Role of adenosine A$_1$ and A$_3$ receptors in regulation of cardiomyocyte homeostasis after mitochondrial respiratory chain injury

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from the closely related A1R subtype. Activation of A1Rs decreased intracellular Ca2+ concentration ([Ca2+]i) and may attenuate mitochondrial Ca2+ accumulation by a mechanism independent from the mitoKATP channel. Ischemic and pharmacological preconditioning exerts cardioprotection by up-regulating endogenous protective mechanisms and may be fully achieved in undamaged, intact cells. The aim of this study was to elucidate the protective effects of AR activation and mitoKATP channel opening in cardiac cells with respiratory chain deficiency. We investigated the roles of A1R and A3R activation on functional tolerance after inhibiting the terminal link of the mitochondrial respiratory chain with sodium azide, which is an inhibitor of cytochrome c oxidase, during a state of normoxia or hypoxia. A comparison of the effects of the mitoKATP channel opener diazoxide and the Ca2+ response after activation of the A1R and A3R might shed light on the pathways of AR signaling in protecting the cardiac cells from conditions of stress.

MATERIALS AND METHODS

The experimental protocol was approved by the Animal Care and Use Committee of Bar-Ilan University. This investigation also conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Pub. No. 85-23, Revised 1996).

Cell culture. Rat hearts (1–2 days old) were removed under sterile conditions and washed three times in phosphate-buffered saline (PBS) to remove excess blood cells. The hearts were minced and then gently agitated in a solution of proteolytic enzymes (RDB; Biological Institute; Ness-Ziona, Israel), which was prepared from a fig tree extract. The RDB was diluted 1:100 in Ca2+- and Mg2+-free PBS at 25°C for a few cycles of 10 min each as described previously (36, 40). Dulbecco’s modified Eagle’s medium that contained 10% horse serum (Biological Industries; Kibbutz Beit Haemek, Israel) was added to the supernatant suspensions that contained dissociated cells. The mixture was centrifuged at 350 g for 5 min. The supernatant phase was discarded, and the cells were resuspended. The suspension of the cells was diluted to 1.0 × 106 cells/ml, and 1.5 ml of the suspension was placed in 35-mm plastic culture dishes on collagen-gelatin-coated coverslips. The cultures were incubated in a humidified 5% CO2–95% air atmosphere at 37°C. Confluent monolayers exhibiting spontaneous contractions were developed in culture within 2 days. Myocyte cultures were washed in serum-free BIO-MPM-1 medium (Biological Industries) that contained 5 mg/ml glucose and were incubated in this medium for an additional 48 h before the experiments were performed.

Hypoxic conditions. Myocyte cultures were washed in serum- and glucose-free medium before incubation in the presence of AR ligands under hypoxic conditions. A 60- or 90-min exposure to N2 (100%) in glucose-free media within a hypoxic chamber was used to simulate hypoxic conditions in primary cardiac myocyte cultures. The hypoxic damage was characterized at the end of the hypoxic period by morphological and biochemical evaluations. Sodium azide (Sigma; St. Louis, MO) was freshly prepared in culture medium for each experiment. Continuous monitoring of [Ca2+]i, or mitochondrial membrane potential during hypoxia was realized in a special barrier well, where cells were protected from oxygen by a laminar counterflow layer of inert argon (100%) gas.

Experiments with A1R and A3R ligands. The A1R agonist 2-chloro-N,N-cyclopentyladenosine (CCPA), the A3R agonist 2-chloro-N,N-iodo-benzyl-5’-N-methylcarbamoxamidoadenosine (CI-IB-MECA), the A2R antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), and the A1R antagonist 5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate (MRS-1523) were added to cell cultures 10 min before the experimental treatment.

Lactate dehydrogenase assay. Cytotoxicity was assessed by spectrophotometric measurement of lactate dehydrogenase (LDH) released into the culture medium. Protein content and LDH activity were determined according to the methods of El-Ani et al. (6). Briefly, 25 μl of the supernatant were transferred to a 96-well dish, and the LDH activities were determined by using LDH-L kits (Sigma) as described by the manufacturer. The results are expressed as a fold of the control in the same experiment. Experiments were done in four to eight replicates each and were repeated at least six times.

Cell death assay. This assay was performed using a modification of the procedure used in our previous work (36). Cells were loaded with propidium iodide, which only stains the nuclei of membrane-compromised cells. To facilitate cell counting, Hoechst-33342 (10 μM) was included to stain the nuclei of all cells. Cell loss (percentage of cell death) was presented as the number of dead (propidium iodide stained) cells/total number of cells (Hoechst-33342 stained).

Measurement of ATP concentration. Cells were washed with ice-cold PBS, frozen in liquid N2, and stored at −80°C until analysis. Cells were resuspended in ice-cold homogenization buffer that consisted of 50 mM potassium fluoride, 10 mM EDTA, and 30% glycerol, pH 7.0. The cell extract was used to measure ATP content with the luciferin-luciferase bioluminescence kit (ATP Bioluminescence Assay Kit CLSII; Boehringer Mannheim) following the manufacturer’s protocol. Values are expressed as nanomoles per milligram of protein (41).

Monitoring mitochondrial retention of DASPMI. Living cells grown on coverslips were exposed to 4-[4-(dimethylamino)styryl]-N-methylpyridinium iodide (DASPMI) dissolved in PBS at a final concentration of 10 μg/ml for 15 min. The coverslips were then washed and mounted on chambers that contained dye-free medium. DASPMI fluorescence was elicited by excitation at 460 nm, and emission was measured using a long-pass filter at 540 nm. For registration of kinetic curves of DASPMI fluorescence, the emitted light was split on the path to the photomultiplier by a dichroic mirror with an input filter at 590 nm. The fluorescence intensity was fed to a SAMPLE program written by Dr. Doron Kaplan (Israel Institute for Biological Research; Ness-Ziona, Israel). DASPMI fluorescence intensity corresponds to a relative polarization of ΔP0. It was shown that distribution of DASPMI ions on the inner mitochondrial membrane occurs in accordance with the Nerst equation. Mitochondrial membrane hyperpolarization and depolarization were induced by sodium succinate (20) and the mitochondrial uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, Ref. 39), respectively.

[Ca2+]i, measurements. We estimated [Ca2+]i from indo-1 fluorescence using a ratio method described elsewhere (41). Continuous monitoring of [Ca2+]i, during hypoxia was performed in a special barrier well where cells were protected from oxygen by a laminar counterflow layer of inert argon (100%) gas.

Chemicals. DASPMI and indo-1 were acquired from Molecular Probes (Eugene, OR). The highly selective A1R agonist CI-1B-MECA was a gift from the National Institute of Mental Health Chemical Synthesis and Drug Supply Program. The highly selective A1R agonist CCPA and the selective A3R antagonist MRS-1523 were purchased from Sigma. Other reagents were purchased from Sigma.

Statistics. Results are expressed as means ± SE. Data were analyzed by ANOVA with application of a post hoc Tukey-Kramer test. P < 0.05 was accepted as indicating statistical significance.

RESULTS

To investigate the role of AR agonists in attenuating myocyte injury during prolonged (90 min) hypoxia, cultured cardiomyocytes were incubated with A1R- and A3R-specific agonists (CCPA and CI-IB-MECA, respectively) for 15 min before

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and during hypoxia. These agonists prevented injury produced by hypoxia according to the level of LDH released from the cells (Fig. 1). Decrease in LDH release suggested that both A1R and A3R agonists reduced hypoxia-induced injury in intact cardiomyocytes. Thus, when intact cardiomyocytes were exposed to hypoxia, the protective effects of A1R and A3R agonists against damage were evident, and protection by A1R activation was more effective. These data agreed well with our earlier findings that CCPA and Cl-IB-MECA attenuated cultured rat cardiomyocyte injury and prevented cell death during hypoxia through activation of ARs (36).

The efficacy of A1R and A3R agonists in protecting damaged cardiomyocytes was studied in the presence of sodium azide, which inhibits the mitochondrial respiratory chain through action at cytochrome c oxidase. Results of LDH release from cardiac myocytes after treatment with 1 mM sodium azide and hypoxia for 60 and 90 min are shown in Fig. 1. Neither the A1R agonist nor the A3R agonist was able to prevent the detrimental effects of sodium azide during 90 min of hypoxia. However, when the cultures were exposed to hypoxia for 60 min in the presence of 1 mM sodium azide, activation of A1Rs abolished the effects of azide, whereas activation of A3Rs was significantly less effective.

Cell death after 60 min of hypoxia was 18 ± 2%. In cardiocytes treated with sodium azide and hypoxia for 60 min, cell death increased to 37.2 ± 3.1%. Activation of A1Rs with Cl-IB-MECA attenuated cell loss to 14.3 ± 2.4%, and activation of A1Rs with CCPA attenuated cell loss only to 30.1 ± 6.1% (Fig. 2). Activation of ARs was not effective in preventing cell death after 90 min of hypoxia with sodium azide (Fig. 2).

The total level of ATP decreased considerably after hypoxia (Fig. 3). However, treatment with 100 nM of the A1R agonist CCPA or 100 nM of the A3R agonist Cl-IB-MECA restricted the decrease in ATP level. Average values for total ATP content after 90 min of hypoxia were 13.54 ± 0.89 nmol/mg protein in the presence of CCPA and 11.18 ± 0.74 nmol/mg protein in the presence of Cl-IB-MECA compared with 7.86 ± 0.36 nmol/mg protein in the hypoxic group. The ATP level of control (untreated) cells was 21.36 ± 2.89 nmol/mg protein.

In cultures treated with sodium azide (1 mM) and hypoxia for 60 min, the average values for total ATP content were 10.01 ± 1.06 nmol/mg protein in the presence of CCPA (100 nM) and 14.06 ± 0.80 nmol/mg protein in the presence of Cl-IB-MECA (100 nM) compared with 8.57 ± 1.23 nmol/mg protein in cells treated with hypoxia and sodium azide together (Fig. 3). Again, activation of A1Rs in the cardiocytes exposed to hypoxia for 60 min in the presence of sodium azide was significantly more effective in protection of the cells. No effects were found when these insults were for 90 min (Fig. 3).

In experiments with cultured cardiomyocytes, sodium azide concentrations of 1–100 mM are usually used (3), which effectively inhibit respiratory activity in a concentration-dependent manner. Treatment with 10 mM sodium azide for 2 h under normoxic conditions induced only moderate LDH release (not shown). However, this treatment caused a consider-
able decrease in the total ATP level (Fig. 4). Blockade of cytochrome c oxidase with 10 mM sodium azide excessively decreased ATP content in cultured cardiomyocytes (3.56 ± 0.52 compared with 21.26 ± 0.69 nmol/mg protein in control cells after a 2-h incubation). Activation of ARs showed a protective action (13.23 ± 1.44 nmol/mg protein after A1R activation and 9.38 ± 1.90 nmol/mg protein after A1R activation). The A1R antagonist DPCPX (1 μM) abolished the protection by CCPA, and the A3R antagonist MRS-1523 (1 μM) abolished the protection by Cl-IB-MECA (Fig. 4). The contribution of mitoK\textsubscript{ATP} channels in injured cells to the protective effects of ARs was examined by assessment of ATP levels in the presence of the mitoK\textsubscript{ATP} channel opener diazoxide. Pretreatment with diazoxide (100 μM) attenuated the decrease in ATP level in cardiomyocytes after 120 min of incubation with sodium azide; however, this protection was less effective than activation of either A1Rs or A3Rs (Fig. 4).

Microscopic observation of mitochondria in cultured cardiomyocytes with the use of the membrane potential indicator DASPMI showed two types of mitochondrial patterns in normoxic conditions. The first displayed longitudinally oriented and stretched mitochondria in subsarcolemmal areas in the cytoplasm, and the second featured oval-shaped mitochondria and stretched mitochondria in subsarcolemmal areas in the moxic conditions. The first displayed longitudinally oriented mitochondrial patterns in normoxic conditions. The protonophore FCCP is able to efficiently collapse the membrane potential (not shown). When hypoxia with 1 mM sodium azide was evoked after the addition of 10 mM sodium succinate (Fig. 6A). The protonophore FCCP is able to efficiently collapse the ΔΨ in intact cells. Upon addition of 5 μM FCCP, the mitochondrial fluorescence decreased monotonically within 20 min (Fig. 6A). Addition of the K\textsuperscript{+} channel opener diazoxide (100 μM), the A1R agonist CCPA (100 nM), or the A3R agonist CI-IB-MECA were effective in retarding a decrease in DASPMI fluorescence and, hence, dissipation of ΔΨ. Pretreatment of the cells with DPCPX before the addition of CCPA or with MRS-1523 before the addition of CI-IB-MECA abolished the protective effects of these agonists (Fig. 6, C and D).

In cardiac myocytes treated with 1 mM sodium azide and 60 or 90 min of hypoxia, the DASPMI fluorescence was decreased, and many myocytes exhibited signs of destructive oncotic alterations with a collapse of ΔΨ (Fig. 5, E and F). Neither the A1R agonist CCPA nor the A3R agonist CI-IB-MECA was able to prevent mitochondrial damage after 90 min of hypoxia and blockade of the terminal link of the respiratory chain (not shown). When hypoxia with 1 mM sodium azide was applied for 60 min, activation of A3Rs prevented dissipation of ΔΨ (Fig. 5G); however, activation of A1Rs only partly protected mitochondrial bioenergetics (Fig. 5H).

For elucidation of the kinetics of DASPMI fluorescence intensity, a microspectrofluorimetric method was used. Sustained and FCCP were applied as standards for mitochondrial energy generation and dissipation. Maximal increase of ΔΨ was evoked after the addition of 10 mM sodium succinate (Fig. 6A). The protonophore FCCP is able to efficiently collapse the ΔΨ in intact cells. Upon addition of 5 μM FCCP, the mitochondrial fluorescence decreased monotonically within 20 min (Fig. 6A). Addition of the K\textsuperscript{+} channel opener diazoxide (100 μM), the A1R agonist CCPA (100 nM), or the A3R agonist CI-IB-MECA (100 nM) did not change DASPMI fluorescence and, hence, ΔΨ during 20 min of observations under normoxic conditions (Fig. 6B). When cultures were placed in hypoxic chambers under a stream of argon (see MATERIALS AND METHODS), the A1R agonist CCPA and the A3R agonist CI-IB-MECA were effective in retarding a decrease in DASPMI fluorescence and, hence, dissipation of ΔΨ. Pretreatment of the cells with DPCPX before the addition of CCPA or with MRS-1523 before the addition of CI-IB-MECA abolished the protective effects of these agonists (Fig. 6, C and D).

In single living cardiomyocytes exposed to hypoxia in the presence of sodium azide, ΔΨ was monitored for 20 s every 10 min during 90 min of the insults (Fig. 6E). The complete depression of ΔΨ by treatment with 1 mM sodium azide during hypoxia took ~90 min. Diazoxide was not effective in protecting the bioenergetics of cardiomyocytes with damaged mitochondria (Fig. 6E). The A1R agonist CCPA retarded the decrease in DASPMI fluorescence during the first 20–25 min of hypoxia. The A3R agonist CI-IB-MECA was more effective in protection of ΔΨ. In the presence of 100 nM CI-IB-MECA, ΔΨ was maintained during 60 min of hypoxia in cells treated with sodium azide (Fig. 6E).

The effects of A1R and A3R activation on Ca\textsuperscript{2+} transients were estimated from indo-1 fluorescence using the ratio method. Control myocytes demonstrated spontaneous, regular beating activity and [Ca\textsuperscript{2+}]i transients in indo-1-loaded cells.

Treatment with sodium azide induced a transient rise in [Ca\textsuperscript{2+}]i, in a dose-dependent manner. Sodium azide at a concentration of 1 mM during normoxia induced only transient acceleration of the beating rate and [Ca\textsuperscript{2+}]i elevation that lasted 10–20 s. Treatment with 10 mM sodium azide induced transient accelerations of the beating rate, elevation of baseline (diastolic) [Ca\textsuperscript{2+}]i, and termination of beating activity after 1–2 h of treatment (Fig. 7A). Pretreatment of cultures with 100 nM CI-IB-MECA prevented the basal Ca\textsuperscript{2+} elevation caused by sodium azide and maintained myocyte contractility (Fig. 7B). The same experiment with the A1R agonist CCPA did not
reveal any protective efficacy of A1R activation (not shown). To confirm the representative data shown in Fig. 7, A and B, we averaged results obtained in six experiments (Fig. 7C). Treatment of cultured cardiac muscle cells with 10 mM NaN3 led to elevation of baseline [Ca2+]i (to 1.40 ± 0.22 vs. 0.62 ± 0.21 in control cells) and disappearance of the [Ca2+]i transient amplitude (0.06 ± 0.02 vs. 1.04 ± 0.14 in control cells). Pretreatment with the A3R agonist restricted elevation of baseline [Ca2+]i and maintained muscle cell contractility (0.90 ± 0.20 and 0.62 ± 0.24, accordingly).

Continuous monitoring of [Ca2+]i during hypoxia in cultures pretreated with 1 mM NaN3 revealed very fast (3–5 min) elevation of [Ca2+]i, decrease of amplitude in [Ca2+]i transients, and cessation of [Ca2+]i oscillations (Fig. 7D). We studied effects of adenosine agonists in cultures pretreated with 1 mM NaN3 during hypoxia when the basal level of [Ca2+]i increased considerably. If at that stage 100 nM Cl-IB-MECA was applied to the cells, [Ca2+]i returned to its normal basal level and beating activity was restored (Fig. 7E). The A1R agonist CCPA in this case was ineffective (Fig. 7F).

In cells pretreated with Cl-IB-MECA 15 min before the addition of sodium azide together with application of hypoxia, contractile activity and [Ca2+]i oscillations, with gradual decrease in oscillation amplitude, were observed during 40–60
A protective effect in this experiment was also achieved when A1Rs were activated with 100 nM CCPA but only during 15–20 min of hypoxia (Fig. 7H). In a total of six experiments, exposure to hypoxia with 1 mM NaN₃ led at 40 min to elevation of baseline $[Ca^{2+}]_i$ (to 1.40 ± 0.22 vs. 0.62 ± 0.21 in control cells) and disappearance of $[Ca^{2+}]_i$ transient amplitude (0.06 ± 0.02 vs. 1.04 ± 0.14 in control cells). Pretreatment with the A3R agonist restricted elevation of baseline $[Ca^{2+}]_i$ (0.90 ± 0.20 vs. 0.62 ± 0.24) and maintained muscle cell contractility (Fig. 7I).

**DISCUSSION**

A crucial mechanism for living cells is mitochondrial oxidative phosphorylation coupled to an electrochemical gradient of H⁺ (or OH⁻) across the inner membrane. Mitochondria support the energy-dependent regulation of several cell functions, e.g., intermediary metabolism and cardiomyocyte contraction. Animal cells derive >90% of their energy from oxidative phosphorylation associated with the inner mitochondrial membrane (26). Thus hypoxia, leading to deprivation of the main electron acceptor, causes perturbation of mitochondrial membrane potentials and decreases the coupling efficiency between oxidation and phosphorylation. This promotes large bioenergetic deficits that lead to the loss of several functions that are vital to the survival of the cell and the organism. The role of adenosine in mediating preconditioning is well recognized (27, 28). In rat and rabbit hearts, protection induced with both A₁R and A₃R agonists is similar to that obtained with adenosine pretreatment (17). Protection of the mitochondrial respiratory chain and its impact on mitochondrial dysfunction (Fig. 6) after AR activation. A: sodium succinate (10 mM) and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, 5 μM) were applied as standards for mitochondrial energy generation and dissipation. B: effects of A₁R agonist CCPA (100 nM), A₃R agonist Cl-IB-MECA (100 nM), and mitochondrial Kᵢ₆₇ channel opener diazoxide (100 μM) on DASPMI fluorescence during normoxia. C and D: A₁R agonist CCPA (100 nM) and A₃R agonist Cl-IB-MECA (100 nM), respectively, were effective in retarding a decrease in DASPMI fluorescence and, hence, dissipation of $ΔΨ$ during hypoxia. Pretreatment of the cells with DPCPX (1 μM) before addition of CCPA or with MRS-1523 (1 μM) before addition of Cl-IB-MECA abolished the protective effects of these agonists. E: effects of AR activation and diazoxide on kinetics of $ΔΨ$. Effects of the A₁R agonist CCPA (100 nM), A₃R agonist Cl-IB-MECA (100 nM), and diazoxide (100 μM) on DASPMI fluorescence in cardiomyocytes treated with sodium azide (1 mM) and exposed to hypoxia. Readings were obtained every 10 min. Each graph is representative of six experiments.
Drial bioenergetics after AR activation may be an important factor associated with increased resistance to hypoxia. As shown in this study, activation of both subtypes of ARs promotes preservation of adequate amounts of ATP and maintenance of mitochondrial metabolism on a level sufficient for cell survival (see Fig. 3).

A possible explanation for the ischemic protection associated with the opening of myocyte mitoK\textsubscript{ATP} channels is that decreasing Δψ promotes the binding of the endogenous ATPase inhibitor IF\textsubscript{1} and hence, the conservation of ATP during ischemia (35). In intact cells, administration of mitoK\textsubscript{ATP} openers or endogenous signaling may lead to moderate K\textsuperscript{+} influx into the mitochondrial matrix. In low-work state cardiomyocytes (high Δψ), influx of K\textsuperscript{+} would cause matrix swelling, matrix alkalization, and increased production of reactive oxygen species (ROS; Ref. 9) during the transition to active mitochondria. In the high-work state, or during ischemia or hypoxia, K\textsuperscript{+} influx through mitoK\textsubscript{ATP} channels will compensate for the decrease in K\textsuperscript{+} diffusion at the lower Δψ, so that matrix and intermembrane space volumes in

**Fig. 7.** Effects of AR activation on intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}],) in cultured cardiomyocytes. A: sodium azide (10 mM) induced transient accelerations of the beating rate, elevation of diastolic [Ca\textsuperscript{2+}], and termination of beating activity after 1.5–2 h of treatment. B: pretreatment of cultures with 100 nM CI-IB-MECA abolished [Ca\textsuperscript{2+}], elevation after treatment with azide and maintained myocyte contractility. C: averaged data obtained from six experiments. Treatment of cultured cardiac muscle cells with 10 mM NaN\textsubscript{3} led to elevation of baseline [Ca\textsuperscript{2+}], and disappearance of [Ca\textsuperscript{2+}], transient amplitude. CI-IB-MECA (100 nM) restricted elevation of baseline [Ca\textsuperscript{2+}], (*P < 0.05 vs. NaN\textsubscript{3} group; n = 18 cells) and maintained muscle cell contractility (**P < 0.01 vs. NaN\textsubscript{3} group; n = 18 cells). D: continuous monitoring of [Ca\textsuperscript{2+}], during hypoxia (Ar) in cultures pretreated with 1 mM NaN\textsubscript{3}. E: application of 100 nM CI-IB-MECA after increase of the basal level [Ca\textsuperscript{2+}], returned it to normal diastolic level, and beating activity was restored. F: A1R agonist CCPA (100 nM) in the same experiment was ineffective. G: application of CI-IB-MECA (100 nM) for 15 min before sodium azide application (NaN\textsubscript{3}) maintained contractile activity and Ca\textsuperscript{2+} oscillations during 40–60 min. H: protective effect of A1R agonist CCPA (100 nM) was observed during 15–20 min of hypoxia after application of 1 mM NaN\textsubscript{3}; I: averaged data obtained from six experiments. Exposure of cultured cardiomyocytes to hypoxia with 1 mM NaN\textsubscript{3} led at 40 min to elevation of baseline [Ca\textsuperscript{2+}], and disappearance of [Ca\textsuperscript{2+}], transient amplitude. Pretreatment with CI-IB-MECA restricted elevation of baseline [Ca\textsuperscript{2+}], (*P < 0.05 vs. NaN\textsubscript{3} group; n = 18 cells) and maintained muscle cell contractility (**P < 0.01 vs. NaN\textsubscript{3} group; n = 18 cells).
mitochondria are maintained (9). The proton pump establishes \( \Delta \psi \), and the terminal sequence of respiratory pump enzymes is critical for ATP production in a state of shortage of the final electron acceptors. However, as shown in this study, activation of the \( A_3Rs \) or \( A_2Rs \) or application of diazoxide does not cause essential dissipation of transmembrane \( \Delta \psi \). Most of the evidence for the involvement of mitoK\(_{ATP} \) channels is pharmacological, based on the selectivity of the openers or blockers for mitoK\(_{ATP} \) channels and their similarities to effects of AR activation. Our finding with diazoxide agrees with other observations (21, 22) showing that diazoxide protects cardiac myocytes during metabolic inhibition without causing mitochondrial depolarization. Moreover, Hanley et al. (11) have shown that diazoxide (100 \( \mu \)M) or the nonselective K\(_{ATP} \) channel opener pinacidil (100 \( \mu \)M) did not change \( \Delta \psi \) in isolated ventricular myocytes (11). They found that diazoxide dose dependently decreased succinate oxidation without affecting NADH oxidation, whereas pinacidil did not inhibit succinate oxidation but selectively inhibited NADH oxidation. Some authors (5, 11, 23, 32) have suggested that partial inhibition of electron transport may explain pharmacological preconditioning and thereby provide an alternative explanation for the preconditioning process (metabolic concept) without assuming the existence of mitoK\(_{ATP} \) channels. Downey et al. (4, 31) have found that most G\( _i \)-coupled receptors trigger protection through the mitoK\(_{ATP} \)-ROS path except for the ARs, which use some other as-yet-unidentified pathway and bypass the mitoK\(_{ATP} \)-ROS path.

The activity of K\(_{ATP} \) channels is tightly regulated by the metabolic state of the cell. The agents that interfere with ATP production via inhibition of energy metabolism are commonly used to activate K\(_{ATP} \) channels (12). Indeed, metabolic inhibition by sodium azide or cyanide has been reported to activate K\(_{ATP} \) channels in many cells including cardiac myocytes (16) and skeletal muscle (1). It is well known that cyanide or sodium azide inhibits oxidative phosphorylation via inhibition of cytochrome c oxidase, which is the final enzyme in the mitochondrial electron transport chain, and thereby results in a rapid depletion of ATP and leads to activation of K\(_{ATP} \) channels (12); however, sodium azide lacks the unfavorable characteristics associated with cyanide (3). It was shown (3) that sodium azide-treated myocytes (1 mM for 12–18 h) remain fully viable after removal of sodium azide from culture medium. Thus, under our conditions, the K\(_{ATP} \) channels were already opened. Therefore, diazoxide or AR activation could not act through modulating this channel activity. The efficiency of A1Rs while in a state of respiratory chain damage points to different or additional pathways of this receptor signaling. Recently, a similar effect was achieved in cardiomyocyte cultures treated with doxorubicin. Activation of the \( A_3 \) subtype but not the \( A_1 \) subtype of ARs attenuated doxorubicin-induced cardiotoxicity (40, 41). It is plausible that the cardioprotective effects of AR activation may also be mediated via activation of K\(_{ATP} \) channels if the A1Rs are similar to the A2Rs in signal transduction downstream of protein kinase C (27, 28). However, the affinity of A1Rs for adenosine is roughly two orders of magnitude lower than for A2Rs (48).

Another possibility is that dissipation of \( \Delta \psi \) decreases the driving force for Ca\(^{2+} \) influx through the Ca\(^{2+} \) uniporter (24). Prevention of Ca\(^{2+} \) accumulation in mitochondria may be a very important mechanism in the protection of mitochondrial structure and function and may be achieved not only through a decrease in mitochondrial energetics. It was shown in several publications that inhibition of Ca\(^{2+} \) influx into the cells by Ca\(^{2+} \) antagonists is beneficial for protecting the heart against mitochondrial disorders. Chen et al. (3) reported that in cultured neonatal rat cardiac myocytes, the Ca\(^{2+} \) antagonist nifedipine inhibited NaN\(_3\)-induced cardiac cell death. Protective effects against cellular and tissue damages induced by this drug were obtained with diltiazem and verapamil (30, 37). Recently, Inomata and Tanaka (13) have shown that Ca\(^{2+} \) antagonists of all groups may protect against NaN\(_3\)-induced cardiac cell death. We (42) have shown that A1R activation (and not A2A or A2AR activation) leads to an increase in cytosolic Ca\(^{2+} \) and its further extrusion. It was shown that extrusion of the elevated cytosolic Ca\(^{2+} \) was achieved via activation of sarcoplasmic reticulum (SR) Ca\(^{2+} \) reuptake and the sarcolemmal Na\(^+\)/Ca\(^{2+} \) exchanger. The increase in SR Ca\(^{2+} \) uptake and Na\(^+\)/Ca\(^{2+} \) exchanger Ca\(^{2+} \) efflux were sufficient not only for compensation of Ca\(^{2+} \) release from SR after A1R activation but also for effective prevention of extensive increase in intracellular Ca\(^{2+} \) and may provide a mechanism against cellular Ca\(^{2+} \) overload. It was shown that Ca\(^{2+} \) unloading of cultured cardiomyocytes after A1R activation is mainly achieved by Ca\(^{2+} \) uptake into the SR Ca\(^{2+} \) pool. We have shown that Ca\(^{2+} \)/calmodulin-dependent protein kinase II-dependent phosphorylation was the only mechanism for sarcoplasmic reticulum Ca\(^{2+} \)-ATPase 2a reactivation induced by A1R signaling (42). In this study, we added CI-IB-MECA or CCPA to cells with elevated [Ca\(^{2+} \)]\(_i\) after hypoxia and treatment with NaN\(_3\). CI-IB-MECA immediately decreased the [Ca\(^{2+} \)]\(_i\), toward diastolic control levels, whereas the A1R agonist was ineffective. This selective activation of A1Rs may be very important for prevention of irreversible damage in cardiomyocytes.

In cardiac myocytes, intracellular Ca\(^{2+} \) overload leads to activation of the proteolytic cleavage of some key cytoskeletal proteins and cell death (10). We showed that A1R signals to increase Ca\(^{2+} \) extrusion mechanisms, and this property allows prevention of the disorders in desmin cytoskeleton and maintenance of the contractile functions of cardiomyocytes after prolonged incubation in high extracellular Ca\(^{2+} \) concentration (42). Activation of both the A1 and A3 subtypes of the ARs can mimic the preventive effects of ischemic preconditioning, whereas the specific protective functions mediated by each receptor remain to be ascertained. It seems to be very important that protective effects of ARs may be achieved not only in intact cells but in cells where the terminal link of the mitochondrial respiratory chain is injured. For a long time, this state of cellular pathophysiology was considered to be “irreversible damage;” therefore, our findings concerning A1R signaling provide new insights into cellular adaptive properties. These properties of A3R signaling may be favorable in protecting heart muscle cells in many diseases accompanied by endotoxicemia (shock, hemorrhage, ischemia, coronary artery bypass surgery, and others), which promotes mitochondrial disorders. These results support our earlier observations that A1R activation protects cardiomyocytes treated with doxorubicin via inhibition of Ca\(^{2+} \) overload (40), and prevents cardiomyocyte death during incubation in high extracellular Ca\(^{2+} \) concentrations (42).
In a recent review, Kloner and Rezkalla (19) ask, “Cardiac protection during acute myocardial infarction: Where do we stand in 2004?” The authors point out that adenosine and AR agonists belong to those classes of pharmacological agents that show promise as adjunctive therapies. Our data establish that adenosine can mediate myocardial protection by acting on A1Rs and A2Rs. Activation of both receptors leads to beneficial effects on high-energy phosphate production and on preservation of mitochondrial integrity. However, the cascade of events involved in cardioprotection may also be distinct for A1R and A2R signaling, and this seems especially important for the development of effective pharmacological agents against ischemia.

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