Chronic Caffeine Ingestion Sensitizes the A_1 Adenosine Receptor-Adenylate Cyclase System in Rat Cerebral Cortex

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Abstract

Caffeine consumption causes significant physiologic effects due to its antagonism of adenosine receptors. The A_1 adenosine receptor is coupled in an inhibitory manner to adenylate cyclase. To study the effects of chronic caffeine ingestion, rats were provided with 0.1% caffeine drinking solution for 28 d. The A_1 adenosine receptor agonist radioligand [^{3}H]phenylisopropyladenosine identifies two affinity states in control rat cerebral cortex membranes with a high affinity dissociation constant (K_H) of 0.40±0.08 nM and low affinity dissociation constant (K_L) of 13.7±3.9 nM, with 33% of the receptors in the high affinity state. In membranes from caffeine-treated animals, all of the A_1 receptors are shifted to the high affinity state with a dissociation constant (K_D) of 0.59±0.06 nM. Guanylyl-imidodiphosphate (10^{-4} M) decreases binding by 43% in control membrane, with no change in K_H or K_L, while membrane binding in caffeine-treated animals decreases by 45% with a threefold shift in K_D to 1.5±0.3 nM. Concomitant with the enhanced high affinity A_1 receptor state and increased sensitivity to guanine nucleotides, membranes from treated animals show a 35% enhancement in (−)-N^6-(*R*-phenylisopropyl)adenosine-mediated inhibition of adenylate cyclase compared with controls ($P < 0.03$). Photoaffinity crosslinking the receptors with [^{125}I]N^6-2-(3-iodo-4-aminophenyl)ethyladenosine reveals that A_1 receptors from both groups migrate as M_r 38,000 proteins. β-adrenergic receptor binding with [^{125}I]iodocyanopindolol shows a decrease in the number of β-receptors from 233±7 fmol/mg protein in control membranes to 190±10 fmol/mg protein in treated membranes ($P = 0.01$). These data indicate that the adenosine receptor antagonist, caffeine, induces a compensatory sensitization of the A_1 receptor-adenylate cyclase system and downregulation of β-adrenergic receptors, and provides a molecular mechanism for the caffeine withdrawal syndrome.

Introduction

The daily consumption of caffeine is estimated at 200 mg per adult (1, 2), approximately equivalent to two cups of coffee/d. Acute caffeine intake is associated with arrhythmias (3), increased heart rate (4, 5) and stroke volume (6), altered blood pressure (5), sleeplessness (7), and other physiologic and behavioral alterations. The physiologic and metabolic effects of chronic caffeine use are less well-documented (8–11) and little is known about the biochemical effects of chronic caffeine ingestion.

Although the pharmacologic action of the methylxanthines was previously thought to be caused by phosphodiesterase inhibition, it is now generally accepted that many of the effects of caffeine are mediated through antagonism of adenosine receptors (AR)[1] (12, 13). Two subtypes of ARs are generally recognized, the A_1 and the A_2. These subtypes can be defined by their relative affinities for adenosine agonist analogs. Thus, the A_1AR displays an agonist potency series of (−)-N^6-(*R*-phenylisopropyl) adenosine (*R*-PIA) > *N*-ethylcarboxyamidoadenosine (NECA) > *S*-PIA, while the A_2 receptor's potency series is NECA > *R*-PIA > *S*-PIA (14). Both of these subtypes are coupled to the enzyme adenylate cyclase: the A_1AR in an inhibitory fashion and the A_2AR in a stimulatory manner. [^{3}H]PIA is an AR agonist radioligand selective for the A_1AR and has been utilized to quantitate A_1AR in a variety of tissues (15). In most adenylate cyclase-coupled receptor systems, agonists distinguish two affinity forms of the receptor, a high affinity form (R_H) and a low affinity form (R_L) (16). The high affinity state is thought to represent a ternary complex of agonist-receptor and guanine nucleotide binding protein (N), while the low affinity form is a binding complex of hormone and receptor (16). The A_1AR appears to be similar to other adenylate cyclase-coupled receptors in that two agonist affinity states are definable; however, A_1 and the inhibitory N protein (N_i) are tightly coupled even in the absence of agonists and some agonist high affinity binding is manifested even in the presence of guanine nucleotides (17). The cellular actions of adenosine, acting via the A_1AR, include inhibition of adenylate cyclase activity in heart, fat, and brain tissue (18, 19), attenuation of epinephrine-dependent adenylate cyclase activity (20), and inhibition of the release of norepinephrine from sympathetic nerve endings (21).

Chronic administration of AR antagonists, such as caffeine, followed by their abrupt withdrawal may produce a pathophysiologic condition characterized by an increased sensitivity to endogenous adenosine. This supersensitivity is likely analogous to that reported in the "propranolol withdrawal syndrome" following the abrupt removal of β-adrenergic antagonists (22). The "propranolol withdrawal syndrome" has been described clinically (22, 23), but the biochemical mechanisms responsible for this syndrome remain a matter of debate (16). A "caffeine withdrawal syndrome" has likewise been described in man with symptoms such as headache, myalgias, fatigue, and anxiety, but the mechanisms responsible for this syndrome remain largely unknown (10, 24–27). In the present study, we chronically administered caffeine to rats and examined its effects on the A_1AR-adenylate cyclase system in rat cerebral cortex. The results in-

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1. *Abbreviations used in this paper:* APNEA, N^6-2-(3-iodo-4-aminophenyl)ethyladenosine; AR, adenosine receptors; CHA, cyclohexyladenosine; DTT, dithiothreitol; Gpp(NH)p, guanylyl-imidodiphosphate; [^{125}I]CYP; (−)-3-[^{125}I]iodocyanopindolol; K_H, high affinity dissociation constant; K_L, low affinity dissociation constant; N, guanine nucleotide binding protein; N_i, inhibitory N protein; *R*-PIA; (−)-N^6-(*R*-phenylisopropyl)adenosine; R_H, high affinity receptor; R_L, low affinity receptor; SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

dicate an antagonist-induced sensitization of the A_1AR-adenylate cyclase system occurs and this sensitization provides a molecular mechanism for the "caffeine withdrawal syndrome."

Methods

Materials. Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories, Wilmington, MA. Caffeine, adenosine deaminase, guanisine triphosphate (GTP), cyclic AMP (cAMP), ATP, deoxyadenosine triphosphate (dATP), creatine phosphate, and creatinine phosphokinase were obtained from Sigma Chemical Co., St. Louis, MO. (−)-N^6-(*R*-phenylisopropyl)adenosine (*R*-PIA), and guanylyl-imidodiphosphate (Gpp(NH)p) were obtained from Boehringer Mannheim, Indianapolis, IN. [^{3}H]L-N^6-1-methyl-2-phenylethyladenosine ([^{3}H]PIA; 49.9 Ci/mmol) and α-[^{32}P]ATP (27 Ci/mmol) were obtained from New England Nuclear, Boston, MA. (−)-3-[^{125}I]Iodocyanopindolol ([^{125}I]CYP; 1950 Ci/mmol) was obtained from Amersham, Arlington Heights, IL. R0-20-1724 was the generous gift of Dr. Peter F. Sorter at Roche Laboratories, Nutley, NJ.

Protocol. Male Sprague-Dawley rats (150–275 g) were divided into two groups. The control group had free access to food and water, while the experimental groups had free access to food and caffeine drinking solution (1 g caffeine/liter H_2O) for a minimum of 28 d. Additional binding experiments were conducted with 600 or 750 mg caffeine/liter H_2O drinking solutions for 12 d with no difference in results. Caffeine administration was continued up to the time of sacrifice. There were no deaths in either group.

Membrane preparations. The rats were decapitated and the cerebral cortex was rapidly dissected out and placed in ice-cold 50 mM Tris-HCl (pH 7.4 at 5°C) with 10^{-4} M phenylmethylsulfonylfluoride. The brain was minced and homogenized with 10 strokes using a motor driven glass-teflon homogenizer on ice. The homogenate was then passed through four layers of cheesecloth, washed in 40 ml of the above buffer, and centrifuged at 39,000 *g* for 10 min. The pellet was resuspended with 40 ml of buffer and recentrifuged (as above). Membranes used for saturation curves were resuspended at 4 ml/cortex in 50 mM Tris-HCl (pH 7.4 at 37°C), 10 mM $MgCl_2$, 1 mM EDTA, and 5.0 U/ml adenosine deaminase, and incubated at 37°C for 30 min. The membranes were then diluted to ~2 mg protein/ml and either utilized immediately or frozen in liquid N_2 and stored at −80°C for a maximum of 2 wk. Membranes used for adenylate cyclase assays were resuspended at 4 ml/cortex in 50 mM Tris-HCl (pH 7.4 at 37°C), 10 mM $MgCl_2$, 1 mM EDTA, 10 U/ml adenosine deaminase, and 4 mM dithiothreitol (DTT), and incubated at 37°C for 15 min. The membranes were rewashed in cold 75 mM Tris (pH 7.4 at 30°C), 12.5 mM $MgCl_2$, and 200 mM NaCl; recentrifuged at 39,000 *g* for 10 min; and diluted to 1 mg protein/ml. Membranes were used immediately in the adenylate cyclase assay. Proteins were measured by the method of Lowry (28) using bovine albumin as standards.

[^{3}H]PIA binding assays. The membranes were washed in 50 mM Tris-HCl (pH 7.4 at 37°C), 10 mM $MgCl_2$, and 1 mM EDTA, and centrifuged at 39,000 *g* for 10 min. Then, they were resuspended to a protein concentration of 600 μg/ml in the above buffer, and adenosine deaminase (1 U/ml) and Gpp(NH)p (1.6×10^{-4} M) were added when indicated. [^{3}H]PIA binding was performed in duplicate in an assay volume of 250 μl consisting of 150 μl of membrane, 50 μl of radioligand, and 50 μl of H_2O or *R*-PIA at 5×10^{-5} M. Final [^{3}H]PIA concentrations varied from 0.1 to 30 nM. Incubations were for 60 min at 37°C and were terminated by rapid filtration over Whatman GF/C glass fiber filters and four washes with 3 ml of buffer. Filters were suspended in 10 ml of scintillation fluid and counted in a Packard scintillation counter (Packard Instrument Co., Downers Grove, IL) with an efficiency of 60%. Saturation curves were analyzed by computer-assisted techniques as previously published and validated (29, 30). Statistical analysis of radioligand binding which compares the "goodness of fit" between two parameters was determined as previously prescribed (30). The more complex 2-state model was retained only if it was found to statistically improve the data ($P < 0.05$).

[^{125}I]CYP binding assays. The membranes were rewashed and centrifuged as described in the [^{3}H]PIA binding assay and diluted to ~100 μg protein/ml. All assays were performed in duplicate in a final volume of 500 μl containing 350 μl of membranes in buffer (50 mM Tris-HCl [pH 7.4 at 25°C], 10 mM $MgCl_2$, 1 mM EDTA), 50 μl of [^{125}I]CYP, and 100 μl H_2O or isoproterenol at 5×10^{-4} M. Incubations were for 1 h at 25°C as previously described (31), and were terminated by rapid vacuum filtration and four washes with 3 ml of buffer over Whatman GF/C filters. Final [^{125}I]CYP concentrations varied from 30 to 400 pM. Filters were counted in a Packard gamma counter (Packard Instrument Co.) with an efficiency of 75%. Saturation curves were analyzed as previously described (29, 30).

Adenylate cyclase assays. Adenylate cyclase assays were performed in triplicate in a final volume of 50 μl consisting of 20 μl of membranes, 20 μl of ATP mix as described below, and 10 μl of H_2O or effector as described. Incubations were for 20 min at 30°C. The final mixture contained ~20-μg membrane protein, 1 μCi [α-^{32}P]ATP, 25 U creatinine phosphokinase, 5 mM creatine phosphate, 1.35×10^{-4} M dATP, 10^{-6} M cAMP, 5×10^{-6} M GTP, 30 mM Tris (pH 7.4 at 30°C), 5 mM $MgCl_2$, 80 mM NaCl, 1 mM DTT, 2 U/ml adenosine deaminase, and 0.1 mM R0-20-1724. The reaction was stopped by placing the assay into an ice bath and adding 1 ml of solution containing 0.4 mM ATP, 0.3 mM cAMP, and [^{3}H]cAMP (~20,000 counts). cAMP was isolated as described by Salomon et al. (32). Curves were constructed as previously described (29, 30) and statistically compared by the *F* test.

Photoaffinity crosslinking. Membranes were prepared as described for radioligand binding except leupeptin (5 μg/ml) was included throughout the above preparation as described. Photoaffinity crosslinking and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the A_1 subtype selective radioligand [^{125}I]N^6-2-(3-iodo-4-aminophenyl)ethyladenosine ([^{125}I]APNEA) as recently described (33). The electrophoresis was performed according to the method of Laemmli (34) using homogenous slab gels. Electrophoresis standards were as follows: phosphorylase *b* (relative molecular weight [M_r] = 94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,100).

Results

Saturation curves constructed with the agonist radioligand [^{3}H]PIA in control membranes demonstrate that the ligand distinguishes between two affinity states of the A_1AR with a high affinity dissociation constant (K_H) of 0.40±0.08 nM and a low affinity dissociation constant (K_L) of 13.7±3.9 nM. As can be seen in Fig. 1 *A*, a two affinity state model significantly improves the fit compared with a one affinity state model ($P < 0.05$). In a series of 14 control animals, membranes from 10 animals demonstrate a two-state fit. In contrast, in membranes derived from caffeine-treated animals, only one high affinity state was evident in 9/9 animals with a dissociation constant (K_D) of 0.59±0.06 (Fig. 1 *B*). This K_D is not significantly different from the K_H in control membranes. This suggests that all of the A_1ARs are shifted to the high affinity state following caffeine treatment. This shift from two affinity states found in control membranes to a single high affinity state in caffeine-treated membranes is statistically significant ($P = 0.02$ by χ^2). Table I lists the [^{3}H]PIA binding parameters. Even though it is difficult to compare the total number of receptors in these two groups because of the one-versus two-state models, the absolute number of receptors in the high affinity state is significantly increased ($P < 0.001$).

Previous studies have demonstrated that A_1AR high affinity agonist binding still occurs in the presence of guanine nucleotides without a shift in K_D (17). The effect of Gpp(NH)p on [^{3}H]PIA binding was therefore assessed in control and "treated" membranes. In control membranes, 10^{-4} M Gpp(NH)p significantly decreased (43%) total [^{3}H]PIA binding, but the same two affinity

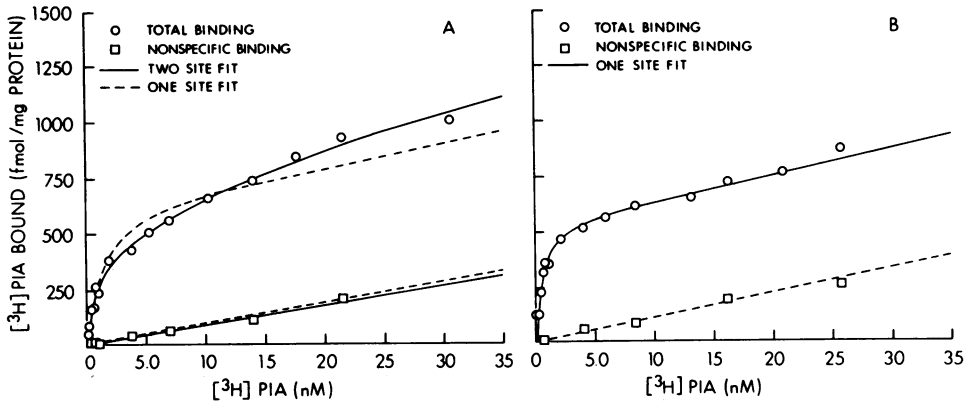

Figure 1. [^{3}H]PIA saturation curves in rat cerebral cortex membranes. (*A*) Membranes were prepared from control animals and binding was performed as described in Methods. [^{3}H]PIA was added at the final concentration shown on the abscissa. Nonspecific binding was defined as the amount of binding which occurred in the presence of 10^{-5} M unlabeled *R*-PIA. The data points are means of duplicate determinations. The curves were drawn with the aid of a computer modeling program based on the law of mass action, assuming either one or two affinity states for the receptor (29). The two-state fit most appropriately describes the data ($P < 0.001$). This experiment is representative of 10 similar experiments. (*B*) Membranes were prepared from caffeine-treated animals and binding was performed as above. This curve is representative of nine similar experiments. Only a single affinity state is observed.

states were evident with no significant change in K_H or K_L (see Table I and Fig. 2 *A*). In contrast, "caffeine-treated" membranes demonstrate a 45% decrease ($P < 0.001$) in total binding with a significant ($P < 0.01$) threefold shift in K_D to lower affinity (1.5±0.3 nM). This suggests that the receptor complex is more sensitive to guanine nucleotides and the A_1-N coupling in the presence of Gpp(NH)p is less tightly associated in treated membranes than in control membranes.

A shift to all high affinity agonist binding and an increased sensitivity to guanine nucleotides in treated membranes would be expected to result in the enhancement of agonist-induced biochemical responses. Therefore, we tested the ability of *R*-PIA to inhibit adenylate cyclase activity in cerebral cortex homogenates. Fig. 3 demonstrates that *R*-PIA-mediated inhibition of basal adenylate cyclase activity is increased by 35% in treated vs. control animals. ($P < 0.03$). Basal activity is exactly the same in the two groups: control, 21.7±2.9 and treated, 22.7±2.9 pmol cAMP/mg protein per min. The effective concentration producing a 50% alteration (EC_{50}) of *R*-PIA demonstrates a leftward shift from 14.8 nM in control to 7.5 nM in treated animals. This change does not, however, reach statistical significance ($P = 0.10$).

Table I. [^{3}H]PIA Binding in Rat Cerebral Cortex of Control and Treated Animals

	Control	Treated	Control plus 10^{-4} M Gpp(NH)p	Treated plus 10^{-4} M Gpp(NH)p
No. of animals	10	9	5	6
K_H (*nM*)	0.40±0.08	0.59±0.06	0.43±0.10	1.49±0.31
K_L (*nM*)	13.68±3.92		15.10±4.30	
R_H (*fmol/mg protein*)	316±46	629±38.6	129±43.5	345±26.7
R_L (*fmol/mg protein*)	568±40		378±59.5	
Percent R_H	33±4	100	25±8	100

Values are given as mean±SEM. Data from treated animals are most appropriately described by a one affinity state model. Percent R_H = percent of receptors in high affinity state.

To assess if the change in agonist binding might be a manifestation of an alteration in the structure of the A_1AR, we photoaffinity crosslinked the receptor with [^{125}I]APNEA in membranes from both groups. Fig. 4 demonstrates the results obtained after SDS-PAGE and autoradiography. The A_1AR's from both control and treated membranes migrate as 38,000 M_r proteins. This suggests that a gross alteration in the receptor-binding subunit is not responsible for the changes seen after caffeine treatment.

Caffeine ingestion has been reported to alter the quantity of β-adrenergic receptors in brain tissue from animals. We, therefore, measured β-adrenergic receptor number in these same animals and found a significant decrease ($P = 0.01$) in treated, 190±10 fmol/mg protein, vs. control, 233±7 fmol/mg protein ($n = 4$) membranes (Table II). There is no change in the K_D for [^{125}I]CYP (control, 62±14 pM, vs. treated, 67±3 pM). This finding is in good agreement with previous studies on the effects of methylxanthine administration (35–37).

Discussion

Chronic therapy with receptor-specific antagonists in animals and man has been reported to induce an enchanced response to agonists following removal of the antagonists (16). The caffeine withdrawal syndrome represents but one example of such sensitization to agonists. The syndrome has been recognized clinically for years with the predominant symptom being characteristic headaches (27). The essential feature of the syndrome is that individuals must consume large quantities of caffeine (~500 mg = 5 cups of coffee) per day until a tolerance develops and then abruptly stop intake (27). Symptoms usually develop within 18–20 h. Furthermore, reinstitution of caffeine intake relieves symptoms within a short period of time. The biochemical mechanisms responsible for these putative "withdrawal syndromes" have remained largely speculative.

In this study, we have probed the dynamic regulation of A_1AR in rat cerebral cortex following chronic treatment with the AR antagonist, caffeine. [^{3}H]PIA saturation curves indicate that in control cerebral cortex membranes, the agonist radioligand can distinguish two discrete affinity states of the A_1AR having K_H and K_L for the agonist. This finding is similar to that reported for the interaction of agonists with a variety of adenylate

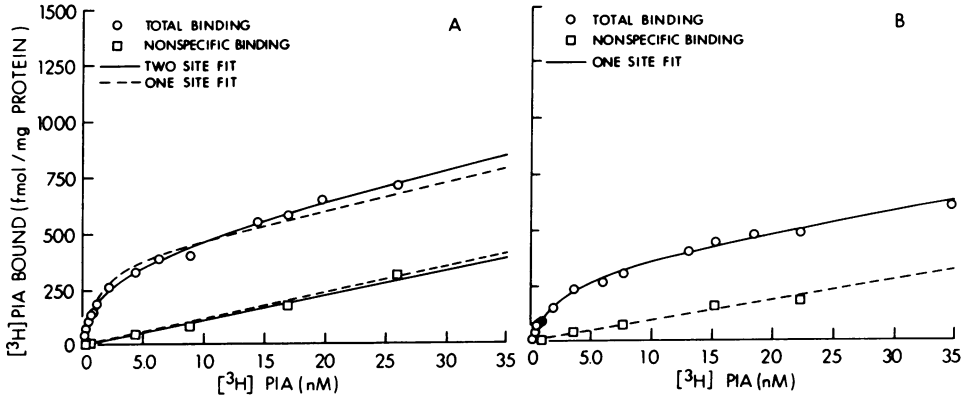

Figure 2. [^{3}H]PIA saturation curves in the presence of 10^{-4} M Gpp(NH)p in rat cerebral cortex membranes. (*A*) Membranes from control animals were prepared and binding was performed as described in Fig. 1. This experiment is representative of five similar experiments. (*B*) Membranes from caffeine-treated animals. This experiment is representative of six similar experiments.

cyclase-coupled receptors (16). Following treatment of animals with caffeine, all of the A_1 receptors are found to display a uniform high affinity for the agonist radioligand such that the unique K_D is indistinguishable from the K_H of control membranes. Thus, chronic occupation of the A_1AR by caffeine (inhibition constant [K_I] = 31 μM [38]) results in a statistically significant shift in the dynamic equilibrium between high and low affinity states to all high affinity binding. This suggests an enhanced ability of A_1AR and N_i proteins to associate with each other, since high affinity agonist binding is thought to be related to the formation of a ternary complex of hormone-receptor N protein. It should be noted that although caffeine is also a phosphodiesterase inhibitor, the concentration required to produce this effect in vitro is 20 to 50 times higher than those needed to block A_1AR (13).

Previous studies have indicated that although guanine nucleotides decrease the amount of the agonist radioligand [^{125}I]iodohydroxyphenylisopropyladenosine bound in cerebral cortex membrane, the K_D of the remaining sites are unaltered (17). This same phenomenon can be seen in the control membranes with [^{3}H]PIA binding wherein both high and low affinity binding are evident in the presence of 10^{-4} M Gpp(NH)p, while the total amount of binding is decreased (see Table I). In contrast, although cerebral cortex membranes from treated animals reveal that high affinity binding is seen in the presence of Gpp(NH)p and total binding decreases by a comparable amount, the K_D has increased threefold, which indicates a decreased affinity of the components of the hormone-receptor-N_i complex. The [^{3}H]PIA-binding data thus suggest that following caffeine treatment, there is an enhancement of the number of the receptors in the high affinity state (316–629 fmol/mg protein) and that these high affinity receptor complexes are more sensitive to guanine nucleotides than the complexes in the control membranes. An enhancement of the high affinity state and an increased sensitivity to guanine nucleotides should be associated with a sensitization of agonist-induced biochemical effector interactions.

Since one of the effects of agonist-A_1AR interactions in the cerebral cortex is to produce inhibition of adenylate cyclase activity, we assessed the ability of *R*-PIA to inhibit adenylate cyclase activity in membranes from control and treated rats. Fig. 3 demonstrates the accentuated effect of *R*-PIA in inhibiting adenylate cyclase activity in treated vs. control membranes. Maximal inhibition is increased by 35%.

These data are all compatible with a sensitization of the A_1AR-adenylate cyclase system following chronic caffeine ingestion. In vivo studies of the A_2AR have suggested a sensi-

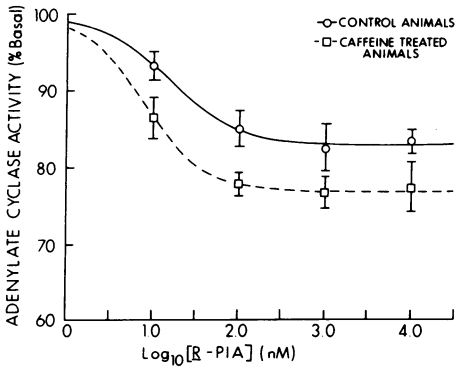

Figure 3. Inhibition of adenylate cyclase activity by *R*-PIA in membranes from rat cerebral cortex. Membranes were prepared from control and caffeine-treated rats as described in Methods. Adenylate cyclase activity was measured as described in Methods. Data are presented as mean±SEM. Basal adenylate cyclase activity was: control, 21.7±2.85 pmol cAMP/mg protein per min (n = 4), and caffeine-treated, 24.7±2.90 pmol cAMP/mg protein per min (n = 5). *R*-PIA was added at the concentration shown on the abscissa. The enhanced inhibition of the treated group was significantly ($P < 0.03$) greater than that seen in the control group.

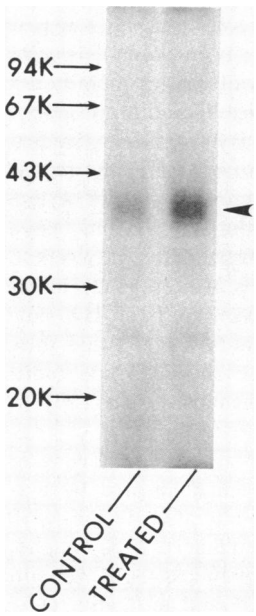

Figure 4. Photoaffinity crosslinking of [^{125}I]APNEA into the A_1AR of rat cerebral cortex membranes. Cerebral cortex membranes were prepared from control and caffeine-treated animals as described in Methods. The membranes were then photoaffinity crosslinked with [^{125}I]APNEA, solubilized, and electrophoresed on a 10% polyacrylamide gel. The gel was then dried and subjected to autoradiography. The M_r scale is calibrated in kilodaltons using iodinated protein standards (see Methods). The arrows indicate the M_r 38,000 A_1AR in control and treated membranes. This experiment was replicated twice.

Table II. [^{125}I]CYP β-Adrenergic Receptor Binding in Rat Cerebral Cortex of Control and Caffeine-treated Animals

	Control	Treated
No. of animals	4	6
K_D (*pM*)	61.7±14.0	66.9±3.06
Receptor binding (*fmol/mg protein*)	233±6.50	190±9.80

Values are given as mean±SEM.

tization of A_2AR responsiveness in rats treated for 3 wk with 0.1% caffeine drinking water (39). These animals demonstrated a significantly enhanced hypotensive response to adenosine compared with control animals. This in vivo caffeine-induced sensitization of the A_2AR, a receptor that is also antagonized by methylxanthines (40), has direct parallels with our in vitro biochemical studies.

Although the number of receptors in the high affinity state has increased ("increased coupling") following caffeine treatment, there is no evidence for an upregulation in the total number of receptors. Previous studies have reported an upregulation of rat cerebral cortical A_1AR following methylxanthine administration (38, 41). Murray (41) describes a 28% increase in [^{3}H]cyclohexyladenosine ([^{3}H]CHA) sites in response to chronic theophylline administration. The data, however, are compatible with our findings. In that study, saturation curves were performed only up to a concentration of 10.5 nM and utilized a one affinity state model. This concentration of [^{3}H]CHA is sufficient to saturate binding at the high affinity site (K_D = 1.1 nM) but not at the low affinity site. Therefore, the binding of [^{3}H]CHA was totally saturated in the membranes from treated animals but not in the control membranes leading to a lower apparent maximum binding capacity in the control membranes. Remodeling our data using a one-site model for both control and caffeine-treated membranes and a maximal [^{3}H]PIA concentration of 8 nM gives an apparent 20% increase in receptor number; these results are similar to those reported by Murray (41). Fredholm (42) also reports an upregulation of A_1AR in response to 1 wk of caffeine administration. The reported K_D for [^{3}H]PIA is 4.7 nM with total control binding of only 325 fmol/mg protein. The low apparent affinity (high K_D) and low receptor number suggest some endogenous adenosine may have been present. Several studies have demonstrated the necessity of treating membranes with adenosine deaminase before radioligand binding (43). Boulenger et al. (44) and Marangos et al. (45) have recently studied the effect of chronic caffeine ingestion on ARs in the mouse brain. They demonstrated that ARs were increased in several brain regions while the nucleoside transporter was not increased and there was a transient increase in the benzodiazepine receptor. As the authors point out (44), there is a discrepancy in the time course and extent of increase in [^{3}H]CHA and [^{3}H]diethylphenylxanthine binding, which suggests that these ligands may be binding to different classes of ARs. These studies point out the need for use of selective ligands, such as [^{3}H]PIA or [^{3}H]CHA.

Alterations in β-adrenergic receptor structure (following agonist-induced desensitization) have been delineated by photoaffinity labeling (16). To assess if an alteration in the A_1AR protein might be responsible for the enhanced R-N_i coupling seen in this study, we photoaffinity crosslinked the A_1AR with [^{125}I]APNEA (33). Following SDS-PAGE and autoradiography, the A_1AR from both control and caffeine-treated membranes comigrate as 38,000 M_r proteins. This suggests that there are no major alterations in receptor structure to account for the enhanced coupling.

Membranes from the treated group demonstrate a significant decrease in the number of cerebral cortex β-adrenergic receptors. This down-regulation of β-adrenergic receptors has been reported by others (35–37). The mechanism responsible for this down-regulation is likely related to the increased levels of norepinephrine and epinephrine induced by caffeine administration (46). It has been established that adenosine can inhibit the release of norepinephrine from sympathetic nerve endings via action on presynaptic terminals (46, 47). The increased catecholamine release probably results from caffeine's antagonism of adenosine's ability to inhibit norepinephrine release.

The biochemical changes in the AR-adenylate cyclase system following chronic caffeine ingestion may provide a molecular mechanism for the "caffeine withdrawal syndrome." In response to caffeine's antagonism of the A_1AR, the A_1AR-N_i protein coupling is shifted to the high affinity state, resulting in the compensatory sensitization of A_1AR-mediated inhibition of adenylate cyclase. The caffeine-induced increase in plasma norepinephrine also causes a β-adrenergic receptor downregulation, and probably attentuation of β-adrenergic adenylate cyclase stimulation. Therefore, chronic caffeine consumption, via its AR antagonism, induces compensatory alterations in the A_1 and β-receptor-adenylate cyclase system, making production of cAMP less favored. Then, after the sudden withdrawal of caffeine, enhanced adenosine-mediated effects predominant and caffeine withdrawal symptoms become evident.

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