A1 or A3 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 A1 or A3 adenosine receptors (ARs) induces late preconditioning (PC) against infarction in conscious rabbits using the selective AR agonists 2-chloro-N6-cyclopentyladenosine (CCPA) and N6-3-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 A1AR and IB-MECA to be ≈20-fold selective for the rabbit A2AR. 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 A2AR 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 A3AR antagonist N-0861; (3) inhibition of nitric oxide synthase (NOS) with Nω-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 (KATP) 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 A1ARs or A3ARs (but not A2ARs) elicits delayed protection against infarction in conscious rabbits and that both A1AR- and A3AR-induced cardioprotection involves opening of KATP channels. However, A3AR-induced late PC uses a NOS-dependent pathway whereas A1AR-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 al1 first demonstrated in an open-chest, anesthetized rabbit model that administration of the A1AR agonist 2-chloro-N6-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 al1 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 (A1, A2A, A2B, and A3), it is generally thought that the A1AR subtype is responsible for mediating the cardioprotective effects of adenosine.5 There is increasing evidence, however, that A3ARs may also exert cardioprotection.6–10 Evidence in support of this hypothesis includes the observations that early PC is not blocked by selective A3AR antagonists but is blocked by nonselective antagonists at high concentrations capable of blocking the A1AR, suggesting that the A3AR can also induce early PC.6,9 Furthermore, activation of A3ARs 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 A3ARs are also expressed in ventric-
ular cardiomyocytes and that they provide protection via a similar mechanism as A\textsubscript{1} ARs. The role of A\textsubscript{3} ARs 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\textsubscript{1} and A\textsubscript{3} ARs 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\textsubscript{1} ARs or A\textsubscript{3} ARs using N\textsuperscript{6}-(4-amino-3-[\textsuperscript{125}I]iodobenzyl)adenosine-5\textsuperscript{\prime}-N-methylcarboxamide ([\textsuperscript{125}I]AB-MECA) as the radioligand. The rabbit A\textsubscript{1} AR cDNA used for transfections was as described by Bhattacharya et al., and the rabbit A\textsubscript{3} AR cDNA was cloned from a rabbit brain cDNA library. Additional binding assays with [\textsuperscript{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\textsubscript{1} ARs or A\textsubscript{3} ARs using radioimmunoassay.

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

mRNA expression of A\textsubscript{1} ARs and A\textsubscript{3} ARs 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 Rabbity, 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 \textmu g/kg), CGS-21680 (100 \textmu g/kg; A\textsubscript{2A} receptor agonist), or IB-MECA (100 \textmu g/kg or 300 \textmu g/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\textsuperscript{-1} · min\textsuperscript{-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 \textmu g/kg bolus) or IB-MECA (300 \textmu g/kg bolus). Groups VII, VIII, and IX were given \textsuperscript{N\textsuperscript{\prime}}-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 \textmu g/kg IV bolus; groups VII and XI), IB-MECA (300 \textmu g/kg IV bolus; groups VIII and X), 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\textsubscript{1} ARs and A\textsubscript{3} ARs

Because the pharmacology of A\textsubscript{1} ARs differs markedly among species, preliminary studies were performed to characterize rabbit A\textsubscript{1} ARs and A\textsubscript{3} ARs using radioligand binding analysis and functional assays.

Binding Studies

Specific binding of [\textsuperscript{125}I]AB-MECA to HEK 293 cell membranes expressing either rabbit A\textsubscript{1} ARs or A\textsubscript{3} ARs fit best to a one-site binding model. The K\textsubscript{s} and B\textsubscript{max} values for the rabbit A\textsubscript{1} AR were 6.18±1.92 nmol/L and 2675±569 fmol/mg membrane protein (n=3), respectively, and for the rabbit A\textsubscript{3} AR were 0.39±0.11 nmol/L and 1033±433 fmol/mg membrane protein (n=3), respectively. No specific binding of [\textsuperscript{125}I]AB-MECA was observed to nontransfected cells (data not shown).

The K\textsubscript{s} values of competing ligands are shown in the Table. The salient findings of these studies can be summarized as...
follows: (1) CCPA is a potent and selective agonist for recombinant rabbit A1 ARs (377-fold); (2) IB-MECA is a potent A3 AR agonist; however, it is only 21-fold selective versus the A1 AR; (3) like rodent A3 ARs,13 the rabbit A3 AR is resistant to blockade by xanthine antagonists and no selective antagonists were identified; (4) the xanthine and adenine antagonists had high affinity for the A1 AR, therefore useful A1 selective antagonists were identified including CPX (7750-fold) and N-0861 (1400-fold); and (5) the A2A AR agonist CGS 21680 and the A2A AR antagonist ZM 241385 had low affinity for both A1 ARs and A3 ARs.

We next measured [125I]AB-MECA binding to membranes prepared from rabbit lung and spleen to determine whether we could detect endogenous expression of A1 ARs and A3 ARs 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 A1 AR transcript exist in spleen,12 and that the brain is a rich source of A3 AR transcript; previous 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 A1 ARs was defined by specific binding displaced by 500 nmol/L CPX, A3 AR binding was defined by the difference in specific binding displaced by 500 nmol/L CPX and 500 nmol/L MRS 1220, and A2A 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 [125I]AB-MECA resulted in 90±1% and 74±1% specific binding, respectively. In brain tissue, 75% of the specific binding sites were A1, 12% were A3, and 13% were A2A. In contrast, 25% of the specific binding sites in spleen were A1, 70% were A3, and 5% were A2A. These data demonstrate that (1) [125I]AB-MECA labels multiple AR subtypes in rabbit brain and spleen, and (2) the majority of [125I]AB-MECA binding in rabbit brain is to A1 ARs whereas the majority of binding in rabbit spleen is to A3 ARs.

Using rabbit brain membranes as a source of A1 ARs and rabbit spleen membranes as a source of A3 ARs, we next performed competition binding assays to compare the affinity of CCPA and IB-MECA for endogenously expressed rabbit ARs. [125I]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

<table>
<thead>
<tr>
<th>Competition by Various Compounds for [125I]AB-MECA Binding to Recombinant Rabbit A1 and A3 Receptors Expressed in HEK 293 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>K Values, nmol/L</td>
</tr>
<tr>
<td>Adenosine analogues (agonists)</td>
</tr>
<tr>
<td>IB-MECA</td>
</tr>
<tr>
<td>CCPA</td>
</tr>
<tr>
<td>NECA</td>
</tr>
<tr>
<td>R-PIA</td>
</tr>
<tr>
<td>CGS 21680</td>
</tr>
<tr>
<td>Xanthine antagonists</td>
</tr>
<tr>
<td>I-ABOPX</td>
</tr>
<tr>
<td>BWA 1433</td>
</tr>
<tr>
<td>CPX</td>
</tr>
<tr>
<td>XAC</td>
</tr>
<tr>
<td>8-SPT</td>
</tr>
<tr>
<td>ZM 241385</td>
</tr>
<tr>
<td>Adenine antagonists</td>
</tr>
<tr>
<td>N-0861</td>
</tr>
<tr>
<td>WRC 0571</td>
</tr>
<tr>
<td>Nonxanthine/nonadenine antagonists</td>
</tr>
<tr>
<td>MRS 1191</td>
</tr>
<tr>
<td>MRS 1220</td>
</tr>
<tr>
<td>L 249313</td>
</tr>
<tr>
<td>Antiasthmatic antagonists</td>
</tr>
<tr>
<td>Theophylline</td>
</tr>
<tr>
<td>Enprofylline</td>
</tr>
</tbody>
</table>

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.
its predicted $K_d$ value for A1ARs and A3ARs, respectively. In addition, 100 nmol/L ZM 241385 was included in all of the assays to inhibit binding to A3ARs, and 500 nmol/L CPX was included in assays of spleen tissue to selectively block A1ARs. Both CCPA and IB-MECA concentration dependently competed for [125I]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 A1AR. 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 A3AR. The IC50 values of CCPA were calculated to be $0.32 \pm 0.06$ nmol/L for the high-capacity binding site in brain (A1AR) and $50.1 \pm 1.8$ nmol/L for the binding site in spleen (A1AR). The IC50 values for IB-MECA were calculated to be $0.78 \pm 0.26$ nmol/L for the single binding site in spleen (A3AR) and $10.1 \pm 2.2$ nmol/L for the high-capacity binding site in brain (A1AR). These data demonstrate that CCPA binds selectively to endogenously expressed rabbit A1ARs (≈160-fold selective) and that IB-MECA binds potently and with moderate selectivity (≈13-fold selective) to endogenously expressed rabbit A3ARs. 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 A1ARs and A3ARs (Figure 3). The EC50 values of CCPA and IB-MECA were calculated to be $0.05 \pm 0.03$ and $59.0 \pm 12.2$ nmol/L, respectively, for HEK 293 cells expressing rabbit A1ARs and $10.3 \pm 4.1$ and $0.14 \pm 0.08$ nmol/L, respectively, for HEK 293 cells expressing rabbit A3ARs. CGS 21680 weakly acted on rabbit A1ARs (EC50=$821 \pm 98.2$ nmol/L) and A3ARs (EC50=$116 \pm 24.6$ nmol/L). These results demonstrate that CCPA and IB-MECA are functional agonists of rabbit A1ARs and A3ARs. 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 A1AR were surmountably blocked by the addition of 10 or 50 μmol/L N-0861 (Figure 3; $K_d=45 \pm 19$ 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 A3AR. Thus, N-0861 acts as a competitive and selective antagonist of A1ARs.

**Detection of A1AR and A3AR Message by RT-PCR**

No bands corresponding to the A1AR or A3AR 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 A1AR (310 bp) and A3AR (415 bp) were detected in both heart tissue and isolated cardiomyocytes by Southern blotting of the RT-PCR reactions and probing with A1AR- or A3AR-specific cDNA radioprobes. These results suggest that both A1AR and A3AR 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.
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 3. Inhibition of isoproterenol-stimulated adenyl cyclase activity in HEK 293 cells transfected with either the rabbit A1AR or A3AR cDNAs. A and B, Effect of CCPA, CGS 21680, and IB-MECA on rabbit A1ARs (A) or A3ARs (B). C and D, Effect of 10 and 50 μmol/L N-0861 on the inhibitory effect of CCPA or IB-MECA on rabbit A1ARs (C) and A3ARs (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 b
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 (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 A1AR 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 A2AARs 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-0861 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 A1ARs 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 (KATP) 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).

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).
The results presented herein demonstrate that, in conscious rabbits, activation of either A1 ARs or A3 ARs (but not A2A 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 A3 AR subtype, in addition to the A1 AR subtype, is capable of triggering the development of late PC, that this effect of the A3 AR is mediated by KATP channels, and that NOS plays a differential role in A1AR-versus A3AR-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 A1 AR agonist CCPA can induce late PC against myocardial infarction, while studies in conscious rabbits suggest that CCPA cannot induce late PC against myocardial stunning. 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, 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 A3 AR is capable of inducing late PC against infarction. Our approach was to compare the effects of CCPA to those of the recently characterized A3 AR agonist IB-MECA. 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 A3 AR 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 A3 ARs compared with rabbit A1 ARs (Table and Figure 2), raises the possibility that IB-MECA may have induced late PC not by interacting with A3 ARs, but rather through nonspecific interactions with the A1 AR. This possibility cannot be addressed using an A3 AR-selective inhibitor because no antagonist is currently available that selectively inhibits rabbit A3 ARs (Table). Therefore, we performed two additional sets of experiments. First, we administered IB-MECA or CCPA in the presence of the A1 AR antagonist N-0861 given at a dose predicted to produce blood levels (20 to 50 μmol/L) that do not block the A3 AR. The finding that N-0861 blocked the actions of CCPA against infarction but not those of IB-MECA (Figure 6) further corroborates the conclusion that activation of A3 ARs in itself induces late PC, indepen-
dent of A1AR activation. The possibility that IB-MECA produced late PC by acting through A2A ARs can be excluded because the selective A2AR agonist CGS 21680 did not reduce infarct size (Figure 6). We can also exclude the possibility that IB-MECA acted through A3ARs, 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 A2ARs.

Although current data indicate that A1ARs can induce both phases of PC, in recent in vivo studies we have observed that mice with genetic disruption of A3ARs exhibit infarcts that are smaller than those in wild-type mice,17 raising the interesting possibility that A3ARs may actually play an injurious role during acute myocardial ischemia in this species. These observations are not in conflict with the idea that A2ARs can trigger PC, since the role of A2ARs 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 A1AR knockout mice need to be confirmed with the use of A2AR-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 A2ARs 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 isofrom of NOS (iNOS).18 Additional studies have demonstrated that the cardioprotective effects of CCPA-induced late PC can be blocked by inhibitors of the KATP 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 KATP channels. We found that the nonselective NOS inhibitor L-NA and the KATP 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 KATP channels. IB-MECA–induced late PC also appears to utilize a mechanism requiring KATP channels; however, upregulation of NOS is not a necessary component. Thus, the results suggest that the mechanisms by which A2ARs and A3ARs induce late PC against infarction involve different pathways that ultimately converge on the KATP channel.

Elucidation of the mechanisms by which ARs modulate PC requires knowledge of the cell types within the heart that express A1ARs and A3ARs. It is well established that A1ARs coupled to inhibition of adenyl cyclase via Gβγ 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 A1ARs induce delayed PC via signaling pathways involving protein kinase C, tyrosine kinases, and mitogen-activated/stress-activated protein kinases.22 With regard to the A3AR, however, little is known regarding the specific cell types within the heart that express this receptor subtype. Strickler et al10 recently demonstrated that A3ARs 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 A3ARs, which may induce PC via similar signaling pathways as the A1AR. This hypothesis is supported by the results of the present investigation in which we were able to detect A3AR mRNA in adult rabbit cardiomyocytes by RT-PCR analysis (Figure 4). The cell types within the heart that express A3ARs may not be limited to cardiomyocytes, however. For example, in other tissues, the A3AR is known to be expressed in resident leukocytes such as macrophages and mast cells.12 A3ARs have also been suggested to be expressed in vascular smooth muscle cells and endothelial cells.23 Based on these observations, it remains possible that A3AR agonists may elicit PC through the release of mediators from nonmyocytic cells. Clearly, additional studies of A3ARs 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 A1ARs or A3ARs can induce a late PC effect. Because A1AR agonists can provide protection with minimal hemodynamic effects, these results imply that targeting the A1AR could be a novel and useful approach to the protection of the ischemic myocardium. Finally, the present study elucidates the molecular mechanisms of A1AR- and A3AR-induced late PC by identifying common and differential roles for the KATP channel and NOS, respectively, in these two forms of delayed cardioprotection.

Acknowledgments

This study was supported in part by National Institutes of Health Grants R01 HL0051 (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.

References


A1 or A3 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 and John A. Auchampach

*Circ Res.* 2001;88:520-528
doi: 10.1161/01.RES.88.5.520

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/88/5/520

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2001/03/12/88.5.520.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org/subscriptions/
ON-LINE MATERIALS AND METHODS

Materials

Phthaloblue dye was purchased from Heucotech; IB-MECA, $N^6$-aminobenzyladenosine-5'-N-methylcarboxamide (AB-MECA), $N^6$-(3-iodo-4-aminobenzyl)adenosine-5'-N-methylcarboxamide (I-AB-MECA), $N^6$-phenylisopropyladenosine (R-PIA), adenosine-5’-N-ethylcarboxamide (NECA), 2-chloro-$N^6$-cyclopentyladenosine (CCPA), CGS 21680, 8-cyclopentyl-1,3-dipropylxanthine (CPX), 8-((4-((2-aminoethyl)aminocarbonyl)methoxy)phenyl)-1,3-dipropylxanthine (XAC), 8-$p$-sulfophenyltheophylline (8-SPT), MRS 1220, MRS 1191, theophylline, enprofylline, and sodium 5-hydroxydecanoate (5-HD) were from Research Biochemicals Incorporated; ZM 243185 was from Tocris; N-0861 and WRC 0571 were gifts from Dr. Pauline Martin (Discovery Therapeutics); 3-(4-amino-3-iodobenzyl)-8-oxycacetate-1-propylxanthine (I-ABOPX) and BWA 1433 were gifts from Dr. Susan Daluge (Glaxo-Wellcome); L-249313 was a gift from Dr. Marlene Jacobson (Merck); $[125]$AB-MECA, Na$^{125}$I, and cAMP radioimmunoassay kits were from Amersham; lipofectamine, taq DNA polymerase, and G-418 were from Gibco BRL; pcDNA3 was from Promega; VENT® DNA polymerase was from New England Biolabs; avian myeloblastosis virus reverse transcriptase, oligo dT primers, and dNTPs were from Invitrogen; adenosine deaminase was from Boehringer-Mannheim Biochemicals; glass fiber filters (GF/C) were from Whatman; human embryonic kidneys cells (HEK 293 cells) were from the American Type Tissue Core facility; the rabbit brain cDNA library was from Clonetech, and oligonucleotides were synthesized by Genosys. All other reagents were purchased from Sigma Chemical Co.

Cloning of the Rabbit $A_3$AR and Pharmacological Characterization of Rabbit $A_1$AR and $A_3$ARs

Molecular Cloning

The rabbit $A_1$AR cDNA has been isolated previously by Bhattacharya et al from rabbit brain and genomic libraries. Although the rabbit $A_3$AR has been cloned previously, a PCR-based method was used to isolate this cDNA and this technique often results in errors due to
incorporation of aberrant nucleotides by taq polymerase. Therefore, we proceeded to isolate the rabbit A3AR from a rabbit brain cDNA library by plaque filter hybridization using a rabbit-specific probe generated by RT-PCR. The probe was a 550 base pair fragment that corresponds to the region spanning the first through the third transmembrane regions. The clone that we isolated, designated rabA311, is 1187 base pairs in length consisting of 227 base pairs of the 5' non-coding region and 960 base pairs of the coding region (the entire sequence can be obtained from Genbank under accession number AF14538). Two nucleotide differences were identified when the sequence of rabA311 were compared to the sequence of the rabbit A3AR cDNA that was reported previously;² both of these differences were located within the coding region at positions 797 and 837 relative to the start codon. Only the nucleotide difference at position 837 resulted in a change in the amino acid sequence: rabA311 encodes a histidine at amino acid 266 located in the seventh transmembrane region whereas a lysine was previously reported to be encoded at this site. To determine the predominant sequence of the rabbit A3AR, we PCR amplified using a high-fidelity taq DNA polymerase (VENT® DNA polymerase, New England Biolabs), subcloned, and sequenced the carboxyl half of the rabbit A3AR from genomic DNA isolated from rabbit spleen. A sequence encoding a histidine at position 266 was observed in DNA clones isolated from three separate rabbits.

Radioligand binding:

Membranes were prepared from HEK 293 cells stably expressing rabbit A1, or A3ARs. The full-length rabbit A1 and A3AR cDNAs were subcloned into the mammalian expression vector pCDNA3 and stably expressed in HEK 293 cells after transfection with lipofectamine and selection in 2 mg/ml G-418. Transfected cells were washed in phosphate-buffered saline, homogenized in buffer A (10 mM Na-HEPES [pH 7.4], 10 mM EDTA, and 0.1 mM benzamidine) and then centrifuged at 35,000 x g at 4°C for 25 minutes. Pellets were resuspended and washed twice in buffer B (10 mM Na-HEPES [pH 7.4], 1 mM EDTA, and 0.1 mM benzamidine) and then resuspended in buffer B containing 10% sucrose at a protein concentration of 1 mg/ml. Protein
concentrations were determined using fluorescamine and BSA standards. Membranes were frozen in aliquots and stored at -80°C. Membranes were also prepared from rabbit brain and spleen using an identical protocol.

Radioligand binding assays were performed by incubating cell membranes (HEK 293 cells, 50 μg; rabbit tissues, 200 μg) for 3 hours at 21°C in buffer B (0.1 ml) containing 5 mM MgCl₂, 5 units/ml adenosine deaminase, and varying concentrations of [³²P]AB-MECA (specific activity = 1,000 Ci/mmol). [³²P]AB-MECA was either purchased from Amersham or prepared by radioiodination of AB-MECA by the chloramine-T method and purification by reverse-phase HPLC, as described by Olah et al. After a 3-hour incubation period at 21°C, the bound and free radioligand were separated by rapid filtration over glass fiber filters; the radioactivity trapped by the filters was measured in a γ counter. For equilibrium binding assays, 6-8 concentrations of [³²P]AB-MECA were added to the incubations. To achieve high concentrations of [³²P]AB-MECA necessary to achieve saturation, the specific activity of the radioligand was diluted 10-20-fold by addition of the non-radiolabeled compound. For competition binding studies, ~ 0.3-6 nmol/L [³²P]AB-MECA was added to the reactions and competing drugs were included over a range of concentrations. The reactions all contained 1% (vol/vol) of dimethylsulfoxide (DMSO). For all studies, non-specific binding was defined by use of 10 μmol/L I-AB-MECA.

Specific binding of [³²P]AB-MECA to recombinant rabbit A₁ and A₂ARs expressed in HEK 293 cells fit optimally to a single site-binding model using Marquardt's non-linear least squares interpolation; from this analysis, dissociation constants (Kₐ) and receptor densities (Bₘₐₓ) were determined. IC₅₀ values of compounds in the competition studies were fit to the equation: Bound = Bₘₐₓ * [I] / (IC₅₀ + [I]), where Bₘₐₓ is specific binding in the absence of the inhibitor and [I] is the concentration of inhibitor. Kᵢ values were calculated from IC₅₀ values, Bₘₐₓ values, the concentration of radioligand, and the Kₐ value, as described previously by Linden. In competition binding studies with rabbit brain tissue and [³²P]AB-MECA, two affinity states were
detected. IC_{S0} values for both affinity states were fit to the equation: \[ \text{Bound} = \left( B_{\text{max}} - (f \times B_{\text{max}}) \right) \times \frac{[I]}{[I]_{501} + [I]} - \left( 1 - f \right) \times \frac{[I]}{[I]_{502} + [I]}, \] where \( B_{\text{max}} \) is specific binding in the absence of inhibitor, \( f \) is the fraction of radioligand bound to the high affinity site, \( 1 - f \) is the fraction of radioligand bound to the low affinity site, \([I]\) is the concentration of inhibitor, and \( [I]_{501} \) and \( [I]_{502} \) are the IC_{S0} values for the two states.

**cAMP assays:**

Cyclic AMP assays were performed on HEK 293 cells transfected with rabbit A_{1} or A_{3}ARs using isoproterenol as the stimulant as described previously.\textsuperscript{7} Cells grown to confluency on 150 mm\textsuperscript{2} cell culture plates were detached by treatment with 5 mM EDTA in PBS for 5-10 min, washed twice with serum-free DMEM buffered with 25 mM HEPES (pH 7.4), and then resuspended in DMEM/HEPES media containing 5 units/ml adenosine deaminase and 20 \( \mu \)M Ro 20-1724 (phosphodiesterase inhibitor). The cells were transferred to polystyrene test tubes in 0.2 ml aliquots (50,000 cells/tube) and then prewarmed to \( 37^\circ \) C for 5 minutes in the presence of antagonists or vehicle. Cells were stimulated by the addition of 5x isoproterenol (50 \( \mu \)mol/L isoproterenol in 50 \( \mu \)l aliquots) in combination with adenosine agonists or vehicle, and then incubated for 15 minutes in a 37\(^{\circ}\) C shaking water bath. Assays were terminated by the addition of 0.5 ml of 0.15 N HCl. cAMP in the acid extracts was quantitated using a radioimmunoassay kit. EC_{S0} values were fit to the equation: \[ E = E_{\text{min}} + \left( E_{\text{max}} - E_{\text{min}} \right) \times \left( 1 + 10^{(C - \log EC_{50})} \right), \] where \( C \) is the concentration of the adenosine agonist, \( E \) is the response, \( E_{\text{max}} \) is the maximal response, \( E_{\text{min}} \) is the minimal response.

**RT-PCR**

Real-time PCR/Southern blotting was performed with total RNA isolated from adult rabbit heart tissue and from \( \sim 100-200 \) adult rabbit ventricular cardiomyocytes. The cardiomyocytes were obtained by picking individual cells from cell suspensions under microscopic inspection after perfusing adult rabbit hearts with collagenase.\textsuperscript{8} Extreme care was taken to pick single ventricular myocytes that were digested free from contaminating cells. For the RT-PCR reaction, 1 \( \mu \)g of
total RNA from heart tissue or the entire amount of total RNA obtained from 100-200 ventricular cardiomyocytes were reverse-transcribed using oligo dT primers and then amplified (50 µl volume; 30-35 cycles) using sequence-specific primers for either the rabbit A₁ or A₂AR. The primers for amplification were the following: A₁AR forward primer (sense 295-314), 5'-TGTTCCTCATCTCACCAGA-3'; A₁AR reverse primer (antisense 586-605), 5'-GCACCCACACGAAGTTGTTG-3'; A₂AR forward primer (sense 85-104), 5'-GAGATTGTACTCGGAGTCTC-3'; A₂AR reverse primer (antisense 481-500), 5'-ACTCCAGGGTGTGCTTCATA-3'. Importantly, for both receptors the primers span the single intron within the coding region located in the second intracellular loop,¹ ⁹ which allows for discrimination between amplification of cDNA and genomic DNA based on the size of the products. Amplification of the A₁AR cDNA yields a 310 bp product and amplification of A₂AR cDNA yields a 415 bp product. Each reaction cycle consisted of incubation at 95° C for 1 minute, 60° for 1 minute, and 72° C for 2 minutes with 0.02 unit/ml taq polymerase. Subsequently, 25 µl of the PCR reaction was electrophoresed and transferred to nylon membranes using denaturing buffer (0.4 N NaOH) and hybridized with specific probes labeled with ³²P. Filters were washed under stringent conditions and exposed to X-ray film. Negative controls consisted of reactions in which water was added instead of RNA, and positive controls consisted of reactions containing 1 µg of total RNA obtained from HEK 293 cells expressing rabbit A₁ or A₂ARs.

**Studies in Conscious Rabbits (Phase II)**

A total of 129 rabbits were used in these experiments. The study was performed in accordance with the guidelines of the Animal Care and Use Committee of the University of Louisville School of Medicine and the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, publication No. [NIH] 86-23).

**Conscious rabbit preparation:**

The conscious rabbit model of myocardial ischemia and reperfusion is as described previously.¹⁰⁻¹³ Briefly, male New Zealand White rabbits (2.2-2.9 kg) were instrumented under
sterile conditionings with placement of a balloon occluder around a major branch of the left coronary artery and bipolar ECG leads on the chest wall. For some studies, a Doppler thickening crystal was sutured to the epicardial surface of the region at risk to measure wall thickening.10-13 The chest wound was closed in layers, and a small tube was left in the thorax for three days to aspirate air and fluids postoperatively. Gentamicin was administered prior to surgery and on the first and second postoperative days (0.7 mg/kg i.m. each day). The rabbits were allowed to recover for a minimum of 10 days after surgery.

*Experimental protocols for ischemia and reperfusion:*

Throughout the experiments, rabbits were kept in a quiet, dimly-lit room. In the studies of myocardial infarction, rabbits were pretreated with diazepam (1 mg/kg) 20 minute before the occlusion to relieve stress caused by the procedure. The ECG were recorded throughout the experiments on a thermal array chart recorder (Gould TA6000). During and after treatment with adenosine receptor agonists, arterial pressure was measured by cannulating the dorsal ear artery with a 22-gauge angiocatheter under local anesthesia (benzocaine), as described previously.10-13

The experimental protocol is depicted in Fig. 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 was measured by dual staining with phthalo blue dye and triphenyltetrazolium hydrochloride.10-13 Rabbits were randomly assigned to twelve treatment groups. Group I (control group) received 1 ml of vehicle (1:1 solution of 0.9% saline and DMSO) as an i.v. bolus 24 hours before the occlusion. In groups II, III, IVa, and IVb, CCPA (A1 agonist; 100 μg/kg in 1 ml of 0.9% saline/DMSO solution), CGS-21680 (A2a agonist; 100 μg/kg in 1 ml of 0.9% saline/DMSO solution), IB-MECA (A3 agonist; 100 μg/kg in 1 ml of 0.9% saline/DMSO solution), IB-MECA IB-MECA (300 μg/kg) were given as boluses 24 hours before the coronary occlusion. After observing a delayed cardioprotective effect following administration of CCPA and IB-MECA (but
not CGS 21680), two additional groups of rabbits were studied to determine whether the A1
receptor antagonist N-0861 affects the cardioprotective effects of CCPA or IB-MECA. 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 0.25 mg/kg/minute which was maintained for 3 hours;
total dose, 52.5 mg/kg); five minutes after the bolus injection of N-0861, the rabbits were
subsequently treated with either CCPA (100 μg/kg iv bolus) or IB-MECA (300 μg/kg iv bolus).
Finally, six additional groups of rabbits were studied to determine whether L-NA, an inhibitor of all
three NOS isozymes, or 5-HD, an inhibitor of the K\textsubscript{ATP} channel that is selective for the
mitochondrial isoform,\textsuperscript{14} blocks the delayed protective effects of CCPA or IB-MECA. Groups VII,
VIII, and IX were given L-NA (13 mg/kg as an iv bolus) and groups X, XI, and XII were given 5-
HD (5 mg/kg iv bolus) five 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). The doses of L-NA and 5-HD that were used
in the study have been demonstrated previously to be effective at blocking nitric oxide synthases
and K\textsubscript{ATP} channels, respectively, in the rabbit.\textsuperscript{10, 11, 15, 16}

Measurement of region at risk and infarct size:

At the conclusion of the study, the rabbits were given heparin (1000 U IV), after which they
were anesthetized with sodium pentobarbital (50 mg/kg i.v.) and then euthanized with KCl. The
hearts were excised, and the size of the ischemic/reperfused region was determined by tying the
coronary artery at the site of the original occlusion and perfusing the aortic root for 2 minutes with
a 5% solution of phthalocyanine dye in normal saline at a pressure of 70 mm Hg using a Langendorff
apparatus. With this technique, the non-ischemic portion of the left ventricle is stained dark blue,
whereas the region at risk remains unstained. The hearts were then frozen at -20°C for 15
minutes and then cut into six or seven transverse slices, which were incubated at 37°C in a 1%
solution of triphenyltetrazolium chloride in phosphate buffer (pH 7.4). All atrial and right
ventricular slices were weighed, fixed in 10% formaldehyde solution, and photographed. For
each slice, the image was magnified 20 times, traced, scanned, and the areas of the infarcted,
ischemic-reperfused, and non-ischemic regions were measured using a software program (Sigmascan); from these measurements, the region at risk was calculated as a percent of the left ventricle, and infarct size was calculated as a percentage of the region at risk.\textsuperscript{10, 13}

**Statistical Analysis**

Data are reported as means ± SEM. Differences in hemodynamic variables in the conscious rabbit studies were analyzed by a two-way repeated-measures ANOVA (time and drug treatment) to determine whether there was a main effect of time, a main effect of treatment, or a time-treatment interaction. If global tests showed a main effect or interaction, post hoc tests between time points or treatments were performed with Student's t tests for unpaired or paired data, as appropriate, with the Bonferroni correction. All of the other data were compared using a one-way ANOVA with the Student's t test and the Bonferroni correction.
ON-LINE REFERENCES


6. Linden J. Calculating the dissociation constant of an unlabeled compound from the concentration required to displace radiolabel by 50%. *J Cyclic Nucleotide Res.*1982;163-172.


Supplemental Data Table 1. Exclusions in the conscious rabbit studies.

<table>
<thead>
<tr>
<th>Group</th>
<th>Assigned</th>
<th>Excluded</th>
<th>Analyzed</th>
<th>Reasons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (control)</td>
<td>12</td>
<td>2</td>
<td>10</td>
<td>fibrillation, small risk region</td>
</tr>
<tr>
<td>Group II (CCPA)</td>
<td>11</td>
<td>1</td>
<td>10</td>
<td>technical problem</td>
</tr>
<tr>
<td>Group III (CGS 21680)</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>fibrillation (2), technical problem (1)</td>
</tr>
<tr>
<td>Group IVa (IB-MECA 100 mg/kg)</td>
<td>10</td>
<td>2</td>
<td>8</td>
<td>fibrillation, small risk region</td>
</tr>
<tr>
<td>Group IVb (IB-MECA 300 μg/kg)</td>
<td>15</td>
<td>5</td>
<td>10</td>
<td>fibrillation (3), small risk region (2)</td>
</tr>
<tr>
<td>Group V (CCPA+N-0861)</td>
<td>7</td>
<td>1</td>
<td>6</td>
<td>fibrillation</td>
</tr>
<tr>
<td>Group VI (IB-MECA+N-0861)</td>
<td>10</td>
<td>1</td>
<td>9</td>
<td>fibrillation</td>
</tr>
<tr>
<td>Group VII (CCPA+L-NA)</td>
<td>8</td>
<td>1</td>
<td>7</td>
<td>fibrillation</td>
</tr>
<tr>
<td>Group VIII (IB-MECA+L-NA)</td>
<td>7</td>
<td>0</td>
<td>7</td>
<td>fibrillation</td>
</tr>
<tr>
<td>Group IX (L-NA)</td>
<td>9</td>
<td>1</td>
<td>8</td>
<td>fibrillation</td>
</tr>
<tr>
<td>Group X (CCPA+5-HD)</td>
<td>11</td>
<td>4</td>
<td>7</td>
<td>fibrillation (3), technical problem (1)</td>
</tr>
<tr>
<td>Group XI (IB-MECA+5-HD)</td>
<td>9</td>
<td>2</td>
<td>7</td>
<td>fibrillation (2)</td>
</tr>
<tr>
<td>Group XII (5-HD)</td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>fibrillation (3)</td>
</tr>
</tbody>
</table>
Table 2. Heart rate on day 2 at baseline, during occlusion, and during the 72 hour reperfusion period.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Pre-occlusion (15 min)</th>
<th>Occlusion</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>Group I (control)</td>
<td>242±8</td>
<td>242±8</td>
<td>256±6</td>
<td>254±9</td>
</tr>
<tr>
<td>Group II (CCPA)</td>
<td>256±7</td>
<td>256±7</td>
<td>265±6</td>
<td>270±8</td>
</tr>
<tr>
<td>Group III (CGS 21680)</td>
<td>252±7</td>
<td>252±7</td>
<td>267±10</td>
<td>259±1</td>
</tr>
<tr>
<td>Group IVa (IB-MECA 100 µg/kg)</td>
<td>241±13</td>
<td>241±13</td>
<td>266±13</td>
<td>264±11</td>
</tr>
<tr>
<td>Group IVb (IB-MECA 300 µg/kg)</td>
<td>255±6</td>
<td>255±6</td>
<td>255±7</td>
<td>251±7</td>
</tr>
<tr>
<td>Group V (CCPA+L-NA)</td>
<td>240±9</td>
<td>200±11*</td>
<td>198±8*##</td>
<td>201±9*#</td>
</tr>
<tr>
<td>Group VI (IB-MECA+L-NA)</td>
<td>244±9</td>
<td>197±8*##</td>
<td>192±8*##</td>
<td>197±9*##</td>
</tr>
<tr>
<td>Group VII (L-NA)</td>
<td>250±5</td>
<td>199±5*##</td>
<td>187±5*##</td>
<td>192±7*##</td>
</tr>
</tbody>
</table>

Beats/minute (means ± SEM)

*P<0.05 versus baseline value.  #P<0.05 versus group I (control).
Supplemental Data Table 3. Risk region and infarct sizes in the conscious rabbit studies.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>N</th>
<th>Area at Risk Size (percent of left ventricle)</th>
<th>Infarct Size (percent of area at risk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>(control)</td>
<td>10</td>
<td>15.8 ± 1.3</td>
<td>56.4 ± 4.0</td>
</tr>
<tr>
<td>Group II</td>
<td>(CCPA)</td>
<td>10</td>
<td>18.5 ± 2.3</td>
<td>38.3 ± 4.1*</td>
</tr>
<tr>
<td>Group III</td>
<td>(CGS 21680)</td>
<td>7</td>
<td>18.9 ± 1.4</td>
<td>61.4 ± 5.5</td>
</tr>
<tr>
<td>Group IVa</td>
<td>(IB-MECA 100 µg/kg)</td>
<td>8</td>
<td>18.0 ± 2.7</td>
<td>31.8 ± 2.8*</td>
</tr>
<tr>
<td>Group IVb</td>
<td>(IB-MECA 300 µg/kg)</td>
<td>10</td>
<td>17.6 ± 1.6</td>
<td>33.7 ± 4.9*</td>
</tr>
<tr>
<td>Group V</td>
<td>(CCPA+N-0861)</td>
<td>6</td>
<td>18.6 ± 1.7</td>
<td>59.8 ± 3.8</td>
</tr>
<tr>
<td>Group VI</td>
<td>(IB-MECA+N-0861)</td>
<td>9</td>
<td>16.7 ± 1.7</td>
<td>41.8 ± 1.9*</td>
</tr>
<tr>
<td>Group VII</td>
<td>(CCPA+L-NA)</td>
<td>7</td>
<td>18.5 ± 2.3</td>
<td>57.7 ± 4.1</td>
</tr>
<tr>
<td>Group VIII</td>
<td>(IB-MECA+L-NA)</td>
<td>7</td>
<td>19.9 ± 3.3</td>
<td>36.6 ± 3.4*</td>
</tr>
<tr>
<td>Group IX</td>
<td>(L-NA)</td>
<td>8</td>
<td>20.1 ± 1.9</td>
<td>55.2 ± 4.4</td>
</tr>
<tr>
<td>Group X</td>
<td>(CCPA+5-HD)</td>
<td>7</td>
<td>17.0 ± 1.7</td>
<td>54.8 ± 5.8</td>
</tr>
<tr>
<td>Group XI</td>
<td>(IB-MECA+5-HD)</td>
<td>7</td>
<td>17.8 ± 1.8</td>
<td>55.0 ± 4.4</td>
</tr>
<tr>
<td>Group XII</td>
<td>(5-HD)</td>
<td>7</td>
<td>17.0 ± 1.8</td>
<td>52.0 ± 5.5</td>
</tr>
</tbody>
</table>

All data are the means ± SEM.

*P<0.05 versus group I.