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Involvement of Adenosine A_1 and A_{2A} Receptors in the Motor Effects of Caffeine after its Acute and Chronic Administration

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The involvement of adenosine A_1 and A_{2A} receptors in the motor effects of caffeine is still a matter of debate. In the present study, counteraction of the motor-depressant effects of the selective A_1 receptor agonist CPA and the A_{2A} receptor agonist CGS 21680 by caffeine, the selective A_1 receptor antagonist CPT, and the A_{2A} receptor antagonist MSX-3 was compared. CPT and MSX-3 produced motor activation at the same doses that selectively counteracted motor depression induced by CPA and CGS 21680, respectively. Caffeine also counteracted motor depression induced by CPA and CGS 21680 at doses that produced motor activation. However, caffeine was less effective than CPT at counteracting CPA and even less effective than MSX-3 at counteracting CGS 21680. On the other hand, when administered alone in habituated animals, caffeine produced stronger motor activation than CPT or MSX-3. An additive effect on motor activation was obtained when CPT and MSX-3 were coadministered. Altogether, these results suggest that the motor-activating effects of acutely administered caffeine in rats involve the central blockade of both A_1 and A_{2A} receptors. Chronic exposure to caffeine in the drinking water (1.0 mg/ml) resulted in tolerance to the motor effects of an acute administration of caffeine, lack of tolerance to amphetamine, apparent tolerance to MSX-3 (shift to the left of its 'bell-shaped' dose–response curve), and true cross-tolerance to CPT. The present results suggest that development of tolerance to the effects of A_1 receptor blockade might be mostly responsible for the tolerance to the motor-activating effects of caffeine and that the residual motor-activating effects of caffeine in tolerant individuals might be mostly because of A_{2A} receptor blockade.
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INTRODUCTION

It is generally accepted that counteraction of an endogenous adenosine tone on central adenosine receptors is the main mechanism of action responsible for the motor-activating effects of caffeine in rodents and primates (Howell *et al*, 1997; Daly and Fredholm, 1998; Fredholm *et al*, 1999). So far, four adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3) have been cloned and pharmacologically characterized (Fredholm *et al*, 2001). Caffeine is a nonselective competitive A_1 and A_{2A} receptor antagonist and both receptors have similar affinities for caffeine (Daly and Fredholm, 1998; Fredholm *et al*, 1999). Adenosine, by acting on both A_1 and A_{2A} receptors, is implicated in the regulation of motor activity. There is clear experimental evidence for a central mediation of the motor-depressant effects of A_1 and A_{2A} receptor agonists (Snyder *et al*, 1981; Katz and Goldberg, 1987; Nikodijevic *et al*, 1990; Barraco *et al*, 1993; Marston *et al*, 1998), independent of their pronounced cardiovascular effects (Appel *et al*, 1995; Mathot *et al*, 1995). In contrast, some contradictory results exist regarding the motor-activating effects of adenosine antagonists and there is no consensus about the relative involvement of A_1 and A_{2A} receptors in caffeine-induced motor activation.

A_1 receptors were initially thought to be the main target mediating the motor-activating effects of caffeine. Hence, Snyder *et al* (1981) showed a significant correlation of the potencies of different methylxanthines in producing motor activation in mice with their potencies in displacing binding of a radiolabeled A_1 receptor agonist. However, with the discovery of the high-affinity A_{2A} receptors and their particularly dense localization in the striatum, and with the availability of more selective compounds, findings from a series of behavioral experiments suggested that A_{2A}

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receptors were, instead, the main target responsible for mediating the motor-activating effects of caffeine in rodents (reviewed in Ferré *et al*, 1992). This hypothesis has been strengthened by recent findings demonstrating the ability of A_{2A} antagonists, and the inability of some A_1 receptor antagonists to reproduce some biochemical and behavioral effects of caffeine (Svenningsson *et al*, 1997; Marston *et al*, 1998; Linskog *et al*, 2002). Furthermore, caffeine was found to be ineffective in producing motor activation in A_{2A} receptor knockout mice (Ledent *et al*, 1997; El Yacoubi *et al*, 2000). However, these results might indicate that A_{2A} receptors are necessary, but not sufficient, for caffeine to produce motor activation, and the role of A_1 receptors cannot be discarded. In fact, Jacobson and co-workers found evidence for the existence of synergism with the motor-depressant effects induced by A_1 and A_{2A} agonists and the motor-activating effects of A_1 and A_{2A} antagonists (Nikodijevic *et al*, 1991; Jacobson *et al*, 1993). The same authors showed that a reliable method for determining central A_1 and A_{2A} receptor antagonism of a compound after systemic administration is to study the differential potency for counteraction of the motor-depressant effects of selective A_1 and A_{2A} receptor agonists (Nikodijevic *et al*, 1991; Jacobson *et al*, 1993; see also, Marston *et al*, 1998). Unfortunately, no clear systematic study comparing counteraction of the motor-depressant effects of selective A_1 and A_{2A} receptor agonists by caffeine has yet been reported.

An additional unresolved issue about caffeine is the strong tolerance that develops to many of its behavioral effects after chronic treatment (Holtzman and Finn, 1988; Howell *et al*, 1997). Cross-tolerance may develop to the behavioral effects of drugs that share common mechanisms of action. In fact, cross-tolerance between the motor-activating effects of caffeine and the nonxanthine, non-selective adenosine antagonist CGS 15943 has been demonstrated (Holtzman, 1991). However, experiments studying the motor effects of selective A_1 and A_{2A} antagonists in caffeine-tolerant animals are lacking.

In the present study, by comparing the effects of caffeine with the effects of the selective A_1 and A_{2A} receptor antagonists CPT and MSX-3 (Maemoto *et al*, 1997; Sauer *et al*, 2000), we obtained evidence for the involvement of both A_1 and A_{2A} receptors in the motor-activating effects of caffeine after its acute administration. Also, by comparing the effects of CPT, MSX-3, and amphetamine (as a motor-activating drug with a different mechanism of action) during chronic exposure to caffeine in the drinking water, we obtained evidence suggesting that development of tolerance to the effects of A_1 receptor blockade might be mostly responsible for tolerance to the motor-activating effects of caffeine, while the residual motor activating effects of caffeine in tolerant individuals might be because of A_{2A} receptor blockade.

METHODS

Subjects

Experimentally naive, male Sprague–Dawley rats (Charles River Lab., Wilmington, MA), weighing 250–280 g at the beginning of the study, were used. Rats were acclimated to laboratory conditions and allowed to drink tap water and feed *ad libitum* for at least 2 weeks before starting of the experiments. All rats were housed in a temperature- and humidity-controlled room with a 12-h light/dark cycle (from 7:00 am to 7:00 pm lights on). Experiments were conducted between 10:00 am and 6:00 pm. Animals used in this study were maintained in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care and all experimentation was conducted in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the Guide for Care and Use of Laboratory Animals (National Research Council, 1996).

Drugs

Caffeine (anhydrate base), D-amphetamine sulfate, the adenosine A_1 receptor agonists N^6-cyclopentyladenosine (CPA) and N^6-cyclohexyladenosine, the adenosine A_{2A} receptor agonist 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS 21680), and the adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPT) were purchased from Sigma Chemical Company (St Louis, MO). The adenosine A_{2A} receptor antagonist 3-(3-hydroxypropyl)-8-(*m*-methoxystyryl)-7-methyl-1-propargylxanthine phosphate disodium salt (MSX-3) was synthesized at the Pharmaceutical Institute, University of Bonn, Germany. All drugs were dissolved in sterile saline (with a few drops of 0.1 N NaOH for MSX-3; final pH: 7.4) and administered intraperitoneally (i.p.) in a volume of 3 ml/kg of body weight. Caffeine was also administered orally in the drinking water (see below).

Chronic Caffeine Exposure

Caffeine was administered chronically for 14 days by giving animals free access to bottles containing either 0.25 or 1.0 mg/ml caffeine anhydrate base solution in tap water (see Gasior *et al*, 2000). Caffeine intake was monitored throughout the experiment. Daily caffeine intake (mg/kg/day) was estimated based on the subject's fluid consumption over a 24-h period and its body weight. Daily water intake in rats not exposed to caffeine (control group) was monitored for comparison.

Motor Activity Recording

A Columbus Instruments Auto-Track system (Coulbourn Instruments, Lehigh Valley, PA) with a resolution of 0.1 s was used to record motor activity. Each of the three sound-attenuation chambers enclosed two clear, Plexiglas cages ($26.4 \times 26.4\ cm^2$, 44 cm in height) bedded with sawdust. The cages were positioned within the chamber, one cage resting on the floor of the chamber, the other on a shelf midway up the chamber. These cages were equipped with a 15×15 array of photocells spaced every 2.4 cm near the base of the cage (horizontal beams). Any movement that interrupted a photobeam was recorded as a motor count, and this event provided no feedback to the rat. A dim red light (power indicator light) was positioned above each cage. General motor activity was defined as the number of horizontal beams that was interrupted by the subject during a

particular interval. All animals tested were brought to the experimental room 1 h prior to the start of the experiment. Depending on the experiment, motor activity was measured in animals habituated (30 min) or nonhabituated to the activity cages. In experiments dealing with adenosine agonists motor activity (total accumulated motor counts) was measured for 30 min. This is the period of time of maximal exploratory activity when rats are exposed to a new environment and, also, the period of maximal effect of adenosine agonists (as found in pilot experiments). In experiments with combined administration of adenosine agonists (CPA or CGS 21680) and adenosine antagonists (caffeine, CPT or MSX-3), the latter were administered in the home cages 10 min before adenosine agonists. In the experiments dealing with adenosine antagonists without coadministration of adenosine agonists motor activity was measured for 60 min. All animals were used only once. Statistical comparisons among differently treated groups were made with one-way ANOVA, followed by Newman–Keuls *post hoc* tests. ED_{50} values were calculated by nonlinear regression analysis (GraphPad Prism Software, Inc., San Diego, CA).

Radioligand Binding Experiments

Saturation experiments with the radiolabeled selective A_1 receptor antagonist [^{3}H]8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX; NEN, Boston MA) and the radiolabeled selective A_{2A} receptor antagonist [^{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; Tocris, Bristol) were performed in striatal membrane preparations from rats chronically treated with caffeine (14 days) and control nontreated animals. The experimental conditions for [^{3}H]DPCPX and [^{3}H]ZM241385 binding were the same as those previously reported for selectively labeling adenosine A_1 and A_{2A} receptors, respectively, in the rat brain (Maemoto *et al*, 1997; Alexander and Millns, 2001). The rats were killed by decapitation and the brain was rapidly removed and the striata dissected out. The striatal membrane preparation was performed as previously described (Ferré *et al*, 1991) with the addition of a preincubation step with adenosine deaminase (5 U/ml; Sigma) for 30 min at 37°C. In the experiments with [^{3}H]DPCPX (120 Ci/mmol), the incubation buffer was: Tris-HCl (50 mM, pH 7.4) containing 2 mM $MgCl_2$. In the experiments with [^{3}H]ZM241385 (17 Ci/mmol), the incubation buffer was: Tris-HCl buffer (50 mM, pH 7.4) containing 120 mM NaCl, 5 mM KCl, and 1 mM EDTA. The final protein concentration was 0.2 mg/ml. Saturation experiments with [^{3}H]DPCPX (final concentration 0.1–6 nM) were made by incubation for 2 h at room temperature and the adenosine A_1 agonist N^6-cyclohexyladenosine (40 μM) was used to define nonspecific binding. Saturation experiments with [^{3}H]ZM241385 (final concentration 0.1–5 nM) were made by incubation for 1 h at room temperature and the preferential adenosine A_{2A} antagonist 3,7-dimethyl-1-propargylxanthine (100 μM) was used to define nonspecific binding. The incubation was stopped by fast filtration through glass-fiber filters (GF/B, Whatman, Millipore, Bedford, MA) by washing three times with 5 ml ice-cold Tris-HCl (50 mM, pH 7.4) with an automatic cell harvester (Brandel, Gaithersburg, MD). The radioactivity content of the filters was detected by liquid scintillation spectrometry. Data from saturation experiments were analyzed by nonlinear regression analysis (GraphPad) for the determination of dissociation constants (K_D) and the number of antagonist binding sites (B_{max}). Statistical comparisons of K_D and B_{max} values from saturation experiments from rat striatal membrane preparations of differently treated groups were made with one-way ANOVA, followed by Newman–Keuls *post hoc* tests.

RESULTS

Motor Activation Induced by Caffeine and the Selective Adenosine A_1 and A_{2A} Receptor Antagonists CPT and MSX-3 in Habituated Rats

As previously described (Gasior *et al*, 2000; for reviews, see Daly and Fredholm, 1998; Fredholm *et al*, 1999), caffeine produced motor activation with a 'bell-shaped' dose–response curve (Figure 1). A similar pattern was also obtained with CPT and MSX-3, compounds which have been previously reported to be very selective A_1 and A_{2A} receptor antagonists, respectively, in membrane preparations of rat striatum (Maemoto *et al*, 1997; Sauer *et al*, 2000). The order of potencies was MSX-3 > CPT > caffeine, with peak effects at 3, 4.8, and 30 mg/kg, respectively. On the other hand, caffeine was more efficient (about a five-fold increase in motor activity, with respect to basal values) than CPT and MSX-3 (about a three-fold increase in both cases) (Figure 1).

Motor Depression Induced by the Selective Adenosine A_1 and A_{2A} Receptor Agonists CPA and CGS 21680 in NonHabituated Rats

As previously described (Rimondini *et al*, 1997; Marston *et al*, 1998), both the A_1 receptor agonist CPA and the A_{2A} receptor agonist CGS 21680 produced a dose-dependent motor depression (Figure 2). CPA was slightly more potent

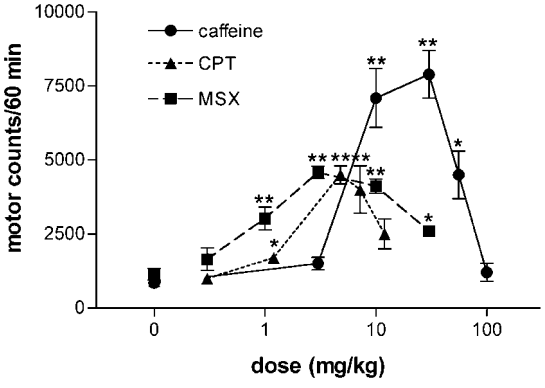

Figure 1 Motor activation produced by caffeine and the selective adenosine A_1 and A_{2A} receptor antagonists CPT and MSX-3 in habituated rats. Results represent means ± SEM of total accumulated counts during the 60-min period of observation. Drugs were administrated after 30 min of habituation of the animals to the activity cages. *, **Significantly different as compared with the corresponding control (0 mg/kg) (ANOVA with *post hoc* Newman–Keuls comparisons, $p<0.05$ and $p<0.01$, respectively; $n=6$–8/group).

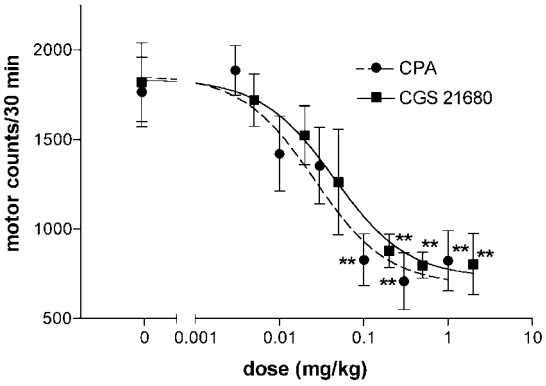

Figure 2 Motor depression produced by the selective adenosine A_1 and A_{2A} receptor agonists CPA and CGS 21680 in nonhabituated rats. Results represent means ± SEM of total accumulated counts during the 30-min period of observation. CPA and CGS 21680 were administrated just before the animals were placed in the activity cages. **Significantly different as compared with the corresponding control (0 mg/kg) (ANOVA with *post hoc* Newman–Keuls comparisons, $p<0.01$; $n=6$–8/group). ED_{50} values for CPA (0.03 mg/kg) and CGS 21680 (0.05 mg/kg) were calculated by nonlinear regression analysis (GraphPad).

than CGS 21680, with ED_{50} values of 0.03 and 0.05 mg/kg, respectively (nonlinear regression analysis; GraphPad). The efficacy of both compounds was similar and the minimal doses with maximal effect were 0.1 and 0.2 mg/kg for CPA and CGS 21680, respectively.

Counteraction of Adenosine Agonist-Induced Motor Depression by the Selective Adenosine A_1 Receptor Antagonist CPT

In nonhabituated rats, CPT dose dependently antagonized the motor-depressant effects of CPA (Figure 3) at the same doses that produced motor activation in habituated rats (Figure 1). On the other hand, only the highest dose of CPT (4.8 mg/kg) could significantly, but partially, counteract the motor-depressant effects of a low, but not high, dose of CGS 21680 (Figure 3). The weak CPT-mediated counteraction of CGS 21680-induced motor depression is most probably related to behavioral competition, since 4.8 mg/kg CPT induced maximal motor activation in habituated rats (Figure 1). Behavioral competition, therefore, might also be a contributing factor in CPT-induced counteraction of CPA-mediated motor depression.

Counteraction of Adenosine Agonist-Induced Motor Depression by the Selective Adenosine A_{2A} Receptor Antagonist MSX-3

MSX-3 produced effects exactly opposite to those of CPT. In nonhabituated rats, MSX-3 dose dependently antagonized the motor-depressant effects of CGS 21680 (Figure 4) at the same doses that produced motor activation in habituated rats (Figure 1). On the other hand, only the highest dose of MSX-3 (3 mg/kg) could significantly, but partially, counteract the motor-depressant effects of a low, but not a high, dose of CPA (Figure 4). Again, the weak MSX-3-mediated counteraction of CPA-induced motor depression might be related to behavioral competition, and behavioral competition might also be a contributing factor in MSX-3-induced counteraction of CGS 21680-mediated motor depression.

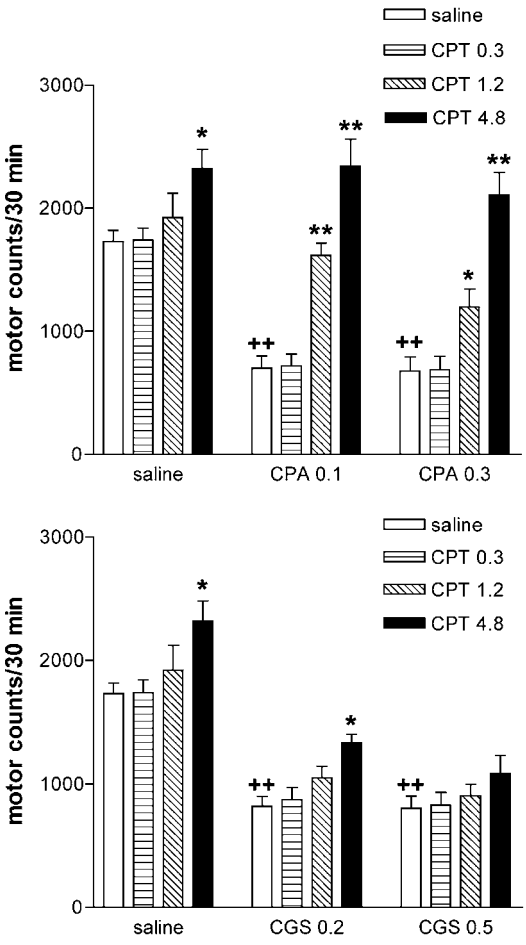

Figure 3 Counteraction of adenosine agonist-induced motor depression by the selective adenosine A_1 receptor antagonist CPT. Results represent means ± SEM of total accumulated counts during the 30-min period of observation. The animals were not habituated to the activity cages. The adenosine A_1 and A_{2A} receptor agonists CPA (0.1 or 0.3 mg/kg) and CGS 21680 (0.2 or 0.5 mg/kg) were administrated just before the animals were placed in the activity cages. CPT (0.3, 1.2 or 4.8 mg/kg) was administered 10 min before CPA or CGS 21680. *, **Significantly different as compared with the group that received the same dose of CPA or CGS 21680 without previous administration of CPT (saline) (ANOVA with *post hoc* Newman–Keuls comparisons, $p<0.05$ and $p<0.01$, respectively). ++Significantly different as compared with the group that received neither CPT nor CPA or CGS 21680 (saline–saline) (ANOVA with *post hoc* Newman–Keuls comparisons, $p<0.01$; $n=6$–8/group).

Counteraction of Adenosine Agonist-Induced Motor Depression by Caffeine

In nonhabituated rats, caffeine dose dependently counteracted the motor depression induced by both CPA and CGS 21680 (Figure 5) at the same doses that produced motor activation in habituated rats (Figure 1). However, caffeine was less effective at counteracting the depressant effects of CGS 21680 than CPA. Also, caffeine was less effective than CPT at counteracting CPA-induced motor depression (compare Figures 5 and 3). These results show that, at the relevant motor-activating doses, caffeine has the profile of a

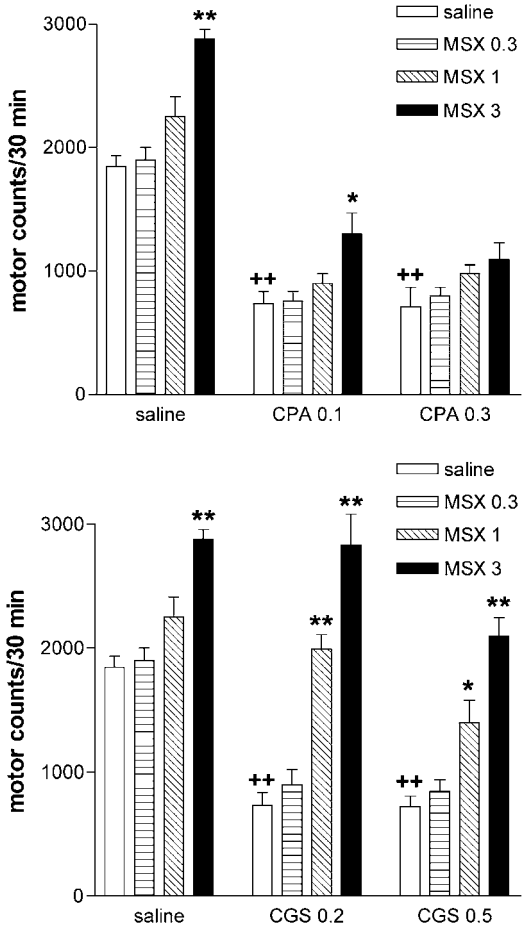

Figure 4 Counteraction of adenosine agonist-induced motor depression by the selective adenosine A_{2A} receptor antagonist MSX-3. Results represent means ± SEM of total accumulated counts during the 30-min period of observation. The animals were not habituated to the activity cages. The adenosine A_1 and A_{2A} receptor agonists CPA (0.1 or 0.3 mg/kg) and CGS 21680 (0.2 or 0.5 mg/kg) were administrated just before the animals were placed in the activity cages. MSX-3 (0.3, 1, or 3 mg/kg) was administered 10 min before CPA or CGS 21680. *, **Significantly different as compared with the group that received the same dose of CPA or CGS 21680 without previous administration of MSX-3 (saline) (ANOVA with *post hoc* Newman–Keuls comparisons, $p<0.05$ and $p<0.01$, respectively). ++Significantly different as compared with the group that received neither MSX-3 nor CPA or CGS 21680 (saline–saline) (ANOVA with *post hoc* Newman–Keuls comparisons, $p<0.01$; $n=6$–8/group).

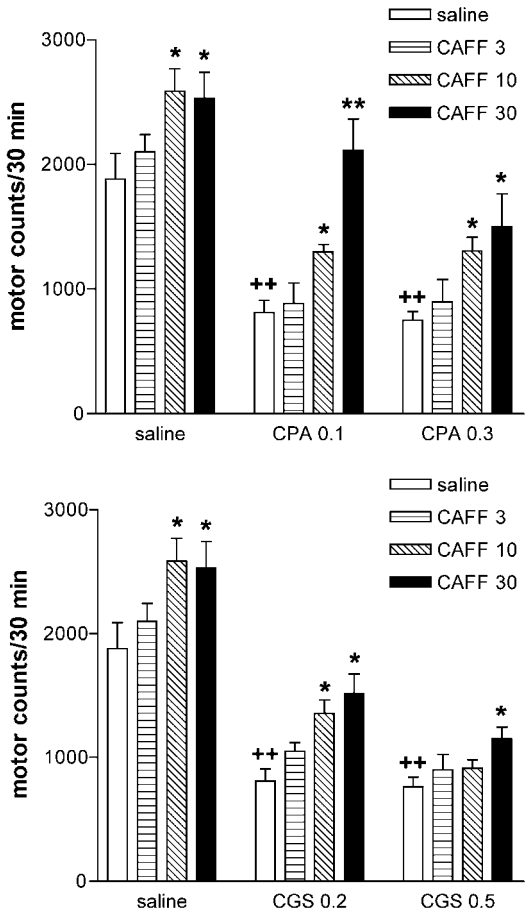

Figure 5 Counteraction of adenosine agonist-induced motor depression by caffeine. Results represent means ± SEM of total accumulated counts during the 30-min period of observation. The animals were not habituated to the activity cages. The adenosine A_1 and A_{2A} receptor agonists CPA (0.1 or 0.3 mg/kg) and CGS 21680 (0.2 or 0.5 mg/kg) were administrated just before the animals were placed in the activity cages. Caffeine (CAFF 3, 10, or 30 mg/kg) was administered 10 min before CPA or CGS 21680. *, **Significantly different as compared with the group that received the same dose of CPA or CGS 21680 without previous administration of caffeine (saline) (ANOVA with *post hoc* Newman–Keuls comparisons, $p<0.05$ and $p<0.01$, respectively). ++Significantly different as compared with the group that received neither caffeine nor CPA or CGS 21680 (saline–saline) (ANOVA with *post hoc* Newman–Keuls comparisons, $p<0.01$; $n=6$–8/group).

nonselective adenosine antagonist, but one with preferential A_1 receptor antagonism.

Motor Activation Induced by Coadministration of the Adenosine A_1 and A_{2A} Receptor Antagonists CPT and MSX-3

A threshold dose of MSX-3 (0.3 mg/kg; see Figure 1) did not potentiate the motor effects of either a low or high dose (1.2 or 4.8 mg/kg, respectively) of CPT in habituated rats (Figure 6). However, a low dose of MSX-3 (1 mg/kg) significantly increased the motor activity induced by the maximal effective dose of CPT (4.8 mg/kg; see Figures 1 and 6). Similarly, a low dose of CPT (1.2 mg/kg), but not a high dose (4.8 mg/kg), significantly increased the maximal effective dose of MSX-3 (3 mg/kg; Figures 1 and 6). The apparently additive effect of the motor effects induced by blockade of A_1 receptors by the concomitant moderate blockade of A_{2A} receptors might, therefore, underlie the motor-activating effects of caffeine.

Effect of Chronic Treatment with Caffeine on the Motor Activation Induced by Caffeine, Amphetamine and the Adenosine A_1 and A_{2A} Receptor Antagonists CPT and MSX-3

No differences were observed in weight gain or fluid intake among the groups exposed to concentrations of 0.0, 0.25, or 1.0 mg/ml of caffeine in the drinking water for 14 days (data not shown). The average caffeine consumption was 33.3 ± 4.7 and 120.7 ± 4.1 mg/kg/day ($n=48$, in both cases)

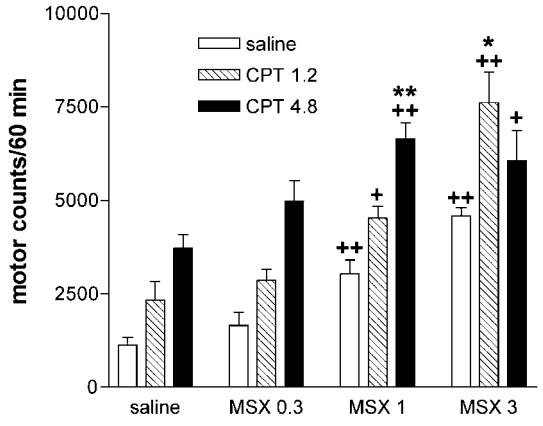

Figure 6 Motor activation induced by coadministration of the adenosine A_1 and A_{2A} receptor antagonists CPT and MSX-3 in habituated rats. Results represent means ± SEM of total accumulated counts during the 60-min period of observation. CPT (1.2 or 4.8 mg/kg) and/or MSX-3 (0.3, 1, or 3 mg/kg) were administrated after 30 min of habituation of the animals to the activity cages. $^{+,\ ++}$Significantly different as compared with the group that received the same dose of CPT without coadministration of MSX-3 (ANOVA with *post hoc* Newman–Keuls comparisons, $p<0.5$ and $p<0.01$, respectively). $^{*,\ **}$Significantly different as compared with the group that received the same dose of MSX-3 without coadministration of CPT (ANOVA with *post hoc* Newman–Keuls comparisons, $p<0.5$ and $p<0.01$, respectively; $n=6$–8/group).

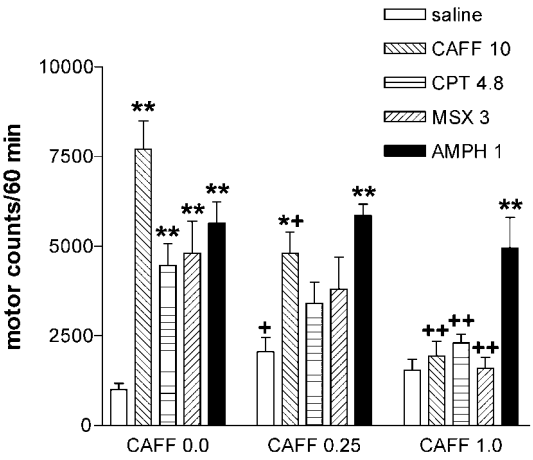

Figure 7 Effect of chronic treatment with caffeine on the motor activation induced by the acute administration of caffeine, the A_1 and A_{2A} receptor antagonists CPT and MSX-3, or amphetamine in habituated rats. The effect of the acute administration of caffeine, CPT, MSX-3, and amphetamine was analyzed after 14 days of chronic treatment with caffeine (0.25 or 1.0 mg/ml in the drinking water, without withdrawal). Results represent means ± SEM of total accumulated counts during the 60-min period of observation. Caffeine (10 mg/kg; CAFF 10), CPT (4.8 mg/kg; CPT 4.8), MSX-3 (3 mg/kg; MSX-3), or amphetamine (1 mg/kg; AMPH 1) were administrated after 30 min of habituation of the animals to the activity cages. $^{*,\ **}$Significantly different as compared with the group that received the same chronic caffeine treatment but only received an acute administration of saline (ANOVA with *post hoc* Newman–Keuls comparisons, $p<0.05$ and $p<0.01$, respectively). $^{+,\ ++}$Significantly different as compared with the group that did not receive chronic caffeine treatment (CAFF 0.0) and received the same acute dose of caffeine, CPT, MSX-3 or amphetamine (ANOVA with *post hoc* Newman–Keuls comparisons, $p<0.05$ and $p<0.01$, respectively; $n=6$–8/group).

after exposure to 0.25 and 1.0 mg/ml solutions, respectively. As previously reported (Holtzman and Finn, 1988), chronic exposure to 1.0 mg/ml of caffeine in the drinking water resulted in an apparent tolerance to the motor-activating effects of an acutely administered dose of caffeine (10 mg/kg), but not of amphetamine (1 mg/kg), in habituated rats (Figure 7). Also in agreement with previous studies (Holtzman and Finn, 1988; Svenningsson *et al*, 1999), but in contrast to some of our recently reported results (Gasior *et al*, 2000), chronic exposure to lower concentrations of caffeine in the drinking water (0.25 mg/ml; 0.3 mg/ml in the study by Svenningsson *et al*, 1999) was associated with partial but significant tolerance (Figure 7). A small increase in motor activity was observed in control animals chronically exposed to caffeine when they were administered saline before recording motor activity, which was only significant for the dose of 0.25 mg/ml (Figure 7). Chronic exposure to 1.0 mg/ml, but not to 0.25 mg/ml, of caffeine in the drinking water also resulted in a significant apparent cross-tolerance to the motor-activating effects of high doses of both CPT (4.8 mg/kg) and MSX-3 (3 mg/kg) in habituated rats (Figure 7). In view of the 'bell-shaped' dose–response curves of caffeine, CPT and MSX-3 (Figure 1), and in order to control an accumulative effect, additional experiments with different acutely administered doses of these compounds were performed in rats exposed to 1.0 mg/ml of caffeine. In fact, a shift to the left of the dose–response curve of MSX-3 was observed, with a significant motor activation induced by 1 mg/kg of MSX-3 (Figure 8). This effect was, however, lower than the maximal effect obtained in animals without chronic exposure with caffeine (with the dose of 3 mg/kg of MSX-3; see Figures 1 and 7). On the other hand, CPT did not produce any significant motor activation within the tested range dose (Figure 8). Although complete tolerance was observed with a 10 mg/kg dose of caffeine (Figure 7), a higher dose of caffeine (30 mg/kg) produced a small but significant motor activation in animals chronically exposed to 1.0 mg/ml of caffeine (Figure 8). The differential cross-tolerance to A_1 and A_{2A} receptor antagonists suggest that tolerance to the motor-activating effects of caffeine mostly involves A_1 receptors. Furthermore, these results suggest that the motor-activating effects of the acute administration of 30 mg/kg of caffeine during chronic exposure with 1.0 mg/ml of caffeine might be mostly mediated by A_{2A} receptor blockade.

Effect of Chronic Treatment with Caffeine on the Binding Characteristics of Striatal Adenosine A_1 and Adenosine A_{2A} Receptors

Saturation experiments with the selective radiolabeled adenosine A_1 antagonist [^{3}H]DPCPX performed in striatal membrane preparations from rats chronically exposed to 1.0 mg/ml of caffeine in their drinking water for 14 days showed a significant increase (of about 25%) in binding sites (B_{max}) without changes in the affinity (K_D), compared to nontreated animals (Table 1). On the other hand, chronic exposure to caffeine was not associated with changes in either the number or affinity of striatal adenosine A_{2A} receptors, labeled with [^{3}H]ZM241385 (Table 1).

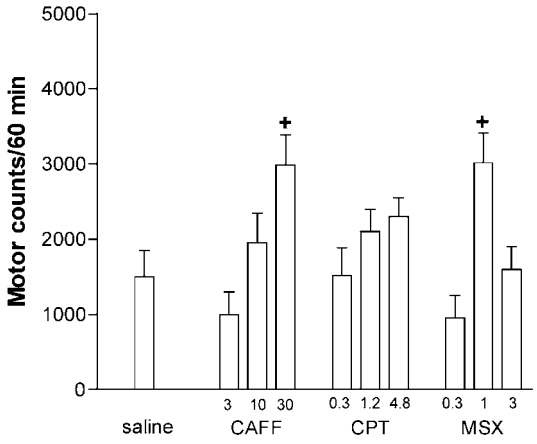

Figure 8 Effect of chronic treatment with caffeine on the motor activation induced by the acute administration of different doses of caffeine and the A_1 and A_{2A} receptor antagonists CPT and MSX-3 in habituated rats. The effect of the acute administration of caffeine, CPT, and MSX-3 was analyzed after 14 days of chronic treatment with caffeine (1.0 mg/ml in the drinking water, without withdrawal). Results represent means ± SEM of total accumulated counts during the 60-min period of observation. Caffeine (3, 10, and 30 mg/kg; CAFF), CPT (0.3, 1.2, and 4.8 mg/kg; CPT), or MSX-3 (0.3, 1, and 3 mg/kg; MSX) were administrated after 30 min of habituation of the animals to the activity cages. $^{+}$Significantly different as compared with the group that received the same chronic caffeine treatment but only received an acute administration of saline (ANOVA with *post hoc* Newman–Keuls comparisons, $p<0.05$; $n=6$–8/group).

Table 1 Saturation Experiments with the Adenosine A_1 Receptor Antagonist [^{3}H]DPCPX and the A_{2A} Receptor Antagonist [^{3}H]ZM24135 in Striatal Membrane Preparations from Rats Receiving 0.0, 0.25, or 1.0 mg/ml of Caffeine (CAFF) for 14 days

		CAFF 0.0	CAFF 0.25	CAFF 1.0
[^{3}H]DPCPX	B_{max}	944 ± 21	1126 ± 53	1237 ± 74**
	K_D	0.22 ± 0.01	0.21 ± 0.01	0.21 ± 0.01
[^{3}H]ZM24135	B_{max}	1509 ± 83	1623 ± 91	1427 ± 76
	K_D	0.61 ± 0.02	0.63 ± 0.01	0.65 ± 0.01

**$p<0.01$ compared with the CAFF 0.0 group (ANOVA).
B_{max} (fmol/mg prot) and K_D (nM) values are expressed as means ± SEM ($n=6$/experiment).

DISCUSSION

The motor-activating properties of CPT and MSX-3 in the present study were related to their selective A_1 and A_{2A} receptor antagonistic properties, respectively. Both CPT and MSX-3 produced motor activation at the same doses that selectively counteracted motor depression induced by the A_1 receptor agonist CPA or the A_{2A} agonist CGS 21680, respectively. This supports previous findings in rodents suggesting that motor activation induced by CPT is because of selective antagonism of central A_1 receptors (Nikodijevic *et al*, 1990, 1991; Baumgold *et al*, 1992; Jacobson *et al*, 1993; Popoli *et al*, 1998; Ferré *et al*, 2001). The present results, however, do not explain the lack of motor activation induced by other A_1 receptor antagonists, such as DPCPX (sometimes referred to as CPX). In fact, like CPT, DPCPX penetrates into the brain in substantial amounts after systemic administration (Baumgold *et al*, 1992) and counteracts centrally mediated, motor-depressant effects of the A1 receptor agonist CPA (Marston *et al*, 1998). The difference in the motor-activating effects of these compounds could depend on the reported ability of DPCPX to bind with high affinity to binding sites other than A_1 receptors, such as a nonstriatal atypical A_{2A} receptor (Cunha *et al*, 1996) or the cystic fibrosis transmembrane conductance regulator (CFTR) (Cohen *et al*, 1997).

As expected from its previously reported nonselective adenosine receptor antagonism in *in vitro* and *in vivo* radioligand binding experiments (Kaplan *et al*, 1996; Daly and Fredholm, 1998; Fredholm *et al*, 1999; El Yacoubi *et al*, 2001), caffeine also produced motor activation at doses that counteracted the motor depression induced by the adenosine agonists CPA and CGS 21680. However, caffeine was less effective than CPT at counteracting CPA and even less effective than MSX-3 at counteracting CGS 21680. On the other hand, when caffeine was administered alone in habituated animals, it produced a stronger motor activation than CPT or MSX-3. If caffeine's adenosine-antagonist profile were the main mechanism of action responsible for its motor-activating effects, these results would indicate a stronger effect on motor activity induced by simultaneous blockade of A_1 and A_{2A} receptors. In fact, Jacobson and coworkers found evidence for synergism in both the motor-depressant effects of A_1 and A_{2A} receptor agonists and the motor-activating effects of A_1 and A_{2A} receptor antagonists in mice (Nikodijevic *et al*, 1991; Jacobson *et al*, 1993). More than a synergistic effect, our results showed evidence for and additive motor activating effect of A_1 and A_{2A} receptor antagonists. Thus, a low dose of the A_{2A} receptor antagonist MSX-3 (1 mg/kg) increased the motor-activating effects of a high dose of the A_1 receptor antagonist CPT (4.8 mg/kg) in rats. This combination of MSX-3 and CPT would show an A_1–A_{2A} receptor antagonistic profile similar to that observed with a full motor-activating dose of caffeine (30 mg/kg), which almost completely counteracted the motor-depressant effects of CPA and weakly counteracted the motor-depressant effects of CGS 21680. The motor-activating effect of a low dose of CPT (1.2 mg/kg) was also increased when combined with a high dose of MSX-3 (3 mg/kg). In contrast, no further significant increase in motor activity was observed when high doses of both A_1 and A_{2A} antagonists were combined. Similar results have been recently reported by El Yacoubi *et al* (2000), who found that DPCPX increased the motor-activating effects of low, but not high, doses of the A_{2A} receptor antagonist SCH 58261 in mice.

The motor-activating effects induced by an acute administration of caffeine in rats, therefore, involve central blockade of both A_1 and A_{2A} receptors. These motor-activating effects most likely involve a combination of presynaptic and postsynaptic mechanisms. In the striatum, adenosine plays an important role as a modulator of both dopamine and glutamate neurotransmission. At a presynaptic level, adenosine, mostly by acting on adenosine A_1 receptors localized in nerve terminals, inhibits dopamine and glutamate release (Wood *et al*, 1989; Okada *et al*, 1996; Golembiowska and Zylewska, 1997; Flagmeyer *et al*, 1997). At a postsynaptic level, adenosine decreases dopaminergic neurotransmission by means of specific antagonistic interactions between adenosine and dopamine receptors, which modulate the function of the two types of striatal

GABAergic efferent neurons (Ferré *et al*, 1992, 1997). A_{2A} receptors and dopamine D_2 receptors antagonistically interact in striatopallidal neurons, while adenosine A_1 and dopamine D_1 receptors antagonistically interact in striatonigro-striatoentopeduncular neurons (Ferré *et al*, 1997). Significantly, formation of specific A_{2A}/D_2 and A_1/D_1 heteromeric receptor complexes has been recently demonstrated in mammalian cell lines (Ginés *et al*, 2000; Hillion *et al*, 2002). Thus, caffeine, by antagonizing the effects of endogenous adenosine, can potentially enhance dopaminergic neurotransmission by stimulating dopamine release and by potentiating the effects of dopamine-receptor stimulation (Ferré *et al*, 1997). Although the latter mechanism is well established, evidence for a dopamine-releasing effect of behaviorally relevant doses of caffeine in the brain regions that may mediate its motor-activating effects was only recently provided by *in vivo* microdialysis experiments (Solinas *et al*, 2002). In this study, only the systemic administration of doses of caffeine producing motor activation (10 and 30 mg/kg) induced dopamine release in the nucleus accumbens (preferentially in the shell), a striatal area clearly involved in motor activation induced by psychostimulants (Wise and Bozarth, 1987; Pontieri *et al*, 1995). These results, however, were not replicated in a recent study by Acquas *et al* (2002). Nevertheless, Solinas *et al* (2002) also found that a motor-activating dose of the A_1 receptor antagonist CPT (4.8 mg/kg), but not of the A_{2A} receptor antagonist SCH 58261 (2 mg/kg), induced significant dopamine and glutamate release in the nucleus accumbens (Solinas *et al*, 2002), supporting the involvement of presynaptic A_1 receptors in the motor-activating effects of caffeine. Thus, both presynaptic and postsynaptic A_1 receptor-mediated mechanisms are probably involved in the motor stimulation produced by caffeine and, together with the postsynaptic A_{2A} receptor-mediated mechanisms, they provide a mechanism for the strong effects of simultaneous A_1 and A_{2A} receptor antagonism on motor activation.

In agreement with previous studies, we found that chronic treatment with caffeine resulted in tolerance to its motor-activating effects (Holtzman and Finn, 1988; Svenningsson *et al*, 1999). In further agreement with previous studies showing pharmacological specificity of tolerance to caffeine-induced motor activation (Holtzman and Finn, 1988) and to caffeine-induced increases in food-reinforced operant responding (Jaszyna *et al*, 1998), in the present study, there was no cross-tolerance to the motor-activating effects of amphetamine. Findings from previous studies also suggest that there are at least two different kinds of tolerance to some behavioral effects of caffeine (Finn and Holtzman, 1987; Holtzman and Finn, 1988). Tolerance to caffeine's motor-activating effects and stimulant effects on operant responding develops rapidly, is insurmountable, and is pharmacologically specific, showing cross-tolerance mostly to other methylxanthines, but not to other nonxanthine psychomotor stimulants (Finn and Holtzman, 1987; Katz and Goldberg, 1987; Holtzman and Finn, 1988; Jaszyna *et al*, 1998). On the other hand, tolerance is usually not seen to the rate-decreasing effects of high doses of caffeine on operant responding (Katz and Goldberg, 1987; Jaszyna *et al*, 1998). In experiments in which tolerance has been reported to the rate-decreasing effects of caffeine on food-reinforced operant responding and to its discriminative stimulus effects, tolerance develops gradually, is surmountable, and extends to many nonxanthine psychomotor stimulants (Holtzman and Finn, 1988).

In the present study, we found evidence for tolerance to the motor-activating effects of caffeine after chronic exposure to 1.0 mg/ml of caffeine and also, although to a lesser extent, to 0.25 mg/ml of caffeine, which agrees with previous studies (Holtzman and Finn, 1988; Svenningsson *et al*, 1999). In a recent study, we reported that chronic exposure to the low concentration of caffeine (0.25 mg/ml) in the drinking water could even potentiate the motor-activating effects of acutely administered caffeine or amphetamine (Gasior *et al*, 2000). The different results might be related to the lower consumption of caffeine in our previous study (around 25 and 85 mg/kg/day after exposure to concentrations of 0.25 and 1.0 mg/ml of caffeine in the drinking water, respectively) compared to the present work (see above). New experiments are in progress in order to reconcile these differences.

Lau and Falk (1994, 1995) and Lau *et al* (1995) have demonstrated the dependence of caffeine pharmacokinetics on the route of administration and the regimen of food (*ad libitum vs* restricted) or caffeine (acute *vs* chronic) administration. Food restriction and chronic treatment are associated with faster metabolism of caffeine, probably because of induction of hepatic microsomal drug-metabolizing enzymes. However, pharmacokinetic factors cannot easily explain differences in the types of compounds that show cross-tolerance with caffeine and, therefore, pharmacodynamic factors cannot be ignored.

In the present study, chronic treatment with caffeine (1.0 mg/ml in the drinking water) resulted in an apparently complete tolerance to the motor activation induced by a high dose of both CPT and MSX-3 (maximal effective doses in animals before chronic exposure to caffeine). However, the results of dose–response experiments demonstrated a differential effect of chronic caffeine exposure on the motor-activating effects of the A_1 and A_{2A} receptor antagonists. There appeared to be the development of an apparent, rather than real, tolerance to MSX-3, suggested by a shift to the left of the MSX-3 dose–response curve, most probably indicating an accumulative effect of the A_{2A} receptor antagonism of caffeine and MSX-3. In contrast, cross-tolerance to CPT was evident at all tested doses, suggesting that tolerance that develops to the effects of A_1 receptor blockade might be mostly responsible for tolerance to the motor-activating effects of caffeine. Nevertheless, since experiments with chronic treatment with A_1 receptor antagonists have not been performed, the existence of A_1 receptor-independent long-term effects of caffeine, which could be responsible for the tolerance to caffeine and cross-tolerance of CPT, cannot be discarded.

The present results also suggest that the small but significant motor activation induced by an acute high dose of caffeine during chronic caffeine exposure might be mostly related to A_{2A} receptor blockade. In fact, in animals chronically exposed to caffeine in the drinking water (1.0 mg/ml) the acute doses of caffeine and MSX-3 that produced a significant motor activation (30 and 1 mg/kg, respectively) had the same A_{2A} receptor antagonist profile (the same efficacy at counteracting the motor depression

induced by the A_{2A} receptor agonist CGS 21680; see Figures 4 and 5). A clearer involvement of A_{2A} receptors was, therefore, observed with the chronic compared with the acute effects of caffeine, most probably because of pharmacokinetic factors. This might be related to previously reported differences in plasma concentrations of caffeine and its main metabolites under these two different conditions, because of the above-mentioned caffeine-induced increases in metabolism (Lau *et al*, 1995; Svenningsson *et al*, 1999; Gasior *et al*, 2002). Thus, chronic exposure to caffeine in the drinking water is associated with marked increases in plasma concentrations of theophylline and paraxanthine, which are even more potent nonselective adenosine antagonists *in vitro* than caffeine (Shi and Daly, 1999; Svenningsson *et al*, 1999). Also, *in vivo*, theophylline effectively counteracts motor depression produced by both A_1 and A_{2A} receptor agonists (Nikodijevic *et al*, 1991; Marston *et al*, 1998). The *in vivo* A_1-A_{2A} antagonism of paraxanthine still needs to be determined.

The present results with MSX-3 agree with recent findings that indicate that tolerance does not develop to A_{2A} receptor antagonists (Halldner *et al*, 2000). Also, in unilaterally 6-OH-dopamine-lesioned rats, chronic treatment with caffeine or repeated administration of the A_{2A} receptor antagonist SCH 58261 was not associated with tolerance to the potentiating effects of the A_{2A} antagonist on turning behavior induced by dopamine agonists (Popoli *et al*, 2000; Pinna *et al*, 2001). Furthemore, in the same animal model, and in agreement with the present findings, chronic treatment with caffeine produced tolerance to the potentiating effects of CPT on turning behavior induced by the dopamine D_1 receptor agonist SKF 38393 (Popoli *et al*, 2000).

At the biochemical level, in most studies, chronic treatment with caffeine is associated with an upregulation of adenosine A_1 receptors, but not A_{2A} receptors (for a review, see Jacobson *et al*, 1996). In a recent study by Svenningsson *et al* (1999), a small, but significant, downregulation of A_{2A} receptors was found. In the present study, we also found evidence for upregulation of striatal A_1 receptors after chronic caffeine treatment (increased antagonist binding) without changes in the binding characteristics of striatal A_{2A} receptors for antagonists. In addition to an increase in receptor density (the usual interpretation of the term 'upregulation'), Ramkumar *et al* (1988) demonstrated that multiple components of the A_1 receptor-adenylyl cyclase system are modified during chronic caffeine ingestion. Thus, caffeine also produces an increase in the proportion of A_1 receptors in the high-affinity state for agonists (resulting in increased receptor-G_i protein coupling) and in the G_i protein content, with a consequent increase in effector efficacy (increase in the maximal inhibition of adenylyl cyclase activity). These studies provide evidence for the existence of biochemical sensitization of A_1 receptors to agonists after chronic treatment with caffeine. On the other hand, similar functional biochemical studies involving A_{2A} receptors have not yet been reported. Both factors, pharmacodynamic (mostly involving A_1 receptors) and pharmacokinetic, may be synergistic, leading to the development of tolerance to the motor-activating effects of caffeine.

In summary, the present results demonstrate that motor activation effects produced by acute administration of caffeine in rats involve central blockade of both A_1 and A_{2A} receptors. During chronic exposure to caffeine in the drinking water, development of tolerance to the effects of A_1 receptor blockade might be mostly responsible for tolerance to the motor-activating effects of caffeine, while the residual motor-activating effects of caffeine in tolerant individuals might be because of A_{2A} receptor blockade.

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