A Novel Pharmacological Approach to Treating Cardiac Ischemia

BINARY CONJUGATES OF A1 AND A3 ADENOSINE RECEPTOR AGONISTS*

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Kenneth A. Jacobson‡§, Rongyuan Xie‡, Laura Young‡, Louis Chang‡, and Bruce T. Liang‡

From the ‡Molecular Recognition Section, Laboratory of Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda, Maryland 20892 and the †Department of Medicine, Cardiovascular Division and Department of Pharmacology, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania 19104

Adenosine released during cardiac ischemia exerts a potent, protective effect in the heart via activation of A1 or A3 receptors. However, the interaction between the two cardioprotective adenosine receptors and the question of which receptor is the more important anti-ischemic receptor remain largely unexplored. The objective of this study was to test the hypothesis that activation of both receptors exerted a cardioprotective effect that was significantly greater than activation of either receptor individually. This was accomplished by using a novel design in which new binary conjugates of adenosine A1 and A3 receptor agonists were synthesized and tested in a novel cardiac myocyte model of adenosine-elicited cardioprotection. Binary drugs having mixed selectivity for both A1 and A3 receptors were created through the covalent linking of functionalized congeners of adenosine agonists, each being selective for either the A1 or A3 receptor subtype. MRS 1740 and MRS 1741, thiourea-linked, regioisomers of a binary conjugate, were highly potent and selective in radioligand binding assays for A1 and A3 receptors (Kd values of 0.7–3.5 nM) versus A2A receptors. The myocyte models utilized cultured chick embryo cells, either ventricular cells expressing native adenosine A1 and A3 receptors, or engineered atrial cells, in which either human A3 receptors alone or both human A1 and A3 receptors were expressed. The binary agonist MRS 1741 coactivated A1 and A3 receptors simultaneously, with full cardioprotection (EC50 = 0.1 nM) dependent on expression of both receptors. Thus, co-activation of both adenosine A1 and A3 receptors by the binary A1/A3 agonists represents a novel general cardioprotective approach for the treatment of myocardial ischemia.

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‡ To whom correspondence should be addressed: Molecular Recognition Section, Bldg. 5A, Rm. B1A-19, Laboratory of Bioorganic Chemistry, NIDDK, NIH, Bethesda, MD 20892-0810. Tel.: 301-496-9024; Fax: 301-480-8422; E-mail: kajacobson@helix.nih.gov.

Isolated heart, by exposure to adenosine agonists selective for either A1 or A3 adenosine receptor subtypes, each of which causes a reduction in damage comparable to that induced by prior exposure to ischemia (2–5).

Although both A1 and A3 receptors can mediate cardioprotection (5–9), the specific mechanism of protection elicited by each receptor appears to be distinct and associated with activation of either phospholipase C or phospholipase D, respectively (10). Furthermore, the co-activation of A1 and A3 receptors may induce a greater protection than activation of each receptor individually. Thus, the protection offered by A1 and A3 receptors is not redundant, and in fact the receptors may act together to produce an additive protection (10).

The additivity of protection offered by A1 and A3 receptor activation has suggested to us that a single agonist capable of activating both A1 and A3, but not A2A, receptors (6), might represent a new class of highly effective cardioprotective agents. Activation of A2A receptors has been shown to increase myocyte death in ischemia (6). Because most of the effort in developing potent adenosine agonists has been aimed at pure subtype selectivity (13), it was not immediately obvious which derivatives of adenosine might be utilized to test this concept. A novel means of achieving mixed selectivity for A1 and A3 adenosine receptors was to covalently link different functionalized congeners of adenosine (14), each of which has agonist selectivity for one of the desired subtypes. The “binary drug” approach based on chemically functionalized congeners (15) has been utilized in synthesis of combinations of adenosine receptor ligands and biologically active peptides, specifically neurokinin agonists.

Using this novel binary conjugate design for adenosine receptor agonists, as well as a newly developed cardiac myocyte model for adenosine-elicited cardioprotection, our objective was to test the novel concept that simultaneous activation of both A1 and A3 receptors exerted a cardioprotective effect that was significantly more potent than the result of activating either receptor individually. The rationale was that it may be possible to achieve tissue selectivity, such that a biological effect would only be observed in cells that have both receptor subtypes.

The models utilized native adenosine A1 and A3 receptors in cultured chick embryo ventricular cells, which exhibited the adenosine-elicited cardioprotection characteristic of that found in the intact heart (3, 5–9, 11, 12), or recombinant human receptors expressed in chick atrial myocytes, in which native A1 receptors were inactivated with a known irreversible antagonist (16). The latter resulted in a recombinant cardiac myocyte model in which the human adenosine receptors were coupled to a well-characterized cardiac cellular response. This study showed that the protective effect mediated by co-activation of both A1 and A3 receptors by a single compound was significantly more potent than that produced by activation of either
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receptor individually. Full protection required stimulation of both receptors and occurred within a therapeutic dose window in which only cells expressing both showed this highly potent response.

**EXPERIMENTAL PROCEDURES**

**Materials**

8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), N 6-[3-(iodobenzyl)adenosine-5'-N'-methylthionamide (IB-MECA), and 3-ethyl-5-benzyl-2-methyl-6-phenyl-4-phenylethyl-1,4(2H)-dipyridinylamine-3,5-dicarboxylate (MRS 1191) were from Research Biochemicals International (Natick, MA). Full-length cDNAs encoding the human adenosine A 1 and A 3 receptors were kindly provided by M. Atkinson, A. Townsend-Nicholson, and P. R. Schofield (Garvan Medical Institute, Sydney, Australia) and were subcloned in the vector pcDNA3 as pcDNA3/hA 1 R and pcDNA3/hA 3 R. Adenosine was obtained from Sigma Chemical Co. (St. Louis, MO). The pcDNA3 vector was obtained from Invitrogen (Carlsbad, CA). Embryonated chick eggs were from Spafas, Inc. (Storrs, CT).

**Synthesis**

The adenosine agonist N 6-[4-(carboxylmethyl)phenyl]adenosine (4) was prepared by our previous method (17). All other materials were obtained from commercial sources. Proton nuclear magnetic resonance (1H NMR) and mass spectrometry were performed in a Finnigan 4600 mass spectrometer, and electron-impact mass spectrometry was performed with a VG7070F mass spectrometer, and chemical shifts (δ) relative to tetramethylsilane are given. Chemical-ionization mass spectrometry was performed with a Finnigan VG7070F mass spectrometer at 6 kV.

**Preparation of the Binary Ligands**

8a (MRS 1740) and 8b (MRS 1741) by Addition of DITC Derivatives (6a and 6b) to ADAC

ADAC, 7 (5.8 mg, 0.01 mmol (16)) was added to a solution of 6a or 6b (0.01 mmol) in 0.2 ml of anhydrous DMF, and the mixture was stirred in a sealed tube under nitrogen at 80 °C for 2 days. Then the reaction mixture was added into 50 ml of ether and refrigerated for about 10 h. The dark brown precipitate from each reaction was collected and recrystallized in methanol. After drying, the pale brown product 8a or 8b was obtained (yields: m-isomer: 11%; p-isomer: 14%). Purity (>95%) was indicated by TLC.

8a (m-DITC-linked) (MRS 1740) — 1H NMR (Me 2SO) δ: 2.72 (s, 3H), 2.34 (t, 2H), 2.05 (s, 2H), 1.79 (d, 2H, 3.74 (m, 2H), 6.15 (d, 2H), 7.34 (d, 2H), 7.90 (s, 1H). HR-MS (FAB): Calculated for C 39H40C 5 N 12 O 9: M +: 630.1706, Found: 630.1747.

8b (p-DITC-linked) (MRS 1741) — 1H NMR (Me 2SO) δ: 2.72 (s, 3H), 3.10 (t, 2H), 3.59 (s, 4H), 3.63 (d, 2H), 3.98 (s, 2H), 4.16 (m, 2H), 4.32 (s, 1H), 4.62 (m, 3H), 4.79 (s, 2H), 5.03 (s, 1H), 5.25 (d, 2H), 5.45 (s, 1H), 5.95 (s, 1H), 7.28 (d, 2H), 7.80 (d, 2H), 8.39 (s, 1H). HR-MS (FAB): Calculated for C 49H 42 N 19 O 11 S: M +: 821.2224, Found: 821.2218.

**Biological Activity**

**Preparation of Cardiac Myocyte Model of Simulation of Ischemia**

Atrial and ventricular cells were cultured from chick embryos 14 days in ovo and maintained in culture as described previously (11, 12, 18). All experiments were performed on day 3 in culture, at which time the medium was changed to a HEPES-buffered medium containing (in mM): 139 NaCl, 4.7 KCl, 0.5 MgCl 2, 0.9 CaCl 2, 5 HEPES, and 2% fetal bovine serum, pH 7.4, at 37 °C, before exposing the myocytes to hypoxic incubation and glucose deprivation in a hypoxic incubator (NuAire), where O 2 was replaced by N 2 as described previously (11, 12). At the end of the 90-min ischemia the extent of myocyte injury was determined, and the myocytes were removed from the hypoxic incubator and re-exposed to room air (normal percentage of O 2). Aliquots of the media were then obtained for creatine kinase activity measurement, which was followed by quantification of the number of viable cells, as determined by the ability to exclude Trypan blue (6). Measurement of the basal level of cell injury was made after parallel incubation of control cells under normal percentage of O 2. The extent of ischemia-induced injury was quantitatively determined by the percentage of cells killed and by the amount of creatine kinase (CK) released.
Co-activation of Adenosine $A_1$ and $A_3$ Receptors

Fig. 1. The synthesis of the binary conjugate MRS 1543. This conjugate is composed of separate adenosine moieties joined covalently and each of which selectively activates either the $A_1$ or $A_3$ receptor. The $A_1$-activating moiety is an $N^6$-phenylcarboxylic congener of adenosine, 4, and the $A_3$ receptor-activating moiety is a phenylpropargylamine derivative, 3a, which is modified at both $N^9$- and 5'-positions. The adenosine receptor affinities are given in Table I. Compound 3b is MRS 1525.

Radioligand Binding Studies

Binding of [3H]R-N'-phenylisopropyladenosine ([3H]R-PIA) to $A_1$ receptors from rat cerebral cortex membranes and of [3H]-2-(4-[(2-carboxyethyl)phenyl]ethylamino)-5'-N-ethylcarboxamidyladenosine ([3H]CGS 21680) to $A_3$ receptors from rat striatal membranes was performed as described previously (20, 21). Adenosine deaminase (3 units/ml) was present during the preparation of the brain membranes, in a preincubation of 30 min at 30 °C, and during the incubation with the radioligands. Binding of [3H]N-(4-amino-3-iodobenzyl)-5'-N-methylcarboxamidyladenosine ([3H]AB-MECA) to membranes prepared from Chinese hamster ovary cells stably expressing the human $A_3$ receptor was performed as described (22). The assay medium consisted of a buffer containing 10 mM MgCl$_2$, 50 mM Tris, and 1 mM EDTA, at pH 8.0. The glass incubation tubes contained 100 ml of the membrane suspension (0.3 mg of protein/ml, stored at −80 °C in the same buffer), 50 ml of a solution of [3H]AB-MECA (final concentration 0.3 nM), and 50 ml of a solution of the proposed antagonist. Nonspecific binding was determined in the presence of 100 μM $N^6$-phenylisopropyladenosine (R-PIA).

All non-radioactive compounds were initially dissolved in Me$_2$SO and diluted with buffer to the final concentration, where the amount of Me$_2$SO never exceeded 2%.

Incubations were terminated by rapid filtration over Whatman GF/B filters, using a cell harvester (Brandell, Gaithersburg, MD). The tubes were rinsed three times with 3 ml of buffer in each case. At least five different concentrations of competitor, spanning three orders of magnitude adjusted appropriately for the IC$_{50}$ of each compound, were used. IC$_{50}$ values, calculated with the nonlinear regression method implemented in the InPlot program (GraphPad, San Diego, CA), were converted to apparent $K_i$ values using the Cheng-Prusoff equation (23) and $K_i$ values of 1.0 nM ([3H]R-PIA); 14 nM ([3H]CGS 21680); 0.59 nM and 1.46 nM ([3H]AB-MECA) at human and rat $A_3$ receptors, respectively.

Preparation of Recombinant Cardiac Myocytes Expressing the Human Adenosine Receptor

Atrial cardiac myocytes were isolated, maintained in culture for 24 h, and then transfected with either the vector pcDNA3, cDNA encoding the human adenosine $A_3$ receptor, or cDNAs encoding both adenosine $A_1$ and $A_3$ receptors (27). 24 h after the transfection, myocytes were treated for 10 min with the irreversible $A_1$ antagonist (m-DITC-XAC, 5 μM, synthesized as reported previously (19)) to inactivate the endogenous $A_1$ receptor as well as any of the exogenous $A_1$ receptors. The cells were washed three times with fresh medium and incubated with medium containing 6% fetal calf serum for 24 h. These myocytes were then exposed to simulated ischemia in the presence or absence of agonist. Because the expression of the exogenous receptor is driven by the constitutive cytomegalovirus promoter, any receptor that appears due to apoptosis (as evidenced by lack of DNA laddering, data not shown).

Radioligand Binding Studies

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RESULTS

Novel Drug Design: Creation of New Agonists Activating Both Adenosine A1 and A3 Receptors

Based on our previous study demonstrating a synergistic action of A1 and A3 adenosine receptors in cardioprotection, we sought to design novel, mixed A1/A3 adenosine receptor agonists using a "functionalized congener" approach (14). Novel derivatives were created by the "binary drug" approach, in which two functionalized congeners of adenosine agonists, each of which was selective for either A1 or A3 adenosine receptor subtypes, were covalently coupled. This was a means of modulating the selectivity ratio to increase and approximately match affinities at A1 and A3 receptors while diminishing affinity at A2A receptors (6).

The synthesis of an amine-functionalized congener, 3a, derived from the A3 receptor-selective agonist IB-MECA is shown in Fig. 1. This amine derivative was intended to serve as a common intermediate for two conceptual approaches: binary drugs (15) (Figs. 1 and 2) and amino acid conjugates (14, 24). The amine derivative 3a was adequately reactive toward acylation to form the desired conjugates. The inclusion of a rigid, narrow molecular "rod" in the form of an ethynyl group in 3a as a means of preserving A3 receptor affinity was predicted by molecular modeling (24). The m- or o-position of the benzyl ring in receptor binding appeared to accommodate long chain extension having minimal steric bulk. This derivative was coupled to an adenosine carboxylic agonist, 4, which was previously shown to form amides that display selectivity for A1 adenosine receptors (17), to form the binary conjugate 5 (MRS 1543, Fig. 1). Compound 3a was also converted to a simple N-acylated derivative, 3b, which was tested for adenosine receptor affinity. In a separate reaction sequence (Fig. 2) the adenosine amine congener (ADAC, 7) was coupled to 3a, leading to binary conjugates 8a (MRS 1740) and 8b (MRS 1741), containing longer spacers than in 5. These two conjugates were regioisomers that differed only in the substitution pattern of the cross-linking moiety (19) phenylene diisothiocyanate (DITC).

Table I

Affinities of novel adenosine derivatives in radioligand binding assays for A1, A2A, and A3 receptors

| Compound     | K<sub>r</sub><sup>a</sup> (nM) | K<sub>rA2A</sub><sup>b</sup> (nM) | K<sub>r</sub><sup>c</sup> (nM) | r<sub>A1</sub>/h<sub>A3</sub> |
|--------------|----------------|----------------|----------------|----------------|
| 3b           | 709 ± 211      | 396 ± 121      | 7.32 ± 3.09    | 97             |
| MRS 1525     | 191 ± 76       | 332 ± 67       | 10.1 ± 1.1     | 19             |
| MRS 1543     | 1.15 ± 0.24    | 336 ± 119      | 0.727 ± 0.261  | 1.6            |
| 8a           | 3.52 ± 0.48    | 197 ± 21       | 1.15 ± 0.25    | 3.1            |
| MRS 1740     | 5              |                |                |                |
| 8b           |                |                |                |                |

<sup>a</sup> Displacement of specific [3H]R-PIA binding in rat brain membranes.
<sup>b</sup> Displacement of specific [3H]CGS 21680 binding in rat striatal membranes.
<sup>c</sup> Displacement of specific [125I]AB-MECA binding at human A<sub>3</sub> receptors expressed in HEK cells, in membranes.

Biological Activity

Selectivity at Adenosine A1 and A3 Receptors—As a first indication of selectivity, the receptor binding affinities of the adenosine derivatives were measured in standard binding assays using rat brain A1 and A2A receptors and recombinant human A3 receptors. K<sub>r</sub> values for the novel agonists are shown in Table I. Compound 3b was A1-receptor-selective, as was the precursor IB-MECA, thus validating the design approach. The amide-linked conjugate MRS 1543, compound 5, was moderately potent and selective for A<sub>1</sub> receptors. The elongated, thiourea-linked, regioisomers MRS 1740 and MRS 1741, 8a and 8b, were highly potent and selective versus A<sub>2A</sub> receptors in radioligand binding assays, with K<sub>r</sub> values of 0.7–3.5 nM at A<sub>1</sub> and A<sub>3</sub> subtypes.

Biological Activity in the Heart Cell: Novel Cardioprotective
FIG. 3. The cardioprotective effect of the binary conjugate MRS 1543 requires the activation of both adenosine A1 and A3 receptors. Cardiac ventricular myocytes were prepared, incubated for 5 min with (A) indicated concentrations of MRS 1543, alone or in the presence of either the A1 receptor antagonist DPCPX (1 μM) or the A3 receptor antagonist MRS 1191 (1 μM), or (B) MRS 1543, alone or in the presence of both DPCPX and MRS 1191. Myocytes were then washed free of the agents and exposed to 90 min of simulated ischemia. The extent of myocyte injury was quantitated as percentage of cardiac cells killed or as CK released (data not shown) at the end of simulated ischemia as described under “Experimental Procedures.” Data are the mean and standard error of four experiments. In A, the percentage of cardiac cells killed in the presence of 10 or 30 nM of MRS 1543 plus either antagonist was significantly different from that determined when myocytes were not exposed to any agent. (The asterisk indicates significant difference from data obtained in cells not pre-exposed to MRS 1543 or DPCPX or MRS 1191.) In B, the percentages of cardiac cells killed in the presence of MRS 1543 plus both DPCPX and MRS 1191 were similar to those obtained when myocytes were not exposed to any agent.

Property—Cultured chick ventricular cells, expressing both native A1 and A3 receptors, were used initially as a myocyte model to determine the cardioprotective property of the conjugates. The N-acetylated amine congener 3b (data not shown) and the binary A1/A3 receptor agonist 5 (Fig. 3) were able to produce a concentration-dependent preconditioning-like effect, simulating the cardioprotective effect induced by a 5-min exposure to ischemia. The EC50 of 5 was approximately 2 nM. The protective response was partially antagonized by the A1 antagonist DPCPX and the A3 antagonist MRS 1191 (Fig. 3A) with a shift of the agonist-mediated dose-response curve to the right. The combination of DPCPX and MRS 1191 caused a complete abolition of the protective response (Fig. 3B). These data suggested that protection mediated by the binary agonist occurs via activation of both adenosine receptors.

To provide further evidence for this concept, a series of experiments were carried out using a novel recombinant cardiac myocyte model expressing only the human adenosine receptor, because endogenous chick A1 receptors were eliminated by incubation with the irreversible A1 receptor antagonist, m-DITC-XAC (16, 19). After such pretreatment, these myocytes were unresponsive to the A1 receptor agonist (data not shown) and remained unresponsive to the agonist following transfection with pcDNA3 (Fig. 4). Because the atrial cardiac myocytes expressed a very low level of native A3 receptors (25, 26), both before and after treatment with the irreversible A1 antagonist in myocytes, they were also unresponsive to the A3 agonist (Fig. 4). Thus, an adenosine receptor-null myocyte was created. Transfection with cDNAs encoding A1 and A3 receptors led to the appearance of a cardioprotective response to either agonist, IB-MECA or ADAC. The protection in myocytes expressing the human adenosine receptors was indicated by a reduction in the percentage of cardiac cells killed and creatine kinase (CK) released at the end of the 90-min ischemia. Thus, the specific protective effect of adenosine A1 or A3 agonist was due to activation of the human receptors.

Using this novel recombinant cardiac myocyte model, we examined the cardioprotective response to a single binary conjugate compound in myocytes expressing both A1 and A3 receptors as compared with myocytes expressing only the A1 or the A3 receptor. The cardioprotective responses to the binary conjugate MRS 1741 (Fig. 5A, IC50 ~ 0.1 nM) or MRS 1543 (data not shown) were significantly more potent and efficacious in myocytes expressing both A1 and A3 receptors than in myocytes expressing only the A1 receptor. Thus, transfection of the atrial cardiac myocyte with pcDNA3/hA1R led to a left shift of the concentration-response curve to MRS 1741, as compared with pcDNA3-transfected myocytes (Fig. 5A). Because pcDNA3/hA3R-transfected atrial myocytes expressed both the A3 recep-
Adenosine is a potent cardioprotective agent, capable of exerting a pronounced reduction in the extent of cardiac myocyte injury incurred during myocardial ischemia (1–6, 8, 9). Previous studies have shown that the cardioprotection offered by either receptor was not redundant (6). In fact, activation of both $A_1$ and $A_3$ receptors appeared to confer an additive anti-isch-

**FIG. 5.** The binary agonist exerts a more pronounced response in myocytes expressing both $A_1$ and $A_3$ receptors than in myocytes expressing either receptor alone. Atrial cardiac myocytes were transfected with (A) pcDNA3 or pcDNA3/hA3R. In B, atrial myocytes were transfected with pcDNA3/hA3R or both pcDNA3/hA1R and pcDNA3/hA3R and then subjected to treatment with m-DITC-XAC. In both A and B, the response to the binary agonist MRS 1741 was then determined as follows. Myocytes were exposed to 5 min of simulated ischemia to precondition the myocytes. Following a 10-min exposure to normal O$_2$, these myocytes were then exposed to 90-min ischemia in the presence of adenosine deaminase alone or with the indicated concentrations of MRS 1741. The percentage of cardiac cells killed and amount of CK released were quantitated at the end of the 90-min ischemia. Data are shown as the percentage of cardiac cells killed and are the mean and standard errors of five experiments. Similar data were obtained when CK released was used as the end point (not shown). The concentration-response curve to MRS 1741 is virtually identical to that obtained in the presence of both ADAC and IB-MECA.

**DISCUSSION**

The cardioprotective effect of a unimolecular, binary agonist that co-activated both receptors was equivalent to that caused by an equimolar mixture of the $A_1$ agonist ADAC and the $A_3$ agonist IB-MECA (Fig. 6). The concentrations shown for incubation with both ADAC and IB-MECA refered to the individual concentration of either agonist in the media bathing the myocytes. The response to each concentration of the binary molecule was virtually identical to the response produced by the same concentration of the two constituent agonists present together.

As compared with myocytes transfected with the vector alone, the binary $A_1$/A3 receptor agonists caused a significant reduction in both the percentage of cells killed and the amount of CK released in myocytes expressing both human adenosine receptor subtypes. This was demonstrated for binary agonists MRS 1543 and MRS 1740 (Fig. 7, A–D, IC$_{50}$ ~ 0.1 nM) and for MRS 1741 (data not shown). Thus, the binary agonists appear to be able to co-activate both receptors at the same time.
emic protective response (10). These data raised the possibility that a single agonist co-activating both receptors might represent a class of new and highly effective cardioprotective agents. Using a combination of novel ligand chemistry, pharmacological, molecular, and cellular approaches, the present study showed that such an agent could concurrently co-activate both receptors. Functionalized congeners, specifically, agonists selective at the adenosine A₁ and A₃ receptors, were combined covalently as binary conjugates. The relatively high molecular weight of the present binary drugs, for compounds 5, 8a, and 8b, would not necessarily be an impediment to bioavailability of the cardioprotective agents, because intravenous administration would be envisioned in the intended application. Pharmacokinetic studies of these and other dual activating adenosine agonists would be appropriate.

The ability of such binary agonists to co-activate both receptors was determined using a newly created recombinant cardiac myocyte model of cardioprotection. The receptor co-activation produced a highly potent anti-ischemic effect within a concentration range in which only cardiac myocytes expressing both receptors responded. Multiple lines of evidence were obtained to support the concept of greater protection upon receptor co-activation. First, in ventricular myocytes, a binary agonist-mediated response was only partially inhibited by either the A₁ receptor-selective antagonist DPCPX or the A₃ receptor-selective antagonist MRS 1191. In contrast, the combined presence of both antagonists caused a complete abolition of the response to the binary agonist.

Second, in a further series of experiments, chick atrial myo-

**FIG. 7.** Binary conjugate of adenosine A₁ and A₃ agonists can mimic the cardioprotective effect of ischemic preconditioning via the human adenosine A₁ and A₃ receptors. Atrial cardiac myocytes were prepared, transfected with pcDNA3 or cDNAs encoding the human adenosine A₁ and A₃ receptor, and subjected to treatment with the irreversible A₁ receptor antagonist to create recombinant myocytes whose functional adenosine receptors are predominantly the human A₁ and A₃ receptors. These myocytes were then exposed to the indicated concentrations of the binary conjugate compounds (A and B) MRS 1543 or (C and D) MRS 1740 for 5 min, washed free of the agent, and then exposed to 90 min of simulated ischemia. The percentage of cells killed (A and C) and the amount of creatine kinase released (B and D) were quantitated at the end of simulated ischemia. Data represent the mean and standard error of four or five experiments. The asterisks represent significant difference from myocytes co-transfected with pcDNA3/hA₁R and pcDNA3/h A₃R.
cytes were engineered to express only human adenosine receptors. The myocytes were made adenosine receptor-null by irreversible blockade of the endogenous adenosine receptor and were transfected with cDNA for human adenosine receptors. If a binary agonist could co-activate both adenosine A<sub>1</sub> and A<sub>3</sub> receptors, it should cause a much more pronounced response in cardiac myocytes expressing both human adenosine A<sub>1</sub> and A<sub>3</sub> receptors than in myocytes expressing only the human A<sub>3</sub> receptor. The data showed that this was indeed the case. Recombinant myocytes expressing either receptor subtype alone or both receptors were then created to determine the response to the binary agonist or the individual constituent agonists. The concentration-response curve of MRS 1741 was significantly left-shifted in myocytes having both receptors compared with myocytes expressing only the A<sub>3</sub> subtype. Similarly, the binary agonist exerted a more potent and efficacious response in myocytes having both receptors compared with myocytes expressing only the A<sub>1</sub> receptor.

Third, if the binary agonist could co-activate both receptors at the same time, the response produced by one molecule of the binary agonist should be equivalent to that elicited by the combined presence of one molecule of each constituent agonist. The response to each concentration of the binary molecule was virtually identical to the response produced by one molecule of the receptor. The data showed that this was indeed the case. Recombinant myocytes expressing only the A<sub>1</sub> receptor.

Species differences in the cardioprotective effects of adenosine and also in the A<sub>3</sub> receptor affinities of various ligands, especially antagonists, have been noted (13). In the present study, the critical question of generality across species of the approach of concurrently activating A<sub>1</sub> and A<sub>3</sub> receptors has been partially satisfied (for chick and human).

The binary drug approach has been demonstrated here for a clearly defined target of A<sub>1</sub> and A<sub>3</sub> receptors. The full cardioprotection induced by the conjugates having mixed selectivity occurred at a low concentration, indicating high potency, and was dependent on both receptors being expressed. The possible mechanism that mediates the potent cardioprotection following co-activation of both A<sub>1</sub> and A<sub>3</sub> receptors remains to be determined. The A<sub>1</sub> and A<sub>3</sub> receptors are differentially coupled to phospholipase C and phospholipase D, respectively (10). Although speculative, the diacetylglycerol derived from the two phospholipases may confer an additive activation of protein kinase C and K<sub>ATP</sub> channels, which in turn causes a greater degree of cardioprotection. Thus, agonists that activate both receptors may be highly protective within a therapeutic dose window in which only cells expressing both receptors respond, thus avoiding or diminishing adenosine-related side effects such as bradycardia and hypotension. Due to the ability of ligands designed using the “functionalized congener” approach (14) to interact potently with these receptors as the intact covalent conjugates, irrespective of the high molecular weight, the possibilities for binary drugs as new therapeutic agents are vast.

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REFERENCES

1. Ely, S. W., and Berne, R. M. (1992) Circulation 85, 893–904
2. Murry, C. E., Jennings, R. B., and Reimer, K. A. (1986) Circulation 74, 1124–1136
3. Downey, J. M. (1992) Trends Cardiovasc. Med. 2, 170–176
4. Li, G. C., Vasquez, J. A., Gallagher, K. P., and Lu, J. C. (1998) Circulation 100, 1455–1468
5. Gross, G. J. (1995) Basic Res. Cardiol. 90, 85–88
6. Strickler, J., Jacobson, K. A., and Liang, B. T. (1996) J. Clin. Invest. 98, 1773–1779
7. Liang, B. T. (1996) Biochem. J. 336, 337–343
8. Auchampach, J. A., Rizvi, A., Qiu, Y., Tang, X.-L., Maldonado, C., Teshner, S., and Bollai, R. (1997) Circ. Res. 80, 809–809
9. Tracey, W. R., Magee, W., Massmann, H., Kennedy, S. P., Knight, D. R., Buchhoz, R. A., and Hill, R. J. (1997) Cardiovasc. Res. 33, 410–415
10. Parsons, M., Young, L., Lee, J.-E., Jacobson, K. A., and Liang, B. T. (2000) FASEB J. 14, 1425–1431
11. Liang, B. T. (1996) Am. J. Physiol. 271, H1769–H1777
12. Stambaugh, K., Jacobson, K. A., Jiang, J.-L., and Liang, B. T. (1997) Am. J. Physiol. 273, H501–H505
13. Jacobson, K. A. (1998) Trends Pharmacol. Sci. 19, 184–191
14. Jacobson, K. A., Kirk, K. L., Padgett, W. L., and Daly, J. W. (1986) Mol. Pharmacol. 29, 126–133
15. Jacobson, K. A., Lipkowski, A. W., Moody, T. W., Padgett, W., Pijl, E., Kirk, K. L., and Daly, J. W. (1987) J. Med. Chem. 30, 1529–1532
16. Dennis, D., Jacobson, K., and Belardini, L., (1992) Am. J. Physiol. 262, H661–H671
17. Jacobson, K. A., Kirk, K. L., Padgett, W. L., and Daly, J. W. (1985) J. Med. Chem. 28, 1341–1346
18. Liang, B. T., and Donovan, L. A. (1990) Circ. Res. 67, 406–414
19. Stiles, G. L., and Jacobson, K. A. (1988) Mol. Pharmacol. 34, 724–728
20. Schwabe, U., and Trust, T. (1988) Naunyn-Schmiedeberg’s Arch. Pharmacol. 313, 179–187
21. Jarvis, M. F., Schutz, R., Hutchison, A. J., Do, E., Sills, M. A., and Williams, M. (1989) J. Pharmacol. Exp. Ther. 251, 888–893
22. Olah, M. E., Gallo-Rodriguez, C., Jacobson, K. A., and Stiles, G. L. (1994) Mol. Pharmacol. 45, 978–982
23. Cheng, Y. C., and Prusoff, W. H. (1973) Biochem. Pharmacol. 22, 3099–3108
24. Siddiqi, S. M., Pearlstein, R. A., Sanders, L. H., and Jacobson, K. A. (1995) Bioorg. Med. Chem. 3, 1331–1343
25. Liang, B. T., and Jacobson, K. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6985–6990
26. Dougherty, C., Barucha, J., Schofield, P., Jacobson, K. A., and Liang, B. T. (1998) FASEB J. 12, 1785–1792
27. Liang, B. T. (1996) Nucleic Acids Res. 24, 1382–1384