Changes in the Biochemical Profiles of Mid-Cervically Located Adenosine A₁ Receptors After Repeated Theophylline Administration in Adult Rats

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Abstract

Background/Objective: Adenosine A₁ receptors localized in the phrenic motoneurons (PMNs), where the axons of the descending bulbospinal respiratory make synaptic contacts, may be involved in theophylline-induced respiratory-related activity in rats. The objective of this study was to characterize the biochemical profiles of adenosine A₁ receptors in 2 groups of rats: (a) naïve and (b) theophylline-treated (3-day oral administration).

Methods: Biochemical binding characteristics of adenosine A₁ receptors in the C3 to C5 (PMN) of adult rats were assessed in naïve (n = 6) and theophylline-treated animals (n = 6) using [³H]-DPCPX (10 pmol/L to 30 nmol/L), the specific adenosine A₁ receptor antagonist in saturation-binding assays. Competition assays used theophylline as the competing ligand (20 mmol/L to 20 pmol/L), and protein concentration was determined with the Bradford assay using a range of standards (0.016–1.0 mg/mL).

Results: In saturation-binding assays in naïve animals, the A₁ receptor was characterized by a single binding site with B_{max} and K_d values of 256.00 ± 32.13 fmol/mg protein and 2.89 ± 0.45 nmol/L, respectively. Analysis of the isotherm in theophylline-treated animals showed 1 site with B_{max} and K_d values of 219.00 ± 26.3 fmol/mg protein and 0.60 ± 0.21 nmol/L, respectively, and a second site characterized by B_{max} and K_d values of 492.6 ± 3.15 fmol/mg protein and 14.09 ± 2.06 nmol/L, respectively.

Conclusions: Theophylline administration revealed 2 binding sites on receptors (characterized by the specific adenosine A₁ antagonist, [³H]-DPCPX) located in the vicinity of phrenic motoneurons (C3–C5). Alteration of the receptor profiles after theophylline may underlie the respiratory-related actions of the drug.

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INTRODUCTION

Adenosine is a neuromodulator that is widely distributed in the central and peripheral nervous systems. Many of the actions of adenosine are mediated through receptors that are coupled with G proteins (1,2). Adenosine receptors have been classified into A₁, A_{2A}, A_{2B}, and A₃ subtypes (1,3). These receptors modulate many of the physiologic actions of adenosine. In the respiratory

system, adenosine receptors have been known to depress or excite activity depending on the site of action. When administered centrally, adenosine depresses respiration through activation of the A₁ receptor subtype (4–8). However, in the periphery, adenosine excites respiratory activity and may be involved in neurotransmitter release through A₂ receptor activation during hypoxia (9,10).

Theophylline is a methylxanthine used clinically in the treatment of asthma, chronic pulmonary obstructive disease (COPD), and premature infants with respiratory deficits. The exact mechanisms of action of the drug are yet to be elucidated. However, blockade of adenosine receptors (A₁ and A₂) is strongly implicated from basic and clinical studies (11–13).

Numerous studies on the biochemical effects of long-term theophylline use have reported changes in whole

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brain adenosine A₁ and A₂ receptors (14–16). Up- or down-regulation of brain adenosine A₁ receptors has been reported depending on the route of drug administration and the method of assessment (15–18). However, there is a paucity of studies that have examined the effect(s) of the drug on adenosine A₁ receptors located in the vicinity of phrenic motoneurons, a site of synaptic contact with the major descending bulbospinal respiratory pathway from the brainstem (19). This is an important issue that needs to be addressed in view of the clinical importance of theophylline in alleviating respiratory impairments, such as COPD, as treatment for apnea in preterm babies, and in patients with spinal cord injuries suffering from respiratory deficits (20–25).

We postulated that the biochemical basis for the actions of theophylline in respiratory activity may be related to its potential to alter the pharmacologic profiles of adenosine receptors, in particular the A₁ subtype. In this study, we tested the hypothesis that, after repeated administration for 3 days in adult rats, theophylline can alter the biochemical profiles of adenosine A₁ receptors. The hypothesis is predicated on the following: (a) that adenosine A₁ receptors are expressed in the phrenic motoneurons (where the major descending bulbospinal respiratory axons make synaptic contacts) and (b) the beneficial effects of theophylline in respiratory activity persist for prolonged periods after a 3-day treatment (13,14).

METHODS

Animal Preparation

Female Sprague-Dawley rats (retired breeders, 250–350 g) were used in this study. An experimental and a control group were used in the study. The experimental group of animals received theophylline orally (18-gauge gavage, Harvard Apparatus, Holliston, MA). A stock solution of theophylline 20 mg/mL in physiologic saline (0.9%) was prepared and administered (20 mg/kg) 3 times daily for 3 days in the experimental group. Control animals received only theophylline vehicle (0.9% saline) 3 times a day for 3 days. Theophylline and vehicle administration was carried out by gavage at 9:00 AM, 1:00 PM, and 5:00 PM. Control and experimental animals were decapitated 16 hours after the last (vehicle or theophylline) administration, respectively (ie, 9:00 AM the following day).

Experimental Protocol

After 3 days of oral administration of theophylline, animals in the experimental group (N = 6) were killed by decapitation, and the cervical cord levels (C3–C5) were removed and quickly stored at –80°C until ready for use. Similarly, control animals (N = 6) were decapitated, and the cord levels were removed and stored until ready for use.

Membrane Homogenate Preparation

For each group, cords were thawed, pooled, and homogenized in 35 mL of buffered 0.32 mol/L sucrose,

using 10 passes of a Polytron Homogenizer (Kinematica, Lucerne, Switzerland) at 3,800g. All procedures were carried out at 4°C unless otherwise indicated. After centrifugation (Sorvall Super, T21 Kendro Labs, Newton, CT) at 111g for 20 minutes, the pellet was discarded. The supernatant was centrifuged (19,712g for 20 minutes) in a Beckman L-40 centrifuge (Beckman Coulter, Inc., Palo Alto, CA), and the resulting pellet was suspended in 5 mmol/L Tris-HCl containing complete EDTA-free protease inhibitor (pH 7.4 at 4°C) using 5 passes of the Polytron Homogenizer. The resulting suspension was kept on ice for 10 minutes to maintain protein integrity and spun (once) at 19,712g for 20 minutes. The supernatant was discarded, and the resulting pellet was washed twice in 5 mmol/L Tris-HCl containing complete EDTA-free protease inhibitor (pH 7.4 at 4°C) at 19,712g for another 20 minutes. The supernatant was discarded, and the resulting pellet was washed twice in 50 mmol/L Tris HCl containing EDTA-free protease inhibitor (pH 7.4 at 4°C) and centrifuged at 7,884g for 15 minutes. The resulting pellet was resuspended in 30 mL 50 mmol/L Tris-HCl containing complete EDTA-free protease inhibitor (pH 7.4 at 4°C), measured out in 1-mL aliquots, and frozen at –80°C until protein concentration was determined.

Protein Assay

Membrane protein concentrations were determined using a 96-well format of the Bradford assay (26). Standard wells (bovine serum albumin standards at concentrations ranging from 0.016 to 1.0 mg/mL), sample wells (membrane samples), and blank wells (buffer 50 mmol/L Tris-HCl) containing complete EDTA-free protease inhibitor (pH 7.4 at 4°C) were all plated out in 5-μL volumes in triplicate. Three-milliliter aliquots of Bradford reagent were added, and the plate was allowed to incubate at room temperature for 10 minutes and counted at 590 nm using a Bio-Tek ELX-800 Spectrophotometer (Fisher Scientific, Chicago, IL).

Chemicals and Drugs

Cyclopentyl-1,3-dipropylxanthine, 8-[dipropyl-2,3-³H(N)]-([³H]-DPCPX) at 120.0 Ci/mmol and “Ultima Gold” scintillation cocktail were obtained from Perkin Elmer (Shelton, CT). Trizma hydrochloride (Tris-HCl), D(+)-sucrose, polyethylenimine (PEI), NaOH solution 1 mol/L, Bradford reagent, bovine serum albumin protein standards, and adenosine were all purchased from Sigma Aldrich (Atlanta, GA). Theophylline was purchased from Research Biochemicals International (Natick, MA). Complete EDTA-free protease inhibitor cocktail tablets were from Roche Applied Science (Indianapolis, IN). Physiological saline was obtained from NERL Diagnostics, (East Providence, RI).

Data Analysis

The calibration curve was fitted, and sample concentrations were calculated using a computer program (KC-Junior, Fisher Scientific, Chicago, IL).

Figure 1. Equilibrium saturation analysis of binding of 0.03 to 17.0 nmol/L [³H]-DPCPX to rat spinal cord (C3–C5) homogenate from naïve rats (▲) and theophylline-treated rats (■). Values represent the means of specific binding ($\pm$ SEM) of three experiments, each performed in triplicate. All values are corrected for NSB as defined with 100 μ mol/L adenosine. The assay was incubated for 1 hour at 4°C. The saturation isotherms were generated by computer-assisted analysis as described in the Materials and Methods section. In this experiment, the 1-site fit described data obtained from the naïve group. The 2-site model fit described data from the theophylline-treated group significantly better than the 1-site model ($F = 13.9$, $P < 0.001$).

Ligand Binding Assays

The specific binding of the adenosine A₁ receptor selective ligand [³H]-DPCPX to spinal cord membranes was determined using a rapid filtration assay (27). Spinal cord membranes were first incubated with adenosine deaminase (ADA; 5 U/mg protein) in 50 mmol/L Tris buffer and 2 mmol/L MgCl₂ at pH 7.4 to remove endogenous adenosine. Thereafter, the membrane suspension (at 0.1 mg/mL protein in a final volume of 500 μ L) was incubated for 1 hour at 4°C with [³H]-DPCPX in the absence of a competing compound. An optimal incubation period was established from preliminary experiments. The equilibrium binding reactions were terminated by vacuum filtration using a Brandel cell harvester (Gaithersburg, MD) and Whatman GF/B filters saturated with 0.5% PEI. Each filter was washed 4 times with 4 mL of cold 50 mmol/L Tris-HCl, pH 7.4. Filter discs were punched into vials using a Deposit/Dispenser (Brandel). Three-milliliter aliquots of “Ultima Gold” Scintillation Cocktail were added to each vial before scintillation counting. Filter-bound radioactivity was determined by liquid scintillation counting using a Tricarb-2800-TR Liquid Scintillation counter (Perkin Elmer). Nonspecific binding was defined in the presence of 100 μ mol/L adenosine. In saturation-binding experiments, [³H]-DPCPX was used (at 8–12 concentrations) from 10 pmol/L to 30 nmol/L. Competition studies used theophylline as the competing ligand at 10 concentrations (20 mmol/L to 20 pmol/L) against a standard concentration of [³H]-DPCPX (1 nmol/L).

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Data and Statistical Analyses of Binding Assay

Ligand-binding data were analyzed using nonlinear least-squares fitting in Prism (Graphpad Software, Inc, San Diego, CA). Saturation isotherms were analyzed by fitting 1-site and 2-site hyperbolic equations to the [³H]-DPCPX binding data. These fits determined the maximal binding ($B_{\max}$) and concentration of [³H]-DPCPX required to reach half-maximal binding (K_d).

Competition curves were analyzed by fitting 1-site and 2-site competition curves to the theophylline/[³H]-DPCPX binding data. These fits were used to determine the concentration of theophylline required to inhibit half-maximal binding of [³H]-DPCPX (IC_{50}). The 1-site and 2-site models were fit on each data set, and the simpler model was selected unless the P value was < 0.05 . The P value was determined using the extra sum-of-squares F test. Competition data were further analyzed by calculating the equilibrium dissociation constant (K_i) of theophylline using the equation of Cheng and Prusoff (28).

RESULTS

Adenosine A₁ Receptor Sites in Naïve and Theophylline-Treated Rats

The results of the binding characterization of [³H]-DPCPX sites in the C3 to C5 spinal cord using saturation studies for naïve and theophylline-treated animals are shown in Figure 1. The results depict receptor saturation isotherms using the concentrations (0.03–25 nmol/L) of the [³H]-DPCPX ligand used (Figure 1). Analysis of the data indicated that, in naïve animals, a single receptor binding site with $B_{\max}$ and K_d values of 256.00 ± 32.13 fmol/mg protein and 2.89 ± 0.45 nmol/L, respectively, was detected in the binding isotherm.

In contrast, analysis of the isotherm for the theophylline-treated group showed that it could be best fit into a 2-site model with distinct binding characteristics. The $B_{\max}$ for 1 of the 2 sites detected was 219.00 ± 26.31 fmol/mg protein, with a K_d of 0.60 ± 0.21 nmol/L. A second binding site characterized by a $B_{\max}$ of 492.6 ± 3.15 fmol/mg protein and a K_d of 14.09 ± 2.06 nmol/L was detected. The binding characteristics of the 2 apparent distinct sites are shown in Figure 2.

The results of competition/displacement studies of [³H]-DPCPX binding from spinal cord (C3–C5) using the adenosine receptor antagonist, theophylline, as the displacement ligand over various (10^{-12} – 10^{-3} mol/L) concentrations are shown in Figure 3. In Figure 3, the characteristic binding parameters of the naïve and theophylline-treated animals are more apparent, with the naïve group depicting a single binding site and the theophylline-treated group depicting a 2-site sigmoidal curve.

Figure 2. (A) Effect of theophylline on $B_{\max}$ of binding sites in naïve (N) and theophylline-treated (NT) rats is depicted in this histogram. Values represent the means of specific binding ($\pm$ SEM) of 3 experiments, each performed in triplicate * $P < 0.05$. (B) Effect of theophylline on K_d of binding sites in naïve (N) and theophylline-treated (NT) rats is shown. Values represent the means of specific binding ($\pm$ SEM) in 3 experiments, each performed in triplicate. * $P < 0.05$, ** $P < 0.01$.

Distribution of Receptor Sites

To assess the distribution of the apparent 2 receptor sites detected after theophylline administration, the second binding site was expressed as a percent of the total receptors detected. Similarly, the site that was detected

Figure 3. Displacement of 1 nmol/L [3]-DPCPX binding to rat spinal cord (C3–C5) homogenate from naïve rats (Δ) and theophylline-treated rats ($\blacksquare$) by 2.7 pmol/L to 27 mol/L theophylline. Values represent the mean specific binding as a percentage increase above basal binding ($\pm$ SEM) of at least 3 experiments, each performed in triplicate. All values are corrected for NSB defined with 100 μ mol/L adenosine. The assay was incubated for 1 hour at 4°C. The competition curves were generated by computer-assisted analysis as described in the Materials and Methods section. In this experiment, the 1-site fit described data obtained from the naïve group. The 2-site fit described data from the theophylline-treated group significantly better than the 1-site fit ($F = 1.31$, $P < 0.05$).

in both the naïve and the theophylline-treated animals was expressed as a percent of total receptors. In theophylline-treated animals, approximately 20% of the receptors (98.4 fmol/mg) detected were of the second type. The percent in the first type amounted to approximately 80% (393.6 fmol/mg; Figure 4).

DISCUSSION

The results of this study describe preliminary findings of the biochemical profiles of adenosine A_1 receptors located in cervical spinal cords in naïve and theophylline-treated rats. Under naïve conditions, the adenosine A_1 receptor is characterized by a single binding site with $B_{\max}$ and K_d values of 256.00 ± 32.13 fmol/mg protein and 2.89 ± 0.45 nmol/L, respectively. After administration of theophylline (for 3 days), the binding characteristics of the site(s) detected changed in 2 significant aspects: (a) theophylline treatment unmasked a second binding site (or alternatively, induced a conformational change in the receptor site) and (b) theophylline administration induced a significant decrease in K_d . The $B_{\max}$ and K_d for the unmasked site were 492.6 ± 3.15 fmol/mg protein and 14.09 ± 2.06 nmol/L.

The characterization of a single binding component for the adenosine A_1 receptor in the naïve cervical spinal cord supports and extends previous findings (29,30). The K_d value in our study (2.89 ± 0.45 nmol/L) is similar to

Figure 4. Histogram of the effect of theophylline on the apparent 2 binding sites (in the vicinity of the phrenic motor neurons) is shown. The respective percentage of the 2 sites detected in naïve (N) and treated (NT) rats is depicted. Values represent means of at least 3 experiments performed in triplicate ($\pm$ SEM). Theophylline administration unmasked another binding site that represented approximately 20% of the total detectable receptor population.

the values of 2.3 to 2.6 and 1.9 to 2.3 nmol/L reported elsewhere (29,30). However, the $B_{\max}$ values obtained in our study (256.00 ± 32.13 fmol/mg protein) are remarkably higher, probably because we used the specific adenosine A_1 receptor antagonist [3 H]-DPCPX as the ligand, whereas Geiger et al (29) used the adenosine A_1 agonist, [3 H]-CHA, and reported values of 176 and 122 fmol/mg protein for the dorsal and ventral spinal cords, respectively. Choca et al (30) similarly used [3 H]-CHA and reported values of 137 fmol/mg protein. It is generally accepted that the use of agonists in binding assays does not allow for a differentiation between a true increase in receptor number (up-regulation) and an increase in coupling to the G protein either as a consequence to the enhanced numbers or to G protein functionality. Thus, while antagonists bind to all receptors detectable, agonists tend to bind to receptors based on affinity states.

In a more recent study, Bantel et al (31) characterized adenosine receptors in the lumbar and thoracic levels in the rat spinal cord and also reported a single binding site for the A_1 receptor. However, the $B_{\max}$ reported in their study is significantly higher than that in this study (probably because of the areas in the cord that the investigators focused on), suggesting that there is a higher density of adenosine A_1 receptors in the lumbar and thoracic regions than in the cervical spinal cord.

The difference in receptor densities may in turn be related to function. It is thus likely that, under naïve or endogenous conditions, the adenosine A_1 receptor at the cervical, thoracic, and lumbar levels of the rat spinal cord is characterized by a single binding site. It must be

stressed, however, that the C3 to C5 regions of the spinal cord are functionally important for the respiratory system in as much as the major descending bulbospinal respiratory axons from the ventral respiratory group from the brainstem make synaptic connections with phrenic motoneurons located in C3 to C5 (19).

Theophylline is a methylxanthine that has been clinically used in respiratory compromise (20,21,23). Although the underlying mechanisms for its clinical benefits are not fully elucidated, adenosine receptors are strongly implicated (3,12,25). In previous studies on the effects of theophylline administration on adenosine receptors, up-regulation of adenosine A_1 receptors in various brain regions of the rat have been reported (15,16,32,33). However, other studies, in which the biochemical profiles of adenosine receptors were investigated with chronic theophylline or caffeine, reported no significant changes in adenosine A_1 receptors (34,35). The results presented in this study provide evidence that, after a 3-day administration of theophylline, significant changes (enhanced affinity and an apparent unmasking of a second binding site) in the profile of adenosine A_1 receptors in the cervical spinal cord region (C3–C5) occur. Although many studies of the effects of a chronic antagonist on receptors show an increase in total number as an adaptive response to the continued presence of the antagonist, our results on the $B_{\max}$ do not support this position; rather, they strongly suggest that a conformational change characterized by an apparent unmasking of another binding site occurs in the C3 to C5 areas of the spinal cord. This is an area where adenosine A_1 receptors, thought to be involved in respiratory activity, are localized (14).

CONCLUSION

In summary, the results of this study show that theophylline administration (compared with vehicle administration) alters biochemical profiles of the adenosine A_1 receptors from a single binding site to a 2-site model. Changes in the A_1 receptor numbers after chronic drug administration have been reported; however, our results with the $B_{\max}$ tend to support a postulate by Georgiev et al (36) that an increase in number would not be expected to manifest itself in vivo in the continued presence of an antagonist such as theophylline. In our study, we provided evidence that theophylline administration unmasks a second binding site and thus changes the distribution of the A_1 receptors into 2 populations. We therefore speculate that the changes in the profile of the receptor may be, at least in part, responsible for the stimulatory effect of theophylline on respiration in the mammalian system. Conversely, altered adenosine receptor structure/function may be implicated in respiratory dysfunction. This is not farfetched in light of a recent study showing that alterations in adenosine receptor numbers and affinity may underlie COPD (37).

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