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Prolonging the Delayed Phase of Myocardial Protection: Repetitive Adenosine A_1 Receptor Activation Maintains Rabbit Myocardium in a Preconditioned State

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Objectives. **This study was designed to examine whether the myocardium can be maintained in a protected state by extending the delayed phase of cardioprotection with chronic, intermittent adenosine A_1 receptor activation.**

Background. **Several recent studies have explored the temporal characteristics of the protective effects of ischemic preconditioning. Two distinct phases of myocardial protection have been described: the short-lived immediate phase, or "classic" preconditioning, and the delayed phase, or "second window of protection" (SWOP). Previous studies have examined the potential for extending the duration of classic preconditioning by repeated application of the preconditioning stimulus. Pretreatment with either multiple episodes of ischemia or continuous infusion of a selective adenosine A_1 receptor agonist, 2-chloro-N^6-cyclopentyladenosine (CCPA), resulted in attenuation of the protective effects of preconditioning, implying downregulation of the receptors involved in triggering classic preconditioning.**

Methods. **Male New Zealand White rabbits were treated with repeated intravenous boluses of CCPA, 100 μg/kg body weight, or 0.9% saline at 48-h intervals. Forty-eight hours after the fifth dose (day 10), the animals were anesthetized and subjected to 30 min of coronary occlusion, followed by 120 min of reperfusion. Infarct size was determined as a percentage of myocardial risk volume using tetrazolium staining. To further explore whether the rabbits had developed tolerance to the effects of adenosine A_1 receptor activation, a subgroup of animals were treated with a further bolus of CCPA, 100 μg/kg, at the end of the reperfusion period, and the hemodynamic response was monitored for 10 min before excision of the heart.**

Results. **Pretreatment with intermittent doses of CCPA resulted in a 42% reduction in the infarct to risk ratio compared with vehicle pretreatment (26.6 ± 3.7% vs. 45.9 ± 5.5%, $p < 0.01$). Furthermore, CCPA treatment at the end of reperfusion resulted in identical hypotension and bradycardia in both groups.**

Conclusions. **We conclude that rabbits can be maintained in a protected state against myocardial infarction by repeated activation of adenosine A_1 receptors, with no evidence of tachyphylaxis to the infarct-limiting or hemodynamic effects of CCPA. This finding suggests that adenosine A_1 receptor activation may hold promise as a new approach to long-term cardioprotection.**

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Ischemic preconditioning (IPC), defined as enhanced myocardial resistance to ischemic necrosis after brief periods of ischemia and reperfusion (1), has been recognized as the most powerful experimental means of attaining myocardial protection. It is now established in most animal species studied that IPC induces a biphasic pattern of myocardial protection. An immediate period of cardioprotection, "classic preconditioning," lasts ~60 to 120 min after the preconditioning stimulus and is then lost (2,3). This is followed, after a delay of 12 to 24 h, by a distinct phase of enhanced tolerance to ischemia (4–7), which, although not as powerful as the early phase, is more prolonged and lasts up to 72 h (8–10). This delayed phase of resistance to ischemia has been termed the "second window of protection" (SWOP) (11).

The precise cellular mechanisms underlying the protective effects of preconditioning are not known. There are several lines of evidence suggesting a role for a number of endogenous paracrine mediators acting as triggers of both phases of myocardial protection (12). In particular, endogenous adenosine, released by myocytes and vascular endothelium during periods of ischemia or acting on adenosine A_1 and A_3 receptors, has been implicated as a trigger of both phases of myocardial protection after IPC. Blockade of adenosine receptors during preconditioning has been reported to abolish both the early (13) and the late (14) cardioprotective effects of IPC. Furthermore, substitution of the IPC with intravenous admin-

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Abbreviations and Acronyms

CCPA = 2-chloro-N^6-cyclopentyladenosine
ECG = electrocardiogram, electrocardiographic
HR = heart rate
I = infarct volume
IPC = ischemic preconditioning
R = risk zone volume
RPP = rate–pressure product
SBP = systolic blood pressure
SWOP = second window of protection
TTC = triphenyltetrazolium chloride

istration of selective adenosine A_1 receptor agonists induces both early (15,16) and late (14) protection against infarction.

All these data point to a therapeutic potential for adenosine A_1 receptor agonists in ischemic heart disease. However, the main shortcoming of such therapy is that it would have to be given as a pretreatment to patients at risk of coronary thrombosis. Such pretreatment could be achieved, however, if the duration of the protection afforded by preconditioning was extended, thereby maintaining the myocardium in a preconditioned state over a long period. A recent study by Tsuchida et al. (17) addressed the possibility of maintaining classic preconditioning by using a continuous infusion of 2-chloro-N^6-cyclopentyladenosine (CCPA) in a rabbit model of infarction, induced by 30 min of regional ischemia followed by 3 h of reperfusion. Rabbits subjected to a 6-h infusion of CCPA showed a 59% reduction in infarct size compared with the saline-treated rabbits. Infarction in a group receiving a 72-h infusion of CCPA, however, was the same as in the 72-h vehicle group. Furthermore, the protective effects of a 5-min ischemic preconditioning stimulus were attenuated after a 72-h infusion of CCPA. The group that received a 72-h infusion of saline, however, was preconditioned with 5 min of ischemia. These investigators concluded that myocytes become desensitized to the protective effects of CCPA with prolonged exposure, and that such tachyphylaxis also abolished the beneficial effects of IPC. In separate experiments, the same group examined the effect of multiple 5-min episodes of regional ischemia in a conscious, chronically instrumented rabbit model (18). Animals subjected to 40 to 65 five-minute coronary occlusions over a 3- to 4-day period showed a marked attenuation in infarct size limitation compared with the animals that had been preconditioned with a single 5-min occlusion.

Although these studies have partially addressed the question of prolonging the *early* phase of myocardial protection, as yet there are no reports of experiments exploring the possible extension of SWOP. We performed preliminary studies to confirm previous observations that delayed cardioprotection is induced after transient adenosine A_1 receptor activation. We then examined whether this delayed phase of myocardial protection can be maintained over a long period by long-term intermittent adenosine A_1 receptor activation. In particular, we wished to establish whether this schedule of long-term dosing would maintain or increase the degree of myocardial protection, or whether tachyphylaxis would result from cumulative dosing.

Methods

Male New Zealand White rabbits weighing 2.2 to 3.5 kg were used in these experiments. All procedures were in accordance with U.K. Home Office guidelines on the Animals (Scientific Procedures) Act of 1986, published by Her Majesty's stationery office. The animals were housed in individual cages and had free access to food and water throughout the preparation period.

Single-dose studies. Preliminary studies were performed to confirm our previous observations that a delayed phase of myocardial protection is induced after transient adenosine A_1 receptor activation (14). CCPA (Research Biochemicals Inc., through Semat, St. Albans, United Kingdom) was dissolved in sterile 0.9% sodium chloride injection *(British Pharmacopoeia)*. Conscious rabbits were randomized to receive a single bolus of CCPA (100 μg/kg body weight) or saline vehicle (0.5 ml) by intravenous injection through a marginal ear vein. Twenty-four or 48-h later, the animals were subjected to 30 min of regional myocardial ischemia and 120 min reperfusion in vivo, and the infarct size was determined as described subsequently (Fig. 1).

Long-term intermittent dosing study. On the basis of these preliminary studies and previous studies from our laboratory characterizing the time course of the delayed protection conferred by A_1 receptor activation (19), the schedule for intermittent dosing of animals was determined. Conscious rabbits were treated with repeated intravenous boluses of CCPA (100 μg/kg) or saline vehicle (0.5 ml) at 48-h intervals. The treatments were administered on a random basis and the animals were returned to their cages with no further manipulation between the treatments. Approximately 48 h after the fifth dose (day 10), the animals were subjected to an infarction procedure in vivo and the infarct size was measured (Fig. 1).

Infarction procedure. At the end of the pharmacologic preconditioning protocols, all animals were subjected to an acute myocardial infarction procedure. Rabbits were premedicated with Hypnorm, 0.15 ml/kg intramuscularly (obtained from Janssen Pharmaceuticals, containing fentanyl citrate [315 μg/ml] and fluanisone [10 mg/ml]) and anesthetized with pentobarbital sodium, 30 mg/kg intravenously. Thereafter, Hypnorm, 0.1 ml/kg intramuscularly, and pentobarbital sodium, 5 to 10 mg/kg intravenously, were administered as required to maintain surgical anesthesia. A midline incision was made in the neck and a tracheostomy was performed. The animals were intubated and mechanically ventilated with a positive-pressure respirator and room air supplemented with oxygen at a rate of 1 Hz (small animal ventilator, Harvard Apparatus). The right common carotid artery was cannulated with a rigid polyethylene cannula connected to a pressure transducer (Lectromed UK Ltd.) for periodic hemodynamic and arterial blood gas measurements. Tidal volume was adjusted as necessary to maintain arterial pH between 7.35 and

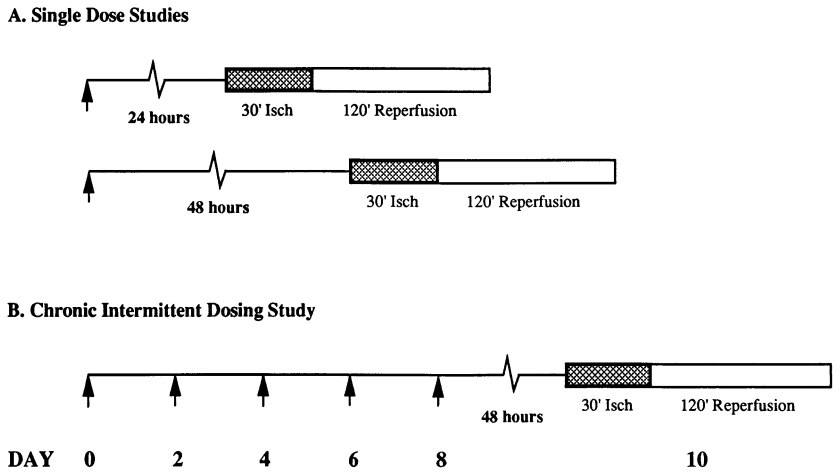


Figure 1. Protocol for pharmacologic preconditioning. **A,** In single-dose studies, the animals were treated with a single 100-μg/kg bolus of CCPA 24 or 48 h before the infarct procedure. **B,** In the long-term intermittent dosing study, the animals were treated at 48-h intervals over a period of 10 days with repeated boluses of CCPA and subjected to an infarction procedure on day 10. **Arrows** represent a single intravenous bolus of CCPA, 100 μg/kg, or saline vehicle. Isch = ischemia.

7.50. The core temperature was measured intermittently by a rectal thermometer and maintained at 38.5 ± 0.5°C by a heating pad. Electrocardiographic (ECG) leads were attached to limbs to obtain a single-channel ECG, which was used for continuous monitoring of heart rate, arrhythmias and ST segment changes. A median sternotomy was performed and the pericardium was incised to expose the heart. A 3-0 silk suture (Mersilk type 546, Ethicon) on an atraumatic needle was passed around a prominent anterolateral branch of the left coronary artery approximately half way between the left atrial appendage and the apex. The ends of the suture were threaded through a polypropylene tube to form a snare. Regional myocardial ischemia was induced by pulling the snare taut against the myocardium and clamping it into position. Ischemia was confirmed by the presence of regional left ventricular hypokinesia and epicardial cyanosis associated with ST segment elevation on the ECG. After 30 min of ischemia, the snare was released and reperfusion confirmed by conspicuous reactive hyperemia of the risk zone and return of ST segments to normal. The myocardium was reperfused for 120 min.

Risk zone and infarct size assessment. At the end of 120 min of reperfusion, the rabbits were anticoagulated with 500 IU of heparin intravenously. The animals were killed with an overdose of pentobarbital and the heart was excised and immediately attached to a Langendorff apparatus through the aortic root and retrogradely perfused with cold saline to remove blood. The coronary suture was ligated and the aortic root perfused with 2 to 4 ml of a 5-mg/ml suspension of 1 to 10 μm of zinc cadmium sulfide microspheres (Duke Scientific) to define the risk zone. Under ultraviolet light the tissue supplied by the occluded branch was nonfluorescent. The hearts were then weighed, frozen at −18°C and cut into 2-mm slices from apex to base perpendicular to the long axis of the heart. After defrosting, the slices were incubated at 37°C in a 1% solution of triphenyltetrazolium chloride (TTC) in phosphate buffer (pH 7.4) for 15 to 20 min and fixed for 24 to 48 h in 4% vol/vol formalin solution. TTC stains normal myocardium red, whereas infarct-related tissue appears pale or gray. The slices were traced on acetate sheets, infarcted areas were outlined and fluorescent and nonfluorescent areas were distinguished under ultraviolet light. The areas of infarct-related tissue (I) and myocardium at risk (R) were determined by using computerized planimetry (Summa Sketch II, Summa Graphics), and the corresponding volumes were calculated by multiplication of each area by slice thickness.

Hemodynamic effects of CCPA. The early hemodynamic effects of an intravenous bolus of CCPA, 100 μg/kg, in anesthetized rabbits have been described by us previously (14). To further explore whether rabbits pretreated with CCPA for 10 days had developed tolerance to the effects of adenosine A_1 receptor activation, a subgroup of animals (6 per group) were treated with a further bolus of CCPA, 100 μg/kg, at the end of the reperfusion period, and the hemodynamic response was monitored for 10 min before excision of the heart. This involved measurement of systolic blood pressure (SBP) and heart rate (HR) at baseline (at the end of 120 min of reperfusion) and at 1, 2, 3, 5 and 10 min after the CCPA bolus.

Statistical analysis. The data are presented throughout as mean values ± SEM. The significance of differences in mean values of I, R and I/R between the two treatment groups was evaluated by the Student unpaired *t* test. Any differences between hemodynamic variables at different time points was assessed by two-way analysis of variance with repeated measures, followed by the Fisher least significant differences test used post hoc for individual differences. The null hypothesis was rejected at $p \leq 0.05$.

Results

A total of 54 rabbits were used for these studies. Thirty were used for the single-dose studies and 24 for the long-term intermittent dosing study. In the single-dose studies, two rabbit hearts were excluded owing to intractable ventricular fibrillation during the infarct protocol (one in 48-h vehicle group and one in 24-h CCPA group); one was excluded owing to failure of the TTC stain (24-h CCPA group); and one was excluded

Table 1. Infarct Size Data for Single-Dose Studies

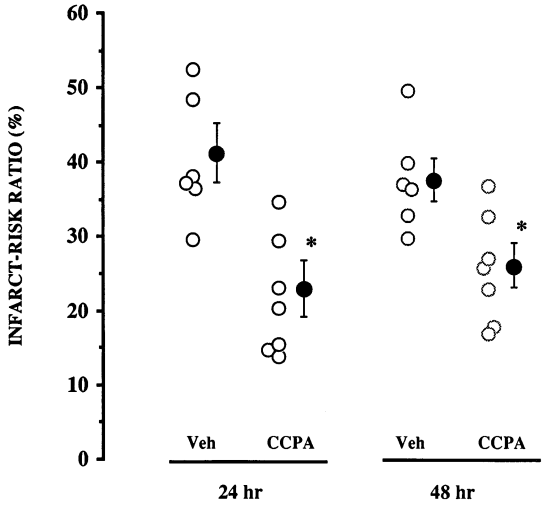
Group	No. of Animals	Body Weight (kg)	Risk Volume (cm^3)	Infarct Volume (cm^3)
Saline				
24 h	6	2.63 ± 0.22	0.94 ± 0.19	0.40 ± 0.09
48 h	6	2.39 ± 0.08	0.95 ± 0.15	0.36 ± 0.07
CCPA				
24 h	7	2.64 ± 0.15	1.07 ± 0.08	0.25 ± 0.05
48 h	7	2.49 ± 0.08	1.06 ± 0.08	0.29 ± 0.05

Data presented are mean value ± SEM. CCPA = 2-chloro-N^6-cyclopentyladenosine.

owing to absence of a risk zone (48-h CCPA group). In the long-term intermittent dosing study, four hearts were excluded: one owing to severe ventricular fibrillation during the infarction protocol, two owing to embolization at the time of microsphere infusion for risk zone determination and one owing to the coronary artery being punctured at the time of insertion of the suture (all in CCPA group). We therefore report data on 46 animals that successfully completed the two studies.

Single-dose studies. Table 1 represents the infarct size and risk volume data for rabbits pharmacologically preconditioned with CCPA at 24 and 48 h before the infarct procedure. Figure 2 represents the I/R ratios graphically. CCPA administration 24 h before infarction resulted in a reduction in the I/R ratio from 41.3 ± 4.4% in the vehicle group to 23.0 ± 3.9% in the CCPA group ($p < 0.05$). The corresponding values for the 48-h CCPA and vehicle groups were 37.7 ± 2.9% and 26.1 ± 3.0%, respectively ($p < 0.05$). There were no differences in hemodynamic variables (Table 2), body temperature or arterial pH (data not shown for brevity) at any time points between the various groups. These data confirm previous results from our laboratory indicating that a delayed phase of myocardial protection is induced 24 and 48 h after transient A_1 receptor activation (19).

Figure 2. Percent infarction of the risk zone in single-dose studies. The figure represents the reduction in infarct size 24 or 48 h after a single intravenous bolus of CCPA, compared with time-matched vehicle (Veh) treatment. **Open circles** indicate individual experiments; **solid circles** represent the mean value ± SEM for each group. *$p <$ 0.05 versus time-matched vehicle-treated group (unpaired Student *t* test).



Long-term intermittent dosing study. *Systemic hemodynamic changes during ischemia-reperfusion.* Table 3 summarizes the changes in HR and rate–pressure product (RPP) during the infarction protocol in animals pretreated for 10 days with intermittent (every 48 h) CCPA/vehicle administration. There were no differences in baseline RPP values between the two groups. There was a small decline in RPP during the 30 min of ischemia with no recovery during reperfusion. This was mainly due to a reduction in SBP, as HR remained relatively unchanged throughout the infarct protocol. The hemodynamic changes with time, however, were very similar in the two groups and are therefore unlikely to have contributed to the differences in infarct size.

Infarct size data. Absolute infarct size and risk zone volumes are presented in Table 4 for saline- and CCPA-pretreated animals. Figure 3 represents infarct size as a percentage of area at risk. Intermittent CCPA pretreatment caused a marked resistance to myocardial infarction compared with vehicle pretreatment (control), indicating that the delayed phase of cardioprotection was present at 10 days of repeated adenosine A_1 receptor activation. The I/R ratio in CCPA-pretreated animals was 26.6 ± 3.7% compared with 45.9 ± 5.5% in control animals ($p < 0.01$). Risk zone volume, a major determinant of infarct size, was similar between the two groups and averaged at 1.1 to 1.2 cm^3. Arterial pH and body temperature were also similar in the two groups at all time points (data not shown).

Early hemodynamic effects of CCPA after long-term treatment. Figure 4 illustrates the early effects of a 100-μg/kg bolus of CCPA administered at the end of the reperfusion period in animals pretreated for 10 days with intermittent CCPA or vehicle. Baseline variables were similar in both groups (CCPA-pretreated group: HR 227 ± 9 beats/min, SBP 75 ± 3 mm Hg; vehicle-pretreated group: HR 237 ± 10 beats/min, SBP 73 ± 4 mm Hg). There was a rapid decline in HR and blood pressure, which was very similar in the two groups. By 10-min after CCPA administration, there was a 20.0 ± 2.4% reduction in SBP and a 28.3 ± 2.8% reduction in HR in the saline-pretreated group compared with 18.9 ± 1.8% and 29.1 ± 3.4%, respectively, in the CCPA-pretreated group (p = NS). This similarity in the hemodynamic response after a bolus of CCPA in the two groups provides strong evidence that rabbits exposed to 10 days of repeated administration had not developed tolerance to the effects of the adenosine A_1 receptor agonist.

Discussion

These studies provide further evidence of a delayed phase of myocardial protection induced 24 and 48 h after transient adenosine A_1 receptor activation and support previous results

Table 2. Hemodynamic Variables During Ischemia-Reperfusion in Single-Dose Studies

	Before Ischemia	5-Min Ischemia	29-Min Ischemia	60-Min Reperfusion	120-Min Reperfusion
24-h group					
HR (beats/min)					
Saline	230.0 ± 3.7	231.7 ± 4.0	241.7 ± 6.5	238.3 ± 7.0	231.7 ± 6.5
CCPA	232.9 ± 7.8	235.7 ± 8.1	252.9 ± 7.1	234.3 ± 9.0	238.6 ± 7.1
SBP (mm Hg)					
Saline	93.8 ± 3.6	88.5 ± 3.8	87.7 ± 7.9	76.7 ± 2.5	70.0 ± 1.0
CCPA	92.3 ± 3.9	81.6 ± 5.0	86.6 ± 5.3	75.1 ± 2.6	72.3 ± 2.7
RPP (mm Hg/min × 10^3)					
Saline	21.6 ± 1.0	20.5 ± 1.1	21.4 ± 2.4	18.3 ± 0.8	16.2 ± 0.4
CCPA	21.6 ± 1.3	19.3 ± 1.5	21.0 ± 1.6	17.7 ± 1.2	17.3 ± 1.0
48-h group					
HR (beats/min)					
Saline	215.0 ± 9.9	221.7 ± 9.1	235 ± 12.0	223.3 ± 11.7	218.3 ± 11.1
CCPA	224.3 ± 6.5	228.6 ± 9.4	232.9 ± 6.1	221.4 ± 8.3	221.4 ± 6.7
SBP (mm Hg)					
Saline	84.3 ± 4.8	74.3 ± 4.4	75.2 ± 3.1	74.7 ± 4.3	74.3 ± 4.5
CCPA	83.3 ± 1.9	71.4 ± 2.2	74.1 ± 1.8	74.0 ± 2.3	73.4 ± 2.7
RPP (mm Hg/min × 10^3)					
Saline	18.3 ± 1.7	16.6 ± 1.4	17.8 ± 1.4	16.8 ± 1.5	16.3 ± 1.3
CCPA	18.6 ± 0.5	16.3 ± 0.9	17.2 ± 0.4	16.4 ± 0.8	16.3 ± 0.7

Data presented are mean value ± SEM. CCPA = 2-chloro-N^6-cyclopentyladenosine; HR = heart rate; RPP = rate–pressure product; SBP = systolic blood pressure.

from our laboratory (19). Furthermore, we have shown for the first time that this SWOP can be maintained over a 10-day period by intermittent pharmacologic preconditioning with an A_1 receptor agonist, with no evidence of tachyphylaxis to the infarct-limiting or hemodynamic effects of CCPA. The resilience against myocardial infarction seen in rabbits preconditioned over the long term with intermittent CCPA was comparable to that seen at 24 and 48 h after a single dose of CCPA. Moreover, after 10 days of pretreatment, the early hemodynamic response to a bolus of CCPA was no different in animals that had received either CCPA or saline pretreatment, providing further evidence that myocytes had not developed tolerance to the effects of the A_1-selective agonist.

No development of tachyphylaxis. In the present study we did not measure the hemodynamic effects of CCPA, 100 μg/kg, in conscious rabbits during the 10-day preconditioning protocol, although it is likely that the early hemodynamic response is less pronounced in conscious animals than in anesthetized animals. Furthermore, in our previous study describing the early hemodynamic response to a 100-μg/kg bolus of CCPA in anesthetized rabbits (14), the bradycardia and hypotension induced by the A_1 receptor agonist had completely resolved by 90 min after administration, implying that the compound is eliminated within a few hours. It is therefore unlikely that the marked anti-infarct effects observed after 10 days of pretreatment with intermittent CCPA had resulted from the transient bradycardia and hypotension induced after the administration of the agonist. In addition, because the hemodynamic variables at the start of the infarction procedure were similar between the two groups, there is no evidence to suggest cumulative hemodynamic effects of the A_1 receptor agonist over the 10-day treatment period.

Table 3. Hemodynamic Variables During Ischemia-Reperfusion in Long-Term Intermittent Dosing Study

	Before Ischemia	5-Min Ischemia	29-Min Ischemia	60-Min Reperfusion	120-Min Reperfusion
HR (beats/min)					
Saline	230.0 ± 6.2	236.0 ± 5.4	251.0 ± 8.6	224.0 ± 7.2	225.0 ± 8.3
CCPA	227.3 ± 5.2	230.9 ± 5.8	233.6 ± 5.8	220.9 ± 7.9	223.6 ± 8.1
SBP (mm Hg)					
Saline	95.0 ± 2.5	88.0 ± 3.1	82.9 ± 1.9	75.5 ± 2.2	73.4 ± 2.7
CCPA	90.6 ± 3.3	81.5 ± 3.3	78.8 ± 2.8	75.5 ± 2.5	73.5 ± 2.5
RPP (mm Hg/min × 10^3)					
Saline	21.9 ± 1.0	20.8 ± 1.0	20.8 ± 0.9	16.9 ± 0.8	16.5 ± 0.7
CCPA	20.5 ± 0.8	18.9 ± 1.0	18.5 ± 0.9	16.8 ± 0.7	16.3 ± 0.6

Data presented are mean value ± SEM. Abbreviations as in Table 2.

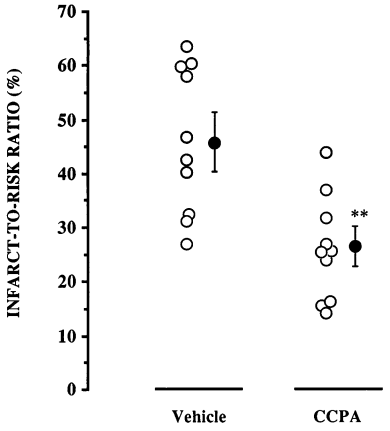
Table 4. Infarct Size Data for Long-Term Intermittent Dosing Study

Group	No. of Animals	Body Weight (kg)	Risk Volume (cm^3)	Infarct Volume (cm^3)
Saline	10	2.71 ± 0.31	1.19 ± 0.10	0.58 ± 0.10
CCPA	10	2.85 ± 0.11	1.12 ± 0.08	0.31 ± 0.05*

*$p < 0.05$ compared with corresponding saline-treated group (unpaired Student *t* test). Data presented are mean value ± SEM. CCPA = 2-chloro-N^6-cyclopentyladenosine.

Previous studies have reported desensitization of adenosine A_1 receptors after long-term exposure to agonists. These include studies examining regulation of adenosine receptors in the brain (20,21), kidney (22) and adipocytes (23–27) of the rat, embryonic myocytes of the chick (28,29) and smooth muscle DDT1 MF-2 cells of the hamster (30,31). Desensitization occurred in a time- and dose-dependent and reversible fashion; thus, long-term exposure to adenosine A_1 receptor agonists resulted in tolerance after 2 to 7 days in rat adipocytes, 24 to 44 h in chick embryo myocytes and 18 to 44 h in hamster DDT1 MF-2 cells. Few studies have explored adenosine A_1 receptor regulation in the mammalian myocardium. Lee et al. (32) reported desensitization of rat atrial adenosine A_1 receptors after a 7-day intravenous infusion of N^6-(phenyl-2*R*-isopropyl)adenosine, a selective A_1 receptor agonist. In the study by Tsuchida et al. (17), the time course of the desensitization was not investigated, but tolerance to the cardioprotective effects of CCPA developed in the rabbit myocardium between 6 to 72 h of a continuous infusion. Furthermore, in that study, the 72-h CCPA group received a total in excess of 3 mg/kg of CCPA over a 3-day period, a 30-fold higher dose than that shown to precondition the rabbit myocardium (14), providing further evidence for a dose-dependent influence on tachyphylaxis. All the cited studies, however, were either undertaken in cell culture models continuously exposed to adenosine analogues or performed in vivo using a continuous intravenous infusion for administering the agonists. The time and dose dependence of A_1 receptor desensitization would imply that reducing the dose frequency of administering the agonist might delay the development of tolerance to its effects. Interestingly, in a recent study by Casati et al. (33), the time course of desensitization to the hemodynamic effects of CCPA was investigated after twice-daily intraperitoneal administration of the agonist to spontaneously hypertensive rats. Tolerance to the bradycardic effect of CCPA, the main A_1 receptor-mediated action, did not develop for 21 days. These results further support the finding of this study that the reduced frequency of exposure to adenosine agonists delays or may even prevent tachyphylaxis.

Figure 3. Percent infarction of the risk zone in long-term intermittent dosing study. Ten days of intermittent treatment with CCPA resulted in marked protection against infarction compared with vehicle pretreatment. **Open circles** indicate individual experiments; **solid circles** represent the mean value ± SEM for each group. **$p < 0.01$ versus vehicle-treated group (unpaired Student *t* test).



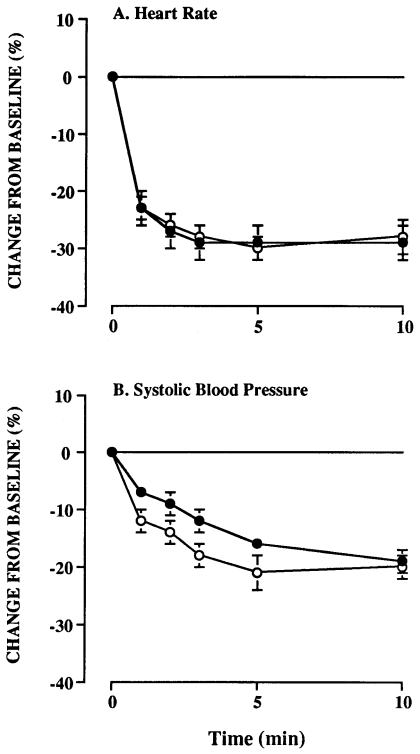


Figure 4. Early hemodynamic effects of a 100-μg/kg bolus of CCPA at the end of reperfusion in rabbits pretreated for 10 days with intermittent CCPA (**solid circles**) or saline vehicle (**open circles**). **A,** Changes in HR. **B,** Changes in SBP. Data shown are percent changes (±SEM) from baseline values.

Time course of delayed preconditioning. The temporal profile of the delayed phase of myocardial protection after ischemic or pharmacologic preconditioning has recently been described for various end points of ischemia-reperfusion injury. The SWOP against myocardial necrosis in the rabbit extends between 24 and 72 h after IPC (8), a time course identical to that observed after pharmacologic preconditioning with CCPA in the same species (19). Meng et al. (34) have recently reported a delayed cardioprotection against postischemic myocardial dysfunction in the rat 4 to 72 h after transient alpha$_1$-adrenoceptor activation with norepinephrine. Further-

more, a delayed protection against ischemia-reperfusion–induced ventricular arrhythmias that extends between 24 to 72 h after brief periods of cardiac pacing has been described in the canine myocardium (9). Late preconditioning against myocardial stunning, recently described by Tang et al. (10), seems to have a similar time course and is protective 12 to 72 h after brief periods of myocardial ischemia in the conscious pig. The prolonged nature of these protective effects, as opposed to the short-lived "classic" preconditioning, which only lasts for 1 to 2 h after the preconditioning stimulus, potentially allows "re-preconditioning" at 48 to 72-hour intervals, a time schedule that maintains the myocardium in a protected state, without development of tolerance, as shown in the present study. We did not examine the protective effects of intermittent CCPA administration beyond 10 days, but as with the bradycardic effect reported by Casati et al. (33), it is likely to be extended to at least 21 days. Furthermore, because the dosing schedule in our study was even less frequent than that used by Casati et al. (33), it is possible that desensitization of adenosine A_1 receptors may be even further delayed or may not occur at all.

Clinical relevance. Patients with unstable angina form a reasonably well defined high risk group that might benefit from pretreatment with agents that trigger or augment myocardial preconditioning over a period of several days or weeks and could maintain the myocardium in a protected state. Despite conventional pharmacologic and interventional approaches, approximately 9% of patients with unstable angina die or suffer from myocardial infarction within 30 days (35). Although a number of these patients may be "naturally" preconditioned by their preceding ischemic episodes and therefore not gain additional benefit from pharmacologic preconditioning, recent evidence suggests that unless the episodes of preinfarct angina occur during a narrow time window in relation to the infarct, this protection is not observed (36,37). So far there has been no direct evidence for the presence of a delayed phase of protection in the human myocardium. However, if such protection does exist, and if its duration can be extended by intermittent pharmacologic preconditioning, the myocardium could be maintained in a protected state, with resultant enhanced tissue tolerance and a slower rate of necrosis in the event the patient has an acute myocardial infarction. Although the ultimate treatment for myocardial infarction is prompt revascularization, such cardioprotective strategies would enhance the time window during which revascularization therapies can be administered, and may improve the outcome in a select group of patients with unstable angina.

Conclusions. We have shown that intermittent activation of adenosine A_1 receptors over a 10-day period, with a highly selective agonist, maintains the rabbit myocardium in a protected or "preconditioned" state against ischemia-reperfusion injury. In contrast to studies exploring maintenance of classic preconditioning, we did not find any evidence of tolerance to the cardioprotective or hemodynamic effects of CCPA over this period. In view of the potential therapeutic application of such a potent and prolonged protection, further studies are warranted to explore the underlying mechanisms.

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