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Protective roles of adenosine A_1, A_{2A}, and A_3 receptors in skeletal muscle ischemia and reperfusion injury

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Abstract

Although adenosine exerts cardio-and vasculoprotective effects, the roles and signaling mechanisms of different adenosine receptors in mediating skeletal muscle protection are not well understood. We used a mouse hindlimb ischemia-reperfusion model to delineate the function of three adenosine receptor subtypes. Adenosine A_3 receptor-selective agonist 2-chloro-N^6-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (Cl-IBMECA; 0.07 mg/kg ip) reduced skeletal muscle injury with a significant decrease in both Evans blue dye staining (5.4 ± 2.6%, $n = 8$ mice vs. vehicle-treated 28 ± 6%, $n = 7$ mice, $P < 0.05$) and serum creatine kinase level (1,840 ± 910 U/l, $n = 13$ vs. vehicle-treated 12,600 ± 3,300 U/l, $n = 14$, $P < 0.05$), an effect that was selectively blocked by an A_3 receptor antagonist 3-ethyl-5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1191; 0.05 mg/kg). The adenosine A_1 receptor agonist 2-chloro-N^6-cyclopentyladenosine (CCPA; 0.05 mg/kg) also exerted a cytoprotective effect, which was selectively blocked by the A_1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.2 mg/kg). The adenosine A_{2A} receptor agonist 2-*p*-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamidoadenosine (CGS-21680; 0.07 mg/kg)-induced decrease in skeletal muscle injury was selectively blocked by the A_{2A} antagonist 2-(2-furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e]

[1,2,4]triazolo[1,5-C]pyrimidin-5-amine (SCH-442416; 0.017 mg/ kg). The protection induced by the A_3 receptor was abrogated in phospholipase C-β2/β3 null mice, but the protection mediated by the A_1 or A_{2A} receptor remained unaffected in these animals. The adenosine A_3 receptor is a novel cytoprotective receptor that signals selectively via phospholipase C-β and represents a new target for ameliorating skeletal muscle injury.

Keywords

wild-type mice; creatine kinase; phosolipase C

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ISCHEMIA AND REPERFUSION can cause significant injury of skeletal muscle, which is the most vulnerable tissue in the extremities (5, 15). Trauma, autogenous skeletal muscle transplantation, surgical incision, vascular clamp application during vascular surgery or musculoskeletal reconstructive surgery, and sustained strenuous exertion can also induce skeletal muscle damage with deleterious systemic consequences (4, 5, 9). Protection of skeletal muscle from ischemia and reperfusion injury is therefore an important therapeutic goal. Although various measures, including a tissue-preserving solution and cold immersion, are used to preserve intact organs and skeletal muscle (18, 34, 38), a more effective method or pharmacological agent to protect skeletal muscle from ischemia-reperfusion injury is needed. Ischemic preconditioning can provide potent protection of the heart (27, 39) as well as the skeletal muscles (7, 8) from ischemia-reperfusion injury. As with cytoprotection of the heart, extracellular adenosine is implicated in mediating the protective effect of preconditioning in skeletal muscle (7, 8, 28). Direct infusion of adenosine can mimic the effect of preconditioning in reducing skeletal muscle injury. Adenosine is an important regulatory agent that exerts its cytoprotective effect via activation of four G protein-coupled receptors: A_1, A_{2A}, A_{2B}, and A_3 subtypes.

N^6-(R-phenyl-2-propyl)adenosine (R-PIA), an adenosine A_1 receptor agonist of low selectivity, exerted an anti-ischemic effect in a pig latissimus dorsi muscle flap model (28). The adenosine A_1 receptor-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) blocked the protection by adenosine in this model. Although these data suggested a role for the adenosine A_1 receptor in mediating protection against ischemia-reperfusion injury in skeletal muscle, it is not known whether other adenosine receptor subtypes also protect skeletal muscle. Activation of the adenosine A_3 receptor has been shown to protect the myocardium against ischemia and reperfusion injury (2, 24, 26). However, the activation of the adenosine A_3 receptor expressed in rodent mast cells stimulates inflammation (30, 37) with a potentially deleterious effect on skeletal muscle. A systematic investigation of the cytoprotective role of adenosine A_1, A_{2A}, and A_3 receptors in skeletal muscle is needed, along with a genetic approach and a detailed pharmacological characterization of selective agonists and antagonists.

Our objective was to define the function of various adenosine receptor subtypes in skeletal muscle ischemia and reperfusion injury. We used a mouse hindlimb ischemia-reperfusion injury model and demonstrated for the first time a novel anti-ischemic cytoprotective role of the adenosine A_3 receptor. A detailed and specific pharmacological characterization of both A_1 and A_{2A} receptors was also carried out. We used phospholipase C (PLC)-β2/β3 knockout mice to determine the signaling role of this enzyme in mediating the cytoprotective role of the adenosine receptor subtypes.

MATERIALS AND METHODS

Mouse hindlimb ischemia and reperfusion model.

After 2.5- to mo-old wild-type (WT; C57BL6 strain) or PLC-β2/β3 knockout(in C57BL6 background) mice, each weighing ~ 23–25 g, were sedated with anesthetic (pentobarbital sodium, 50 mg/kg ip), their right or left hindlimbs (used randomly) were elevated briefly to minimize retained blood before being subjected to ischemia as previously described (1). Ischemia was induced by placement of a constrictor band (Latex O-Rings, Miltex Instruments, York, PA) above the greater trochanter with a McGiveney Hemorrhoidal Ligator (7 in. long; Miltex) according to a modification of a previously described method (1, 11). After 90 min of warm ischemia at 37°C, the constrictor was removed to allow reperfusion for 24 h. The mice were continuously maintained on a 37°C warming pad (Physitemp Instruments, Clifton, NJ) during the reperfusion. After the mice were euthanized by anesthetic overdose, the gastrocnemius muscle was quickly frozen, cut into three slices separated by 2 to 3 mm, and embedded in Shandon Cryomatrix (10% polyvinyl alcohol and 4% polyethylene glycol; Anatomical Pathology, Pittsburgh, PA). Each slice was processed as one 10-μm section on a Thermo Electron/Shandon Cryotome (Anatomical Pathology), fixed in ice-cold acetone, air dried, and washed in phosphate-buffered saline (PBS). Each 10-μm section had seven fields. Gastrocnemius was used because of its high proportion of fast-twitch muscle, which is prone to ischemia and reperfusion injury (1, 11).

Quantification of skeletal muscle injury.

Evans blue dye (EBD), prepared as a 1% wt/vol solution to yielding 1 mg of EBD/10 g body wt, was given via a separate intraperitoneal injection 2.5 h before the induction of ischemia. EBD stained only injured muscle, and EBD-positive cells were quantified according to a previously described method (14). The percentage of EBD-positive cells in each field was averaged with those from all seven fields within one 10-μm section. The averaged fraction of EBD-positive cells in each 10-μm section was similar among the three sections. Each 10-μm section was also stained with rabbit polyclonal anti-skeletal muscle actin antibodies (ab15265; Abcam, Cambridge, MA) and goat polyclonal anti-rabbit IgG conjugated with fluorescein isothiocyanate. Sections were mounted, and their cross sections were viewed with fluorescent microscopy (EBD-positive cells via a DM580 band-pass filter 510–560 nm with emission of 590 nm; fluorescein isothiocyanate cells via a DM510 filter of 450–490 nm with emission at 520 nm). Each field was counted at ×20 magnification, and their images were captured via the two filters for quantification of muscle injury as follows. Images were acquired and stored as. jpeg files with a Macrofire camera (Macrofire 1.0, Optronics, Goleta, CA). The ImageProPlus Program (version 5.0, Media Cybernetics, Silver Spring, MD) was used for the quantitative determination (31). The percentage of EBD-positive areas was calculated by dividing the area of EBD staining by the total muscle cells, a quantity defined as the total area stained by anti-skeletal muscle actin antibodies and was identical to the total area in each field minus the area not occupied by any cell. The actin-stained area included the EBD-positive area, as shown in superimposed EBD- and actin antibody-stained images. Serum creatine kinase (CK) activity was measured with a previously described procedure (13). The fraction of skeletal muscle staining positive for EBD represented a direct determination of the muscle that was injured, and the serum CK level provided a circulating index of the extent of skeletal muscle injury. Both are established and accepted methods to quantify skeletal muscle injury.

Protocol for administration of adenosine receptor agonist and antagonist.

Adenosine receptor agonists [0.07 mg/kg for 2-chloro-*N*^{6}-(3-iodobenzyl)adenosine-5’-*N*-methyluronamide (Cl-IBMECA) and 2-*p*-(2-carboxyethyl)phenethylamino-5’-*N*-

ethylcarboxamidoadenosine (CGS-21680), 0.05 mg/kg for 2-chloro-N^6-cyclopentyladenosine (CCPA) and R-PIA], antagonists {0.2 mg/kg for DPCPX, 0.05 mg/kg for 3-ethyl-5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1, 4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1191), 0.017 mg/kg for 2-(2-furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo [4,3-e][1,2,4]triazolo[1,5-C]pyrimidin-5-amine (SCH-442416)}, or vehicle (0.1% DMSO in PBS) was administered in a sterile 0.1-ml volume by intraperitoneal injection 2 h before induction rather than at the onset of ischemia. This protocol allowed time for the absorption of adenosine ligands and for their presence in the circulation before the beginning of ischemia. Previous studies demonstrated that intraperitoneal injection of similar doses of adenosine receptor agonists produced potent pharmacological myocardial protection in the mouse (40, 42). Similar intraperitoneal doses of adenosine receptor antagonists (MRS-1191 and DPCPX) were also given, and only MRS-1191 blocked the myocardial protection afforded by the A_3 receptor agonist Cl-IBMECA (41). When both antagonist and agonist were administered, the antagonist was given 30 min before the agonist. Unless otherwise indicated, data are shown as means ± SE. One-way ANOVA followed by posttest Newman-Keuls comparison was used to analyze the statistical significance of differences in more than two groups. $P < 0.05$ was considered statistically significant.

Materials and chemicals.

The adenosine receptor ligands DPCPX, MRS-1191, CGS-21680, CCPA, Cl-IBMECA, and R-PIA were obtained from Sigma Chemicals (St. Louis, MO). The adenosine A_{2A} receptor antagonist SCH-442416 was from Tocris Bioscience (Ellisville, MO).

PLC-β2/β3-deficient mice.

PLC-β2/β3 null mice were bred as previously described (21). C57BL6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for research and approved by the Institutional Animal Care and Use Committee of the University of Connecticut Health Center.

RESULTS

Role of adenosine A_1 receptors in anti-ischemic skeletal muscle protection.

Ischemia followed by reperfusion resulted in significant limb skeletal muscle injury in PBS vehicle-treated mice. The extent of injury was quantified by an increase in the EBD staining of the skeletal myocytes (Fig. 1, *A* and *B*). The fraction of total skeletal muscle cross sections that stained positive for EBD was 28 ± 6% (n = 7 mice, means ± SE, Fig. 1*B*). Administration of the relatively nonselective adenosine receptor agonist R-PIA before ischemia and reperfusion caused a significant reduction in the extent of injury (data not shown). To elucidate the cytoprotective role of different adenosine receptor subtypes, we found that a highly A_1 receptor-selective agonist, CCPA, induced a large decrease in the extent of muscle injury (Fig. 1*C*; EBD-positive area in CCPA-treated mice: 10.8 ± 2%, n = 12 mice vs. PBS vehicle-treated mice: 28 ± 6%, n = 7 mice, $P < 0.05$). The A_1 receptor-selective antagonist DPCPX completely abrogated the cytoprotective response to CCPA

(Fig. 1*C*; DPCPX and CCPA treatment: 22 ± 3.3%, $n = 9$ mice, $P < 0.05$ vs. CCPA treatment). The adenosine A_3 receptor-selective antagonist MRS-1191 did not affect CCPA-mediated protection (Fig. 1*C*; MRS-1191 and CCPA treatment: 12.2 ± 2.4%, $n = 23$ mice, $P > 0.05$ vs. CCPA treatment), indicating that the adenosine A_1 receptor protects skeletal muscle from ischemia-reperfusion injury. The contralateral limb not subjected to ischemia-reperfusion showed no EBD staining (data not shown), indicating an absence of muscle injury in the nonischemic limb.

A novel anti-ischemic protective role of adenosine A_3 receptors in skeletal muscle.

Our data demonstrated that the A_3 receptor agonist Cl-IBMECA induced a significant reduction in EBD-positive cells (Fig. 2*A*; Cl-IBMECA treatment: 5.4 ± 2.6%, $n = 8$ mice, $P < 0.05$ vs. PBS treatment). This reduction was sensitive to antagonism by MRS-1191 (Fig. 2*A*; MRS-1191 and Cl-IBMECA treatment: 21.5 ± 3.5%, $n = 22$ mice, $P < 0.05$ vs. Cl-IBMECA treatment) but not by DPCPX (Fig. 2*A*; DPCPX and Cl-IBMECA treatment: 4 ± 1.6%, $n = 9$ mice, $P > 0.05$ vs. Cl-IBMECA treatment). Neither MRS-1191 (23 ± 4.4%, $n = 15$ mice) nor DPCPX (19.3 ± 4.4%, $n = 8$ mice) alone had any effect on the extent of ischemia-reperfusion-induced skeletal muscle injury.

Mice pretreated with the adenosine A_{2A} receptor-selective agonist CGS-21680 showed reduced muscle injury compared with PBS vehicle-treated animals (6.6 ± 3.5%, $n = 9$ mice, $P < 0.05$ vs. PBS treatment; Fig. 2*A*). The protective effect of CGS-21680 was attenuated by DPCPX (DPCPX and CGS-21680 treatment: 14.7 ± 2.3%, $n = 10$ mice, $P > 0.05$ vs. DPCPX alone). The A_3 antagonist MRS-1191 could not inhibit the CGS-21680-induced skeletal muscle protection (MRS-1191 and CGS-21680 treatment: 2.4 ± 1.25%, $n = 8$ mice, $P < 0.05$ vs. MRS-1191 alone). The adenosine A_{2A} receptor-selective antagonist SCH-442416 completely abrogated the CGS-21680-induced protection (Fig. 2*A*). Animals treated with SCH-442416 and CGS-21680 showed significantly larger EBD-positive areas (26 ± 4%, $n = 5$ mice, means ± SE) than mice treated with CGS-21680 alone ($P < 0.05$).

We measured CK levels as another method to quantify skeletal muscle injury induced by ischemia and reperfusion. Cl-IBMECA, CGS-21680, and CCPA were able to reduce these levels when each agonist was administered individually before ischemia and reperfusion (Fig. 2*B*). CK in Cl-IBMECA-treated mice was 1,840 ± 910 U/L, $n = 13$ mice. In CCPA-treated mice, CK was 2,340 ± 710 U/L, $n = 11$ mice. CK in CGS-21680-treated mice was 838 ± 243 U/L, $n = 10$ mice ($P < 0.05$ for any agonist vs. vehicle-treated mice, which had a serum CK level of 12,600 ± 3,300 U/L, $n = 14$ mice). The protection against CK release induced by A_1, A_{2A}, or A_3 receptors was blocked by an antagonist of each adenosine receptor subtype. In mice treated with MRS-1191 and Cl-IBMECA, serum CK was 14,400 ± 2,900 U/L ($n = 15$ mice, $P < 0.05$ vs. Cl-IBMECA alone). Serum CK in mice treated with DPCPX plus CCPA was 11,300 ± 2,200 U/L ($n = 9$ mice, $P < 0.05$ vs. CCPA alone). CK in mice treated with SCH-442416 plus CGS-21680 was 15,180 ± 4,420 U/L, $n = 8$ mice. The data obtained on serum CK activity, derived from the same method of agonist and antagonist administration in the same ischemia-reperfusion injury model, complement those obtained through EBD staining.

Specificity of protection induced by each adenosine receptor subtype.

The specificity by which each adenosine receptor agonist mediates its protective response was further illustrated by testing the effect of the agonist in PLC-β2/β3 null mice. Cl-IBMECA-induced protection was completely abrogated in PLC-β2/β3 null mice (Cl-IBMECA treatment: 22 ± 3.6%, $n = 8$ mice, $P > 0.05$ vs. PBS treatment: 23.4 ± 4%, $n = 9$ mice; Fig. 3, *A*-*C*), whereas protection induced by CCPA was unaffected (CCPA treatment: 8.5 ± 2.5%, $n = 10$ mice, $P < 0.05$ vs. PBS treatment). PLC-β2/β3 deficiency alone had no effect on ischemia-reperfusion-induced skeletal muscle injury (PBS vehicle-treated WT mice had 28 ± 6% EBD-positive area, $n = 7$; PLC-β2/β3 null mice had 23.4 ± 4% EBD-positive area, $n = 9$, $P > 0.05$; Figs. 1*C* and 3*C*, respectively).

The A_{2A} agonist CGS-21680 was also able to cause cytoprotection in PLC-β2/β3 null mice (CGS-21680 treatment: 4 ± 1.1%, $n = 6$ PLC-β2/β3 null mice, $P < 0.05$ vs. PBS treatment: 23.4 ± 4%, $n = 9$ PLC-β2/β3 null mice; Fig. 3*C*). Because the A_{2A} receptor is coupled to stimulation of adenylyl cyclase activity and cAMP accumulation, it was not unexpected that the absence of PLC-β2/β3 had no effect on the cytoprotective effect of the A_{2A} receptor. The cytoprotective action of adenosine A_{2A} receptors in skeletal muscle is independent of and separate from the salutary effect mediated by adenosine A_1 or A_3 receptors in that tissue.

DISCUSSION

Ischemia and reperfusion of the skeletal muscle can cause significant injury with deleterious consequences. Effective therapies that reduce such injury will have significant benefits in treatment of trauma, autogenous skeletal muscle transplantation, and vascular and musculoskeletal reconstructive surgery. As with anti-ischemic myocardial protection, adenosine and its receptors have been implicated in protecting the skeletal muscle against ischemia and reperfusion injury. The present study demonstrated for the first time that the adenosine A_3 receptor can induce potent cytoprotection of the skeletal muscle against ischemia and reperfusion injury. The adenosine A_3 receptor, but not the A_1 or A_{2A} receptor, signals via PLC-β2/β3 to achieve its skeletal muscle protective effect.

Several lines of evidence clearly delineate the cytoprotective role of adenosine A_1, A_{2A}, and A_3 receptors. The highly A_1 receptor-selective agonist CCPA decreased skeletal muscle ischemia and reperfusion injury. The protective effect was blocked only by the A_1 receptor-selective antagonist DPCPX but not by the A_3 receptor-selective antagonist MRS-1191. Conversely, the A_3 receptor-selective agonist Cl-IBMECA reduced skeletal muscle injury, and this protective effect, although insensitive to blockade by the A_1 receptor antagonist DPCPX, was completely abrogated by the A_3 receptor antagonist MRS-1191, which was shown to antagonize the adenosine A_3 receptor in mice (3, 38, 39). Similarly, the cytoprotective effect of A_{2A} receptor agonist CGS-21680 was selectively blocked by its antagonist SCH-442416 and was insensitive to the A_3 antagonist MRS-1191. The A_1 antagonist DPCPX, at the current dosage, was able to attenuate the CGS-21680-induced skeletal muscle protection. Several explanations are possible. Given the interaction between A_1 and A_{2A} receptors, it is possible that A_{2A} receptor-mediated effect could be potentiated by A_1 receptor activation. A positive interaction between adenosine A_1 and A_{2A} receptors was recently demonstrated in rat heart (23). Since DPCPX could inhibit the protective effect of CGS-21680 in the current skeletal muscle ischemia-reperfusion injury model, it is possible that A_1 receptor activation contributed to the CGS-21680-induced skeletal muscle protection. Recent evidence suggests that protein kinase C (PKC) activation can potentiate the adenosine A_{2B} receptor signaling during reperfusion in the heart (22). Thus another possible explanation is that PKC activation, induced by adenosine A_1 receptor, may also increase the responsiveness of the adenosine A_{2A} receptor signaling during reperfusion in skeletal muscle. Overall, the present data provided detailed characterization of antagonists and agonists associated with A_1, A_{2A}, and A_3 receptors in the current in vivo model of skeletal muscle ischemia-reperfusion injury. The study confirmed their selectivity at each adenosine receptor subtype and indicates that the cytoprotection afforded by each receptor agonist was due to activation of that specific receptor.

PLC-β2/β3 deficiency selectively abrogated the protective effect of A_3 receptor agonist Cl-IBMECA and had no effect on CCPA- or CGS-21680-induced protection. It was unlikely that PLC-β2/β3 deficiency affected the bioavailability or pharmacokinetic properties of Cl-IBMECA for the following reason. CCPA and CGS-21680 have similar size and molecular weights as Cl-IBMECA. Both CCPA and CGS-21680 were fully capable of protecting against skeletal muscle injury in the PLC-β2/β3 knockout mice. In WT mice, CGS-21680 induced anti-ischemic skeletal muscle protection in a manner that was insensitive to blockade by MRS-1191 but was completely abolished by the A_{2A} receptor-selective antagonist SCH-442416. The protective effect of CGS-21680 remained unaffected and intact in PLC-β2/β3 null mice. The exact bioavailability or pharmacokinetic property of Cl-IBMECA in WT or PLC-β knockout mice remains to be determined.

Although PLC-β2/β3 is not involved in mediating the protective effect of A_1 or A_{2A} receptors in the skeletal muscle, ATP-sensitive K^+ channels appear to be an important effector mechanism for the anti-ischemic effect of the A_1 receptor (28). The adenosine A_{2A} receptor serves an important nonredundant role in suppressing immune and lymphoid cells and thus in protecting against inflammatory tissue damage (25, 32, 33). Since activation of adenosine A_{2A} receptors on $CD4^+$ T cells mediated potent protection against renal ischemia-reperfusion injury (12), it is possible that the same mechanism is also responsible for its protection in the skeletal muscle in vivo. Activation of the A_3 receptor in rodent immune cells such as mast cells is proinflammatory and may be damaging (10, 20). A genetic absence or antagonism of adenosine A_3 receptors augmented an increase in coronary flow or hypotension mediated by adenosine or an A_{2A} receptor agonist (36, 43), pointing to a vasoconstrictive role of the vascular A_3 receptor. Activated mast cells and neutrophils mediate skeletal muscle ischemia-reperfusion injury (6, 16, 17). The present data could not determine whether the adenosine A_3 receptor-mediated protection was due to direct activation of A_3 receptors on the skeletal muscle or the result of an anti-inflammatory action of A_3 receptors on the immune cells. EBD is a dye that accumulates in injured tissues as a result of an increase in vascular permeability (35) and a disruption of sarcolemmal integrity of the tissue (such as muscle) supplied by the vasculature. It is possible that a decrease in EBD staining was due to a decrease in vascular permeability induced by one or all of the adenosine receptor subtypes studied. Differentiating a protective effect of adenosine receptor subtypes at the levels of vasculature, circulating immune cells, and skeletal muscle is needed. Bone marrow transplant from adenosine receptor knockout mice, or possible creation of vascular or skeletal muscle specific knockout of adenosine receptor subtypes, would provide a more definitive answer to this question.

That the adenosine A_3 receptor exerts a potent cytoprotective effect in mouse skeletal muscle is consistent with its cardioprotective action in the mouse heart (19). Although PLC is currently shown to have an important role in mediating the A_3 effect in skeletal muscle, a previous study suggested that PLD, but not PLC, mediated the cardioprotective effect of adenosine A_3 receptors in chick embryo ventricular myocytes (29). The reasons for this apparent difference are not clear; however, several plausible explanations are offered. First, species and age differences (chick embryos vs. adult mouse) may be important. Second, the coupling of adenosine A_3 receptors to PLC vs. PLD may be different in skeletal than in cardiac muscles. Third, our skeletal muscle ischemia-reperfusion injury preparation was an in vivo and intact animal model, whereas the model used by Parsons et al. (29) was an isolated cell culture model. The present gene knockout approach rendered all cells completely deficient in PLC-β2/β3, including skeletal muscle and circulating immune cells capable of mediating anti-and proinflammatory. It is possible that PLC mediated an anti-inflammatory effect of A_3 receptors on circulating immune cells. In this scenario, knockout of PLC would eliminate the anti-inflammatory effect of A_3 receptors on immune cells and thus abrogated their cytoprotective effect on skeletal muscles.

The combined use of receptor pharmacological tools and a gene ablation approach delineated, for the first time, a distinct anti-ischemic protective role of adenosine A_1, A_{2A}, and A_3 receptor subtypes in skeletal muscle. Although both adenosine A_1 and A_{2A} receptors have shown anti-ischemic protective properties, agonists at either receptor caused pronounced decreases in blood pressure or heart rate. Our data provide convincing evidence that the adenosine A_3 receptor is a novel cytoprotective receptor in skeletal muscle. Because the A_3 receptor agonist is not associated with cardiac or hemodynamic depression (2), the A_3 receptor represents a potential therapeutic target because of its ability to ameliorate skeletal muscle injury.

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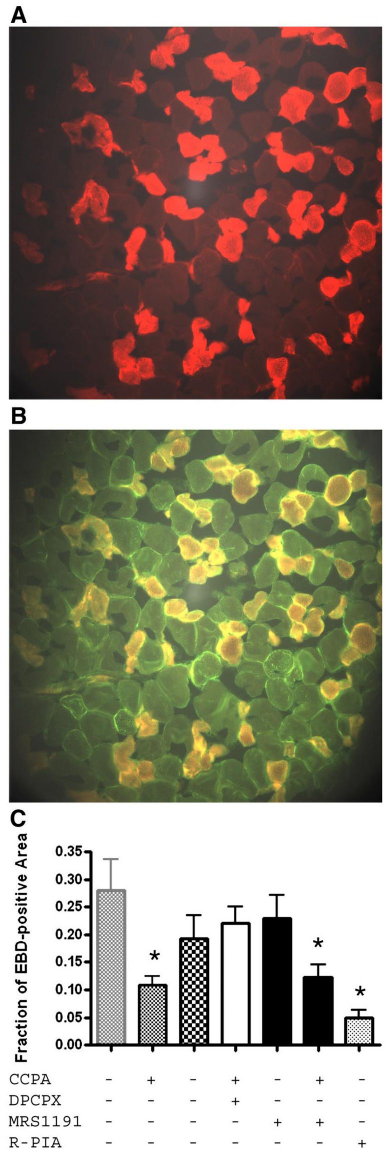

Fig. 1.

Cytoprotective action of adenosine in a quantitative model of mouse hindlimb ischemia-reperfusion (I/R) injury. Adult wild-type (WT) mice were injected with various adenosine ligands, they were subjected to I/R injury, and their skeletal muscle injuries were quantified as described in MATERIALS AND METHODS. A: extent of Evans Blue dye (EBD) staining is shown in mice treated with vehicle (0.1% DMSO in phosphate-buffered saline, pH 7.4, *n* = 7 mice). The contralateral leg not subjected to I/R showed no EBD uptake. *B*: the same section was stained with rabbit polyclonal anti-skeletal muscle actin antibodies followed by staining with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate. *C*: 2-chloro-N^6-cyclopentyladenosine (CCPA) decreased the percentage of EBD-positive area (*n* = 12 mice); this reduction was reversed in mice injected with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) before CCPA treatment (*n* = 9 mice). Effect of 3-ethyl-5-benzyl-2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS-1191) on CCPA-induced decrease in EBD staining is also shown (*n* = 23 mice). Average EBD staining (means ± SE) of skeletal muscle sections following treatment with vehicle or adenosine ligand were quantified by blinded observers (*n* = 8 mice for DPCPX alone and 15 mice for MRS-1191 alone). $^*P < 0.05$ for CCPA or MRS-1191 plus CCPA or N^6-(R-phenyl-2-propyl)adenosine (R-PIA) vs. any of the following groups: treatment with PBS, DPCPX, MRS-1191, or CCPA plus DPCPX (Newman-Keuls posttest comparison). $P > 0.05$ for any comparison between CCPA, MRS-1191 plus CCPA, and R-PIA.

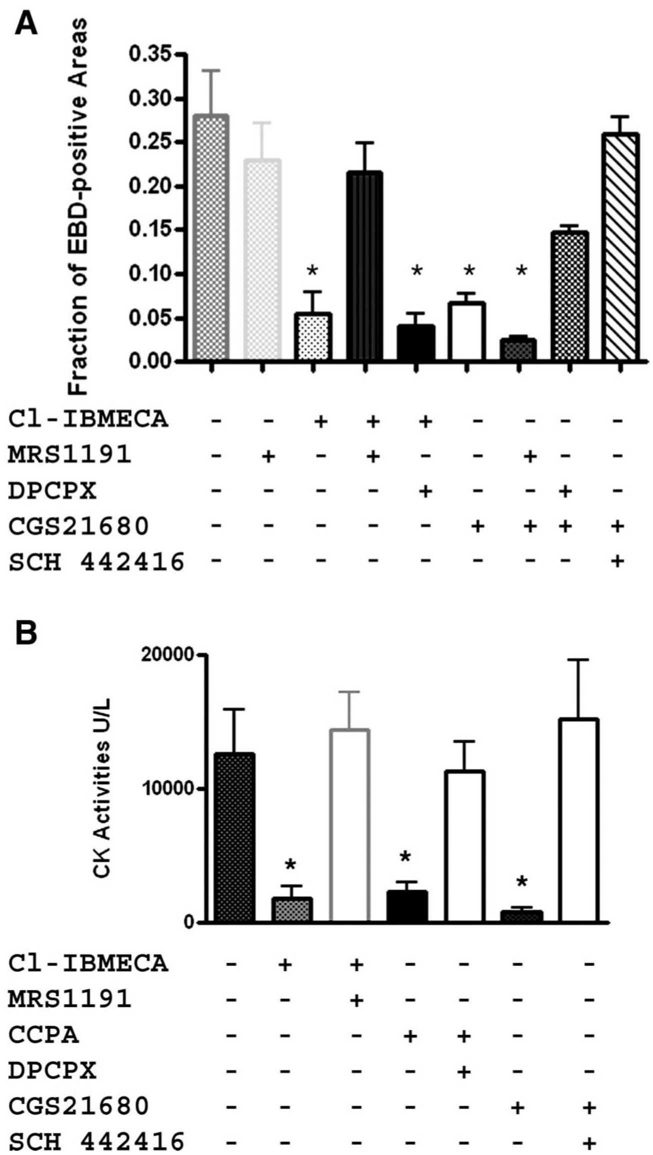

Fig. 2.

Adenosine A_{2A} and A_3 receptors mediate a potent anti-ischemic protective response distinct from that mediated by the A_1 receptor. Adult WT mice were injected with various adenosine ligands, subjected to I/R injury, and their skeletal muscle injuries were quantified as described in Fig. 1. *A*: effects on EBD staining following treatment with 2-chloro-N^6-(3-iodobenzyl) adenosine-5'-*N*-methyluronamide (Cl-IBMECA) or 2-*p*-(2-carboxyethyl)phenethylamino-5'-*N*-ethylcarboxamidoadenosine (CGS-21680) in the presence or the absence of DPCPX, 2-(2-furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-C]pyrimidin-5-amine (SCH-442416) or MRS-1191 were determined at the indicated combinations. Average EBD staining (means ± SE) of skeletal muscle sections following treatment with vehicle or adenosine ligand is shown. $P > 0.05$ for comparison between any pair of the following groups: Cl-IBMECA ($n = 8$ mice), DPCPX plus Cl-IBMECA ($n = 9$), CGS-21680 ($n = 9$), CGS-21680 plus MRS-1191 ($n = 8$), or

CGS-21680 plus DPCPX ($n = 10$). *$P < 0.05$ for Cl-IBMECA, DPCPX plus Cl-IBMECA, CGS-21680, or CGS-21680 plus MRS-1191 vs. any of the following groups: PBS, MRS-1191 (15 mice), CGS-21680 plus SCH-442416 ($n = 5$ mice), or MRS-1191 plus Cl-IBMECA ($n = 22$) in a Newman-Keuls posttest comparison. *B*: adenosine receptor agonists and antagonists were administered as in the experiments described in Fig. 1. Creatine kinase (CK) was used as a marker of muscle injury. A_3 receptor agonist Cl-IBMECA caused a reduction in the serum CK, as did the A_1 receptor agonist CCPA or the A_{2A} receptor agonist CGS-21680 (one-way ANOVA and posttest Newman-Keuls comparison, *$P < 0.05$ for Cl-IBMECA, CCPA, or CGS-21680 vs. any of the others). Data are means ± SE of 14 mice (PBS), 13 mice (Cl-IBMECA), 15 mice (MRS-1191 plus Cl-IBMECA), 9 mice (DPCPX plus CCPA), 11 mice (CCPA), 10 mice (CGS-21680), and 8 mice (CGS-21680 plus SCH-442416).

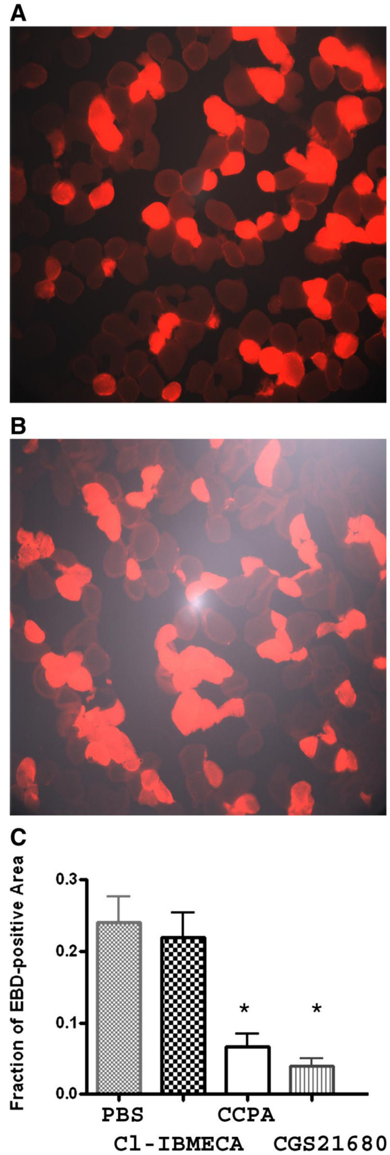

Fig. 3.

The adenosine A_3 receptor signals through PLC-β2/β3 to cause its anti-ischemic skeletal muscle protective response. Adult PLC-β2/β3 null mice were injected with vehicle ($n = 9$ mice), Cl-IBMECA ($n = 8$ mice), CGS-21680 ($n = 6$ mice), or CCPA ($n = 10$ mice); they were subjected to I/R, and the extent of skeletal muscle injury was subsequently quantified as in WT mice. In PLC-β2/β3 null mice not subjected to I/R, skeletal muscles did not show any EBD staining. A: a typical EBD staining in a vehicle-injected PLC-β2/β3 null mouse following I/R is shown. The extent of EBD staining was similar to that obtained in vehicle-injected WT mice after I/R (see Fig. 1*C*, $P > 0.05$). *B*: a representative EBD staining in a Cl-IBMECA-treated mouse is shown. *C*: treatment with CCPA or CGS-21680 reduced EBD staining, but Cl-IBMECA did not. Average EBD staining (means ± SE) of skeletal muscle sections from PLC-β2/β3 null mice following treatment with vehicle or adenosine ligand is shown. *$P < 0.05$, CCPA, CGS-21680 vs. either PBS or Cl-IBMECA. $P > 0.05$, PBS vs. Cl-IBMECA; $P > 0.05$, CCPA vs. CGS-21680.