A_1 or A_3 Adenosine Receptors Induce Late Preconditioning Against Infarction in Conscious Rabbits by Different Mechanisms

Hitoshi Takano, Roberto Bolli, Richard G. Black, Jr, Eitaro Kodani, Xian-Liang Tang, Zequan Yang, Samita Bhattacharya, John A. Auchampach

Abstract—We investigated whether activation of A_1 or A_3 adenosine receptors (ARs) induces late preconditioning (PC) against infarction in conscious rabbits using the selective AR agonists 2-chloro-N^6-cyclopentyladenosine (CCPA) and N^6-3-iodobenzyladenosine-5′-*N*-methylcarboxamide (IB-MECA). In vitro radioligand binding and cAMP assays demonstrated CCPA to be ≈200- to 400-fold selective for the rabbit A_1AR and IB-MECA to be ≈20-fold selective for the rabbit A_3AR. We observed that (1) pretreatment of rabbits 24 hours earlier with CCPA (100 μg/kg IV bolus) or IB-MECA (100 or 300 μg/kg) resulted in an ≈35% to 40% reduction in the size of the infarct induced by 30 minutes of coronary artery occlusion and 72 hours of reperfusion compared with vehicle-treated rabbits, whereas pretreatment with the selective A_{2A}AR agonist CGS 21680 (100 μg/kg) had no effect; (2) the delayed cardioprotective effect of CCPA, but not that of IB-MECA, was completely blocked by coadministration of the highly selective A_1AR antagonist N-0861; (3) inhibition of nitric oxide synthase (NOS) with N^{ω}-nitro-L-arginine during the 30-minute occlusion abrogated the infarct-sparing action of CCPA but not that of IB-MECA; and (4) inhibition of ATP-sensitive potassium (K_{ATP}) channels with sodium 5-hydroxydecanoate during the 30-minute occlusion blocked the cardioprotective effects of both CCPA and IB-MECA. Taken together, these results indicate that activation of either A_1ARs or A_3ARs (but not A_{2A}ARs) elicits delayed protection against infarction in conscious rabbits and that both A_1AR- and A_3AR-induced cardioprotection involves opening of K_{ATP} channels. However, A_1AR-induced late PC uses an NOS-dependent pathway whereas A_3AR-induced late PC is mediated by an NOS-independent pathway. **(*Circ Res*. 2001;88:520-528.)**

Key Words: adenosine receptors ■ ischemia/reperfusion injury ■ myocardial infarction ■ ATP-dependent potassium channels ■ nitric oxide synthase

Although it is well established that stimulation of adenosine receptors (ARs) can induce early preconditioning (PC), it is controversial whether ARs can elicit the late phase of PC.[1,2] Studies by Baxter et al[1] first demonstrated in an open-chest, anesthetized rabbit model that administration of the A_1AR agonist 2-chloro-N^6-cyclopentyladenosine (CCPA) limited infarct size after a 30-minute coronary artery occlusion produced 24 to 72 hours later. On the other hand, we found in conscious rabbits that a 24-hour pretreatment with CCPA did not attenuate myocardial stunning.[2] One interpretation of these results is that stimulation of ARs is capable of inducing late PC against infarction, but not late PC against myocardial stunning. This hypothesis is not implausible, because myocardial infarction and myocardial stunning are pathophysiologically distinct.[3,4] However, since the studies by Baxter et al[1] were performed in open-chest, anesthetized rabbits whereas our studies were performed in conscious rabbits, differences in the model systems that were used is another potential explanation for the disparate results. Given the lack of uniformity of the studies described above, the ability of ARs to induce late PC remains uncertain.

Of the four subtypes of ARs known to exist (A_1, A_{2A}, A_{2B}, and A_3), it is generally thought that the A_1AR subtype is responsible for mediating the cardioprotective effects of adenosine.[5] There is increasing evidence, however, that A_3ARs may also exert cardioprotection.[6–10] Evidence in support of this hypothesis includes the observations that early PC is not blocked by selective A_1AR antagonists but is blocked by nonselective antagonists at high concentrations capable of blocking the A_3AR, suggesting that the A_3AR can also induce early PC.[6,9] Furthermore, activation of A_3ARs has been suggested to reduce injury in several different models of ischemia/reperfusion injury.[7,8,10] Based on these results, it has been hypothesized that A_3ARs are also expressed in ventric-

Original received March 3, 2000; resubmission received October 12, 2000; revised resubmission received January 17, 2001; accepted January 17, 2001.

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This manuscript was sent to James T. Willerson, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

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ular cardiomyocytes and that they provide protection via a similar mechanism as A_1ARs. The role of A_3ARs in late PC, however, is unknown.

The present study was undertaken to address these issues and to further characterize the cardioprotective actions of AR stimulation. The results demonstrate, for the first time, that activation of A_1 and A_3ARs induces late PC against infarction via different mechanisms.

Materials and Methods

Radioligand Binding Assays and cAMP Assays

Binding assays were performed with membranes prepared from HEK 293 cells expressing recombinant rabbit A_1ARs or A_3ARs using N^6-(4-amino-3-[^{125}I]iodobenzyl)adenosine-5′-*N*-methylcarboxamide ([^{125}I]AB-MECA) as the radioligand. The rabbit A_1AR cDNA used for transfections was as described by Bhattacharya et al,[11] and the rabbit A_3AR cDNA was cloned from a rabbit brain cDNA library. Additional binding assays with [^{125}I]AB-MECA were performed with membranes prepared from rabbit brain and spleen. Inhibition of isoproterenol-induced cAMP accumulation by adenosine agonists was performed on HEK 293 cells expressing rabbit A_1ARs or A_3ARs using radioimmunoassay.

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Analysis

mRNA expression of A_1ARs and A_3ARs was determined by RT-PCR of total RNA obtained from rabbit heart or 100 to 200 ventricular rabbit cardiomyocytes isolated by enzymatic digestion.

Studies in Conscious Rabbits

New Zealand White rabbits (2.2 to 2.9 kg; Myrtle's Rabbitry, Thompson Station, Tenn) were instrumented with a balloon occluder around a major branch of the left coronary artery for occlusion and reperfusion and bipolar ECG leads on the chest wall. In some studies (groups I through IV; see below), a Doppler thickening crystal was sutured to the epicardial surface of the region at risk to measure wall thickening (WTh). The rabbits were allowed to recover for a minimum of 10 days after surgery. All animal experiments conformed to the guidelines established by the University of Louisville.

The experimental protocol is depicted in Figure 1. All rabbits were subjected to 30 minutes of coronary artery occlusion and 3 days of reperfusion. At the end of the experiments, infarct size and the area-at-risk size were measured by dual staining with phthalo blue dye and triphenyltetrazolium hydrochloride. Rabbits were randomly assigned to 12 treatment groups. Group I (control group) received 1 mL of vehicle as an IV bolus 24 hours before the occlusion. In groups II, III, and IV, CCPA (100 μg/kg), CGS-21680 (100 μg/kg; A_{2A} receptor agonist), or IB-MECA (100 μg/kg or 300 μg/kg) were given as boluses 24 hours before the coronary occlusion. Groups V and VI were treated 24 hours before the occlusion with N-0861 (7.5 mg/kg IV bolus followed immediately by an intravenous infusion of 0.3 mg · kg^{-1} · min^{-1}, which was maintained for 3 hours; total dose, 55.5 mg/kg); 5 minutes after the bolus injection of N-0861, the rabbits were treated with either CCPA (100 μg/kg IV bolus) or IB-MECA (300 μg/kg IV bolus). Groups VII, VIII, and IX were given N^{ω}-nitro-L-arginine (L-NA) (13 mg/kg as an IV bolus) and groups X, XI, and XII were given 5-hydroxydecanoate (5-HD) (5 mg/kg IV bolus) 5 minutes before the 30-minute occlusion in rabbits pretreated 24 hours earlier with either CCPA (100 μg/kg IV bolus; groups VII and X), IB-MECA (300 μg/kg IV bolus; groups VIII and XI), or vehicle (groups IX and XII).

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

Results

Pharmacological Characterization of Rabbit A_1ARs and A_3ARs

Because the pharmacology of A_3ARs differs markedly among species,[12] preliminary studies were performed to characterize

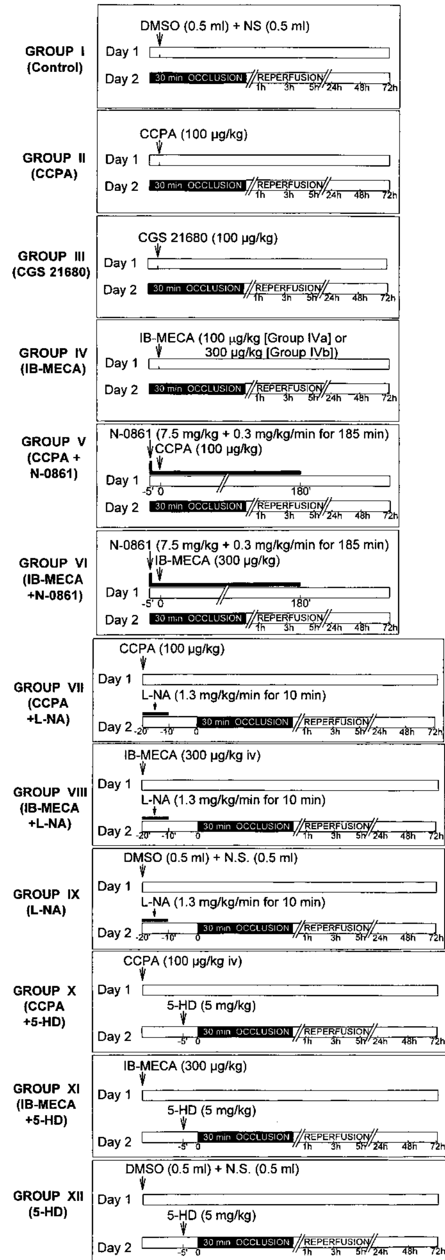

Figure 1. Experimental protocol for the conscious rabbit studies.

rabbit A_1ARs and A_3ARs using radioligand binding analysis and functional assays.

Binding Studies

Specific binding of [^{125}I]AB-MECA to HEK 293 cell membranes expressing either rabbit A_1ARs or A_3ARs fit best to a one-site binding model. The K_d and B_{max} values for the rabbit A_1AR were 6.18±1.92 nmol/L and 2675±569 fmol/mg membrane protein (n=3), respectively, and for the rabbit A_3AR were 0.39±0.11 nmol/L and 1033±433 fmol/mg membrane protein (n=3), respectively. No specific binding of [^{125}I]AB-MECA was observed to nontransfected cells (data not shown).

The K_i values of competing ligands are shown in the Table. The salient findings of these studies can be summarized as

Competition by Various Compounds for [^{125}I]AB-MECA Binding to Recombinant Rabbit A_1 and A_3 Receptors Expressed in HEK 293 Cells

	K_i Values, nmol/L		Selectivity Ratios	
	A_1	A_3	A_3/A_1	A_1/A_3
Adenosine analogues (agonists)				
IB-MECA	12.6±4.92	0.59±0.13	...	21
CCPA	0.10±0.03	37.7±8.19	377	...
NECA	0.21±0.08	18.2±5.42	87	...
R-PIA	0.12±0.03	28.5±8.98	238	...
CGS 21680	120±31.1	673±86	6	...
Xanthine antagonists				
I-ABOPX	22.9±7.42	428±38	19	...
BWA 1433	17.4±2.09	7060±809	406	...
CPX	0.68±0.49	5270±921	7750	...
XAC	1.80±0.49	299±69	166	...
8-SPT	1730±506	493 000±9670	285	...
ZM 241385	566±30	467±24	...	...
Adenine antagonists				
N-0861	31.9±8.71	45 000±8260	1411	...
WRC 0571	6.34±1.46	2010±698	317	...
Nonxanthine/nonadenine antagonists				
MRS 1191	16%*	54%*	...	...
MRS 1220	37.5±6.21	3.65±0.94	...	10
L 249313	3800±508	556±30	...	7
Antiasthmatic antagonists				
Theophylline	5550±986	50%†	...	...
Enprofylline	62 000±8600	44%†	...	...

Data are mean±SEM; n=3 to 5.

*Percent inhibition of specific binding at 1 μmol/L; †percent inhibition of specific binding at 1 mmol/L.

follows: (1) CCPA is a potent and selective agonist for recombinant rabbit A_1ARs (377-fold); (2) IB-MECA is a potent A_3AR agonist; however, it is only 21-fold selective versus the A_1AR; (3) like rodent A_3ARs,[13] the rabbit A_3AR is resistant to blockade by xanthine antagonists and no selective antagonists were identified; (4) the xanthine and adenine antagonists had high affinity for the A_1AR, therefore useful A_1 selective antagonists were identified including CPX (7750-fold) and N-0861 (1400-fold); and (5) the A_{2A}AR agonist CGS 21680 and the A_{2A}AR antagonist ZM 241385 had low affinity for both A_1ARs and A_3ARs.

We next measured [^{125}I]AB-MECA binding to membranes prepared from rabbit lung and spleen to determine whether we could detect endogenous expression of A_1ARs and A_3ARs and to confirm that endogenous rabbit ARs expressed in tissues are pharmacologically similar to recombinant rabbit receptors expressed heterologously in HEK 293 cells. Previous studies have demonstrated that high levels of A_3AR transcript exist in spleen,[12] and that the brain is a rich source of A_1ARs. These studies could not be performed in heart, because the level of expression of all of the adenosine receptor subtypes is too low to be detected accurately in heart tissue with agonist radioligands. For these studies, binding to A_1ARs was defined by specific binding displaced by 500 nmol/L CPX, A_3AR binding was defined by the difference in specific binding displaced by 500 nmol/L CPX and 500 nmol/L MRS 1220, and A_{2A}AR binding was defined by specific binding displaced by 100 nmol/L ZM 241385. As shown in Figure 2, incubation of rabbit brain or spleen membranes with ≈0.3 nmol/L [^{125}I]AB-MECA resulted in 90±1% and 74±1% specific binding, respectively. In brain tissue, ≈75% of the specific binding sites were A_1, ≈12% were A_3, and ≈13% were A_{2A}. In contrast, ≈25% of the specific binding sites in spleen were A_1, ≈70% were A_3, and <5% were A_{2A}. These data demonstrate that (1) [^{125}I]AB-MECA labels multiple AR subtypes in rabbit brain and spleen, and (2) the majority of [^{125}I]AB-MECA binding in rabbit brain is to A_1ARs whereas the majority of binding in rabbit spleen is to A_3ARs.

Using rabbit brain membranes as a source of A_1ARs and rabbit spleen membranes as a source of A_3ARs, we next performed competition binding assays to compare the affinity of CCPA and IB-MECA for endogenously expressed rabbit ARs. [^{125}I]AB-MECA was included at a concentration of 6 nmol/L for assays of brain membranes and 0.3 nmol/L for assays of spleen membranes, ie, concentrations equivalent to

its predicted K_d value for A_1ARs and A_3ARs, respectively. In addition, 100 nmol/L ZM 241385 was included in all of the assays to inhibit binding to A_{2A}ARs, and 500 nmol/L CPX was included in assays of spleen tissue to selectively block A_1ARs. Both CCPA and IB-MECA concentration dependently competed for [^{125}I]AB-MECA binding in rabbit brain and spleen tissues (Figure 2). In brain tissue, the CCPA and IB-MECA competition binding data fit best to a two-site binding model; the high-capacity binding site reflected binding to the A_1AR. In spleen tissue when 500 nmol/L CPX and 100 nmol/L ZM 241825 were added to the assays, the data fit best to a single-site binding model reflecting binding to the A_3AR. The IC_{50} values of CCPA were calculated to be 0.32±0.06 nmol/L for the high-capacity binding site in brain (A_1AR) and 50.1±18.1 nmol/L for the binding site in spleen (A_3AR). The IC_{50} values for IB-MECA were calculated to be 0.78±0.26 nmol/L for the single binding site in spleen (A_3AR) and 10.1±2.2 nmol/L for the high-capacity binding site in brain (A_1AR). These data demonstrate that CCPA binds selectively to endogenously expressed rabbit A_1ARs (≈160-fold selective) and that IB-MECA binds potently and with moderate selectivity (≈13-fold selective) to endogenously expressed rabbit A_3ARs. These results are similar to those obtained with CCPA and IB-MECA in binding studies using recombinant rabbit ARs expressed in HEK 293 cells (see Table).

cAMP Assays With HEK 293 Cells

CCPA and IB-MECA were found to inhibit isoproterenol-stimulated cAMP accumulation in HEK 293 cells transfected with recombinant rabbit A_1ARs and A_3ARs (Figure 3). The EC_{50} values of CCPA and IB-MECA were calculated to be 0.05±0.03 and 59.0±12.2 nmol/L, respectively, for HEK 293 cells expressing rabbit A_1ARs and 10.3±4.11 and 0.14±0.08 nmol/L, respectively, for HEK 293 cells expressing rabbit A_3ARs. CGS 21680 weakly acted on rabbit A_1ARs (EC_{50}=821±98.2 nmol/L) and A_3ARs (EC_{50}=116±24.6 nmol/L). These results demonstrate that CCPA and IB-MECA are functional agonists of rabbit A_1ARs and A_3ARs. None of the agonists influenced isoproterenol-induced increases in cAMP levels in nontransfected HEK 293 cells (data not shown).

The cAMP-lowering effects of CCPA in HEK 293 cells transfected with the A_1AR were surmountably blocked by the addition of 10 or 50 μmol/L N-0861 (Figure 3; K_d=45±19 nmol/L by Schild regression analysis; Figure 3). N-0861 did not antagonize the inhibitory effects of IB-MECA in HEK 293 cells transfected with the A_3AR. Thus, N-0861 acts as a competitive and selective antagonist of A_1ARs.

Detection of A_1AR and A_3AR Message by RT-PCR

No bands corresponding to the A_1AR or A_3AR were detected in heart tissue or isolated ventricular cardiomyocytes after RT-PCR, electrophoretic separation of the reactions through agarose gels, and staining with ethidium bromide. However, as shown in Figure 4, distinct bands corresponding to the A_1AR (310 bp) and A_3AR (415 bp) were detected in both heart tissue and isolated cardiomyocytes after Southern blotting of the RT-PCR reactions and probing with A_1AR- or A_3AR-specific cDNA radioprobes. These results suggest that both A_1AR and A_3AR mRNAs are expressed in whole heart tissue as well as ventricular cardiomyocytes. Note that no bands were detected in negative control samples in which water was included in the RT-PCR reactions instead of RNA, excluding the possibility that the reactions were contaminated with foreign DNA.

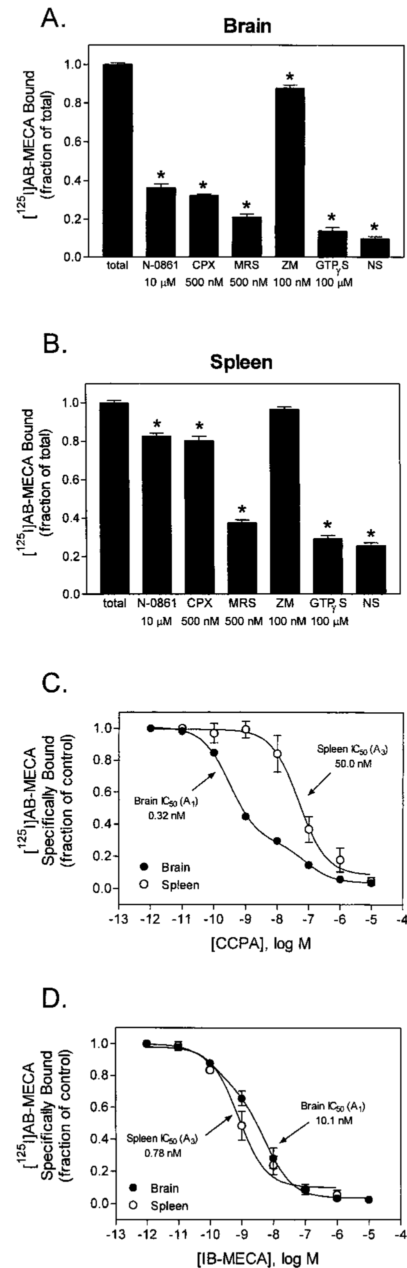

Figure 2. Radioligand binding to membranes from rabbit tissues. A and B, Effects of various antagonists on [^{125}I]AB-MECA (≈0.30 nmol/L) binding to rabbit brain (A) or spleen (B) tissue. C and D, Competition by CCPA (C) and IB-MECA (D) for [^{125}I]AB-MECA (brain ≈6.0 nmol/L; spleen ≈0.30 nmol/L) binding to rabbit brain or spleen tissue. In panels C and D, 100 nmol/L ZM 241825 was included in the assays to inhibit binding to A_{2A}ARs and 500 nmol/L CPX was included in assays with spleen tissue to inhibit binding to A_1ARs. All assays included 200 μg membrane protein per tube. NS indicates nonspecific binding defined by 10 μmol/L I-AB-MECA. Data are mean±SEM of 3 to 4 experiments performed in triplicate.

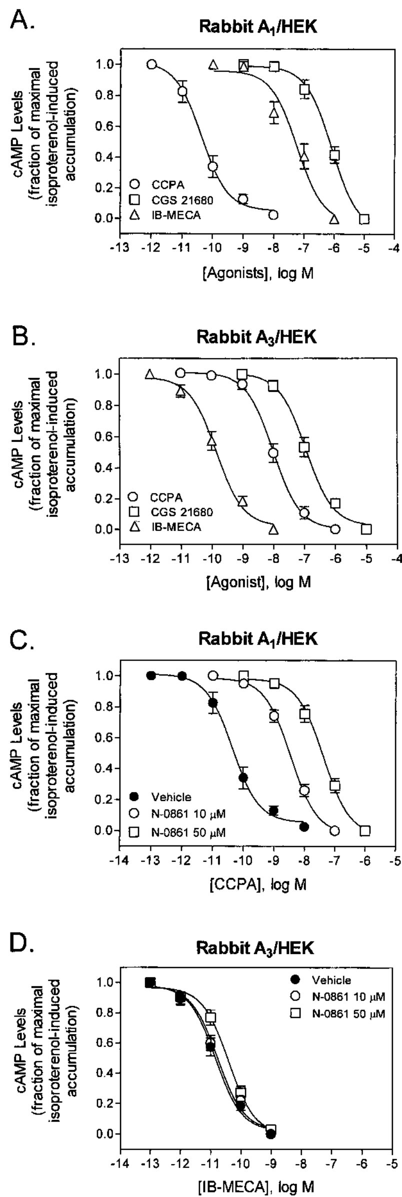

Figure 3. Inhibition of isoproterenol-stimulated adenylyl cyclase activity in HEK 293 cells transfected with either the rabbit A_1AR or A_3AR cDNAs. A and B, Effect of CCPA, CGS 21680, and IB-MECA on rabbit A_1ARs (A) or A_3ARs (B). C and D, Effect of 10 and 50 μmol/L N-0861 on the inhibitory effect of CCPA or IB-MECA on rabbit A_1ARs (C) and A_3ARs (D). Data are presented as the fraction of maximal inhibition of isoproterenol-induced cAMP accumulation caused by the AR agonists. Data are mean±SEM of 3 experiments performed in triplicate.

Delayed Cardioprotection by CCPA and IB-MECA in Conscious Rabbits

Exclusions

Of the 129 rabbits instrumented for the studies of myocardial infarction, 26 were excluded because of ventricular fibrillation, technical problems, or a small risk region (<10% of left ventricular weight; see Table 1 in the online data supplement, available at http://www.circresaha.org).

Hemodynamic Variables on Day 1

Hemodynamic data for groups II, III, IVa, IVb, V, and VI on day 1 are presented in Figure 5. At baseline, heart rate and mean arterial blood pressure were similar in all of the treatment groups (which ranged from 241 to 255 bpm and 74 to 82 mm Hg, respectively). In group II, administration of CCPA (100 μg/kg) produced a transient reduction in heart rate and mean arterial blood pressure (maximal decreases of 24% and 14%, respectively). In group III, administration of

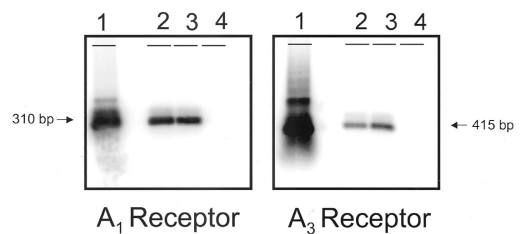

Figure 4. Detection of A_1AR (310-bp band) and A_3AR (415-bp band) message by RT-PCR/Southern blotting in total RNA obtained from rabbit heart ventricular myocardium (lane 2) or from ≈100 to 200 ventricular cardiomyocytes obtained by enzymatic digestion with collagenase (lane 3). The primer sequences and reaction conditions are as described in the online Materials and Methods. Positive control reactions included total RNA (lane 1) obtained from HEK 293 cells transfected with either the rabbit A_1AR or A_3AR. Negative controls (lane 4) included an equivalent volume of water in place of RNA in the RT-PCR reaction. Identical results were observed in a total of 5 independent experiments.

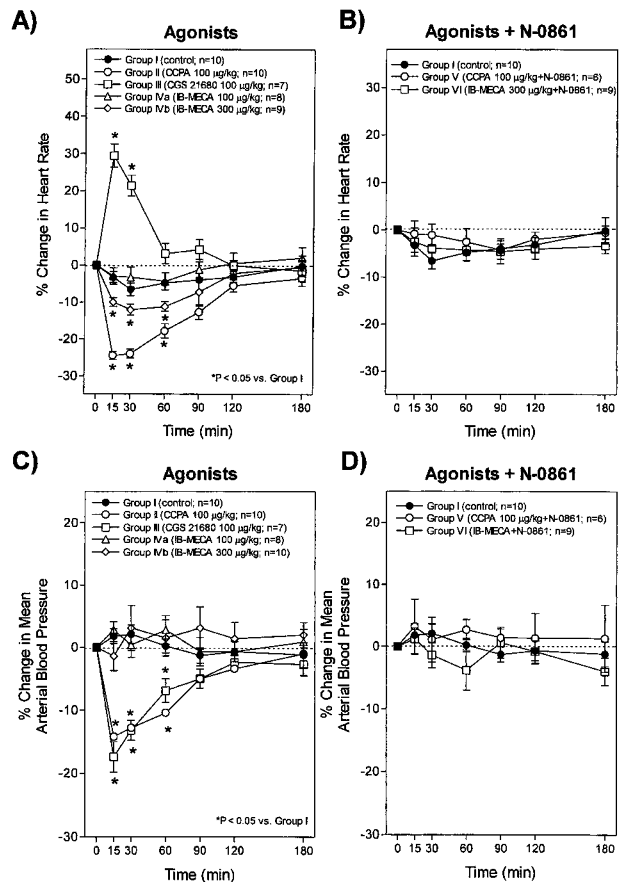

Figure 5. Heart rate (A and B) and mean arterial blood pressure (C and D) expressed as a percentage change from baseline in the conscious rabbit studies on day 1 in groups I through VI. Data are mean±SEM.

100 μg/kg CGS 21680 increased heart rate by 29% and reduced mean arterial blood pressure by 17%. In group IVb, administration of IB-MECA at 300 μg/kg caused an 11% decrease in heart rate but did not cause any appreciable changes in mean arterial blood pressure. However, IB-MECA at a dose of 100 μg/kg (group IVb) did not influence either hemodynamic parameter. In group V and VI in which 100 μg/kg CCPA and 300 μg/kg IB-MECA were administered in animals treated concurrently with the A_1AR antagonist N-0861, there were no appreciable changes in heart rate or blood pressure at any time, indicating that N-0861 completely blocked the hemodynamic actions of CCPA and IB-MECA.

Heart Rate on Day 2

There were no significant differences in heart rate among any of the groups during the 30-minute coronary occlusion or during the 72-hour reperfusion period, except for an ≈20% decrease in heart rate in all of the groups of rabbits treated with L-NA (see Table 2 in the online data supplement).

Region at Risk and Infarct Size

There were no significant differences among the groups with respect to the weight of the region at risk (which ranged from 15.8% to 20.1% of the left ventricle; see Table 3 in the online data supplement), indicating that this important determinant of infarct size is similar among the treatment groups. However, the average infarct size was 33% smaller in group II (CCPA 100 μg/kg group), 44% smaller in the group IVa (IB-MECA 100 μg/kg group), and 40% smaller in group IVb (IB-MECA 300 μg/kg group) compared with the control group (38.3±4.1%, 33.7±4.9%, and 31.8±2.9% versus 56.4±4.0% of the risk region, respectively), indicating that both CCPA and IB-MECA (the latter even at a hemodynamically inert dose of 100 μg/kg) elicited protection against infarction 24 hours later (Figure 6). The average infarct size in group III (CGS 21680 100 μg/kg group; 61.4±5.5%) did not differ from the control group, indicating that activation of $A_{2A}ARs$ does not produce late PC against infarction. In group V (CCPA 100 μg/kg+N-0861 group), infarct size (59.8±3.8%) was indistinguishable from that measured in the control group and significantly larger than that measured in the CCPA group (Figure 6), indicating that N-0681 completely blocked the infarct-sparing effect of CCPA pretreatment. In group VI (IB-MECA 300 μg/kg+N-0861), infarct size (41.8±1.9%) was significantly smaller than that of the control group and was not significantly larger than that observed in the IB-MECA–pretreated group (Figure 6). Thus, the same dose of N-0861 that blocked the infarct-sparing effect of CCPA failed to block the infarct-sparing effect of IB-MECA, implying that activation of A_3ARs alone is sufficient to produce late PC against infarction.

Having found that both CCPA and IB-MECA induced late PC against infarction, we examined the effect of a nitric oxide synthase (NOS) inhibitor and an ATP-sensitive potassium (K_{ATP}) channel blocker on the cardioprotective responses. In group VII (CCPA 100 μg/kg+L-NA), infarct size (57.7±4.1%) was significantly greater than in group II (CCPA 100 μg/kg) and essentially indistinguishable from controls (Figure 7), indicating that L-NA abrogated the protective effect of CCPA. However, in group VIII (IB-MECA 300 μg/kg+L-NA) infarct size (36.6±3.4%) was significantly smaller than the control group and similar to that in group IVb (IB-MECA 300 μg/kg), indicating that L-NA failed to block the protective effect of IB-MECA pretreatment. In groups X (CCPA 100 μg/kg+5-HD) and XI (IB-MECA 300 μg/kg+5-HD), infarct size (54.8±5.8% and 55.0±4.4%) was greater than in groups II (CCPA 100 μg/kg) and IVb (IB-MECA 300 μg/kg), respectively, and essentially indistinguishable from the control group (Figure 7). Thus, administration of 5-HD on day 2 completely blocked the protection provided by pretreatment with either CCPA or IB-MECA. In groups IX (L-NA) and XII (5-HD), infarct size did not differ significantly from that in the control group (Figure 7), indicating that administration of L-NA or 5-HD did not affect infarct size in vehicle-treated myocardium (55.0±4.4% and 52.0±5.5%, respectively).

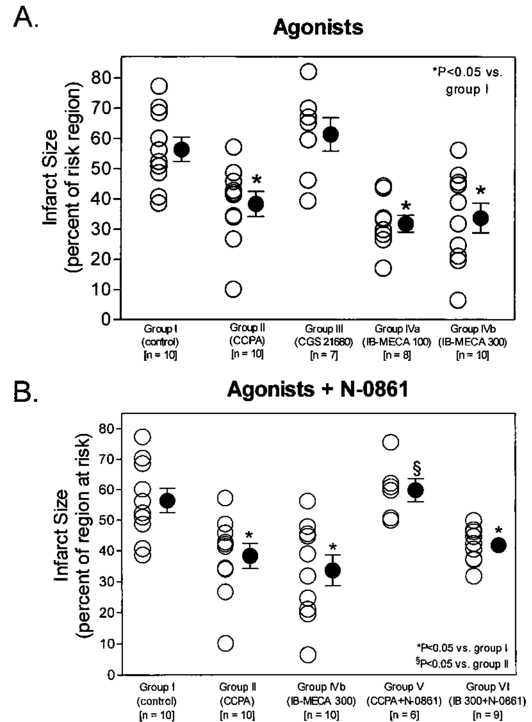

Figure 6. Myocardial infarct size expressed as a percentage of the risk region in groups I (control), II (CCPA), III (CGS 21680), IVa (IB-MECA 100 μg/kg), and IVb (IB-MECA 300 μg/kg) (A) and groups I (control), II (CCPA), IVb (IB-MECA 300 μg/kg), V (CCPA+N-0861), and VI (IB-MECA 300 μg/kg+N-0861) (B). ○, Individual rabbits. ●, Mean±SEM.

Functional Recovery

The recovery of regional contractile function in the ischemic-reperfused region was measured in groups I through IV. In keeping with the infarct size data, the recovery of systolic WTh was improved in rabbits pretreated with 100 μg/kg CCPA, 100 μg/kg IB-MECA, or 300 μg/kg IB-MECA after 72 hours of reperfusion compared with that measured in the control group (3.5±3.9%, 5.5±9.4%, and 7.3±8.3% of baseline, respectively, versus −16.2±6.4% of baseline; $P<0.05$). In the CGS 21680-treated group, the recovery of systolic WTh at 72 hours was not significantly improved (−15.4±4.2% of baseline).

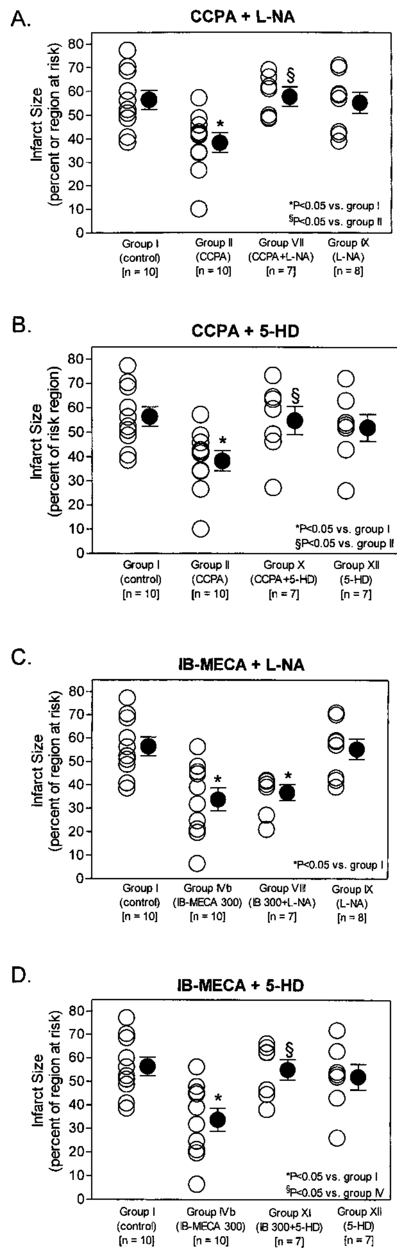

Figure 7. Myocardial infarct size expressed as a percentage of the risk region in groups I (control), II (CCPA), VII (CCPA+L-NA), and IX (L-NA) (A), groups I (control), II (CCPA), X (CCPA+5-HD), and XII (5-HD) (B), groups I (control), IVb (IB-MECA 300 μg/kg), VIII (IB-MECA 300 μg/kg+L-NA), and IX (L-NA) (C), and groups I (control), IV, (IB-MECA 300 μg/kg) XI (IB-MECA 300 μg/kg+5-HD), and XII (5-HD) (D). ○, Individual rabbits. ●, Mean±SEM.

Discussion

The results presented herein demonstrate that, in conscious rabbits, activation of either A_1ARs or A_3ARs (but not A_{2A}ARs) can induce late PC against infarction acting via distinct mechanisms. To the best of our knowledge, this is the first investigation to demonstrate that ARs can induce late PC in a conscious rabbit model. This is also the first investigation to demonstrate that the A_3AR subtype, in addition to the A_1AR subtype, is capable of triggering the development of late PC, that this effect of the A_3AR is mediated by K_{ATP} channels, and that NOS plays a differential role in A_1AR- versus A_3AR-induced late PC.

The ability of AR agonists to induce late PC is controversial. Studies in barbital-anesthetized, open-chest rabbits support the concept that the A_1AR agonist CCPA can induce late PC against myocardial infarction,[1] whereas studies in conscious rabbits suggest that CCPA cannot induce late PC against myocardial stunning.[2] One of the goals of the present investigation was to gain insights into this apparent discrepancy. Specifically, we sought to distinguish between two basic possibilities: (1) ARs can induce late PC against infarction but not late PC against myocardial stunning, or (2) the ability of AR agonists to induce late PC is dependent on the system in which they are studied. Our results provide evidence that the former hypothesis is correct. Using the same conscious rabbit model in which we previously observed that CCPA did not induce late PC against myocardial stunning,[2] we found that the same dose of CCPA induced late PC against myocardial infarction (Figure 6). To the best of our knowledge, this is the first identification of a late PC stimulus that provides selective protection against a specific type of ischemic injury. Our results imply that there are important differences between the mechanism of late PC against myocardial stunning and late PC against infarction.

A second major goal of the present investigation was to determine whether the A_3AR is capable of inducing late PC against infarction. Our approach was to compare the effects of CCPA to those of the recently characterized A_3AR agonist IB-MECA.[14] We found that IB-MECA, at a dose of 300 μg/kg, produced reductions in infarct size that were equivalent in magnitude to those elicited by CCPA (Figure 6), suggesting that the A_3AR is also capable of inducing late PC against infarction. These results, however, must be interpreted with caution, because IB-MECA at a dose of 300 μg/kg produced a modest decrease in heart rate (Figure 5). This finding, coupled with the results of our in vitro radioligand binding studies showing that IB-MECA is only 13- to 21-fold more potent at binding to rabbit A_3ARs compared with rabbit A_1ARs (Table and Figure 2), raises the possibility that IB-MECA may have induced late PC not by interacting with A_3ARs, but rather through nonspecific interactions with the A_1AR. This possibility cannot be addressed using an A_3AR-selective inhibitor because no antagonist is currently available that selectively inhibits rabbit A_3ARs (Table). Therefore, we performed two additional sets of experiments. First, we administered IB-MECA or CCPA in the presence of the A_1AR antagonist N-0861 given at a dose predicted to produce blood levels (20 to 50 μmol/L)[15] that do not block the A_3AR. The finding that N-0861 blocked the actions of CCPA against infarction but not those of IB-MECA (Figure 6) demonstrates that IB-MECA is capable of eliciting delayed cardioprotection through a mechanism independent of the A_1AR, thereby implicating the involvement of the A_3AR. Second, we administered IB-MECA at a lower dose (100 μg/kg), which had no effect on heart rate (Figure 5), indicating that it did not activate A_1ARs. The finding that this lower dose of IB-MECA exerted a robust delayed cardioprotective action (Figure 6) further corroborates the conclusion that activation of A_3ARs in itself induces late PC, indepen-

dent of A_1AR activation. The possibility that IB-MECA produced late PC by acting through $A_{2A}ARs$ can be excluded because the selective $A_{2A}AR$ agonist CGS 21680 did not reduce infarct size (Figure 6). We can also exclude the possibility that IB-MECA acted through $A_{2B}ARs$, because it has extremely low affinity for this receptor subtype.[16] Thus, our results indicate that, similar to early PC, the late phase of PC can also be induced by activation of A_3ARs.

Although current data indicate that A_3ARs can induce both phases of PC, in recent in vivo studies we have observed that mice with genetic disruption of A_3ARs exhibit infarcts that are smaller than those in wild-type mice,[17] raising the interesting possibility that A_3ARs may actually play an injurious role during acute myocardial ischemia in this species. These observations are not in conflict with the idea that A_3ARs can trigger PC, since the role of A_3ARs in modulating injury during acute myocardial ischemia is distinct from their role in eliciting PC before ischemia. It is also important to keep in mind that the findings obtained with A_3AR knockout mice need to be confirmed with the use of A_3AR-selective antagonists, because chronic disruption of these receptors may produce compensatory changes in other genes and/or signaling pathways resulting in protection from ischemic injury. In addition, there are marked differences in the properties and tissue expression of A_3ARs among species,[12] such that observations in mice should not be extrapolated to rabbits.

We also investigated the mechanisms by which CCPA and IB-MECA induce the delayed cardioprotection against infarction. We have previously found that both the anti-stunning and anti-infarct effects of ischemia-induced late PC are mediated by increased activity of NOS, specifically, the inducible isoform of NOS (iNOS).[18] Additional studies have demonstrated that the cardioprotective effects of CCPA-induced late PC can be blocked by inhibitors of the K_{ATP} channel and are absent in iNOS knockout mice.[19,20] Based on these observations, we hypothesized that late PC induced by both CCPA and IB-MECA is also the result of increased NOS activity and increased function of K_{ATP} channels. We found that the nonselective NOS inhibitor L-NA and the K_{ATP} channel antagonist 5-HD completely abrogated the protective effects of CCPA when they were administered immediately before the 30-minute coronary occlusion on day 2 (Figure 7). On the other hand, IB-MECA–induced late PC was not blocked by L-NA but was completely abrogated by 5-HD (Figure 7). It appears, therefore, that CCPA-induced late PC against infarction involves a mechanism similar to that of ischemia-induced late PC[18]; that is, cardioprotection is due to enhanced production of NO via induction of NOS and enhanced function of K_{ATP} channels. IB-MECA–induced late PC also appears to utilize a mechanism requiring K_{ATP} channels; however, upregulation of NOS is not a necessary component. Thus, the results suggest that the mechanisms by which A_1ARs and A_3ARs induce late PC against infarction involve different pathways that ultimately converge on the K_{ATP} channel.

Elucidation of the mechanisms by which ARs modulate PC requires knowledge of the cell types within the heart that express A_1ARs and A_3ARs. It is well established that A_1ARs coupled to inhibition of adenylyl cyclase via $G_{i/o}$ proteins are expressed in cardiomyocytes in both atria and ventricles. These receptors classically are known to counteract the positive inotropic actions of catecholamines.[21] Recent data indicate that cardiac A_1ARs induce delayed PC via signaling pathways involving protein kinase C, tyrosine kinases, and mitogen-activated/stress-activated protein kinases.[22] With regard to the A_3AR, however, little is known regarding the specific cell types within the heart that express this receptor subtype. Strickler et al[10] recently demonstrated that A_3ARs are expressed in cultured embryonic chicken cardiomyocytes and that activation of these receptors produces protection against cell death induced by simulated ischemia and reperfusion. Thus, it is possible that cardiomyocytes also express A_3ARs, which may induce PC via similar signaling pathways as the A_1AR. This hypothesis is supported by the results of the present investigation in which we were able to detect A_3AR mRNA in adult rabbit cardiomyocytes by RT-PCR analysis (Figure 4). The cell types within the heart that express A_3ARs may not be limited to cardiomyocytes, however. For example, in other tissues, the A_3AR is known to be expressed in resident leukocytes such as macrophages and mast cells.[12] A_3ARs have also been suggested to be expressed in vascular smooth muscle cells and endothelial cells.[23] Based on these observations, it remains possible that A_3AR agonists may elicit PC through the release of mediators from nonmyocytic cells. Clearly, additional studies of A_3ARs in the heart are warranted.

In conclusion, the present study demonstrates that activation of ARs induces a long-lasting cardioprotective effect in conscious rabbits, suggesting that AR agonists could be used to maintain patients in a protracted preconditioned state. Our results further demonstrate that selective agonists of either A_1ARs or A_3ARs can induce a late PC effect. Because A_3AR agonists can provide protection with minimal hemodynamic effects, these results imply that targeting the A_3AR could be a novel and useful approach to the protection of the ischemic myocardium. Finally, the present study elucidates the molecular mechanisms of A_1AR- and A_3AR-induced late PC by identifying common and differential roles for the K_{ATP} channel and NOS, respectively, in theses two forms of delayed cardioprotection.

Acknowledgments

This study was supported in part by National Institutes of Health Grants R01 HL60051 (J.A.A.), HL-43151 (R.B.), and HL-55757 (R.B.); by American Heart Association (AHA) National Center Grant 9630083N (J.A.A.); by AHA Kentucky Affiliate Grant KY 9804558 (H.T.), and by the Jewish Hospital Research Foundation, Louisville, Ky.

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