Antagonism of adenosine receptors by caffeine and caffeine metabolites in equine forebrain tissues

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Objective—To determine the presence of adenosine receptor subtypes A_1 and A_{2a} in equine forebrain tissues and to characterize the interactions of caffeine and its metabolites with adenosine receptors in the CNS of horses.

Sample Population—Brain tissue specimens obtained during necropsy from 5 adult male research horses.

Procedure—Membrane-enriched homogenates from cerebral cortex and striatum were evaluated by radioligand binding assays with the A_1-selective ligand [^{3}H]DPCPX and the A_{2a}-selective ligand [^{3}H]ZM241385. Functional responses to adenosine receptor agonists and antagonists were determined by a nucleotide exchange assay using [^{35}S]-guanosine 5'-(γ-thio) triphosphate ([^{35}S]GTPγS).

Results—Saturable high affinity [^{3}H]DPCPX binding (A_1) sites were detected in cerebral cortex and striatum, whereas high-affinity [^{3}H]ZM241385 binding (A_{2a}) sites were detected only in striatum. Caffeine and related methylxanthines had similar binding affinities at A_1 and A_{2a} sites with rank orders of drug binding affinities (theophylline > paraxanthine ≥ caffeine >> theobromine) similar to other species. [^{35}S]GTPγS exchange revealed that caffeine and its metabolites act as pure adenosine receptor antagonists at concentrations that correspond to A_1 and A_{2a} receptor binding affinities.

Conclusions and Clinical Relevance—Results of our study affirm the presence of guanine nucleotide binding protein linked adenosine receptors (ie, high-affinity A_1 and A_{2a} adenosine receptors) in equine forebrain tissues and reveal the antagonistic actions by caffeine and several biologically active caffeine metabolites. Antagonism of adenosine actions in the equine CNS by these stimulants may be responsible for some central actions of methylxanthine drugs, including motor stimulation and enhanced racing performance. (*Am J Vet Res* 2003;64:216–224)

Caffeine and related plant alkaloids of the methylxanthine family are present as natural constituents or additives in numerous human foods and beverages and are commonly detected as minor contaminants in domestic animal feeds.[1] In view of the widespread prevalence of methylxanthine-related compounds in everyday products, many domestic species are likely to ingest or otherwise be exposed to these agents and thereby may contain detectable drug concentrations in tissues or body fluids. Caffeine is the archetype agent for the methylxanthine class and is considered to produce the broadest range of pharmacologic actions, including bronchial, cardiovascular, respiratory, and CNS stimulatory effects. Because the results of several studies[2-4] indicate that caffeine can delay the onset of fatigue and increase the capacity for work during prolonged exercise, caffeine use is monitored closely or, in some instances, banned by agencies that oversee human and nonhuman athletic competitions. Caffeine has been classified as a class 2 agent in racehorses by the Association of Racing Commissioners International, with no acceptable amount permitted.

Although methylxanthine-related agents have distinct pharmacokinetic properties and elicit a unique spectrum of pharmacologic actions, in general, these drugs are considered to interact with a common set of molecular or cellular targets. Three predominant mechanisms have been described for methylxanthine action, including mobilization of intracellular calcium, inhibition of intracellular phosphodiesterase enzymes, and antagonism of cellular adenosine receptors.[5,6] Although the results of several studies[7-10] have revealed an association between selected behavioral or cardiovascular effects and the 2 former mechanisms of action, most of the effects of caffeine appear to derive from adenosine receptor blockade.[11] Within the CNS, a link exists between caffeine-induced psychomotor stimulation and competitive blockade of adenosine receptors in selected forebrain pathways. In light of such findings, studies of methylxanthine-induced behavioral changes have focused primarily on the roles of CNS adenosine receptors.

Molecular cloning studies in mammalian tissues have uncovered 4 major classes of adenosine receptors that are designated A_1, A_{2a}, A_{2b}, and A_3.[12] Results of complimentary pharmacologic studies[13,14] have confirmed the presence of distinct adenosine receptor subtypes in many tissues and have revealed striking differences in the drug binding properties among these receptors.One interesting and potentially important observation is the low affinity of A_{2b} and A_3 receptors for the endogenous agonist adenosine. This observation may be relevant to the action of methylxanthine drugs and other adenosine receptor antagonists, because under normal conditions, it appears that extracellular adenosine concentrations are insufficient to activate A_{2b} and A_3 receptors. In view of this, A_1 and A_{2a} receptor subtypes are considered the primary mediators of methylxanthine drug actions in most tissues.[11,13]

Received July 1, 2002.
Accepted September 6, 2002.
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Funded by a grant from the State of Florida Pari-Mutuel Wagering Trust Fund.
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Previous reports[6,13-15] have described the binding affinities and pharmacologic actions of caffeine and its congeners at A_1 and A_{2a} receptors in CNS tissues from several species, including rats, mice, guinea-pigs, cows, and humans. However, similar studies of adenosine receptors and methylxanthine drug actions have not been reported for equine forebrain tissues. Nevertheless, in view of concerns over nonmedical uses of caffeine and related agents as possible performance-enhancing drugs in racing horses, our investigation was undertaken to delineate the nature of methylxanthine drug interactions with equine CNS adenosine receptors.

Materials and Methods

Tissue preparation—Brains were obtained from adult male horses (Thoroughbreds and Quarter Horses; age range, 3 to 8 years old) that were euthanatized by barbiturate overdose[a] by personnel of the Anatomic Pathology Service at the University of Florida, College of Veterinary Medicine. None of the horses were afflicted with any known CNS disease or dysfunction. Following removal, brains were packed in ice, and samples of cerebral cortex and striatum were dissected, weighed, and homogenized in 30 volumes of ice-cold Tris-HCl buffer (50mM, pH 7.4) with a tissue homogenizer[b] at setting 4 for two 15-second bursts. Tissue homogenates were centrifuged immediately at 48,000 × *g* for 15 minutes at 4°C, pellets were washed twice by removal of the supernatant resuspended in fresh cold buffer (30 volumes), and tissues were recollected by centrifugation. Washed tissue pellets were homogenized in 5 volumes of fresh buffer and stored at –80°C until use for radioligand binding or nucleotide exchange assays. Samples were analyzed for total protein according to the Lowry method.

Radioligand binding assays—Radioligand binding experiments were performed by use of 8-[dipropyl-2,3-^{3}H(N)]-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX, 111.6 Ci/mmol)[c] or [2-^{3}H](4-(2-[7-amino-2 (2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol) ([^{3}H]ZM241385, 17 Ci/mmol)[d] to label A_1 and A_{2a} adenosine receptors, respectively. For determination of A_1 receptor binding, tissue homogenates were thawed and diluted in cold Tris-HCl buffer (50mM) to a final concentration of 30 to 40 µg protein/mL (cortex) or 40 to 50 µg protein/mL (striatum). Binding reactions (0.5mL total volume) were performed in Tris-HCl (50mM, pH 7.4) buffer containing adenosine deaminase[e] (0.1 U/mL) at 25°C for 15 minutes. The binding reaction was initiated by addition of [^{3}H]DPCPX (0.05 to 10nM final concentration) and terminated by rapid separation of tissue-bound radioligand by vacuum filtration onto glass fiber filter strips[f] with a cell harvester.[g] Filters were rinsed 3 times with 2 mL of ice-cold buffer, transferred to scintillation vials, and tissue was digested in 2 mL of NaOH (0.2 N). Samples were acidified with excess HCl (0.5 N). and tritium content was determined by liquid scintillation counting[h] using a commercial scintillation fluid.[i] Total and nonspecific radioligand binding was measured in duplicate tubes in the presence of vehicle (1% dimethyl sulfoxide) or the nonxanthine adenosine receptor antagonist 9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo[1,5]quinazolin-5-imine monomethane sulfonate[j] (CGS15943, 5µM final concentration), respectively. Specific binding was calculated as the arithmetic difference between total and nonspecific binding and represented > 92% of the bound radioactivity. For competition binding experiments, membrane suspensions were incubated with a fixed concentration of [^{3}H]DPCPX (1nM, final concentration) and 9 concentrations of each methylxanthine agent.

Estimation of A_{2a} receptor binding in equine forebrain membranes was performed using the A_{2a}-selective ligand [^{3}H]ZM241385. Binding reactions (0.5 mL final volume) were performed in Tris-HCl buffer (50mM; pH 7.4) containing NaCl (120mM), KCl (5mM), adenosine deaminase (1 U/mL), and membrane protein (300 to 500 µg) for 30 minutes at 25°C. The binding assay was initiated by addition of [^{3}H]ZM241385 (0.05 to 20nM final concentration) and terminated as described for [^{3}H]DPCPX binding, except for the substitution of cold Tris-NaCl-KCl buffer as the rinse buffer. For competition binding experiments, membrane suspensions were incubated with a fixed concentration of [^{3}H]ZM241385 (3nM, final concentration) and 9 concentrations of each methylxanthine agent. Nonspecific binding was determined in the presence of CGS15943 (5µM in 1% dimethyl sulfoxide) and used to estimate specific radioligand binding as already described.

Guanine nucleotide exchange assay—Adenosine receptor regulated exchange of membrane-bound guanine nucleotides was determined in the presence of the nonhydrolyzable analog guanosine-5'-O-(3-[^{35}S]thiotriphosphate)-tetralithium ([^{35}S]GTPγS, 1,250 Ci/mmol)[c] according to a modification of a published technique.[16] Membrane preparations from cerebral cortex (approx 70 µg protein/assay) and striatum (approx 50 µg protein/assay) were incubated for 20 minutes at 37°C to eliminate endogenous adenosine. Immediately thereafter, membrane homogenates were diluted in Tris-HCl (50mM, pH 7.4) containing NaCl (80mM), $MgCl_2$ (4mM), EDTA (1mM), dithiothreitol (1mM), guanosine diphosphate (10µM), bovine serum albumin (0.5%, wt/vol), [^{35}S]GTPγS (0.2nM), and adenosine (0.2nM to 15µM) in a final volume of 300 µL. Reaction mixtures were incubated for 30 minutes at 25°C, and tissue-bound [^{35}S]GTPγS was separated by rapid filtration and rinsing as already described for [^{3}H]DPCPX binding experiments. Nonspecific binding was determined in the presence of excess nonradioactive GTPγS[k] (20µM). To assess the efficacy and potency of adenosine receptor ligands, each drug was tested at multiple concentrations alone and in the presence of adenosine (4µM) to test for possible agonist and antagonist actions, respectively. Data from [^{35}S]GTPγS exchange experiments were calculated as the percent of maximal drug-induced stimulation of [^{35}S]GTPγS binding relative to the maximal effect by adenosine.

Data analysis—In each experiment, all assays were performed in duplicate tubes, and experiments were repeated over 4 to 5 separate runs. Unless specified otherwise, all results are expressed as arithmetic means ± 1 SEM. For saturation binding experiments, values of specific radioligand binding were transformed with a computer-assisted curve fitting algorithm[l] into standard Scatchard plots (bound vs bound/free) and used to determine **dissociation binding constants (K_D)** and **maximum binding capacities (B_{MAX})** for each radioligand. For competition binding experiments, transformed data were fitted to a 4-parameter logistic equation by weighted nonlinear regression curve fitting[l] and used to determine values of IC_{50} (drug concentration that inhibits 50% of ligand binding). **Inhibition binding constants (K_I)** were derived from IC_{50} values according to the relationship described previously.[17] Values of K_D and K_I are reported as geometric means in view of the log-normal distribution of these parameters.[18] Statistical comparisons of drug binding data were carried out using a Student's t-test or 1-way ANOVA. A value of $P < 0.05$ was considered significant.

Results

Binding properties of forebrain A_1 and A_{2a} adenosine receptors—Receptor binding studies were per-

formed in washed homogenates from equine cerebral cortex and striatum with the A_1-selective radioligand [^{3}H]DPCPX and the A_{2a}-selective radioligand [^{3}H]ZM241385. Prior to undertaking binding assays in equine forebrain tissues, preliminary studies were conducted to identify and optimize appropriate assay conditions (buffer, temperature, incubation time, etc) that yielded equilibrium binding of both radioligands in tissue homogenates. Specific binding of both ligands was determined to be directly proportional to protein concentration over an 8- to 10-fold range of total tissue protein (data not shown), and binding was unaffected following storage of washed tissue homogenates for up to several weeks at –20°C. Specific binding for each

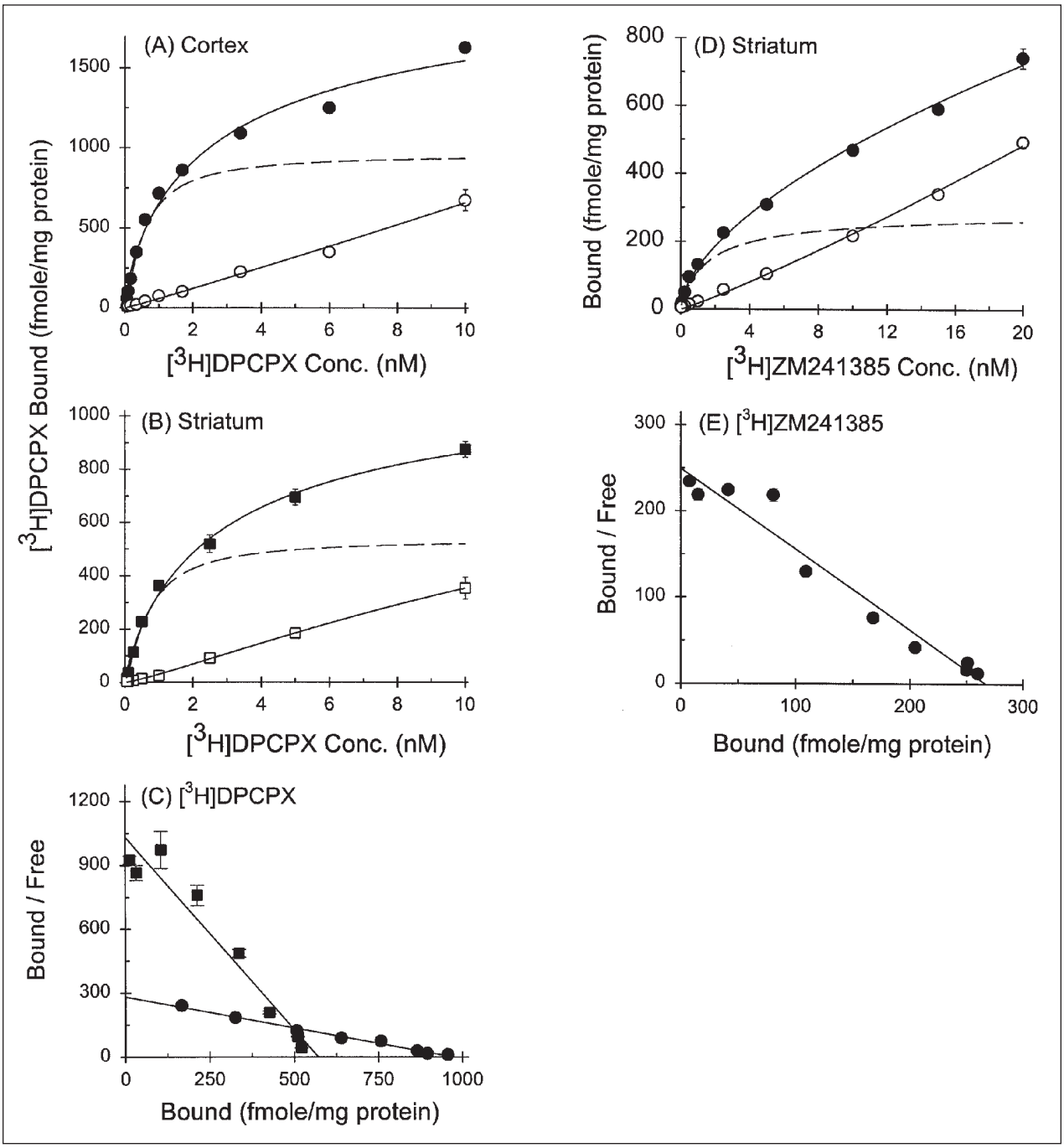

Figure 1—Saturation binding and Scatchard plots for [^{3}H]DPCPX and [^{3}H]ZM241385 in equine forebrain homogenates. Data are plotted as arithmetic means ± SEM. Panels A and B—[^{3}H]DPCPX (0.05 to 10nM) nonspecific (open symbols) and total (closed symbols) binding in the cortex and striatum. Fitted curves for specific [^{3}H]DPCPX binding (dashed lines) were obtained by a least-squares nonlinear algorithm. Panel C—Scatchard analysis of specific high-affinity [^{3}H]DPCPX binding revealed a single population of binding sites in the cerebral cortex (circles) and striatum (squares) with correlation coefficients for linear regressions of 0.98 and 0.92, respectively. Panel D—Total (closed circles), nonspecific (open circles), and specific (dashed line) binding of [^{3}H]ZM241385 (0.05 to 20nM) to equine striatal membranes. Panel E—Scatchard analysis indicated a homogenous population of high affinity [^{3}H]ZM241385 binding sites in striatal homogenates with a correlation coefficient for the regression line of 0.94.

radioligand achieved a rapid equilibrium (< 10 minutes for [^{3}H]DPCPX and < 20 minutes for [^{3}H]ZM241385) and, under the specified assay conditions, remained stable for > 2 hours. Saturation binding experiments were conducted with both ligands in cerebral cortical and striatal membranes (**Fig 1**). For the A_1-selective ligand [^{3}H]DPCPX, saturable high-affinity specific binding sites were detected in the cerebral cortex and striatum. Scatchard analysis of specific binding data revealed that cerebral cortical homogenates contained a significantly higher density of [^{3}H]DPCPX binding sites than striatal homogenates with apparent B_{MAX} values of 972 ± 24 and 580 ± 15 fmol/mg protein, respectively. In contrast, the equilibrium K_D values derived from Scatchard plots revealed a 2-fold higher binding affinity for [^{3}H]DPCPX in striatum (0.29nM), compared with cerebral cortex (0.58nM). For both tissues, the linearity of bound versus bound/free plots was consistent with the presence of a single homogenous population of noninteracting A_1 adenosine receptor binding sites.

A parallel set of experiments was performed with the A_{2a}-selective radioligand [^{3}H]ZM241385, although no results were presented for cerebral cortical homogenates because little or no specific binding could be detected (data not shown). For striatal tissues, [^{3}H]ZM241385 bound to a finite population of high-affinity binding sites (Fig 1) with an apparent K_D of 0.9nM. Scatchard analysis unveiled a linear binding relationship with an apparent density of striatal [^{3}H]ZM241385 binding sites (B_{MAX}, 274 ± 19 fmol/mg protein) that was markedly lower than the density of A_1 binding sites in the cortex or striatum. The presence of high-affinity binding sites for [^{3}H]ZM241385 and [^{3}H]DPCPX in striatal tissue, coupled with the near absence of [^{3}H]ZM241385 binding sites from the cerebral cortex, provided an opportunity for additional investigations of A_1 and A_{2a} receptor sites within these tissues. By using the unlabeled form of the A_{2a}-selective ligand ZM241385, binding sites labeled with [^{3}H]DPCPX in cortex and striatum, as well as striatal sites labeled with [^{3}H]ZM241385, were compared with respect to the K_I for this highly selective compound. ZM241385 inhibited striatal [^{3}H]ZM241385 binding with high affinity (apparent K_I, 0.48nM; **Fig 2**), a finding that agrees reasonably well with the K_d obtained from direct saturation binding analysis. In contrast, when tested against [^{3}H]DPCPX-labeled tissues, ZM241385 had nearly 600-fold lower affinity in the cerebral cortex (apparent K_I, 260nM) and striatum (apparent K_I, 275nM). In all cases, the pseudo-Hill slope values were close to unity, thereby lending support to the view that homogenous but separate populations of membrane sites are labeled by [^{3}H]DPCPX and [^{3}H]ZM241385 in equine forebrain tissues. The high selectivity of ZM241385 in our experiments agrees closely with results reported previously[19-22] for other animal species and indicates that binding sites identified here represent pharmacologically distinct adenosine receptor populations.

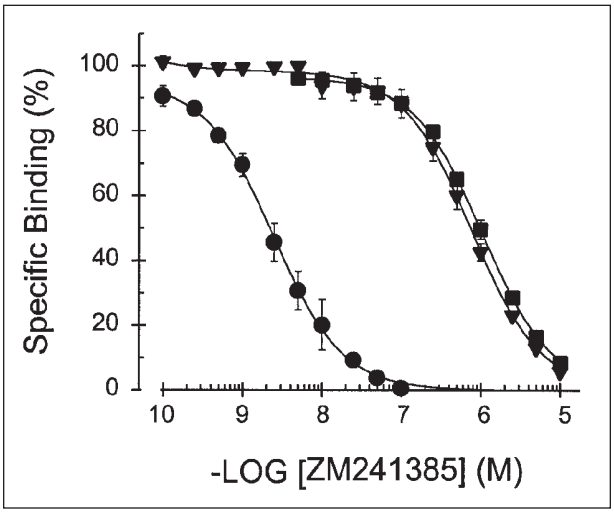

Figure 2—Selective interaction of ZM241385 with putative A_{2a} adenosine receptor binding sites in equine forebrain. Nonradioactive ZM241385 (0.1nM to 10mμM) was tested for inhibition of [^{3}H]ZM241385 binding to striatal membranes (circles) and [^{3}H]DPCPX binding to striatal (squares) and cortical membranes (triangles). Results are plotted as mean ± SEM of duplicate measurements from 3 independent experiments. Hill values for ZM241385 were 0.99, 0.98, and 0.98 for inhibition of [^{3}H]ZM241385 and [^{3}H]DPCPX in striatum and cortex, respectively.

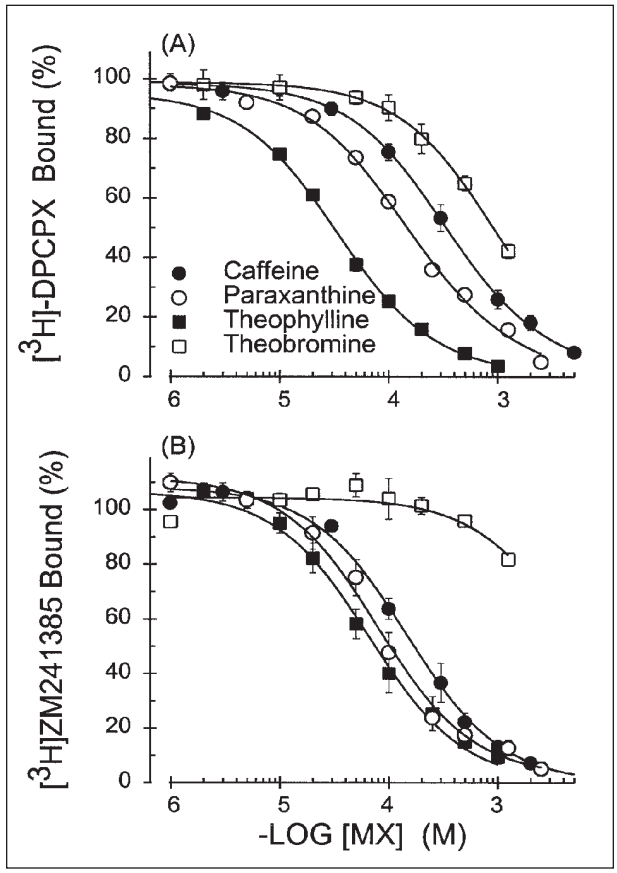

Figure 3—Competitive inhibition of A_1 and A_{2a} adenosine receptor binding in equine forebrain by selected methylxanthine (MX) agents. Membrane homogenates from the cerebral cortices (panel A) and striata (panel B) from horse forebrains were labeled with [^{3}H]DPCPX (1nM) and [^{3}H]ZM241385 (3nM), respectively. Competing drugs were tested in duplicate tubes at 8 to 9 concentrations, and results are plotted as mean ± SEM from 3 to 4 independent experiments. Best-fit curves were generated by use of a 4-parameter logistic equation.

Binding of methylxanthine drugs to equine adenosine receptors—Competitive radioligand binding experiments were used to determine the receptor binding affinities of 4 methylxanthine derivatives (caffeine, theophylline, theobromine, and paraxanthine) at A_1 and A_{2a} adenosine receptors in the cerebral cortex and striatum, respectively. All 4 compounds produced concentration-dependent displacement of radioligand from cortical A_1 ([^{3}H]DPCPX-labeled) and striatal A_{2a} ([^{3}H]ZM241385-labeled) adenosine receptors (**Fig 3 and 4**). The rank order for drug potencies appeared to be similar in both tissues (ie, theophylline > paraxan-

Table 1—Estimates of binding constants for selected methylxanthine drugs at [^{3}H]DPCPX-labeled sites in equine cortex and [^{3}H]ZM241385-labeled sites in equine striatum

	[^{3}H]DPCPX (cortical A_1 sites)		[^{3}H]ZM241385 (striatal A_{2a} sites)		
Agents	**IC_{50}***	**K_i†**	**IC_{50}***	**K_i†**	**A_1/A_{2a} selectivity**
Caffeine	344 ± 44	77 (71–84)	178 ± 28	38 (31–49)	2.0
Theophylline	31 ± 2	7 (6–9)	76 ± 18	16 (10–31)	2.3
Theobromine	934 ± 98	209 (193–258)	> 10,000	ND	ND
Paraxanthine	153 ± 5	35 (32–39)	104 ± 13	22 (17–29)	1.6

*IC_{50} values are mean (± SEM) values from 3 to 4 experiments. †Estimates of K_i values are derived from IC_{50} values according to the Cheng-Prusoff relationship[17] and are reported as geometric means with 95% confidence limits shown in parentheses. *†K_i and IC_{50} values are given in micromolar units.

ND = Not determined. IC_{50} = Drug concentration that inhibits 50% of ligand binding. K_i = Inhibition binding constant.

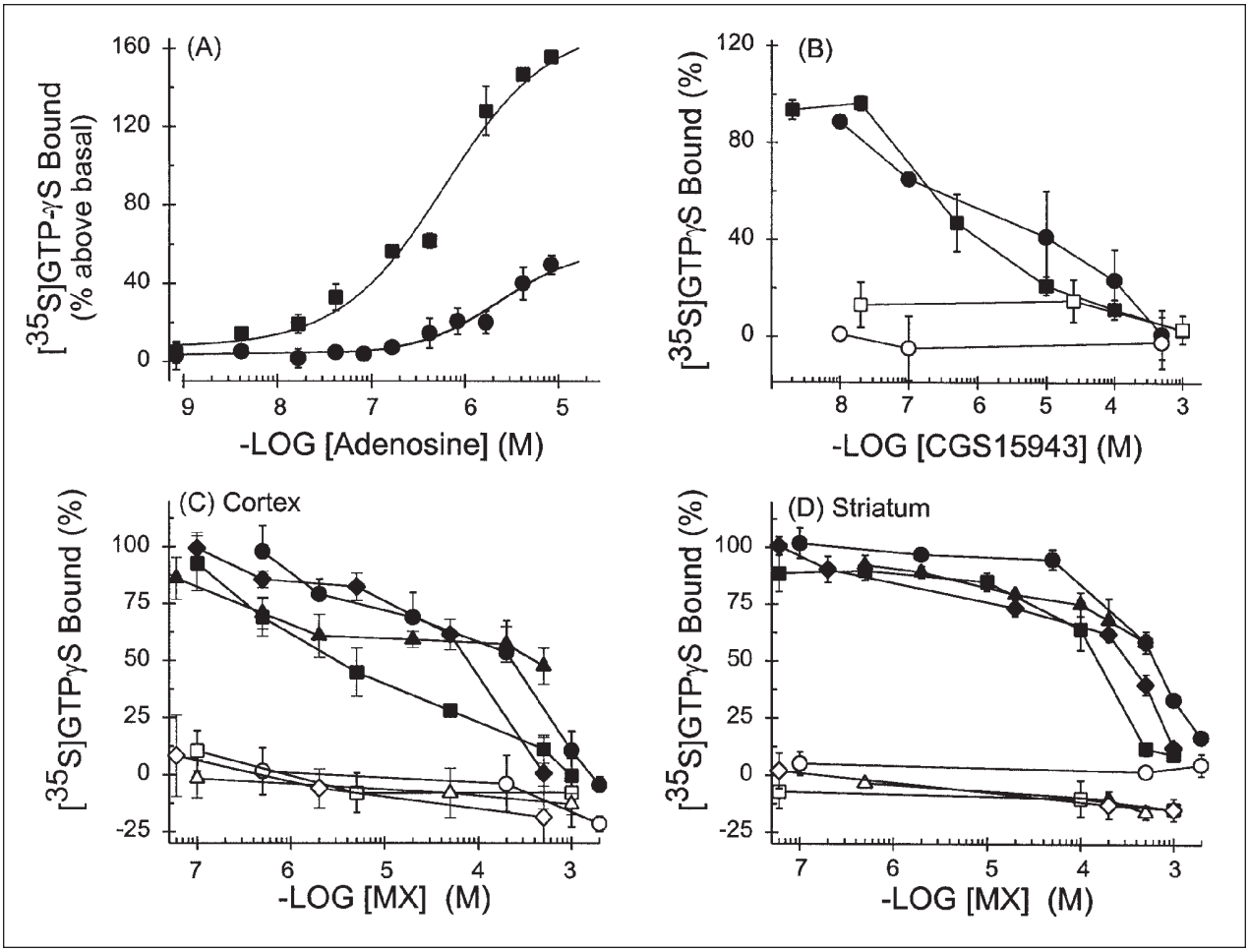

Figure 4—Adenosine receptor regulation of guanine nucleotide exchange in equine forebrain membranes. Data are expressed as mean ± SEM from 3 to 4 independent experiments. Nonspecific binding was determined in the presence of 20μM GTPγS. Panel A—Membrane homogenates from cerebral cortex (circles) and striatum (squares) were incubated with adenosine (0.2nM to 15μM) and the radiolabeled guanine nucleotide [^{35}S]GTPγS (0.2nM). Results are expressed as the percent increase in [^{35}S]GTPγS binding relative to adenosine-free controls. Panel B—Effect by CGS15943 on adenosine-stimulated [^{35}S]GTPγS binding in cerebral cortex (circles) and striatum (squares). Multiple concentrations of CGS15943 were tested alone (open symbols) and in the presence of 4μM adenosine (closed symbols). Panel C and D—Antagonistic effects by selected methylxanthine agents (caffeine, circles; theophylline, squares; theobromine, triangles; paraxanthine, diamonds) in the cerebral cortex and striatum. Each agent was tested at several concentrations alone (open symbols) and in the presence of 4μM adenosine (closed symbols).

thine ≥ caffeine >> theobromine), with theobromine having the lowest apparent affinity and causing incomplete inhibition at the highest concentration (1.5mM) that was tested. Quantitative analysis of fitted inhibition curves revealed that theophylline had the highest affinity among the methylxanthine compounds with apparent K_I values of 7 and 16μM at A_1 and A_{2a} binding sites, respectively (**Table 1**). With the possible exception of theobromine, none of the methylxanthine agents appeared to have any substantial selectivity toward A_1 or A_{2a} receptors, although caffeine and paraxanthine had modest (approx 2-fold) preference for A_{2a} receptors, and theophylline had an equally modest preference for A_1 receptors.

Adenosine receptor-regulated guanine nucleotide exchange—Previous studies[23,24] have established that drug binding to **guanine nucleotide binding protein (G protein)**-coupled adenosine receptors can stimulate the exchange of GTP or selected analogs that are bound to the receptor-G protein complex. In view of this, we used [^{35}S]GTPγS, a metabolically stable radiolabeled analog of GTP, to delineate the nature of selected drug actions on adenosine receptors in equine forebrain tissues (Fig 4). Under the conditions already described, the natural agonist adenosine stimulated [^{35}S]GTPγS exchange in homogenates of the cerebral cortex and striatum in a potent and concentration-dependent manner. Significant increases in [^{35}S]GTPγS exchange were detected at submicromolar concentrations (as low as 20nM adenosine), and maximal stimulation in both tissues occurred at 10μM adenosine. Fitting of data to a logistic equation revealed modest differences in the potency and maximal effect by adenosine in these tissues. Specifically, adenosine produced an approximately 3-fold significantly greater stimulatory effect in the striatum (158 ± 14%), compared with the cerebral cortex (53 ± 9%). In addition, exogenous adenosine was slightly more potent in striatum as indicated by significant differences in the concentrations that produced a half-maximal (50%) effect (EC_{50}) in striatum (0.4μM) versus the cerebral cortex (1.6μM). Parallel studies with the nonxanthine adenosine receptor antagonist CGS15943 revealed no significant changes in [^{35}S]GTPγS exchange when this agent was tested alone at concentrations up to 1mM. However, CGS15943 caused a concentration-dependent and total inhibition of adenosine-stimulated [^{35}S]GTPS binding in both tissues with estimated IC_{50} values of 0.4μM (striatum) and 1.6μM (cerebral cortex). On the basis of these results, [^{35}S]GTPγS exchange was used to delineate the intrinsic activities of methylxanthine-related agents at adenosine receptors in equine striatum and cerebral cortex. Caffeine, theophylline, theobromine, and paraxanthine acted as pure adenosine receptor antagonists in the cerebral cortex and striatum insofar as all 4 agents caused complete inhibition of adenosine (4μM)-stimulated [^{35}S]GTPγS exchange while causing little or no stimulatory effect when tested alone. Quantitative analysis of the inhibitory effects by these drugs revealed that, in general, the rank orders of drug potencies were similar to results obtained from binding studies (Table 1; Fig 3), although no direct relationship was established between drug receptor binding and [^{35}S]GTPγS exchange.

Discussion

The purposes of our study were to demonstrate the existence of specific high-affinity adenosine receptors in equine forebrain tissues and to evaluate possible actions by caffeine and related methylxanthine agents on these important CNS drug targets. To achieve this goal, we used 2 conventional pharmacologic approaches, radioligand binding and guanine nucleotide exchange assays, as a means to characterize the drug binding properties and pharmacologic effects on selected adenosine receptor subtypes. The results from our study provide the first direct evidence that caffeine and related compounds act as competitive antagonists at central equine adenosine receptors and, furthermore, indicate that the cellular actions of methylxanthine drugs in horses have striking similarities to actions reported in other species, including humans.

To identify and study putative adenosine receptors in equine brain tissues, radioligand-binding techniques were developed by use of 2 high-affinity ligands that have been used previously in other animal species. Putative A_1 adenosine-receptor binding sites were studied with [^{3}H]DPCPX, a high-affinity ligand for A_1 receptors that binds with > 500-fold selectivity at A_1 versus A_{2a} receptors in rat tissues.[15,25,26] In equine forebrain tissues, [^{3}H]DPCPX binds with high affinity to homogenous populations of membrane binding sites in the cerebral cortex and striatum with affinity constants (apparent K_D values of 0.58nM and 0.29nM for cortex and striatum, respectively; Fig 1) that are within the range of K_D values reported for brain membranes from other species, including sheep (0.2nM), cows (0.2nM), rats (0.5nM), hamsters (0.5nM), rabbits (0.7nM), pigs (0.8nM), and guinea pigs (2.1nM).[27] The higher density of [^{3}H]DPCPX binding sites in equine cerebral cortex relative to striatum parallels regional distribution patterns for A_1 receptors in other species,[15,25,27] although the absolute density of A_1 binding sites is substantially higher in the striatum of horses relative to most species. Taken together, our results for [^{3}H]DPCPX binding in equine brain tissues are consistent with data reported for other species and provide strong support for the presence of specific high-affinity A_1 adenosine receptors in the equine CNS.

Similar experiments were performed by use of the nonxanthine adenosine antagonist [^{3}H]ZM241385 to identify possible A_{2a} adenosine receptors in equine CNS tissues. On the basis of the results of previous functional studies,[19,20] as well as investigations using tissues or cell lines derived from rats, guinea pigs, dogs, and humans,[19-22] [^{3}H]ZM241385 is considered to be 1 of the most selective, high-affinity ligands available for labeling A_{2a} adenosine receptors. [^{3}H]ZM241385 labeled a homogenous population of binding sites in equine striatal membranes that had a binding affinity (apparent K_D, 0.9nM) comparable to that reported for other species (Fig 1).[21,22] The large excess of [^{3}H]ZM241385 binding sites in horse stria-

tum relative to cerebral cortex (undetectable in most samples) also parallels the reported distribution of A_{2a} adenosine receptors in the forebrains of other mammalian species, although low densities of A_{2a} binding sites have been detected within rat cerebral cortex.[28-30] On the basis of these findings, we conclude that [^{3}H]ZM241385 is a suitable radioligand for labeling high-affinity A_{2a} adenosine receptors in the equine CNS. Nevertheless, in view of the coexistence of substantial amounts of A_1 and A_{2a} adenosine receptors in equine striatal tissues (B_{MAX} values of 580 ± 15 and 274 ± 19 fmol/mg protein, respectively), additional experiments were performed to differentiate the drug-binding properties of these putative receptor subtypes. Under identical experimental conditions, unlabeled ZM241385 was found to inhibit binding of both radioligands in striatum, as well as [^{3}H]DPCPX in cerebral cortex. However, ZM241385 had nearly 600-fold higher affinity for A_{2a} sites, compared with A_1 sites labeled with [^{3}H]DPCPX in striatal and cortical membranes (Fig 2). On the basis of this marked selectivity by a well-recognized A_{2a}-selective adenosine receptor antagonist, we conclude that ZM241385 represents a suitable pharmacologic tool for A_{2a} receptor binding studies in equine tissues and could easily provide a useful probe for distinction between A_{2a} and A_1 adenosine receptor-mediated effects in horses.

Interactions of caffeine and related methylxanthine agents with equine CNS adenosine receptors were characterized by use of competitive ligand binding and a functional second messenger-based assay. In tests for potential drug interactions with A_1 adenosine receptors ([^{3}H]DPCPX binding in cerebral cortex) and A_{2a} adenosine receptors ([^{3}H]ZM241385 binding in striatum), all 4 methylxanthine drugs had strong inhibitory actions with all agents except theobromine, causing complete inhibition of ligand binding. For both tissues, a similar rank order potency was obtained with a ranking of theophylline > paraxanthine ≥ caffeine >> theobromine (Fig 3). Although the absolute values for drug-inhibition constants (K_I values) differed slightly for each drug between horses (Table 1) and other species, overall these agents had no substantial selectivity between A_1 and A_{2a} adenosine receptor subtypes in the equine forebrain. When compared with similar studies[19,21,25,27,28] in other species and tissues, including rats, cows, and humans, our results reveal remarkable concordance with drug inhibition constants and rank order potencies for the methylxanthine drugs across several mammalian species. However, some discrepancies were observed between our results and results from several older studies[31,32] that used agonist radioligands or ligands with poor binding selectivity profiles for A_1 or A_{2a} adenosine receptor subtypes. Despite these minor differences, we conclude that caffeine and 2 of its active metabolites (theophylline and paraxanthine) bind with high affinity at adenosine receptors in equine forebrain tissue and, therefore, are likely to exert substantial pharmacologic actions in the equine CNS.

Although radioligand-binding approaches can be used to determine important quantitative measures of drug-receptor interactions, it is often impossible to ascertain the functional consequences of receptor occupancy (ie, agonism vs antagonism) through the use of radioligand binding studies alone. Therefore, to determine the intrinsic activities of methylxanthine drugs at equine forebrain adenosine receptors, experiments on function were undertaken on the basis of the presumed coupling of these receptors with functional G proteins. Studies[33,34] in other species have established that adenosine receptors are coupled to G proteins through which intracellular signaling pathways may be regulated. For example, A_1, A_{2a}, and A_{2b} adenosine receptors are likely to associate selectively with distinct families of G proteins and either inhibit (A_1) or stimulate (A_{2a} and A_{2b}) adenylate cyclase activity.[13,35,36] Although previous studies[35] have revealed the utility of monitoring adenosine receptor-regulated cyclic AMP production as a means to characterize drug-receptor interactions, such measures can be limited by the need for tissues that are fresh and contain intact viable cells. In light of these and other limitations, we used adenosine-regulated exchange of the GTP analog [^{35}S]GTPγS as a functional measure for drug interactions with equine adenosine receptors. Similar approaches have been used previously in freeze-thawed or cell-free tissue homogenates and appear to provide a sensitive and accurate indicator for drug intrinsic activities at adenosine receptors.[16,23,24] Under our assay conditions, adenosine stimulates [^{35}S]GTPγS exchange in the cerebral cortex and striatum (Fig 4), which supports the hypothesis that adenosine receptors are coupled to G proteins in equine forebrain tissues. Although it is noteworthy that adenosine produces a maximal effect nearly 3-fold greater in striatum and is slightly more potent in that tissue, our data provide no clear explanation for this difference. Nevertheless, several possible reasons could account for the disparity between cerebral cortex and striatum. For example, differences in the concentration of endogenous substances that regulate [^{35}S]GTPγS exchange could alter the basal rate for this process and thereby affect the net response to exogenous adenosine. In addition, the coupling efficiency between receptors and G proteins could be greater in striatum, or the combined effect by A_1 and A_{2a} receptors in striatal tissue might supersede the stimulatory effect mediated by A_1 receptors alone in cerebral cortex. With regard to the latter point, studies[16] in bovine tissues have failed to detect any substantial effects on guanine nucleotide exchange by A_{2a} adenosine receptors. Nevertheless, despite these regional differences, it appears that adenosine-induced stimulation of [^{35}S]GTPγS exchange is mediated entirely by specific adenosine receptors in both tissues. This conclusion is based on the observation that CGS15943, a nonselective synthetic adenosine receptor antagonist,[37] completely blocks the effect of adenosine in both tissues. Using the same approach in our study, all 4 methylxanthine compounds were determined to act as adenosine receptor antagonists on the basis of their ability to inhibit adenosine-stimulated [^{35}S]GTPγS exchange while having no effect when tested alone. Therefore, in conjunction with results from radioligand binding analyses, our results indicate that caffeine, theophylline, paraxanthine, and theobromine

can competitively antagonize adenosine actions in the equine CNS, although their abilities to do so in vivo is likely to vary owing to differences among their pharmacokinetic profiles.

Finally, although our study focused on the interactions of caffeine and its analogs with G protein-coupled A_1 and A_{2a} receptors in the equine forebrain, it is important to consider additional possible actions by these drugs. Based on evidence obtained from studies[34,38] in rodents and other species, there is abundant evidence that adenosine and adenosine receptors may have a central role in numerous CNS-related phenomena, including sleep-arousal mechanisms, seizure susceptibility, and analgesia. The putative roles of various adenosine receptor subtypes, including those that influence neuronal cation-selective channels (notably, potassium and calcium channels), are complex and reflect the broad spectrum of functions influenced by CNS adenosine receptors.[33,39,40] In addition, blockade of adenosine A_{2a} receptors within the basal ganglia is now recognized to disrupt γ-aminobutyric acid-mediated regulation of striatal dopaminergic neurons and thereby alter spontaneous motor activity.[41-43] In view of the broad array of actions associated with adenosine and its various receptors, it is likely that caffeine actions in the equine forebrain have a similar degree of diversity and complexity.

The results of our study provide the first direct evidence that specific A_1 and A_{2a} adenosine receptors are present in equine CNS tissues. Furthermore, our results indicate that normal activation of these receptors by adenosine can be blocked by caffeine, theophylline, paraxanthine, theobromine, and related methylxanthine analogs and thereby may explain in part some of the behavioral effects of these agents, including motor stimulation. Although our study does not address any specific pharmacologic actions by these agents in horses in vivo, previous investigations have affirmed a pronounced motor stimulant effect following systemic caffeine administration.[44,45] In view of our results that reveal potent antagonistic effects by theophylline and paraxanthine at equine adenosine receptors, the possibility exists for these caffeine metabolites to contribute substantially to the overall spectrum of CNS actions associated with caffeine administration.

[a]Beuthanasia D-Special, Brandel Inc, Gaithersburg, Md.
[b]Polytron homogenizer model PT 10/35, Brinkmann Instruments, Westbury, NY.
[c]New England Nuclear Corp, Boston, Mass.
[d]Tocris Cookson Inc, Ballwin, Mo.
[e]Boehringer Mannheim Corp, Indianapolis, Ind.
[f]Whatman GF/B filters, Brandel Inc, Gaithersburg, Md.
[g]Brandel Cell-Harvester, model M-24R, Brandel Inc, Gaithersburg, Md.
[h]LKB Rackbeta model 1214, LKB-Wallac, Tukow, Finland.
[i]EcoLume, ICN Biomedicals Inc, Costa Mesa, Calif.
[j]Novartis Pharmaceutical Corp, Summit, NJ.
[k]Research Biochemical International, Natick, Mass.
[l]SigmaPlot, SPSS Inc, Richmond, Calif.

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