Multiple Components of the A_1 Adenosine Receptor-Adenylate Cyclase System Are Regulated in Rat Cerebral Cortex by Chronic Caffeine Ingestion

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Abstract

The effects of chronic caffeine on the A_1 adenosine receptor-adenylate cyclase system of rat cerebral cortical membranes were studied. Caffeine treatment significantly increased the number of A_1 adenosine receptors as determined with the A_1 adenosine receptor antagonist radioligand [^{3}H]xanthine amine congener (XAC). *R*-PIA (agonist) competition curves constructed with [^{3}H]XAC were most appropriately described by a two affinity state model in control membranes with a K_H of 2.1±0.8 and a K_L of 404±330 nM with 50±4% of receptors in the high affinity state (%R_H). In contrast, in membranes from treated animals, there was a marked shift towards the high affinity state. In three of seven animals all of the receptors were shifted to a unique high affinity state which was indistinguishable from the K_H observed in membranes from control animals. In four of seven animals the %R_H increased from 50 to 69% with K_H and K_L indistinguishable from the control values. Thus, the agonist specific high affinity form of the receptor was enhanced following caffeine treatment. Maximal inhibition of adenylate cyclase activity in cerebral cortical membranes by *R*-PIA (1 μM) was significantly increased by 28% following caffeine treatment, consistent with an increased coupling of receptor-G_i protein with adenylate cyclase. Importantly, the quantity of G_i (α_i) in rat cerebral cortex, determined by pertussis toxin-mediated labeling, was also increased to 133% of control values by this treatment. Thus, multiple components and interactions of the A_1 adenosine receptor-adenylate cyclase complex are regulated by caffeine. These changes are likely compensatory measures to offset blockade of A_1 receptors in vivo by caffeine and lead to a sensitization of this inhibitory receptor system.

Introduction

Caffeine, found in coffee, tea, and cola, is one of the most widely consumed drugs in the world. The average daily consumption of caffeine is estimated at about 200 mg per adult (1, 2), or approximately two cups of coffee per day. Upon acute ingestion, the drug affects the cardiovascular system (3–7) and brain (8–11). Like other methylxanthines, caffeine is an antagonist of adenosine receptors (8–13) and blockade of these receptors by the drug might account for its pharmacological effects (3–11). The brain contains both subtypes of adenosine receptors, termed A_1 and A_2 adenosine receptors (10, 14). Generally, A_1 receptors mediate inhibition of adenylate cyclase while A_2 receptors stimulate this enzyme (10, 14). In addition to differential effects on adenylate cyclase, these receptors show different selectivities for adenosine analogues (10, 14–16).

Tolerance develops rapidly to both the peripheral and central effects of caffeine in man (17) and rodents (18). Chronic ingestion of caffeine, followed by abrupt cessation of drug intake, leads to a number of symptoms, such as headaches, myalgias, fatigue, and anxiety (19–21). In an attempt to understand the mechanisms underlying the "caffeine withdrawal syndrome" and the development of tolerance to this drug, we have recently begun to assess changes in the A_1 adenosine receptor-adenylate cyclase system of rat cerebral cortex following caffeine administration (22).

Recent studies using A_1 selective agonist radioligands have demonstrated increases in A_1 receptor binding in rat cerebral cortex following chronic caffeine ingestion (22–26). Concurrent enhancement of *R*-phenylisopropyladenosine (*R*-PIA)[1] mediated inhibition of adenylate cyclase was also observed following similar treatment conditions (22). The use of agonist radioligands, however, does not permit the differentiation between two potential mechanisms responsible for the increase in agonist binding: one, a true increase in receptor number (up-regulation) or two, an increase in receptor coupling to the guanine nucleotide regulatory protein (G_i) either as a consequence of changes in adenosine receptor or G_i protein quantity or functionality.

The recent development of a high affinity antagonist radioligand, tritiated xanthine amine congener (8-{4-[({[(2-amino-ethyl)amino]carbonyl}methyl)oxyl]phenyl}-1,3-dipropylxanthine ([^{3}H]XAC) (27) now permits the definitive assessment of whether receptor number and/or receptor-G protein coupling are altered as a consequence of chronic caffeine ingestion. Very recent studies from our laboratory have documented that chronic exposure to *R*-PIA in rats is associated with the phenomenon of desensitization in the A_1 adenosine receptor-adenylate cyclase system and that one mechanism responsible for this is the modulation of G_i (28).

In the present study, therefore, we have quantitated receptor number, receptor coupling and the quantity of the inhibitory guanine nucleotide regulatory (G_i) protein (α_i subunit) in

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1. *Abbreviations used in this paper:* G_i and G_s, inhibitory and stimulatory guanine nucleotide regulatory proteins, respectively; K_H, high affinity dissociation constant; K_I, equilibrium dissociation constant; K_L, low affinity dissociation constant; R_H and R_L, receptors in the high and low affinity states, respectively; *R*-PIA, (−)-N^6-(R-phenylisopropyl)adenosine; XAC, (8-{4-[({[(2-amino-ethyl)amino]carbonyl}methyl)oxyl]phenyl}-1,3-dipropylxanthine.

rat cerebral cortex. We now report alterations in all three parameters following chronic caffeine treatment.

Methods

Materials. Adenosine deaminase, cyclic AMP (cAMP), guanosine triphosphate (GTP), ATP, deoxyadenosine triphosphate (dATP), creatine phosphokinase and papaverine were from Sigma Chemical Co., St. Louis, MO. Creatine phosphate and (−)-N^6-(*R*-phenylisopropyl)-adenosine (*R*-PIA) were from Boehringer Mannheim, Indianapolis, IN. 2,8-[^{3}H]cAMP (40 Ci/mmol), [α-^{32}P]ATP (27 Ci/mmol) and [^{3}H]XAC (130 Ci/mmol) were from New England Nuclear, Boston, MA. [^{32}P]NAD (250 Ci/mmol) was from ICN Nutritional Biochemicals, Cleveland, OH. Crude collagenase was from Worthington Biomedical, Freehold, NJ. Bovine serum albumin (fraction IV) was from Armour Pharmaceutical Co., Kankakee, IL. Cholera and pertussis toxins were from List Biological Laboratories, Campbell, CA.

Drug treatment protocol. Male Sprague-Dawley rats (275–300 g) were administered either normal tap water (control group) or water containing 1 g caffeine/liter (treated group) for 28 d. Rats were then killed by decapitation, their brains rapidly excised, hemisectioned, and cerebral cortices were dissected out and rapidly frozen on dry ice. Cerebral cortices were frozen for periods of no longer than 2 wk prior to performing experiments.

For pertussis toxin and cholera toxin-mediated labeling of G_i and G_s, respectively, in rat adipocytes, animals were sacrificed as above and adipocyte membranes were prepared from epididymal fat pads as described below.

Membrane preparations. Rat cerebral cortical membranes were prepared by homogenizing the cortices (~ 0.5 g each) in 50 ml Tris-HCl buffer (pH 7.4 at 37°C) containing 10 mM $MgCl_2$ and 1 mM EDTA, using 10 up and down strokes on ice. Homogenates were centrifuged for 10 min at 1,000 *g*, followed by centrifugation of the supernatant at 47,000 *g* for 20 min. The resulting pellet was then resuspended in 5 ml of Tris-HCl buffer and incubated at 37°C for 10 min in presence of 5.0 U/ml adenosine deaminase. After incubation, the membrane suspensions were centrifuged for 15 min at 47,000 *g* and resuspended in the appropriate buffers for [^{3}H]XAC binding, adenylate cyclase assays or [^{32}P]NAD labeling of the α_i subunit of G_i. For radioligand binding assays brain membranes were resuspended in 50 mM Tris-HCl buffer (pH 7.4 at 37°C) containing 10 mM $MgCl_2$, 1 mM EDTA, and 5 U/ml adenosine deaminase to give a protein concentration of ~ 1 mg/ml. Adenylate cyclase assays were performed using membranes resuspended in 75 mM Tris HCl buffer (pH 7.4 at 30°C) containing 12.5 mM $MgCl_2$, 200 mM NaCl, and 2.5 mM DTT to give a protein concentration of ~ 0.3–0.5 mg/ml. Membranes used for bacterial toxin-catalyzed labeling were resuspended in 25 mM Hepes buffer (pH 7.4) containing 2.5 mM $MgCl_2$ and 0.3 mM EDTA to give a protein concentration of 2 mg/ml.

Rat adipocyte membranes were prepared from epididymal fat pads as previously described (29) with the following modifications: leupeptin and soybean trypsin inhibitors were omitted during the collagenase digestion; both of those and phenylmethylsulfonylfluoride were omitted from the hypotonic buffer. Membrane pellets obtained from centrifugation were resuspended in 25 mM Hepes buffer (pH 7.4) containing 2.5 mM $MgCl_2$ and 0.3 mM EDTA to give a protein concentration of ~ 1–2 mg/ml and were pretreated with adenosine deaminase as above.

[^{3}H]XAC binding. Rat cerebral cortical membranes were incubated with five to eight concentrations of [^{3}H]XAC (0.25–10 nM) for 1 h at 37°C. The total volume of the reaction mixture was 250 μl of 50 mM Tris-HCl buffer containing 10 mM $MgCl_2$ and 1 mM EDTA. *R*-PIA (10 μM) was included to define nonspecific binding which averaged ~ 30% of total binding at [^{3}H]XAC concentrations approximating the K_D. After incubation, membranes were rapidly filtered over 25-mm glass fiber filters (#32; Schleicher and Schuell, Keene, NH) by vacuum and washed three times with ice-cold buffer containing 0.01% Chaps. Filters were allowed to extract for at least 6 h in toluene-based scintillation fluid before counting.

For competition experiments cerebral cortical membranes were incubated with a single concentration of [^{3}H]XAC (~ 2.5 nM) and increasing concentrations of *R*-PIA.

Adenylate cyclase assays. Adenylate cyclase activity in cerebral cortical membranes was determined as previously described (22). Briefly, 20 μl of membranes suspended in 75 mM Tris-HCl buffer (pH 7.4 at 30°C), containing 12.5 mM $MgCl_2$, 200 mM NaCl and 2.5 mM DTT, were incubated with 20 μl Lomix (0.14 mM dATP, 5 mM phosphocreatine, 1 μM cAMP, 30 U/ml creatine phosphokinase, ~ 1.5 μCi of [^{32}P]ATP and 10 μl of H_2O or drug. Papaverine (0.1 mM) was used in all experiments to provide adequate phosphodiesterase inhibition. Assay mixtures were incubated for 15 min at 30°C. Adenylate cyclase assays were terminated by addition of 1 ml ice-cold stop solution, containing ~ 20,000 cpm [^{3}H]cAMP (internal standard), 0.3 mM cAMP and 0.4 mM ATP. Cyclic AMP was then isolated by the method of Salomon et al. (30).

Toxin labeling. Bacterial toxin-catalyzed labeling of rat cerebral cortical membranes was performed as described previously by Owen et al. (31) with slight modifications. Membranes, prepared as described above, were incubated with 25 mM Hepes buffer (pH 7.4) containing the following constituents: 2.5 mM $MgCl_2$, 0.3 mM EDTA, 10 mM thymidine, 1 mM ATP, 1 mM DTT, and 5 μM [^{32}P]NAD. Pertussis and cholera toxins were activated in buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$ and 50 mM DTT for 15 min at 37°C and added to the reaction mixtures just before incubation. The final concentrations of pertussis toxin were 16 or 32 μg/ml, while the final concentration of cholera toxin was 200 μg/ml. Incubations were for 15 min at 30°C in a total volume of 0.1 ml Hepes buffer.

Following incubation, membranes were washed twice with ice-cold buffer containing 50 mM Tris-HCl (pH 7.4 at 37°C), 10 mM $MgCl_2$, and 1 mM EDTA and pelleted in a microfuge. Membrane pellets were then solubilized in buffer (10% SDS, 10% glycerol, 20 mM Tris-HCl, 6% beta-mercaptoethanol; pH 6.5) for 1 h and then subjected to SDS-PAGE.

SDS-PAGE. Electrophoresis was performed according to the method of Laemmli (32) using homogeneous gels with the stacking gel containing 3% acrylamide and the separating gel 12% acrylamide. Electrophoresis was performed at a constant current of 25 mA. Premixed SDS-PAGE standards from Pharmacia Fine Chemicals, Piscataway, NJ, radiolabeled using the chloramine T method, included phosphorylase B, M_r = 94,000; albumin, M_r = 67,000; ovalbumin, M_r = 43,000; carbonic anhydrase, M_r = 30,000; soybean trypsin inhibitor, M_r = 20,000. Electrophoresis reagents were from Bio-Rad Laboratories, Richmond, CA. Following electrophoresis, gels were dried using a Bio-Rad gel dryer. Gels were then used for autoradiography at −80°C, using Kodak x-ray film (XAR-5) and Cronex Lightening Plus intensifying screens from Dupont. Relative labeling of α_i from control and caffeine-treated rats was determined by densitometric scanning of the autoradiographs.

Data analysis and protein determination. Saturation and competition curves were analyzed using a nonlinear least squares curve filling technique with statistical analysis as previously described (33, 34). Protein concentrations were determined by the method of Bradford (35).

Results

Administration of caffeine to rats for 28 d results in an increase in the number of A_1-adenosine receptors in the cerebral cortex compared to controls, as determined with the new high affinity antagonist radioligand [^{3}H]XAC (Fig. 1). B_{max} values for control and caffeine-treated rats were (mean±SEM) 699±83 and 982±101 (n = 8), respectively (statistically significant difference, P = 0.05). No change in K_D values was observed, these

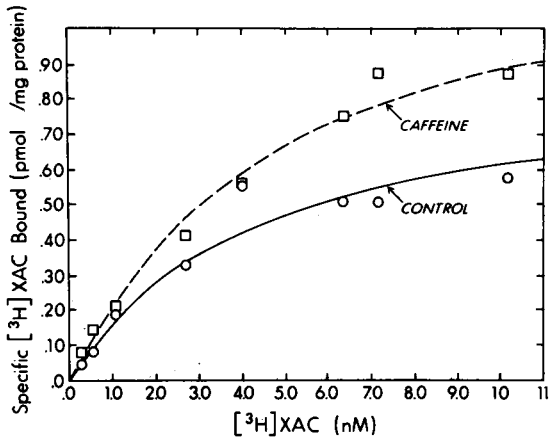

Figure 1. [^{3}H]XAC saturation curves in cerebral cortex membranes from control and caffeine-treated rats. Cerebral cortical membranes prepared from controls and rats treated with caffeine for 28 d were incubated with different concentrations of [^{3}H]XAC in the absence or presence of 1×10^{-5} M *R*-PIA and experiments were performed as described in Methods. The ordinate represents specific binding in nanomolars. Nonspecific binding at 3 nM was ~ 30%. The data points are means of triplicate determinations. The curves were fitted with the aid of a computer modeling program (33, 34) based on the law of mass action, assuming a one-affinity state, which most appropriately describes the data. This is a representative experiment of seven experiments showing similar results.

values averaging 2.9±0.3 and 2.7±0.3 nM for control and caffeine-treated rats, respectively.

To assess receptor-G protein coupling, *R*-PIA competition curves were constructed versus [^{3}H]XAC in control and caffeine-treated rat cortical membranes and are shown in Fig. 2. These competition curves are shallow, with slopes (mean±SEM) of 0.52±0.08 and 0.62±0.08 for control and caffeine-treated rats, respectively. Shallow curves with slopes significantly less than unity suggest the interaction of the agonist with multiple affinity states of the receptor. Computer-assisted analysis of competition data (33, 34) was used to determine high and low affinity constants (K_H and K_L) as well as the proportion of receptors in each affinity state. In control membranes a two affinity state model most appropriately described the data ($P < 0.001$). K_H and K_L values averaged 2.1 and 404 nM, respectively, with the percentage of receptors in these respective affinity states being 50.4 and 49.6% (Table I). Caffeine treatment produced a leftward shift in the *R*-PIA competition curve (Fig. 2). In four of the animals studied, a two affinity state model was most appropriate ($P < 0.001$). In contrast with the control situation, however, 69% of the receptors were in the high affinity state compared to ~ 50% in control membranes (Table I). There was no significant change in K_H and K_L between the controls and these caffeine-treated rats. Membranes from the remaining three treated animals could be analyzed only with a single affinity state model, and in these cases the single K_I was similar to the high affinity state (K_H) found in the two state model, suggesting that all the receptors (within a ~ 10% error margin) were in the high affinity state. A shift to all high affinity state is consistent with previous findings from our laboratory using the agonist radioligand [^{3}H]PIA (22).

It has previously been observed that caffeine treatment increased the inhibitory actions of *R*-PIA on adenylate cyclase in rat cerebral cortex (22). Using 10 μM *R*-PIA in the cAMP assay mixture, we have confirmed this observation. In control membranes, 10 μM *R*-PIA inhibited adenylate cyclase activity by 14.4±1.2% ($n = 9$), while in cerebral cortical membranes obtained from caffeine-treated rats the inhibitory effect was increased to 18.5±1.4 ($n = 9$) (statistical significant difference, $P < 0.05$). No significant changes in basal, forskolin- and fluoride-stimulated adenylate cyclase activity between control and caffeine-treated rats were observed (not shown).

The observation that caffeine treatment increased the percentage of receptors in the agonist specific high affinity state and the maximal inhibitory effect of *R*-PIA suggested to us

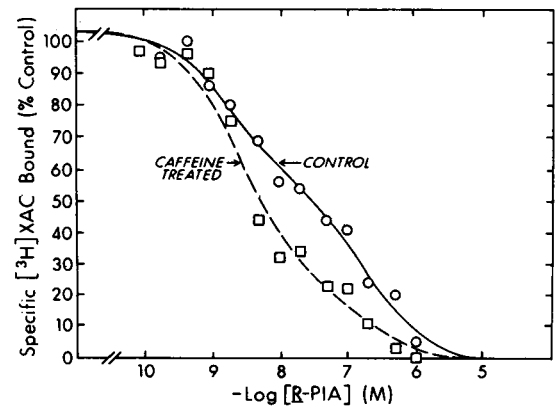

Figure 2. *R*-PIA competition curves in cerebral cortical membranes obtained from control and caffeine-treated rats. Membranes (~ 50 μg protein) were incubated with 2.5 nM of [^{3}H]XAC and increasing concentrations of *R*-PIA using experimental conditions as described in Methods. The curves were fitted by computer modeling (33, 34), assuming a two affinity state model which most appropriately describes the data ($P < 0.001$). This is a representative experiment of four to seven experiments with similar results.

Table I. Effect of Caffeine Treatment on R-PIA Competition Curves in Rat Cerebral Cortex

Treatment	Binding parameters				
	K_I	K_H	K_L	$\%R_H$	$\%R_L$
		nM			
Control					
2-state fit (7)		2.1±0.8	404±330	50.4±4.1	49.6±4.1
Caffeine					
1-state fit (3)	3.9±0.7*			100	
2-state fit (4)		1.4±0.2	161±56	68.9±7.4‡	31.1±7.4‡

Values are reported as the mean±SEM. K_I is the equilibrium dissociation constant obtained assuming a 1-state fit. K_H and K_L are the equilibrium dissociation constants for the agonist high- and low-affinity sites, while $\%R_H$ and $\%R_L$ are the percent of receptors in the respective affinity states. These latter values are obtained assuming a 2-state fit. A two-affinity state model was retained only when it significantly improved the fit over a one-affinity state model. Data obtained from three of seven caffeine-treated rats could only be analyzed using a 1-state model. All control competition curves were most appropriately analyzed by a 2-state fit (2-state fits were preferred; $P < 0.001$).

* Unique high affinity state found that is not significantly different from the K_H values obtained from control and caffeine-treated rats.

‡ Statistically significant from the respective control value ($P < 0.04$).

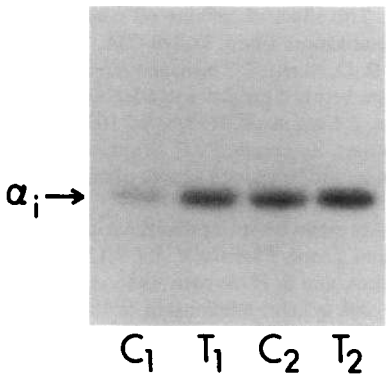

Figure 3. Pertussis toxin-catalyzed labeling of G_i in rat cerebral cortical membranes from control and caffeine treated animals. Membranes (~ 60 μg protein) from both control (C) and treated (T) rats were incubated at 30°C for 15 min in presence of 16 μg/ml (C_1, T_1) or 32 μg/ml (C_2, T_2) of activated pertussis toxin and 5 μM [^{32}P]NAD. Details of other experimental conditions are described in Methods. Labeled membranes were pelleted, solubilized, and subjected to SDS-PAGE. Densitometric scanning of the autoradiograph show increases in labeling of G_i induced by caffeine of 200% (T_1) and 135% (T_2) over the respective controls (C_1 and C_2). Similar increases in labeling of G_i were observed in seven of eight cortices tested.

that the inhibitory guanine nucleotide regulatory protein (G_i) might also be regulated by chronic administration of this drug. Therefore, we quantitated the levels of G_i (α_i subunit) in cerebral cortices from control and caffeine-treated rats by pertussis toxin-catalyzed [^{32}P]NAD incorporation into this protein. Fig. 3 shows the results of one such experiment, utilizing two different concentrations of pertussis toxin. Laser densitometric scanning of the autoradiographs shows increases in labeling of G_i by 200 and 135% of control, using 16 and 32 μg/ml of pertussis toxin, respectively. Increases in G_i labeling in cerebral cortices of caffeine-treated rats, using 32 μg/ml pertussis toxin averaged 132.9±8.3% (mean±SEM; n = 7), with values ranging from 128 to 170%. These increases were statistically different from control ($P < 0.01$). Labeling of G_s by cholera toxin in the brain was also attempted to test possible changes in the level of this protein. However, we, like others (36), were unable to label this protein in brain membranes. This likely relates to the very high levels of NADase activity in brain and the fact that the K_m of NAD for cholera toxin catalyzed ADP-ribosylation is ~ 1 mM (37) while that for the pertussis toxin catalyzed reaction is only 10–20 μM (38).

Labeling of both G_s and G_i in rat adipocyte membranes was undertaken in order to determine whether the increase in G_i observed in the brain following caffeine ingestion was observed in other tissues. No significant changes in the levels of these G proteins were observed. The levels of G_s and G_i were 84.9±12.9 and 99.5±29% (mean±SEM; n = 5) of the respective control values. This suggests that the changes in the brain are likely specific and not simply a nonspecific effect of caffeine in general.

Discussion

The present results indicate that chronic caffeine treatment regulates the A_1 adenosine receptor-adenylate cyclase system of the rat cerebral cortex at multiple levels. Not only are receptor number and receptor-G protein coupling modulated, but the quantity of the α_i subunit of the G_i protein is also perturbed. These changes may represent a concerted effort of the brain to offset the receptor antagonistic effects of caffeine at A_1 adenosine receptors.

Adenosine plays a significant role in the functioning of the central nervous system. The purine produces marked sedation (24, 39–41) and anticonvulsant activity (40). In addition, administration of adenosine analogues to animals produces analgesia (24, 42, 43). These behavioral effects are purportedly mediated by activation of adenosine receptors since they are attenuated by methylxanthines (8, 24), which are antagonists of these receptors (8–13). In vitro, adenosine inhibits spontaneous firing of central neurons (44) and the release of various neurotransmitters (10, 45–49). Again, these effects are likely mediated by adenosine receptors (10, 48, 49) of the A_1 subtype (48, 49). Due to their important role in the central nervous system, it is expected that regulation of adenosine receptors and/or the adenosine receptor-adenylate cyclase system will significantly affect brain functions.

The initial focus of this study was to determine whether caffeine treatment produces an increase in total A_1 receptor number (up-regulation) and/or agonist high affinity sites. For these studies we employed [^{3}H]XAC, an antagonist radioligand for the A_1 adenosine receptors. In contrast to agonists, antagonists do not discriminate between different affinity states of the receptor and the B_{max} determined from an antagonist radioligand reflects the total population of receptors. Thus, with the use of this radioligand we could differentiate between increases in receptor number versus enhanced coupling. As observed in Fig. 1, caffeine treatment significantly increased the binding of [^{3}H]XAC in cerebral cortices, suggesting that the methylxanthine increased the total number of A_1 adenosine receptors. R-PIA competition curves were generated to determine the effects of caffeine on agonist high (K_H) and low (K_L) affinity states, along with the percentage of receptors in each state (R_H and R_L, respectively). Previous studies, using agonist radioligands, have shown that caffeine treatment increases agonist high affinity states of the A_1 receptor (22, 23). The use of agonist radioligands provides only limited information concerning the low affinity state since the concentrations of radioligand required to saturate this site are associated with high levels of nonspecific binding. Our results indicate that caffeine treatment selectively increases the population of A_1 adenosine receptors in the high affinity state (Table I), in agreement with previous studies (22, 23). No change in the estimated K_H and K_L were observed (Table I). Three of seven competition curves obtained from caffeine-treated rats were most appropriately described by a one state fit with unique K_I values corresponding to the K_H of control membranes (Table I). Thus, in these three cases, a complete shift to high affinity state was effected.

In accordance with the observed increase in agonist high affinity sites induced by caffeine, data from our laboratory demonstrate an augmented inhibitory effect of R-PIA on adenylate cyclase (the present data, 22) in treated over control animals. This most likely reflects the increase in receptor-G_i coupling inferred from the R-PIA competition curves described above. The effect of such a change at the cellular level would be to restore intracellular cyclic AMP to normal levels. It is possible that this might account, in part, for the development of tolerance to the physiological effects of caffeine (17, 18). Furthermore, abrupt withdrawal of caffeine from animals chronically treated with the drug will expose the sensitized A_1 adenosine receptor-adenylate cyclase complex to maximal

stimulation by endogenous adenosine, leading to increased inhibition of adenylate cyclase and possibly accounting for caffeine withdrawal syndrome described clinically (19–21).

Regulation of the quantity and function of G proteins represents a novel means by which the cell adapts to prolonged drug administration. Infusion of *R*-PIA for six days, for example, leads to a significant reduction in pertussis toxin-mediated labeling of the α_i subunit of G_i, together with a reciprocal change in cholera toxin-mediated labeling of G_s in rat adipocytes (28). It is known that G proteins couple receptors to their effectors and since the present data suggest enhanced coupling of A_1 adenosine receptors to G_i following caffeine treatment, we were interested in determining whether G_i was regulated by this treatment. As observed in Fig. 3, a significant increase in pertussis toxin labeling of G_i in rat cerebral cortex could be demonstrated following caffeine treatment. This might account for the observed increase in agonist high affinity state, in addition to the enhancement in *R*-PIA inhibition of adenylate cyclase. Whether changes in G_s also occur is not known, since we, like others (36), were unable to quantitate this G protein by cholera toxin labeling in rat cerebral cortex.

In summary, the present data indicate regulation of the A_1 adenosine receptor inhibitory system by caffeine at three levels: the A_1 adenosine receptor, A_1 receptor-G protein coupling and α_i. Caffeine increases the total number of A_1 adenosine receptors together with the proportion of these receptors in the high affinity state recognized by *R*-PIA, the latter effect reflecting increased receptor-G_i coupling. Taken together, these changes could account for the sensitization of the A_1 receptor inhibitory system to endogenous adenosine. These different means of adaptation might account for the development of tolerance to the central actions of caffeine along with the central nervous system manifestation of caffeine withdrawal syndrome. Future experiments in this area are required before such a connection can be established.

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