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# Inherent Hyporesponsiveness to Methylxanthine-Induced Behavioral Changes Associated With Supersensitivity to 5′-N-Ethylcarboxamidoadenosine (NECA)

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SEALE, T. W., K. A. ABLA, W. CAO, K. M. PARKER, O. M. RENNERT AND J. M. CARNEY. *Inherent hyporesponsiveness to methylxanthine-induced behavioral changes associated with supersensitivity to 5′-N-ethylcarboxamidoadenosine (NECA).* PHARMACOL BIOCHEM BEHAV **25**(6) 1271–1277, 1986.—Two inbred mouse strains, SWR and CBA, differed significantly in their susceptibility to acute dose dependent theophylline- and caffeine-induced stimulation of locomotor activity. The efficacy of both methylxanthines was reduced in the SWR strain compared to the CBA strain. When brain levels of theophylline were determined at a dose (32 mg/kg IP) which gave maximal behavioral separation of the two strains, no significant differences were found between them (SWR levels 12.5±1.9, CBA levels 14.3±1.7 μg/g wet weight brain). The dose dependent ability of several adenosine agonists ($N^6$-cyclohexyladenosine, (−)-$N^6$-phenylisopropyladenosine, 5′-N-ethylcarboxamidoadenosine) to depress locomotor activity was investigated. SWR mice were found to be significantly more sensitive to NECA-induced depression of locomotor activity and the NECA-induced hypothermia than were CBA mice (respective $ED_{50}$ values for inhibition of activity, 11.6 and 30.5 nmoles/kg IP). No differences were found in brain [$^3$H]-NECA levels at doses which produced marked differences in behavioral effects between the two strains. The differences in adenosine agonist sensitivity between the strains were both agonist- and behavior-specific. These data indicate that an inherited alteration in behavioral responsiveness to methylxanthine administration can be inversely associated with inherent alterations in susceptibility to the action of specific adenosine analogs. An adenosine A-2 receptor sub-class may be involved in these changes in *in vivo* pharmacological susceptibility to the action of both methylxanthines and adenosine agonists on locomotor activity.

Adenosine receptor agonists Adenosine receptors 5′-N-ethylcarboxamidoadenosine $N^6$-cyclohexyladenosine (−)-$N^6$-phenylisopropyladenosine Caffeine Theophylline Locomotor activity Hypothermia Inbred mice Behavioral genetics

METHYLXANTHINES such as caffeine and theophylline induce a variety of central excitatory effects including stimulation of locomotor activity [19,29], disruption of sleep [5, 14, 18] and induction of convulsions [22, 24, 32, 33]. Behavioral effects which occur at low doses of these stimulants have been attributed to the action of the compounds on adenosine receptors [6, 7, 10, 30]. The ability of a variety of methylxanthines to stimulate activity levels *in vivo* is highly correlated with their potency to specifically antagonize radiolabeled agonist binding to adenosine receptors [16,29]. Caffeine and theophylline are non-selective in their ability to compete with radioligands binding to adenosine A-1 and A-2 receptors [7,11] and in their capacity to antagonize the responses mediated by either A-1 or A-2 receptors [2,20]. Thus, *in vivo* responsiveness to the behavioral effects of caffeine and theophylline at low doses is thought to depend on the level of released endogenous adenosine and its interaction with adenosine receptors [7, 10, 30].

Susceptibility to behavioral actions of methylxanthines varies widely among individual subjects in both man and animals [9, 12–14, 19, 24, 27, 31]. These differences in responsiveness do not necessarily reflect differences in systemic concentrations of these compounds [3, 9, 27]. Recently we have initiated a systematic investigation of inherent variation in methylxanthine responsiveness in the inbred mouse. The purpose of these studies is to identify the occurrence of inherently altered behavior in response to administration of the methylxanthines and to determine the pharmacological and neurochemical basis for these differences in neuropharmacological responsiveness. Using the inbred

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mouse as a model system, we have identified a variety of intrinsic alterations in susceptibility to behavioral changes brought about by acute administration of caffeine at both high [3, 24, 27] and low [19,25] doses. Relative susceptibility to one behavioral effect of caffeine is not necessarily predictive of relative responsiveness of a second behavioral trait [19, 24, 25, 28]. The SWR strain of inbred mice is hyporesponsive to the locomotor activity-stimulating effects of both caffeine and theophylline compared to the CBA strain [19]. This intrinsic difference in methylxanthine efficacy (but not potency) is inherited in a complex, polygenic manner [25]. To determine whether such inherent differences in responsiveness to caffeine and theophylline actually involve a change in an aspect of the adenosine receptor system, we investigated the relative *in vivo* susceptibility of these two strains of mice to the behavioral actions of several adenosine agonists. Here we report that relative to the CBA strain, SWR mice are significantly more responsive to adenosine 5'-N-ethylcarboxamidoadenosine (NECA). This difference in responsiveness to NECA is specific since other adenosine agonists, e.g., $N^6$-cyclohexyladenosine (CHA), are equally potent in inhibiting locomotor activity in both inbred strains of mice.

## METHOD

### *Subjects*

Ten week old male mice of two inbred strains, SWR and CBA, obtained from the Jackson Laboratory, Bar Harbor, ME, were housed in groups of 5 animals per cage (dimensions 18×29 cm) on a continuous 12 hours light-dark cycle under constant humidity and temperature (19–21°C). Animals were drug naive and were used only a singe time for drug administration. The litter used was aspen wood chips (Sanichips, P. J. Murphy). Free access to a standard pelleted rodent food (Lab/Blox, Wayne) and water was given.

### *Drug Sources and Administration*

Adenosine agonists, $N^6$-cyclohexyladenosine (CHA), 5'-N-ethylcarboxamidoadenosine (NECA) and (−)-$N^6$-phenylisopropyladenosine (L-PIA) were obtained from Boehringer Mannheim Biochemicals. Caffeine and theophylline were obtained from Sigma Chemical Co. Caffeine and theophylline were dissolved in 0.9% NaCl solution containing approximately 0.2 mM NaOH. Adenosine analog solutions were prepared by dissolving the compounds in a small volume of 0.2 M HCl and diluting them appropriately with 0.9% NaCl solution. Intraperitoneal (IP) injections of each compound were made so that each dose was administered in a fixed volume to animal weight ratio (0.1 ml/10 g).

### *Locomotor Activity Testing*

Locomotor activity was monitored in eight activity chambers. Each activity chamber consisted of a 2 foot diameter circular arena, 10 inches high, equipped with two photocell detectors. Each detector was illuminated by a 25 W light bulb (General Electric No. 25R14N) placed outside the arena with the light beam directed through a 1/2 inch hole in the side of the arena. To minimize background light, each bulb was shielded in a metal box. The bulbs were the only source of lighting within the chamber. A Rockwell AIM 65 microprocessor system was used for data acquisition. Data recorded for each 1 hour activity session consisted of 10 minute interval counts and cumulative total counts for the 60 minute period. Activity sessions were conducted daily from 0800 to 1530 hours. The mice were allowed to habituate to the activity chambers for a period of 3 days before drug testing began.

### *Measurement of Core Temperature*

Adenosine analog-induced changes in core temperature were determined in the following way. Unrestrained animals were put singly into plastic mouse cages without litter for 60 minutes prior to NECA or CHA administration. Room temperature was maintained at 20±0.5°C during the course of the experiments. Rectal temperatures were determined immediately prior to drug administration to establish basal values and were determined at 15 minute intervals for 2 hours following drug injection. For each experimental and control determination, 5 mice were used. Rectal temperature was measured with a YSI-44TA tele-thermometer (Yellow Springs Instrument Co.). The thermister probe (YSI-402), lubricated with glycerol, was inserted 2.6 cm into the rectum, and the temperature was recorded when a stable value was reached (i.e., about 30 seconds after insertion of the probe). Animals were evaluated between the hours of 0900 and 1600.

### *Extraction and Quantitation of Methylxanthines and NECA From the Brain*

Theophylline levels in the brains of SWR and CBA inbred mice by the method described by Snyder *et al.* [26]. Theophylline (32 mg/kg IP) was injected and the animals (n=5 for each strain) were sacrificed by decapitation 30 minutes post dosing. This dose was chosen because it maximized the behavioral difference between strains. This time was chosen because it represented the midpoint of the period used for behavioral assessment. Brains were homogenized (Polytron, 30 seconds, setting 7) in 5 volumes (v/w) of 0.01 M HCl, and the homogenate was extracted 5 times with 5 ml chloroform. To each combined chloroform extract an internal standard of beta-hydroxethyltheophylline was added, and the specimens were dried under nitrogen. The residue was dissolved in 1.0 ml of the mobile phase used for chromatography (0.1 M sodium acetate, pH 4.0 containing 10% acetonitrile). Standards used to quantitate methylxanthine levels included caffeine, theophylline and the internal standard. High pressure liquid chromatography separation were performed on an MPLC-RP-18 SPHER1-5 OD-MP column (Brownlee Laboratories) using a Schoeffel ultraviolet detector at 280 nm.

[$^3$H] NECA levels in brains of the two strains were determined in the following manner. A dose of unlabeled NECA which maximized the behavioral differences between the two strains (4.6 μg/kg IP) was mixed with [$^3$H] NECA (New England Nuclear, specific activity 20 Ci/mmol) to give a final specific activity of 8.3 Ci/mmol. Thirty minutes after injection, the mice (n=5 for each strain) were sacrificed by decapitation, and the brains extracted three times in 3 ml 0.9% saline after homogenization with a Polytron cell disrupter (30 seconds, setting 7). After centrifugation at 5000×g for 15 minutes, a fixed volume of the extract (200 μl) was added to 8 ml Beckman Ready-Solv-EP for liquid scintillation counting. Control experiments established that this method of extraction had an efficiency of approximately 85%. From the known specific activity of the NECA solution, the known amount injected and the brain weights, amount of NECA present per g wet weight of brain was calculated.

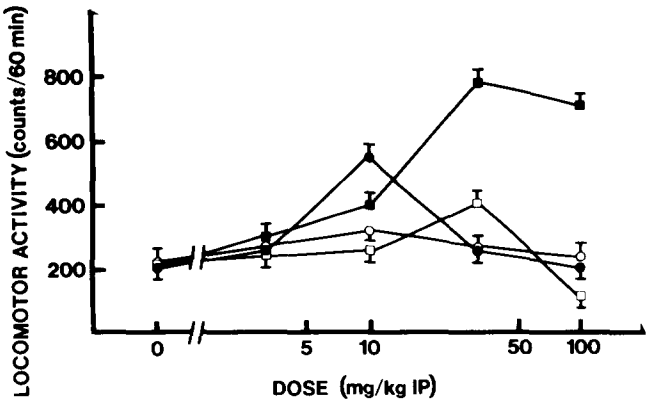


FIG. 1. Methylxanthine-induced alteration of locomotor activity in SWR and CBA inbred mice. Dosage dependence of caffeine-induced increases in locomotor activity in (○) SWR mice and (●) CBA mice. Dosage dependence of theophylline-induced increases in locomotor activity in (□) SWR mice and (■) CBA mice. Each point represents the mean±S.E.M. of the activity of six mice.

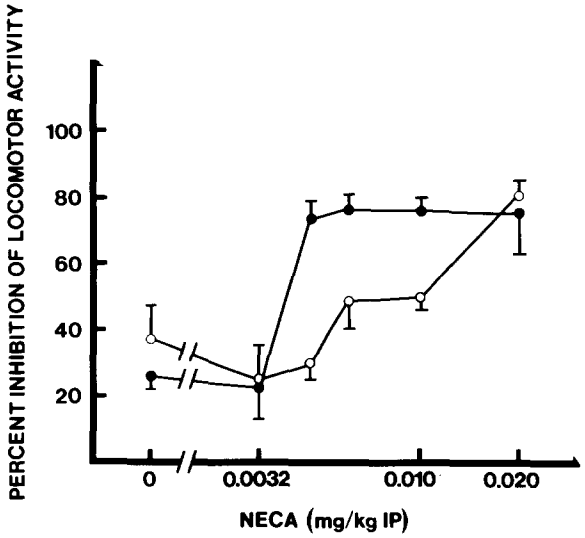


FIG. 2. Dosage dependence of NECA-induced inhibition of locomotor activity in SWR and CBA mice. Each point represents the mean±S.E.M. of the activity of six mice. *Indicates a significant difference ($p<0.05$) in the responsiveness of the two strains of mice. (●) SWR mice; (○) CBA mice.

*Statistics*

Comparison of individual strain responses to adenosine analogs and methylxanthines was made by Student's $t$-tests [17]. Statistically significant results were considered to occur if a $p$ value less than 0.05 was calculated.

## RESULTS

*Methylxanthine-Induced Changes in Locomotor Activity of CBA and SWR Inbred Mice*

Differences in the susceptibility of CBA and SWR inbred mice to the dose dependent stimulatory effects induced by theophylline and caffeine are shown in Fig. 1. The theophylline doses of 3.2–100 mg/kg IP significantly ($p<0.05$) stimulated locomotor activity in CBA mice compared to their uninjected or vehicle treated controls. In contrast, SWR mice were significantly ($p<0.05$) stimulated only at a theophylline dose of 32 mg/kg IP. Doses higher than 32 mg/kg IP did not further stimulate activity levels in either strain. Each of the strains was inhibited to about the same extent by a dose of 100 mg/kg IP. CBA and SWR mice also differed in their susceptibilities to the dose dependent stimulation of locomotor activity by caffeine. A dose of 3.2 mg/kg IP caused an insignificant increase in locomotor activity of both strains. A dose of 10 mg/kg IP caused the activity of CBA mice to be significantly ($p<0.05$) stimulated compared to their saline injected controls or compared to the SWR strain. Although this dose of caffeine produced the maximal increase in locomotor activity in the CBA strain, the magnitude of the increase in locomotor activity was significantly ($p<0.05$) less than that achieved with theophylline at 32 and 100 mg/kg IP. Neither strain showed significantly increased locomotor activity (compared to basal levels) at the highest caffeine dosages employed. Under these conditions the activity of the SWR strain was not significantly increased at any of the doses of caffeine employed. Thus, the SWR strain was significantly hyporesponsive, compared to the CBA strain, to the locomotor activity-stimulating effects of both caffeine and theophylline. This behavioral difference in methylxanthine responsiveness appears to be an efficacy limited one.

To determine whether these differences in methylxanthine responsiveness were due to pharmacokinetic or pharmacodynamic changes, brain levels of theophylline were quantitated in the two strains of mice. The dose administered maximized the differences in behavioral responsiveness between the two strains (32 mg/kg). Animals were sacrificed 30 minutes after methylxanthine administration, the midpoint for the time period used to determine the action of these compounds on locomotor activity. Theophylline levels were found to be respectively 12.5±1.9 and 14.3±1.7 μg/g brain wet weight in the SWR and CBA strains. These levels were not significantly different between the mouse strains.

*Adenosine Agonist-Induced Inhibition of Locomotor Activity in CBA and SWR Inbred Mice*

To investigate whether the differences in methylxanthine responsiveness of the two mouse strains involved an alteration in the adenosine-mediated regulation of activity, we investigated the responsiveness of these two strains of mice to several adenosine analogs. We first observed a marked difference between the two strains in the ability of NECA to inhibit locomotor activity. Figure 2 shows a detailed comparison of the dose dependent responses of the two strains to NECA administration. The two strains differed significantly at doses >3.2 μg/kg IP. SWR mice were significantly more responsive to NECA than were mice of the CBA strain ($ED_{50}$ values were respectively 3.8 and 10 μg/kg IP; $p<0.01$). The slope of the dose response curve was also altered in CBA mice. It was much more shallow than that seen in the SWR strain. However, NECA had equal efficacy for the inhibition of locomotor activity (≥80%) in the two strains of mice.

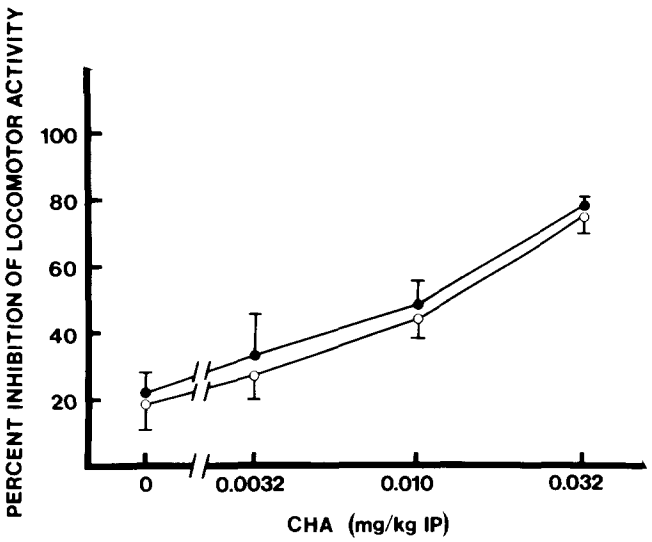


FIG. 3. Dosage dependence of CHA-induced inhibition of locomotor activity in SWR and CBA mice. Each point represents the mean± S.E.M. of the activity of six mice. (●) SWR mice; (○) CBA mice. No significant differences in response between the strains were observed at any dose.

To determine if the differences in NECA susceptibility were specific to this adenosine analog or generalized to other adenosine agonists, we examined the response of these two mouse strains to several other adenosine congeners (Fig. 3 and Table 1). The inhibition of locomotor activity by CHA, an adenosine analog with partial selectivity for the A-1 adenosine receptor, is shown in Fig. 3. The dose dependent susceptibility to this analog of the two strains did not differ. $ED_{50}$ values for both strains were approximately 10 μg/kg IP. As found with the NECA dose response curve, there was no difference in efficacy of CHA-induced inhibition of locomotor activity between the two strains. Dose response curves were also carried out for L-PIA-induced inhibition of locomotor activity (dose response curve not shown). $ED_{50}$ values for this analog also did not differ between SWR and CBA mice (Table 1). The only difference in susceptibility of the SWR and CBA mouse strains was found to be in the potency of the NECA-induced inhibition of locomotor activity. Susceptibility of the two strains to each of the other adenosine analogs did not differ significantly. No potency or efficacy differences were observed for the other adenosine derivatives.

*Adenosine Agonist-Induced Hypothermia in CBA and SWR Inbred Mice*

Another assay to monitor the *in vivo* responsiveness of mice to adenosine agonists is the induction of hypothermia. The dosage dependent changes in core temperature brought about in the two mouse strains by NECA and by CHA are shown in Figs. 4 and 5. High doses of both adenosine analogs induce the rapid onset of acute hypothermia (−9–10°C) at high doses. There is no significant difference in the maximal efficacy of the two adenosine agonists on these two strains (data not shown). However, significant potency differences for the induction of hypothermia were observed. Figure 4 shows that NECA was significantly more potent in inducing

TABLE 1

$ED_{50}$ VALUES FOR THE INHIBITION OF LOCOMOTOR ACTIVITY BY ADENOSINE ANALOGS IN SWR AND CBA INBRED MICE

| Adenosine analog | Inbred Mouse Strain SWR | CBA |
|---|---|---|
| NECA | 11.6 (0.0038)* | 30.5 (0.010) |
| CHA | 28.6 (0.010) | 28.6 (0.010) |
| L-PIA | 83.0 (0.032) | 83.0 (0.032) |

Values in this table are $ED_{50}$ values expressed in n moles/kg IP (or in mg/kg IP, shown in parentheses). An asterisk indicates that the observed $ED_{50}$ values were significantly different ($p<0.01$) between the two strains of mice.

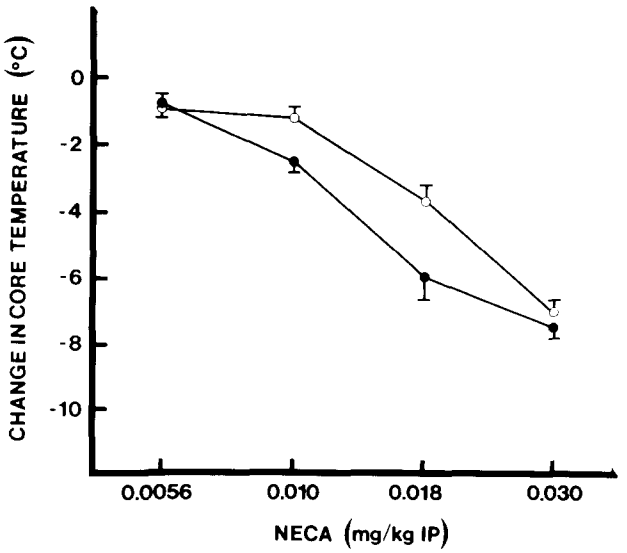


FIG. 4. Dosage dependence of NECA-induced hypothermia in SWR and CBA mice. Each point represents the mean±S.E.M. of the maximal hypothermia occurring within a two hour period of administration. *Indicates a significant difference ($p<0.05$) in the responsiveness of the two strains of mice. (●) SWR mice; (○) CBA mice.

hypothermia in the SWR strain than it was in CBA mice. However, the respective $ED_{50}$ values of 46 and 64 nmoles/kg IP (Table 2) indicated that the magnitude of the difference in the responsiveness of the two mouse strains was considerably smaller than for that observed in the locomotor activity behavioral assay. CHA responsiveness of the two mouse strains also differed (Fig. 5). The SWR strain was again slightly more susceptible to CHA-induced hypothermia than was the CBA strain (respectively 1400 nmoles/kg IP versus 2600 nmoles/kg IP). The CHA/NECA ratios for the $ED_{50}$ values describing the induction of hypothermia were 30.4 in the SWR strain and 40.6 in the CBA strain (Table 2).

*NECA Uptake Into Brain*

NECA uptake into the brains of these two strains of mice

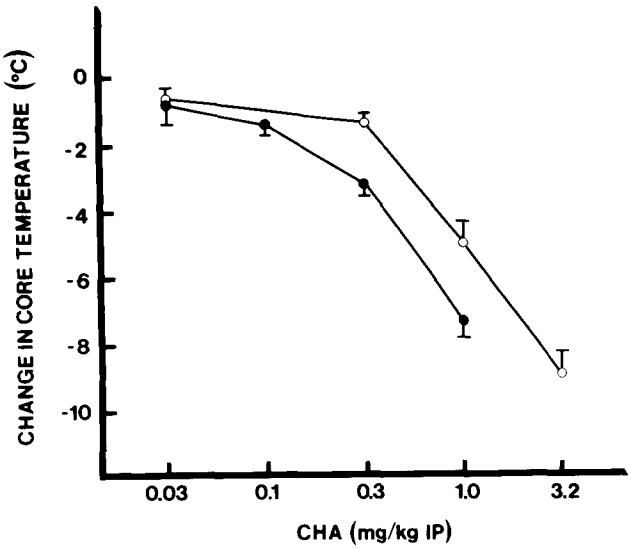


FIG. 5. Dosage dependence of CHA-induced hypothermia in SWR and CBA mice. Each point represents the mean±S.E.M. of the maximal hypothermia occurring within a two hour period of administration. *Indicates a significant difference ($p<0.05$) in the responsiveness of the two strains of mice. (●) SWR mice; (○) CBA mice.

TABLE 2

$ED_{50}$ VALUES FOR ADENOSINE ANALOG-INDUCED HYPOTHERMIA IN CBA AND SWR INBRED MICE

| Adenosine analog | Inbred Mouse Strain SWR | CBA |
|---|---|---|
| NECA | 46 (0.015)* | 64 (0.021) |
| CHA | 1400 (0.50)* | 2600 (0.90) |
| CHA/NECA ratio | 30.4 | 40.6 |

Values in this table are $ED_{50}$ values expressed in n moles/kg IP (or mg/kg IP, shown in parentheses). Asterisks indicate the two strains differ significantly ($p<0.05$) in their responsiveness to the adenosine agonist.

also was investigated. [$^3$H]NECA was added to a dose of unlabeled NECA (4.6 μg/kg IP) which maximized the differences between CBA and SWR for the inhibition of activity. Based upon the recovery of [$^3$H]NECA from extracts of the brains (n=5 from each of the two mouse strains), respective NECA levels were 73.4±6.1 and 72.5±7.2 pmol/g brain in CBA and SWR mice. These values do not differ significantly. These data indicate the NECA levels in the brains of the two strains of mice did not differ under dosage conditions which caused distinct behavioral effects in them.

## DISCUSSION

The results presented here suggest a direct association between *inherent* behavioral responsiveness to the methylxanthines, caffeine and theophylline, and the adenosine receptor agonist, NECA. In the two mouse strains that we characterized pharmacologically, the relationship between intrinsic responsiveness to methylxanthines and to NECA is an inverse one. Hyporesponsiveness to caffeine and theophylline is associated with relative supersensitivity to NECA.

This difference in susceptibility to NECA-induced depression of locomotor activity is pharmacologically specific. Other adenosine agonists such as L-PIA and CHA are equally potent in reducing locomotor activity in the two strains of mice. Pharmacokinetic explanations of these differences in pharmacological responsiveness can be ruled out. Brain levels of both theophylline and NECA did not differ in the two strains at doses of each compound which elicited significantly different behavioral effects in each strain.

Previous evidence indicated that adenosine receptor agonists are potent inhibitors of locomotor activity [1, 16, 29]. Their action appears to be directly upon the CNS [1]. Methylxanthines which stimulate locomotor activity are effective antagonists of specific radioligand binding to adenosine receptors [7, 8, 16, 29]. From the rank order of potencies of adenosine analog-induced inhibition of locomotor activity of rodents, it has not yet been possible to establish which type of adenosine receptor has a predominant role in the regulation of locomotor activity. It seems possible that both A-1 and A-2 receptors may influence locomotor activity behavior. NECA is an adenosine agonist with partial selectivity for the adenosine A-2 receptor [6, 15, 18, 21, 23, 30]. NECA is 2.5 more potent than CHA and 7.1 more potent than L-PIA in inhibiting locomotor activity in the SWR strain. These observations are consistent with a major role for A-2 receptor-mediated effects in the SWR strain since the potency of NECA is higher at A-2 receptors than are the potencies of CHA and L-PIA [6, 8, 23]. In contrast, NECA is significantly less potent in reducing locomotor activity in the CBA strain than it is in the SWR strain. NECA and CHA are about equally potent in inducing this behavioral change in CBA mice. Our findings identifying the coincident alteration of behavioral susceptibility specifically to NECA and to the two methylxanthines between CBA and SWR mice suggest that the observed differences in behavioral responsiveness are due to a genetically specified alteration in the structure, number or function of adenosine A-2 receptors in these mouse strains.

Additional support for the involvement of two different adenosine receptor subtypes in the inhibiting action of adenosine agonists on locomotor activity comes from our recent attempt to differentially antagonize the effects of NECA and CHA. We found that theobromine, at doses which themselves had no direct effect on behavior, completely blocked CHA-induced suppression of spontaneous locomotor activity but were without effect on NECA-induced decreases in this behavior in mice [4]. This *in vivo* finding is consistent with the *in vitro* biochemical effects of theobromine on adenosine receptors [17]. Theobromine has a reduced efficacy compared to other methylxanthines in its capacity to antagonize adenosine A-2 receptor-mediated increases in cAMP production in striatal slices [7]. Thus, both pharmacological data (selectivity of blockade) and genetic data (intrinsic specific changes in potency of NECA between mouse strains) suggest a significant role for the A-2 receptor in control of locomotor activity behavior. Daly *et al.* [8] have shown that two classes of adenosine A-2 receptors exist in the rodent brain,

a low affinity ($EC_{50}$ 10–20 $\mu$M) site found in all brain regions and a higher affinity site ($EC_{50}$ 0.5 $\mu$M) which is present in the striatum. Because the striatum is known to have an important role in regulating motor behavior and in drug-induced alteration of locomotor activity, the strain specific differences in methylxanthine and NECA responsiveness shown here may involve inherited changes in striatal A-2 receptors.

To investigate whether the genetic change effecting altered susceptibility to the inhibiting effects of NECA on locomotor activity also extended to other adenosine agonist-induced behavioral changes, we investigated the ability of NECA and CHA to induce hypothermia in SWR and CBA mice. Recently we have shown that low doses of adenosine analogs induce rapidly occurring, profound hypothermia in mice [4,26]. NECA is 30 times more potent than CHA in its hypothermic action in DBA/2 mice [4]. The ability of these adenosine analogs to induce hypothermia is completely blocked by low doses of methylxanthines. Mouse strains with specifically altered susceptibility to either NECA- or CHA-induced hypothermia have been identified [26]. Table 2 shows that the NECA to CHA potency ratio in SWR mice is similar to that found in other strains of mice but that there exists a significant potency reduction for both NECA- and CHA-induced hypothermia in the CBA strain. The magnitude of the relative NECA supersensitivity of the SWR strain compared to the CBA strain is not as large as that found for NECA-induced locomotor activity suppression. However, when hypothermia is the behavioral assay, CBA mice are about 2-fold less sensitive to CHA than are SWR mice. No change in CHA potency for inhibition of locomotor activity was found between the two strains. Therefore, susceptibility of individual behaviors (behavioral markers) to modulation by adenosine agonists appears to be controlled by distinct, separable genetic determinants.

The data reported here identify that altered responsiveness of individuals to the behavioral effects of methylxanthines can be associated with intrinsic changes in behavioral susceptibility to adenosine agonists. These observed *in vivo* associations provide indirect genetic evidence for the concept that methylxanthines exert their behavioral activating effects *via* their antagonistic action on the interaction of endogenous adenosine with adenosine receptors. Inbred mouse strains such as SWR and CBA which have marked, intrinsic, analog-specific differences in behavioral responsiveness may prove to be useful tools for the development of adenosine agonists and antagonists possessing selectivity for certain adenosine receptor subclasses.

ACKNOWLEDGEMENTS

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