishes at 37°C in 5% CO_2/95% air and in Dulbecco's modified Eagle's medium [0.3 mg/ml G418, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 10% fetal calf serum (FCS)]. All cells were split three times a week at a ratio of 1:5. These cells overexpress A_{2b}AR making them a sensitive and selective assay system for A_{2b}AR-active ligands.

Prior to their use, HEK-A_{2b} cells were maintained overnight in medium deprived of serum. They were washed two times with calcium-free Tyrode's solution, and then stimulated with AMP579, BAY 60-6583, 2-chloro-N^6-cyclopentyladenosine (CCPA), CGS 21680, and 2-Cl-N^6-(iodobenzyl) adenosine-5′-*N*-methyluronamide (2-CI-IB-MECA) at 37°C in calcium-free Tyrode's solution for 10 min. When used, the selective A_{2b} antagonists MRS1754 or PSB1115 were added for 20 min before stimulation by agonists. After two washes in ice-cold phosphate-buffered saline, 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 aprotinin, 5 μg/ml leupeptin) and cellular debris were removed by centrifugation. Samples were denatured with Laemmli buffer and protein content analyzed using the Bradford method. Fifty microgram of protein was loaded on each lane of a 10% polyacrylamide gel. Proteins were separated with electrophoresis and then transferred to polyvinylidene difluoride membranes. To analyze ERK1/2 phosphorylation membranes were blocked in 5% dry milk powder in PBST and incubated with mouse monoclonal phospho-specific anti-phospho Thr202/Tyr204 ERK1/2 antibodies (Cell Signaling 9106) overnight on a shaker at 4°C. After incubation with anti-mouse horseradish peroxidase-coupled secondary antibody, bands were visualized with Immobilon™ western chemiluminescent HRP substrate according to the manufacturer's instructions. Equal loading of the lanes was confirmed by Ponceau stain of the membranes and by stripping the membranes and reprobing them with an anti-ERK1/2 antibody (Cell Signaling 9107).

We measured the expression of A_{2b}AR in the plasma membranes of transfected and non-transfected HEK cells. Cultured cells were lysed in Tris–HCl (5 mM, pH 7.4) by freezing and thawing three times and then centrifuged for 1 h at 100,000 *g*. The pellet was re-suspended in Tris–HCl and used for western blot analysis as described above by incubating with a primary anti-A_{2b}AR antibody (SC-28996f, Santa Cruz, CA), and 50 μg of protein was loaded on each lane.

Immunofluorescence

Stably transfected HEK cells seeded on coverslips were washed once in PBS and then fixed in 4% paraformaldehyde and permeabilized by incubation in 0.1% Triton X-100/PBS. After washing, cells were blocked for 1 h at room temperature in 5% FCS in PBS. They were then incubated with either the above-mentioned primary anti-A_{2b}AR antibody (1:100) or affinity-purified rabbit IgG in a corresponding concentration (negative control) overnight at 4°C. After washing with PBS, cells were incubated with a secondary antibody conjugated to Alexa Fluor 488 for an additional hour at room temperature. After extensive washing, coverslips were mounted on microscope slides (DAKO mounting medium, Carpinteria, CA, USA) and observed with a confocal laser scanning microscope (Chromaphor Analysen Technik, Duisburg, Germany). A CCD camera and VoxCell software from VisiTech International (Sunderland, UK) were used for analysis.

Isolated heart model

All animal care satisfied published guidelines [21], and procedures were approved by institutional committees. New Zealand White rabbits of either sex weighing 2–3 kg were

anesthetized with sodium pentobarbital (30 mg/kg) and ventilated with 100% oxygen. Hearts were exposed through a left thoracotomy, and a suture was passed around a branch of the left coronary artery. The heart was removed and perfused on a Langendorff apparatus with modified Krebs–Henseleit bicarbonate buffer that contained (mM) 118.5 NaCl, 24.7 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, and 10.0 glucose. The buffer was gassed with 95% O_2 and 5% CO_2. A fluid-filled latex balloon was inserted into the left ventricle to measure pressure. All hearts were allowed to equilibrate for 20 min before the protocol was started.

Protocol for infarct studies

Six groups of hearts were studied (Fig. 1). All hearts were subjected to 30 min of regional ischemia and 120 min of reperfusion. Control hearts received no treatment. The second group of hearts was treated with AMP579 (500 nM) starting 5 min before reperfusion and continuing for 60 min. The third group of hearts was treated with NECA (100 nM) for an equal amount of time. In the fourth group, PSB1115 (500 nM), a highly selective A_{2b} antagonist, was infused for 20 min beginning 5 min before the release of the coronary occlusion. In the fifth and sixth groups, PSB1115 infusion was combined with either AMP579 or NECA (100 nM) administered for 60 min from 5 min before the onset of reperfusion.

Measurement of infarct size

At the end of the experiment, the coronary artery was re-occluded, and 2–9 μm fluorescent microspheres (Micro-genics, Fremont, CA, USA) were infused to delineate the ischemic zone (region at risk) as the area of tissue without fluorescence. The heart was cut into 2-mm thick slices which were incubated in 1% triphenyltetrazolium chloride in sodium phosphate buffer (pH 7.4) at 37°C for 10 min. The slices were immersed in 10% formalin to preserve the tissue. The risk zone was identified by illuminating slices with ultraviolet light. The areas of infarct and risk zone were determined by planimetry of each slice, and volumes were calculated by multiplying each area by slice thickness and summing them for each heart. Infarct size is expressed as a percentage of the risk zone.

Materials

Cell culture media and FCS were obtained from Sigma-Aldrich. AMP579 was a gift from Aventis Pharm. MRS1754, PSB1115, CCPA, CGS 21680, and 2-Cl-IB-MECA were purchased from Tocris Bioscience. Mouse monoclonal anti-phospho Thr202/Tyr204 ERK1/2, anti-ERK1/2, and anti-mouse horseradish peroxidase-coupled antibodies were obtained from Cell Signaling Technology. Immobilon™ western chemiluminescent HRP substrate was purchased from Millipore. The anti-A_{2b}AR antibody was purchased form Santa Cruz.

Statistics

All data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) with Student–Newman–Keuls post hoc test was performed on baseline hemodynamic variables, risk zone, infarct size, and western blot band densities. $P < 0.05$ was considered significant.

Results

HEK-A_{2b} cell studies

Figure 2a shows the stably transfected HEK cells subjected to immunofluorescence with an anti-A_{2b}AR primary antibody. Transfected cells show intense membrane staining. No fluorescence staining could be observed in cells overexpressing A_{2b} receptors but not exposed to A_{2b} antibody, and, therefore, without bound A_{2b}-receptor–antibody–fluorochrome complex indicating that the fluorescence did reflect antibody binding (Fig. 2b). We then subjected a

membrane fraction of transfected and non-transfected cells to western blotting with the anti-A_{2b}AR antibody. A single band was observed at the expected molecular weight of 36 kDa in the transfected cells (Fig. 2c). No band was detected in the wild-type cells.

Ischemic preconditioning increases phosphorylation and activation of both ERK isoforms in the first minutes of reperfusion [9]. Since inhibiting ERK at reperfusion blocks the protection of IPC, it has been assumed that this kinase is involved in the protective signaling [9]. In our preliminary investigations, we had observed that A_{2b} agonists induced phosphorylation of ERK 1/2 in HEK cells that had been stably transfected with human A_{2b} receptors. We, therefore, used ERK phosphorylation as an A_{2b} assay system to test whether AMP579 is an A_{2b}-receptor agonist. Because the cells express human receptors, the selectivity and potency of the ligands we used are well known.

To test for other surface ARs the cells were incubated with highly selective agonists for all four AR subtypes: CCPA (1 nM) for A_1 receptors, CGS 21680 (300 nM) for A_{2a} receptors, BAY 60-6583 (100 nM) for A_{2b} receptors [14], and 2-CI-IB-MECA (20 nM) for A_3 receptors. The doses of agonists were approximately 10-fold higher than their published K_ds. Figure 3a shows there was an increase in phosphorylation of both isoforms of ERK after exposure of cells to all four agonists, although the increase in phosphorylation was much higher after BAY 60-6583 which would be expected since the cells are overexpressing A_{2b}AR. Therefore, although A_{2b} receptors are dominant in the HEK-A_{2b} cells, the other three AR subtypes appear to be expressed at a small, but detectable level. Therefore, we also had to show that A_{2b}-selective antagonists would block a response to confirm A_{2b} binding.

We tested the specificity of the antagonist MRS1754 by administering it prior to adding the agonist. The increases in ERK phosphorylation induced by CCPA and CGS 21680 (Fig. 3b) were not affected by MRS1754 (20 nM); neither were those triggered by 2-CI-IB-MECA (data not shown). But the increased phosphorylation from the highly selective A_{2b}AR agonist BAY60-6583 was strongly attenuated (Fig. 3c). PSB1115 (500 nM), another selective A_{2b}AR antagonist, also blocked BAY 60-6583-induced phosphorylation (Fig. 3c).

AMP579 could also induce ERK1/2 phosphorylation and both A_{2b}-selective antagonists, MRS1754, and PSB 1115, dramatically attenuated the phosphorylation (Fig. 3d). This is strong evidence that AMP579 is an agonist of human A_{2b} ARs. Figure 4 shows that the application of increasing concentrations of AMP579 induces a dose-dependent increase in ERK1/2 phosphorylation with an EC_{50} of about 250 nM.

Hemodynamics in isolated hearts

Having established that AMP579 is an A_{2b}AR-potent agonist, we next tested whether PSB1115 could block protection from AMP579 in an isolated rabbit heart undergoing ischemia/reperfusion. No group differences in heart rate, developed pressure, or coronary flow were observed at baseline (Table 1). Coronary branch occlusion caused an expected decrease in left ventricular developed pressure and coronary flow in all groups. Both AMP579 and NECA significantly increased coronary flow during the last few minutes of the coronary occlusion following their addition to the perfusate, and this increase was attenuated by PSB1115. There was a partial recovery of both left ventricular developed pressure and coronary flow following reperfusion. The increased coronary flow caused by NECA was also seen during reperfusion. PSB1115 had no significant independent effect on coronary flow.

Infarct size in isolated hearts

There was no significant difference in body weight, heart weight, or risk zone volume among the groups (Table 2). Control hearts undergoing 30 min of regional ischemia and 2 h of

reperfusion had 32.0 ± 1.9% infarction of the risk zone (Fig. 5). AMP579 started 5 min before reperfusion and continued for 60 min decreased infarction to 12.9 ± 2.2% ($P < 0.05$ vs. control). Because we have previously shown that AMP579 limits infarct size in this model [28], we studied only four additional hearts in this group (white symbols in Fig. 5) to verify that our drug was still active. The previous data points are included in Fig. 5 for comparison (gray symbols). The non-selective, but A_{2b}-potent, AR agonist NECA has been shown to limit infarct size when administered just before reperfusion through an A_{2b}AR-dependent mechanism [31]. We confirmed NECA's infarct-sparing effect in four additional hearts (white symbols in Fig. 5). Again, previous data points (gray symbols) are included for comparison. We tested whether protection from NECA, structurally very similar to AMP579, could also be blocked by a highly selective A_{2b} antagonist PSB1115. PBS1115 blocked the protective effect of both AMP579 and NECA (32.2 ± 3.1 and 38.7 ± 2.4% infarction, respectively). PSB1115 administered alone at reperfusion had no significant effect on infarction (32.6 ± 1.8%).

Discussion

The present study demonstrated that the limitation of infarct size by AMP579 administered just prior to reperfusion of ischemic rabbit hearts is dependent on adenosine A_{2b} receptors. Furthermore, using phosphorylation of ERK in HEK 293 cells that were stably transfected with human A_{2b} receptors as our end-point, we could demonstrate that AMP579 is a potent agonist of human adenosine A_{2b} receptors with an EC_{50} of about 250 nM. The conclusion that AMP579 protects ischemic hearts from infarction by activation of adenosine A_{2b}AR is consistent with our previous findings that A_{2b} receptors control the protective signal transduction pathway in ischemically preconditioned hearts in the early reperfusion period [25]. As a result, A_{2b}-selective agents mimic IPC's anti-infarct effect when infused at reperfusion [14].

We measured ERK phosphorylation in HEK-A_{2b} cells because activation of ERK through phosphorylation in the reperfusion period is believed to be central to the mechanism of preconditioning's protection [9]. In addition, A_{2b} agonists given at reperfusion not only protect against infarction, but also increase ERK phosphorylation in the heart [14]. ERK and Akt are often referred to as "survival kinases" since their activation opposes infarction [9]. The studies in HEK cells indicate that this pathway also exists in cell types other than heart muscle. It is likely that AMP579 protects by triggering activation of these survival kinases, but we did not measure phosphorylation of these kinases in the rabbit hearts to confirm this hypothesis since this has already been demonstrated for other A_{2b} agonists, i.e., NECA [31] and BAY 60-6583 [14]. In support of our hypothesis, Kis et al. [12] showed ERK inhibitors blocked AMP579's anti-infarct effect.

AMP579 limits infarct size in pigs [24], rabbits [30], and dogs [2] when administered just before reperfusion. MRS1754 infused at reperfusion blocks protection from IPC [25], thus supporting the involvement of the A_{2b} receptor. The A_{2b}AR has been proposed to mediate preconditioning's protection at the time of reperfusion [3]. Kuno et al. [14] reported that IPC sensitizes the heart to A_{2b}-selective agonists through a PKC-dependent pathway. They concluded that preconditioning allows endogenous adenosine to populate the normally low-affinity A_{2b}AR at reperfusion and initiate signaling leading to the activation of the survival kinases. Those activated kinases likely protect by preventing opening of the mitochondrial permeability transition pore in the first minutes of reperfusion [10]. This high conductance pore spans the inner and outer mitochondrial membrane and uncouples mitochondria, which stops ATP production at a time when the heart needs it the most. If enough mitochondria are affected, the cell will die in the first minutes of reperfusion. Eckle et al. [5] recently evaluated the ability of IPC to protect mice with selective deletion of either A_1, A_{2a}, A_{2b}, or A_3 receptors. Protection from IPC persisted in the A_1, A_{2a}, and A_3 knockout strains of mice. Only in A_{2b}-knockout mice was IPC's protection aborted.

In a previous study, we found that AMP579 needed to be administered immediately on reperfusion; delaying administration of AMP579 for 10 min after the onset of reperfusion aborted its protection [29]. Similarly, the drug had to be present for an extended period of time. A 60-min infusion of AMP579 was protective, but stopping the infusion after only 30 min of reperfusion resulted in loss of protection. Finally, transiently blocking either survival kinases or the A_{2b}AR for only 15 min at any time during the first hour of reperfusion aborted protection from IPC [25]. We suggested forces are trying to open the transition pores from the onset of reperfusion and that a 60-min convalescence period is required before those forces subside and protection from either a drug or the survival kinases can be withdrawn. Accordingly, we had to interrupt the protection from A_{2b}AR's signaling for only 15 min to cause irreversible injury, presumably because of pore opening.

It is interesting that AMP579 was tested in a small-scale clinical trial in patients with acute myocardial infarction [13]. In that study, an infusion of AMP579 was started several minutes after an open artery was confirmed. Because a loading dose was not used a protective blood level would not have been achieved until about 30 min after reperfusion. Clearly, they failed to establish the drug's schedule requirements before implementing the trial [11].

Although NECA is a potent A_{2b} agonist, it is not selective and can activate other AR subtypes including A_{2a} and A_1. In the present study, NECA's ability to increase phosphorylation and reduce infarct size at reperfusion was completely abolished by co-infusion of the highly selective A_{2b}AR antagonist PSB1115 which indicates that NECA protected through an adenosine A_{2b} receptor. AMP579 is closely related to NECA with a similar structure and pharmacology. Figure 6 shows the structure of the two molecules. They differ only by the side groups on the adenine moiety. It is noteworthy that AMP579 with an EC_{50} of about 250 nM for the A_{2b} receptor is about 100 times more potent than NECA [4]. The highly A_{2b}-selective BAY 60–6583 is even more potent with an EC_{50} of about 10 nM [14].

The cardioprotective effect of AMP579 could also be blocked by co-infusion of PSB1115, again supporting our hypothesis. We also have reported that BAY 60-6583, a highly selective A_{2b}-receptor agonist, given at reperfusion reduced infarct size by an amount similar to that seen with NECA, and the A_{2b}-selective antagonist MRS1754 blocked its protection as well [14].

The advantage of A_{2b} agonists is that few tissues express these receptors so that intravenous administration of BAY 60-6583 is hemodynamically silent in rabbits (unpublished observation). AMP579 does have some bradycardic and hypotensive effects related to its A_1 and A_{2a} activity, but those side effects are minimal when AMP579 is administered at a dose that is protective. Intravenous AMP579 has already been shown to be safe in humans when it was tested in a small-scale study in patients undergoing reperfusion therapy for acute myocardial infarction [13]. Unfortunately that study did not show that AMP579 was protective, most likely because the study had a serious flaw in its design. As noted above, blood levels of drug did not reach a protective concentration until nearly 30 min after reperfusion. To inhibit lethal transition pore formation postconditioning agents must be present in the first minutes of reperfusion, and that has been specifically shown for AMP579 [29]. In conclusion, our data indicate that the anti-infarct effect of AMP579 derives from its action as a potent A_{2b} AR agonist.

Acknowledgments

This work was supported by grant HL-20468 from the Heart, Lung, and Blood Institute of the National Institutes of Health.

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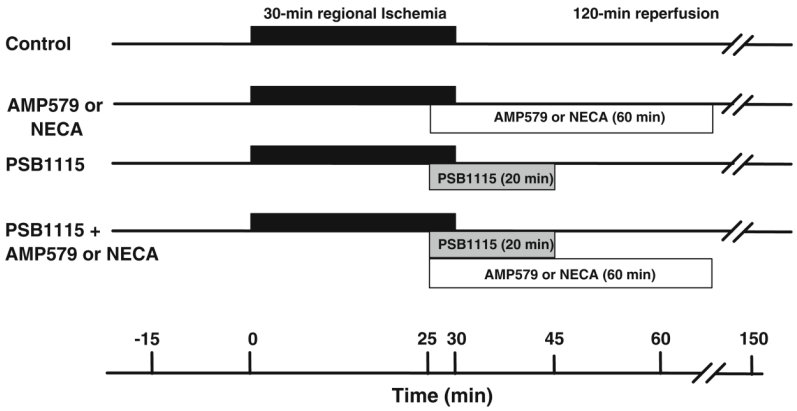

Fig. 1.
Experimental protocols

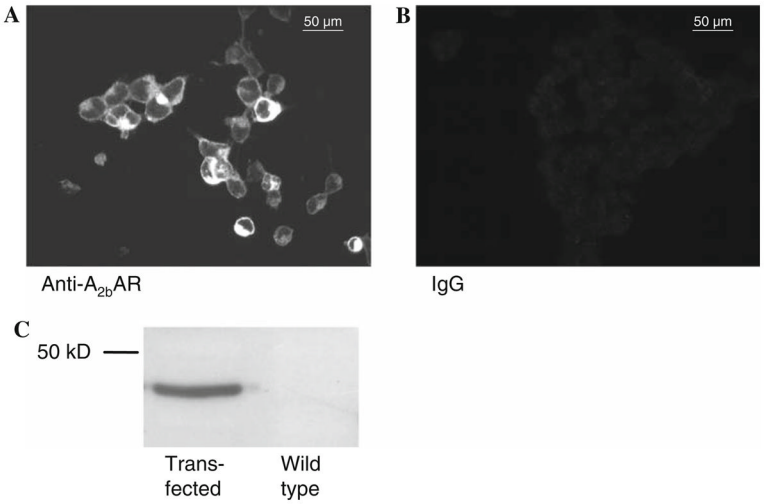

Fig. 2.

Transfected HEK cells overexpressing A_{2b} receptors treated with A_{2b} antibodies coupled to a fluorochrome show intense fluorescence of the plasma membrane (**a**), while no fluorescence is seen in negative control cells (**b**) where IgG was substituted for the primary anti-A_{2b} antibody. **c** Representative western blot of A_{2b}-transfected HEK and unaltered HEK cells showing detection of adenosine A_{2b}AR only in the transfected cells

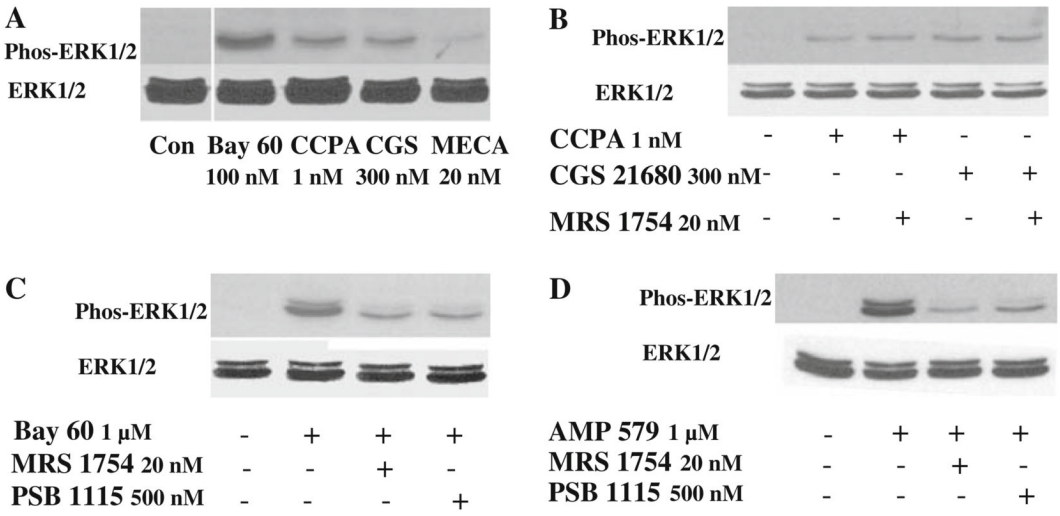

Fig. 3.

a Agonists of A_1 (CCPA), A_{2a} (CGS), A_{2b} (Bay 60), and A_3 (MECA) adenosine receptors induced ERK1/2 phosphorylation, but the A_{2b} agonist BAY 60-6583 was the most potent. **b** Phosphorylation of ERK1/2 by either CCPA or CGS 21680 was not affected by the A_{2b}-selective antagonist MRS 1754. **c** PSB 1115 and MRS 1754 could both dramatically attenuate phosphorylation of ERK1/2 induced by Bay 60. **d** AMP579-induced phosphorylation of ERK 1/2 could also be attenuated by both A_{2b}-selective antagonists MRS 1754 and PSB 1115. In **b–d** total ERK 1/2 was unchanged by any intervention. *Con* control

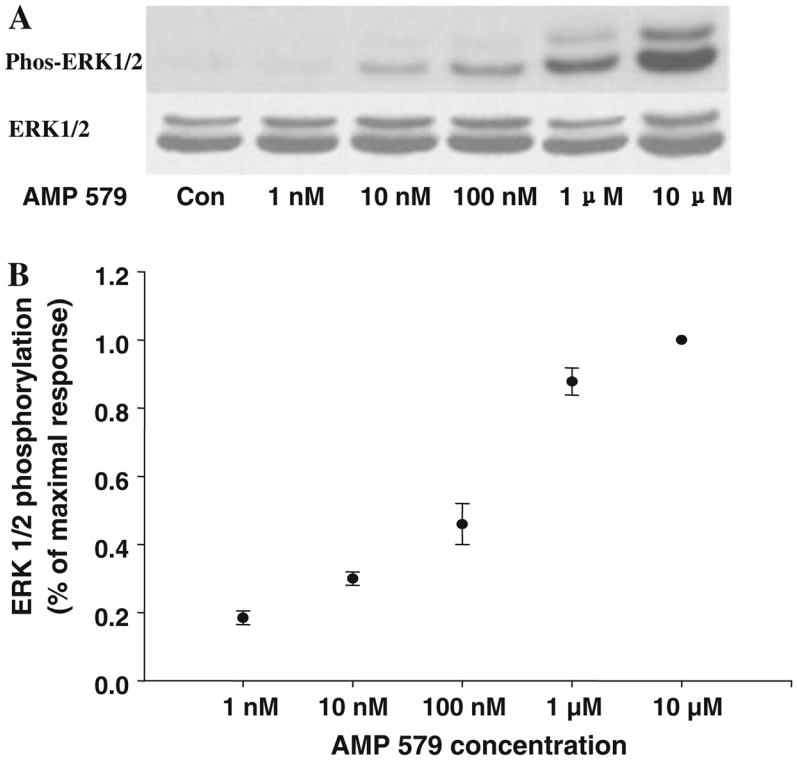

Fig. 4.

a Dose-dependent increase in ERK1/2 phosphorylation with increasing concentrations of AMP579 in an individual experiment. Total ERK 1/2 is unchanged as AMP concentrations are increased. **b** Summary of ERK 1/2 phosphorylation data following increasing doses of AMP579. The plot of blot density against concentration indicates the EC_{50} for ERK1/2 phosphorylation by AMP579 is about 250 nM (n = 4)

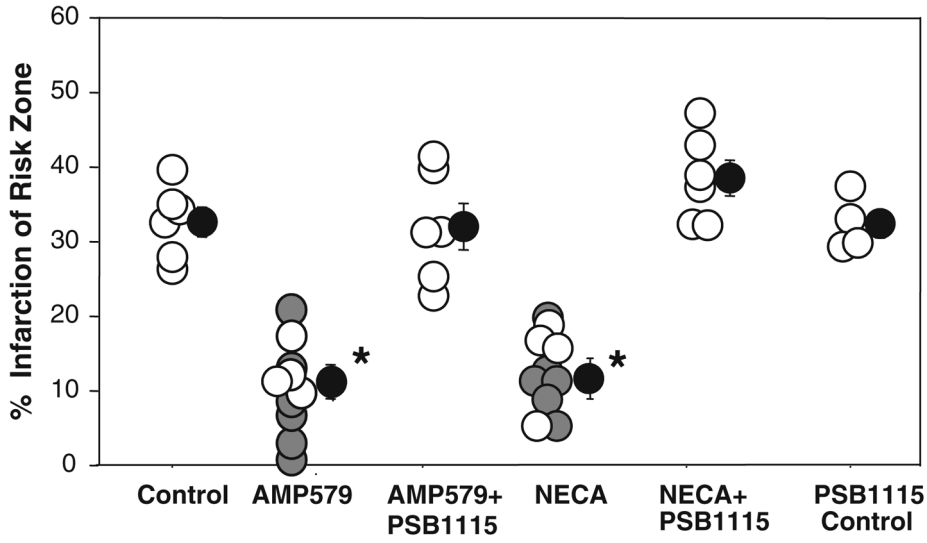

Fig. 5.

Myocardial infarct size expressed as a percentage of risk zone in isolated rabbit hearts treated with AMP579 alone or in addition to the selective A_{2b} receptor blocker PSB1115. *Open* and *gray circles* represent individual experiments while *black circles* depict group mean ± SEM. *Gray circles* depict previously obtained data with AMP579 [28] or NECA [31] and are presented for comparison. Infusion of AMP579's at reperfusion was protective. PSB115 blocked both AMP579s and 5′-(*N*-ethylcarboxamido) adenosine's (NECA) protection. PSB1115 alone had no effect on infarct size. Thus, A_{2b} receptors are involved in the AMP579 signaling that leads to protection. *$P < 0.05$ versus control

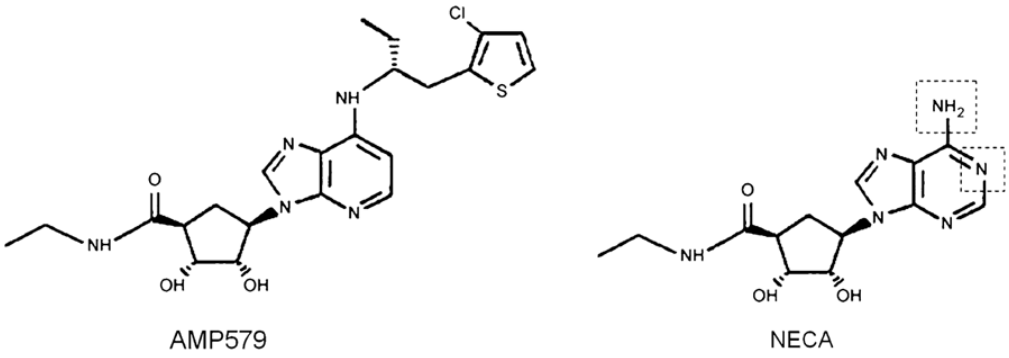

Fig. 6.

Structure of AMP579 and 5′-(*N*-ethylcarboxamido) adenosine (NECA). The *dotted lines* on the NECA structure show where the molecules differ

Table 1

Hemodynamic data

	Baseline			25′ Occlusion			30′ Occlusion			15′ Reperfusion			30′ Reperfusion		
	HR (bpm)	**LVDP (mmHg)**	**CF (ml/min/g)**	**HR (bpm)**	**LVDP (mmHg)**	**CF (ml/min/g)**	**HR (bpm)**	**LVDP (mmHg)**	**CF (ml/min/g)**	**HR (bpm)**	**LVDP (mmHg)**	**CF (ml/min/g)**	**HR (bpm)**	**LVDP (mmHg)**	**CF (ml/min/g)**
Control	204 ± 12	114 ± 6	9.3 ± 0.5				192 ± 13	38 ± 5*	5.2 ± 0.3*				188 ± 14	73 ± 3*	5.8 ± 0.5*
AMP579	215 ± 10	110 ± 3	8.4 ± 0.2	202 ± 14	37 ± 9*	4.9 ± 0.3*	230 ± 0	52 ± 7*	7.2 ± 0.0*				227 ± 3	83 ± 4*	6.5 ± 0.3*
NECA	213 ± 13	114 ± 6	9.4 ± 0.2	208 ± 18	36 ± 3*	4.2 ± 0.3*	204 ± 4	41 ± 2*	7.8 ± 0.8*				211 ± 7	86 ± 6*	9.4 ± 0.2
PSB1115	218 ± 12	114 ± 7	9.1 ± 0.2	225 ± 19	53 ± 1*	4.5 ± 0.2*	213 ± 16	51 ± 2*	4.3 ± 0.2*	203 ± 13	70 ± 12*	6.1 ± 0.2*	208 ± 16	66 ± 14*	6.0 ± 0.1*
AMP579 + PSB1115	215 ± 11	113 ± 3	9.2 ± 0.4	207 ± 11	50 ± 6*	5.6 ± 0.6*	218 ± 17	47 ± 8*	7.0 ± 0.7*	246 ± 8	7.3 ± 7*	7.1 ± 0.7*	244 ± 8	78 ± 3*	7.6 ± 0.6*
NECA + PSB1115	199 ± 6	111 ± 4	7.9 ± 0.4	191 ± 6	45 ± 5*	4.9 ± 0.1*	172 ± 6	52 ± 6*	5.5 ± 0.4*	184 ± 16	73 ± 6*	6.7 ± 0.5*	180 ± 20	75 ± 5*	6.5 ± 0.6*

Values are mean ± SEM

bpm beats per minute, *CF* coronary flow, *HR* heart rate, *LVDP* left ventricular developed pressure, *NECA* 5′-(*N*-ethylcarboxamido) adenosine

Statistical significance:

* $P < 0.05$ between experimental points and baseline

Table 2

Infarct size data

	n	Body weight (kg)	Heart weight (g)	Risk zone volume (cm^3)	Infarct volume (cm^3)	I/R (%)
Control	6	2.3 ± 0.1	7.2 ± 0.2	1.24 ± 0.06	0.47 ± 0.03	38.0 ± 2.1
AMP579	4	2.5 ± 0.0	8.5 ± 0.2	1.22 ± 0.13	0.16 ± 0.04*	12.9 ± 2.2*
PSB1115	4	2.4 ± 0.0	7.8 ± 0.4	1.48 ± 0.10	0.49 ± 0.05	32.6 ± 1.8
NECA	4	2.4 ± 0.2	7.5 ± 0.4	1.45 ± 0.10	0.21 ± 0.05*	14.3 ± 0.3*
AMP579 + PSB1115	6	2.5 ± 0.1	8.1 ± 0.2	1.47 ± 0.18	0.49 ± 0.09	32.2 ± 3.1
NECA + PSB1115	6	2.5 ± 0.0	8.5 ± 0.2	1.43 ± 0.14	0.55 ± 0.07	38.7 ± 2.4

Values are mean ± SEM

see Table 1; *I/R* infarction as a percentage of risk zone, *n* number of animals

Statistical significance of difference between experimental and control groups:

* $P < 0.05$