Supplementary Experimental Procedures

BAY-60 treatment of isolated cardiomyocytes

Rat cardiomyocytes were isolated as described above and treated with 500 nM BAY60-6583 for 10 min. A sample was taken as control. After washing three times with PBS the cells underwent 3 cycles of freezing and thawing. The cytosolic fraction was obtained after 10 min of centrifugation at 10,000 g. BAY60-6583 content was measured by the Department of Drug Metabolism and Pharmacokinetics, Bayer HealthCare GmbH, Wuppertal, Germany. After solution in acetonitrile, BAY60-6583 was separated and detected using liquid chromatography-mass spectrometry. Fig. S1 summarizes the data. Clearly, initial BAY60-6583 concentration was reduced in the resulting supernatant and enriched in the cell lysate, confirming the ability of BAY60-6583 to enter cardiomyocytes and potentially act on intracellular targets.

Biochemical studies

Human embryonic kidney (HEK) 293 cells stably transfected with human A_{2b}AR (HEK293-A_{2b}AR, selected in G418 [0.3 mg/ml]-containing DMEM) were seeded on coverslips and grown to confluence, harvested by trypsinization, and washed once in cold PBS.

Hearts and liver from wild type C57BL/6J mice or $A_{2b}AR^{-/-}$ mice were removed after sodium pentobarbital injection (70 mg/kg i.p.), rinsed in ice cold 0.9 % NaCl and snap frozen in liquid nitrogen. Cell pellets and pulverized organs were homogenized in ice-cold lysis buffer containing [mM] 20 Tris-HCl (pH 7.5), 150 NaCl, 1 Na_2EDTA, 1 EGTA, 1 PMSF, 2.5 sodium pyrophosphate, 1 β-glycerophosphate, and 1 Na_3VO_4 as well as 1% Triton and 1 µg/ml leupeptin. The samples were centrifuged for 15 min at 13,000 g and the protein content of the supernatant was determined by the BCA protein assay (Pierce Biotechnoligy, Rockford, IL). After homogenization the samples were immediately analyzed by standard SDS-PAGE methods. Samples were electrophoresed on a 12% polyacrylamide gel followed by transfer to a nitrocellulose membrane. Fifty µg of total protein were loaded in each lane. After blocking with 5% milk, the membranes were treated with the primary A_{2b}AR antibody (1:500) followed by the secondary antibody (1:5000) conjugated to horseradish peroxidase. Immunoreactive proteins were detected by enhanced chemiluminescence with LumiGLO (Cell Signaling Technology, Beverly, MA).

Immunofluorescence of HEK293 and HEK293-A_{2b}AR cells

HEK293 and HEK293–A_{2b}AR cells were grown in 75 cm^3 flasks to approximately 80% confluence, washed once in PBS, and then fixed in 4% paraformaldehyde and permeabilized by incubation in 0.1% Triton X-100/PBS. After being washed, cells were blocked for one hour at room temperature in 5% FCS in PBS. They were then incubated with either primary anti-A_{2b}AR antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA) or affinity-purified rabbit IgG in a corresponding concentration (negative control) overnight at 4°C. After being washed 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) 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.

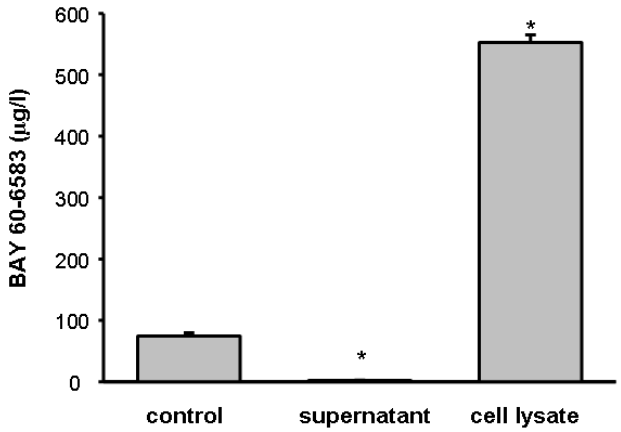
Additionally, saline rinsed hearts from C57BL/6J or $A_{2b}AR^{-/-}$ mice were used for cryosection preparations and A_{2b}AR immunofluorescent staining. Nuclei were stained with DAPI (0.2 µg/ml, Roth, Karlsruhe, Germany). Pictures were taken with identical settings with a Nikon Eclipse Ti microscope and NIS-Elements software.

Percoll-purified mitochondria

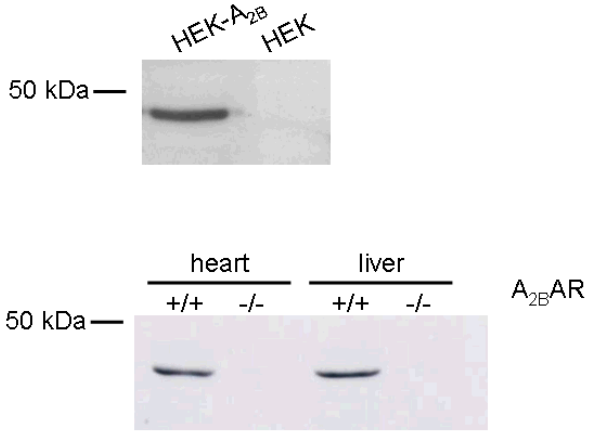
Whole heart mitochondria from either Wistar rats or C57BL/6J mice were isolated as described in Methods. One portion was stored for western blot analysis, and one for further purification. Mitochondria were layered on top of a 37 % percoll solution containing 250 mM sucrose, 10 mM Tris-HCl, and 0.1 mM EGTA (pH 7.4). Samples were centrifuged at 50,000 g for 40 min at 4°C, and the lower band was diluted 1:5 with ice cold solution B and then centrifuged at 17,000 g for 15 min. The pellet containing purified mitochondria was washed once more in solution B and

centrifuged at 9,000 g for 10 min. Mitochondria were then resuspended in solution B and prepared for western blot analysis. For comparison, whole heart lysates from rats and mice were used.

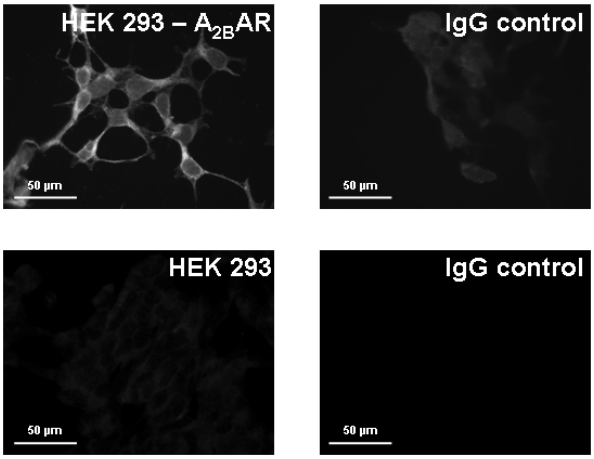
Supplementary Figures



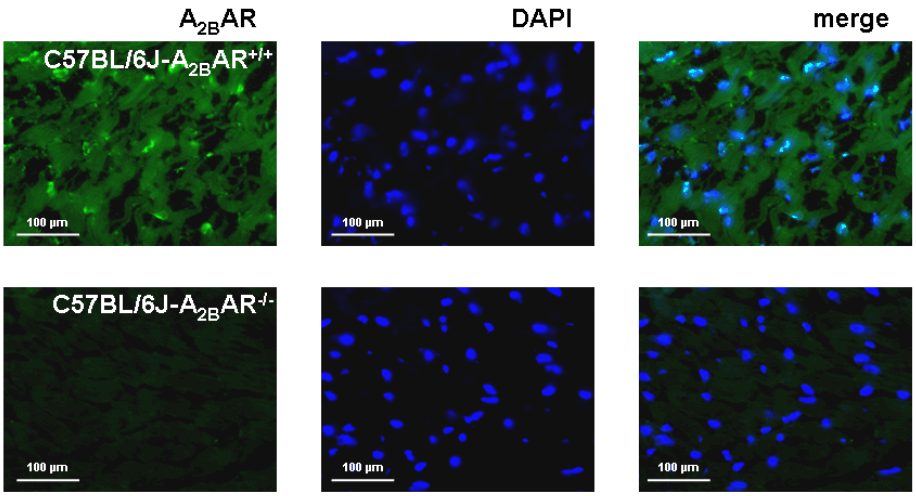
Supplementary Figure S1. BAY60-6583 concentration (mean±SEM) in cardiomyocyte lysate and corresponding supernatant compared to the initially applied BAY60-6583 solution (control). *$p<0.01$ vs. control.



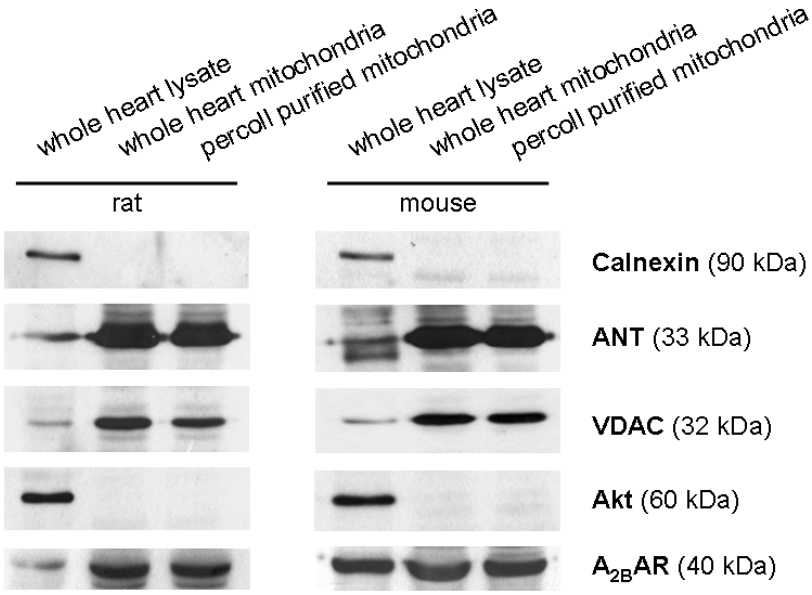
Supplementary Figure S2. HEK293 cells overexpressing the human A_{2b}AR show a single clear band for A_{2b}AR while normal HEK293 did not express any A_{2b}AR (upper panel). The lower panel reveals that the whole heart as well as liver homogenate of C57BL/6J mice show a comparable single band, while it was absent in the A_{2b}AR knockout animals.



Supplementary Figure S3. Normal and A_{2b}AR overexpressing HEK293 cells were immunostained with the A_{2b}AR antibody. HEK293-A_{2b}AR cells show clear membrane staining which was not present in either the HEK293 cells or IgG controls.



Supplementary Figure S4. Cryosections from hearts of either C57BL/6J or $A_{2b}AR^{-/-}$ mice were immunostained with the $A_{2b}AR$ antibody coupled to a fluorochrome. Nuclei were co-stained with DAPI. While wild-type mice showed broad staining of the nuclei and the cytosol, the $A_{2b}AR$ staining was minimal in the knock-out animals.



Supplementary Figure S5. Comparison of different heart preparations revealed mitochondrial localization of $A_{2b}AR$ protein in both rat and mouse hearts. Mitochondrial preparations were free of contaminations from sarcoplasmic reticulum (calnexin) and cytosol (Akt). Further percoll purification did not show any additional effect.