Adenosine receptors and second messenger signaling pathways in rat cardiac fibroblasts

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Adenosine receptors and second messenger signaling pathways in rat cardiac fibroblasts. Am J Physiol Cell Physiol 296: C1171–C1177, 2009. First published February 25, 2009; doi:10.1152/ajpcell.00290.2008.—The ability of adenosine (ADO) to inhibit proliferation and protein synthesis in (particular, collagen synthesis) in cardiac fibroblasts (CF) may ameliorate adverse cardiac remodeling and fibrosis seen in heart failure patients. However, little is known about the signaling pathways that ADO may modulate in CF to alter cell phenotype. Accordingly, this study was designed to identify ADO receptors (AR) and the signaling pathways linked to them in primary cultures of adult rat CF. Quantitative RT-PCR data indicate that the mRNAs for all four known ARs (A1R, A2aR, A2bR, and A3R) are present in rat CF, with a greater prevalence of A2 receptor subtypes. No coupling of AR to the Gα, phospholipase C signaling pathway or to mobilization of calcium is measurable. Studies using subtype specific agents imply that the A2aR and A2bR couple to Gs-adenylyl cyclase and A1R couple weakly to Gi-adenylyl cyclase. 2-Chloroadenosine, 5′-N-ethylcarboxamidoadenosine, and other agents that elevate cellular cAMP stimulate extracellular signal-regulated kinase 1/2 activity in a pertussis toxin-insensitive manner. We conclude that a combination of cAMP-dependent signals generated via A2a and A2b receptors likely mediate ADO signaling in adult rat CF.

G protein-linked receptors; extracellular matrix; myocardial fibrosis

An important participant in the development of adverse remodeling of the heart involves cardiac fibroblasts (CF), which produce excess extracellular matrix (ECM) proteins (8) leading to focal or diffuse fibrosis (4). Myocardial fibrosis can alter the mechanical properties of the heart, impairing cardiac function and contributing to the development of adverse chamber remodeling and heart failure (6, 25, 26).

The ability of the nucleoside adenosine (ADO) to inhibit CF proliferation, protein, and/or collagen synthesis (7, 11, 12, 37) may ameliorate myocardial fibrosis and chamber remodeling. Although the modulation of these effects by ADO appears to occur predominantly via A2 receptors (7, 11, 13), the results have not been carefully validated and the participant signaling pathways in CF have not been unequivocally established. ADO can modulate cell function via stimulation of G protein-linked receptors; four subtypes are known (A1R, A2aR, A2bR, and A3R). A1R and A3R appear to signal through Gi/Go in an increase in inositol phosphate (IP) accumulation and Ca2+ mobilization (2, 33). Activation of the A2bR can also result in increases in intracellular Ca2+ levels via coupling to Gαs (15) or via Gi coupling that results in the activation of a Ca2+ channel (16).

The message for all four adenosine receptor (AR) subtypes is present in rat CF (7), indicating that all AR may be functionally present in these cells. Thus ADO applied to CF could bind AR that couple to Gi/Go, Gs, and/or Gαq, simultaneously activating multiple downstream signaling pathways. The purpose of this study was to characterize AR signaling in primary cultures of isolated adult rat CF as a prelude to understanding how ADO may modulate cardiac fibrosis. A companion article (36a) presents data indicating that cAMP-dependent regulation of collagen synthesis occurs via exchange factor directly activated by cAMP (Epac) rather than protein kinase A.

Materials and Methods

8-Bromo-cAMP (8-Br-cAMP), 2-chloroadenosine (CADO), forskolin (FSK), isoproterenol, lysophosphatidic acid (LPA), 5′-N-ethylcarboxamidoadenosine (NECA), and uridine triphosphate (UTP) were purchased from Sigma-Aldrich. Pertussis toxin (PTX) was purchased from EMD-Biosciences Calbiochem. 2-[p-(2-carboxyethyl)-phenylethylamino]-5′-N-ethyl-carboxamidoadenosine (CGS-21680 or CGS), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), N-(4-acetylphenyl)-2-(4-[2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl]phenoxo)acetamide (MR5-1706 or MR5), N-(4-methoxyphenyl)-N′-(3-pyridyl)quinazolin-4-yl)urea (VUF-5574 or VUF), and 4-[2-(7-amino-2-[2-furyl]-1,2,4-triazolo[2,3-a][1,3,5]triazin-5-yl amino)ethyl]phenol (ZM-241385 or ZM) were all purchased from Tocris Bioscience. All other biochemicals were of reagent grade from Sigma-Aldrich, Bio-Rad Laboratories, or as noted below.

Isolation of adult rat ventricular cardiac fibroblasts. Animal protocols were approved by the Animal Subjects Committee of UCSD, which is AALAC accredited, and CF were isolated from Sprague-Dawley rats (male, 250 g) by modification of a previously described protocol (38). Briefly, six hearts were removed, minced, and placed into spinner flasks with 110 U/ml collagenase (Worthington Biochemicals) and 0.6 mg/ml pancreatic (MP Biomedicals). The ventricles were digested repeatedly (15–20 min at 37°C), with cells released by the second-sixth digestions pooled, pelleted, and resuspended in HEPES-buffered DMEM (Invitrogen Gibco) supplemented with 10% FBS (Omega Scientific) and 100 U/ml penicillin-streptomycin-fungizone (Gemini Bioproducts). The cells were plated for 45 min to allow for preferential attachment of CF, after which unattached cells were removed by aspiration, and fresh medium was added. Cells were passaged by trypsinization. All CF used in the experiments were from the second or third passage, grown to a density of 70% confluency unless noted otherwise, and cultured in serum-free medium for 2 h before any experimental treatment. The purity of these cultures is >95% as determined by immunostaining (38).

Quantification of transcripts by real-time PCR. Quantification of mRNA transcripts was performed via real-time PCR analysis on a DNA engine Opticon 2 machine (MJ research) (5). Total RNA was isolated from CF using an RNeasy kit (Qiagen). The RNA was...
subjected to RT-PCR using the ThermoScript RT-PCR system with platinum Taq DNA polymerase (Invitrogen) and the following AR-specific primers: A1R, sense 5'-TCATGTCGTGTTCGTCTG-3', antisense 5'-CAAGGGAGAGAAATCCAGCAG-3'; A2aR, sense 5'-TCCGAGAAGTGCAACTCTT-3', antisense 5'-GTGGCGGTCTTCTGACTGC-3'; A2bR, sense 5'-GTTCCCGTCTTGTCCTTGCT-3', antisense 5'-GTCATTGCCTTGGTCTCTGTG-3'; A3R, sense 5'-GCTTTGGGTGTGTCCTTCC-3', antisense 5'-CAAAA-CAGGGGAAGAGGAGGGATCTTG-3'; A2aR, sense 5'-GGCTATGATTTG-GGCACTT-3', antisense 5'-GACAATGATAATGGCGTG-3'; A2bR, sense 5'-GCTTCCATCATGTGCTTTGCT-3', antisense 5'-GTGCAGTCCACGCAAGAGG-3'.

Calcium mobilization. CF (6 × 10^5 cells) were plated on 25-mm coverslips in DMEM plus 10% FBS for 8 h and then cultured in serum-free DMEM for 16 h. Cells were washed with HEPES-buffered saline (HBS) and incubated (30 min, 37°C) in HBS containing 5 mM CaCl_2, 1 mM EGTA, 10 mM HEPES, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, 10 mM NaF, and 30 mM β-glycerol-phosphate, pH 7.0). Equal amounts of total protein were processed by SDS-PAGE. Phosphoextracellular signal-regulated kinase 1/2 (ERK1/2) and total ERK1/2 were detected on Western blots with immunoblotting reagents (Amersham Life Sciences). Protein content was estimated by the method of Bradford, with bovine serum albumin as a standard.

Statistical analysis. Data were graphed and analyzed using Prism 3.0 (GraphPad Software, San Diego, CA). All data are expressed as means ± SE unless otherwise noted. Statistical analysis of the data was performed using Student’s unpaired t-test or analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison posttest. Differences were considered significant with P < 0.05.

RESULTS

Quantification of AR mRNAs in cardiac fibroblasts. Real-time RT-PCR was used to quantify levels of the four AR mRNA transcripts. A1R was least abundant (7.9 ± 4.1 copy numbers per nanogram RNA; Fig. 1). The A2aR was 1.8 times more abundant than the A1R, whereas the A2bR and A3R were 24.2 and 55.9 times more abundant, respectively. Although mRNAs for the A2R are the most abundant in adult rat CF, message for all subtypes is detectable, suggesting that all four receptor subtypes could be present. Accordingly, we proceeded to assess receptor-effector coupling to deduce which AR receptors are functional in CF.

No detectable AR coupling to a Gq-PLC pathway. Published results indicate that activation of the A2aR increases intracellular calcium levels via activation of a calcium channel or stimulation of the Gq-PLC pathway (15, 16). Experiments were performed to assess this possibility. Treatment of CF with either UTP (100 μM) or ANG II (1 μM) caused 16.5-fold and 7.4-fold increases, respectively, in IP accumulation (Table 1), indicating that the Gq-PLC pathway is functional in CF. However, exposure of cells to either CADO (10 μM) or NECA (100 μM) did not affect IP accumulation (Table 1). Stimulation with UTP (100 μM) was also able to increase calcium mobilization in CF, but neither CADO (10 μM) nor NECA (100 μM) had any effect (Table 1). These results indicate that in rat CF, no AR couples either to the Gq-PLC-IP-Ca^2+ pathway or to the activation of a calcium channel, within the limits of our detection.

An AR couples to the Gα12 signaling pathway. Inhibitors of cyclic nucleotide phosphodiesterases (PDEs) are commonly used experimentally to magnify small increases in cellular cAMP levels. However, many PDE inhibitors [e.g., 3-isobutyl-1-methylxanthine (IBMX)] are methylxanthines that structurally resemble ADO and may antagonize or mimic ADO binding to AR. In the present experiments, we have utilized rolipram, a PDE4 inhibitor that does not structurally resemble ADO. Control experiments indicated that 10 μM rolipram has −60% the efficacy of 1 mM IBMX in adult rat CF (14) and is likely a superior PDE inhibitor to use when studying AR signaling. Treatment of CF with CADO (10 μM) increased cAMP levels 3.2-fold (Fig. 2), indicating there is AR coupling to the Gα12-AC-cAMP pathway. IBMX (1 mM, a maximally effective concentration when assessing β-adrenergic responsiveness in these cells)(14) doubled the effect of CADO; however, rolipram (10 μM) more than tripled the response (Fig. 2). The greater increase in CADO-stimulated cAMP accumulation in the presence of rolipram suggests IBMX may antagonize the Gs-coupled AR to a greater extent than does rolipram; alternatively, ADO may stimulate cAMP accumula-
As seen in Fig. 3, NECA (100 μM) also significantly increased intracellular cAMP. The responses to CADO and NECA were maximal by 5 min after stimulation (Fig. 3). These data indicate that functional AR couple to the Gs-AC-cAMP pathway in rat CF.

Evidence for an AR-Gi/Go-AC pathway. In experiments performed in heterologous expression systems, A1R and A3R can signal through a Gi/Go-AC signaling pathway (2, 32). We examined this possibility by comparing cAMP accumulation in response to NECA or CADO in untreated cells and in cells treated with PTX to inhibit signaling through Gi/Go. Pretreatment with PTX enhanced NECA-stimulated cAMP accumulation significantly but only slightly (by 11 ± 2%, n = 6, P < 0.05) over the control response. This small, but significant, effect suggests there is an AR present in CF (A1R, A3R, or both) that couples weakly to the Gi/Go-AC signaling pathway. Treatment with PTX presumably removes signaling through the Gi pathway, thereby enhancing the effect of AR agonists to stimulate cAMP production via the A3R. Thus, when we observe an overall increase in cellular cAMP in CF in response to ADO congeners, we are observing the combined effect of activation of both the Gs and Gi pathways, and the Gs effect predominates.

Activation/phosphorylation of ERK1/2 downstream of ADO treatment occurs in a PTX-independent manner. Having detected an apparent coupling of ARs to the Gi pathway (above), we looked for additional evidence of signaling via Gi. Frequently, activation of Gi leads to activation of the ERK pathway (23). Treatment of CF with either CADO (10 μM), NECA (100 μM), or LPA (10 μM) (used as a positive control for PTX-dependent ERK1/2 phosphorylation) led to a significant increase in ERK1/2 phosphorylation (Fig. 4). However, pretreatment with PTX inhibited only the effect of LPA on ERK1/2 phosphorylation and had no effect on CADO- and NECA-stimulated ERK1/2 activation (Fig. 4). Thus CF express an LPA-sensitive Gi/Go signaling pathway that can be completely abolished by pretreatment with PTX. The effects of ADO congeners on ERK1/2 phosphorylation must occur by another mechanism, however, one that is insensitive to PTX. Stimulation of CF with FSK (1 μM) or 8-Br-cAMP (100 μM) significantly increased ERK1/2 phosphorylation (data not shown), mimicking the effects of CADO and NECA. Thus, it is possible that CADO and NECA work via a Gi/Go-AC-cAMP signaling pathway to activate ERK1/2 in CF. Together, these

Fig. 2. Effects of phosphodiesterase (PDE) inhibitors on cAMP accumulation. CF were pretreated in the absence or presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX) or 10 μM rolipram for 15 min and then stimulated with or without 10 μM 2-chloroadenosine (CADO) for 10 min. Pretreatment with IBMX leads to a greater global inhibition of PDE activity than pretreatment with rolipram. However, when cAMP levels were stimulated with CADO, pretreatment with rolipram leads to a greater increase in cAMP accumulation vs. IBMX (data are means ± range; n = 2).

Fig. 3. cAMP accumulation in response to adenosine (ADO) agonists. A: CF were preincubated with 10 μM rolipram for 15 min and then treated with 10 μM CADO, 100 μM 5’-N-ethylcarboxamido-adenosine (NECA), or 1 μM isoproterenol (Iso; as a positive control) for 5 min. All three agonists are capable of significantly increasing cAMP levels above basal (n ≥ 10; ***P < 0.001 vs. control, **P < 0.01 vs. control, *P < 0.05 vs. control). B: CF were preincubated with 10 μM rolipram for 15 min and then treated with 10 μM CADO or 100 μM NECA for the times indicated (data are means ± range; n = 2).

Table 1. No detectable coupling of adenosine receptors to Gq-PLC pathway

<table>
<thead>
<tr>
<th>Condition</th>
<th>IP Accumulation (Fold Change of Control)</th>
<th>Ca²⁺ Mobilization (Peak Fura-2/AM 340/380 Ratio)</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.00 ± 0.06</td>
<td>0.572 ± 0.022</td>
</tr>
<tr>
<td>UTP (100 μM)</td>
<td>16.50 ± 3.12*</td>
<td>1.144 ± 0.064*</td>
</tr>
<tr>
<td>ANG II (1 μM)</td>
<td>7.35 ± 0.68†</td>
<td></td>
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<tr>
<td>CADO (10 μM)</td>
<td>1.29 ± 0.14†</td>
<td>0.620 ± 0.017</td>
</tr>
<tr>
<td>NECA (100 μM)</td>
<td>1.10 ± 0.08</td>
<td>0.625 ± 0.024</td>
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Angiotensin (ANG II) significantly increased inositol phosphate (IP) accumulation and UTP significantly increased both IP accumulation and Ca²⁺ mobilization in cardiac fibroblasts (CF). However, neither 2-chloroadenosine (CADO) nor 5’-N-ethylcarboxamido-adenosine (NECA) had an effect on either IP accumulation or Ca²⁺ mobilization. (For IP accumulation: n ≥ 6; for Ca²⁺ mobilization: n = 30; *P < 0.001 vs. control, †P < 0.01 vs. control.)
data suggest that although there is slight coupling of an AR to the Gi/o-AC signaling pathway, stimulation of an A2R and the Gs-AC signaling pathway predominates.

ADO agonist concentration-dependence curves. Since the A2Rs appear to be the predominant functioning ARs in adult rat CF, experiments were performed to determine the relative contributions of A2aR and A2bR. Cells were treated with CADO, NECA (both general ADO agonists), or CGS-21680 (a specific A2aR agonist) over a wide range of concentrations (Fig. 5A). Each of the three agonists increased cAMP levels in a concentration-dependent manner. NECA was more efficacious than either CADO or CGS, with a 40% greater maximal cAMP accumulation. The EC50 values obtained are in the approximate range expected for A2Rs: 2.5 \( \mu \)M for CADO, 1.1 \( \mu \)M for NECA, 271 nM and for CGS-21680, although published data on this point are quite variable (17, 18, 24).

ADO antagonist concentration-dependence curves. To further elucidate which A2R are responsible for increases in cAMP levels, we performed experiments utilizing AR antagonists to block cAMP accumulation by the general agonist NECA (5 \( \mu \)M). The A1R antagonist DPCPX caused an initial 40% increase in cAMP levels above that achieved with NECA treatment alone (data not shown), suggesting that activation of the A1 receptor by NECA may be inhibiting cAMP production. Further experiments (data not shown) indicated this initial increase was due to the vehicle used to dissolve DPCPX (50% ethanol, 10% DMSO, diluted 1:100 in the experiment; control experiments indicated that ethanol was the active ingredient). As the concentration of DPCPX increased >10 nM, the initial increase was ultimately followed by an inhibition of the NECA-induced cAMP accumulation (IC50 \( \approx \) 2.7 \( \mu \)M) (data not shown). The capacity of DPCPX to inhibit cAMP accumulation suggests that at higher doses DPCPX functions to inhibit an A2R. Indeed, DPCPX is known to interact with the rat A2\( \beta \)R with an affinity \( \approx \) 340 nM (17).

The treatment of CF with the A3R antagonist ZM-241385 (ZM) led to a complete inhibition of the NECA-mediated increases in cAMP (IC50 \( = \) 20 nM) (Fig. 5B), substantiating the involvement of A2\( \beta \)R. By contrast, the A2\( \beta \)-specific antagonist MRS-1706 inhibited only 60% of the NECA-induced increases in cAMP content (IC50 \( = \) 10 nM) (Fig. 5B). These data suggest that NECA activates both the A2\( \beta \)R and the A2\( \beta \)R and that activation of the A2\( \beta \)R accounts for 60% of the observed increase in cAMP levels, whereas activation of the A2aR accounts for the remaining 40%.

DISCUSSION

The literature on ARs in rat heart cells is replete with inconsistencies and unknowns. In whole rat hearts (Wistar) the mRNA for A1R is most highly expressed, the ratio for the

Fig. 4. ADO agonists lead to extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation independent of Gi/o. CF were incubated with or without 0.1 \( \mu \)g/ml pertussis toxin (PTX) for 16 h and then treated with 10 \( \mu \)M lysophosphatidic acid (LPA), 10 \( \mu \)M CADO, or 100 \( \mu \)M NECA for 5 min. A: representative phospho-ERK1/2 immunoblots. B: quantitation of ERK1/2 phosphorylation. LPA increases ERK1/2 phosphorylation in a PTX-sensitive manner, whereas NECA and CADO increase ERK1/2 phosphorylation in a PTX-insensitive manner (n \( \geq \) 8; ***P < 0.001 vs. control, **P < 0.01 vs. control, *P < 0.05 vs. control, #P < 0.001 vs. LPA).

Fig. 5. ADO agonist and antagonist cAMP concentration-dependence curves. CF were preincubated with 10 \( \mu \)M rolipram for 15 min and then treated with either the indicated doses of CADO, NECA, or CGS-21680 for 10 min (n = 6) (A) or the indicated doses of ZM-241385, MRS-1706, or VUF-5574 (B).
various mRNAs being ~1.3 (A1):0.5 (A2b):1.0 (A2b):0.3 (A3) (21). On the cardiac myocytes the mRNAs for A2b and A3 receptors are enriched as 1.0 (A1):0.4 (A2b):2.0 (A2b):0.8 (A3) (21). Grden et al. (20) reported yet a different expression of mRNA for ARs in rat CF as 0.9 (A1):0.2 (A2b):1.0 (A2b):0.4 (A3). Our real-time PCR results indicate that in rat CF (Sprague-Dawley) express the mRNAs for A2a and A2b in much greater abundance then those for A1 and A3 receptors (Fig. 1). Despite the inconsistencies among studies, it seems likely that different cardiac cells express different abundances of the mRNAs and protein for the AR. If the presence of all four AR transcripts in CF indicates the presence of all four AR proteins in quantities proportional to the prevalence of the transcripts, then the A1R would be in greatest abundance in CF. Indeed, our data and that in the literature suggest that the A2R are largely responsible for regulating CF functions such as cell division, protein, and collagen synthesis. Unfortunately, the expression of A1R proteins could not be confirmed or quantified since available antibodies are insufficiently specific (31). Thus we have assessed functional coupling to signaling pathways as a means to determine the presence of functional receptors.

A1R, A2bR, and A3R all couple to pathways that can result in an increase in intracellular Ca²⁺ levels (1, 2, 15, 16, 34). However, in the experiments, treatment of CF with ADO agonists CADO (10 μM) and NECA (100 μM) had no effect on either IP or Ca²⁺ levels, indicating there is no functional AR that couples to a PLC signaling pathway or calcium channel in these cells. Reports of such responses may reflect characteristics specific to the artificial overexpression system, tissue, or species used.

Treatment of CF with ADO agonists increases intracellular cAMP levels, indicating the presence of a functioning A2R coupling to the Gi/o-AC signaling pathway. This is in agreement with reports in the literature (7, 13). Our data demonstrate that when assessing cAMP accumulation in response to AR stimulation, methylxanthine inhibitors of PDE such as IBMX may reduce the response, presumably by antagonizing agonist interaction with the ADO receptor. We find that a nonmethylxanthine PDE inhibitor, such as the PDE4-specific inhibitor rolipram, works well in CF, where PDE4 seems to be the dominant isoform (22). Thus, whereas IBMX enhances the β-adrenergic responses to CF better than other PDE inhibitors, rolipram is optimal for responses to ADO and its congener. The use of IBMX in many AR studies (13, 27, 36) likely confuses quantitative interpretations of the data.

Surprisingly, studies of cAMP accumulation in the presence of PTX reveal a functional AR coupled to the Gi/o-AC signaling pathway in CF. Pretreatment with PTX results in a small but significant increase in the response to A2 agonists, suggesting that ARs coupled to the Gi/o-AC signaling pathway are in low abundance (as the mRNA data may indicate for A1R and A2R), couple poorly to the Gi/o protein, or are masked by the more predominant A3R that coupled to the Gi/o-AC signaling pathway. The identity of the receptor mediating this modest Gi-coupled response is not yet clear, although A1R is a candidate (10, 19).

Attempts to verify this using an A1-specific antagonist DPCPX were not successful due to obscuring effects of the drug diluent at low concentrations. The downward phase of the DPCPX curve likely relates to its actions at A3R: the observed IC₅₀ values (between 1 and 2.7 μM) correspond to an apparent Kᵢ in the 200–599 nM range, close to the published value of the affinity of DPCPX at A3R, 340 nM (17). A previous study reported functional A1R in adult rat CF; the receptor appeared to be involved in the regulation of extracellular ADO levels, since treatment of isolated fibroblasts with selective A1R antagonists resulted in an increase in extracellular ADO (3). The question of functional consequences of A1R activation cannot be resolved until further studies are performed with specific agonists for this receptor (29).

A number of previous studies, including work in heterologous expression systems, have assessed AR coupling to cell signaling pathways. Studies in Chinese hamster ovary (CHO) cells overexpressing human ARs also indicate that stimulation of any of the four subtypes can lead to ERK1/2 phosphorylation. In CHO cells overexpressing the human A1R or A3R subtype, both the A1R and the A3R activate ERK1/2 in a Gi/o-dependent, PTX-sensitive manner (10, 19). In CHO cells overexpressing the human A2aR or A2bR, both A2Rs activate ERK1/2 in a Gi-cAMP-dependent manner (33, 35). Our studies of ERK1/2 activation by ADO agonists provide additional support for the hypothesis that Gi-coupled ARs predominate functionally in rat CF. CADO or NECA significantly increased ERK1/2 phosphorylation in a PTX-insensitive manner; FSK and 8-Br-cAMP mimicked this effect on ERK1/2 phosphorylation, indicating that ERK1/2 activation likely results from cAMP accumulation. Curiously, in a study performed by Dubey et al. (13), treatment of adult rat CF with either CADO (1 μM) or NECA (1 μM) had an inhibitory effect on mitogen-activated protein kinase activity. However, this result may not reflect of ERK1/2 activity, since ERK1/2 were not immunoprecipitated from cellular lysates (13).

In an effort to elucidate whether the A2aR, A2bR, or both are responsible for the observed increases in cAMP and whether the A1R or A2R is coupling to a Gi/o-AC signaling pathway, we employed the most specific agonists and antagonists that are commercially available; there is limited knowledge of the efficacies and affinities of these drugs on rat ARs. In our experiments, CADO, NECA, and CGS (an A2aR-specific agonist) all increased intracellular cAMP in a concentration-dependent manner, with NECA being 40% more efficacious than either CADO or CGS, which had similar maximal responses. Two interpretations of the data seem plausible. First, it is possible that CADO is not acting as a general agonist in rat CF, but instead stimulates only the A2aR. Conversely, NECA may activate both of the A2Rs, yielding a higher increase in cAMP levels. Second, NECA may act as a full agonist, whereas CADO acts as a partial agonist that is incapable of maximally stimulating cAMP production. Distinguishing these models must await the development of more specific compounds such as an A2bR agonist and the capacity to conduct binding studies on AR receptors of rat CF. The known specificities of AR agonists and antagonists are generally based on studies of human receptors that do not always apply across other species (24).

Our antagonist concentration-dependence studies suggest that stimulation with NECA leads to activation of both the A2aR and A2bR. NECA-induced cAMP accumulation was completely abolished by pretreatment with ZM-241385 (an A2aR antagonist), whereas pretreatment with MRS-1706 (an A2b antagonist) led to only a 60% inhibition of cAMP accumula-
tion; presumably the 40% of the response not inhibited by MRS-1706 represents cAMP accumulation due to activation of the A2aR. Thus our data support the hypothesis that A2aR play important roles in ADO-mediated regulation of CF functions via the upregulation of intracellular cAMP, but the participa-
tion of A2bR cannot be eliminated. We find no evidence of
functional A1R that affect cAMP metabolism in these cells. A limitation associated with our observations is the possibility
that as the cells are cultured from their initial isolation into
passage 2–3, their phenotype may shift to become more similar
to that of myofibroblasts. Indeed, in our cultures we find that
expression of the ED-A isoform of fibronectin and smooth
muscle embryo proteins do increase modestly at passage 2
and 3 (data not shown), suggesting a phenotype shift in the
cardiac fibroblasts.

In summary, our results indicate the mRNAs of all four
known ARs (A1R, A2aR, A2bR, and A3R) are present in adult
rat CF. There is no coupling of an AR to the Gq-PLC signaling
pathway, as well as slight coupling of an AR to the Gi/o-AC
signal-
way. Currently available pharmacological tools are insufficient for determining the cellular consequences fol-
lowing activation of each ADO receptor type. At present, we
conclude that a combination of cAMP signals via A2a and
A2b receptors likely mediate adenosine’s effects an cyclic nucleo-
tide signaling in rat CF. As we show in accompanying paper
(36a), cAMP, acting via Epac, can alter aspects of collagen
metabolism in these cells.

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