Selective A2-Adenosine Receptor Agonists Do Not Alter Action Potential Duration, Twitch Shortening, or cAMP Accumulation in Guinea Pig, Rat, or Rabbit Isolated Ventricular Myocytes

John Shryock, Yejia Song, Desuo Wang, Stephen P. Baker, Ray A. Olsson, and Luiz Belardinelli

In this study, the hypothesis that mammalian ventricular myocytes possess A2-adenosine receptors was tested. Electrophysiological, contractile, and cAMP responses to the selective A2-adenosine receptor agonists 2-[2-(4-methylphenyl)ethoxy]adenosine (WRC-0090) and 2-(2-cyclohexylethoxy)adenosine (WRC-0013) and the nonselective adenosine receptor agonist 5′-(N-ethylcarboxamido)adenosine (NECA) were measured using ventricular myocytes isolated from guinea pig, rabbit, and rat hearts. Pertussis toxin pretreatment and/or the selective A2-adenosine receptor antagonists 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and (±)N′-endonorbornan-2-yl-9-methyladenine (N-0861) were used to prevent activation of A1-adenosine receptors on these cells. Action potential duration at 50% repolarization was not altered by WRC-0090, NECA, or WRC-0013 with or without 0.1 μM DPCPX or pertussis toxin pretreatment, and WRC-0090 and NECA failed to prolong the action potential duration of myocytes exposed to 0.1 or 1 μM forskolin. WRC-0090 alone or with 0.1 μM DPCPX did not increase the amplitude of shortening of pertussis toxin–treated or untreated myocytes, and WRC-0090 or NECA did not significantly increase cAMP accumulation. In contrast to these results with myocytes, in the smooth muscle cell line DDT,MF-2, the effect of both selective A2-agonists on cAMP accumulation was biphasic: low concentrations (<0.3 μM) increased but higher concentrations decreased accumulation of cAMP. The decreased cAMP accumulation seen at higher agonist concentrations was completely abolished by either 0.1 μM DPCPX or pretreatment of cells with pertussis toxin. In summary, the results of the present study do not provide evidence for A2-adenosine receptors on mammalian ventricular cardiomyocytes but confirm reports of the coexistence of both A1 and A2 subtypes of adenosine receptors on DDT,MF-2 cells. (Circulation Research 1993;72:194–205)

KEY WORDS • myocardium • receptors, purinergic • cardiac electrophysiology • adenylyl cyclase • adenosine

Cell surface adenosine receptors are classified as A1 or A2 according to pharmacological and functional criteria.1,2 Activation of A1- and A2-adenosine receptors leads to inhibition and stimulation of adenylyl cyclase, respectively, by guanine nucleotide–binding proteins (G proteins). The agonist potency profile for the A2-adenosine receptor on rat fat cells is (−)N′-(2-phenylisopropyl)adenosine (R-PIA)>adenosine>5′-(N-ethylcarboxamido)adenosine (NECA).3 A reverse order of potency is seen at the A2-adenosine receptor, where NECA is more potent than adenosine or R-PIA. A2-Adenosine receptors are further subdivided into “high” and “low” affinity, or A2a and A2b subtypes, respectively.3,4 A3a-Receptors have been found in the striatal cortex of the brain, whereas A3b-receptors are present throughout the brain and in many other tissues of the body. Several types of cells appear to possess both A1- and A2-adenosine receptor subtypes.5–7

In the heart, A1-adenosine receptors mediate an inhibition of catecholamine- and forskolin-stimulated adenylyl cyclase activity in both atrial and ventricular myocytes.8–11 This action is the basis for the “antiadrenergic” effects of adenosine on heart function, including attenuation of catecholamine-induced increases of calcium inward current, ventricular action potential duration, and contractility.12,13 In addition, adenosine directly increases a potassium outward current in sinoatrial and atrial cells by a G protein–mediated mechanism that is independent of cAMP.14

Analogue of adenosine have also been reported to stimulate adenylyl cyclase activity in cardiac tissue, cell, or membrane preparations.15–21 A2-Adenosine receptors may mediate this action. Experimental evidence suggests that adenylyl cyclase activity in ventricular myocytes isolated from rat and guinea pig ventricles19,20

From the Departments of Medicine and Pharmacology, University of Florida College of Medicine, Gainesville, and the Department of Internal Medicine, University of South Florida, Tampa. Supported by National Heart, Lung, and Blood Institute grant RO1 HL-35272 and by Whitby Research, Inc., Richmond, Va.

Address for reprints: Luiz Belardinelli, MD, Department of Medicine, P.O. Box 100277, Gainesville, FL 32610-0277.

Received October 11, 1991; accepted October 2, 1992.
and from embryonic chicken hearts is both inhibited by A1-adenosine receptors and stimulated by A2-adenosine receptors. A2-Adenosine receptor-mediated stimulation of adenylyl cyclase activity by NECA was masked by concomitant activation of A1-adenosine receptors that inhibited the enzyme. NECA was found to stimulate adenylyl cyclase activity in embryonic chick ventricular myocytes pretreated with pertussis toxin or incubated with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) to attenuate A1-adenosine receptor-mediated actions. Interestingly, NECA does not appear to increase contractility of guinea pig isolated ventricular myocytes, and functional responses associated with activation of adenylyl cyclase, such as increased calcium inward current, positive inotropy, or lengthening of action potential duration (APD), have not been convincingly demonstrated in a mammalian cardiac cell incubated with adenosine or with adenosine analogues.

Because a large number of cells are needed to perform cAMP or adenylyl cyclase assays, contamination of myocyte preparations with nonmyocytes could explain results indicating the presence of A1-adenosine receptors in cardiac preparations. Thus, functional responses recorded from single cells under visual observation are needed to conclusively demonstrate the existence of A1-adenosine receptors on mammalian ventricular myocytes.

Highly selective agonists or antagonists with which to assess the presence and function of the A1-adenosine receptor in cardiac tissues are not commercially available. Recently, however, the synthesis and selectivities of two novel series of adenosine derivatives were described. We selected two of these derivatives for experimental use: 2-(2-cyclohexylethoxy)adenosine, a 2-alkoxyadenosine (referred to as No. 3m by Ueeda et al., but renamed WRC-0013); and 2-[2-(4-methylphenyl)ethoxy]adenosine, a 2-aralkoxyadenosine (referred to as No. 19 by Ueeda et al., but renamed WRC-0090). They were, respectively, 8,700-fold and 44,000-fold more potent in increasing coronary flow (an A2-mediated effect) of the guinea pig isolated perfused heart than in causing prolongation of the stimulus-QRS interval (an A1-mediated effect).

The goal of the present study was to investigate the actions of these two selective A1-adenosine receptor agonists on ventricular myocytes isolated from guinea pig, rabbit, and rat hearts. Electrophysiological and contractile responses and cAMP content were measured. Because even selective A1-adenosine receptor agonists might not be sufficiently A1 selective in myocytes, for the purpose of this study, it was necessary to attenuate A1-adenosine receptor-mediated responses, which may obscure responses to activation of A2-adenosine receptors. Two experimental approaches were used. In some experiments, A1-adenosine receptor-mediated effects were attenuated by using the A1 subtype-selective antagonists (+)-XN-endoonorborn-2-yl-9-methyladenine (N-0861) and DPCPX. In other experiments, animals or isolated myocytes were pretreated with pertussis toxin to uncouple the A1-adenosine receptor from inhibitory G proteins. The validity of these experimental approaches was demonstrated by parallel studies using DDT,MF-2 cells. In these cells, the presence of both A1- and A2-adenosine receptor subtypes has been demonstrated by use of radioligand binding with adenosine receptor subtype-selective probes and adenylyl cyclase assays. The results of our experiments do not provide evidence for functional A1-adenosine receptors on mammalian ventricular myocytes but demonstrate their presence on DDT,MF-2 cells.

**Materials and Methods**

**Chemicals**

Two analogues of adenosine with high selectivity for the A1-adenosine receptor, WRC-0090 and WRC-0013 were synthesized and provided by Dr. R.A. Olsson, University of South Florida, Tampa, with the permission of Whitby Research, Richmond, Va. Stock solutions (10 mM) were prepared in dimethyl sulfoxide and diluted at least 1,000-fold in saline for use in experiments. The selective A1-adenosine receptor antagonist N-0861 was a gift from Whitby Research, Richmond, Va. The selective A1-adenosine receptor antagonist DPCPX and the nonselective adenosine receptor antagonist xanthine amine congener were purchased from Research Biochemicals, Natick, Mass. Pertussis toxin was a gift from Dr. Eric Hewlett, University of Virginia, Charlottesville. Forskolin, adenosine deaminase, and bovine serum albumin were from Sigma Chemical Co., St. Louis, Mo. Collagenase was purchased from Worthington Biochemical, Freehold, N.J.; dispase was from Boehringer Mannheim Corp., Indianapolis, Ind.; and trypsin was from Serva Fine Chemicals, Heidelberg, FRG. Rolipram, a phosphodiesterase inhibitor selective for the low Kₘ cAMP-specific form(s) of phosphodiesterase, was a gift from Berlex Labs, Cedar Knolls, N.J., and was prepared and stored as a 50-mM stock solution in dimethyl sulfoxide.

**Preparation of Isolated Ventricular Myocytes**

Adult guinea pigs and rats of either sex were anesthetized with methoxyflurane. Rabbits were anesthetized with ketamine and acepromazine. The chest was opened, and the heart was quickly excised and placed into an ice-cold modified Krebs-Henseleit (K-H) solution containing (mM) NaCl 127, KCl 4.6, CaCl₂ 2, MgSO₄ 1.1, sodium pyruvate 2, glucose 10, creatine 10, taurine 20, ribose 5, adenine 0.01, allopurinol 0.1, and sodium HEPES 5, pH 7.4. The aorta was cannulated, and retrograde perfusion of the heart was begun with warmed (35°C), oxygenated (100% O₂) solutions at a rate of 8 ml/min for guinea pig and rat hearts and 16 ml/min for rabbit hearts. The following solutions were perfused in sequence: 1) modified K-H solution for 10 minutes, 2) Ca⁡⁺⁺⁺⁺free K-H solution for 10 minutes, 3) Ca⁡⁺⁺⁺⁺free solution containing (mg/ml) collagenase type II 0.4, dispase 0.04, trypsin 0.04, and albumin 2 for 20 minutes. The heart was then removed from the cannula, and the ventricles were minced with scissors. The minced tissue was further incubated at 35°C in a shaker bath to dissociate cells. Dissociated cells were stored at room temperature in modified K-H solution containing 0.1 mM Ca⁡⁺⁺⁺⁺ until use. Only quiescent rod-shaped myocytes with clear striations were used for electrophysiology or contraction studies.

In some experiments, collagenase (0.6 mg/ml) alone was used to digest guinea pig isolated hearts to avoid possible proteolysis of cell surface adenosine receptors by trypsin. The freshly dissociated ventricular myocytes
were incubated for 2 hours at 22°C in a modified KB medium 20 to prevent development of calcium overload and facilitate cellular recovery of calcium tolerance. This medium contained (mM) KCl 80, K$_2$HPO$_4$ 30, MgSO$_4$ 5, Na$_3$ATP 5, pyruvic acid 5, β-hydroxybutyric acid 5, creatine 5, taurine 20, and glucose 20, with pH adjusted to 7.0. During the second hour of incubation, free calcium concentration of the medium was increased in two steps to 100 μM. Cells were allowed to settle, and the supernate (containing damaged cells) was aspirated. Cells were then incubated in a Petri dish in an atmosphere of 5% CO$_2$–95% air for 3 hours at 37°C (calcium concentration, 1.8 mM) with 2% bovine calf serum, 1 mM ascorbic acid, and 1 unit/ml adenosine deaminase. When appropriate, pertussis toxin (10 μg/ml) was added to the incubation medium to uncouple A$_2$-adenosine receptors from inhibitory G proteins. Cells were transferred to a glass tube and allowed to settle, and the supernate was aspirated and replaced with modified K-H solution at room temperature containing 2 mM calcium and 1 unit/ml adenosine deaminase; this step was repeated twice in 1 hour. In two experiments with guinea pig atrial myocytes, 50 μM theophylline and 1 unit/ml adenosine deaminase were added to all media (isolation, recovery, and pretreatment) to reduce the potential for desensitization of A$_2$-adenosine receptors by adenosine. Cells exposed to theophylline were then washed three times with fresh K-H solution before use.

Two modifications of the above procedure were made to increase myocyte stability for measuring cell shortening in response to drugs. After incubation in medium 199, cells were resuspended in a physiological saline solution (see “Measurement of Cell Shortening”) containing 0.1 mM calcium, rather than 2 mM calcium, before use in experiments. Also, the addition of 1.5 mM Na$_2$EDTA to medium 199 to reduce the free calcium concentration during the 3-hour control/pertussis toxin treatment was found to enhance subsequent inotropic responses of myocytes to isoproterenol.

Myocyte electrophysiological, contractile, or cAMP responses to A$_2$-adenosine receptor agonists were qualitatively similar comparing myocytes isolated by the “collagenase alone” and the “collagenase/disrase/trypsin” protocols.

Electrophysiology

Isolated ventricular myocytes were placed into an experimental chamber (volume, 0.5 ml) fixed to the stage of a Zeiss inverted microscope and superfused at a rate of 2 ml/min with modified K-H solution (2 mM Ca$^{2+}$) warmed to 35°C. For experiments with rat ventricular myocytes, the potassium concentration of the superfusate was raised to 15 mM to prevent depolarization of cells upon impalement with a microelectrode. Single microelectrodes (resistance, 30–50 MΩ) filled with 3 M KCl were used for both stimulation and recording of transmembrane resting and action potentials. The ranges of resting membrane potentials of ventricular myocytes isolated from guinea pigs, rabbits, and rats were −83 to −87, −80 to −87, and −40 to −54 mV, respectively. Action potentials were elicited by brief (5-msec) rectangular current pulses applied at a rate of 0.5 Hz between the intracellular electrode and a grounded bath electrode. Membrane voltage was amplified with an Axoclamp-2A (Axon Instruments, Burlingame, Calif.) and recorded by a strip-chart recorder (model 2200S, Gould, Cleveland, Ohio). Recordings of membrane action potentials were obtained before and after the addition of drugs. Cells were exposed for 4 minutes each to various concentrations of drugs. APD was measured at 50% repolarization (APD$_{50}$).

Measurement of Cell Shortening

Freshly isolated ventricular myocytes from guinea pig, rat, or rabbit hearts were suspended in a physiological saline solution containing (mM) NaCl 118, KCl 4.78, CaCl$_2$ 1.2, MgCl$_2$ 1.1, glucose 10, sodium HEPES 10, pH 7.4. An aliquot of cell suspension was placed in a recording chamber (volume, 1–1.5 ml) mounted on the stage of a Nikon Diaphot inverted microscope. The microscope image was captured using an Ikegami ITCT- Vita videon CCTV camera and displayed on a 17-in. CCTV picture monitor (Ikegami Electronics, Haywood, N.J.). The unloaded contraction of a single myocyte was quantitated using a video edge detector (Crescent Electronics, Logan, Utah) to measure the extent of movement of a cell edge along a selected raster line segment of the TV image. The voltage output of the edge detector was displayed on an oscilloscope (Nicolet Instrument Corp., Madison, Wis.) and recorded by a Gould strip-chart recorder. New position data was obtained every 16.7 msec. The amplitude of cell wall motion was measured from the strip-chart recording and calibrated to obtain motion in microns. The amplitude of cell wall motion is an index of contractility. 30

Twitch shortening of myocytes was elicited by rectangular current pulses (0.1–0.2 mA in amplitude and 2.5–5 msec in duration at a rate of 40 pulses per minute) applied between an extracellular micropipette and a remote silver-wire electrode. The extracellular glass micropipette was filled with physiological saline solution (see above) and positioned with its tip 10–25 μm from the myocyte to be stimulated. Myocytes were superfused with 35°C physiological saline solution at 2 ml/min. Exposures of myocytes to drugs were kept as short as possible (approximately 2–3 minutes) to avoid desensitization but still allow attainment of an acute maximal response.

**DDT,**MF-2 Cells

DDT,MF-2 (a cell line from a Syrian hamster ductus deferens leiomyosarcoma) cells were obtained from the American Type Culture Collection, Rockville, Md., and grown at 37°C on 150-mm plastic culture dishes (Falcon) in Dulbecco’s modified Eagle’s medium supplemented with fetal bovine serum (5%), penicillin G (100 units/ml), streptomycin (0.1 mg/ml), and amphotericin B (2.5 μg/ml) equilibrated with an atmosphere of 5% CO$_2$–95% air. Cells were seeded at 0.5×10$^6$ cells/cm$^2$ and subcultured twice weekly after detachment by using 1 mM EDTA in phosphate-buffered saline. One-day preconfluent cultures were used for experiments.

**Measurement of cAMP Content**

Freshly isolated guinea pig ventricular myocytes were suspended in modified K-H solution containing adenosine deaminase (1 unit/ml) and 50 μM rolipram (to inhibit cAMP phosphodiesterase). Aliquots (400 μl) of the cell suspension, containing approximately 10$^6$ cells,
were warmed to 34–35°C for 10 minutes. Drugs (or vehicle only) were added, and cells and drugs were incubated for 6 minutes. Hydrochloric acid (final concentration, 50 mM) was added to terminate the incubation. Samples were stored at 4°C until assay of cAMP content by the automated radioimmunoassay method of Brooker et al.31

DDT, MF-2 cells were rinsed twice with Hanks’ balanced salt solution (HBSS), harvested with a cell lifter, pelleted by centrifugation at 1,000g for 5 minutes, and resuspended in HBSS. Aliquots of the cell suspension were warmed to 37°C for 10 minutes in HBSS containing 100 μM rolipram. Drug incubation, termination, and assay of cAMP were carried out as stated for myocytes. Protein content of cell suspensions was determined using the Lowry method with bovine serum albumin as standard.

**Pertussis Toxin Treatment**

Adult guinea pigs were injected with pertussis toxin (150 μg/kg) via the jugular vein. Seventy-two hours after the injection, the animals were killed, and cardiac myocytes were isolated (see “Preparation of Isolated Ventricular Myocytes”). To test the efficacy of pertussis toxin treatment, the effect of adenosine on APD50 of isolated myocytes was determined. The APD50 values of control myocytes were increased by isoproterenol (10 nM), and adenosine (100 μM) abolished the prolongation of APD50 caused by isoproterenol. In contrast, adenosine (100 μM) failed to shorten the prolongation of the APD50 caused by isoproterenol in myocytes from pertussis-intoxicated animals.

In a separate series of experiments, isolated guinea pig ventricular myocytes rather than whole animals were pretreated for 3 hours with pertussis toxin (10 μg/ml), as stated in “Preparation of Isolated Ventricular Myocytes.”

Cultures of DDT-MF-2 cells were pretreated by addition of pertussis toxin (25 ng/ml) to the culture media 18 hours before cells were to be used in experiments. Pretreatment with pertussis toxin abolished the inhibitory effect of 10 μM N6-cyclopentyladenosine (an A3-adenosine receptor agonist) on isoproterenol-stimulated cAMP accumulation (not shown).

**Isolated Perfused Hearts**

Hearts of adult guinea pigs (n=5) were excised and perfused as described above (“Preparation of Isolated Ventricular Myocytes”) with oxygenated warmed (35°C) K-H solution. Left ventricular pressure (LVP) was measured using a fluid-filled latex balloon placed in the left ventricle and connected to a pressure transducer (Gould P23ID). Coronary perfusion pressure was measured using a pressure transducer connected to the perfusion line via a T connector. Pressures were recorded using an ink-chart recorder (Gould RS3400). Each heart was paced at a rate 5–10% greater than its intrinsic rate by using a bipolar stimulating electrode placed on the base of the right ventricle. Electrical pacing was momentarily discontinued at appropriate times to allow measurement of spontaneous heart rate (HR). HR was determined from the recording of LVP.

The experimental protocol for determination of the effects of WRC-0090 on LVP and HR was as follows. After an equilibration period of ≥30 minutes, control recordings were obtained. Hearts were then sequentially perfused for 4 minutes each with 1-, 10-, 100-, and 1,000-nM concentrations of WRC-0090 in K-H buffer. After a 10-minute washout period, hearts were exposed a second time to each concentration of WRC-0090 in the presence of DPCPX (0.1 μM). A final washout period completed each experiment. Measurements of LVP and spontaneous HR were obtained during the final minute of each period.

**Data Analysis**

Data are presented as mean±SEM unless otherwise noted. Statistical significance of differences between individual means in an experiment with several treatment groups was determined by analysis of variance (ANOVA) and Newman-Keuls multiple comparison test by using the computer program PHARM/PCS (version 4, R.J. Tallarida and R.B. Murray, Microcomputer Specialists, Philadelphia, Pa.). Differences between mean values were considered significant at p<0.05. Values of APD50 (Figures 1–5) and cAMP content of isolated ventricular myocytes (Figures 11 and 12) have been plotted as percentages of the control values recorded in the absence of tested drugs.

**Results**

**Effect of A2-Adenosine Receptor Agonists on Ventricular Myocyte Action Potential Duration**

APDs of ventricular myocytes were unaltered by exposure of myocytes to either the selective A2-adenosine receptor agonists WRC-0090 and WRC-0013 or by exposure to the unselective adenosine agonist NECA. Control values of APD50 for ventricular myocytes isolated from guinea pig (n=20), rabbit (n=3), and rat (n=3) hearts were 191±13 msec (mean±SEM, n=57), 162±12 msec (n=14), and 20±1 msec (n=12), respectively. Results of experiments using guinea pig isolated ventricular myocytes are shown in Figure 1. Neither WRC-0090 (0.1 nM–10 μM) nor WRC-0013 (1–1,000 nM) caused a significant change in APD50 compared with control data (Figure 1A). Likewise, WRC-0090 or WRC-0013 did not alter either action potential amplitude or cellular resting membrane potential (data not shown). Because a possible A2-adenosine receptor–mediated increase in APD50 may have been “masked” by an A3-adenosine receptor–mediated decrease in APD50, DPCPX (0.1 μM) or N-0861 (10 μM) was used to inhibit a possible activation by WRC-0090 and WRC-0013 of the A3-adenosine receptor. However, there was no effect of either A2 agonist or of NECA on APD50 in the presence of DPCPX (Figure 1B) or N-0861 (data not shown). Results similar to these with guinea pig myocytes were obtained with myocytes from rat (n=3) and rabbit (n=3) hearts (Figure 2). In contrast, isoproterenol (10 nM) caused increases of APD of 30–40% in guinea pig and 120% in rat ventricular myocytes compared with control data (data not shown).

The effect of WRC-0013 on APD was tested further by using ventricular myocytes isolated from hearts of guinea pigs (n=4) pretreated for 48–72 hours with pertussis toxin to uncouple the A2-adenosine receptor from inhibitory G proteins. The effectiveness of pertussis toxin treatment was demonstrated by the observation that adenosine (100 μM) did not oppose the action of isoproterenol (10 nM) to increase APD50 from 161±10
FIGURE 1. Graphs showing the lack of effect of A2-adenosine receptor agonists 2-[2-(4-methylphenyl)ethoxy]adenosine (WRC-0090), 2-(2-cyclohexylethoxy)adenosine (WRC-0013), and 5'-N-ethylcarboxamidoadenosine (NECA) on duration of the action potential of guinea pig ventricular myocytes. Action potential duration at 50% repolarization (APD50) was measured in the absence (panel A) and presence (panel B) of the selective A2-adenosine receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 0.1 μM) and plotted as a percentage of the mean APD50 in the absence of adenosine receptor agonist, which was 191±13 msec (n=57). Each point represents the mean of two to 16 determinations, for which myocytes from 20 hearts were used. Error bars indicate SEM. Differences among means are not significant by analysis of variance. DPCPX alone did not alter APD50 (data not shown).

to 236±15 msec (n=8) (data not shown). The A2-adenosine receptor agonist WRC-0013 did not increase APD90 in cells (n=20) from pertussis toxin–pretreated animals, even in the presence of 10 μM N-0861, a selective A1-adenosine receptor antagonist (Figure 3). Similar results were found when guinea pig isolated myocytes rather than whole animals were pretreated with pertussis toxin. WRC-0090 (1 μM) in the presence of 0.1 μM DPCPX did not significantly increase APD50 in either untreated or pertussis toxin–treated myocytes (Figure 4). In contrast, isoproterenol (5 nM) increased APD50 in both untreated and treated myocytes (Figure 4). As expected, pertussis toxin treatment nearly abolished the action of adenosine (100 μM) to attenuate an isoproterenol-mediated increase of APD90 (Figure 4).

The absence of an A2-adenosine receptor–mediated increase of APD90 could be the result of receptor desensitization by endogenous adenosine. To test this possibility, responses to adenosine and NECA were measured with cells that were isolated from two guinea pig hearts by using collagenase in the presence of theophylline (50 μM) and adenosine deaminase (1 unit/ml). Cells were pretreated with pertussis toxin (10 μg/ml), also in the presence of theophylline and adenosine deaminase, for 3 hours at 37°C and then washed three times to remove theophylline. The response of these cells to adenosine (10 and 100 μM) was not different from the control response. The APD90 of control cells (n=6) exposed to 0.1 μM DPCPX alone was 173±9 (mean±SEM) msec, and the APD90 values of cells exposed to 10 and 100 μM adenosine in the presence of 0.1 μM DPCPX were 172±8 msec (n=6) and 170±9 msec (n=6) respectively. Similarly, NECA (10 and 100 μM) did not significantly increase APD50 of the theophylline and pertussis toxin–treated cells (n=6).

Forskolin directly activates adenylyl cyclase and potentiates stimulation of the enzyme by receptors coupled to the adenylyl cyclase–stimulatory G protein (Gs). Accordingly, forskolin alone (150 nM) was reported to increase calcium inward current and APD11.

FIGURE 2. Bar graph showing lack of effect of the A2-adenosine receptor agonist 2-(2-cyclohexylethoxy)adenosine (WRC-0090, 0.5 μM) on action potential duration at 50% repolarization (APD50) of rabbit and rat ventricular myocytes. The durations of control action potentials for rabbit and rat myocytes were 162±12 msec (n=14) and 20±1 msec (n=12), respectively. 1,3-Dipropyl-8-cyclopentylxanthine (DPCPX, 0.1 μM) or (+)N6-endonorbornan-2-yl-9-methyladenine (N-0861, 10 μM) was used to block A1-adenosine receptors. Bars indicate mean and SEM of determinations from seven to 14 myocytes.

FIGURE 3. Graph showing lack of effect of the A2-adenosine receptor agonist 2-(2-cyclohexylethoxy)adenosine (WRC-0013) on duration of the action potential of pertussis toxin–treated guinea pig ventricular myocytes. Ventricular myocytes were isolated from guinea pigs (n=4) that were injected with pertussis toxin 48–72 hours earlier (see “Materials and Methods” for details). Action potential duration at 50% repolarization (APD50) was measured in the absence (open circles) and presence (filled circles) of the selective A2-adenosine receptor antagonist (+)N6-endonorbornan-2-yl-9-methyladenine (N-0861, 10 μM). Each point represents the mean of determinations from two to nine myocytes. Error bars indicate SEM. Differences among means are not significant by analysis of variance.
Figure 4. Bar graph showing the effects of 2-[(4-methylphenyl)ethoxy]adenosine (WRC-0090), isoproterenol (ISO), and adenosine (ADO) on action potential duration at 50% repolarization (APD<sub>50</sub>) of control and pertussis toxin (PTX)-treated guinea pig ventricular myocytes. Control APD<sub>50</sub> values for untreated and PTX-treated myocytes were 128±11 msec (n=5) and 155±8 msec (n=6), respectively. Ventricular myocytes isolated from guinea pig hearts by collagenase digestion were preincubated for 3 hours at 37°C with adenosine deaminase (1 unit/ml) in the absence or presence of PTX (10 μg/ml) and then superfused with modified Krebs-Henseleit buffer (see “Materials and Methods”) containing the indicated drugs. Duration of each drug exposure was 3 minutes. Bars indicate mean and SEM of values from four to six myocytes from three hearts. Drug concentrations were as follows: WRC-0090, 1 μM; ISO, 5 nM; ADO, 100 μM; and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 0.1 μM. NS indicates no significant difference from corresponding ISO value. *p<0.01 vs. control, by Newman-Keuls test. +p<0.01 vs. ISO.

In the present study, forskolin (1 μM) significantly prolonged the APD<sub>50</sub> of guinea pig isolated ventricular myocytes from 177±8 to 228±13 msec. Isoproterenol (10 nM) further prolonged the APD<sub>50</sub> by 31±8% (n=4, p<0.05 versus forskolin) in the presence of 1 μM forskolin and occasionally led to the appearance of afterpotentials and triggered activity. In contrast, the A<sub>1</sub>-adenosine receptor agonist WRC-0090 not only failed to prolong the APD<sub>50</sub> of isolated ventricular myocytes exposed to 1 μM forskolin but, at the highest tested concentration (10 μM), significantly decreased it (Figure 5). This action of WRC-0090 was blocked by 0.1 μM DPCPX (Figure 5). Because the prolongation of APD<sub>50</sub> by 1 μM forskolin may have hindered a demonstration of its ability to potentiate responses to activation of adenyl cyclase–coupled stimulatory receptors, a lower concentration of forskolin was tested. Whereas 1 μM forskolin alone prolonged the APD<sub>50</sub>, 0.1 μM forskolin did not (Figure 6). However, 0.1 μM forskolin potentiated the response of guinea pig isolated myocytes to 5 nM isoproterenol (Figure 6). In contrast, neither NECA (10 μM) nor WRC-0090 (0.5 μM) caused any change in APD<sub>50</sub> of myocytes in either the presence or absence of 0.1 μM forskolin (Figure 6), even though 10 μM N-0861 was present to block the activation of A<sub>1</sub>-adenosine receptors. Also, APD<sub>50</sub> was not prolonged by WRC-0090 (0.5 μM) in guinea pig isolated ventricular myocytes (n=5) exposed to the phosphodiesterase inhibitor rolipram (50 μM) (data not shown). Rolipram alone increased the duration of the action potential by 19±6% (n=5, p<0.05 versus control).

Effects of WRC-0090 on Shortening of Isolated Ventricular Myocytes

The selective A<sub>1</sub>-adenosine receptor agonist WRC-0090 (0.1–1,000 nM) did not increase the amplitude of shortening of guinea pig (Figures 7 and 8) or rat (Figure 8) isolated ventricular myocytes. In contrast, isoproterenol (10 nM) elicited an increase in the amplitude of shortening (Figures 7–9). A high concentration of WRC-0090 (10 μM) attenuated the action of isoproterenol (Figure 9). Attenuation by WRC-0090 of the isoproterenol-induced increase in shortening was antagonized by 0.1 μM DPCPX (Figure 9). NECA (10 μM) in the presence of 0.1 μM DPCPX did not increase shortening of guinea pig isolated ventricular myocytes (data not shown). Similar results were obtained using ventricular myocytes from rabbits and rats (n=11 and 19 myocytes from two and four hearts, respectively; data not shown). Twitch amplitude of guinea pig isolated myocytes pretreated for 3 hours with pertussis toxin (10 ng/ml) and adenosine deaminase (1 unit/ml) was unaltered either by WRC-0090 (1 μM) (Figure 10) or by NECA (10 μM, not shown), whereas isoproterenol (10 nM) increased the twitch amplitude of the same cells by more than fourfold (Figure 10). A high concentration of NECA (100 μM) decreased the amplitude of shortening of guinea pig isolated myocytes to 89±8% (mean±SEM, n=9 myocytes from two hearts) of the control value.

Effects of WRC-0090 on cAMP Accumulation in Guinea Pig Isolated Myocytes and in Cultured DDT;MF-2 Cells

WRC-0090 (1–1,000 nM) did not significantly increase cAMP accumulation in guinea pig isolated ventricular myocytes treated with rolipram (50 μM) in
either the absence or the presence of the selective A1-adenosine receptor antagonist N-0861 (10 μM) (Figure 11). Furthermore, pretreatment of myocytes with pertussis toxin and adenosine deaminase did not “uncover” a stimulatory A2-adenosine receptor–mediated response. Although both WRC-0090 (1 μM) and NECA (10 μM) slightly increased cAMP accumulation in pertussis toxin–treated myocyte preparations in the presence of rolipram and N-0861, these increases did not reach statistical significance (Figure 12). In contrast, isoproterenol (0.1 μM) caused a threefold increase of cAMP content in pertussis toxin–treated myocytes that was not antagonized by 5 μM N6-cyclopentyladenosine, a selective A1-adenosine agonist (Figure 12).

**FIGURE 8.** Bar graph showing the effects of 2-[2-(4-methylphenyl)ethoxy]adenosine (WRC) and isoproterenol (ISO) on the amplitude of shortening of guinea pig and rat isolated ventricular myocytes. Shortening amplitude is plotted as a percentage of control amplitude in the absence of drug. Drug concentrations were as follows: WRC, 1 μM; 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 0.2 μM; and ISO, 1 nM for guinea pig, 10 nM for rat. Number of myocytes is indicated in parentheses; error bars indicate SEM. Myocytes were from four guinea pig and four rat hearts. Twitch amplitude in the presence of ISO was significantly greater than control (p<0.01).

**FIGURE 9.** Recordings showing A1-adenosine receptor–mediated inhibition by 2-[2-(4-methylphenyl)ethoxy]adenosine (WRC) of isoproterenol (ISO)–stimulated shortening of an isolated guinea pig ventricular myocyte. Panel I: Amplitude of shortening in the absence of drug (control) and in the presence of ISO (1 nM) and ISO+WRC (10 μM). Panel II: Same cell showing reversal by the selective A1-adenosine receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 0.1 μM) of WRC-mediated inhibition of ISO’s effect on shortening. Duration of exposure to each different drug treatment was approximately 3 minutes. Chart speed was varied from slow to fast to allow visualization of recordings of single twitches. Results are representative of experiments with three myocytes from one heart.

**FIGURE 7.** Lack of effect of the A1-adenosine receptor agonist 2-[2-(4-methylphenyl)ethoxy]adenosine (WRC) on the amplitude of shortening of a guinea pig isolated ventricular myocyte. Each panel (a–h) is a recording of shortening amplitude versus time, obtained at slow, medium, and fast chart speeds. Panel a: Absence of drug (control). Panels b–f: WRC at concentrations of 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-6} M. Panel g: Isoproterenol (ISO, 10^{-8} M). Panel h: Absence of drug. The duration of each intervention was 2–3 minutes. The experiment was repeated a second time using a different myocyte preparation with similar results.
Adenosine increased cAMP content of myocytes slightly but not significantly. In two experiments, guinea pig ventricular myocytes were isolated in the presence of adenosine deaminase (1 unit/ml) and theophylline (50 nM) (see “Preparation of Isolated Ventricular Myocytes”) and studied after incubation with pertussis toxin (10 µg/ml). cAMP contents of aliquots of cells incubated with 5 µM erythro-9-(2-hydroxy-3-nonyl)adenine (an inhibitor of adenosine deaminase), 0.1 µM DPCPX, and 50 µM rolipram in the absence or presence of 10 µM or 100 µM adenosine were 7.89±0.25 pmol (mean±SEM, n=6), 9.05±0.38 pmol (n=5), and 9.79±0.15 pmol (n=6), respectively (p>0.05 versus control by ANOVA). In contrast, cAMP content of cells incubated with 0.1 µM isoproterenol was increased significantly to 27.9±1.5 pmol (n=5). The A1-adenosine receptor agonist N6-cyclopentyladenosine did not attenuate the isoproterenol-induced increase of cAMP accumulation in these pertussis toxin–treated cells. cAMP content of cells incubated with N6-cyclopentyladenosine (5 µM) and isoproterenol (0.1 µM) was 28.1±2.7 pmol (n=5). This result suggests that A1-adenosine receptor–mediated responses were absent. In summary, although adenosine, NECA, and WRC-0090 (above) increased cAMP accumulation by a small amount, because the increase was not statistically significant, its relevance is questionable.

The effects of WRC-0090 and of WRC-0013 on cAMP accumulation in DDT1MF-2 cells were biphasic. cAMP accumulation increased as WRC-0090 concentration was raised from 1 to 300 nM and then fell when WRC-0090 concentration was increased further (Figure 13). When DPCPX (0.1 µM) was present to antagonize the action of WRC-0090 at A1-adenosine receptors or when cells were pretreated with pertussis toxin to uncouple A1-adenosine receptors from inhibitory G proteins, WRC-0090 concentrations from 1 nM to 100 µM caused cAMP content to increase, with a maximum response at 0.3 µM WRC-0090 (Figure 13). The action of WRC-0090 to increase cAMP accumulation in DDT1MF-2 cells was not antagonized to a significant extent by either 10 µM N-0861 or 0.1 µM DPCPX (Figure 14). On the other hand, 5 µM xanthine amine congener and 0.2 µM DPCPX significantly reduced the cAMP response to 0.1 µM WRC-0090 by 75% and 12%, respectively (Figure 14).
FIGURE 13. Graphs showing dose–response effect for the biphasic action of 2-[2-(4-methylphenyl)ethoxy]adenosine (WRC-0090) on cAMP content of cultured DDT,MF-2 cells in the absence and presence of 0.1 μM 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, panel A) and for control and pertussis toxin (PTX)–treated cells (panel B). Points represent means of single determinations on each of four wells of cells; error bars indicate SD. Data are from a single experiment that was repeated a second time (using a different passage of cells) with similar results. cAMP contents are expressed as picomoles per milligram cell protein. Curves were fit to the data by hand.

Effects of WRC-0090 on Isolated Perfused Hearts

To determine the effects of WRC-0090 on the guinea pig isolated perfused heart, measurements of LVP and spontaneous HR were made during exposures of hearts (n = 5) to WRC-0090 (1–1,000 nM) in the presence and absence of the A1-adenosine receptor antagonist DPCPX. WRC-0090 alone (1–1,000 nM) or in combination with 0.1 μM DPCPX caused no significant change (either increase or decrease) in LVP or HR compared with control values of 48±6 mm Hg (mean±SD, n = 20) and 261±13 beats per minute (n = 17), respectively. As expected,24 WRC-0090 decreased coronary vascular resistance. The decrease in vascular resistance was maximal at WRC-0090 concentrations ≥10 nM. Isoproterenol (10 nM) caused increases of LVP and spontaneous HR in all hearts (data not shown).

Discussion

This study was designed to test the hypothesis that mammalian ventricular myocytes possess A2-adenosine receptors. The A2-adenosine receptor is functionally defined as an adenosine receptor that mediates activation of adenyl cyclase. Stimulation of adenyl cyclase activity and accumulation of cAMP in cardiac myocytes are associated with increases in calcium inward current, contractility, and lengthening of the APD.11,33 Our results clearly demonstrate the absence of statistically significant electrophysiological, contractile, or cAMP responses to A2-adenosine receptor agonists in ventricular myocytes from guinea pig, rat, and rabbit hearts and thus do not support the hypothesis tested. This conclusion is based on three observations: 1) Two selective A2-adenosine receptor agonists and two non-selective agonists (NECA and adenosine) did not significantly alter APD, amplitude of twitch shortening, or cAMP content of isolated myocytes. 2) Attenuation of inhibitory responses mediated by A1-adenosine receptor agonists did not uncover a stimulatory response to A2-adenosine receptor agonists. 3) The selective A2-adenosine receptor agonist WRC-0090 did not increase duration of the action potential in the presence of either forskolin or rolipram (a phosphodiesterase inhibitor). Furthermore, unpublished observations from our laboratory using the whole-cell patch-clamp technique to study guinea pig isolated ventricular myocytes indicate that WRC-0090 (1 μM) alone or in combination with N-0861 (10 μM) does not increase peak calcium inward current.

The anticipated responses to activation of putative A2-adenosine receptors were not revealed in mammalian ventricular myocytes by attenuating the opposing responses mediated by A1-adenosine receptors. Pertussis toxin treatments of either whole animals or isolated myocytes attenuated the actions of A1-adenosine receptor agonists to reduce isoproterenol-mediated increases of either APD50 or cAMP accumulation. However, after myocytes were pretreated with pertussis toxin, neither APD50 (Figures 3 and 4) nor contractile amplitude (Figure 10) nor cAMP content (Figure 12) was significantly elevated by the A2-adenosine receptor agonists tested (WRC-0090, WRC-0013, NECA, and adenosine). Similarly, A1-adenosine receptor agonists did not alter APD50 (Figures 1–4), contractile amplitude (Figures 8 and 10), or cAMP content (Figures 11 and 12) in the presence of the A2-adenosine receptor antagonists.
agonists in have been suggested. Thus, our inability to demonstrate that these adenosine receptor antagonists did not significantly reduce a WRC-0090–mediated elevation of cAMP content (an A2-adenosine receptor–mediated response). Thus, our inability to demonstrate functional responses to A2-adenosine receptor agonists using either pertussis toxin pretreatment of myocytes, selective A2-agonists, or both does not appear to be the result of either an incomplete attenuation of A2-adenosine receptor–mediated responses or an inadvertent antagonism of the putative A2-adenosine receptor.

Because proteolytic digestion of the heart to isolate myocytes may have caused damage to adenosine receptors, we tested the effect of the selective A2-adenosine receptor agonist WRC-0090 on the guinea pig isolated perfused heart. WRC-0090 (1–1,000 nM) had no significant effects on HR or LVP in either the presence or absence of 0.1 μM DPCPX. This result suggests that guinea pig cardiac myocytes in situ do not possess functional A2-adenosine receptors. Also, the lack of A2-adenosine receptor–mediated responses in isolated myocytes did not appear to be an artifact of trypsin or dispase digestion of the heart. When hearts were digested with collagenase alone and myocytes were allowed to recover in medium 199 at 37°C for 3 hours, A2-adenosine receptor–mediated responses were absent (Figures 4, 10, and 12), whereas responses to isoproterenol and adenosine (A1) were present. Furthermore, desensitization of A2-adenosine receptors by exogenous adenosine during isolation of myocytes did not appear to explain our negative findings. We were unable to demonstrate electrophysiological responses to adenosine (10 and 100 μM) or NECA (10 and 100 μM) with guinea pig myocytes that had been isolated in the presence of theophylline (50 μM) and adenosine deaminase (1 unit/ml) (to prevent desensitization of adenosine receptors and accumulation of adenosine, respectively) and pretreated with pertussis toxin to uncouple activation of A2-adenosine receptors from functional responses. However, despite precautions to prevent loss of putative A2-adenosine receptors and measures to reduce A2-adenosine receptor–mediated inhibitory responses, the possibility still exists that the experimental conditions and/or drugs chosen for this study were not ideal for demonstration of A2-adenosine receptors on ventricular cardiomyocytes.

The A2-adenosine receptor agonists used in this study were determined to be highly selective in the guinea pig isolated perfused heart. We have confirmed this selectivity using the DDT, MF-2 cell line. Both WRC-0090 and WRC-0013 potently increased cAMP content of DDT, MF-2 cells (Figure 13 and text). However, at high concentrations (1–100 μM), either compound inhibited the increased cAMP accumulation caused by a lower (<0.3 μM) concentration of the same compound. The inhibition of cAMP accumulation by 1–100 μM WRC-0090 was abolished in the presence of either 0.1 μM DPCPX (Figure 13A) or pertussis toxin (Figure 13B). The results indicate the coexistence of both A1 and A2-adenosine receptors on DDT, MF-2 cells and suggest that, in the presence of an A2-receptor antagonist or after pretreatment with pertussis toxin, the increased accumulation of cAMP in DDT, MF-2 cells incubated with WRC-0090 or WRC-0013 was due to a selective activation of the A2-adenosine receptor.

Adenosine at high concentrations (0.1 and 1 mM) has been reported to exert a small (6–20%) positive inotropic effect on papillary muscles isolated from rat,34 guinea pig,35 and human36 ventricles. The adenosine analogues NECA and R-PIA do not appear to elicit this effect.35–37 To our knowledge, the positive inotropic effect of adenosine in mammalian ventricles has not been demonstrated to be receptor mediated, and its mechanism is unknown, although a stimulation of inositol phosphate formation may be involved.34 The fact that a significant adenosine receptor–mediated positive inotropic effect in ventricular myocardium has not been consistently demonstrated suggests that the physiological importance of this effect is minor.

The presence of adenosine receptors stimulating adenylyl cyclase activity in myocytes was suggested by investigators studying responses of guinea pig myocardial slices,15 crude homogenates of cultured myocytes,17 sarcolemmal membrane preparations,18,19 and isolated intact myocytes20 to unselective adenosine receptor agonists. Using membranes prepared from adult rat ventricular myocytes, Romano et al19 found that adenylyl cyclase activity was increased by NECA (1–100 μM) in the presence but not the absence of DPCPX. Behnke et al20 measured both the contractile response and the cAMP content of guinea pig isolated ventricular myocytes after exposure of cells to adenosine analogues in the presence of isoproterenol (0.1 μM). R-PIA decreased both the contractile response and the cAMP content of myocytes. In contrast, NECA decreased the contractile response to isoproterenol, but cAMP content of cells either was not changed (in the presence of NECA alone), increased (NECA with 0.3 μM DPCPX), or decreased (NECA with the A2-adenosine receptor antagonist CGS 15943A). The authors concluded that guinea pig ventricular myocytes possessed both A1-adenosine receptors that inhibited the contractile and cAMP responses to isoproterenol and A2-adenosine receptors that increased cAMP content but did not alter contractility.20 However, some investigators using mammalian isolated ventricular myocyte preparations exposed to adenosine analogues have observed only A1-adenosine receptor–mediated responses.38,39 and Wilken et al40 reported that basal cAMP levels were not altered in guinea pig ventricular myocytes by NECA or its A2-selective analogue CGS 21680 in either the presence or the absence of 1 μM DPCPX. Most recently, Xu et al21 found that both membrane adenylyl cyclase activity and shortening amplitude of isolated ventricular myocytes cultured from embryonic chicken hearts were stimulated by A2-adenosine receptor agonists after myocytes were either pretreated with pertussis toxin or were incubated with DPCPX to block A1-adenosine receptors. The increased adenylyl cyclase activity in myocyte membranes correlated with an increased twitch amplitude of isolated myocytes.

Conflicting results indicating the presence or absence of A2-adenosine receptor–mediated increases of adenylyl cyclase activity in mammalian ventricular myocytes may be explained if it is assumed that nonmyocytes
containing A₂-adenosine receptors are a contaminant of myocyte preparations. Hybridization of A₁-adenosine receptor cDNA revealed the presence of a single hybridizing transcript in rat heart; however, the cellular localization of this transcript was not reported. Microscopic observation of preparations of isolated cardiac myocytes after nuclear staining has revealed the presence of nonmyocytes closely adherent to myocytes. The identity of these cells is unknown, but endothelial cells, fibroblasts, blood cells, mast cells, and macrophages are all potential contaminants of myocyte preparations, and all are reported to have adenosine receptors coupled to stimulation of adenyl cyclase. Thus, for a functional demonstration of adenyl cyclase–coupled A₁-adenosine receptors, pure preparations of cardiac myocytes or pure myocyte cell lines are needed.

An alternative explanation of the conflicting results is that A₁-adenosine receptors and/or receptor-mediated responses are rendered nonfunctional in some experiments by unknown processes during the enzymatic isolation or recovery of myocytes or during the preparation of membranes. For example, proteolytic damage to receptors, uncoupling of the receptor from responses, activation and desensitization of receptors by endogenous adenosine, and potentiation or inhibition of effects of adenosine (by elevated intracellular calcium?) may potentially occur. On the other hand, damage to or inhibition of function of the inhibitory A₁-adenosine receptor may facilitate demonstration of an A₂-adenosine receptor–mediated response.

In conclusion, the results of experiments using intact hearts, isolated cells, several different techniques, and three species of animals do not provide support for the presence of A₁-adenosine receptors on mammalian cardiomyocytes.

Acknowledgments

The authors thank Sheryl Thedford for studies of the effects of WRC-0090 on membrane currents of guinea pig ventricular myocytes and Kathleen Duvall for preparation of the manuscript.

References

1. van Calker D, Müller M, Hamprecht B: Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. J Neurochem 1979;33:999–1005
34. Leggser A, Fregoli J, Renard D, Vassort G: ATP and other adenosine compounds increase mechanical activity and inositol trisphosphate production in rat heart. J Physiol (Lond) 1988;401:185–199
Selective A2-adenosine receptor agonists do not alter action potential duration, twitch shortening, or cAMP accumulation in guinea pig, rat, or rabbit isolated ventricular myocytes.

J Shryock, Y Song, D Wang, S P Baker, R A Olsson and L Belardinelli

Circ Res. 1993;72:194-205
doi: 10.1161/01.RES.72.1.194

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1993 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/72/1/194