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Evidence for an intracellular localization of the adenosine A_{2B} receptor in rat cardiomyocytes

Karina Grube¹, Julia Rüdebusch¹, Zhelong Xu³, Thomas Böckenholt¹, Carmen Methner⁷, Tobias Müller², Friederike Cuello⁴, Katrin Zimmermann¹, Xiulan Yang⁵, Stephan B. Felix¹, Michael V. Cohen^{5,6}, James M. Downey⁵, and Thomas Krieg⁷

¹Department of Cardiology, Ernst-Moritz-Arndt University, Greifswald, Germany

²Department of Anatomy, Ernst-Moritz-Arndt University, Greifswald, Germany

³Department of Anesthesiology, University of North Carolina, Chapel Hill, NC

⁴Cardiovascular Division, King's College London, London, UK

⁵Department of Physiology, University of South Alabama, Mobile, AL

⁶Department of Medicine, University of South Alabama, Mobile, AL

⁷Department of Clinical Pharmacology Unit, University of Cambridge, Cambridge, UK.

Abstract

Aim—Protection achieved by ischemic preconditioning is dependent on A_{2B} adenosine receptors (A_{2B}AR) in rabbit and mouse hearts and, predictably, an A_{2B}AR agonist protects them. But it is controversial whether cardiomyocytes themselves actually express A_{2B}AR. The present study tested whether A_{2B}AR could be demonstrated on rat cardiomyocytes.

Methods and Results—Isolated rat hearts experienced 30 min of ischemia and 120 min of reperfusion. The highly selective, cell-permeant A_{2B}AR agonist BAY60-6583 (500 nM) infused at reperfusion reduced infarct size from 40.4±2.0 % of the risk zone in control hearts to 19.9±2.8 % indicating that A_{2B}AR are protective in rat heart as well. Furthermore, BAY60-6583 reduced calcium-induced mitochondrial permeability transition in isolated rat cardiomyocytes. A_{2B}AR protein could be demonstrated in isolated cardiomyocytes by western blotting. In addition, message for A_{2B}AR was found in individual cardiomyocytes using quantitative RT-PCR. Surprisingly, immunofluorescence microscopy did not show A_{2B}AR on the cardiomyocyte's sarcolemma but rather at intracellular sites. Co-staining with MitoTracker Red in isolated cardiomyocytes revealed A_{2B}AR are localized to mitochondria. Western blot analysis of a mitochondrial fraction from either rat heart biopsies or isolated cardiomyocytes revealed a strong A_{2B}AR band.

Conclusions—Thus the present study demonstrates that activation of A_{2B}AR is strongly cardioprotective in rat heart and suppresses transition pores in isolated cardiomyocytes, and A_{2B}AR are expressed in individual cardiomyocytes. However, surprisingly, A_{2B}AR are present in or near mitochondria rather than on the sarcolemma as are other adenosine receptors. Because A_{2B}AR signalling is thought to result in inhibition of mitochondrial transition pores, this convenient location may be important.

Address correspondence to Thomas Krieg, Clinical Pharmacology Unit, University of Cambridge, Addenbrooke's Hospital, Box 110, Cambridge, CB2 2QQ, UK, Tel: +44 1223 762584, Fax: +44 1223 762576, tk382@medschl.cam.ac.uk.

Conflict of interest

None.

Keywords

adenosine A_{2B} receptors; cardioprotection; mitochondria

Introduction

Ischemic preconditioning (IPC) triggers powerful endogenous signalling that ultimately makes the heart resistant to infarction from ischemia. A key step in preconditioning's signal transduction pathway is activation of adenosine A_{2B} receptors (A_{2B}AR) early in reperfusion [8,10,25,43]. Ischemic postconditioning, another endogenous cardioprotective phenomenon, also depends on activation of A_{2B}AR [33]. Analogously the highly selective A_{2B} agonist BAY60-6583 given at reperfusion reduces infarct size in rabbit hearts following prolonged myocardial ischemia [21].

A_{2B}AR are G protein-coupled receptors (GPCR) that communicate through both G_{αs} and G_{αq}, which in turn lead to increased cytosolic cAMP, inositol trisphosphate and Ca²⁺ [23]. There is agreement that coronary endothelial cells contain A_{2B}AR [14,39], but there is discrepant information about the expression of A_{2B} on cardiomyocytes, the cells that IPC must ultimately protect. To address this question, Yang et al. [47] replaced exon 1 of the A_{2B}AR gene in transgenic mice with a reporter construct containing β-Gal. They did not detect β-Gal expression in heart muscle indicating that expression of the receptors was either absent or too low to detect. On the other hand, Morrison and colleagues saw a positive inotropic response to the potent, albeit nonselective, A_{2B}AR agonist 5'- (N-ethylcarboxamido) adenosine (NECA) in A_{2a}AR knockout mice suggesting that some A_{2B}AR must reside on or within cardiomyocytes [30]. Furthermore, Liang and Haltiwanger [22] studied contractility and cAMP production of cultured chick embryonic ventricular cells and again found responses to A_{2B}AR agonists. Finally, a recent report by Xin *et al.* [45] contributed to the controversy; they could find no functional evidence for sarcolemmal A_{2B}AR in isolated rabbit cardiomyocytes.

In the present study we address the question whether A_{2B}AR are expressed in rat ventricular cardiomyocytes. First we tested whether a selective A_{2B}AR agonist is as protective in intact rat hearts and isolated cardiomyocytes as it is in rabbits. Then, using antibodies to A_{2B}AR we performed immunofluorescence, FACS analysis, and western blotting to try to localize the receptors.

Methods

The present study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The experimental protocols were approved by the local authorities of Mecklenburg-Vorpommern (Germany) and the University of North Carolina (USA).

Isolated rat hearts and infarct size measurement

Adult Wistar rats of either sex were used. Heart isolation and determination of infarct size were performed as described previously in detail [26]. Control hearts (n=6) were exposed to 30 min of regional ischemia by occluding a snared coronary artery and then 120 min of reperfusion. The second group (n=10) was treated with 500 nM BAY60-6583 for 65 min starting 5 min before reperfusion. The third group (n=6) received 500 nM BAY60-6583 in the presence of the highly selective A_{2B}AR antagonist MRS1754 (20 nM). At the end of the experiment risk zone was determined with green fluorescent microspheres injected into the aorta perfusate after religation of the snared coronary artery and infarct size was determined

with triphenyltetrazolium chloride staining of the sliced left ventricle. Infarct size is presented as a percentage of the risk zone. BAY60-6583 and MRS1754 were dissolved in dimethyl sulfoxide (DMSO) before being further diluted in perfusion buffer, resulting in DMSO concentrations lower than 0.01 %. The ability of BAY60-6583 to enter cardiomyocytes has been demonstrated (supplemental Fig. S1).

Isolated rat cardiomyocytes and assessment of mitochondrial membrane potential $\Delta\Psi_m$

Hearts were excised and retrogradely perfused with Krebs-Henseleit-buffer containing 25 μM calcium and collagenase type II at 37°C as previously described [12]. Viable ventricular myocytes were separated by centrifugation in a 4% bovine serum albumin gradient, seeded on laminin coated four-well plates and cultured in medium M199 containing 0.2% BSA, 5 mM creatine, 2 mM L-carnithine, 5 mM taurine and penicillin and streptomycin (each 100 U/ml). Four hours after isolation cells were washed once and loaded for 20 min with 100 nM tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes Inc., Eugene, OR, USA) which causes cells to fluoresce in proportion to their mitochondrial membrane potential ($\Delta\Psi_m$). A reduction in TMRE fluorescence served as an indicator of the loss of $\Delta\Psi_m$, which would occur with mitochondrial permeability transition pore (mPTP) formation. If required, BAY 60-6583 (100 nM) and MRS1754 (100 nM) were added 5 min before TMRE loading to mimic a preconditioning protocol. Afterwards cells were washed with TMRE-free medium and then incubated with the selective calcium ionophore calcimycin (1 μM), which is known to induce mPTP formation similar to that seen at reperfusion following myocardial ischemia [32]. Fluorescence intensity was measured at 582 nm (FL-2 channel) using a Becton Dickinson (Heidelberg, Germany) FACSCalibur and CellQuest software after 35 min of calcimycin exposure and expressed as the mean fluorescence for each measurement. Four groups of TMRE-loaded myocytes were studied: treatment with calcimycin alone or with the additional agents BAY 60-6583, MRS1754, or BAY 60-6583 plus MRS1754 (each n=9).

Isolated mitochondria from intact rat hearts and isolated cardiomyocytes

Cardiac mitochondria were isolated using standard centrifugation techniques. Briefly, hearts were minced in ice-cold solution A (250 mM sucrose, 10 mM Tris-HCl, 0.1 mM EGTA and 0.5% BSA, pH 7.4). Tissue pieces were then homogenized using a Potter S (Braun Biotech International, Melsungen, Germany) at 1000 rpm and subjected to stepwise centrifugation. The homogenate was centrifuged at 500 g and the supernatant at 9,000 g. The resulting pellet (crude mitochondrial fraction) was resuspended in ice-cold solution B (250 mM sucrose, 10 mM Tris-HCl, 0.1 mM EGTA, pH 7.4). To further purify mitochondria the resulting suspension was alternately spun at 9,000g and washed 5 times.

Mitochondria were harvested from isolated cardiomyocytes at the end of the cell isolation procedure by directly suspending the cells in solution A. The rest of the preparation was identical to that described above.

Isolation and purification of cardiac sarcolemma

To obtain purified sarcolemma two different protocols were used. First, hearts were homogenized as described above and the homogenate centrifuged at 9,000 g to remove intracellular organelles including mitochondria. The supernatant was centrifuged at 100,000 g and the pellet was resuspended in Tris-HCl (5 mM, pH 7.4) and further used for Western blot analysis. The second protocol included an additional pellet purification step with a sucrose gradient (38 % sucrose in 5 mM HEPES, pH 7.4). The pellet was centrifuged at 280,000 g and the interphase containing purified sarcolemma was washed in Tris-sucrose (10 mM and 250 mM, pH 7.4) prior to Western blot analysis.

A_{2B}AR expression by quantitative RT-PCR

Isolated rat cardiomyocytes were plated in a glass chamber and observed at 30 X using an Olympus BX-51 microscope. Under micromanipulator control cardiomyocytes (5-10 cells) were harvested by drawing them into micropipettes pulled from 1.5 mm diameter borosilicate glass (Drummond Scientific Company, Broomall, PA). Care was taken to exclude all other cell types. After collection, cells were immediately transferred to a 500 μ L microcentrifuge tube containing protease K and RNAase inhibitors by breaking the pipette tip against the bottom of the tube and expelling the contents of the pipette. Total mRNA was isolated using Trizol (Invitrogen) reagent. RNA was converted to cDNA using reverse transcriptase. GAPDH and A_{2B}AR mRNA sequences were identified using Rat Genome Resources of NCBI (<http://www.ncbi.nlm.nih.gov>). Primer pairs for GAPDH (forward: CAGGTTGTCTCTCGACTT, reverse: ATGTAGGCCATGAGGTCCAC) and A_{2B}AR (forward: CCAAGGACAAGCCCAAATG, reverse: CCGTCTGGCAGAGAACGTAT) were designed using Primer3 program. Gene expressions were assessed by quantitative RT-PCR using QuantiTect SYBR Green PCR Kit (Qiagen) and ABI 7900HT Fast Real-Time PCR System. Relative quantities of message were calculated from cycle threshold (Ct) measurements. Ct values were standardized to GAPDH for each sample and then subjected to standard calculations. Dissociation curves were also initially evaluated for each qPCR sample to qualitatively confirm assay integrity. The amplified product was analyzed on an agarose gel to confirm its expected molecular size. Additionally, two target bands were selected from two samples and purified with Wizard SV gel and PCR clean-up system (Promega, WI). The PCR products (10 ng) and the primers (10 pmol) were used for RNA sequencing at the University of North Carolina Genome Analysis Facility. The raw data were processed and analyzed with Sequencher (Version 4.8.) and ChromasPro.

Immunofluorescence

Either cryosections of the heart, isolated cardiomyocytes, or mitochondria were studied. Cryosections were prepared after rinsing the heart of blood with ice cold saline (0.9%) delivered retrogradely through the aorta. After cutting the heart from base to apex into parallel slices which were snap-frozen in liquid nitrogen, 5 μ m-thick cryosections were cut from the snap-frozen slices (Leica CM 1900, Leica Microsystems, Nussloch, Germany). Heart sections were fixed on SuperFrost Plus microscope slides (R. Langenbrinck, Teningen, Germany) in acetone and permeabilized in 0.5% Triton X-100 in PBS. After a blocking step in 5% FCS in PBS for one hour, staining was carried out overnight at 4°C using either the primary A_{2B}AR antibody or affinity-purified IgG in a corresponding concentration. A secondary antibody conjugated to Alexa Fluor 488 was used. Staining of the nuclei was performed by using propidium iodide (1 μ g/ml, Sigma, St. Louis, MO). Adult ventricular cardiomyocytes were isolated as described above. Cells were plated on laminin-coated coverslips and incubated in modified CCT-medium 199 as previously described [34]. After 4 h of incubation cardiomyocytes were stained with a mitochondria-specific dye (50 nM MitoTracker Red CMXRos, Invitrogen, Karlsruhe, Germany) and fixed in 2% paraformaldehyde. Finally, the protocol described above for cryosections was used. MitoTracker-loaded cells were first exposed to the A_{2B}AR primary antibody. Native cells were incubated with either a combination of primary antibodies against A_{2B}AR and α -actinin or against A_{2B}AR and Na⁺/K⁺-ATPase and then exposed to secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen, Karlsruhe, Germany). Staining of the nuclei was performed by 4'-6-diamidino-2-phenylindole (DAPI) incubation (0.2 μ g/ml, Roth, Karlsruhe, Germany). Fluorescent micrographs were taken with a confocal laser scanning microscope (Leica SP5, Wetzlar, Germany). Representative pictures from at least four independent heart or cell preparations are shown. Additionally human embryonic kidney (HEK) 293 cells overexpressing human A_{2B}AR were treated with the A_{2B} antibody

and then a fluorochrome-linked secondary antibody. These experiments are more fully described in the online supplement.

Mitochondria were isolated as described above, blocked in Solution A containing BSA, and incubated with primary antibodies (0.1 $\mu\text{g}/200 \mu\text{g}$ mitochondria) against $A_{2B}AR$, G_{α_s} , and markers of mitochondria [voltage-dependent anion channel (VDAC), an outer membrane mitochondrial protein, and adenine nucleotide transporter (ANT), an inner membrane mitochondrial protein] and cytosol (Akt). In control experiments the primary antibody was omitted. After washing in Solution A probes were incubated with secondary antibody conjugated to Alexa Fluor 488, washed again, and analysed using the FL-1 flow cytometer channel (Becton Dickinson FACSCalibur, Heidelberg, Germany) and CellQuest software. Data were gated to exclude debris, and fluorescence of 20,000 particles was computed. Representative FL-1 histograms from three independent mitochondria isolations are presented.

Western blot analysis

Transmural biopsies of the left ventricle were snap-frozen in liquid nitrogen and tissue lysate was prepared as described previously [12]. Accordingly, cell lysates from isolated cardiomyocytes were prepared after trypsinization and sonication. Mitochondrial and sarcolemmal samples were isolated as described above. In 50 μg samples of extracts proteins were separated by standard SDS-PAGE methods and immunoreactive bands were detected by enhanced chemiluminescence with LumiGLO (Cell Signaling Technology, Beverly, MA). Representative western blots from at least three independent preparations are presented. In addition, in preliminary experiments western blotting in HEK293 cells overexpressing human $A_{2B}AR$ and in $A_{2B}AR$ knockout mice were done to document the selectivity of the anti- A_{2B} antibody and are more fully described in the online supplement.

Preparation of mitoplasts with digitonin digestion

Digitonin (Sigma, Saint Louis, MO) was prepared as described elsewhere [41] with slight modifications. Briefly, digitonin was recrystallized with hot ethanol and dried at 100°C to produce a fine powder. A 2% stock solution was then made by adding an appropriate volume of solution B. Aliquots of mitochondrial suspensions (200 $\mu\text{g}/100 \mu\text{l}$ solution B) were placed on ice and 10 μl digitonin were added every minute up to 10 times, resulting in final concentrations of 0, 0.75, 1.5, 2.25, 3.0 and 3.75 mg digitonin/mg mitochondrial protein. The suspensions were incubated on ice for 30 min with oxygen access and gentle stirring and were diluted with solution B. The diluted mitochondrial solutions were again fractionated by centrifugation as described above and prepared for western blotting with antibodies to $A_{2B}AR$, ANT, and VDAC. Experiments were repeated twice and representative western blots are presented.

Antibodies

The following antibodies and concentrations were used: rabbit polyclonal anti-human $A_{2B}AR$ (1:50 for immunofluorescence microscopy and 1:500 for western blotting), goat polyclonal anti-human calnexin (1:500), goat polyclonal anti-human ANT (1:500), goat polyclonal VDAC-1 (1:500), rabbit polyclonal anti-rat G_{α_s} (1:500), and donkey anti-goat horseradish peroxidase linked IgG (1:5000). All were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-mouse Akt (1:1000), normal rabbit IgG, horse anti-mouse horseradish peroxidase linked IgG, and goat anti-rabbit horseradish peroxidase linked IgG (1:5000) were from Cell Signaling Technology (Beverly, MA), while Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 568-conjugated goat anti-mouse IgG (1:200 each) were from Invitrogen (Karlsruhe, Germany). Monoclonal mouse anti-rabbit Na^+/K^+ -ATPase (1:250 for immunofluorescence and 1:2000 for western blotting)

was from Millipore (Temicula, CA) and monoclonal mouse anti α -actinin (1:2000) from Sigma (St. Louis, MO).

Statistics

Data are presented as mean \pm SEM. Differences in infarct sizes, risk zones and TMRE fluorescence amongst groups were compared by one-way ANOVA with Fisher LSD post hoc testing. $P < 0.05$ was considered to be significant.

Results

Infarct size measurements after $A_{2B}AR$ activation

As shown in Fig. 1A treating rat hearts at reperfusion with the highly selective $A_{2B}AR$ agonist BAY60-6583 protected against ischemia/reperfusion injury. Protection was blocked by co-administration of the selective $A_{2B}AR$ antagonist MRS1754, thus, A_{2B} agonists are also protective in the rat heart.

Assessment of mitochondrial membrane potential $\Delta\Psi_m$ in isolated rat cardiomyocytes

Isolated cardiomyocytes were stressed with the calcium ionophore calcimycin which causes mPTP formation related to calcium overload. Fig. 1B illustrates that treatment with BAY60-6583 preserved $\Delta\Psi_m$ compared to calcimycin-treated control cells and protection was blocked by MRS1754. These results reveal an $A_{2B}AR$ -mediated response in individual cardiomyocytes.

$A_{2B}AR$ mRNA expression in individual rat cardiomyocytes

Single cardiac myocytes were selected under a microscope and placed in the PCR tube to insure that no other cell types were included. Quantitative RT-PCR analysis demonstrated $A_{2B}AR$ mRNA expression in rat cardiomyocytes (Fig. 2). $A_{2B}AR$ mRNA was expressed as a percentage of the housekeeping gene GAPDH, and easily detected in all of the tubes tested. The agarose gel revealed that the amplified product was the predicted size based on our primers. Finally, sequences from two of our samples were analysed. The sequencing results showed 100 % homology with the A_{2B} sequence in the GeneBank [gi|8392863|ref|NM_017161.1 Rattus norvegicus adenosine A_{2B} receptor (Adora2b) mRNA].

Immunofluorescence

Intracellular distribution of $A_{2B}AR$ in rat heart and isolated rat cardiomyocytes was analyzed by immunofluorescence microscopy. A western blot of heart homogenate with the antibody used for immunohistochemistry reveals a single strong band making nonspecific staining unlikely (Fig. 3A). In response to the same antibody, HEK293 cells overexpressing human $A_{2B}AR$ showed a similar single band which was not present in normal HEK293 cells (supplemental Fig. S2). Furthermore, heart homogenate of $A_{2B}AR$ knockout mice no longer showed the clear single band seen in wildtype animal hearts (supplemental Fig. S2), suggesting that the antibody used indeed immunodetects $A_{2B}AR$. Fig. 3B shows punctate staining of the myocyte's cytosol and its nuclei but no staining of the sarcolemma. In comparison, an immunofluorescent micrograph of HEK293 cells overexpressing $A_{2B}AR$ recorded at the same magnification showed receptor localization at the plasma membrane (Fig. 3C and supplemental Fig. S3). To make sure the absence of membrane staining was not an artefact of the collagenase digestion used to disperse the myocytes, we examined a cryosection of a rat ventricle. Figure 3D shows anti- $A_{2B}AR$ staining in green, and Fig. 3E shows propidium iodide staining the nuclei red. Fig. 3F is the merged image. In addition to the cytosolic and nuclear staining, string-like structures between the cells stained for $A_{2B}AR$ and were presumed to be capillaries. Again there was no staining of cell membranes.

Incubation of cardiomyocytes with purified IgG showed no staining (Fig. 3G). Cryosections from wild-type mouse hearts showed a similar pattern which was fully absent in A_{2B}AR knock-out animals (supplemental Fig. S4), further indicating specificity of the signal.

Staining of cardiomyocytes with anti-A_{2B}AR antibody (green), DAPI (blue) to label nuclei, and anti- α -actinin antibody (red) to localize Z bands demonstrates integrity of isolated myocytes and shows clear localization of staining within the nuclei, but there is no association with the α -actinin-labelled Z bands (Fig. 4, upper panels). To test the hypothesis that A_{2B}AR might be located on mitochondria, co-staining with anti-A_{2B} antibody and MitoTracker Red was performed. As shown in the merged picture in Fig. 4, middle panel, there was good co-localization of A_{2B}AR and MitoTracker suggesting the receptors are indeed on or very near the mitochondria. The lower panels of Fig. 4 showed only weak co-staining of A_{2B}ARs and the sarcolemmal Na⁺/K⁺-ATPase, additionally demonstrating the intracellular A_{2B}AR localization. As expected, no immunoreactivity was detected in control experiments with the corresponding IgG, and only the nuclei stained with DAPI were visible (Fig. 4, lower panel, right).

Mitochondrial localization of A_{2B}ARs was further demonstrated by FACS analysis of isolated mitochondria treated with antibodies recognizing A_{2B}AR and marker proteins of different compartments. As shown in Fig. 5A, A_{2B}AR antibody binding to isolated mitochondria was recognized by a shift of fluorescence intensity (population II) when compared to controls (no primary antibody, population I). To further demonstrate that the analyzed particles are most likely mitochondria, we treated them with antibodies against mitochondrial marker proteins (VDAC, ANT). As seen in Fig. 5B, there is staining of A_{2B}AR, VDAC and ANT, and there is a rightward shift towards population II cells. The same was true for G_{αs} (Fig. 5C), but not for the cytosolic marker Akt (Fig. 5D).

Western blot analysis

Fig. 6 shows that A_{2B}AR immunoreactivity was detected in western blots of mitochondrial preparations from left ventricular biopsies as well as isolated rat cardiomyocytes. In all preparations the A_{2B}AR band appeared at approximately 40 kDa, its expected size. In addition to the A_{2B}AR band in the mitochondrial fractions, the mitochondrial membrane proteins ANT and VDAC were also evident and enriched compared to whole heart and cell lysates. G_{αs} is known to couple to A_{2B}AR and was detected in all fractions. In contrast, neither mitochondrial fraction (whole heart or cardiomyocyte) demonstrated immunoreactivity to the plasma membrane marker Na⁺/K⁺-ATPase, the sarcoplasmic reticulum marker (calnexin), or the cytosolic marker (Akt). Mitochondria purification using an additional percoll gradient showed no decrease in A_{2B}AR content (supplemental Fig. S5). Furthermore, two different sarcolemmal preparations were analysed, a 100,000 g pellet in which mitochondrial contamination should be reduced by an upstream centrifugation step, and a 100,000 g pellet further purified by a sucrose gradient. The high Na⁺/K⁺-ATPase immunoreactivity of this preparation purified by sucrose gradient demonstrates sarcolemmal enrichment (Fig. 6). While the normal 100,000 g pellet still contained mitochondrial (ANT, VDAC) and SR (calnexin) contamination, this was much less the case when the sucrose gradient step was added. Hence, the sucrose gradient was able to reduce mitochondrial contamination, while SR was still present. A clear reduction of A_{2B}AR in the sucrose purified fraction supports our hypothesis of mitochondrial A_{2B}AR localization. Not surprisingly, strong signals for G_{αs} were present in both preparations related to the abundant presence of GPCRs in the sarcolemma.

Mitochondria were incubated with increasing concentrations of digitonin to remove the outer mitochondrial membrane (Fig. 7). Higher digitonin concentrations dissolve the outer membrane of mitochondria as is confirmed by the loss of VDAC, a protein only present in

the outer membrane. Conversely, the inner mitochondrial membrane marker ANT persists at all concentrations of digitonin. Increasing concentrations of digitonin also caused loss of A_{2B}AR suggesting that they are also predominantly located in or near the outer mitochondrial membrane.

Discussion

Considerable evidence indicates activation of A_{2B}AR is cardioprotective. A_{2B}AR selective agonists limit infarct size in hearts subjected to ischemia/reperfusion [10,21,46]. In the present study BAY60-6583 also protected the isolated rat heart when infused at the time of reperfusion. Protection from postconditioning initiated with either ischemia or a PKC activator could be blocked by co-administration of MRS1754 [21,33] which is a highly selective inhibitor of A_{2B}AR [19]. While these data imply that functional receptors must reside in the heart, there is debate as to whether cardiomyocytes actually express them in their plasma membranes. We were able to detect A_{2B}AR mRNA transcripts using quantitative RT-PCR in individually selected cardiomyocytes uncontaminated with other cell types. Furthermore, western blot analysis of both heart biopsy and isolated cardiomyocyte lysate showed a strong A_{2B}AR band.

Since the A_{2B}AR is a GPCR, we assumed that immunofluorescence would reveal plasma membrane staining. To our surprise, the A_{2B}AR antibody did not bind to the cardiomyocyte sarcolemma as expected, but rather caused structures at intracellular sites to fluoresce. Co-staining with propidium iodide or DAPI revealed high expression in nuclei. There was also a chain-like pattern of fluorescent staining of intracellular structures with the A_{2B} antibody which resembled the pattern seen with mitochondrial stains in myocytes. The merged image of A_{2B}AR staining with staining generated by MitoTracker Red, a mitochondria-specific dye, indicated co-localization of A_{2B}AR and mitochondria. To further assess the likelihood that A_{2B}AR were in the sarcolemma, we exposed isolated cardiomyocytes simultaneously to antibodies to A_{2B}AR and Na⁺/K⁺-ATPase and found almost no co-localization of these two proteins. In ventricular muscle cells surface receptors are often located on the T-tubules which overlay the Z-lines deep in the cell. However, co-localization of A_{2B}AR with Z-lines stained with anti-actin antibody was also not observed.

In contrast to the findings in cardiomyocytes, HEK293 cells overexpressing A_{2B}AR indeed showed typical membrane staining by fluorochrome-linked A_{2B}AR antibodies. While our observations are consistent with recent reports of A_{2B}AR localization [27,31], we have no explanation for the differences between these cell models of A_{2B}AR overexpression and the pattern seen in primary adult cardiomyocytes.

Although it has been generally assumed that GPCRs are only trafficked to the plasma membrane, there is evidence that GPCR can also localize and function in intracellular compartments, such as the nucleus for the β-adrenergic receptor [7]. Also, mitochondrial Ca²⁺ transport has been linked to the presence of P2Y-like receptors on mitochondria [2] and G_{αi} subunits have been found in mitochondria of HEK293T cells [24]. Furthermore, mitochondria are known to contain non-GPCR for estrogens, glucocorticoids, androgens, and thyroid hormones, as well as the retinoid receptor α and the peroxisome proliferator-activated receptor β [36].

Western blot analysis of mitochondrial preparations from hearts as well as isolated cardiomyocytes showed A_{2B}AR immunoreactivity (Fig. 6). The mitochondrial fractions appeared to be nearly free of contamination with sarcolemma and sarcoplasmic reticulum as revealed by a lack of reactivity to anti-Na⁺/K⁺-ATPase and anti-calnexin antibodies, respectively. To further demonstrate an intracellular A_{2B}AR localization we tried to isolate

sarcolemma free of mitochondria and SR, which we hypothesized would not contain A_{2B}AR. First, a simple preparation including a centrifugation step at lower speed followed by a high speed centrifugation step to pellet the sarcolemma was not sufficient because we still saw contamination by mitochondria and SR proteins and, therefore, also A_{2B}AR. Further treatment with a sucrose gradient markedly reduced mitochondrial contamination. Since the A_{2B}AR signal also decreased without affecting SR content, A_{2B}AR localization in or near mitochondria is likely. Our digitonin studies additionally suggest A_{2B}AR may reside in the outer mitochondrial membrane. Nevertheless our studies do not unequivocally prove that A_{2B}AR reside within mitochondria; they may in fact only be loosely attached on the outside. Moriyama and Sitkovsky [29] recently showed that A_{2B}AR are poorly trafficked to the membrane in some cells leaving many receptors in the endoplasmic reticulum of the Golgi. However, the location of the receptors in our study is not consistent with a peri-nuclear Golgi locus.

The molecular weight of our A_{2B}AR band was near 40 kDa which closely approximates the expected 38.18 kDa size. Previous studies have reported a tissue- and species-dependent protein size between 31 and 50 kDa. For example, hexahistidine/FLAG-tagged human A_{2B}AR recombinantly expressed in HEK293 cells have molecular weights of 34.8 kDa and after deglycosylation 31.3 kDa [23]. Puffinbarger *et al.* [37] demonstrated human A_{2B}AR with differing sizes, 35 kDa in small intestine, and 50-52 kDa in thymus, colon and placenta. Jackson *et al.* [18] presented evidence for a 52 kDa A_{2B}AR in rat aorta and preglomerular microvasculature. It is difficult to explain the observed differences, but it is suspected that the higher molecular weights were the result of antibodies cross-reacting with some non-receptor epitope. In the present study the fact that the A_{2B} band disappeared in tissue from A_{2B} knockout mice argues against non-specific binding of our antibody to another protein or receptor subtype.

Functional relevance of cardiac A_{2B}AR could be demonstrated by a BAY60-6583-mediated decrease in infarct size after ischemia/reperfusion, and this protection was blocked with the highly-selective A_{2B}AR antagonist MRS1754. To test if cardiomyocytes, the cells that must be protected at reperfusion, respond to A_{2B}AR stimulation, we used a cell model of intracellular calcium stress which mirrors the detrimental calcium increase occurring at reperfusion [32]. Cardiomyocytes were stained with TMRE, and it is well accepted that a loss in TMRE fluorescence is correlated with a loss of mitochondrial membrane potential ($\Delta\Psi_m$), which in turn presumably indicates mPTP opening. When BAY60-6583 was added before the calcium ionophore, cells were less prone to calcium-induced depolarization of $\Delta\Psi_m$. This effect was inhibited by MRS1754. Confirmation of our results comes from a recent study in which NECA, a potent A₂AR agonist, suppressed mitochondrial transition pore formation in isolated rat cardiomyocytes and, again, this protection could be blocked with MRS1754 [44].

We do not know how A_{2B}AR might be directed to mitochondria. Like 99% of the mitochondrial proteins A_{2B}AR are not part of the mitochondrial genome. Rather they are encoded in the nuclear DNA. After being synthesized in the cytosol they must be transported to the mitochondria. Most mitochondrial proteins carry importing sequences which are recognized by cytosol-exposed receptors present in the mitochondrial outer membrane called “translocase of the outer membrane” (TOM). Depending on the imported protein's target they are transported by TOM and possibly by TIM (translocase of the inner membrane) complexes [28]. Mitochondrial targeting moieties are quite variable and can be present anywhere in the poly-peptide structure. However, as analyzed by either TargetP or MITOPROT programs the A_{2B}AR amino acid sequence does not contain any known importation sequence [11]. This does not exclude the possibility that mitochondria will incorporate them, however, as some imported mitochondrial proteins do not contain any of

these sequences. This was recently found to be the case for connexin 43 which is present in myocardial mitochondria [4,6,38].

Although still controversial, the survival kinases ERK, Akt and GSK-3 β are central to the protection conferred by pre- and postconditioning in the rat heart [13,15,42] and they can localize in mitochondria as well. As reviewed by Poderoso *et al.* [35] activation of protein kinase A (PKA), MEK1/2 and ERK1/2 leads to signal complex formation at the mitochondrion surface. GSK-3 β which is cardioprotective when inhibited by phosphorylation is present in mitochondria [17], and Akt reportedly accumulates in mitochondria following PI3-kinase activation [3]. In our study we used Akt as a cytosolic marker and did not see immunoreactivity in our mitochondrial preparations. But our cells were in a basal, unstimulated state.

A_{2B}AR couple through stimulatory G proteins (G_{as}) which in turn are known to produce cAMP via adenylyl cyclase. Indeed several G proteins were identified in mitochondria of HEK293T cells [24]. And we found G_{as} in our mitochondrial preparation. Sardanelli *et al.* [40] showed PKA in mitochondria from rat hearts, while Acin-Perez *et al.* [1] demonstrated cAMP production within mitochondria and the subsequent activation of mitochondrial PKA. Furthermore, nitric oxide synthase and its product nitric oxide could also be demonstrated within mitochondria [20]. Although the presence of A_{2B}AR in or near mitochondria is likely, the demonstration of a functional relevance of these receptor complexes surely requires further study.

There is growing evidence that protection afforded by pre- and postconditioning is related to kinase- and signalling molecule-dependent inhibition of mitochondrial transition pores upon reperfusion [5,9,16] and that A_{2B}AR control them [33]. It therefore seems quite conceivable that A_{2B}AR localized on mitochondria could be involved in this signalling cascade.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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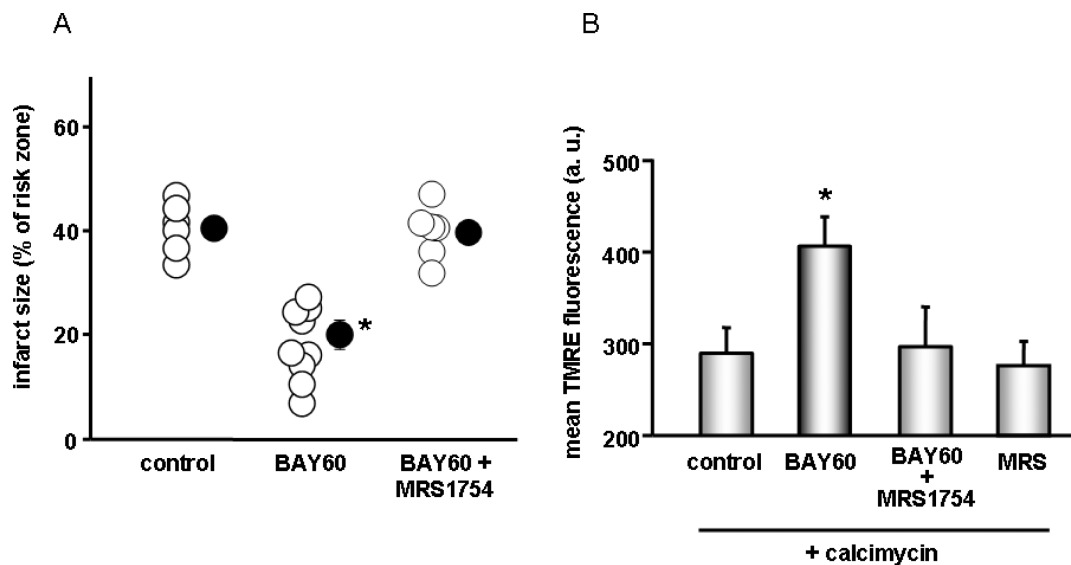


Figure 1.

A. Infarct size in isolated rat hearts expressed as a percentage of the risk zone. Open circles denote individual experiments, while filled-in circles are group means \pm SEM. * $p < 0.001$. B. Assessment of mitochondrial membrane potential $\Delta\Psi_m$ in isolated cardiomyocytes. Whereas control cells were treated with only calcimycin, other cells were also exposed to BAY 60-6583 alone or a combination of BAY 60-6583 and MRS1754. * $p < 0.05$

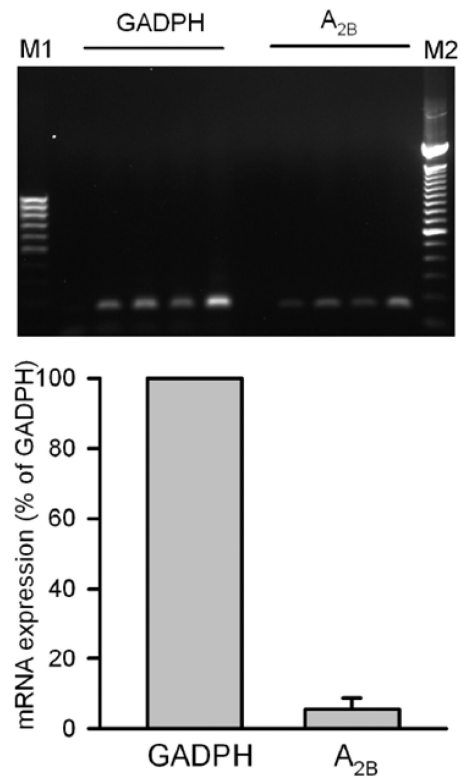


Figure 2. A_{2B}AR-mRNA expression in isolated rat cardiomyocytes. Quantitative RT-PCR showed A_{2B}AR mRNA copy count was low but measurable as compared with GAPDH. The gel demonstrates that the amplified transcripts were of the size these primers were expected to produce.

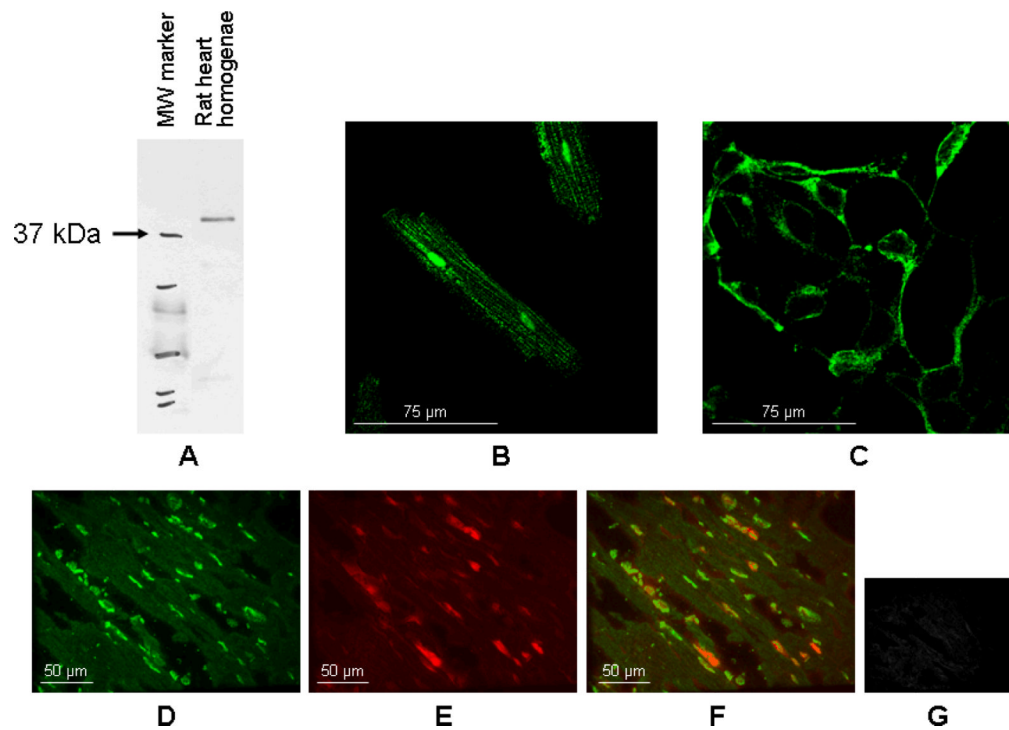


Figure 3.

A. Western blot of heart homogenate with anti- A_{2B} antibody revealing a single strong band. In Fig. 3B-3G cardiomyocytes, HEK293 cells overexpressing A_{2B} AR, and cryosections of rat heart slices were stained with A_{2B} AR antibody coupled to a fluorochrome (green). B. Isolated rat cardiomyocytes with punctate intracellular and nuclear staining, but no staining of sarcolemma. C. Plasma membrane staining in HEK293 cells overexpressing A_{2B} AR at identical magnification as in Fig. 3B. D. Slices of rat left ventricle showing diffuse intracellular and nuclear A_{2B} AR staining. E. Propidium iodide staining of the nuclei. F. Merged image of Figs. 3D and 3E. G. Control with cardiomyocytes incubated with IgG.

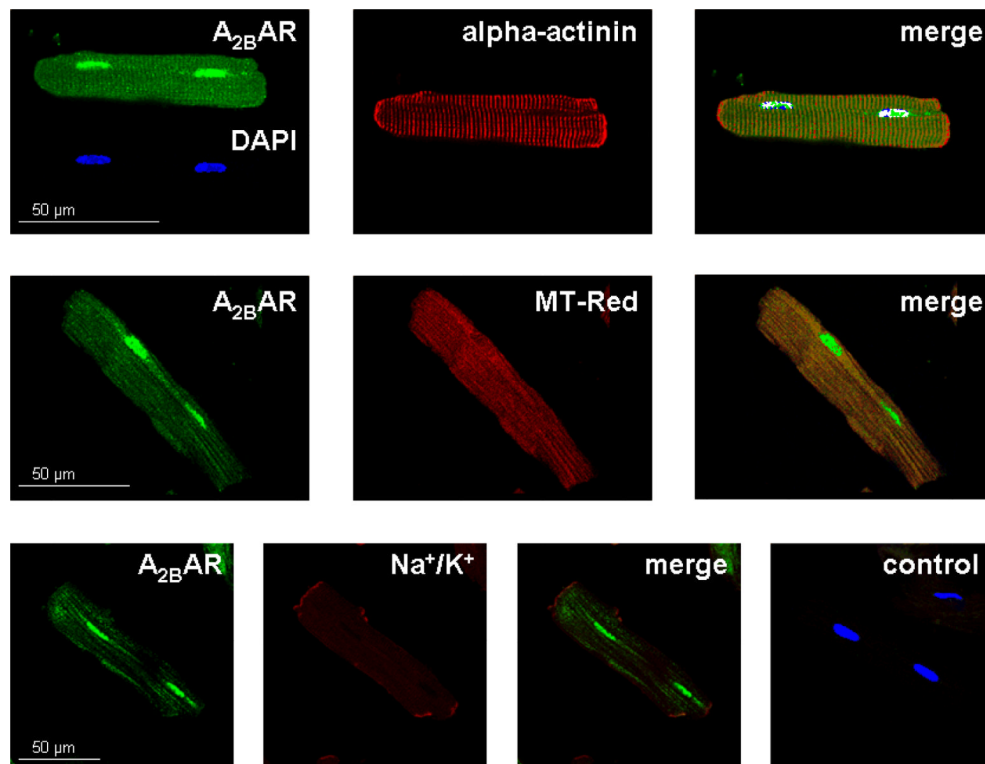


Figure 4.

Isolated rat cardiomyocytes were stained with $A_{2B}AR$ antibody coupled to a fluorochrome (green). Additionally, nuclei, Z bands, mitochondria and Na^+/K^+ -ATPase were detected using DAPI, α -actinin antibody, MitoTracker Red and Na^+/K^+ -ATPase antibody, respectively. $A_{2B}AR$ green fluorescence was observed in nuclei but not in plasma membranes or Z bands (upper panel). Intracellular staining for $A_{2B}AR$ localized to mitochondria (middle panel). Intracellular $A_{2B}AR$ staining did not co-localize with sarcolemmal Na^+/K^+ -ATPase (lower panel). DAPI-stained nuclei are visible in control cardiomyocytes incubated with IgG instead of specific primary antibody (lower panel, right image).

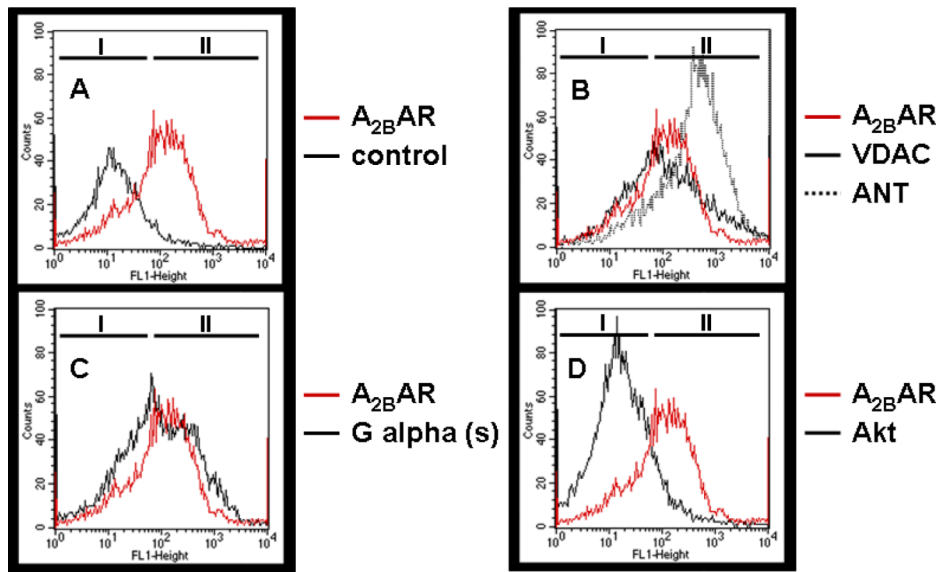
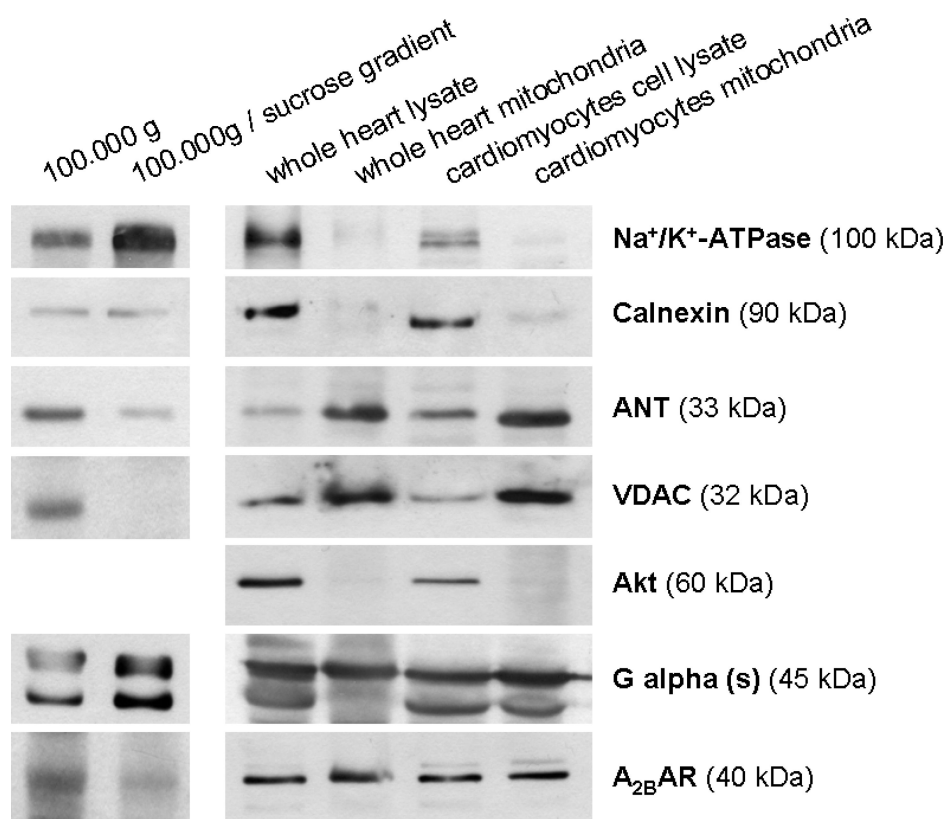


Figure 5.

Isolated mitochondria were stained with different primary antibodies and corresponding secondary antibody coupled to green fluorescent 488 dye and subjected to FACS analysis. A. Anti-A_{2B} antibody-stained mitochondria showed a marked rightward shift. Mitochondria stained with mitochondrial marker proteins VDAC and ANT (B) and G_{αs} (C) similarly showed rightward shifts, suggesting mitochondrial A_{2B}AR and G protein co-localization. D. The purity of the mitochondrial preparation was demonstrated by using Akt as a cytosolic marker which had no effect.

**Figure 6.**

Western blot of different rat heart preparations revealed mitochondrial localization of A_{2B}AR protein. Purity of mitochondrial samples from hearts and isolated cardiomyocytes was demonstrated by co-staining of different marker proteins. The heart mitochondria samples were free of plasma membrane (Na⁺/K⁺-ATPase), sarcoplasmic reticulum (calnexin), and cytosol (Akt). Absence of A_{2B}AR in sarcolemma was demonstrated by two different membrane preparations in which Na⁺/K⁺-ATPase was enriched. 100,000 g pellets still contained SR and mitochondria and, therefore, stained positive for A_{2B}AR, while further purification with a sucrose gradient greatly reduced mitochondrial contamination together with A_{2B}AR staining.

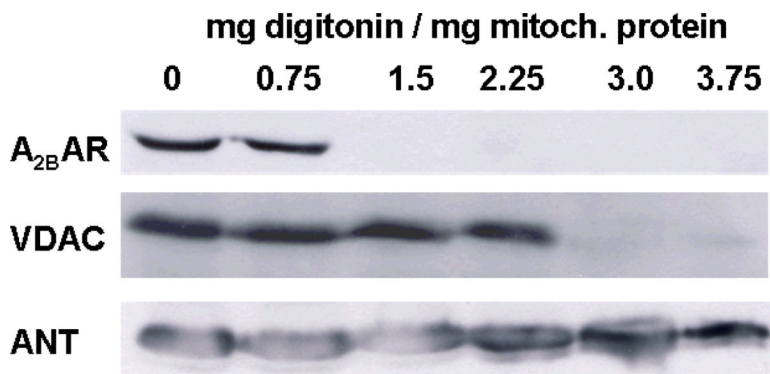


Figure 7. Western blot analysis of mitochondrial preparations treated with increasing digitonin concentrations. The signal for the outer membrane marker voltage-dependent anion channel (VDAC) is lost at higher concentrations, while the inner membrane marker protein adenine nucleotide transporter (ANT) is still present even at the highest digitonin concentration. Since A_{2B}AR and VDAC bands were similarly affected by digitonin, a location for the former in the outer mitochondrial membrane is assumed.