Cardiac myocyte adenosine A$_{2a}$ receptor activation fails to alter cAMP or contractility: role of receptor localization

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Kilpatrick, Eric L., Prakash Narayan, Robert M. Mentzer, Jr., and Robert D. Lasley. Cardiac myocyte adenosine A$_{2a}$ receptor activation fails to alter cAMP or contractility: role of receptor localization. Am J Physiol Heart Circ Physiol 282: H1035–H1040, 2002. First published November 29, 2001; 10.1152/ajpheart.00808.2001.—Adenosine A$_{2a}$ receptors are not present in cardiac myocytes. The purpose of this study was to determine whether A$_{2a}$ receptors are expressed in rat ventricular myocytes and what physiological effects are mediated via activation of these receptors. Western blot analysis with a polyclonal antibody raised against a peptide sequence specific to the carboxy terminus of the A$_{2a}$ receptor revealed the presence of a band at ~45 kDa. However, the immunoreactivity was located in the nonmembrane fraction of the cell lysate. The membrane fraction only exhibited an immunoreactive band ≥50 kDa. Treatment of isolated myocytes with the adenosine A$_{2a}$ agonist 2-[(4-[(2-carboxyethyl)-phenylethylamino]-5′-N-ethylcarboxamido)adenosine (CGS-21680) exerted no effects on cAMP levels or myocyte twitch amplitude. These results indicate that although rat ventricular myocytes appear to express adenosine A$_{2a}$ receptors, stimulation with an A$_{2a}$ agonist exerts no functional effects, possibly because of the subcellular localization of the A$_{2a}$ receptor.

phosphodiesterase inhibitor; Western blot

ADENOSINE EXERTS A VARIETY of effects in the heart by the activation of purinergic P$_1$ receptor subtypes. Activation of the myocyte A$_1$ receptor alters sinoatrial and atrioventricular conduction and attenuates the metabolic and contractile effects of β-adrenergic receptor stimulation (3, 35–37). Adenosine A$_1$ receptor activation before ischemia also exerts significant protective effects in ischemic-reperfused myocardium (15). Stimulation of A$_{2a}$ receptors located on coronary endothelial cells and vascular smooth muscle mediates coronary vasodilation (17, 33). Additional reports indicate that coronary A$_{2a}$ receptor stimulation during reperfusion may exert beneficial effects (16, 47).

There is universal agreement that A$_1$ receptor agonists alter neither contractility nor cAMP accumulation in normal ventricular myocardium; however, there are conflicting reports on the effects of adenosine A$_{2a}$ receptor agonists. Initial studies in several species (dog, rat, guinea pig) indicated that the mixed agonist 5′-(N-ethylcarboxamido)adenosine (NECA) had no effect on cAMP accumulation (11, 30, 38). Subsequent studies indicated that more selective adenosine A$_{2a}$ agonists exerted no effects on cAMP accumulation or contractility, suggesting that ventricular cardiomyocytes do not express adenosine A$_{2a}$ receptors (40, 43). In contrast, there are additional reports that A$_{2a}$ receptor agonists increase adenyl cyclase activity, myocyte twitch amplitude, or cAMP accumulation in isolated guinea pig, rat, and fetal chick ventricular myocytes (4, 6, 18, 37, 41, 46).

In addition to the conflicting pharmacological evidence for the existence of myocyte A$_{2a}$ receptors, molecular studies have yielded disparate findings. The first report of the presence of adenosine A$_{2a}$ receptor mRNA in ventricular myocardium was based on Northern blotting of rat myocyte lysates (46). Subsequently, the A$_{2a}$ receptor protein was detected by immunoblotting in isolated porcine myocytes (24). However, a more recent investigation with RT-PCR concluded that adenosine A$_{2a}$ receptor transcript was absent in porcine myocardial homogenate (10).

Although it has been reported that adult rat ventricular myocytes contain A$_{2a}$ receptor mRNA, there have been no reports of the expression of A$_{2a}$ receptor protein in this species. Additionally, the pharmacological evidence for functional myocyte A$_{2a}$ receptors is controversial. Therefore, the purpose of this study was to ascertain the effects of the A$_{2a}$ receptor agonist CGS-21680 on ventricular myocyte cAMP levels and twitch amplitude. Furthermore, Western blot analysis was used to investigate the presence of A$_{2a}$ receptors in these cells.
METHODS

All animals in this study received humane care according to the guidelines set forth in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council (National Institutes of Health Publication No. 85-23, 1996) and according to the guidelines of the Department of Laboratory Animal Resources, University of Kentucky.

Isolation of myocytes. Ventricular myocytes were enzymatically dissociated from male Sprague-Dawley rats (350-400 g) by previously described methods (28, 29) with minor modifications. Rats were heparinized (500 U i.p.) and anesthetized with pentobarbital sodium (65 mg/kg ip). The hearts were rapidly excised and retrogradely perfused for 4 min at constant pressure (70 mmHg) with calcium-free modified HEPES buffer (10 mM HEPES and 2 mM pyruvate, pH 7.2, gassed with 100% O₂ at 37°C). After addition of Liberase Blendzyme (0.14 mg/ml; Roche Diagnostic, Indianapolis, IN), the perfusate was recirculated for 15–17 min. Calcium was gradually added during the final 10 min of enzyme recirculation to reach 500 μM. The ventricles were minced and placed in a shaking water bath (37°C) with 20 ml of HEPES-buffered enzyme solution for 10 min followed by gentle tituration. The digestion mixture was filtered through 250-μm nylon mesh, washed twice with enzyme-free buffer containing 500 μM Ca²⁺, and resuspended in HEPES buffer (1 mM CaCl₂, pH 7.4). This protocol typically yielded >75% rod-shaped myocytes. All cells were used within 6 h of isolation.

cAMP measurement. cAMP was measured as previously described with modifications (28). Myocytes were incubated at room temperature (30 min) in HEPES buffer supplemented with adenosine deaminase (1 U/ml) to degrade endogenous adenosine. The myocyte suspensions from 10 separate isolations were divided into 1.5-ml aliquots and assigned to the following groups: control (DMSO vehicle), the cAMP phosphodiesterase (PDE) inhibitor rolipram (50 μM), the adenosine A₂a receptor agonist CGS-21680 (100 nM), and CGS-21680 + rolipram. In three of these isolations, additional myocyte aliquots were treated with isoproterenol (10 nM) or isoproterenol + rolipram. Cells were initially treated with rolipram or vehicle (10 min, 37°C) followed by application of CGS-21680, isoproterenol, or vehicle for 10 min. After treatments, the cells were pelleted by centrifugation (1 min, 1,000 g) and the supernatant was discarded. Myocytes were lysed in 500 μl of HCl (0.1 N, 10 min) and vortexed. The suspension was centrifuged (4 min, 3,000 g), and the supernatant was assayed for cAMP (ELISA kit; R&D Systems, Minneapolis, MN). The pellet was solubilized with 1 N NaOH, and protein was assayed by the Lowry method. Intracellular cAMP was expressed as picomoles per milligram of protein.

Myocyte contractility measurements. Myocytes were adhered to the laminin coated-glass coverslip floor of the recording chamber (RC-24; Warner Instrument, Hamden, CT) and mounted on the stage of an inverted microscope (IX-70; Olympus America, Melville, NY). Cells were suffused with HEPES buffer (100% O₂, pH 7.4, 1 ml/min) from an inline heater (37°C, TS 28; Warner Instrument, Hamden, CT), field stimulated (0.5 Hz, SD9 stimulator; Grass Instruments, Quincy, MA), and imaged with a charge-coupled device camera. Changes in cell length were quantified via edge-motion detection with a video dimension analyzer (Coyote Bay), and twitch amplitude was expressed as a percentage of diastolic length. Baseline measurements were obtained after a 10-min equilibration period to ensure the presence of steady-state contractions.

The effect of CGS-21680 at different concentrations was analyzed in three groups: group 1, 100 nM (n = 8 myocytes from 4 hearts); group 2, 1 μM (n = 11 myocytes from 4 hearts); and group 3, vehicle (n = 7 myocytes from 4 hearts). The myocytes were exposed to CGS-21680 or vehicle (10 min) followed by washout. Isoproterenol (2 min, 5 nM) was used as a positive control. Two further groups tested the effects of the PDE inhibitor rolipram on twitch amplitude. Myocytes were treated with either rolipram (50 μM; n = 17 myocytes from 3 hearts) or vehicle (DMSO; n = 9 from 3 hearts) for a total of 20 min (twitch amplitude measured every 5 min) followed by 10-min washout.

Western blot analysis. Myocytes were centrifuged twice (3 min, 60 g, 4°C) with intervening resuspension in 10 ml of PBS. The pellet was resuspended in 1 ml of buffer A (in mM: 250 sucrose, 5 Tris-HCl, 0.4 MgCl₂, pH 7.5) with the addition of aprotinin (2 μg/ml), leupeptin (2 μg/ml), pepstatin A (2 μg/ml), benzamidine (0.1 mM), and phenylmethylsulfonyl fluoride (0.1 mM) and received 20 strokes with a Teflon Dounce on ice. The homogenate was centrifuged (10 min, 200,000 g for 1 h). The supernatant containing soluble proteins was reserved, and the pellet was washed twice with buffer A followed by resuspension in buffer B (in mM: 50 Tris-HCl and 4 MgCl₂ containing protease inhibitors). Proteins were assayed by the Lowry method. Fifty micrograms of protein from the soluble and particulate fractions was subjected to 10% SDS-PAGE (25 μA) under reducing conditions as described by Laemmli (14). After electrophoresis, the proteins were electroblotted (200 mA, 2 h) to polyvinylidene difluoride (PVDF) membranes (0.45 μm). The membranes were blocked by rocking incubation (1 h, room temperature) with PBS containing 5% milk. Incubation (2 h, room temperature) of primary anti-A₂a peptide antiserum (1:1,000) in PBS-milk was followed by four 10-min washes with PBS containing 0.05% Tween-20. Secondary antibody (1:7,500) conjugated with horseradish peroxidase was incubated (1 h, room temperature) with PBS (1% milk + 0.05% Tween-20) followed by four 10-min washes. The membrane was incubated with enhanced chemiluminescent substrate (Pierce, Rockford, IL) and exposed to Kodak X-OMAT film for up to 5 min. Specificity of the primary antibody for the rat A₂a receptor protein was verified by cross-reaction with the heterologously expressed human A₂a receptor (RBI, Natick, MA). In addition, rat A₁ receptor and rat A₃ receptor from NEN Life Science (Boston, MA) were used as negative controls.

Materials. Isoproterenol, CGS-21680, adenosine deaminase, and DMSO were purchased from Sigma (St. Louis, MO). Rolipram was obtained from Alexis Biochemical (San Diego, CA). Antiserum from rabbit raised against a peptide sequence from the carboxy terminus of the canine adenosine A₂a receptor was a kind gift of Dr. S. J. Mustafa (East Carolina University, Greenville, NC). The secondary antibody was goat anti-rabbit IgG from Santa Cruz Biotechnology (Santa Cruz, CA). Lowry protein assay kit was purchased from Bio-Rad (Hercules, CA).

Data analysis. Data are expressed as means ± SE and analyzed by two-way ANOVA with mixed factors or repeated measures, and Tukey honestly significant difference test was used for post hoc analysis with Statistica software (StatSoft, Tulsa, OK). Significance was regarded as P < 0.05.

RESULTS

Effect of CGS-21680 on isolated myocyte cAMP accumulation. Basal myocyte intracellular cAMP levels were 11.7 ± 0.8 pmol/mg protein. As shown in Fig. 1A, treatment with the adenosine A₂a agonist CGS-21680
(100 nM) for 10 min had no effect on cAMP (11.3 ± 1.3 pmol/mg). Although the combination of CGS-21680 + rolipram elevated cAMP levels to 13.2 ± 1.2 pmol/mg (P < 0.05), this effect was not different from treatment with rolipram alone (14.0 ± 1.0 pmol/mg). In contrast to the lack of effect of CGS-21680, isoproterenol (10 nM, 10 min) increased cAMP levels from 9.3 ± 1.4 to 20.3 ± 6.0 pmol/mg (P < 0.05; Fig. 1B). The addition of rolipram potentiated the effects of isoproterenol on cAMP as levels increased to 44.3 ± 3.5 pmol/mg.

**Effect of CGS-21680 on myocyte contractility.** The effects of two doses of CGS-21680 on myocyte twitch amplitude were tested (Fig. 2). Basal twitch amplitudes in vehicle- and CGS-21680-treated myocytes were similar (6.3 ± 0.7%, 5.7 ± 0.6%, and 6.1 ± 0.5% of resting cell length, respectively). Neither 100 nM nor 1 μM CGS-21680 treatment for 10 min had any effect on contractility. After washout of the CGS-21680, myocytes were exposed to isoproterenol (5 nM) as a positive control. Twitch amplitude in vehicle- and CGS-21680-treated myocytes increased similarly with isoproterenol (53 ± 6%; P < 0.05). In an additional group of myocytes, 20-min application of rolipram (50 μM) exerted no effects on contractility (Fig. 3).

**Western blot analysis of adenosine A2a receptor.** Whole antiserum was used for Western blotting. An enhanced chemiluminescence blot of PVDF membrane probed with adenosine A2a receptor antiserum is shown in Fig. 4. Protein from the soluble fraction of isolated rat ventricular myocytes showed a major immunoreactive band at ~45 kDa that comigrated with the human A2a receptor. This band was not detectable in the lane containing the particulate fraction, although a distinct immunoreactive band was observed at a higher molecular mass (Mr) (~50 kDa). Protein from heterologously expressed rat A1 and A3 receptors did not demonstrate any cross-reactivity, confirming the specificity of the antibody for the A2a receptor.
DISCUSSION

The results of the present study indicate that the adenosine $A_2a$ receptor agonist CGS-21680 did not increase accumulation of intracellular cAMP in isolated rat ventricular myocytes in the presence or absence of the PDE inhibitor rolipram. Similarly, application of the $A_2a$ agonist did not alter myocyte twitch amplitude. Western blot analysis revealed that ventricular myocytes expressed $A_2a$ receptors in both the soluble and particulate fractions, albeit at different molecular masses. These findings indicate that, despite the presence of $A_2a$ receptors within rat ventricular myocytes, their activation is not associated with increases in cAMP or inotropy. The lack of functional effects of adenosine $A_2a$ receptor stimulation may be caused by compartmentation of the receptor or inefficient coupling to G proteins.

The best-known mechanism of $A_2a$ receptor signaling is the accumulation of cAMP. However, studies examining cAMP accumulation with $A_2a$ agonists in isolated myocytes have yielded inconsistent results. There are several reports that NECA and the more selective $A_2a$ agonists CGS-21680 and 2-[2-(4-methylphenyl)ethylamino]adenosine (WRC-0090) do not alter cAMP levels in ventricular myocytes (11, 23, 30, 38, 40, 43). In contrast, there are additional reports that these same agonists do increase cAMP, either by directly activating adenylyl cyclase or indirectly by inhibiting cAMP PDE (1, 4, 6, 18, 37, 41, 46). Two of these studies, in guinea pig and rat (41, 46), reported increases in cAMP accumulation with 100 nM CGS-21680, but another, in the rat (6), did not. The results of myocyte studies with PDE inhibitors with $A_2a$ agonists have consistently reported no changes in cAMP (11, 30, 38, 40, 43), whereas those without PDE inhibitors have reported increases (4, 6, 41, 46). Although the presence or absence of PDE inhibitors could potentially explain these divergent results, none of the above studies directly tested the effect of PDE inhibition on $A_2a$ receptor-mediated increases in cAMP accumulation. Our results indicate that the $A_2a$ agonist CGS-21680 did not affect cAMP accumulation whether or not the PDE inhibitor rolipram was present. Rolipram alone increased cAMP content by 21%, consistent with previous reports (4, 25, 27). Isoproterenol (10 nM) increased cAMP by 118%, which was potentiated by rolipram (238% increase), confirming the ability of the myocytes to accumulate cAMP and to respond to PDE inhibition. The results of the present study thus indicate that adenosine $A_2a$ receptor stimulation does not alter ventricular myocyte cAMP levels.

Previous studies of $A_2a$-mediated positive inotropic effects in isolated myocytes have also yielded conflicting results. Both nonselective and selective $A_2a$ agonists have been reported to have no effect on guinea pig myocyte twitch amplitude (30, 40, 41). In contrast, there are reports showing that NECA and CGS-21680 increase twitch amplitude in chick and rat ventricular myocytes (6, 18, 19, 44, 46). In our hands, neither 100 nM nor 1 µM CGS-21680 altered rat ventricular myocyte twitch amplitude. The PDE inhibitor rolipram, which did increase cAMP levels, also did not alter myocyte twitch amplitude, in agreement with previous observations (12, 13). Despite the lack of response to CGS-21680 and rolipram, the myocytes responded robustly to isoproterenol (5 nM), confirming their ability to respond to inotropic agents. Our present results are consistent with the lack of inotropic effect of CGS-21680 in normal intact guinea pig, rat, and porcine myocardium (8, 16, 26). We also observed no effects of CGS-21680 (1–1,000 nM) on left ventricular developed pressure in isolated rat hearts despite dose-dependent increases in coronary flow (data not shown). Given that we observed no increase in cAMP, and that it has been reported that CGS-21680 did not alter intracellular Ca$^{2+}$ in rat ventricular myocytes (43), it is not surprising that CGS-21680 had no effect on myocyte contractility.

There are several possible explanations for the lack of CGS-21680 effects in the present study. It is possible that we did not use an adequate dose of the agonist. However, this seems unlikely, given that we used 100 nM and 1 µM concentrations of CGS-21680, and the EC$_{50}$ values for this agonist on cAMP accumulation in PC12 cells and human neutrophils have been reported to be <20 nM (9, 42). Several reports have suggested that possible cross-activation of the myocyte A$_1$ receptor may mask $A_2a$ stimulatory effects on cAMP and contractility (6, 18, 19, 37, 41, 46). The results of additional studies, performed in the presence of the A$_1$ antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and/or pertussis toxin (to block inhibitory G proteins), have indicated no effects of $A_2a$ agonists on adenylyl cyclase activity, cAMP accumulation, or myocyte inotropism.
ropy (23, 40, 43). Therefore, it does not appear that our inability to demonstrate CGS-21680 effects in the present study could be due simply to omission of DPCPX or pertussis toxin.

A third possibility for the lack of CGS-21680 effects is that ventricular myocytes lack adenosine A2a receptors. In fact, the results of several negative pharmacological studies have led to the conclusion that A2a receptors are not expressed on cardiac myocytes (40, 43). However, Western blot analysis of our whole cell lysates indicated an immunoreactive band at ~45 kDa that comigrated with heterologously expressed human A2a receptors but had no cross-reactivity with either rat A1 or A3 receptors. These results are consistent with the M, reported in membranes from human and porcine heart (24) and rat striatum (34) and kidney (48). Interestingly the 45-kDa band was found exclusively in the soluble, rather than the membrane, fraction of myocyte lysates. We did observe immunoreactivity in the particulate fraction, but this band appeared ≥50 kDa. The location of this band is consistent with the higher mass (~52 kDa) reported for the A2a receptor in canine liver plasma membranes (32) and the canine thyroid-derived receptor (~60 kDa) transfected into CHO cells (31). Thus, as originally concluded by Palmer et al. (32), there appear to be both tissue- and species-dependent differences in the size of the adenosine A2a receptor.

The Western blot results may provide an explanation for why the A2a agonist CGS-21680 was unable to increase cAMP accumulation or alter myocyte contractility. Localization of the 45-kDa form of the A2a receptor in the soluble fraction of the myocytes would preclude receptor interaction with membrane-bound stimulatory G proteins and adenylyl cyclase. It is possible that our soluble fractions were contaminated with intracellular organelles such as endosomes or microsomes or other small vesicles. The movement of A2a receptors into light vesicles has been proposed to be a possible explanation for receptor desensitization (31). Alternatively “cytosolic” adenosine A2a receptors have been observed in human skeletal muscle (22) and PC12 cells (2). In the latter study cytosolic A2a receptors appeared to translocate to the plasma membrane during anoxia, an effect that was associated with a fivefold decrease in EC50 for CGS-21680 stimulation of cAMP accumulation (2). Although our observation that the 45-kDa immunoreactivity resided exclusively in the soluble fraction was unexpected, the A2a receptor is unique among adenosine receptors in lacking a cysteine palmitoylation site (20), which appears to play a key role in targeting and anchoring proteins to membranes (7).

Although the 50-kDa band was found in the membrane fraction, it is possible that the higher-mass form of the protein may not effectively couple to Gs, because immunoreactivity provides no information regarding appropriate coupling or functionality of the identified protein. Rat striatal adenosine receptors have been shown to have both a high- and a low-affinity state, with the majority of receptors being of low affinity and not G protein coupled (21). Alternatively, A2a receptor stimulation may have increased cAMP in a subcellular compartment that does not lead to increased inotropy. The compartmentation of cAMP in cardiac myocytes was first reported nearly two decades ago (5), when it was reported that prostaglandin E1 increased cAMP in the soluble, but not the particulate, fraction of rabbit ventricular myocytes, whereas the β-adrenergic agonist isoproterenol increased cAMP in both fractions. More recently, it has been determined that increases in particulate cAMP levels correlate with the inotropic effects of β-adrenergic receptor stimulation (45) and adenylyl cyclase is compartmentalized in cardiac myocyte plasma membranes within caveolae (39). Thus it is possible that CGS-21680 increased cAMP in the soluble fraction, which is not associated with an inotropic effect.

In summary, we showed that although the adenosine A2a receptor protein is expressed in rat ventricular myocytes, its activation does not lead to changes in inotropy. The lack of an A2a receptor inotropic effect is consistent with the lack of effect of CGS-21680 on myocyte cAMP accumulation in the presence or absence of a PDE inhibitor. In conclusion, adenosine A2a receptor stimulation in rat ventricular myocytes does not increase whole cell intracellular cAMP levels or alter twitch amplitude, possibly because of receptor subcellular localization and/or poor receptor-transduction coupling.

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