Published in final edited form as:
J Pharmacol Exp Ther. 1991 October ; 259(1): 286–294.

Behavioral Effects of A_1- and A_2-Selective Adenosine Agonists and Antagonists: Evidence for Synergism and Antagonism

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Abstract

The locomotor effects in mice of selective A_1 and A_2 adenosine agonists, antagonists and combinations of agonists were investigated using a computerized activity monitor. The A_2-selective agonist 2-[(2-aminoethylamino)carbonylethylphenylethylamino]-5'-N-ethylcarboxamidoadenosine (APEC), an amine derivative of 2-(carboxyethylphenylethylamino)adenosine-5'-carboxamide, was a more potent locomotor depressant than its amide conjugates. The rank order of potency after i.p. injection for adenosine agonists was 5'-N-ethylcarboxamidoadenosine (NECA) (ED_{50}, 5.8 nmol/kg) > APEC (ED_{50}, 25 nmol/kg) > N^6-cyclohexyladenosine (CHA) (ED_{50}, 270 nmol/kg). An A_1-selective, centrally acting, adenosine antagonist, 8-cyclopentyltheophylline (10 mg/kg), completely reversed the locomotor depressant effects of CHA (A_1-selective) and NECA (nonselective) at doses of agonists as high as twice the ED_{50}, and shifted the dose-response curves to the right, suggesting a primary involvement of A_1 receptors. 8-cyclopentyltheophylline did not affect the depressant effects of APEC at the ED_{50}, consistent with the A_2-selectivity of APEC. The locomotor effects of APEC and CHA were completely reversed by theophylline, but not by the peripherally active 8-p-sulfophenyltheophylline, indicating central action of the adenosine agonists. The depressant effects of APEC, but not of NECA or CHA, were reversed significantly by an A_2-selective adenosine receptor antagonist, 4-amino-8-chloro-1-phenyl-[1,2,4]triazol[4,3-a]quinoxaline. Low or subthreshold doses of CHA potentiated the depressant effects of APEC. A subthreshold dose of CHA did not alter the depressant effect of NECA, whereas a subthreshold dose of APEC increased the depressant effects of low doses of NECA. Thus, it appears that A_1- and A_2-selective adenosine agonists have separate central depressant effects, which can be potentiative. The relatively high potency of NECA *in vivo* could be due to a synergism between central A_1 and A_2receptor activation by this nonselective agonist.

Adenosine is a modulator of many physiological functions. In the CNS adenosine depresses neuronal activity and causes behavioral depression (Snyder, 1985; Dunwiddie, 1985; Dunwiddie *et al.,* 1986; Phillis *et al.,* 1986; Fredholm and Dunwiddie, 1988; Durcan and Morgan, 1989a). At least two classes of adenosine receptors have been defined: A_1-adenosine receptors inhibit, whereas A_2-adenosine receptors stimulate adenylate cyclase (Van Calker *et al.,* 1979; Hamprecht and Van Calker, 1985). A_1 receptors also can inhibit calcium fluxes (Cerbai *et al.,* 1988) and stimulate potassium fluxes (Belardinelli and Isenberg, 1983). Effects of A_1 receptors on phosphoinositide breakdown also have been reported (Linden and Delahunty, 1989). The relevance of A_1 and A_2 receptors to CNS function is under active investigation.

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A_1-selective agonists such as CHA and R-PIA, and the non-selective agonist NECA, are potent locomotor depressants in rodents (Snyder *et al.,* 1981; Seale *et al.,* 1988; Bruns *et al.,* 1988; Heffner *et al.,* 1989). Alkylxanthines, such as theophylline and caffeine, which act as CNS stimulants, are adenosine antagonists and reverse the behavioral depression elicited by adenosine analogs (Snyder *et al.,* 1981; Barraco *et al.,* 1983, 1984; Katims *et al.,* 1983; Glowa *et al.,* 1985). The locomotor depressant actions of adenosine agonists appear to be centrally mediated, because they are reversed by theophylline, but not by xanthines such as 8-PST that poorly penetrate the blood-brain barrier (Katims *et al.,* 1983; Seale *et al.,* 1988; Nikodijevic *et al.,* 1990; Durcan and Morgan, 1989b). The depressive effects of N^6-cyclopentyladenosine, a close analog of CHA, are reversed by highly A_1-selective antagonists such as CPT, indicating that A_1 receptors activated by N^6-cycloalkyladenosines subserve behavioral depression (Bruns *et al.,* 1988). However, the potencies of adenosine agonists in locomotor depression were recently found to correlate to the potencies of the analogs at A_2 adenosine receptors and not to potencies at A_1 adenosine receptors (Durcan and Morgan, 1989a), leading to the proposal that primarily A_2 receptors are involved in these effects. Spealman and Coffin (1986) also concluded that A_2 receptors were involved in disrupting schedule-controlled behavior in monkeys. However, in similar studies in rats, the 100- to 300-fold greater potency of R-PIA relative to S-PIA is more consonant with the involvement of A_1 receptors (Goldberg *et al.,* 1985).

Although A_1-selective agonists have been developed, adenosine agonists or antagonists truly selective for A_2 adenosine receptors for use as physiological probes have been difficult to identify. CGS21680 is A_2-selective in competitive binding experiments at central A_1 (measured in cortex) and A_2 (measured in striatum) adenosine receptors by a factor of 140, and was shown to be A_2-selective in the cardiovascular system (Hutchison *et al.,* 1989; Jarvis *et al.,* 1989). CGS21680 contains a carboxylic acid functionality, which is expected to limit its passage across the blood-brain barrier. Using a functionalized congener approach, a series of long chain derivatives of CGS21680 that retain A_2 potency and selectivity and do not contain the carboxylic functionality was synthesized (Jacobson *et al.,* 1989). An amine derivative, APEC (table 1, compound 1) had a *Ki* value of 6 nM *vs.* [^{3}H]NECA at central A_2 receptors compared to 235 ± 54 nM *vs.* [^{3}H]R-PIA at central A_1 adenosine receptors in rat brain (unpublished data). APEC served as a synthetic intermediate for molecular probes for A_2 adenosine receptors (Jacobson *et al.,* 1989; unpublished data). Recently, we reported that APEC is a potent locomotor depressant in mice and that the pharmacological profile of this *in vivo* action suggests activation of A_2 adenosine receptors (Nikodijevic *et al.,* 1990).

Few antagonists with even modest selectivity for A_2 receptors have been developed. However, from a series of triazoloquinoxalines, CP-66,713 proved to be aselective A_2 antagonist (Sarges *et al.,* 1990). CP-66,713 has IC_{50} values of 21 nM *vs.* [^{3}H]NECA and 270 nM *vs.* [^{3}H]*R*-PIA at rat brain A_2 and A_1 adenosine receptors, respectively. The purpose of the present study is to characterize the A_1 and A_2 receptor-related components of locomotor depression induced by adenosine analogs, using CHA as an A_1-selective agonist, CPT as an A_1-selective antagonist, NECA as a nonselective agonist, APEC as an A_2-selective agonist and CP-66,713 as an A_2-selective antagonist.

Materials and Methods

Chemicals—NECA, CHA, 8-PST, CPT, and hydroxypropyl-γ-cyclodextrin were obtained from Research Biochemicals, Inc. (Natick, MA). CGS 21680C (Na salt) was the generous gift of Dr. A. Hutchison of CIBA-Geigy Corp. (Summit, NJ). APEC and derivatives 2 through 5 (table 1) were synthesized as described (Jacobson *et al.,* 1989). CP-66,713 was prepared by Pfizer Central Research (Groton, CT) according to the procedure of Sarges *et al.* (1990).

Animal Studies

Subjects—Adult male mice of the NIH (Swiss) strain weighing 25 to 30 g were housed in groups of 12 animals per cage. In the animal room and in the experimental room, mice were kept on a 12/12 h dim lighting/dark cycle. The animals were given free access to standard pellet food and water and were habituated for 24 h in laboratory conditions before testing. Each animal was used only once in the activity monitor.

Locomotor activity—The behavioral effects in mice elicited by selective A_1 and A_2 adenosine agonists and antagonists administered peripherally were investigated. Individual animals were studied in a Digiscan activity monitor (Omnitech Electronics Inc., Columbus, OH) equipped with an IBM-compatible computer. The monitor included multiple activity monitor cages (40 × 40 × 30.5 cm), each of which is surrounded by horizontal and vertical sensors, which were not detectable by the rodent. The cages were connected to a Digiscan Analyzer and printer, which collects, sorts and prints out the locomotor activity information collected over a specified time period. The data were automatically written to disk at the end of the period, and group data were tabulated. The measurements made by the monitor represent multivariate locomotor analysis with specific measures, such as simultaneous measurements of ambulatory, rearing, stereotypical and rotational behaviors. Two nonequivalent parameters (Sanberg *et al.,* 1985) were analyzed: 1) horizontal activity, which represents the total number of beam interruptions in the horizontal direction and 2) total distance traveled, which indicated the distance in cm traveled by the animal. The latter is dependent on the path taken. For most purposes in this study, only total distance traveled is presented, because changes in horizontal activity were highly correlated with total distance traveled. Mice were tested individually, in a sound- (white noise) and light- (low level fluorescent light from above) controlled area. Data were collected between 9 A.M. and 1 P.M. for three consecutive intervals of 10 min each and analyzed as a group for a 30-min sampling period, except for figure 1. Statistical analysis was performed using the Student *t* test. The results are reported as mean ∓ S.E. for each point. The number of mice in each experimental group was $n = 6$ to 33, except for the control group, where $n = 35$.

ED_{50} values (figs. 3–7) were determined by fitting the data with nonlinear regression to sigmoidal curves using the computer program GraphPad (ISI) and represent the dose which produced half the maximal effect observed (100% value for all curves corresponds to the control in the absence of drug and 0% is the maximum response elicited by agonist). Due to a limited number of points on the curves for a combination of theophylline and agonist (figs. 3 and 4), it was not possible to calculate a precise ED_{50}. ED_{50} values varied somewhat between experimental sets. Control values were determined separately for each experiment.

Drug administration—All drugs were dissolved in a 20:80 v/v mixture of Emulphor EL-620 (GAF Chemicals Corp., Wayne, NJ) and phosphate-buffered saline and administered i.p. in a volume of 5 ml/kg body weight. Warming and sonication aided in dissolving the drugs. CP-66,713 was of very low aqueous solubility, and this necessitated increasing the volume injected to 10 ml/kg. Where applicable, the antagonist was injected 10 min before the agonist. When a combination of agonists was administered, there was a 5-min delay between injections. Immediately after the final injection, the mouse was placed in the activity monitor cage, and data collection was begun after a delay of 10 min.

Isobolographic Analysis

To determine whether the locomotor depressant effects of CHA and APEC are additive or greater than additive (synergistic), three subseries of experimental groups were used. First, three dose-effect curves were determined: two with APEC or CHA given alone, and a third with a combination of approximately equipotent doses of CHA and APEC. Five points ($n =$

6–12 for each) were used to determine the dose-response curve in each subseries of experiments. In the subseries for the combination, the ratio of doses of APEC and CHA was between 1:4 and 1:6, because in preliminary experiments it was found that APEC was approximately 4 to 6 times as potent as CHA in locomotor depression. The construction of the dose-response curves and the determination of ED_{50} values were based on the Probit procedure (Finney, 1971; Kissin *et aL.,* 1990). The resulting ED_{50} values were then plotted in the form of an isobologram (fig. 9). The ED_{50} value for each drug alone was plotted on the *x* and *y* axes, and the combined ED_{50} value was placed in the dose field.

Algebraic (fractional) analysis (Kissin *et al.*, 1990) was based on the relationship of ED_{50} doses for APEC and CHA in combination (A_c and C_c, respectively) to the doses of these drugs that produce the same effect when given separately (A_s and C_s, respectively). The value of the sum of fractional doses ($A_c/A_s + C_c/C_s$) was interpreted to indicate synergism (<1.0), antagonism (>1.0) or additivity (=1.0), based on a previous application of this method (Berenbaum, 1977).

Results

Locomotor depression elicited by novel adenosine analogs

APEC and other 2-substituted-amino-adenosine-5′-carboxamide derivatives, including conjugates of APEC, were examined for locomotor effects in varying doses. Table 1 shows the results for APEC, compound 1, and conjugates of APEC (Jacobson *et al.*, 1989), in which the amino group has been condensed with the following: *p*-hydroxyphenylpropionic acid (compound 2) or 2-thienylacetic acid (compound 3), both of which are iodinatable for potential radiolabeling, and *p*-sulfophenylisothiocyanate (compound 4), which is charged for potentially restricting activity to the periphery. Another derivative, consisting of a higher homolog of APEC coupled to the *p*-fluoromethylbenzoyl group, a prosthetic group for radiofluorination (compound 5), is included. All of these conjugates, which were reported to be A_2-selective in binding assays in the range of 50- to 350-fold (Jacobson *et al.*, 1989), were found to depress locomotor activity at low doses indicating *in vivo* potency. We did not determine ED_{50} values for each compound, except for APEC (compound 1). Nevertheless, for each compound there was a definite trend of the dose dependence of the locomotor depression. At a dose of 30 nmol/kg, APEC was the most potent, followed in order of potency by compounds 5 > 4 > 2 > 3. At a 10-fold higher dose, the conjugates were nearly equipotent, yet still less potent than APEC. The locomotor depression induced by derivative 4 was not reversible by a high dose of the nonselective peripherally acting antagonist, 8-*p*-sulfophenyltheophylline, suggesting that the action of the agonist was not limited to the periphery in spite of the presence of the charged sulfonate group. This might be a result of *in vivo* hydrolysis of derivative 4. We previously reported that the precursor of APEC, CGS21680, was only very weakly active as a locomotor depressant when administered peripherally (Nikodijevic *et al.*, 1990).

Locomotor depression elicited by an A_2-selective adenosine agonist, APEC

Mice were injected i.p. with 30 nmol/kg (found previously to be the ED_{50}) of APEC, or with vehicle, and locomotor activity was measured for six successive periods of 10 min beginning 10 min after the administration of the drug or a vehicle. Figure 1 indicates that the depressant effects of APEC on the total distance traveled were intense and remained constant during the 1-h monitoring period. Depression elicited by APEC, in both parameters, significantly differed from vehicle controls at all time points ($P < .001$). In vehicle-treated mice, locomotor activity decreased gradually by approximately 20% between 10 and 60 min after the beginning of monitoring.

Effects of theophylline on locomotor activity in mice

Initially, the nonselective centrally active adenosine antagonist, theophylline, was used to demonstrate that APEC was acting *in vivo* at adenosine receptors. It was first necessary to determine the effect of theophylline alone, injected i.p. in mice, as a behavioral stimulant. Figure 2 shows a dose-dependent stimulation of total distance traveled at doses ranging from 1 to 30 mg/kg ($P < .005$). However, at the highest dose tested (100 mg/kg), theophylline produced a significant depressant effect on locomotor activity compared to the control value ($P < .05$). A dose of 10 mg/kg theophylline was used to investigate reversal of adenosine agonist-induced behavioral depression.

Effects of theophylline on locomotor depression elicited by selective adenosine agonists

Reversal of the centrally elicited locomotor depressant effects of adenosine agonists by the nonselective adenosine antagonist theophylline, a well-documented phenomenon (Snyder *et al.*, 1981; Barraco *et al.*, 1983; Glowa *et al.*, 1985), was examined. Locomotor depressant effects of adenosine agonists selective for A_1 or A_2 adenosine receptors, CHA and APEC, respectively, were measured in the presence and absence of theophylline (10 mg/kg), injected i.p. Figures 3 and 4 show the effect of a fixed dose of theophylline on the dose-response curves for locomotor depression by CHA and APEC. The curve for APEC (fig. 3) was clearly shifted to the right (EC_{50} shifted from 25.2 nmol/kg to approximately 266 nmol/kg) in the presence of theophylline, suggesting a pharmacological antagonism, as would be predicted from the large body of biochemical experiments with theophylline/caffeine and adenosine analogs carried out previously in other laboratories. However, the stimulant effects of theophylline complicate interpretation of interactions of the two agents. The curve for CHA (fig. 4) was shifted markedly to the right in the presence of theophylline. The EC_{50} was shifted from 268 nmol/kg in the absence of theophylline to an undetermined greater value. Theophylline completely blocked the depressant effect of CHA relative to control at doses up to 1000 nmol/kg, as well as the effect of APEC at doses up to 25 nmol/kg. Again the stimulant effects of theophylline complicated interpretation of the interactions of theophylline and the adenosine analogs. At the highest dose of CHA tested (3000 nmol/kg) there was a statistical difference ($P < .001$) between the values for locomotor activity with agonist alone or agonist and theophylline combined. Remarkably, at the lowest dose of CHA there was no longer any locomotor stimulation elicited by theophylline.

Effects of an A_1-selective antagonist (CPT) on adenosine agonist-induced locomotor depression

The rank order of potency (figs. 5–7) after i.p. injection for adenosine analogs was NECA (ED_{50}, 5.8 nmol/kg) > APEC (ED_{50}, 25 nmol/kg) > CHA (ED_{50}, 270 nmol/kg)

CPT, an A_1-selective antagonist, has been reported to antagonize the centrally induced locomotor depressant effects of adenosine agonists, such as N^6-cyclopentyladenosine (Bruns *et al.,* 1988). To further explore this finding, we tested a single dose of CPT with different doses of the adenosine agonists CHA, NECA and APEC. At a dose of 10 mg/kg (at its maximum aqueous solubility), CPT, like theophylline, caused a behavioral stimulation (figs. 5–7). At this dose CPT significantly reversed the dose-related depressant effects of both CHA and NECA, but there was no reversal of the effects of APEC administered at a dose equal to the previously determined ED_{50}. Dose-response curves for CHA- and NECA-treated mice were shifted to the right in the presence of CPT. Reversal elicited by the antagonist CPT was complete for both CHA (fig. 5) and NECA (fig. 6) at doses of agonists up to twice the ED_{50} dose. The EC_{50} for CHA was shifted from 172 to 620 nmol/kg in the presence of CPT. At the highest dose of CHA tested (1000 nmol/kg) there was a stastical difference ($P < .001$) between the values for locomotor activity with agonist alone or agonist and CPT combined. The EC_{50} for NECA was shifted from 5.76 to 43.8 nmol/kg in the

presence of CPT. These results might suggest that the locomotor depressant effects of CHA and NECA are primarily the result of the stimulation of central A_1 adenosine receptors. In contrast, the locomotor depressant effects of an A_2 receptor-selective adenosine analog, APEC (Nikodijevic *et al.,* 1990), indicated that activation of A_2 adenosine receptors also could result in locomotor depression. Consistent with this conclusion, the A_1-selective antagonist CPT was unable to reverse the locomotor depression induced by APEC (fig. 7).

The effect of an A_2-selective adenosine antagonist (CP-66,713) on adenosine agonist-induced locomotor depression

An A_2-selective adenosine antagonist, CP-66,713, was recently developed (Sarges *et al.,* 1990). CP-66,713 has a 13-fold selectivity for A_2 receptors in binding studies in rat brain and has a K_i value of 12 nM at rat brain A_2 receptors (*vs.* binding of [^{3}H]CGS21680, unpublished data). CP-66,713 was used as an A_2-selective antagonist for further characterization of the locomotor depressant effects of APEC. Results presented in figure 8 show a reversal by CP-66,713 of the locomotor depression in mice induced by APEC. CP-66,713, at a dose of 1.5 mg/kg administered i.p., significantly reversed the dose-dependent depressant effects of APEC at doses as high as twice the ED_{50} dose (fig. 8). The ED_{50} of APEC in this experiment shifted from 8.47 to 66.4 nmol/kg in combination with CP-66,713. However, at higher doses of APEC (≥180 nmol/kg), CP-66,713 failed to diminish the locomotor depression. Higher doses of CP-66,713 could not be administered because of a low solubility of approximately 1 μM in aqueous buffers. In the Emulphor injection medium the maximum solubility of CP-66,713 was found by ultraviolet analysis to be 0.4 mM. Use of a 40% solution of the solubilizer hydroxypropyl-γ-cyclodextrin as a cosolvent failed to increase the solubility of CP-66,713 significantly. CP-66,713 dissolved in dimethylsulfoxide/aqueous mixtures could not be used, because dimethylsulfoxide alone, present in the vehicle at a concentration of only 15%, elicited a locomotor depressant effect in mice (data not shown). At a dose of 1.5 mg/kg, CP-66,713 alone did not cause a significant stimulation of behavior (fig. 8).

Peripherally administered CP-66,713 appears to be selective as a central A_2 adenosine receptor antagonist in the mouse. Thus, CP-66,713, at a dose of 1.5 mg/kg. did not block the locomotor depression induced by doses equal to the previously determined ED_{50} doses of the A_1-selective agonist CHA or of the nonselective agonist NECA (table 2).

Locomotor effects of combinations of adenosine agonists

Combinations of adenosine agonists, both CHA and APEC (selective A_1 and A_2 agonists, respectively) as well as NECA (a nonselective adenosine agonist), were administered in order to explore possible facilitatory effects of A_2 receptor activation on A_1 receptor-elicited depression and *vice versa*. Results from this study are presented in figure 9 and tables 4 through 6.

The A_1-selective agonist CHA, at a dose of 29 nmol/kg, which by itself has only a small effect on locomotor activity in mice, resulted in a significant potentiation of the depressant effect of APEC (table 3) when administered together with APEC at either a noneffective dose (3.7 nmol/kg) or at the previously determined ED_{50} dose. In both cases, the effect of the combination was greater than additive ($P < .001$).

The potentiation of locomotor depressant effects was also observed in mice treated with a combination of CHA and APEC, administered at their respective, previously determined ED_{50} doses. The isobolographic analysis comparing APEC and CHA ED_{50} doses when the drugs were given in combination *vs.* separately is presented in figure 9. The ED_{50} values for the combination were 6.1 nmol/kg for APEC and 54 nmol/kg for CHA. The point

corresponding to the ED_{50} doses for the combination deviates to the left of the line of additivity, clearly indicating statistically valid synergism. An algebraic (fractional) analysis of these data is presented in table 4. With the combination, the sum of the fractions (0.53) indicated a degree of synergism of 1.89.

A combination of CHA at a subthreshold dose with a minimum effective dose (3.2 nmol/kg) or previously determined ED_{50} (6.5 nmol/kg) dose of NECA did not potentiate the depressant effect of NECA (table 5).

In order to see if the A_2-selective adenosine agonist APEC could potentiate the locomotor depression induced by NECA, further experiments were conducted using a combination of APEC (3.7 nmol/kg, a dose that was without effect on locomotor activity) and NECA (at two different doses: at 3.2 nmol/kg, nearly the lowest effective dose, or at 6.5 nmol/kg, the previously determined ED_{50} for NECA). Results of these experiments are presented in table 6. APEC at a noneffective dose potentiated the locomotor depression in mice induced by 3.2 nmol/kg of NECA. The effect of the combination on total distance traveled was greater than additive ($P < 0.001$). There was no potentiative effect of the same dose of APEC in combination with the previously determined ED_{50} dose of NECA.

Discussion

Adenosine agonists elicit a profound behavioral depression in mammals (Snyder *et al.*, 1981; Glowa *et al.*, 1985; Durcan and Morgan, 1989a; Yarbrough and McGuffin-Clineschmidt, 1981). Similar locomotor depressions are elicited after either parenteral or intracerebroventricular administration and appear to be centrally mediated. The main evidence for a central site of action is that adenosine antagonists, such as 8-PST, that do not cross the blood-brain barrier do not reverse the behavioral effects of adenosine analogs (Katims *et al.*, 1983; Seale *et al.*, 1988; Durcan and Morgan, 1989b). Our preliminary study (Nikodijevic *et al.*, 1990) also supported an assumption that locomotor depressant effects of adenosine analogs are not peripheral because they could not be blocked by 8-PST or 1,3-dipropyl-*p*-sulfophenylxanthine, two potent, but nonselective, peripherally acting adenosine antagonists. A 10 mg/kg dose of the standard peripheral antagonist 8-PST was chosen because it has been shown to effectively block the peripheral actions of adenosine agonists in other studies (see above).

The nature of the receptors subserving the centrally mediated depression has not been resolved. Selective A_1 agonists such as CHA and N^6-cyclopentyladenosine elicit behavioral depression, and selective A_1 antagonists do reverse the effects of adenosine analogs in mice. However, the *in vivo* potencies of several adenosine analogs did not correlate with potencies in binding at a rat brain A_1 receptor, but did correlate with potencies at a rat striatal A_2 receptor (Durcan and Morgan, 1989a). The lack of behavioral effects of *R*-PIA in one strain of mice was attributed to low levels of brain A_1 receptors (Fredholm *et al.*, 1985), but levels of A_2 receptors were not ascertained. In two mouse strains, a selective increase in sensitivity to NECA, but not to CHA was observed (Seale *et al.*, 1986). In one strain, NECA was more potent than CHA, suggesting that A_2 receptors were important in this strain, whereas in the other strain NECA and CHA were equipotent. Recently we reported that the selective A_2 agonist APEC can elicit a centrally mediated locomotor depression (Nikodijevic *et al.*, 1990). Preliminary data on behavioral depression elicited by a somewhat A_2-selective agonist, 2-phenylaminoadenosine (CV 1808), indicated that A_2 receptors were involved (Brans *et al.*, 1988). Thus, it appears that either activation of A_1 or A_2 receptors can lead to locomotor depression. The importance and possible interactions of such receptor pathways to the effects of very potent nonselective agonists, such as NECA, remain unknown.

Our previous report of behavioral effects of a recently developed A_2-selective adenosine agonist (APEC) strongly suggested possible involvement of central A_2 receptors in the locomotor depression that is induced by adenosine and certain active analogs. The results in screening other A_2-selective agonists (table 1) in locomotor depression have revealed that APEC is highly potent *in vivo* in relation to other analogs and, therefore, an appropriate choice for the present study. The K_i values at A_2 receptors from binding studies using [^{3}H]CGS21680 (Jarvis *et al.*, 1989) in the rat brain range from 12 for APEC to 26 nM for compound 4 (values from Jacobson *et al.*, 1989). Some of these analogs are more A_2-selective than APEC and still cause behavioral depression. CGS21680 was relatively weak as a locomotor depressant. Doses of 30 nmol/kg and 1000 nmol/kg resulted in 13 and 62% decreases, respectively, in total distance traveled. This likely reflects poor penetration of the blood-brain barrier by CGS21680, which contains a carboxylate group.

The present study provides evidence that potentiative interactions between A_1 and A_2 adenosine receptor components can contribute to the locomotor depressant effects of adenosine and its analogs. The potency of CPT (an A_1-selective adenosine antagonist) in inhibiting the locomotor depression elicited by CHA or NECA (A_1-selective and nonselective adenosine agonists, respectively) provided strong evidence for an A_1 receptor involvement in locomotor depression induced by these two adenosine agonists. Similarly, the lack of reversal by CPT of the locomotor depression elicited by APEC (a 41-fold A_2-selective adenosine agonist) indicates that an A_1 receptor is not involved in the action of this adenosine agonist. Theophylline did block depressant effects of CHA at doses up to 1000 nmol/kg and APEC at doses up to 25 nmol/kg. The pronounced right shift in the depressant effects of CHA and APEC by theophylline indicates that adenosine receptors are involved. However, theophylline has behavioral stimulant activity alone (fig. 2), and this complicates interpretation of its antagonistic activity *vs.* adenosine analogs. Curiously, we have observed that a low dose of CHA abolishes the stimulatory effect of theophylline (fig. 3b).

The present dose of theophylline (10 mg/kg) was chosen to avoid possible contributions from the depressant effects of this xanthine that begin to manifest themselves at doses >30 mg/kg (fig. 2). The depressant effect of methylxanthines at very high doses has been noted previously with caffeine and other xanthines (Thithapandha *et al.*, 1972; Kaplan *et al.*, 1989).

The A_2-selective adenosine antagonist CP-66,713, a triazoloquinoxaline derivative, is selective for A_2 receptors by a factor of 13 in binding assays (Sarges *et al.*, 1990). It was used to further investigate the nature of adenosine receptors involved in behavioral depression. CP-66,713 blocked locomotor depression in mice induced by APEC at twice the ED_{50} dose. It was not possible to block higher depressant doses of APEC with CP-66,713, presumably because the low solubility of the antagonist precluded administration of higher dosages. Pretreatment of mice with CP-66,713 did not block the depressant effects of NECA and CHA at previously determined ED_{50} doses (table 2). This is consistent with our previous report (Nikodijevic *et al.*, 1990), which suggested that primarily A_1 receptors are involved in the depression elicited by both CHA and NECA. Antagonism of the locomotor depressant effects of APEC by CP-66,713 further supports the conclusion that the depressant effects of APEC are due to the stimulation of A_2 adenosine receptors in the brain.

The relative potencies of NECA and CHA did not correlate to their A_1 receptor affinities even though both were antagonized by the A_1-selective antagonist CPT. NECA was much more potent than CHA as a locomotor depressant, whereas the K_i values for a rat brain A_1 receptor are 6.3 nM and 1.3 nM, respectively. At rat striatal A_2 receptors, the K_i values were 10.3 and 514 nM, respectively (Bruns *et al.*, 1986). The better correlation of locomotor effects with A_2 receptor affinities have led to the proposal that A_2 receptors are involved

(Durcan and Morgan, 1989a), although the antagonism by selective A_1 antagonists, such as CPT, would appear to contradict such a conclusion. Pharmacokinetics might play a role in the relatively high potency of NECA. However, it is possible that the high potency of NECA compared to CHA is due to the fact that NECA is a potent nonselective agonist and may elicit depression through summation of both A_1 and A_2 receptor activation, with A_1 activation being essential and A_2 activation potentiative. Therefore, we have experimented with combinations of A_1- and A_2-selective adenosine agonists in the same mouse. Injection of a noneffective dose of CHA together with subthreshold or previously determined ED_{50} doses of APEC, increased locomotor depression in mice in a potentiative manner (table 3). However, the same dose of CHA did not potentiate the locomotor depressant effects induced by NECA. Administration of CHA and APEC at these ED_{50} doses to the same animal produced a greater than additive depressant effect in mice. When a combination of NECA and APEC (at a noneffective dose) was used, a significant potentiation of depressant effects was observed at the lowest effective dose of NECA, but not at its previously determined ED_{50} dose (table 3).

These results indicate that either A_1- or A_2-selective adenosine agonists are central depressants and that they can act in a synergistic (potentiative) manner through activation of A_1 and A_2 adenosine receptors in the brain. This synergism between A_1 and A_2 agonists may account for the high potency of NECA as a locomotor depressant, observed in this study and in others (Seale *et al.*, 1988; Durcan and Morgan, 1989b), in relation to its affinity for either A_1 or A_2 adenosine receptors. It appears likely that the depression due to activation of A_1 receptors is of primary importance, and that A_2 receptor activation is mainly potentiative. However, the lack of effect of the A_2 selective antagonist CP-66,713 on the depression elicited by NECA (table 2) seems inconsistent with this interpretation. It could be that doses of NECA that activate the A_1 receptors are fully saturating the A_2 receptors and it is, therefore, difficult to block NECA activation of A_2 receptors with CP-66,713. Higher doses of CP-66,713 could not be used because of low solubility. Certainly, at the previously determined ED_{50} dose for NECA, APEC could not further enhance the depressant effect of NECA (table 6). In summary, the present results indicate that both $A_1$and A_2 receptors can be involved in the regulation of behavioral states by adenosine agonists. The results with APEC indicate that a clearly A_2-selective agonist can elicit full behavioral depression.

The nature of interaction of A_1 and A_2 receptor activation *in vivo* is unknown. Synergistic interactions of A_1 and A_2 receptors have not been demonstrated *in vitro* with cells or membranes. It likely represents interactions at the level of different neuronal pathways involved in behavior, some of which are regulated by A_1 receptors, others of which are regulated by A_2 receptors.

ABBREVIATIONS

APEC	2-[(2-aminoethylamino)carbonylethylphenylethylamino]-5′-N-ethylcarboxamicloadenosine
CGS21680	2-(carboxyethyl-phenylethylamino)adenosine-5′-carboxamide
CHA	N^6-cyclohexyladenosine
CNS	central nervous system
CPT	8-cyclopentyltheophylline
CP-66	713, 4-aminc-8-chloro-1-phenyl-[1,2,4]triazolo[4,3-a]quinoxaline
NECA	5′-N-ethylcarboxamidoadenosine

8-PST	8-*para*-sulfophenyltheophylline
***R*-PIA**	N^6-*R*-(phenylisopropyl)adenosine.

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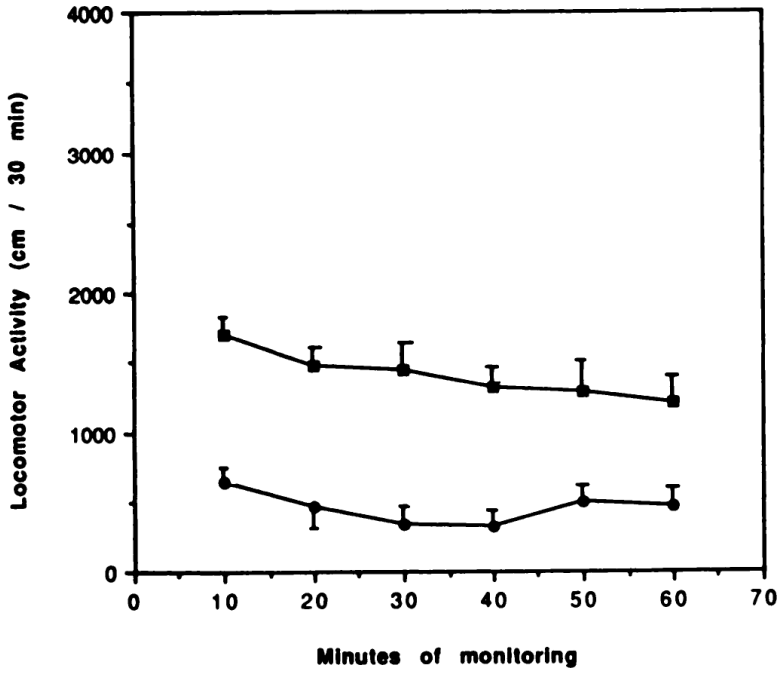


Fig. 1.

Locomotor activity over 60 min in control animals (■) and for animals injected i.p. with APEC (30 nmol/kg), an A_2selective adenosine agonist (●). Activity counts for total distance traveled are shown in relation to time elapsed since the beginning of monitoring in a Digiscan activity monitor, initiated 10 min after injection i.p. ($n = 6$). This dose of APEC in other experiments (see fig. 7) corresponded to the ED_{50} dose.

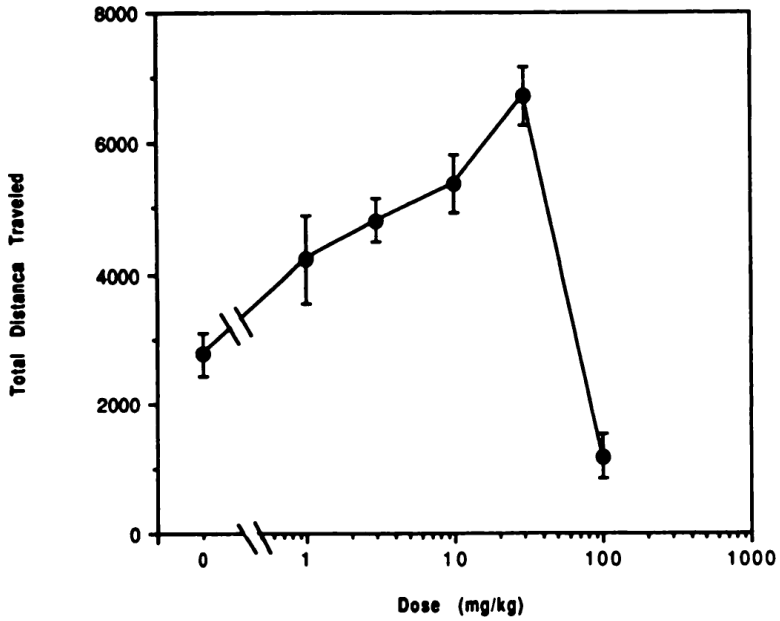


Fig. 2.

Dose-response curves for locomotor stimulation and depression in mice by the adenosine antagonist theophylline. Activity counts for total distance traveled (mean ± S.E.) over a 30-min period are shown as a function of dose of theophylline injected i.p (n = 6–8).

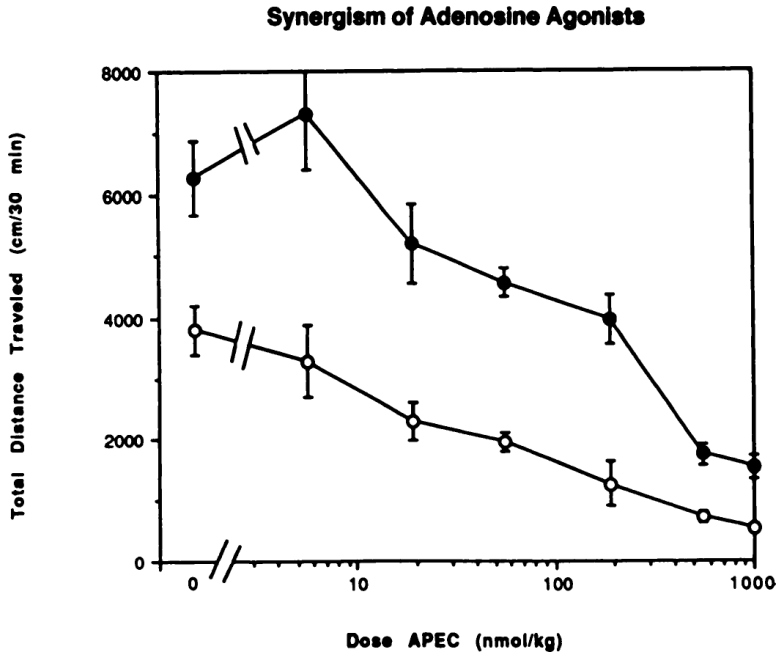


Fig. 3.

Reversal by theophylline (10 mg/kg) of locomotor depression elicited by the A_2-selective adenosine agonist APEC. The antagonist or vehicle was injected 10 min before the agonist. The activity reflects the total distance traveled (mean ± S.E.) over a 30-min period, begun 10 min after the agonist injection in the absence (◯) or presence (●) of theophylline (n = 6–12).

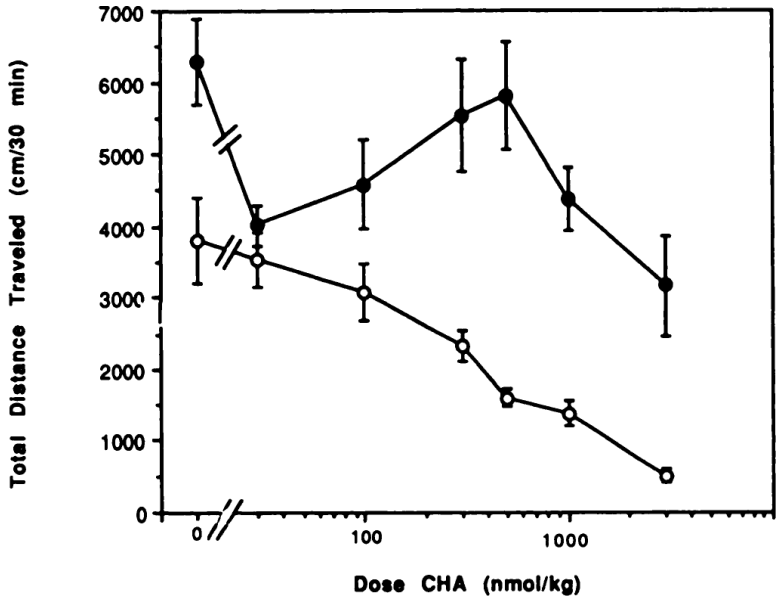


Fig. 4.

Reversal by theophylline (10 mg/kg) of locomotor depression elicited by the A_1-selective adenosine agonist CHA. The antagonist or vehicle was injected 10 min before the agonist. The activity reflects the total distance traveled (mean ± S.E.) over a 30-min period, begun 10 min after the agonist injection in the absence (◯) or presence (●) of theophylline (n = 6–12).

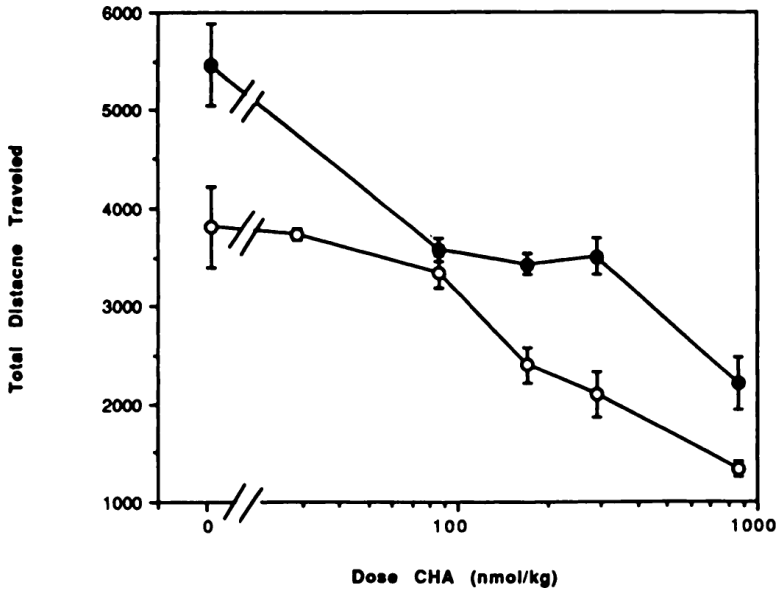


Fig. 5.

Dose-response curves for locomotor depression in mice by the adenosine agonist CHA in the absence (◯) or presence (●) of the A_1-selective antagonist CPT (10 mg/kg). The antagonist or vehicle was injected 10 min before the agonist. The activity reflects the total distance traveled (mean ± S.E.) over a 30-min period (n = 6–12).

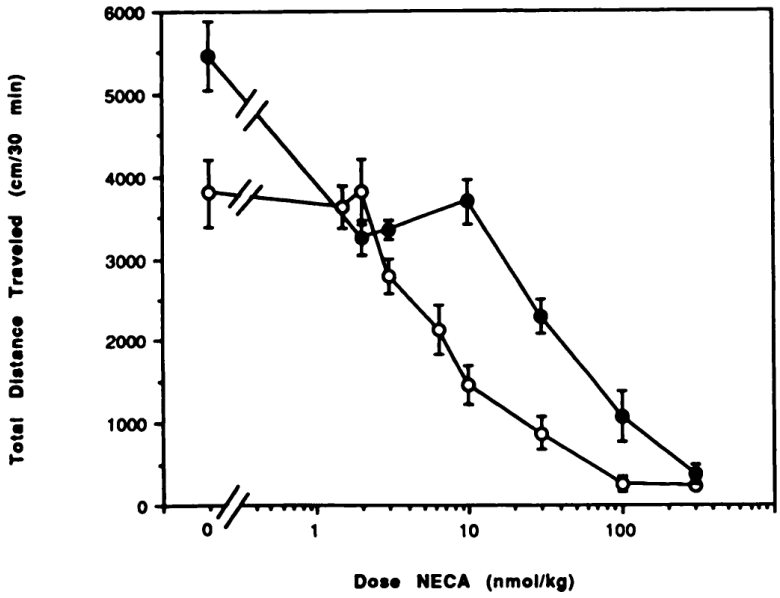


Fig. 6.

Dose-response curves for locomotor depression in mice by the adenosine agonist NECA in the absence (◯) or presence (●) of the A_1-selective antagonist CPT (10 mg/kg). The antagonist or vehicle was injected 10 min before the agonist. The activity reflects the total distance traveled (mean ± S.E.) over a 30-min period (n = 6–12).

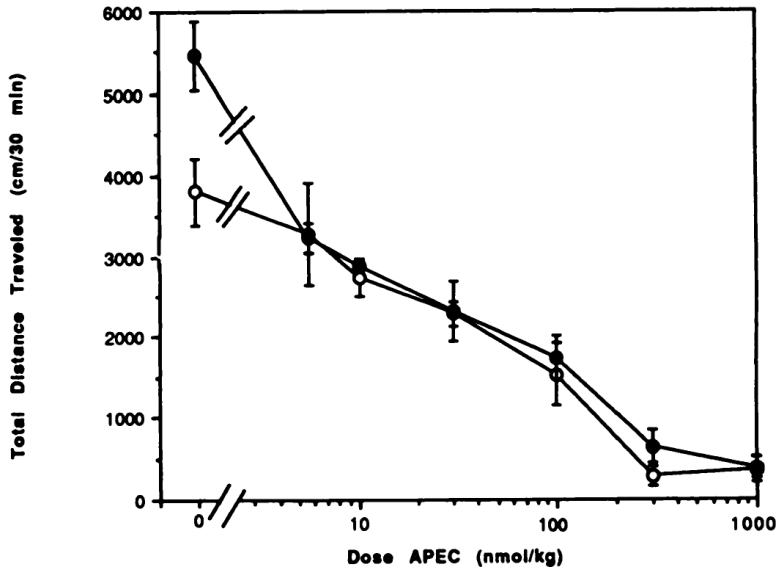


Fig. 7.

Dose-response curves for locomotor depression in mice by the adenosine agonist APEC in the absence (◯) or presence (●) of the A_1-selective antagonist CPT (10 mg/kg). The antagonist or vehicle was injected 10 min before the agonist. The activity reflects the total distance traveled (mean ± S.E.) over a 30-min period (n = 6–12).

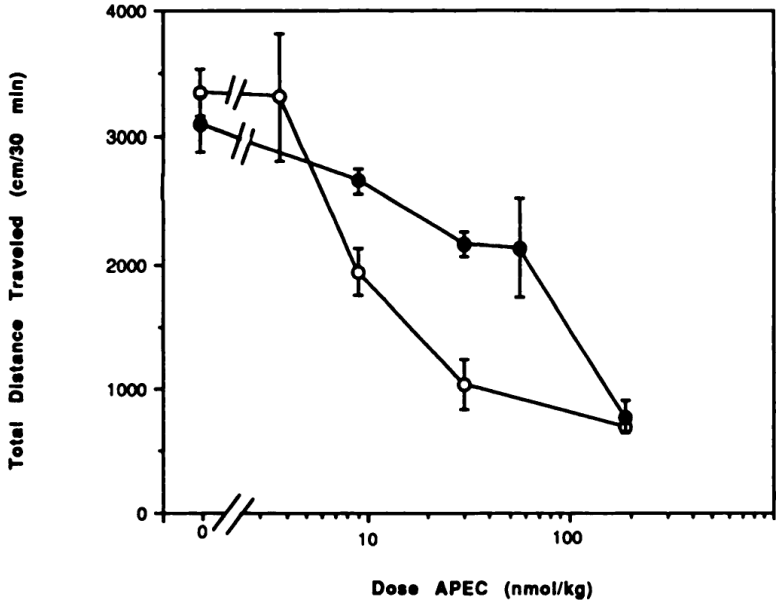


Fig. 8.

Dose-response curves for locomotor depression in mice by the adenosine agonist APEC in the absence (◯) or presence (●) of an A_2-setective adenosine antagonist, CP-66,713 (1.5 mg/kg). The antagonist was injected 10 min before the agonist. The activity reflects the total distance traveled (mean ± S.E.) over a 30-min period ($n = 8$). In this set of experiments and those of table 2, APEC was more potent than in others (see fig. 7, tables 1 and 3).

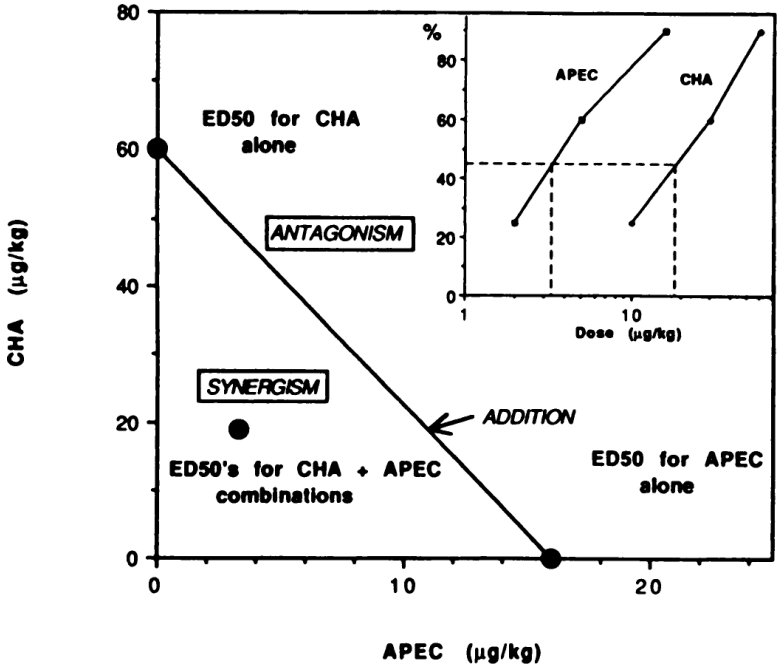
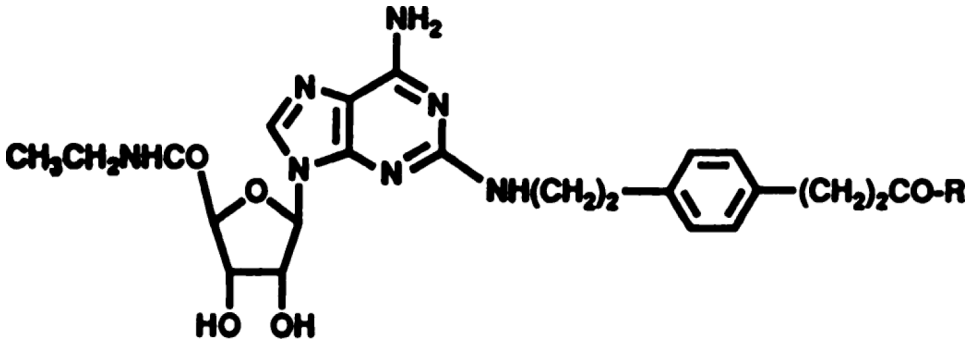


Fig. 9.

An isobotogram depicting the effects of combinations of adenosine agonists at various doses, which indicates that the interaction of A_1 and A_2 agonists is synergistic. The construction of the dose-effect curves and determination of ED_{50} values was based on the probit procedure (Kissin *et al.,* 1990; Finney, 1971). A CHA-APEC subseries, in which the ratio of doses was between 4:1 and 6:1 (combinations of approximately equieffective doses), was administered. The ED_{50} values for the combination were 6.1 nmol/kg for APEC and 54 nmol/kg for CHA. The inset shows the data from which the ED_{50} values were determined. Percent locomotor depression is plotted as a function of the dose of each agonist in the combination subseries.

TABLE 1

Summary of locomotor depression in mice elicited by various 2-substitiited-5′-carboxamidoadenosine analogs[a]



Compound R	A_2 selectivity[b]	Dose	Horizontl Activity	Total Distance Traveled
		nmol/kg		
1. −$NH(CH_2)_2NH_2$ (APEC)	20	30	2420 ± 121 (↓62%)	1600 ± 96 (↓52%)
		300	320 ± 22 (↓95%)	170 ± 11 (↓95%)
2. −$NH(CH_2)_2NHCO(CHL_2)_2$-ϕ-OH	73	23	5220 ± 605 (↓18%)	2346 ± 431 (↓30%)
		230	3568 ± 572 (↓44%)	1985 ± 338 (↓40%)
3. $NH(CH_2)_2NHCOCH_2$-2-thiophene	93	24	6756 ± 565 (↓6%)	3351 ± 249 (↑1%)
		240	3248 ± 620 (↓49%)	1686 ± 298 (↓91%)
		2400	569 (↓91%)	283 (↓91%)
4.[c] $NH(CH_2)_2NHCSNH$-ϕ-SO_3Na	145	21	4764 ± 528 (↓25%)	2466 ± 204 (↓38%)
		210	3756 ± 460 (↓41%)	2068 ± 233 (↓38%)
5. −$NH(CH_2)_4NHCO$-ϕ-CH_2F	77	23	3786 ± 395 (↓40%)	2140 ± 255 (↓36%)
		230	3170 ± 281 (↓50%)	1436 ± 126 (↓57%)

[a] Locomotor activity was indicated by counts, expressed as the mean ± S.E.M. for horizontal activity and total distance in a Digiscan activity monitor (control values, $n = 35$, were 6370 ± 478 and 3340 ± 218, respectively). Percent depression relative to vehicle control is given in parentheses. Adenosine derivatives were injected i.p. at the dose indicated in mg/kg b.wt., in a vehicle consisting of 20:80 v/v mixture of Emulphor EL-620 and phosphate-buffered saline and administered i.p. in a volume of 5 ml/kg b.wt. Monitoring was initiated 10 min after injection, and carried out for 30 min ($n = 6–7$, except where noted).

[b] A_2 selectivity ratios are equal to the ratio of K_I values at A_1/A_2 receptors from binding studies using [^{3}H]N^6-phenyliSopropyladenosine and [^{3}H]CGS21680 (Jarvis *et al.*, 1989) in the rat brain (values from Jacobson *et al.*, 1990). The K_I values at A_2 receptors range from 12 (APEC) to 26 nM (compound 4).

[c] Combined with a 10 mg/kg dose of the peripheral antagonist 8-PST, the locomotor activity folowing a dose (21 nmol/kg) of compound 4 was 4733 ± 560 counts (↓25%)for horizontal activity and 2339 ± 220 counts (↓30%) for total distance traveled ($n = 4$).

TABLE 2

Locomotor effects of an A_2-selective adenosine antagonist, CP-66,713, alone or in combination with CHA, NECA, or APEC

Drug(s)[a]	*n*	Locomotor Activity[b]	Depression(↓)
			%
None[b]	27	3344 ± 184	0
CP-66,713	10	3104 ± 218	6↓
CHA	8	2073 ± 145	38↓
CHA + CP-66,713	7	2006 ± 120	40↓
NECA	20	1806 ± 99	46↓
NECA + CP-66,713	9	1538 ± 92	54↓
APEC	16	1237 ± 87	63↓
APEC + CP-66,713	8	2408 ± 72	28↓

[a] Dose of CP-66,713 was 1.5 mg/kg i.p. The antagonist was injected 10 min before each agonist. The doses of CHA (170 nmol/kg), NECA (6.5 nmol/kg) and APEC (30 nmol/kg) were chosen to approximate the ED_{50}.

[b] Vehicle control.

[c] Mean total distance traveled (cm/30 min) ± S.E.M., and depression relative to vehicle control, are shown.

TABLE 3

Potentiative depressant effects of adenosine agonists in combination[a]

Dose		Locomotor Activity	Depression
APEC	CHA		
nmol/kg			%
0	0	3400 ± 184	0
3.7	0	3468 ± 35	2↑
30	0	2142 ± 129	37↓ **
0	29	3264 ± 68	4↓
0	170	1972 ± 122	42↓ **
3.7	29	2414 ± 221	29↓ **
30	29	1632 ± 85	52↓ **
30	170	204 ± 8	94↓ **

[a]The activity reflects the mean total distance traveled (cm) ± S.E.M. over a 30-min period, begun 10 min after the last injection (CHA). Injections of agonists in the same animal were separated by a 5-min interval, $n = 6–33$.

** $P < .01$ compared to vehicle control.

TABLE 4

Algebraic fractional analysis of locomotor depression elicited by combinations of CHA and APEC. Fractions represent an expression of the doses of the drugs in combination that produce the same effect when given separately (*i.e.*, equieffective). Antagonism is indicated if the sum of the fractions is more than 1.0, whereas synergism is indicated if the sum of the fractions is less than 1.0 (see Kissin *et al.*, 1990). With the combination of APEC and CHA, the sum of the fractions indicated a degree of synergism of 1.89.

Subseries	Fraction		Sum of Fractions[a]
	CHA Component	APEC Component	
	ED_{50} in nmol/kg		
CHA alone	1.00/170	0.00	1.00
APEC alone	0.00	1.00/30	1.00
CHA + APEC combination	0.32/54	0.21/6.1	0.53

[a]The sum of the fractions is defined as [(ED_{50} APEC, in combination/ED_{50} APEC, alone) + (ED_{50} CHA, in combination/ED_{50} CHA, alone)].

TABLE 5

Depressant effects of NECA and CHA in combination

Dose		Locomotor Activity	Depression
CHA	NECA		
nmol/kg			%
0	0	3400 ± 211	0
0	3.2	2380 ± 102	30↓ **
0	6.5	2142 ± 128	37↓ **
29	0	3264 ± 68	4↓ **
29	3.2	2244 ± 187	34↓ **
29	6.5	1904 ± 133	44↓ **

[a] The activity reflects the mean total distance traveled (cm) ± S.E.M. over a 30-min period, begun 10 min after the last injection (CHA). Injections of agonists in the same animal were separated by a 5-min interval (n = 9–12).

** $P < .01$ compared to vehicle control.

TABLE 6

Locomotor effects of combinations of NECA and APEC at various doses

Dose		Locomotor Activity	Depression
APEC	NECA		
nmol/kg			%
0	0	3400 ± 211	0
3.7	0	3468 ± 35	2↑
0	3.2	2380 ± 71	30↓ **
0	6.5	2142 ± 86	37↓ **
3.7	3.2	2006 ± 99	41↓ **
3.7	6.5	1972 ± 59	42↓ **

[a] The activity reflects the mean total distance traveled (cm) ± S.E.M. over a 30-min period, begun 10 min after the last injection (APEC). Injections of agonists in the same animal were separated by a 5-min interval (n = 8–14).

** $P < .01$ compared to vehicle control or 3.7 nmol/kg APEC.