Expression and Pharmacological Characterization of a Stimulatory Subtype of Adenosine Receptor in Fetal Chick Ventricular Myocytes

David Xu, Haeyoung Kong, and Bruce T. Liang

Ventricular and atrial myocytes cultured from chick embryos 14 days in ovo were used as model systems to study cardiac adenosine receptors. In membranes of ventricular cultures, blocking of the A1-adenosine receptor pathway by the A1-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) or by pertussis toxin treatment of the myocyte resulted in a significant adenosine agonist–mediated stimulation of the adenylyl cyclase activity. The maximal increases in adenylyl cyclase activity caused by the equipotent or the A2-adenosine receptor–selective agonists (from 52.1±3% to 63±10% [mean±SEM]) were significantly greater than those caused by the A1-selective agonists (from 11±5% to 34.6±5%) (p<0.01 , by t test, n=4–8). However, in membranes of atrial myocytes, when A1-subtype had been blocked, the various adenosine agonists had no effect on the adenylyl cyclase activity. Whether the stimulatory adenylyl cyclase–coupled adenosine receptor is also capable of stimulating contractility in the intact ventricular myocyte was next investigated. In ventricular but not in atrial cells, the various adenosine agonists caused an increase in the contractile amplitude in the presence of DPCPX or in myocytes preexposed to pertussis toxin. The increase in contraction amplitude caused by each agonist was expressed as percent of maximum (maximum is the increase in contractility caused by 2.4 mM calcium). In the pertussis toxin–treated myocyte, the maximal increases caused by the equipotent or A2-agonists (NECA, MECA, CV-1808, and CGS21680, from 49.6±3% to 52.5±6%, n=8–12) were significantly greater than those elicited by the A1-agonists (2-CADO, S-PIA, R-PIA, and DCCA, from 12±4% to 37±3%, n=8) (p<0.05, by t test). These data demonstrated that a stimulatory adenosine receptor, likely the A2-adenosine receptor, was present on the ventricular but not the atrial myocytes and was linked directly to a stimulation of the cardiac contractility. The functional effects mediated by the A2-subtype became manifest in the presence of isoproterenol, as evidenced by an inhibition of the isoproterenol-stimulated increases in adenylyl cyclase activity and in cardiac contractility by adenosine agonists. Thus, both subtypes of adenosine receptors, each mediating opposing responses, were present on the ventricular myocytes, whereas only the A1-subtype was found in the atria. The presence of a stimulatory functional A2-adenosine receptor may help explain the absence of a direct negative inotropic response to adenosine in the ventricle. (Circulation Research 1992;70:56–65)

In cardiac myocytes, the negative inotropic effect is mediated by adenosine receptors of the A1 subtype.1–3 In atria, the adenosine receptor agonist causes a direct, negative inotropic effect in the absence of a β-adrenergic receptor agonist. However, in ventricular myocytes, the negative inotropic effect of the adenosine receptor agonist is only evident when the myocyte contractility has first been stimulated by a β-adrenergic agonist. In the absence of a β-adrenergic receptor agonist, adenosine or an adenosine agonist causes a slight increase or no increase in the contractility of the ventricular myocyte. In studies of the regulation of adenylyl cyclase

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activity, adenosine agonists cause an inhibition of the cyclase activity stimulated by GTP or by isoproterenol in atria, whereas in the ventricles, the effects of adenosine agonists on cAMP level or on adenylate cyclase activity have been variable, with both an increased or a decreased level of the adenylate cyclase activity. Thus, the contractile and the adenylate cyclase responses of the ventricle to adenosine agonist differ from those of the atrium. The reason for the different contractile responses to adenosine between atria and ventricles was thought to be the coupling of the A₁-adenosine receptor to a potassium channel in atria but not in ventricles. Another possibility was the presence of another subtype of adenosine receptor that mediated a stimulatory rather than an inhibitory effect on the adenylate cyclase and the cardiac contractility. Recent studies have demonstrated the presence of a separate subtype of adenosine receptor, the A₂-adenosine receptor, that was coupled to an increase in the cAMP level in acutely dispersed ventricular myocytes from guinea pig and to a stimulation of the adenylate cyclase in membranes of ventricular myocytes cultured from adult rat or from chick embryo. These data were based on the ability of the equipotent agonist N-ethyladenosine-5'-uronic acid (NECA) to induce an increase in cAMP level or in adenylate cyclase activity in the presence of the A₁-adenosine receptor-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). However, a pharmacological characterization of this stimulatory adenylate cyclase receptor subtype and the relative roles of the stimulatory and the inhibitory adenosine receptor subtypes in modulating the GTP basal and the isoproterenol-stimulated adenylate cyclase activity has not been carried out. Further, although the ability of NECA to inhibit the isoproterenol-stimulated increase in contractility was partially reversed by an A₁-selective antagonist, it is not known whether the presence of an A₁-antagonist will unmask a stimulatory effect of NECA on the basal contractile state in the absence of isoproterenol. It is not clear whether the A₂-subtype has a functional role in modulating the contractility of the ventricular myocyte and whether the presence of the A₂-subtype in ventricles could help explain the ventricular contractile response to adenosine. In the present study, atrial and ventricular myocytes cultured from chick embryos, in which the A₁-adenosine receptor can be blocked by DPCPX or uncoupled from its functional effect(s) by pertussis toxin (PTX) treatment of the culture, were used to 1) investigate whether activation of a different subtype of the adenosine receptor, which could mediate a stimulation of the adenylate cyclase, is capable of stimulating or modulating the cardiac contractility and 2) carry out pharmacological characterization of this adenosine receptor subtype. A further objective of the present study was to test the notion that these stimulatory adenosine receptors are preferentially expressed on the ventricular but not the atrial myocytes. The present data demonstrated that, in ventricular myocytes, adenosine receptor agonists, whether selective for the A₁- or the A₂-subtypes or equipotent, were all capable of causing an increase in the myocyte contractility as well as in the adenylate cyclase activity when the A₁-adenosine receptor pathway had been blocked by either DPCPX or PTX. Both adenosine receptor subtypes coexisted on the ventricular myocyte, and each subtype mediated opposing functional responses, whereas only the A₁-subtype was present on the atrial myocyte.

**Materials and Methods**

**Materials**

ATP, GTP, lima bean trypsin inhibitor, soy bean trypsin inhibitor, leupeptin, L-isoproterenol, and alamethicin were from Sigma Chemical Co., St. Louis, Mo. Adenosine deaminase (AD) was obtained from Boehringer Mannheim Corp., Indianapolis, Ind. [³H]ATP, [³²P]NAD+, and [¹⁴C]-cAMP were from Du Pont Pharmaceuticals, Wilmington, Del. Embryonic chick eggs were purchased from Spafas Inc., Storrs, Conn. PTX was available from List Biological Laboratories, Inc., Campbell, Calif. The adenosine analogues used were N6-R-phenyl-2-propyladenosine (R-PIA), N6-S-phenyl-2-propyladenosine (S-PIA), DPCPX, NECA, 8-(p-sulfophenyl)theophylline (8-PST), N-methyladenosine-5'-uronic acid (MECA), 2-chloroadenosine (2-CAĐO), 2-phenylaminoadenosine (CV-1808), 2-[p-(2-carboxyethyl)-phenethylamino]-5′-N-ethylcarboxamido adenosine (CGS 21680), and 1-deaza-2-chloro-N⁶-cyclopentyladenosine (DCCA).

**Methods**

**Tissue preparation.** Atrial and ventricular myocytes from chick embryos 14 days in ovo were maintained in culture as previously described. Briefly, atrial and ventricular cells were isolated in calcium- and magnesium-free Hanks’ balanced buffer containing trypsin (0.025%) (GIBCO, Grand Island, N.Y.). The trypsin was neutralized by medium containing 50% heat-inactivated horse serum and the same Hanks’ solution described above. After centrifugation, the cells were resuspended in culture medium containing the following: 6% heat-inactivated fetal bovine serum, 40% Medium 199 (GIBCO), 0.1% penicillin streptomycin antibiotic solution, and a salt solution containing (mM) NaH₂PO₄, 1.0, NaCl 116, MgSO₄ 0.8, KCl 1.18, NaHCO₃ 26.2, CaCl₂ 0.88, and glucose 5.5. Ventricular cells were plated at a density of 4×10⁶ cells per milliliter and atrial cells at 5×10⁶ cells per milliliter. The cultures were incubated in a humidified 5% CO₂–95% air mix at 37°C. Confluent monolayers of cells developed by day 2–2.5. In the various experiments described, cells were used after 2½–3 days in culture. For assay of adenylate cyclase activity, cells were broken and Dounce-homogenized in buffer containing 50 mM Tris-HCl, 5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 32 μg/ml leupeptin, 32 μg/ml lima bean trypsin inhibitor, 32 μg/ml soybean trypsin inhibitor, and 0.01% CHAPS, pH 7.4. Cell
homogenates were then pretreated with 20 μg/ml of alamethicin and with 5 units/ml ADA at 37°C for 10 minutes before the assay. Unless otherwise indicated, experiments were carried out in the presence of the protease inhibitors, alamethicin, ADA, and CHAPS.

Adenylate cyclase and Lowry assays. Assays of adenylate cyclase activity were carried out as previously described.3 In the adenylate cyclase assay, ADA was added at 5 units/ml to help minimize the effects of endogenous adenosine. Control experiments were carried out; these experiments demonstrated that the formation of cAMP under control, isoproterenol-stimulated, and adenosine agonist–stimulated conditions increased linearly as a function of both the time of incubation as well as the membrane protein concentration. Doubling the concentration of ADA to 10 units/ml in the assay did not affect the maximal increase or the EC50 of the NECA-stimulated adenylate cyclase activity. Proteins were determined according to the method of Lowry et al,13 using bovine serum albumin as the standard.

Contractility. Measurement of contractile amplitude in cultured atrial and ventricular myocytes was performed with an opticovideo motion detection system as previously described.3,11,12 The cultured heart cells attached on a glass coverslip were placed in a perfusion chamber with temperature maintained at 37°C. The chamber was placed on the stage of an inverted phase-contrast microscope (Nikon) with both an inlet and an outlet that allowed infusion and removal of medium containing the various drugs. The contractile motion of attached cells was monitored by an opticovideo motion detecting system as previously described12 using a video motion analyzer (Colorado Video, Boulder, Colo.). The perfusion medium contained the following (mM): HEPES 4 (pH 7.4), NaCl 137, KCl 3.6, MgCl2 0.5, CaCl2 0.6, glucose 5.5, and horse serum at 6% as well as the various drugs at the indicated concentrations. The mean±SEM rate of contraction was 135±14 beats per minute (n=104). The mean±SEM increase in contractile amplitude produced by raising the calcium concentration from 0.6 to 2.4 mM in the superfusion medium was 46.8±3.1% (n=50) and represented the maximal increase in contraction amplitude. The increase in the contraction amplitude by each agonist was normalized to the increase caused by 2.4 mM calcium as percent maximum.

PTX–mediated ADP-ribosylation. The [32P]ADP–ribosylation of G proteins was carried out at 37°C for 40 minutes in membrane homogenates of cultured myocytes as previously described.3 The culture was treated with 5 ng/ml PTX, and the ability of PTX to cause subsequent [32P]ADP–ribosylation of the G proteins in membranes prepared from these cultures was determined.

Data Analysis

EC50 values were calculated according to the method previously described using a nonlinear regression program in BASIC.14,15 Statistical analyses were carried out by both unpaired and paired t tests and by one-way analysis of variance (ANOVA), followed by group comparison.

Results

Stimulation of Adenylate Cyclase in Ventricular Myocytes by Adenosine Receptor Agonists

Adenosine receptor agonists, A1- or A2-selective or equipotent, were capable of stimulating the adenylate cyclase activity in membranes of the culture where the A1-receptor pathway had been blocked by prior treatment of the culture with PTX (5 ng/ml for 12 hours) (Figure 1). The levels of PTX-mediated [32P]ADP–ribosylation in membrane homogenates prepared from both control and PTX-treated cultures were compared. In membrane homogenates prepared from PTX-treated cells, the level of PTX-catalyzed [32P]ADP–ribosylation was negligible, indicating that the α-subunit of G proteins involved was already ADP-ribosylated by the endogenous NAD+ in the intact cell (Figure 2). In membranes of these PTX-treated ventricular cultures, the EC50 of the stimulation of adenylate cyclase activity caused by each agonist was similar (by ANOVA, F=1.65, p=0.2); however, the maximal stimulation of adenylate cyclase activity caused by the A2-selective or the equipotent agonists was significantly greater than

![Graph showing effects of various adenosine receptor agonists on the adenylate (adenosyl) cyclase activity in membranes of ventricular myocytes. R-PIA, N7-R-phenyl-2-propyladenosine; NECA, N-ethyladenosine-5'-uronic acid; S-PIA, N7-S-phenyl-2-propyladenosine; 2-cADO, 2-chloroadenosine. Preparation of membranes and assay of adenylate cyclase activity were carried out as described in "Methods." The level of stimulation of adenylate cyclase activity caused by the maximum concentration of each agonist was termed 100% (maximal stimulation) and represented the difference in adenylate cyclase activity determined in the presence of GTP (0.1 mM) and GTP plus the maximal agonist concentration. The stimulation produced at each concentration of adenosine agonist was normalized to the maximal stimulation as percent of maximum. Data were mean±SEM of four to eight experiments.](http://circres.ahajournals.org/doi/abs/10.1161/01.RES.70.1.58.CIR.12.21.1992)
that caused by the A1-selective agonists (Table 1) (p<0.01, unpaired t test). The maximal increase in the adenosine agonist–stimulated adenylate cyclase activity was 60% above that obtained in the presence of GTP (100 μM) and represented about 40% of the maximal isoproterenol-stimulated adenylate cyclase activity. The values for maximal percent increase in adenylate cyclase activity caused by the A1-agonists were similar to one another (by one-way ANOVA, F=0.0034, p=0.99). The values for maximal percent increase caused by the A1-agonists were similar to one another except among the R-PIA versus S-PIA and the R-PIA versus DCCA comparisons (ANOVA followed by group comparisons, the difference was significant at the 95% confidence level). The stimulation of adenylate cyclase activity in these PTX-treated ventricular cells was blocked by the presence of 8-PST (300 μM) (data not shown), indicating that the increase in adenylate cyclase was secondary to activation of an adenosine receptor. These data suggest that a stimulatory adenosine receptor subtype, distinct from the inhibitory A1-subtype, was also present on the cultured ventricular myocyte. Parallel studies were carried out in membranes prepared from cultured chick atrial myocytes. In contrast to the ventricular myocyte, the various adenosine receptor agonists inhibited the GTP-mediated adenylate

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Adenylate cyclase activity (pmol cAMP/10 min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP</td>
<td>438±19</td>
</tr>
<tr>
<td>GTP+NECA</td>
<td>725±12</td>
</tr>
<tr>
<td>GTP+MECA</td>
<td>650±15</td>
</tr>
<tr>
<td>GTP+CV-1808</td>
<td>685±8</td>
</tr>
<tr>
<td>GTP+2-CADO</td>
<td>573±30</td>
</tr>
<tr>
<td>GTP+5-PIA</td>
<td>462±7</td>
</tr>
<tr>
<td>GTP+R-PIA</td>
<td>550±17</td>
</tr>
</tbody>
</table>

Increase in adenylate cyclase activity (%)  

<table>
<thead>
<tr>
<th>Agonists</th>
<th>EC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2-Agonists</td>
<td></td>
</tr>
<tr>
<td>NECA</td>
<td>1.27±0.4</td>
</tr>
<tr>
<td>MECA</td>
<td>1.3±0.6</td>
</tr>
<tr>
<td>CV-1808</td>
<td>0.6±0.35</td>
</tr>
<tr>
<td>CGS21680</td>
<td>1.4±0.55</td>
</tr>
<tr>
<td>A1-Agonists</td>
<td></td>
</tr>
<tr>
<td>2-CADO</td>
<td>0.87±0.6</td>
</tr>
<tr>
<td>S-PIA</td>
<td>0.83±0.3</td>
</tr>
<tr>
<td>R-PIA</td>
<td>0.71±0.3</td>
</tr>
<tr>
<td>DCCA</td>
<td>1.3±0.4</td>
</tr>
</tbody>
</table>

Values are mean±SEM of triplicate determinations of typical experiment for top part of table and mean±SEM of four to eight similar experiments for bottom part of table. NECA, N-ethylenosine-5'-uronic acid; MECA, N-methyladenosine-5'-uronic acid; CV-1808, 2-phenylaminoadenosine; CGS21680, 2-[p-(2-carboxyethyl)-phenethylamino-5'-N-ethylcarboxamido-adenosine; 2-CADO, 2-chloroadenosine; S-PIA, N'-S-phenyl-2-propyladenosine; R-PIA, N'-R-phenyl-2-propyladenosine; DCCA, 1-deaza-2-chloro-N'-cyclopentyladenosine. Cultured ventricular myocytes were prexposed to pertussis toxin (5 ng/ml for 12 hours), and assay of adenylate cyclase activity was carried out in the presence of the agonists indicated as described in “Methods.” The values for the percent increase in adenylate cyclase caused by the equipotent or the A2-agonists were similar to one another (by one-way analysis of variance, F=1.034, p=0.9912) but were significantly greater than values for the percent increase stimulated by the A1-agonists (p<0.01, unpaired t test comparing each pair of the A2- and A1-agonists). GTP was present at 100 μM, and each analogue was also present at 100 μM.
TABLE 2. Comparison of the Adenylate Cyclase Response to Adenosine Agonists Between Atrial and Ventricular Myocytes

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Adenylate cyclase activity (pmol cAMP/10 min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ventricle</td>
</tr>
<tr>
<td>Control</td>
<td>1,071±21</td>
</tr>
<tr>
<td>+DPCPX</td>
<td>1,018±14</td>
</tr>
<tr>
<td>GTP (100 μM)</td>
<td>964±19</td>
</tr>
<tr>
<td>GTP+NECA</td>
<td>1,018±14</td>
</tr>
<tr>
<td>GTP+MECA</td>
<td>1,020±19</td>
</tr>
<tr>
<td>GTP+R-PIA</td>
<td>1,011±18</td>
</tr>
</tbody>
</table>

Values are mean±SEM of triplicate determinations and are representative of two other experiments. DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; NECA, N-ethyladenosine-5'-uronic acid; MECA, N-methyladenosine-5'-uronic acid; R-PIA, N6-R-phenyl-2-propyladenosine. Atrial and ventricular cultures were performed and assay of adenylate cyclase activity was carried out as described in “Methods.” In membranes of ventricles, each agonist (1 μM) caused an increase in the adenylate cyclase activity in the absence (p<0.05 vs. GTP, paired t test) or the presence (p<0.01 vs. DPCPX and GTP) of DPCPX (0.5 μM). However, the percent increase in the adenylate cyclase activity induced by each agonist in the presence of DPCPX was significantly greater than the percent increase in the absence of DPCPX (p<0.01, paired t test). In membranes of atrial cells, the same adenosine agonists caused an inhibition of the GTP-mediated adenylate cyclase activity (p<0.01, paired t test) in the absence of DPCPX.

cyclic AMP (cAMP)

Adenosine Agonists

- 2-propyladenosine.
described
percent
the
absence (p<0.05 increase
NECA,
membranes
In
agonists
on
the cardiac
subtype
that,
suggest
in the presence
however,
agonists (either DPCPX
or PTX, paired t test)
activation of the A1-subtype was able to cause an inhibition of the isoproterenol-stimulated increase in the adenylate cyclase activity.

Stimulation of Ventricular Contractility by the Adenosine Receptor Agonist

We next examined whether the A2-adenosine receptor was also capable of coupling to a stimulation of the myocardial contractility. The various adenosine receptor agonists tested, whether A1- or A2-selective or equipotent, caused a significant increase in the cardiac contractility when the inhibitory A1-adenosine receptor pathway had been blocked by

TABLE 3. Effects of Adenosine Receptor Agonists on the Isoproterenol-Stimulated Adenylate Cyclase Activity in Membranes of Ventricular Myocytes

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Adenylate cyclase activity (pmol cAMP/10 min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP (100 μM)</td>
<td>442±13</td>
</tr>
<tr>
<td>GTP+ISO (10 μM)</td>
<td>916±15</td>
</tr>
<tr>
<td>GTP+ISO+NECA</td>
<td>840±11</td>
</tr>
<tr>
<td>GTP+ISO+MECA</td>
<td>861±17</td>
</tr>
<tr>
<td>GTP+ISO+R-PIA</td>
<td>806±19</td>
</tr>
</tbody>
</table>

Values are mean±SEM of triplicate determinations and are typical of three other experiments. ISO, isoproterenol; NECA, N-ethyladenosine-5'-uronic acid; MECA, N-methyladenosine-5'-uronic acid; R-PIA, N6-R-phenyl-2-propyladenosine. The decreases in ISO-stimulated adenylate cyclase activity caused by each adenosine agonist (1 μM) were statistically significant (p<0.01, paired t test).

TABLE 4. Reversal of N6-R-Phenyl-2-propyladenosine-Induced Inhibition of Adenylate Cyclase Activity and Myocyte Contractility by Pretreatment With Pertussis Toxin

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Adenylate cyclase activity (% of maximum)</th>
<th>Contractile amplitude (% of maximum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>82±2.9 (n=4)</td>
<td>58±9 (n=10)</td>
</tr>
<tr>
<td>PTX-treated</td>
<td>104±4 (n=4)</td>
<td>102±3 (n=6)</td>
</tr>
</tbody>
</table>

Values are mean±SEM. PTX, pertussis toxin. The effects of 1 μM N6-R-phenyl-2-propyladenosine on the isoproterenol-stimulated increases in contractile amplitude and adenylate cyclase activity in control and PTX-treated ventricular cells were compared. The percent of maximum, where maximum is the increase elicited by isoproterenol, in adenylate cyclase activity or contractility in PTX-treated cells was significantly different from the corresponding percent of maximum in control ventricular cells (p<0.01, paired t test). The stimulatory response in adenylate cyclase elicited by isoproterenol was equal to the adenylate cyclase activity obtained in the presence of isoproterenol and GTP minus that obtained in the presence of GTP alone. The stimulatory contractile response caused by isoproterenol was the contraction amplitude obtained in the presence of isoproterenol minus the amplitude obtained before the superfusion of medium containing isoproterenol.

Adenylate cyclase activity

(%) of maximum

- 82±2.9 (n=4)
- 104±4 (n=4)
- 58±9 (n=10)
- 102±3 (n=6)

Myocyte Contractility

(%) of maximum

- 82±2.9
- 104±4
- 58±9
- 102±3

TABLE 4. Reversal of N6-R-Phenyl-2-propyladenosine-Induced Inhibition of Adenylate Cyclase Activity and Myocyte Contractility by Pretreatment With Pertussis Toxin

Control  | PTX-treated
---------|---------------------
Adenylate cyclase activity (% of maximum) | 82±2.9 (n=4)  | 104±4 (n=4) |
Contractile amplitude (% of maximum) | 58±9 (n=10)  | 102±3 (n=6) |

Values are mean±SEM. PTX, pertussis toxin. The effects of 1 μM N6-R-phenyl-2-propyladenosine on the isoproterenol-stimulated increases in contractile amplitude and adenylate cyclase activity in control and PTX-treated ventricular cells were compared. The percent of maximum, where maximum is the increase elicited by isoproterenol, in adenylate cyclase activity or contractility in PTX-treated cells was significantly different from the corresponding percent of maximum in control ventricular cells (p<0.01, paired t test). The stimulatory response in adenylate cyclase elicited by isoproterenol was equal to the adenylate cyclase activity obtained in the presence of isoproterenol and GTP minus that obtained in the presence of GTP alone. The stimulatory contractile response caused by isoproterenol was the contraction amplitude obtained in the presence of isoproterenol minus the amplitude obtained before the superfusion of medium containing isoproterenol.

Adenylate cyclase activity

(%) of maximum

- 82±2.9
- 104±4
- 58±9
- 102±3

Myocyte Contractility

(%) of maximum

- 82±2.9
- 104±4
- 58±9
- 102±3

Stimulation of Ventricular Contractility by the Adenosine Receptor Agonist

We next examined whether the A2-adenosine receptor was also capable of coupling to a stimulation of the myocardial contractility. The various adenosine receptor agonists tested, whether A1- or A2-selective or equipotent, caused a significant increase in the cardiac contractility when the inhibitory A1-adenosine receptor pathway had been blocked by
prior treatment with PTX (Figures 3 and 4). Because of potential differences in the loading of these monolayer cultured ventricular myocytes, the increase in contractility caused by each adenosine agonist was normalized to the increase in contractile amplitude produced by 2.4 mM calcium in the superfusion medium as percent of maximum. This concentration of calcium in the superfusion medium elicited the maximal increase in contractile amplitude in dose-response experiments using sequentially higher calcium concentration (data not shown). The various adenosine agonists elicited an increase in the contractile amplitude in the PTX-treated cell in a dose-dependent fashion (Figure 4). The maximal stimulation of myocyte contractility elicited by the A2-selective or the equinot agonist was significantly greater than that induced by the A1-selective agonists (p<0.05, unpaired t test), whereas the EC50 of the various agonists causing the increase in contractility were similar to one another (by ANOVA, F=1.43, p=0.26) (Table 5 and Figure 4). The greatest stimulation of cardiac contractility, induced by the A2-agonists, represented about a 23% increase in the contractile amplitude above the basal level and was similar to the maximal increase caused by isoprenaline (1 μM) (21±2.5% increase above basal or 57±4.5% of the calcium response, n=21). The values for the maximal percent stimulation of contractility by the A2-agonists were similar to one another (by ANOVA, F=0.64, p=0.59). The values for the maximal percent stimulation caused by the A2-agonists were similar to one another except in the R-PIA versus S-PIA and the R-PIA versus DCCA comparisons and in the 2-CADO versus S-PIA and 2-CADO versus DCCA comparisons (by ANOVA followed by multiple group comparison). The contractile effects of NECA were blocked by the theophylline analogue 8-PST (data not shown), indicating that the stimulation of cardiac contractility was mediated by a cell surface adenosine receptor. Blocking of the A1-adenosine receptor by the selective antagonist DPCPX in the presence of NECA also unmasked a NECA-elicited increase in the contractile amplitude (51.7±4% of the response evoked by 2.4 mM calcium, n=15). These experiments demonstrated that a stimulatory subtype of adenosine receptor was present on the ventricular myocyte and was capable of coupling to an increase in the myocardial contractility. To investigate whether the stimulatory adenosine

### Figure 3
Tracings showing contractile response to adenosine receptor agonist in control and pertussis toxin (PTX)-treated ventricular myocytes. NECA, N-ethyladenosine-5'-uronic acid; MECA, N-methyladenosine-5'-uronic acid; ADO, adenosine; R-PLA, N°-R-phenyl-2-propyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylanthine. After the equilibration period, the myocyte was superfused with HEPES-buffered medium containing 100 μM of one of the adenosine receptor agonists, and changes in contractile amplitude were determined (arrows) in control myocyte with or without DPCPX and in PTX-treated (5 ng/ml for 12 hours) myocytes. Data were typical of eight to 12 tracings.

### Table 5. Effects of Adenosine Receptor Agonists on the Cardiac Contractility in Pertussis Toxin–Treated Ventricular Myocytes

<table>
<thead>
<tr>
<th>Stimulation of contractile amplitude (% of maximum)</th>
<th>EC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A2-Agonists</strong></td>
<td></td>
</tr>
<tr>
<td>NECA</td>
<td>49.7±5</td>
</tr>
<tr>
<td>MECA</td>
<td>50.5±4</td>
</tr>
<tr>
<td>CV-1808</td>
<td>52.5±6</td>
</tr>
<tr>
<td>CGS21680</td>
<td>49.6±3</td>
</tr>
<tr>
<td><strong>A1-Agonists</strong></td>
<td></td>
</tr>
<tr>
<td>2-CADO</td>
<td>37±3</td>
</tr>
<tr>
<td>S-PIA</td>
<td>17.6±5</td>
</tr>
<tr>
<td>R-PIA</td>
<td>34.8±2.5</td>
</tr>
<tr>
<td>DCCA</td>
<td>12±4</td>
</tr>
</tbody>
</table>

Values are mean±SEM of eight to 12 experiments. NECA, N-ethyladenosine-5'-uronic acid; MECA, N-methyladenosine-5'-uronic acid; CV-1808, 2-phenylaminoadenosine; CGS21680, 2-[2-carboxyethyl]-phenethylamine]-5'-N-ethylcarboxamido adenosine; 2-CADO, 2-chloroadenosine; S-PIA, N°-S-phenyl-2-propyladenosine; R-PIA, N°-R-phenyl-2-propyladenosine; DCCA, 1-deaza-2-chloro-N°-cyclopentyladenosine. Cultured ventricular cells were preexposed to pertussis toxin (5 ng/ml for 12 hours), and the contractile response to the various agonists was determined as described in “Methods.” The contractile amplitude under the control condition before infusion of the adenosine agonists was 1.23±0.25 μM (n=32). The increase in contractile amplitude caused by the adenosine agonists (100 μM) was normalized to the increase produced by 2.4 mM calcium in the superfusing buffer as percent of maximum. The values for the stimulation of contractile amplitude by the A2-agonists were similar to one another (by analysis of variance, F=0.64, p=0.59) but were significantly greater than the values for the stimulation caused by the A1-agonists (p<0.05, unpaired t test).
receptor was also present on atrial myocytes, we carried out parallel studies to determine whether adenosine receptor agonists were able to stimulate the cardiac contractility in the presence of the A₁-adenosine receptor–selective antagonist DPCPX. None of the adenosine receptor agonists tested, whether A₁- or A₂-selective or equipotent, could induce an increase in the cardiac contractility in the presence of DPCPX in the atrial myocytes (Figure 5). Similarly, in PTX-treated atrial culture where the A₁-adenosine receptor was uncoupled from its functional effects, NECA (100 μM) failed to stimulate any increase in the contractile amplitude (control amplitude, 1.4±0.3 μm, n=12; NECA-stimulated amplitude, 1.35±0.6 μm, n=11). The completeness of PTX treatment of the atrial culture was determined by a [³²P]ADP-ribosylation experiment (data not shown) similar to that performed for the ventricular culture in Figure 2. These results provide further evidence that A₂-adenosine receptors are not present on the cultured atrial myocytes.

![Graph showing effects of varying concentrations of adenosine receptor agonists on the contractile amplitude in pertussis toxin–treated ventricular myocytes.](http://circres.ahajournals.org/)

**FIGURE 4.** Graph showing effects of varying concentrations of adenosine receptor agonists on the contractile amplitude in pertussis toxin–treated ventricular myocytes. NECA, N-ethyladenosine-5'-uronic acid; MECA, N-methyladenosine-5'-uronic acid; R-PIA, N⁶-R-phenyl-2-propyladenosine; 2-cADO, 2-chloroadenosine; S-PIA, N⁶-S-phenyl-2-propyladenosine. Ventricular myocytes pretreated with pertussis toxin (5 ng/ml for 12 hours) were exposed to varying concentrations of each agonist, and the amplitude of cell motion was measured as described in “Methods.” The increase in the contractile amplitude produced at each concentration of the agonist was normalized to the increase in contractile amplitude caused by 2.4 mM calcium in the superfusion medium as percent maximum response. Data were plotted as percent maximum response versus the concentration of each adenosine receptor agonist. Data were mean ± SEM from eight to 12 myocytes.

![Tracings showing contractile response of the cultured atrial myocyte to adenosine receptor agonists.](http://circres.ahajournals.org/)

**FIGURE 5.** Tracings showing contractile response of the cultured atrial myocyte to adenosine receptor agonists. DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; NECA, N-ethyladenosine-5'-uronic acid; MECA, N-methyladenosine-5'-uronic acid; ADO, adenosine; R-PIA, N⁶-R-phenyl-2-propyladenosine. After a 20-minute equilibration, myocytes were superfused with HEPES-buffered medium containing 0.3 μM DPCPX and then exposed to medium containing DPCPX and the adenosine receptor agonist (100 μM) indicated. Changes in the amplitude of cell motion were determined (arrows). Tracings of the steady state for each condition were presented and were typical of six other similar experiments.

**The Role of Adenosine Receptor Subtypes in Modulating the Contractile State of the Ventricular Myocyte**

To investigate the possible coexistence of the inhibitory A₁- and the stimulatory A₂-adenosine receptors on the ventricular myocyte and to study their potential roles in modulating the cardiac contractility, the effects of various agonists on the myocardial contractility in the presence or the absence of isoproterenol were investigated. In the absence of isoproterenol, the adenosine agonists (100 μM) caused a slight stimulation of the contractility (from 6.6±3% to 18±4%, n=8–10, where increase in contractility is percent of maximum contractility produced by 2.4 mM calcium). The degree of the adenosine agonist–induced increase in the contractile amplitude, however, was significantly enhanced in the presence of DPCPX or in PTX-treated cells (Figure 3, Table 5), indicating the presence of an inhibitory effect mediated by the A₁-adenosine receptor when DPCPX or PTX was not used. In the presence of isoproterenol, the agonists caused a significant inhibition of the increase in contractility produced by isoproterenol (Figure 6). Prior treatment of the culture with PTX
abolished the adenosine agonist–induced inhibition, indicating that the A₁-adenosine receptor mediated the antagonism of isoproterenol-stimulated increase in contractility (Table 4 and Figure 6).

Discussion

The myocardial depressant effects of adenosine are mediated by adenosine receptor of the A₁ subtype.1–3 Whereas adenosine agonist induces a direct negative inotropic effect in the atrial cell, it is capable of inducing a negative inotropic effect in the ventricular cell only after the myocyte contractility has been stimulated by a cAMP-raising agent. The reason for the difference in contractile response to the adenosine receptor agonist between atrial and ventricular myocytes is thought to be the activation of a potassium channel by adenosine in atria with a consequent abbreviation of the action potential duration and the duration of calcium influx.1,6,16,17 Recent studies7–9 have demonstrated that a separate subtype of the adenosine receptor coupled to stimulation of adenylate cyclase was present in ventricles. These data raised the possibilities that the stimulatory adenosine receptor may be preferentially present in ventricles but not in atria and that this preferential expression of the stimulatory adenosine receptor, opposing the inhibitory A₁-adenosine receptor, may also explain the lack of a direct negative inotropic effect of adenosine in the ventricular myocyte. However, it is not clear whether the stimulatory adenylate cyclase–coupled adenosine receptor is able to couple to a stimulation of the ventricular contractility, which could serve to counteract the inhibition of contractility mediated by the A₁-adenosine receptor. Our preliminary data9 suggested that in membranes of ventricular myocytes cultured from chick embryos, a distinct subtype of the adenosine receptor was present and was coupled to an increase in the adenylate cyclase activity. The objective of the present study, using cultured chick cardiac myocytes as a model system, is to further characterize pharmacologically the stimulatory adenylate cyclase–coupled adenosine receptor and to test the hypotheses that these adenosine receptors are capable of coupling to an increase in the contractility of the ventricle and are present only on ventricular but not on atrial cells.

To unmask the functional effects mediated by the stimulatory adenosine receptor, we used two methods to inactivate the inhibitory A₁-adenosine receptor pathway. The first method used PTX to uncouple the A₁-adenosine receptor from its inhibitory effect on the adenylate cyclase activity and the myocyte contractility. The second method involved blocking the inhibitory A₁-adenosine receptor by a highly selective A₁-adenosine receptor antagonist, DPCPX. In the pharmacological characterization of these A₁-adenosine receptors, a potential advantage of using PTX treatment to inactivate the A₁-adenosine receptor pathway is the ability of PTX to uncouple all inhibitory receptors including possible subtype(s) of the A₁-adenosine receptor that may not be blocked by DPCPX. The second possible benefit of using PTX rather than DPCPX is that, at the high concentration of the adenosine agonist, 0.3 μM DPCPX may not be adequate in blocking all the A₁-adenosine receptors. Adenosine receptor agonists had a slight stimulatory effect on the GTP-dependent basal adenylate cyclase activity in membranes of cultured ventricular myocytes. Prior treatment of the cultured ventricular cell with PTX led to markedly greater increases in the extent of stimulation of the adenylate cyclase by these agonists. Blocking of the A₁-adenosine receptor by the A₁-selective antagonist DPCPX also resulted in a significant increase in the abilities of the various adenosine receptor agonists to stimulate adenylate cyclase activity. These data indicate the presence of a stimulatory effect of adenosine agonists on the adenylate cyclase activity, which became manifested by the uncoupling of A₁-adenosine receptors from the cyclase. Thus, in ventricles, a different subtype of the adenosine receptor is present and couples, in a stimulatory rather than an inhibitory fashion, to the adenylate cyclase. The potency of the R-PIA effect on the adenylate cyclase activity in these PTX-treated cells was similar to the potency of NECA or S-PIA, indicating that the receptor subtype involved is not the A₁-adenosine receptor. The cou-
pling of this subtype of adenosine receptor to a stimulatory adenylate cyclase response suggested that the subtype was the A$_2$-adenosine receptor.

The similarity in the potency of the adenosine agonists in stimulating the adenylate cyclase is in contrast to the classical rank order of potency described for the effects mediated by the A$_2$-adenosine receptor. The reason for such a difference is not known but may be related to the heterogeneity of the A$_2$-adenosine receptors$^{18,19}$ or to the differences between the coupling of the A$_2$-adenosine receptor to adenylate cyclase in these cultured ventricular myocytes. An alternative explanation is that the relatively small magnitude of increase in the adenylate cyclase activity makes it difficult to obtain and compare accurately the difference in EC$_{50}$ for each agonist. It is curious that the A$_2$-selective or the equipotent agonists were capable of causing a greater stimulation of the adenylate cyclase activity than were the A$_1$-adenosine receptor–selective agonists. Taken together, these experiments provide direct evidence that a separate subtype of the adenosine receptor, probably the A$_2$-subtype, is present and is coupled to a stimulation of the adenylate cyclase activity in the ventricular myocyte.

Prior studies by others$^8$ demonstrated that the nonselective agonist NECA was capable of stimulating an increase in the cAMP level in guinea pig ventricular myocytes in the presence of DPCPX. The increase in the cAMP level elicited by NECA in the presence of DPCPX represented a further increase in the cAMP level, above that produced by isoproterenol. This NECA-induced increase in the cAMP level was associated, however, with a NECA-mediated inhibition of the contractility stimulated by the same concentration of isoproterenol. The reason for the discrepancy between the NECA effects on the cAMP level and the myocyte contractility is not clear but may be related, perhaps, to compartmentalization of intracellular cAMP, as suggested by Behnke et al.$^8$ An issue that was not addressed in the previous study$^8$ relates to the question of whether activation of the A$_2$-subtype is capable of stimulating the basal contractile state of the ventricle in the absence of β-adrenergic stimulation. In the present study, we examined whether the adenosine receptor agonists, under conditions that caused a stimulation of the adenylate cyclase activity, were also capable of eliciting an increase in the basal contractility of the cultured chick ventricular myocyte. Because these ventricular cells contract spontaneously, they afford a unique opportunity to investigate the modulation of ventricular contractile state by adenosine. In contrast to the atrial myocyte, in which adenosine agonists caused a direct negative inotropic effect, the same agonists produced a slight increase in the cardiac contractility in the ventricular myocyte. In ventricular but not in atrial cells, blocking of the A$_1$-adenosine receptor by DPCPX or uncoupling of the A$_1$-adenosine receptor from its effector(s) by PTX resulted in a further significant increase in contractile amplitude in response to adenosine or adenosine agonists. Thus, a separate subtype of the adenosine receptor is present on the ventricular myocyte and is coupled to stimulation of the myocardial contractility, in parallel with a stimulation of the adenylate cyclase activity.

The EC$_{50}$s for each agonist-induced stimulation of the myocyte contractility were all similar, consistent with the similarity of the EC$_{50}$s of these agonists in stimulating the adenylate cyclase activity. The maximal increases in contractile amplitude produced by the A$_2$-adenosine receptor–selective or equipotent agonists were significantly greater than those evoked by the A$_2$-adenosine receptor agonists, also similar to the findings on the maximal stimulation of adenylate cyclase by these agonists. These data are compatible with the possibility that the subtype of adenosine receptor, the A$_2$-adenosine subtype, that mediated the stimulation of myocyte contractility was the same subtype that mediated the stimulation of adenylate cyclase activity. Since the cultured heart cell preparation contains fibroblasts (<7%),$^{20}$ the present study cannot rule out the possibility that the stimulation of adenylate cyclase activity by the adenosine agonist is partially due to activation of the A$_2$-subtype present in membranes of the fibroblasts in the ventricular culture. However, such a possibility is unlikely, because the atrial culture, which also contained fibroblasts, did not exhibit any adenosine agonist–induced stimulation of adenylate cyclase activity or cardiac contractility.

To investigate the potential role of each subtype in modulating the adenylate cyclase activity and the cardiac contractility of the ventricular cell, the abilities of adenosine agonists to regulate the basal and the isoproterenol-stimulated responses were examined. Under the basal condition in the absence of isoproterenol, adenosine agonists caused a slight increase in the adenylate cyclase activity, with a concomitant stimulation of the cardiac contractility. Blocking of the A$_1$-adenosine receptor resulted in a markedly enhanced stimulation of these two responses by the adenosine agonist. These results demonstrate that the A$_2$-adenosine receptor–mediated stimulatory effects are masked by the inhibitory effects of the A$_1$-subtype. These data raised the possibility, although speculative, that the inhibitory contractile effect mediated by the A$_1$-adenosine receptor was counterbalanced by the positive stimulatory effect of the A$_2$-adenosine receptor in response to adenosine, which could activate both receptor subtypes. In the presence of isoproterenol, adenosine agonists caused an inhibition of the isoproterenol-stimulated increase in adenylate cyclase activity and in myocyte contractility, an effect mediated by the A$_1$-subtype. These data are compatible with those of Behnke et al.$^8$ which demonstrated an inhibition of isoproterenol-stimulated increase in contractile amplitude by both the equipotent agonist NECA and the A$_2$-selective agonist R-PIA, an inhibition that was antagonized by the A$_1$-selective antagonist DPCPX. The present data on the ventricular myocyte are in
contrast to the findings in the atrial myocyte, in which adenosine agonists have a marked inhibitory effect on contractility and on the adenylate cyclase activity regardless of whether isoproterenol is present. The cardiac contractile and adenylate cyclase responses to adenosine appear to correlate with the presence (ventricles) or the absence (atria) of the A_{2a}-adenosine receptor. The present study thus raises the possibility that the ability of adenosine agonists to modulate adenylate cyclase activity or myocardial contractility is a function of the activation of a particular adenosine receptor subtype, the expression of which is atrium and ventricle specific.

Taken together, these results indicate that both the A_{1a} and the A_{2a}-subtypes of the adenosine receptor are present on the ventricular myocyte and that each subtype mediates opposing biochemical and physiological responses, whereas only the A_{1a}-subtype is present on the atrial myocyte. The absence of the stimulatory A_{2a}-adenosine receptor subtype on the atrial cell, in addition to the ability of the atrial A_{1a}-adenosine receptor to couple to a potassium channel, may help explain the ability of adenosine to cause a direct negative inotropic effect in the atrium. It is not known at the present time whether the stimulatory A_{2a}-receptor in the embryonic chick ventricular cell is similar to the A_{2a}-adenosine receptor in the adult mammalian heart cell. Nevertheless, the presence of both adenosine receptor subtypes, which are functionally coupled to cardiac responses in these cultured ventricular myocytes, suggests that these cultures may be a good model system for the study of cardiac adenosine receptors.

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