Cloning and pharmacological characterization of the equine adenosine A2A receptor: a potential therapeutic target for the treatment of equine endotoxemia


INTRODUCTION

Adenosine is an endogenous purine nucleoside that has evolved to modulate many physiologic processes. Cellular signaling by adenosine occurs through four known adenosine receptor subtypes (A1, A2A, A2B, and A3), all of which are seven transmembrane spanning G-protein coupled receptors (Hettinger et al., 1998; Olah & Stiles, 2000). These four receptor subtypes are further classified based on their ability to either stimulate or inhibit adenylate cyclase activity. The A2A and A2B receptors couple to GaS and mediate the stimulation of adenylate cyclase, while the A1 and A3 adenosine receptors couple to Gas which inhibits adenylate cyclase activity (Cronstein, 1994; Linden, 2001). Additionally, A1 receptors couple to Gs, which has been reported to mediate adenosine inhibition of Ca2+ conductance, whereas A2B and A3 receptors also couple to Gs and stimulate phospholipase activity.

Extracellular adenosine concentrations from normal cells are approximately 300 nM (Hirschhorn et al., 1981); however, in response to cellular damage (e.g. in inflammatory or ischemic

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The aim of the current study was to clone the equine adenosine A2A receptor gene and to establish a heterologous expression system to ascertain its pharmacologic profile via radioligand binding and functional assays. An eA2A-R expression construct was generated by ligation of the eA2A-cDNA into the pcDNA3.1 expression vector, and stably transfected into human embryonic kidney cells (HEK). Binding assays identified those clones expressing the eA2A-R, and equilibrium saturation isotherm experiments were utilized to determine dissociation constants (Kd), and receptor densities (Bmax) of selected clones. Equilibrium competition binding revealed a rank order of agonist potency of ATL > CV-1808 > NECA > 2-CADO > CGS21680, and a rank order of antagonist potency as ZM241385 > 8-phenyltheophylline > p-sulfophenyltheophylline > caffeine. Furthermore, adenylate cyclase assays using selective A2A-R agonists revealed that the eA2A-R functionally coupled to GaS as indicated by an increase in intracellular [3H]cAMP upon receptor activation. Finally, NF-κB reporter gene assays revealed a CGS21680 concentration-dependent inhibition of NF-κB activity. These results indicate that the heterologously expressed eA2A-R has a pharmacological profile similar to that of other mammalian A2A receptors and thus can be utilized for further characterization of the eA2A-R to ascertain whether it can serve as a suitable pharmacological target for equine inflammatory disease.

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tissue), these concentrations are quickly elevated (600–1200 nM) (Cronstein et al., 1995). Thus, in regard to stress or injury, the function of adenosine is primarily that of cytoprotection preventing tissue damage during instances of hypoxia, ischemia, and seizure activity (Ibayashi et al., 1988). Activation of A<sub>2A</sub> receptors produces a constellation of responses that in general can be classified as anti-inflammatory (Sullivan & Linden, 1998; Salvatore et al., 2000).

Inflammation represents a host defense response to a variety of harmful stimuli and is crucial to the survival of an organism (Lukashev et al., 2004). The inflammatory cascade is mediated by proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), lymphotoxin-α, and interferon-gamma as well as many others; the interplay among these cytokines and cytokine-induced chemokines results in the activation of macrophages, lymphocytes, and neutrophils (Rosenberg & Gallin, 2003). Whereas the primary function of these proinflammatory molecules is to remove the invading pathogen, it is not without its consequences as local tissue damage and prolonged inflammation can also occur. One of the most potent of these cytokines is TNF-α, and in some instances of inflammatory disease its expression can become dysregulated via activation of the NF-κB pathway. NF-κB is a nuclear transcription factor that activates the transcription of a host of inflammatory genes – including that of TNF-α. Remarkably, NF-κB activation is self-limiting; while it transcribes proinflammatory genes, it also drives the transcription of its own inhibitory protein – inhibitory-κB (I-κB). Thus, NF-κB is active for approximately 30 min; after which I-κB is translated and binds NF-κB forming an inactive complex in the cytoplasm. However, the expressed TNF-α can also bind to and activate its own receptor on certain cell types and this also activates the NF-κB pathway in some instances of inflammatory disease. Therefore, in this situation, NF-κB activity is no longer self-limiting; thus TNF-α transcription continues constitutively.

It has been shown that activation of adenosine A<sub>2A</sub> receptors strongly inhibits the NF-κB pathway, as well as the production of TNF-α (Bshesh et al., 2002). With this in mind, it seems reasonable to assume that administration of adenosine may be of therapeutic value. However, due to the relatively short biological half-life of adenosine and potential adverse side effects (e.g. hypotension, bradycardia, and hypothermia), its therapeutic usefulness may be limited. To that end, research into the effects due to their specificity for individual adenosine receptor subtypes.

Thus, the long-term goal stemming from our laboratory is to characterize fully the equine adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>-R) to ascertain if it can be utilized as a therapeutic target for the treatment of equine endotoxemia. Thus, the present study was designed to characterize the heterologously expressed equine A<sub>2A</sub> receptor via radioligand binding and functional assays using selective receptor antagonists and an array of adenosine analogs. It is our belief that results from this study will provide the foundation for future research to determine the effects of these agonists on cytokine production by equine monocytes challenged with LPS. To this end, the objectives of this study were (i) to heterologously express equine A<sub>2A</sub>-R in human embryonic kidney 293 (HEK) cells; (ii) to characterize the pharmacological signature of this receptor utilizing radioligand binding assays; (iii) to determine the ability of adenosine analogs to alter adenylate cyclase activity and intracellular concentrations of cAMP; and (iv) to determine what effect eA<sub>2A</sub>-R activation plays on signaling components of the inflammatory cascade.

**MATERIALS AND METHODS**

**Cloning and sequencing of the equine A<sub>2A</sub> Receptor cDNA**

An equine monocyte cDNA library was constructed as described in the Uni-ZAP II cDNA library construction kit (Stratagene, LaJolla, CA, USA), and subsequently screened for the equine A<sub>2A</sub> receptor cDNA by hybridization with a <sup>32</sup>P end-labeled rat A<sub>2A</sub> oligonucleotide as a probe. A single hybridization positive plasmid, isolated by alkali lysis over Qiagen columns (Valencia, CA, USA), contained an EcoRI/Xhol insert of the estimated size for the full-length equine A<sub>2A</sub> receptor, and was selected for sequencing. The plasmid was sequenced in either direction with an ABI Prism BigDye<sup>™</sup> Terminator Cycle Sequencing Ready Reaction Kit (ver. 3.0) in both 96- and 384-well format. Sequencing reactions included 4 μL of plasmid DNA (100 ng), 44 pmol of primer, 0.70 μL of BigDye<sup>™</sup>, 0.58 μL ddH<sub>2</sub>O, 0.34 μL DMSO, 1.4 μL 5x reaction buffer (5x = 10 mm MgCl<sub>2</sub>, 400 mm Tris-Cl pH 9.0), resulting in a total reaction volume of 7.2 μL. Thermal cycling reactions were performed on a 96-well GeneAmp<sup>®</sup> PCR System (Applied Biosystems, Foster City, CA, USA) and an Autoloid Dual 384-well GeneAmp<sup>®</sup> PCR System 9700 (Applied Biosystems). The purified extension products were separated on an ABI Prism<sup>®</sup> 3700 DNA Analyzer (Applied Biosystems).

The eA<sub>2A</sub>-R-containing plasmid was then amplified by transformation into bacterial cells (Top10 cells; Invitrogen, Carlsbad, CA, USA), which were grown overnight at 37 °C on LB agar plates containing 50 μg/mL ampicillin (Sigma, St Louis, MO, USA). Ampicillin-resistant colonies were then inoculated in 300 mL of LB broth (Sigma) containing 50 μg/mL ampicillin and shaken overnight at 37 °C, 5% CO<sub>2</sub>. Plasmid purification of the resulting culture was achieved using an endotoxin-free plasmid purification kit (Endo-Free Maxi Kit; Qiagen) as described previously (Meyer, 1990). A BamHI/DraI restriction fragment of the eA<sub>2A</sub>-R cDNA was then directionally subcloned into a BamHI/NotI restriction site of the pcDNA3.1 expression vector (Invitrogen) using a Takara DNA ligation kit (Fisher Scientific, Suwannee, GA, USA). Specifically, the eA<sub>2A</sub>-R cDNA was digested with BamHI and DraI, while the pcDNA3.1 expression vector was digested with BamHI and NotI; the digestion reactions were carried out in separate tubes at 37 °C for 3 h. Prior to ligation, the pcDNA3.1 vector was treated further with calf intestinal alkaline phosphatase to prevent re-circularization of the plasmid DNA; after which both...
the eA2A-R cDNA and pcDNA3.1 were extracted in phenol-
chloroform to remove extraneous salts and proteins. The resulting eA2A-R BamHI/DraI restriction fragment and BamHI/
NotI-digested pcDNA3.1 were resolved on an agarose gel, 
purified and ligated together in a reaction using T4 DNA ligase 
at 16 °C for 24 h. The eA2A-R cDNA was sequenced fully from 
either end on an ABI 3700 automated sequencer (Amersham 
Biosciences, Piscataway, NJ, USA) using the Transposon based 
EZ::TN insertion kit (Epitentre, Madison, WI, USA) with the Kan-
2 FP-1 and Kan-2 RP-1 primers supplied by the manufacturer. 
Sequence fragments were then assembled into a contig using 
AssemblyLIGN software (Accelrys, Norwalk, CT, USA), and 
translation start and stop codons identified via sequence analysis 
utilizing MACVECTOR 6.5.3 software (Accelrys). The resulting 
coding region of the eA2A-R cDNA was then translated into its 
corresponding amino acid sequence and aligned with other 
mammalian A2A-R proteins (MACVECTOR). Conserved amino acid 
residues were then divided by the total alignment score to 
determine sequence homology.

**Heterologous expression system**

For stable transfection, HEK293 were seeded in six-well microt-
iter plates (Becton-Dickinson, Bedford, MA, USA) at a density of 
6 × 10^4 cells/well, and cultured in complete medium containing 
Minimal Essential Media (MEM; Sigma), 10% fetal calf serum 
(FCS), and 1% penicillin–streptomycin to approximately 75% 
confluence. Transfection of the eA2A-R cDNA was performed 
using Polyfect transfection reagent (Qiagen). Initially, 2 μg of the 
eA2A-R pcDNA plasmid were added to Eppendorf tubes, and 
brought to a final volume of 100 μL with TE buffer (Tris–EDTA), 
ph 7.5. Polyfect transfection reagent (40 μg) was then added 
and allowed to incubate at room temperature for 10 min. HEK 
cell growth media was removed and replaced with 1.5 mL 
complete media in the absence of antibiotics, followed by the 
addition of the eA2A-R cDNA/Polyfect reaction complex. The transfection reaction was incubated for 6 h at 37 °C, 5% CO₂, 
and 95% relative humidity, after which the transfection media 
was replaced with MEM supplemented with 10% FCS and 1% 
penicillin–streptomycin. At 24 h post-transfection, the selection 
media, comprised the complete media that included 0.25 mg/mL 
of the antibiotic geneticin (G418, Invitrogen), was added. To 
isoalte individual eA2A-R clones, plaques were isolated using 
6.4 × 8 mm cloning cylinders (Fisher Scientific), trypsinized, and 
transferred to separate culture flasks. Individual eA2A-R-express-
ing HEK cell clones were then maintained at 37 °C, 5% CO₂, 
95% relative humidity until pharmacological characterization 
experiments were undertaken.

[^H]ZM241385 Radioligand Binding Assay

To screen individual clones for membrane receptor expression, 
binding assays were performed on eA2A-R/HEK cell membrane 
preparations. Initially, eA2A-R-expressing HEK cells were grown 
to confluence in 150 × 15 mm Nunc culture plates (Fisher), 
after which cells were harvested via cell scraping in ice cold 
25 mM Tris, pH 7.5. Equine A2A/HEK cell membrane prepara-
tions were then obtained by Dounce homogenization (20 strokes), 
and the resulting membranes centrifuged at 40 000 g for 
20 min. The membranes were then resuspended in Tris buffer 
and washed two additional times prior to the binding assays.

For the binding assays, 100 μg of eA2A-R/HEK cell membrane 
were incubated in the presence of 1.0 nM of the selective A2A 
analyst [^H]ZM241385 (Tocris Cookson, Ellisville, MO, USA) 
to define total binding and with the addition of 10 μM 2-
chlordoadenosine (2-CADO) to define nonspecific binding. Prior to 
use in binding assays, cell membrane preparations were 
incubated with 2.0 μM adenosine deaminase for 30 min at 
22 °C. The final reaction volume was 500 μL. The binding 
reaction was incubated for 45 min at room temperature, after 
which the membranes were harvested via rapid filtration onto 
Brandell GF/B filters using a 48-well cell harvester (Brandell, 
Gaithersburg, MD, USA). Four milliliters of scintillation cocktail 
was added to each filter, and counts determined on a Beckman 
LS6000 scintillation counter (Beckman Coulter, Fullerton, CA, 
USA). Specific binding was determined by subtracting nonspec-
cific binding from that of the total. Data were analyzed using 
Graphpad Prism Software (Prism 4.0; Graphpad Software Inc., 
San Diego, CA, USA).

**Equilibrium saturation isotherms**

To determine the antagonist affinity (Kᵦ) and receptor density 
(B_max) of selected clones, equilibrium saturation isotherms 
were performed. Briefly, 100 μg of eA2A-R/HEK cell membranes 
were incubated with increasing concentrations of [^H]ZM241385 
(0.0625–2.0 nM) in the presence or absence of 10 μM 2-CADO to 
define nonspecific and total binding, respectively. Data were 
alanaed utilizing nonlinear regression analysis with Graphpad 
Prism Software (Prism 4.0).

**Adenylate cyclase assays**

Adenylate cyclase assays were performed to determine whether 
the heterologously expressed eA2A-R functionally coupled to 
intracellular heterotrimeric G-proteins. Briefly, eA2A-R/HEK cells 
were plated in six-well plates at a density of 7.5 × 10^4 cells/well 
and cultured for 24 h. The cells were then washed three times in 
serum-free media, and preloaded with [^3H]-adenine at a final 
concentration of 1.2 μCi/mL for 5 h. Cells were then washed three 
times in serum-free media followed by the addition of 10 μL of 
individual A2A agonists (CGS21680, ATL303, NECA, and 
2-CADO) in MEM containing 50 μM of the phosphodiesterase 
inhibitor rolipram (Sigma) and incubated for 40 min at 37 °C, 
and 5% CO₂. The cells were then lysed, and the supernatant 
transferred to 1.5 mL microcentrifuge tubes and centrifuged at 
10 000 g for 15 min at 4 °C. The resulting supernatant was 
washed over Dowex (50W-X4 Resin; Bio-Rad, Hercules, CA, USA) 
followed by alumina columns, and [^3H]-cAMP was eluted from the 
alumina columns by the addition of 0.1 M imidazole buffer and 
counts determined. Data were analyzed utilizing nonlinear 
regression analysis with Graphpad Prism Software (Prism 4.0).
Equilibrium competition binding

To determine the pharmacological signature of the heterologously expressed e-A2A-Rs, equilibrium competition experiments were performed. Briefly, 100 µg of e-A2A/HEK cell membranes were incubated with 0.25 nM [3H]ZM241385 and increasing concentrations of either A2A agonists or antagonists. Agonists utilized in these experiments were ATL303, NECA, 2-CADO, CGS21680, and CV-1808, and the antagonists utilized were caffeine, 8-phenyltheophylline, p-sulfophenyltheophylline and unlabeled ZM241385. Data were analyzed utilizing nonlinear regression analysis with Graphpad Prism Software (Prism 4.0).

Reporter gene and electrophoretic mobility shift assays

To determine the effect of receptor activation on downstream signal transduction, NF-kB reporter gene assays were performed. The eA2A-F receptor clone was seeded in a 96-well microtiter plate at a density of 2.5 × 10^4 cells per well at 37 °C, 5% CO2, and 95% relative humidity until approximately 75–80% confluence was achieved. The cells were then transiently transfected with 50 ng/well of the Endothelial Leukocyte Adhesion Molecule (ELAM) NF-kB-dependent firefly luciferase reporter construct (a generous gift from Dr Douglas Golenbock; University of Massachusetts Medical School, Worcester, MA, USA), and 5 ng/well of the synthetic Renilla luciferase reporter plasmid (Promega, Madison, WI, USA) with the Polyfect transfection reagent (Qiagen). Initially, 100 ng total DNA (50 ng ELAM, 5 ng Renilla, 45 ng empty vector [pcDNA]) was added to individual tubes, followed by the addition of serum-free media to a final volume of 20 µL DNA/well. The transfection reaction Polyfect was then added (3 µg/well), and incubated at room temperature for 10 min, after which MEM + 10% FCS was added to a final volume of 100 µL/well. After the DNA-Polyfect incubation period, media were removed and replaced with 100 µL complete media containing 10% FCS, 1% penicillin–streptomycin, followed by the addition of 100 µL/well of the ELAM/Renilla/pcDNA-Polyfect reaction mixture. The transfection reaction was allowed to proceed for 6 h, after which the cells were washed in serum-free media. The media was then replaced with MEM containing 10% FCS, 1% penicillin–streptomycin, and 0.25 mg/ml G418. The cells were allowed to recover for 48 h and then stimulated with human recombinant TNF-α at a final concentration of 10 ng/mL for 4 h in the presence of the selective A2A agonist CGS21680 at a concentration range from 10 nM to 100 µM. Following the stimulation reaction, cells were lysed with 50 µL passive lysis buffer and assayed for firefly and Renilla luciferase activities, respectively using the Dual-Luciferase Reporter Assay kit (Promega). Luciferase activity was normalized relative to the Renilla luciferase activity to control for differences in transfection efficiencies.

To determine the effect of eA2A-F receptor activation on NF-kB nuclear translocation, electrophoretic mobility shift assays were performed. Briefly, cells were plated in 15 mm culture plates (Becton-Dickinson) and allowed to reach 75–80% confluency. The cells were then stimulated as in the reporter gene assays for 50 min. Growth media utilized were either MEM + 10% FCS or MEM + 10% FCS including 50 µM of the phosphodiesterase inhibitor rolipram. Following stimulation, cells were trypsinized, washed 2x in ice cold PBS, and the nuclear extracts obtained by incubation in a hypotonic HEPES buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF, pH 7.9), followed by the addition of 10% NP-40. Cells were then centrifuged, supernatants discarded, and the nuclear pellet suspended in a hypotonic HEPES buffer (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, pH 7.9), extracted at 4 °C for 30 min with mixing, followed by centrifugation at 1.4 × 10^5 g, 4 °C for 5 min. Equivalent amounts of nuclear extract were then incubated with a [γ32P] end-labeled NF-kB oligonucleotide for 20 min at room temperature. A one-tenth volume of 10x gel loading buffer consisting of 250 mM Tris/HCl, pH 7, 0.2% bromophenol blue, and 40% glycerol was then added to each reaction tube, and the protein/oligonucleotide complexes were resolved on a 4% nondenaturing polyacrylamide gel. After protein migration, the gel was dried and opposed to a phosphoimager for at least 24 h; the resulting bands were then analyzed utilizing a 170–7856 Molecular Imager FX Pro (Bio-Rad).

RESULTS

Equine A2A-R cloning and sequencing

The cloning and sequencing of the eA2A-R cDNA revealed a full-length transcript complete with start and stop codons, 5’ and 3’ untranslated regions (UTR), as well as a poly-adenylation signal in the 3’ UTR. ClustalW alignment of the eA2A-R cDNA indicated this receptor cDNA had a high degree of sequence similarity with that of other mammalian A2A receptor transcripts. The position of the start codon was conserved in the sequences for the equine and human A2A-R cDNAs, while the position of the translation start sequence for the mouse, rat, and guinea pig was 10 bp downstream from that of the equine receptor. The termination signal was conserved for the equine, human, and guinea pig, while that of the rat and mouse were located 27 bp downstream. Furthermore, BLAST homology screening of the deduced amino acid sequence of the eA2A-R with that of other mammalian A2A-R proteins revealed a similar degree of homology (Fig. 1; Table 1).

Evaluation of eA2A receptor expression

Initially, eA2A-R/HEK clones were screened for membrane expression utilizing radioligand binding assays to document displaceable binding of 1 nM [3H]ZM241385 in the presence of 10 µM 2-CADO. Ten individual G418-resistant plaque-isolated clones were screened and compared with membranes from rat striatum which served as a positive control. From these 10 G418-resistant clones, four were identified that expressed the eA2A-R at levels greater than untransfected HEK293 cells as indicated by the specific binding of [3H]ZM241385 (Fig. 2a).
Based on these levels of receptor expression, four clones (A2A-D, A2A-E, A2A-F, and A2A-G) were selected from the original pool of 10 to utilize in further characterization experiments (Fig. 2b).

Equilibrium saturation isotherms

To further characterize the four selected eA2A-HEK clones, equilibrium saturation isotherm experiments were performed.

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**Table 1.** Comparison of the equine adenosine A2A receptor homology with that of other mammalian species

<table>
<thead>
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<th>A2A protein sequence</th>
<th>% Overall homology</th>
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<tr>
<td>Human</td>
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<tr>
<td>Rat</td>
<td>77.4</td>
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</tr>
</tbody>
</table>

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**Fig. 1.** ClustalW protein alignments of the equine adenosine A2A receptor with that of other mammalian species.

**Fig. 2.** (a) One point-binding assay of eA2A-R transfected HEK293 cells to screen for receptor membrane expression. Total binding was defined with 1.0 nM [3H]ZM241385, and nonspecific binding with [3H]ZM241385 in the presence of 10 μM 2-chloroadenosine (arrows denote those clones selected for further characterization). (b) Replot of the data in histogram 2A showing specific membrane receptor densities of the four selected clones; data are expressed in pmol/mg protein bound. Membranes from rat striatum (Str., in both panels) served as the positive control.
Results from these studies revealed that [3H]ZM241385 bound saturably and with high affinity to membranes derived from the stably transfected HEK cells (Fig. 3a). All four clones had dissociation constants that were within the published range of 0.79–0.92 nM for rat striatal membranes (Alexander & Millns, 2001). Isotherm analysis of nontransfected HEK cells revealed no displaceable binding, indicating that binding occurring in the four selected clones was due to the presence of the eA2A-R. Additionally, a Scatchard replot of these data resulted in a linear transformation indicating specific binding to a homogeneous population of binding sites (Fig. 3b). Based on a similar affinity for [3H]ZM241385 between clone eA2A-F and that of rat striatum (0.76 nM vs. 0.74 nM, respectively) coupled with that clone’s high level of receptor expression (1.575 pmol/mg vs. 0.7620 pmol/mg), the eA2A-F clone was utilized in all subsequent characterization experiments (Fig. 3b).

Equilibrium competition binding

To determine the rank order of agonist potency of clone eA2A-F, equilibrium competition binding assays were performed. The specific A2A agonists used in these experiments were ATL303, NECA, CV-1808, 2-CADO, and CGS21680. Nonlinear regression analysis of these data revealed that ATL303 and CV-1808 displacement data were better fit ($P < 0.05$) with a two-site competition model. The remaining three compounds (2-CADO, CGS21680, and NECA) were adequately fit by a one-site competition model (Fig. 4). The rank order of high-affinity binding of these agonists at the equine A2A receptor was ATL303 > CV-1808 > NECA > 2-CADO > CGS21680 (Table 2).

To determine the rank order of potency for antagonists, competition experiments were performed using the antagonists caffeine, 8-phenylethylphylline, p-sulfophenylethylphylline, and unlabeled ZM241385 in competition with [3H]ZM241385. Additionally, the selective A3 antagonist MRS1220 was included to document further the pharmacologic profile of the equine A2A receptor. The deduced rank order of antagonist potency was ZM241385 > 8-phenylethylphylline > p-sulfo-

Adenylate cyclase assays

To document the functionality of the heterologously expressed equine A2A receptors, adenylate cyclase assays were performed.
The selected A2A agonists used in these experiments were ATL303, NECA, CGS21680, and 2-CADO at concentrations ranging from 1 nM to 300 nM. The rank order of potency of these agonists for production of [3H]cyclic AMP accumulation was ATL303 > CGS21680 > NECA > 2-CADO (Fig. 7).

Adenylate cyclase assays were then performed in the presence of the A2A antagonist ZM241385 (1 nM) to determine whether the A2A agonist stimulation of adenylate cyclase was due to eA2A-R activation. In these experiments, CGS21680 was incubated with the HEK293 cells expressing the eA2A-R in the presence or absence of 1 nM ZM241385. Addition of ZM241385 resulted in a rightward shift in the concentration–response curve by approximately one order of magnitude (Fig. 8).

Reporter gene and electrophoretic mobility shift assays

In these experiments, eA2A-R activation by CGS21680 resulted in a concentration-dependent inhibition of TNF-α-stimulated NF-κB activity, with maximal inhibition (74%) occurring at 100 μM CGS21680. A return to baseline (e.g. cells stimulated with 10 ng/mL TNF-α alone) was observed in cells co-incubated with 10 nM CGS21680 and TNF-α. Furthermore, the inhibitory effect of CGS21680 (1 μM) was largely reversed with the addition of 10 μM of the A2A selective antagonist ZM241385. Inhibition of the effects of TNF-α by CGS21680 was apparently mediated via cyclic AMP as this inhibitory effect was enhanced by the addition of the phosphodiesterase inhibitor rolipram. Incubation of the cells with 10 μM forskolin, a direct activator of adenylate cyclase, in the presence of TNF-α...
also resulted in almost complete inhibition of NF-κB activity (Fig. 9).

Data from the electrophoretic mobility shift assays indicated that the inhibitory effect of CGS21680 on TNF-α-stimulated NF-κB activity was not due to an inhibition of nuclear translocation as NF-κB subunits were identified in nuclear extracts from TNF-α-stimulated cells at all concentrations of CGS21680 (Fig. 10; Table 3). Furthermore, to show that the bands visualized in the electrophoretic mobility shift assays in the TNF-α-treated cells were activated NF-κB subunits, nuclear extracts were incubated with a polyclonal antibody to the p65 subunit of NF-κB. Addition of this antibody shifted the band to a higher molecular weight, providing evidence that the TNF-α-activated complex consisted of the p65 (RelA) subunit. Additionally, the elimination of the band by incubating nuclear extracts with excess cold oligonucleotide lends further support for the presence of NF-κB subunits in the nuclear extracts of TNF-α-stimulated cells.

DISCUSSION

Tumor necrosis factor-α plays an important role in the response of mammals to proinflammatory substances such as the lipopolysaccharide (LPS) component of the outer cell wall of gram-negative bacteria. Interactions among LPS and its receptors on the surface of mononuclear phagocytes result in activation of the NF-κB pathway leading to the transcription of a host of inflammatory genes, including TNF-α. The TNF-α protein is then secreted to the extracellular space where it can bind to TNF-α receptors on target cells, and in turn cause activation of the NF-κB pathway. In this situation, TNF-α...
expression becomes dysregulated and can result in dire consequences to the host, including septic shock and death. Thus, agents capable of inhibiting the response to TNF-α may have enormous therapeutic potential for the treatment of inflammatory disease.

The results obtained in this study indicate that activation of the eA2A-R with the A2A-selective agonist CGS21680 reduces TNF-α-induced NF-κB-driven reporter gene expression in a concentration-dependent manner without altering nuclear translocation of NF-κB (Figs 9 & 10; Table 3). Specifically, we observed a 74% inhibition of NF-κB activity at the highest concentration of CGS21680 (100 μM) (Fig. 9), and this effect was potentiated by the addition of the phosphodiesterase inhibitor rolipram. These findings are consistent with a cyclic AMP-mediated inhibitory effect on NF-κB activation. The strong inhibition of NF-κB activity by forskolin alone also supports a role for cyclic AMP. Increased concentrations of cyclic AMP have been reported to inhibit NF-κB-mediated transcription of TNF-α in human monocyctic and endothelial cells (Ollivier et al., 1996).

In this earlier report, forskolin and dibutyryl cyclic AMP inhibited LPS-stimulated TNF-α gene expression and NF-κB activity without altering nuclear translocation of NF-κB heterodimers. We demonstrate herein an eA2A-R-mediated concentration-dependent inhibition of TNF-α activation of NF-κB reporter gene activity, that was potentiated by inhibition of phosphodiesterase and occurred in the absence of changes in NF-κB nuclear translocation. These results strongly suggest that the inhibition of TNF-α-stimulated NF-κB activity via eA2A-R activation is a cyclic AMP-mediated effect. This hypothesis is further supported by the results of a recent report summarizing the effects of A2A-R activation in equine articular chondrocytes (Tesch et al., 2002). In that study adenosine, the A2A-selective agonist N(6)-[(dimethoxyphenyl)-ethyl]adenosine (DMPA), and forskolin alone, significantly increased intracellular concentrations of cyclic AMP, and suppressed LPS-stimulated nitric oxide production by articular chondrocytes. Moreover, similar to our results, inhibition of phosphodiesterase potentiated actions of DMPA and forskolin.

Using the electrophoretic mobility shift assays, we identified NF-κB subunits in nuclear extracts of TNF-α-stimulated cells in both the presence and absence of an A2A receptor agonist (Fig. 10; Table 3). Additionally, supershift assays using a p65 polyclonal antibody (Santa Cruz Biotech, Inc., Santa Cruz, CA, USA) clearly indicated that the DNA binding complex induced by TNF-α contained the active p65 NF-κB subunit (Fig. 9; Table 3). The inhibitory effect on NF-κB is occurring through a route other than that of nuclear translocation; this effect has been reported previously for forskolin-mediated modulation of LPS-stimulated NF-κB activity (Majumdar & Aggarwal, 2003). Additional work will be required to determine the precise mechanism by which eA2A-R activation inhibits NF-κB transcriptional activity. However, the ability of A2A-R agonists to inhibit both the early induction of NF-κB activity by LPS as reported by Ollivier et al. (1996), as well as later induction of NF-κB activity by TNF-α that we report here, would seem to imply great potential in the treatment of endotoxemia.

Additionally, the results obtained from the reporter gene assays performed in the present study suggest possible species and/or cell-type-dependent activation of the NF-κB pathway.

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**Table 3. Densitometry analysis of electrophoretic mobility shift assays (EMSA) data.** Band densities were measured and converted to percentage NF-κB translocation.

<table>
<thead>
<tr>
<th>Lane no.</th>
<th>Cell treatment</th>
<th>Scan analysis</th>
<th>% Translocation</th>
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<tbody>
<tr>
<td>1</td>
<td>Media control</td>
<td>171 (0)</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2 ng/mL TNF</td>
<td>2153 (1982)</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>10 μM CGS21680 in 0.5% DMSO</td>
<td>152 (−19)</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>50 μM Rolipram</td>
<td>193 (22)</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>TNF + 10 μM CGS21680</td>
<td>1954 (1783)</td>
<td>99</td>
</tr>
<tr>
<td>6</td>
<td>TNF + 10 μM CGS21680 + RO</td>
<td>2243 (2072)</td>
<td>105</td>
</tr>
</tbody>
</table>

Numbers in parentheses are relative optical densities with background subtracted.
For example, the results of a recent study using LPS-stimulated RAW 246.7 cells reported that adenosine regulates macrophase function independently of NF-κB activity (Nemeth et al., 2003). In that study, neither adenosine nor adenosine receptor agonists inhibited NF-κB binding in mobility shift assays; furthermore, adenosine treatment of LPS-stimulated cells failed to inhibit NF-κB-specific activity as assessed by reporter gene assays. A possible explanation for the discrepancy in NF-κB activity between that study and the results reported herein may be that RAW 246.7 cells express A₁ receptors but not the A₂A receptor subtype. The fact that A₂B receptors are relatively low affinity receptors when compared with A₂A may account for the lack of an inhibitory effect on TNF-α-stimulated NF-κB activity. In contrast, the inhibitory effect of adenosine analogs in our eA₂A heterologous expression system was marked. In agreement with our findings are the results of a study by Bshesh et al. (2002) in which CGS21680 inhibited NF-κB activity in LPS-stimulated human monocyctic leukemia cells (THP-1). The results of that study also indicated that inhibition of NF-κB activity occurred in the absence of an alteration in nuclear translocation of NF-κB. These authors concluded that adenosine receptor activation resulted in activation of PKA, phosphorylation of CREB, and a reduction in TNF-α production, most likely via inhibition of NF-κB-dependent transcription of the TNF-α gene. Additionally, the results of a recent study performed in human myeloid KBM-5 cells indicate that adenosine suppresses TNF-α-stimulated NF-κB-driven reporter gene expression (Majumdar & Aggarwal, 2003). To define which adenosine receptor subtypes were mediating these effects, these authors incubated KBM-5 cells with adenosine in the presence of either the A₇-selective (DPCPX) or A₂-selective (DMPX) antagonists. Results from these experiments revealed that the A₂-selective antagonist DMPX reversed the adenosine-mediated inhibition of TNF-α-stimulated NF-κB activity in a concentration-dependent manner, while the A₁ antagonist had no effect. Thus, the inhibitory effect on NF-κB activity was mediated through the A₂-receptor subtype. In contrast to our results, adenosine treatment of TNF-α-stimulated KBM-5 cells significantly reduced NF-κB nuclear binding as evidenced by mobility shift assays. This may imply that the cellular context is the primary determinant for the mechanism of adenosine receptor-mediated regulation of TNF-α production.

It has been suggested by Majumdar and Aggarwal (2003) that the effects of adenosine are selective as it had no effect on NF-κB activity induced by other inflammatory agents (phorbol-12-myristate-13-acetate [PMA], LPS, H₂O₂, and ceramide), all of which activate the NF-κB pathway. This finding suggests that the mechanism by which these agents activate NF-κB differs from the pathway used by TNF-α. Taken together, the contradictory findings regarding adenosine regulation of the NF-κB pathway suggest that this inhibitory signal transduction pathway may be species and/or cell-type specific, as well as dependent on the subtype of adenosine receptor involved. By using cells heterologously expressing equine A₂A-R, we demonstrated that adenosine A₂A-receptor-specific agonists modulate TNF-α-induced activation of NF-κB.

The results of equilibrium saturation isotherm experiments revealed that [³H]ZM241385 bound saturably and with high affinity to the eA₂A-F clone. The affinity of clone eA₂A-F for [³H]ZM241385 (0.74 nM) was comparable with that for rat striatum (0.84 nM) (Alexander & Milins, 2001). Similarly, the affinity of [¹²⁵I]ZM241385 for bovine striatal A₂A-R is 1.4 nM and 1.6 nM for canine A₂A-R expressed in CHO cells (Palmer et al., 1995). In a more recent study, [³H]ZM241385 had a binding affinity of 1.2 nM in the equine striatum (Chou & Vickroy, 2003). Thus, the A₂A-selective antagonist [³H]ZM241385 binds to the equine A₂A receptor with an affinity comparable with that of other mammalian species.

Equilibrium competition binding assays revealed an agonist rank order of potency to be ATL303 > CV-1808 > NECA > 2-CADO > CGS21680 (Fig. 4, Table 2), and the antagonist rank order as ZM241385 > 8-phenyltheophylline > p-sulfophenyltheophylline > caffeine (Fig. 5). Results from adenylate cyclase assays revealed a rank order of agonist potency to be ATL303 > CGS21680 > NECA > 2-CADO (Fig. 7). Overall, the pharmacological characteristics of the equine A₂A receptor are similar to those of other species, with the exception of a somewhat higher affinity for CV-1808. The pharmacological signature of the eA₂A-R is most similar to the human A₂A-R (Table 4), which is to be expected inasmuch as the eA₂A-R shares 90% amino acid sequence homology with the human A₂A-R (Fig. 1; Tables 1 & 4).

In summary, the cloned equine adenosine A₂A receptor stably expressed in HEK293 cells provides a unique heterologous expression system to characterize the receptor. In these experiments we have demonstrated that: (i) the eA₂A-R is expressed in HEK293 cell membranes and binds the selective A₂A antagonist [³H]ZM241385 with both high affinity and

<table>
<thead>
<tr>
<th></th>
<th>eA₂A-R (HEK293) (nM)</th>
<th>rA₂A-R (PC12) (nM)</th>
<th>hA₂A-R (CHO) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATL303</td>
<td>0.83 (0.15–4.47)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>CV-1808</td>
<td>53.8 (39.78–72.66)</td>
<td>949 (589–1,530)</td>
<td>76 (62–93)</td>
</tr>
<tr>
<td>NECA</td>
<td>74.8 (63.62–87.92)</td>
<td>160 (110–234)</td>
<td>66 (40–110)</td>
</tr>
<tr>
<td>2-CADO</td>
<td>208.1 (147.24–294.00)</td>
<td>879 (722–1,070)</td>
<td>164 (92–293)</td>
</tr>
<tr>
<td>CGS21680</td>
<td>243.6 (167.66–351.99)</td>
<td>298 (216–412)</td>
<td>221 (156–311)</td>
</tr>
</tbody>
</table>

Rank order values for the rat and human A₂A-R are taken from Kull et al. (1999). 95% confidence intervals for each mean value are denoted in parentheses.
selectivity, as is characteristic of other mammalian A2A receptors; (ii) equilibrium competition binding and cyclase data demonstrate a rank order of potency that is similar to that of other mammalian adenosine A2A receptors; (iii) the expressed receptor is functional as indicated by the activation of adenylate cyclase; and (iv) adenosine receptor activation results in a concentration-dependent inhibition of TNF-α-stimulated NF-κB activity. These results are encouraging for the further characterization of the equine A2A receptor as a molecular target for the treatment of equine endotoxia.

ACKNOWLEDGMENTS

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