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# CHRONIC EXPOSURE TO SUBCUTANEOUSLY IMPLANTED METHYLXANTHINES

## DIFFERENTIAL ELEVATION OF $A_1$-ADENOSINE RECEPTORS IN MOUSE CEREBELLAR AND CEREBRAL CORTICAL MEMBRANES

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**Abstract**—Upregulation of brain adenosine receptors in DBA/2J mice as affected by theophylline and caffeine, adenosine antagonists, was examined following subcutaneous drug implantation to ensure chronic exposure. Scatchard analysis of binding to membranes of cerebral cortex and cerebellum from individual mice showed a differential upregulation of (−)-$N^6$-*R*-[G-$^3$H]phenylisopropyladenosine ([$^3$H]-L-PIA) binding density by theophylline. After 14 days of exposure to theophylline (serum concentration of 1.2 ± 0.01 μg/ml measured by HPLC analysis), the $B_{max}$ for L-PIA binding to cerebellar membranes increased 22% over the control mice (statistically significant at $P < 0.01$ level). Theophylline had no effect on the $B_{max}$ for L-PIA binding to cerebral cortical membranes. The observed increases in $B_{max}$ values of cerebellar (13.2%) and cerebral cortical membrane binding (14.2%) on chronic exposure to caffeine (7.1 ± 0.5 μg/ml) were not statistically significant at the $P \leq 0.05$ level. Neither methylxanthine affected the dissociation constant, $K_D$, for L-PIA. The increased potential for adenosine receptor upregulation by theophylline compared to caffeine following chronic, low level exposure suggests that caffeine treatment for sleep apnea may be preferred to the standard theophylline therapy.

The use of theophylline to treat apnea in infants has become a standard medical practice. Infants expressing idiopathic apnea are stabilized when plasma levels of theophylline are between 5 and 13 μg/ml [1–3]. Caffeine has also been used to treat apnea in both preterm and full-term infants with fewer toxic effects [4, 5]. While the mechanism of methylxanthine inhibition of idiopathic apnea in the human infant is not understood, these compounds are known antagonists of adenosine receptors in the central nervous system [6, 7] where they are associated with psychostimulation [8], increased motor activity [6], increased respiratory stimulation [9], anxiety [10] and seizures [11].

Due to the effects of methylxanthines on the CNS and their known interactions with adenosine receptors, it is of particular interest to determine the effects of long-term exposure to methylxanthines on the adenosine receptors in brain. Recently, several laboratories have reported upregulation of adenosine receptors in rat and mouse following exposure to caffeine [12–15] or theophylline [16] either via 1× or 2× daily i.p. injections of methylxanthines or via a methylxanthine-containing diet. Serum caffeine and theophylline levels were not determined in the test animals, and receptor density was determined from assays at one ligand concentration or from Scatchard analyses using pooled brain regions. Since both theophylline and caffeine are used in the treatment of apnea, we have examined the effects of continuous exposure to these methylxanthines on adenosine receptor densities in both cerebellum and cortex of individual mice. The route of delivery was via subcutaneously placed silastic tubing implants containing powdered caffeine or theophylline to mimic the continuous exposure to methylxanthine that might be experienced in the administration of sustained-use tablets. Throughout the experimental period, levels of caffeine and theophylline were monitored by HPLC to determine serum methylxanthine concentrations.

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### METHODS

*Chemicals.* (−)-$N^6$-*R*-[G-$^3$H]Phenylisopropyladenosine ([$^3$H]-L-PIA) (sp. act. 46 Ci/mmol) was obtained from Amersham. RIA Solve II was purchased from Research Products International, Inc.

*Drug treatment.* Male DBA/2J mice (Jackson Laboratory, Bar Harbor, ME), aged 10 weeks with an average weight of 28 g, were given implants under anesthesia, of a 4.5-cm segment of Dow Corning silastic tubing, 0.058 inches i.d. and 0.077 inches o.d. (VWR), filled with 37.5 ± 0.3 mg caffeine, 42.4 ± 0.4 mg theophylline, or left empty (N = 30) according to the procedure outlined by Zielke and Zielke [17]. Animals treated with theophylline received a second implant on day 6 and caffeine-treated mice received a second implant on day 7 to ensure sustained delivery of methylxanthine. On day 14, no statistical difference in body weight was observed in the treatment groups.

Blood was collected daily from mice (N = 2) of each treatment group from the tail vein after warming the animals with an infrared lamp. Immediately after decapitation, the cerebellum and cerebral cortex were dissected on a cold plate, weighed, frozen in liquid nitrogen, and stored at −70°. On day 14, immediately before the animals were decapitated, blood samples were collected from all animals in the study.

*Determination of caffeine and theophylline in serum.* Serum levels of methylxanthines were determined by HPLC after precipitation with a 10% volume of 60% perchloric acid. A total of 20–100 μl of the deproteinized and filtered serum was injected into a Bioanalytical System HPLC equipped with a Waters model 441 absorbance detector and integrator set for a scale range of 0.005 absorbance units at 265 nm. Samples were chromatographed on a Waters μBondapak C-18 reverse phase column (3.9 mm × 30 cm) using a mobile phase of 10 mM acetate buffer, pH 4.0, containing 11% acetonitrile for caffeine separation and 6.5% acetonitrile for theophylline separation.

*Preparation of membranes.* Crude washed mouse brain membranes were prepared according to the procedure of Bruns *et al.* [18]. The individually frozen brain sections from cerebral cortex and cerebellum were thawed and disrupted for 10 sec in ice-cold buffer, 50 mM Tris–HCl at pH 7.4 (20 ml/g wet tissue weight), utilizing a Branson sonicator, model 200 (setting 3) equipped with a micro probe. The homogenate was centrifuged in a Sorvall centrifuge at 50,000 *g* for 30 min. The membrane pellet was washed twice in ice-cold Tris buffer and centrifuged after each wash. The washed pellet was resuspended in 50 mM Tris–HCl at pH 7.4 (37°) with 2 I.U. adenosine aminohydrolase (EC 3.5.4.4, Sigma Type VI) per ml, incubated for 30 min at 37°, recentrifuged, and stored at −70°. All individual brain samples from cerebral cortex or cerebellum were prepared at the same time.

*Determination of* $K_D$ *and* $B_{max}$ *for* [$^3$H]-L-*PIA.* Frozen membranes were thawed and suspended in 50 mM Tris-HCl, pH 7.4 (37°), 1 mM $MgCl_2$, 1 mM EDTA, and 2 I.U. adenosine aminohydrolase per ml. After preincubation for 30 min at 37°, 300-μl aliquots of membrane suspension were incubated for an additional 30 min with [$^3$H]-L-PIA in a final volume of 0.5 ml to determine total binding. For determination of nonspecific binding, 100-μl aliquots of 50 μM non-radioactive L-PIA were added to selected tubes. Total and nonspecific binding measurements were run in quadruplicate for each concentration of [$^3$H]-L-PIA. The reaction was stopped by the addition of 5.0 ml of Tris–HCl buffer (pH 7.4 at 20°) to each sample tube followed by immediate filtering on Whatman GF/B fiber glass sheets under reduced pressure using a Brandell M-24R Cell Harvester. Filters were washed an additional two times with 5.0-ml aliquots of Tris buffer. The radioactivity bound to the filters was measured in an LKB liquid scintillation counter after the addition of 5.5 ml of RIA Solve II to filters contained in 6-ml minivials. Samples were rigorously shaken for 15 min to disperse counts.

For the cerebral cortical samples, binding was determined in a range of 0.1 to 6 nM [$^3$H]-L-PIA with 13 to 15 ligand concentrations per Scatchard analysis. Protein concentration ranged from 37 to 62 μg per assay.

With the cerebellar samples, binding was determined in a range of 0.39 to 20 nM [$^3$H]-L-PIA at protein concentrations between 18 and 28 μg per assay. Ten to twelve ligand concentrations were used for each Scatchard analysis.

Protein concentration was determined by the method of Lowry *et al.* [19] with an aliquot of thawed membranes resuspended at 2.0 mg protein/ml in Tris buffer and diluted 1:4 with water before protein analysis.

Similar $B_{max}$ and $K_D$ values were obtained for cerebral cortical samples using either 10 μM or 1 μM L-PIA or 10 μM 2-choloroadenosine as the non-radioactive ligand for determination of nonspecific binding (unpublished observations).

*Data analysis.* The data were analyzed by both the linear regression program EBDA [20] and the non-linear curve-fitting program LIGAND [21] adapted for the IBM-PC by McPherson [20]. Analysis of the binding isotherm from the individual cerebral cortical or cerebellar samples without background subtraction using the LIGAND program gave results essentially similar to those from the EBDA analysis of these samples assuming a one-site or a three-parameter model. A two-site model could not be fitted to either the individual binding isotherms or to a simultaneous analysis of all the files from each treatment group according to the method of Munson and Rodbard [21]. The mean ± SEM (N = 8) for each treatment group within a brain region was subjected to one-way analysis of variance and Tukey's test [22] to determine statistical differences between any two means.

*Association–dissociation kinetics.* Washed membranes from drug-naive animals were incubated at 37° in 50 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM EDTA and [$^3$H]-L-PIA. Aliquots (1 ml) were removed at indicated times, diluted with 4 ml of 50 mM Tris–HCl buffer (4°), and immediately filtered under vacuum through 2.4 cm GF/B filters. Filters were washed twice with 4-ml aliquots of ice-cold buffer. At 60 min, dissociation was initiated by the addition of cold L-PIA (10 μM final concentration), and 1-ml aliquots were removed at timed intervals for filtration. Nonspecific binding was determined in a separate sample with the simultaneous addition of non-radioactive and radioactive L-PIA at zero time. The dissociation rate constant was calculated by linear regression analysis from a plot of ln $[B/B_e]$ versus time in min where $B$ = dpm specifically bound at time $t$ and $B_e$ = dpm specifically bound at equilibrium. The association rate constant was determined from the relationship $k_{+1} = (k_{obs} - k_{-1})/[L]$ where $k_{obs}$, the pseudo first-order rate constant, was determined by linear regression analysis from a plot of ln $[B_e/(B_e - B)]$ versus time in min.

## RESULTS

*Levels of caffeine and theophylline in serum.* One day after implantation of the silastic tubing, caffeine

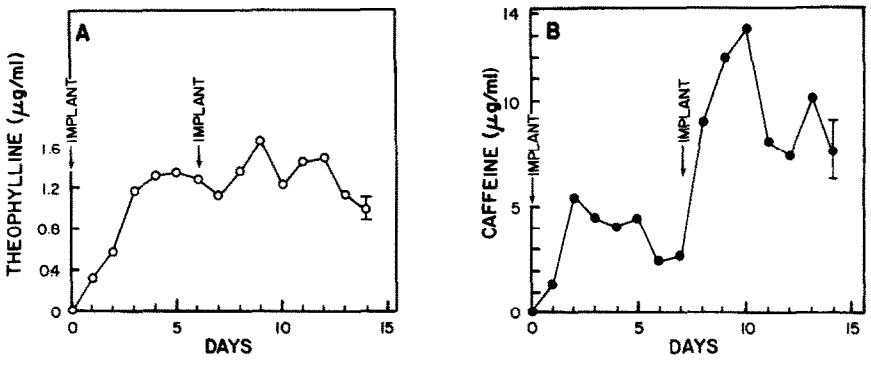


Fig. 1. Serum levels of theophylline (A) and caffeine (B). Levels were determined daily as described in Methods. No single mouse was bled more than two times. On day 14, all mice were killed, and the serum levels of the drugs were re-determined. Daily weighing of animals indicated no difference in body weight. The brackets on the data for day 14 indicate the mean ± SEM (N = 8).

and theophylline were detectable in serum. Equilibrium was established within 2–3 days after implantation (Fig. 1). The 50% decline in serum levels of caffeine on day 6 indicated a need for a second implant. Indeed, all the caffeine was solubilized in the original implant by day 7, whereas the theophylline was not solubilized completely until 2 weeks after the implantation. Animals on the theophylline implant maintained an average serum theophylline level of 1.2 ± 0.01 μg/ml (SEM) during the 14-day study. Although the serum levels for caffeine averaged 7.1 ± 0.5 μg/ml, or 3–7 times the level of theophylline on day 14, there was a greater fluctuation in the daily level of caffeine.

*Adenosine receptor upregulation.* Binding of [$^3$H]-L-PIA to membranes of cerebral cortex and cerebellum for individual mice from each treatment group was analyzed using the EBDA linear regression program as described in Methods. Figure 2 (panels A and B) presents typical Scatchard plots for membranes from individual samples of cerebellum and cerebral cortex, respectively, from the control treatment groups. Binding was saturable, and nonspecific binding was linear. The computed means (± SEM) for the $K_D$ and $B_{max}$ from eight experiments for each treatment group are summarized in Table 1.

The 22% increase in $B_{max}$ observed in cerebellar membranes of the theophylline treatment group was statistically significant at the $P < 0.01$ level. Surprisingly, theophylline had no effect on $B_{max}$ values for cerebral cortical membrane samples from the same group of mice. The increases observed for the caffeine treatment group (13.2 and 14.2% for cerebellum and cortex, respectively) were significant at the $P < 0.05$ level when a less rigorous statistical treatment such as Duncan's Multiple Range Test [22] was used. Thus, upregulation by caffeine under conditions of this study was marginal.

The dissociation constant, $K_D$, of both cerebellar and cerebral cortical membranes for [$^3$H]-L-PIA binding was unaffected by long-term exposure to theophylline or caffeine, as has been observed by other investigators [12–16]. An independent determination of $K_D$ for L-PIA using cerebellar and cerebral cortical membranes prepared from drug-naive mice was conducted by displacement of bound ligand with excess unlabeled L-PIA (Fig. 3, A and B). Association was complete in less than 10 min, and binding was fully reversible. The $K_D$ values calculated from the ratio of the first-order rate constants, $k_{-1}$ and $k_{+1}$, were 0.60 nM for the cerebellar and 1.29 nM for the cortical membranes. These values agree reasonably well with the mean $K_D$ values calculated for cerebellar (1.33 nM) and cerebral cortical (1.04 nM) membranes in the equilibrium binding studies. At 37°, the $T_{\frac{1}{2}}$ values for dissociation of L-PIA from membranes of cerebellum and cortex were approximately 9 and 3 min respectively.

## DISCUSSION

Although the initial dose level of theophylline in the silastic tubing was similar to that of caffeine, theophylline levels in plasma were markedly lower than caffeine levels. The theophylline concentration, however, remained very constant during the 2-week treatment period, indicating that a steady state among solubilization, uptake, metabolism, and excretion had been achieved. The greater solubility of caffeine in aqueous solution may have contributed to the higher serum levels observed for caffeine delivered subcutaneously and, thus, to the need to implant additional caffeine after 6 days. Differences in metabolism and excretion rates for the two methylxanthines would also have affected their serum equilibrium levels. The concentration of theophylline observed (1.2 to 1.6 μg/ml) is compatible with levels of theophylline required to reverse L-PIA locomotor depressions and with the lower limit of theophylline stimulation of locomotor activity in the mouse [6, 7]. The average serum level for caffeine (7.1 μg/ml) was likewise sufficient to elicit locomotor stimulation in the mouse [6, 7]. Furthermore, the

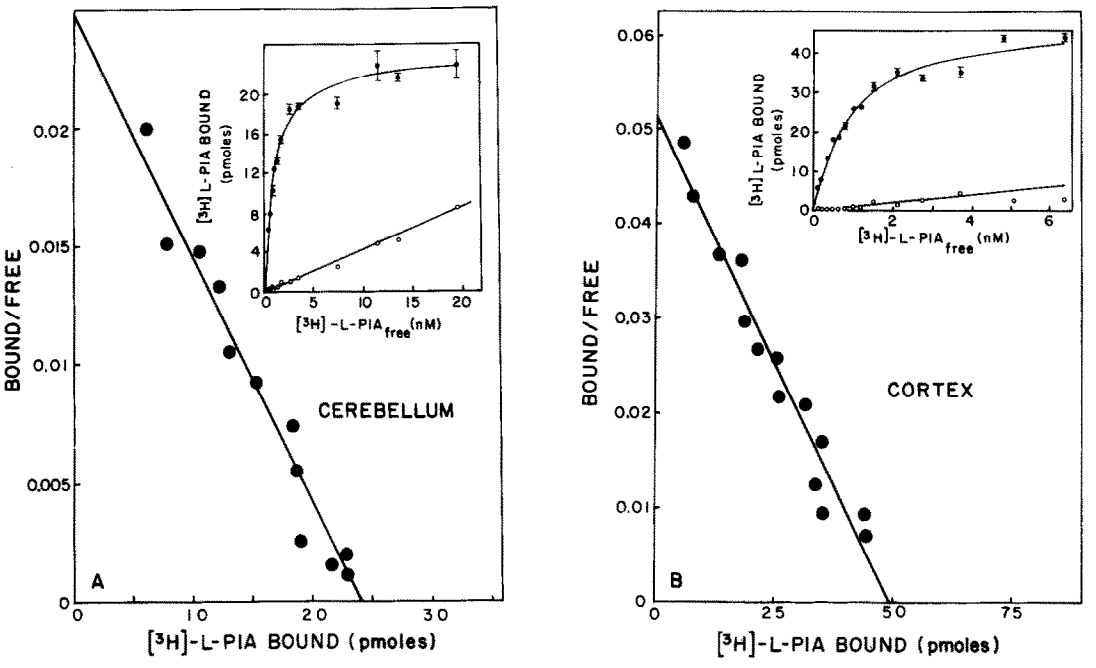


Fig. 2. Typical Scatchard plots for [$^3$H]-L-PIA binding to mouse brain cerebellar (A) and cerebral cortical (B) membranes from the control treatment group. Specific binding of [$^3$H]-L-PIA to mouse brain membranes was determined as described under Methods and is plotted as BOUND/FREE versus BOUND. The $K_D$ and $B_{max}$ values determined from linear regression analysis were 0.95 nM and 447 fmol/mg protein for cerebellar and 0.96 nM and 537 fmol/mg protein for cerebral cortical membranes. The saturation plots (inserts) of the same data are presented along with the observed nonspecific binding for membranes of each brain region. The points for specific binding represent the mean ± SEM (N = 4), where the standard error of the difference of two means was calculated as the square root of the sum of the squares of the standard errors for total and nonspecific binding. The protein concentrations in these samples were 27 μg and 46 μg per 0.5 ml assay for membranes of cerebellum and cerebral cortex respectively. Key: (●——●) specific [$^3$H]-L-PIA binding; and (○——○) nonspecific [$^3$H]-L-PIA binding in the presence of 10 μM cold L-PIA.

serum theophylline and caffeine levels obtained using the silastic tubing implants were sufficient to ensure significant binding to brain adenosine receptors, based on published $IC_{50}$ and $K_D$ values for these methylxanthines in brain tissue of both rodents and humans [23–26] and on the known distribution of methylxanthine in serum and brain [27]. In a recent study on adenosine receptor upregulation in whole mouse brain, Ahlijanian and Takemori [28] measured the concentration of caffeine in brain following its chronic administration in drinking water. They observed a total of 2.1 μg caffeine/g brain tissue after 14 days of ingestion of 221.7 mg caffeine/kg/day.

Preliminary to initiation of this study, several modes of methylxanthine administration were

Table 1. Upregulation of [$^3$H]-L-PIA binding sites in mouse cerebellum and cortex following subcutaneous implantation of caffeine and theophylline

| | Cerebellum | | Cortex | |
|---|---|---|---|---|
| Compound | $K_D$ (nM) | $B_{max}$ (fmol/mg protein) | $K_D$ (nM) | $B_{max}$ (fmol/mg protein) |
| Control | 1.33 ± 0.11* | 511 ± 20 | 1.04 ± 0.03 | 507 ± 10 |
| Caffeine | 1.46 ± 0.12 (+9.2)‡ | 578 ± 20† (+13.2) | 1.06 ± 0.03 (+1.9) | 579 ± 22† (+14.2) |
| Theophylline | 1.44 ± 0.09 (+8.3) | 624 ± 22§ (+22.2) | 1.10 ± 0.05 (+5.8) | 538 ± 28† (+6.1) |

* Mean ± SEM N = 8.
† Not significant ($P > 0.05$, Tukey's Test).
‡ Percent increase over control value.
§ Significant ($P < 0.01$, Tukey's Test).

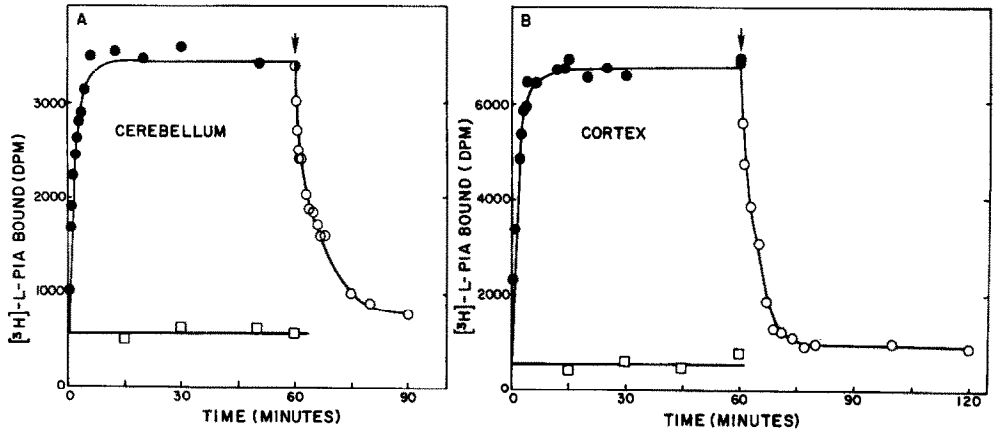


Fig. 3. Association and dissociation kinetics for [$^3$H]-L-PIA binding to adenosine receptors of membranes of cerebellum (A) and cerebral cortex (B). Membranes were prepared and washed as described in Methods. The arrow indicates addition of nonradioactive L-PIA to initiate the off reaction. The concentrations of [$^3$H]-L-PIA were 2.71 and 2.43 nM for cerebellum and cortex respectively. The values for $k_{-1}$ were 0.204 $min^{-1}$ (cortex) and 0.086 $min^{-1}$ (cerebellum). The association rate constants ($k_{+1}$) were 0.158 $min^{-1}$ $nM^{-1}$ (cortex) and 0.14 $min^{-1}$ $nM^{-1}$ (cerebellum). Key: (●——●) total [$^3$H]-L-PIA binding; (○——○) total [$^3$H]-L-PIA binding after the addition of non-radioactive L-PIA (10 $\mu$M final concentration); and (□——□) nonspecific [$^3$H]-L-PIA binding.

tested. Delivery by osmotic pump proved unsatisfactory since the serum concentrations for caffeine and theophylline were below the detection limit of the HPLC analysis system (unpublished observations). Administration via diet at a dosage of 400 mg methylxanthine/kg lab chow resulted in serum levels of 1.4 $\mu$g/ml and 3.0 $\mu$g/ml for theophylline and caffeine respectively (unpublished observations). Although the serum level of caffeine was similar to that observed in humans drinking 3 or more cups of coffee per day [29], this level was below the reported threshold level for locomotor stimulation in the mouse [6, 7]. Consequently, both methylxanthines were administered via the silastic tubing implants.

Theophylline, at serum levels one-fifth that of caffeine, increased $B_{max}$ for PIA binding to the cerebellar membranes, but not to the cerebral cortical membrane samples. A similar differential effect was observed by Marangos *et al.* [14] in their study on adenosine receptor upregulation by caffeine in five regions of the mouse brain using a one-point assay. More than 2 weeks administration of caffeine was required before upregulation of 3.75 nM [$^3$H]-1,3-diethyl-8-phenylxanthine ([$^3$H]DPX) binding was observed to cerebral cortical membranes. The cerebellar membranes, however, showed significant upregulation at 16 days of exposure [14]. These data do not necessarily differentiate between effects of caffeine on the $A_1$ receptor versus the $A_2$ receptor since [$^3$H]DPX binds to both receptors [30]. Upregulation of [$^3$H]-$N^6$-cyclohexyladenosine ([$^3$H]CHA) [13] and [$^3$H]-L-PIA [28] binding site densities has also been reported for whole mouse brain membrane after 2 weeks of exposure to caffeine via drinking water. Indeed, these studies may reflect increases in receptor densities in the cerebellar membranes rather than in whole brain membranes.

In our study, caffeine-exposed mice showed no upregulation of adenosine receptor density in either cerebellar or cerebral cortical membranes despite the higher serum levels of this methylxanthine. However, the higher levels of caffeine occurred only after day 6 when the second caffeine implant was inserted. Since stimulation of motor activity in the mouse is not evident below 10 $\mu$mol/kg [6, 7], the exposure time to caffeine levels high enough to effect the adenosine receptor upregulation may have been too short to observe a statistically significant increase in our study using Tukey's test for statistical significance. Although we failed to see upregulation of PIA binding sites in mouse cerebral cortical or cerebellar membranes with exposure to high levels of caffeine, Chou *et al.* [15] observed a significant increase in [$^3$H]CHA binding site density in rat mesencephalic reticular formation membranes following a 2-week exposure to low doses of caffeine (5–10 mg/kg/24 hr). In the caffeine treated animals both the single neuron activity level of the reticular formation and the spontaneous motor activity indicated tolerance to the stimulatory action of caffeine. The effect of such low level exposure on adenosine receptor density in other brain regions was not reported.

Among our objectives was to examine adenosine receptor density in individual mice rather than in pooled samples, in order to determine individual responses to these methylxanthines. In both brain regions the experimentally determined $B_{max}$ and $K_D$ values were highly reproducible (5–10% variability) with control and caffeine-treated groups. The cerebral cortical samples from the theophylline treatment group exhibited greater variability (15%), which may reflect differences in individual responses to this methylxanthine.

In a previously reported study on receptor upregulation in the mouse [28], the $K_D$ for PIA binding was approximately 4-fold higher (5.1 to 7.2 nM), and the $B_{max}$ was about one-half (188–341 fmol/mg protein) that observed by us. These differences may reflect the presence or absence of $Mg^{2+}$ and/or genetic variability in $K_D$ and $B_{max}$ values for PIA binding. Goodman *et al.* [31] observed that magnesium significantly increases [$^3$H]CHA binding at low ligand concentrations in bovine brain. We observed a similar increase in [$^3$H]PIA binding in whole mouse brain (unpublished observations). While the presence of $Mg^{2+}$ may explain the greater affinity for PIA observed in our study, the higher $B_{max}$ may be related to variability in receptor density among different strains of mice.

It would appear from our data and that of others that adenosine receptors in different brain regions may be differentially sensitive to upregulation by methylxanthines and that the necessary exposure time and the limiting serum concentrations of methylxanthines may vary from region to region. Whether or not these differences are due to subtle differences in receptor subtype between brain regions, e.g. differences in $K_i$ for antagonists, or to differences in antagonist concentration in brain regions cannot be determined from the present data. Characterization of variations in receptor subtype with respect to brain region, agonist and antagonist kinetic parameters, and genetic variability in total receptor density may lend further insight to the differential response to methylxanthines.

Our studies using a mouse model suggest that the density of adenosine receptors may increase in patients under long-term theophylline treatment, since theophylline, at serum levels one-third the lowest physiologic effective level for sleep apnea patients, caused a statistically significant increase in PIA binding sites in mouse cerebellum. In sleep apnea, the alternative caffeine treatment with its fewer side effects [8] and reduced potential for adenosine receptor upregulation may be the treatment of choice.

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