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Differential Antagonism of the Behavioral Depressant and Hypothermic Effects of 5′-(N-ethylcarboxamide) Adenosine by Theobromine

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CARNEY, J. M., W. CAO, L. LOGAN, O. M. RENNERT AND T. W. SEALE. *Differential antagonism of the behavioral depressant and hypothermic effects of 5′-(N-ethylcarboxamide) adenosine by theobromine.* PHARMACOL BIOCHEM BEHAV **25**(4) 769–773, 1986.—The methylxanthine, theobromine (3,7-dimethylxanthine), was tested in mice, to determine whether theobromine could function *in vivo* as an adenosine receptor antagonist, in keeping with its reported *in vitro* effects as a blocker of agonist binding to the adenosine A-1 receptor. Theobromine doses, which themselves had no direct effects on spontaneous locomotor activity, completely blocked N^6-cyclohexyladenosine-induced suppression of locomotor activity but were without effect on 5′-N-ethylcarboxyamido adenosine (NECA)-induced decreases in motor activity. In contrast to the specific antagonism, theobromine blocked the hypothermia induced by both of these adenosine analogs. These results demonstrate that theobromine is an active *in vivo* adenosine receptor antagonist and that the antagonism of N^6-cyclohexyladenosine sensitive systems occurs even though theobromine does not stimulate spontaneous locomotor activity. Thus, the behavioral stimulant effects of methylxanthines may be more related to effects on NECA-sensitive systems, which are not blocked by theobromine. The use of *in vivo* differences in the effects xanthine may provide a useful tool in the development of compounds to probe the mechanisms of caffeine induced CNS effects.

Theobromine Methylxanthines Adenosine receptors 5′-(N-ethylcarboxamide) adenosine
N^6-Cyclohexyladenosine Locomotor activity Hypothermia Inbred mice

METHYLXANTHINES produce a variety of behavioral, physiological and biochemical effects. Although a number of neurochemical mechanisms have been proposed to underlie these effects [2, 3, 7, 11, 13], including inhibition of phosphodiesterase isozymes [11,23], more recent data has suggested that the actions of methylxanthines arise through blockade of adenosine receptor systems [7–9]. Caffeine (1,3,7-trimethylxanthine) and theophylline (1,3-dimethylxanthine) have been shown to block extracellular membrane bound receptors for adenosine at concentrations which themselves produce significant behavioral effects.

The *in vivo* pharmacology of methylxanthines largely has been limited to studies on the effects of caffeine and theophylline. These xanthines increase spontaneous motor activity [14,20], alter schedule controlled (operant) behavior [4,25] cause changes in body temperature [5, 17, 19] and at high doses induce seizures [20,21]. Relatively little work has been conducted on the effects of theobromine (3,7-dimethylxanthine). In contrast to caffeine, theophylline and paraxanthine (1,7-dimethylxanthine), theobromine fails to stimulate spontaneous locomotor activity in rodents [12], does not increase variable interval responding in rats [5] and does not appear to produce the same discriminative stimulus (subjective) properties as caffeine or theophylline [16]. Consistent with this rather inert behavioral profile, Daly *et al.* [9] reported that theobromine is considerably less effective *in vitro* as an A-2 adenosine receptor antagonist, compared to its ability to block the A-1 adenosine receptor. This suggests that A-2 rather than A-1 receptors play a major role in methylxanthine induced behavioral effects.

The present study was conducted to determine if theobromine could function as an adenosine receptor antagonist *in vivo* at both the A-1 and A-2 sites. Based upon the previously cited *in vitro* binding and related biochemical studies, we hypothesized that the inability of theobromine to elicit behavioral stimulant effects is due to its inability to blockade

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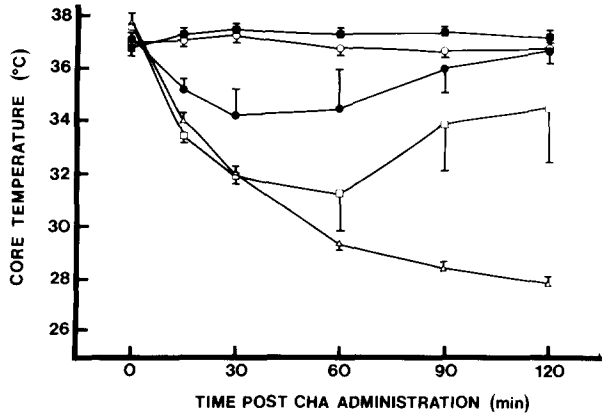

FIG. 1. Time course and dosage dependence of CHA-induced hypothermia in DBA/2 mice (n=5 for each dose). Values are mean reductions in core temperature±S.E.M. (■) Saline control; (○) 0.03, (●) 0.1, (□) 0.32 and (△) 1.0 mg/kg IP.

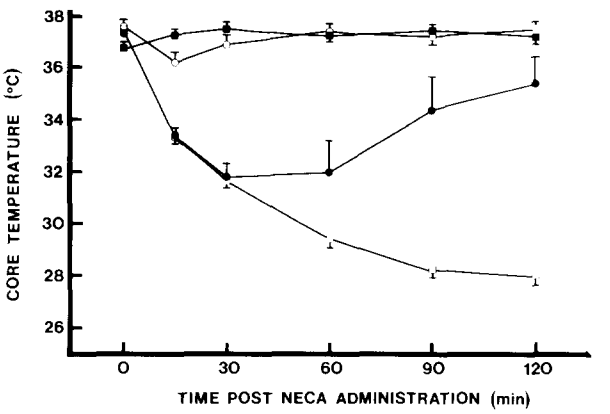

FIG. 2. Time course and dosage dependence of NECA-induced hypothermia in DBA/2 mice (n=5 for each dose). Values are mean reductions in core temperature±S.E.M. (■) Saline control; (○) 0.003, (●) 0.01 and (□) 0.03 mg/kg IP.

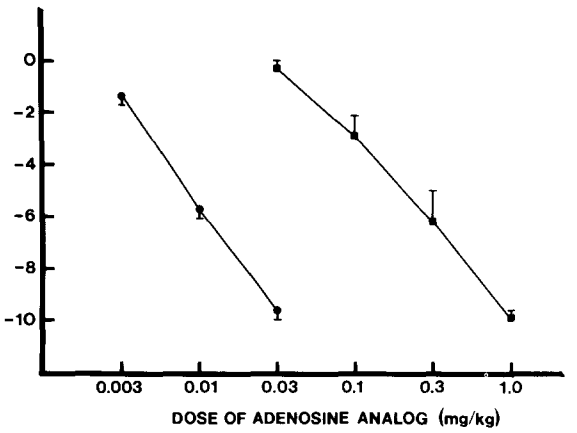

FIG. 3. Comparison of the dose response curves for CHA- and NECA-induced hypothermia in DBA/2 mice. Values are mean maximal reductions in core temperature±S.E.M. achieved within 2 hours of drug administration. (●) NECA; (■) CHA.

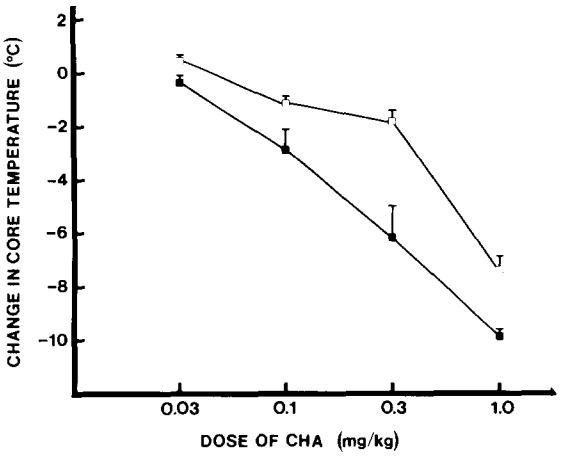

FIG. 4. Blockade of CHA-induced hypothermia by theobromine (100 mg/kg IP) in DBA/2 mice. Values are mean reductions±S.E.M. in core temperature in the absence (■) or presence (□) of theobromine pretreatment.

A-2 adenosine receptors. We now report that theobromine is an effective antagonist of both N^6-cyclohexyladenosine (CHA) and 5'-(N-ethylcarboxamide) adenosine (NECA) induced hypothermia. In contrast to this effect, theobromine failed to antagonize the decrease in locomotor activity produced by NECA (a partially selective A-2 agonist) at doses that did block the effects of CHA (a partially selective A-1 agonist).

METHOD

Subjects

Male DBA/2J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice weighed approximately 20 g at the time of the study and were housed in groups of 5 per cage. Water and food (Lab/Blox, Wayne) were continuously available in the home cage and the litter used was kiln dried aspen wood chips (Sani chips, P. J. Murphy). Mice were maintained under a 12 hour light/dark cycle under constant humidity and temperature.

Body Temperature Determination

Mice (n=5) were placed into individual plastic cages (28×17×12.5 cm) that had a wire mesh cover but did not contain any litter and were allowed to acclimate to the individual housing conditions for 60 minutes prior to any experimentation. Food and water were not available in the individual cages. Ambient temperature was maintained between 20 and 22°C.

Rectal temperature was determined using a Yellow Springs Instruments telethermometer (YSI-44TA) and a thermister probe (YSI-402). The probe was lubricated with glycerine and inserted in the rectum to a depth of 2.5 cm. Temperatures were recorded when a stable value was achieved (i.e., about 30 seconds after probe insertion). Temperatures were determined immediately prior to intraperitoneal drug or saline injection and at 15, 30, 45, 60, 90 and 120 minutes following injection. In the case of theobromine antagonism studies, theobromine was injected 15 minutes before injection of the adenosine analog and body temperature

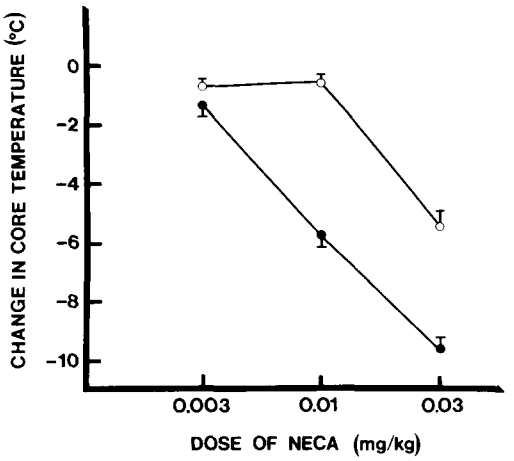

FIG. 5. Blockade of NECA-induced hypothermia by theobromine (100 mg/kg IP) in DBA/2 mice. Values are mean reductions±S.E.M. in core temperature in the absence (●) or presence (○) of theobromine pretreatment.

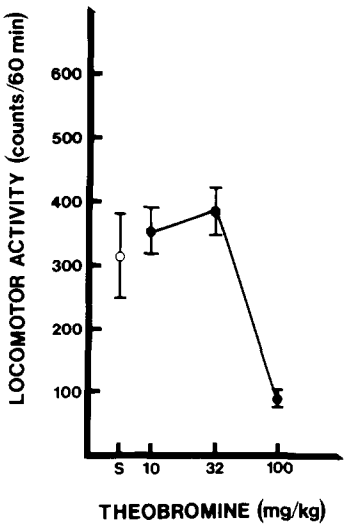

FIG. 6. Dose dependent action of theobromine on locomotor activity in DBA/2 mice. Values are mean activities±S.E.M. compiled during a period of one hour post dosing. (○) saline control; (●) theobromine injected.

was monitored for the 120 minute period after the adenosine analog injection.

Spontaneous Motor Activity Determination

Activity was monitored using photocell activity chambers. Each chamber consisted of a circular arena 2 ft in diameter and 10 in. in height. A screen cover prevented escape and allowed for circulation of air. Two orthagonally placed photocell beams were used to monitor activity. Interruption of a single photocell beam defined an activity count. Each activity chamber was interfaced to the Rockwell International AIM-65 microprocessor system that was dedicated to recording the activity counts from the 12 chambers. Mice were placed in individual activity chambers for a 60 minute session. Drug and vehicle testing were conducted on the same day. Each of the experimental groups consisted of 5 mice.

Drugs

N^6-Cyclohexyladenosine and 5'-(N-ethylcarboxamide)-adenosine were purchased from Research Biochemical Incorporated (Wayland, MA). Theobromine was purchased from Sigma Chemical Co. (St. Louis, MO). The adenosine analogs were dissolved in 0.1 N HCl in saline at a stock solution concentration of 50 mg/ml. Serial dilutions with saline were made to reach the final concentrations used in this study. Theobromine (base) was suspended in 0.5% methylcellulose. All compounds were injected IP. The volume of injection was held constant at 0.2 ml/20 g.

Statistical Analysis

Data for both changes in body temperature and spontaneous activity are presented as the mean±S.E.M. Statistical significance of these drug effects was determined by ANOVA. Effects were taken as statistically significant if a p-value <0.05 was calculated.

RESULTS

Blockade of Hypothermia Induced by Adenosine Analogs

One behavioral action of adenosine analogs is their dose dependent ability to rapidly induce hypothermia in mice. The time course and dose dependence for CHA- and NECA-induced decreases in core temperature are shown respectively in Figs. 1 and 2. Both of these analogs were effective in producing substantial decreases in body temperature. At relatively low doses, the maximum fall in temperature occurred during the first 60 minutes following dosing, and body temperature returned toward control values during the second hour of the test session. At the highest dose of CHA and NECA, body temperature remained depressed at the end of the 120 minute session. Comparison of the dose effect curves for maximum change in body temperature during the 120 minute session indicated that NECA was about 30 times more potent than CHA in eliciting hypothermia (Fig. 3). The ED_{50} values for maximal induction of hypothermia were respectively 0.002 and 0.018 mg/kg IP for NECA and CHA. The two adenosine analogs had the same efficacy in decreasing core temperature. Maximal hypothermia occurred at the high dosages shown in Fig. 3. Increasing the dose of NECA or CHA above these doses resulted in no further increase in hypothermia. All animals survived the nearly 10 degree drop in body temperature brought about by high doses of NECA and CHA.

Having established the dosage and time dependence of NECA- and CHA-induced hypothermia, we then examined the ability of theobromine to antagonize this effect. Theobromine alone at doses up to 100 mg/kg IP produced insignificant changes in core temperature over the 120 minute test period (data not shown). Pretreatment with theobromine (100 mg/kg IP) 15 minutes prior to the administration of CHA or NECA significantly blocked the ability of *both* of these adenosine analogs to induce hypothermia (Figs. 4 and 5). Dose effect curves for both of the adenosine analogs were shifted about 3-fold to the right by theobromine. It is impor-

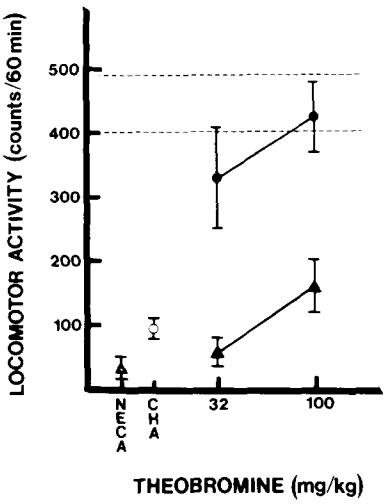

FIG. 7. Theobromine blockade of adenosine analog-induced inhibition of locomotor activity in DBA/2 mice. Values are mean activities±S.E.M. compiled over a one hour period. (△) NECA alone (0.01 mg/kg IP); (▲) NECA following a 15 minute pretreatment with theobromine; (○) CHA alone (0.1 mg/kg IP); (●) CHA following a 15 minute pretreatment with theobromine. Dotted lines represent ±1 S.E.M. for saline controls.

tant to note that theobromine was equally effective as an antagonist of NECA- and CHA-induced hypothermia.

Blockade of Locomotor Activity Depression Induced by Adenosine Analogs

Another behavioral action of adenosine and its analogs is to significantly inhibit locomotor activity. Because this behavioral effect of adenosine analogs is more well characterized than others [1], we chose to use it as an additional assay for the behavioral actions of theobromine. NECA is significantly more potent than CHA in inhibiting locomotor activity (ED_{50} respectively 0.002 and 0.018 mg/kg IP), but the two analogs have similar efficacy. Doses of these analogs producing 80–90% inhibition of locomotor activity were used to investigate the ability of theobromine to act as an antagonist *in vivo*.

Theobromine is generally considered to be behaviorally inert. However, before undertaking the blockade experiments, we assessed its direct effect on locomotor activity to insure that it had no unexpected actions in this strain of inbred mice. Figure 6 indicates that doses up to 32 mg/kg IP are without effect; a higher dose (100 mg/kg IP) caused significant inhibition of locomotor activity.

With the direct behavioral actions of theobromine and the adenosine analogs established, their interactions were investigated. The ability of theobromine to block the inhibition of locomotor activity by CHA and NECA is shown in Fig. 7. At a dose of 32 mg/kg IP, which itself has no demonstrable direct effect on locomotor activity, theobromine had no significant effect on NECA-mediated inhibition of this behavior. However, this dose of theobromine significantly blocked CHA-induced inhibition of locomotor activity. This specificity it its ability to block inhibition of locomotor activity by the adenosine analogs was maintained at a dose of 100 mg/kg IP. These observations are in contrast to theobromine's lack of specificity for the blockade of NECA- and CHA-induced hypothermia (Figs. 4 and 5). It also is notable that complete blockade of CHA-induced locomotor activity decreases occurred at 100 mg/kg of theobromine, a dose that produced a substantial decrease in activity where tested alone.

DISCUSSION

Theobromine is devoid of behavioral stimulant activity and other CNS direct effects that are characteristic of caffeine and other xanthines. It does not produce the same cue properties as caffeine or theophylline in trained subjects [16], it does not increase spontaneous activity [12,24] and it fails to increase schedule controlled behavior in rodents [5]. If xanthines produce their effects through blockade of adenosine receptor systems, then theobromine must fail to interact with one or more subclasses of these receptors. Receptor binding data identifies a potency difference between the caffeine (IC_{50}=90–110 μM) and theobromine-induced blockade (IC_{50}=210–280 μM) of [^{3}H]-CHA binding [9]. In contrast to the apparent potency difference for competition at A-1 binding sites [4], theobromine does not appear to be block (IC_{50}>1000 μM) the adenosine A-2 receptor-mediated increases in cAMP production in striatal slices [9]. On the other hand, caffeine is non-specific with regard to adenosine receptor types and antagonizes the *in vivo* effects of both CHA and NECA [8,22]. Caffeine (IC_{50}=120 μM) and related xanthines produced a dose related blockade of NECA-stimulated increases in adenylate cyclase activity (an A-2 receptor mediated effect) which did not occur on theobromine (IC_{50}>1000 μM) addition. This lack of adenosine A-2 receptor blockade (based upon receptor functional assays rather than binding criteria) by theobromine occurred in striatal slices. In view of the important role of the striatum in regulating motor behavior, and in determining the effects of drugs on motor activity, the observed biochemical differences may be the basis for both the lack of efficacy of theobromine as a direct behavioral stimulant and its inability to antagonize NECA inhibition of locomotor activity that we observed in this study.

Snyder [24] described one other adenosine antagonist, isobutylmethylxanthine (IBMX), which did not directly stimulate locomotor activity when administered alone, but was effective in the blockade of adenosine radioligand binding *in vitro*. IBMX was highly active in reversing PIA-induced depression of locomotor activity. Thus, IBMX and theobromine have similar behavioral actions.

A number of biochemical and physiological effects of adenosine have been identified. Attempts have been made to categorize these effects into either A-1 or A-2 adenosine receptor-mediated effects [8]. However, it is difficult to resolve A-1 from A-2 receptors with currently available adenosine receptor radio-ligands. The difficulty lies in the lack of availability of highly specific A-2 receptor ligands. Given the difficulty in specifically separating adenosine A-1 and A-2 receptors types, it has not been possible to unequivocally assign adenosine analog-induced behavioral responses to one or the other receptor type. One way to overcome the problem of relatively non-specific receptor agonists is to develop receptor-specific antagonists. Theobromine possesses both *in vivo* and *in vitro* properties which may indicate its potential value as a compound with differential action on A-1 and A-2 receptors. Our locomotor activity data indicate that theobromine fails to block NECA-mediated decreases in activity but effectively antagonizes CHA-induced decreases in activity. Since relative responsiveness to NECA, CHA and other adenosine analogs have

been used previously to indicate the likelihood of A-2 and A-1 receptor mediated effects [4,8], our data are consistent with the interpretation that theobromine is an A-1 receptor-selective antagonist. This *in vivo* result in consistant with the *in vitro* biochemical data on Daly *et al.* [9].

In contrast to the observed separation in motor activity, the hypothermic effect of CHA and NECA appear to be similar in their sensitivity to blockade by theobromine. This lack of specificity for the antagonism of adenosine agonist-induced hypothermia by theobromine is also seen with caffeine [22]. One explanation for the lack of specificity of theobromine as an antagonist of adenosine analog-mediated hypothermia would be that the NECA-induced hypothermia arises by its action on A-1 receptors. However, this seems unlikely because NECA is 30 times more potent than CHA in inducing hypothermia. Further this potency difference also occurs in NECA- and CHA-inhibition of locomtor activity. Based upon the selective blockade of CHA-induced hypoactivity by theobromine, a pharmacokinetic explanation for this difference in behavioral selectivity of theobromine's antagonist effects also appears unlikely. Alternatively, the NECA-sensitive binding sites in striatum and other selected motor areas of the brain may be pharmacologically unique in their sensitivity/binding capacity for theobromine. This hypothesis can be explored by behavioral analyses and membrane binding experiments employing analogs of theobromine and other methylxanthine congeners that would more clearly identify structurally or functionally distinct forms of adenosine receptors. The present study demonstrates the valuable role in *in vivo* pharmacological experiments in the dissection of complex adenosine receptor systems.

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