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[^{3}H]5′-N-ethylcarboxamide adenosine binds to both R_a and R_i adenosine receptors in rat striatum

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Summary. Adenosine analogs such as 5′-N-ethylcarboxamide adenosine and N^6-cyclohexyladenosine stimulate or inhibit adenosine cyclase activity in preparations of rat striatum depending on the assay conditions. N^6-cyclohexyladenosine inhibits but does not stimulate adenosine cyclase activity in preparations of hippocampus. These findings suggest that the striatum contains both R_a (stimulatory) and R_i (inhibitory) adenosine receptors while the hippocampus contains only R_i receptors. We have previously shown that [^{3}H]N^6-cyclohexyladenosine binds to R_i receptors in rat hippocampus (Yeung and Green 1983). Comparisons of the characteristics of [^{3}H]5′-N-ethylcarboxamide adenosine and [^{3}H]N^6-cyclohexyladenosine binding to hippocampus show that [^{3}H]5′-N-ethylcarboxamide adenosine also binds to R_i receptors with high affinity. [^{3}H]5′-N-ethylcarboxamide adenosine binds to R_i receptors in the striatum and to a second site that is present in striatum but not hippocampus. High affinity binding of both ligands to R_i receptors can be blocked by treatments with N-ethylmaleimide that do not markedly affect [^{3}H]5′-N-ethylcarboxamide adenosine binding to the second site in the striatum. The pharmacological characteristics of the second site indicate that it is the R_a adenosine receptor.

Key words: Adenosine receptors – [^{3}H]5′-N-ethylcarboxamide adenosine – [^{3}H]N^6-cyclohexyladenosine – Rat striatum – N-ethylmaleimide

Introduction

Ligand binding to R_i adenosine receptors which mediate the inhibition of adenylate cyclase activity has been convincingly demonstrated using [^{3}H]N^6-cyclohexyladenosine (CHA) (Bruns et al. 1980), [^{3}H]N^6-(L-phenylisopropyl)adenosine (L-PIA) (Schwabe and Trost 1980; Trost and Schwabe 1981), and [^{3}H]2-chloroadenosine (Williams and Risley 1980; Wu et al. 1980). All three of these agonists have K_D values for R_i receptors in the nanomolar range. Attempts to measure ligand binding to R_a receptors which mediate the stimulation of adenylate cyclase activity have been largely unsuccessful. Daly et al. (1979) studied the binding of [^{3}H]5′-N-cyclopropylcarboxamide adenosine to rat cerebral cortical membranes but concluded that the binding was not exclusively to *R* type adenosine receptors. The original work of Bruns et al. (1980) on the binding of the *R* receptor antagonist [^{3}H] diethylphenylxanthine (DPX) suggested that this ligand may bind with high affinity to both R_a and R_i receptors. We initiated the studies presented here in an attempt to demonstrate the binding of [^{3}H]5′-N-ethylcarboxamide adenosine (NECA) to R_a receptors in rat striatum. [^{3}H]NECA was chosen for study because it was known to be a potent R_a site agonist (Londos et al. 1980). The rat striatum was chosen because it was known to contain adenylate cyclase activity that is stimulated by adenosine analogs and thus by definition contains R_a receptors (Premont et al. 1977; Anand-Srivastava and Johnson 1980). These studies were expanded to include studies on the hippocampus, a tissue that contains only R_i receptors (Yeung and Green 1983), and studies with [^{3}H]CHA, a specific ligand for R_i receptors (Bruns et al. 1980), when it became clear that [^{3}H]NECA binds to more than one site or affinity state of a single site in the striatum. These ligand binding experiments along with adenylate cyclase assays show: (1) the rat striatum contains both R_a and R_i receptors, and (2) [^{3}H]NECA binds with high affinity to both R_a and R_i receptors. In addition, experiments are presented which show that high affinity agonist binding to R_i receptors is blocked by treatments with N-ethylmaleimide (NEM) that do not detectably affect [^{3}H]NECA binding to R_a receptors. Thus under appropriate experimental conditions [^{3}H]NECA can be used to study binding to R_a adenosine receptors.

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The abbreviations used are: NEM, N-ethylmaleimide; Gpp(NH)p, 5′-guanylylimidodiphosphate; NECA, 5′-N-ethylcarboxamide adenosine; L-PIA, N^6-(L-phenylisopropyl)adenosine; D-PIA, N^6-(D-phenylisopropyl) adenosine; DPX, 1,3-diethyl-8-phenylxanthine.

Materials and methods

Materials. N^6-(Adenine-2,8-[^{3}H])cyclohexyladenosine (11.5 Ci/mmol), and 1,3-(phenyl-4-[^{3}H])-diethyl-8-phenylxanthine (13.4 Ci/mmol) were obtained from New England Nuclear (Boston, MA, USA). [α-^{32}P]dATP was purchased from ICN (Irvine, CA, USA). [8-H^3]5′-N-ethylcarboxamide adenosine was prepared by catalytic tritiation of 8-bromo-5′-N-ethylcarboxamide adenosine by ICN (Irvine, CA, USA). The specific activity of the purified product was 12.7 Ci/mmol as determined by bioassay on C-1300 neuroblastoma adenylate cyclase. L-PIA and CHA were obtained from Boehringer (Mannheim, FRG). NECA and 5′-ethylcarboxylate adenosine were gifts from Dr. H. Stein, Abbott Laboratories, North Chicago, IL, USA; DPX and D-PIA were kindly supplied by Dr. John Daly, NIH, Bethesda, MD, USA; all other chemicals were from Sigma (St. Louis, MO, USA) or other standard sources. All compounds studied with the

exception of DPX and 8-phenyltheophylline were dissolved in distilled water; DPX and 8-phenyltheophylline were dissolved in dimethylsulfoxide (Yeung and Green 1983). Sprague Dawley rats (150–350 g; either sex) were sacrificed by decapitation, the hippocampi and striata rapidly dissected, and the tissue homogenized to make crude membrane preparations for immediate study or frozen and stored intact in liquid nitrogen for later study. Only unfrozen tissues were used to make synaptic membrane preparations.

Methods. Preparation of membranes. Crude membrane preparations of striatum and hippocampus were prepared by a modification of the procedure of Anand-Srivastava and Johnson (1980). Briefly, striata or hippocampi were homogenized (glass homogenizer-teflon pestle) in 10 mM imidazole (pH 7.5) containing 1 mM EDTA (Buffer A) (4–6 striata or hippocampi/ml), incubated with adenosine deaminase (5 U/ml) for 10 min at 37° C (to metabolize endogenous adenosine to inosine), diluted further with Buffer A and centrifuged at 4° C (1,000 $g \times 10$ min). The particulate fraction was resuspended in 1 mM $NaHCO_3$ + 1 mM EDTA, pH 7.5, allowed to remain on ice for 30 min and then recentrifuged (27,000 $g \times 10$ min). The particulate fraction was resuspended in Buffer A at a concentration of approximately 6–8 mg protein/ml and used for all binding studies. Synaptic membrane preparations were made using striata and hippocampi from 6–15 rats using the method of Jones and Matus (1974) and stored in liquid nitrogen. All adenylate cyclase assays were performed on these synaptic membrane preparations. Protein was determined by the method of Lowry et al. (1951).

Adenylate cyclase assays. [^{32}P]Deoxycyclic AMP formed from [^{32}P]dATP was isolated as described by Cooper and Londos (1979). Adenylate cyclase activity was assayed in 100-µl samples under two different conditions. Incubation mixture 1 contained 10 mM L-histidine (pH 7.5), 0.1 mM papaverine, 0.5 mM $MgCl_2$, 0.1 % BSA, 50 µM [^{32}P]dATP (50–150 cpm/pmol), 50 µM deoxycyclic AMP, 100 µM EGTA, (pH 7.5), 5 U/ml adenosine deaminase, 2 mM creatine phosphate, 100 µg/ml creatine phosphokinase, 100 µM GTP, 100 mM NaCl, synaptic membrane preparation (2–20 µg protein) and various concentrations of CHA or NECA. Incubations with assay mixture 1 were performed for 15 min at 24° C. Incubation mixture 2 differed from 1 in that it did not contain NaCl and the GTP concentration was reduced to 0.3 µM. Incubations in assay mixture 2 were performed at 30° C for 10 min. All assays were performed in triplicate.

Ligand binding assay. Incubation mixtures (100 µl) contained 30 µl membrane preparation (180–250 µg protein), 50 mM glycylglycine, pH 7.5, 4 mM $MgCl_2$, 5 U/ml adenosine deaminase, [^{3}H]ligand and other additions as noted. Incubations were performed in triplicate for 15 min at 37° C at which time 3 ml of ice cold wash buffer (1 mM glycylglycine, pH 7.5, 1 mM $MgCl_2$) was added and the sample rapidly poured onto a 24-mm Whatman GF/A filter under reduced pressure and washed 3 times with 5-ml volumes of the same buffer. Samples containing 0.1 mM L-PIA were used to correct for nonspecific binding. Samples were counted in a Packard Tricarb scintillation counter at approximately 30 % efficiency.

Data analysis. Hill coefficients or slope factors (n_H) and IC_{50} values were determined from Hill plots by linear regression (Bennett 1978). The Cheng and Prusoff equation was used to calculate K_i values from IC_{50} values (Bennett 1978). Kinetic constants were calculated by methods described by Bennett (1978). Biphasic dissociation curves were analyzed using standard techniques described by Riggs (1963). Binding parameters (K_D and B_{max} values) were determined by least squares analysis of Scatchard plots. Computer analysis of the untransformed data with the nonlinear multipurpose curve fitting program LIGAND was performed to determine if the binding data were best fit by a one site or two site model (Munson and Rodbard 1980). All values are summarized as means ± S.E. (N) where N is the number of values determined in separate experiments. Statistical evaluations were performed with Students t-test.

Results

Adenylate cyclase activities in synaptic membrane preparations of striatum and hippocampus were performed under two different conditions. Condition 1 (low temperature, high GTP and added monovalent cation) was designed to favor the detection of a receptor-mediated inhibition of adenylate cyclase (Londos et al. 1978; Limbird 1981; Cooper 1982). Under these assay conditions both CHA and NECA inhibited adenylate cyclase activity in preparations of striatal synaptic membranes. CHA was more potent that NECA (Fig. 1A). The inhibitory effect of CHA was antagonized by 8-phenyltheophylline (control = 0.1 %, DMSO = 100 %; 3 µM phenyltheophylline 98 ± 2 %; 1 µM CHA 76 ± 2 %; CHA + 8-phenyltheophylline 98 ± 6 %, $N = 3$). These results show that the effect is not via P sites (Londos and Wolff 1977) and thus directly establish the presence of R_i receptors in striatum.

Both CHA and NECA stimulated adenylate cyclase activity in striatal synaptic membranes when assay condition 2 was employed (Fig. 1B). NECA was more potent than CHA. Figure 1C shows that the adenylate cyclase activity in hippocampal synaptic membranes was inhibited under condition 1 but minimally inhibited or unaffected under condition 2. These results confirm our earlier report (Yeung and Green 1983) that the hippocampus contains R_i receptors but is devoid of R_a receptors.

Preliminary experiments with [^{3}H]NECA showed that we could measure specific binding of [^{3}H]NECA to striatum at low ligand concentrations. The specific binding of [^{3}H]NECA was proportional to the amount of membrane protein (50–250 µg/sample) and increased to reach a plateau at 10 min (data not shown). Specific binding was stable for at least 30 min; all samples in succeeding experiments were filtered after a 15-min incubation.

Figure 2 summarizes several experiments showing the inhibition of the binding of 20 nM [^{3}H]NECA by adenosine analogs and methylxanthines. All of these compounds are known to be either agonists or antagonists at R type adenosine receptors (Bruns 1980; Londos et al. 1981; Daly 1982). All of the inhibition curves in Fig. 2A had n_H values of > 0.8. These data show that 8-phenyltheophylline was more potent than isobutylmethylxanthine and theophylline and that 5′-ethylcarboxylate adenosine was less potent than the corresponding amide, NECA. The inhibition curves shown in Fig. 2B were shallow ($n_H < 0.8$) and, in some cases, appeared biphasic. All of these compounds appeared to inhibit one component of [^{3}H]NECA binding at concentrations much lower than those necessary to inhibit a second component. Other experiments (data not shown) showed that: (1) 2′,5′-dideoxyadenosine, a compound with high affinity for P sites

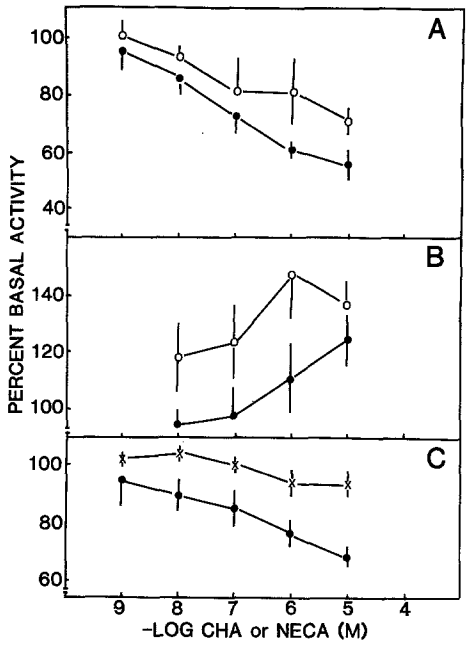

Fig. 1A–C. Effects of CHA and NECA on adenylate cyclase activities in striatal and hippocampal synaptic membrane preparations. *Ordinate:* adenylate cyclase activity expressed as percent of basal activity. *Abscissa:* -log CHA or NECA. All values are means from experiments on three different synaptic membrane preparations. Bars represent S.E. **A** Effect of CHA (●) and NECA (○) on adenylate cyclase activity in striatal membrane preparations under assay condition 1 (mean basal activity 220 fmol/mg/min). **B** Same as panel A but assay condition 2 (mean basal activity 267 fmol/mg/min). **C** Effect of CHA on adenylate cyclase activity in hippocampal membrane preparations studies under condition 1 (●) and condition 2 (×). The mean basal activities for conditions 1 and 2 were 71 and 151 fmol/mg/min, respectively

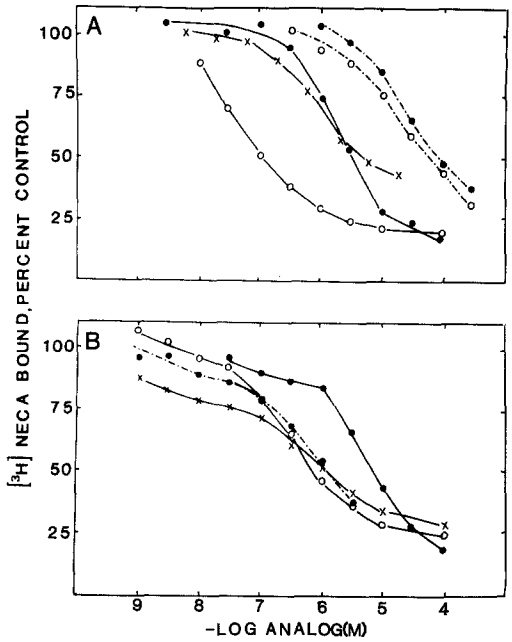

Fig. 2A,B. Effects of various compounds on [^{3}H]NECA binding to striatum. Analogs were incubated simultaneously with 20 nM [^{3}H]NECA for 15 min at 37° C before filtration. *Ordinate:* [^{3}H]NECA bound as percent of control total radioactivity. *Abscissa:* -log of the molar concentration of the competing compound. **A.** ○——○, NECA; ●——●, 5′-ethylcarboxylate adenosine; ×——×, 8-phenyltheophylline; ○- - -○, 1-methyl-3-isobutylxanthine; ●——●, theophylline. **B.** ×——×, L-Pia; ●——●, CHA; ○——○, 2-chloroadenosine; ●——● 5′-deoxy-5′-methylthioadenosine

(Londos and Wolff 1977; Londos et al. 1981; Daly 1982), (2) dipyridamole, an inhibitor of nucleoside transport, and (3) adenine, 5′-AMP, inosine, and tubercidin, compounds devoid of activity at *R* sites (Londos and Wolff 1977; Bruns 1980; Londos et al. 1981; Daley 1982), all minimally affected [^{3}H]NECA binding. These results therefore suggested that the binding of [^{3}H]NECA was to *R* type adenosine receptors. Although it is not shown in Fig. 2 we tested the effect of 100 μM L-PIA on [^{3}H]NECA binding in all of these experiments. With the exception of compounds modified in the 5′ position, all compounds inhibited the same proportion of ligand binding as did 100 μM L-PIA. The 5′-modified analogs depressed binding to a slightly greater extent. Scatchard plots of binding experiments using higher [^{3}H]NECA concentrations and cold NECA to correct for nonspecific binding revealed a high capacity, low affinity site which has been excluded from the present analysis by using L-PIA to define nonspecific binding. The significance of this site has not been investigated. As it is advantageous to use a chemically distinct compound rather than cold ligand itself to define nonspecific binding (Bennett 1978) and L-PIA is an agonist for stimulating adenylate cyclase activity in striatum (Premont et al. 1977) the use of L-PIA to define nonspecific binding is clearly justified.

The shallow inhibition curves shown in Fig. 2B suggested that [^{3}H]NECA bound to more than one site in striatum. This could reflect binding to more than one type of adenosine receptor (both R_a and R_i receptors) or the existence of multiple affinity states of a single type of adenosine receptor. The observation that part of the [^{3}H]NECA binding was inhibited by very low concentrations of L-PIA or CHA suggested that [^{3}H]NECA was binding to R_i sites and we thus favored the first explanation. We therefore studied the binding of [^{3}H]NECA to preparations of hippocampus as the hippocampus contains R_i receptors but is devoid of R_a receptors (Yeung and Green 1983; present results Fig. 1C). Scatchard plots of [^{3}H]NECA binding to hippocampus were monophasic and gave $K_D = 12.3 \pm 1.8$ nM and $B_{max} = 510 \pm 21$ fmol/mg protein ($N = 7$). Kinetic analysis of the binding gave $k_1 = 0.012 \pm 0.003\ min^{-1}\ nM^{-1}$, $k_2 = 0.058 \pm 0.003\ min^{-1}$ and $K_D\ (k_2/k_1) = 5.4 \pm 1.3$ nM ($N = 3$). Thus the K_D determined by kinetic analysis was in reasonable agreement with the K_D determined by Scatchard analysis.

Previous work in our laboratory showed that [^{3}H]CHA binds to three affinity states of R_i receptors in rat hippocampus depending on the assay conditions. [^{3}H]CHA binds to a single middle affinity state of the R_i receptors in rat hippocampus when 100 μM Gpp(NH)p is present (Yeung and Green 1983). We therefore determined the IC_{50} of NECA for inhibiting [^{3}H]CHA binding under this condition and calculated a K_i of 80 ± 13 nM ($N = 3$). The affinities of compounds for a low agonist affinity state of the R_i receptor can be calculated from competition curves of [^{3}H]DPX binding to N-ethylmaleimide-pretreated preparations of hippocampus

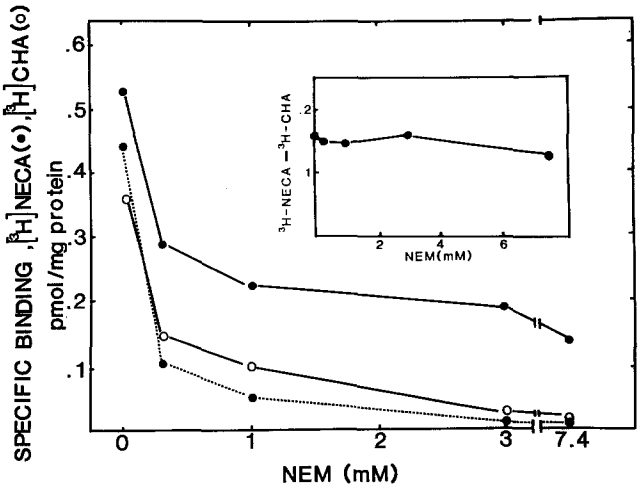

Fig. 3. Effect of pretreatment with NEM on [^{3}H]NECA binding to hippocampus (●——●) and striatum (●——●) and [^{3}H]CHA binding to striatum (○——○). Inset shows differences between [^{3}H]NECA and [^{3}H]CHA binding in striatum. NEM treatments were performed after the initial slow speed centrifugation (see "Materials and methods"). The low speed particulate fraction was resuspended in homogenization buffer, NEM added to give the stated final concentration and incubated for 15 min at 37° C. At the end of the incubation period the samples were diluted 10-fold with cold homogenization buffer and centrifuged (27,000 g × 10 min). The particulate fraction was resuspended in $NaHCO_3$ + EDTA and processed as described in "Materials and methods"

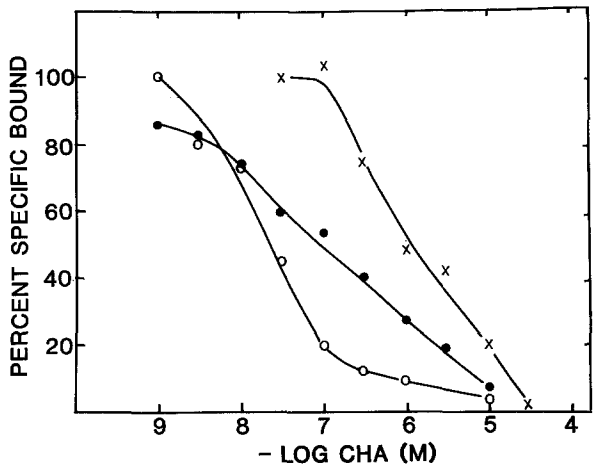

Fig. 4. Inhibition curves for CHA on [^{3}H]NECA binding to hippocampus (○——○), control striatum (●——●), and NEM-pretreated striatum (×——×). *Ordinate:* Specific binding as percent control (no CHA). *Abscissa:* -log CHA concentration. The [^{3}H]NECA concentration was 72 nM, the specific binding in the absence of CHA in hippocampus, control striatum, and NEM-pretreated striatum in fmol/mg protein were 534, 544 and 214, respectively

(Yeung and Green 1983). The affinity (K_i) of NECA using this procedure was 2,250 ± 200 nM ($N = 3$). Thus NECA like CHA binds to preparations of hippocampus with three different affinities depending on the experimental conditions convincingly demonstrating that NECA binds to R_i adenosine receptors. This being the case it is most likely that part of the binding of NECA to preparations of striatum is to R_i receptors.

Previous work from this laboratory showed that [^{3}H]CHA binding in preparations of hippocampus is markedly reduced by treatment with NEM (Yeung and Green 1983). We therefore determined the effect of NEM-pretreatment on [^{3}H]CHA binding in striatum and [^{3}H]NECA binding in hippocampus and striatum in the hope that we could block high affinity binding to R_i receptors while sparing high affinity binding to R_a receptors (Fig. 3). While almost all of the [^{3}H]CHA binding in striatum and [^{3}H]NECA binding in hippocampus appeared to be susceptable to inhibition by NEM, the binding of [^{3}H]NECA in striatum appeared to be composed of NEM-sensitive and NEM-insensitive components. The inset of Fig. 3 shows that the difference between the amounts of [^{3}H]NECA and [^{3}H]CHA bound to striatum was not reduced from the control value except possibly at the highest concentration of NEM studied. These results suggested that appropriate NEM treatments block high affinity binding of [^{3}H]NECA to R_i sites without appreciably affecting high affinity binding to R_a sites. We therefore performed numerous experiments using the general experimental design used in the experiment summarized in Fig. 3 to attempt to maximally inhibit high affinity [^{3}H]NECA binding to R_i sites while minimally affecting high affinity R_a site binding. Variations in temperature, NEM concentration and time of treatment failed to improve the results over those obtained with a 15-min treatment with 3 mM NEM (37° C) (data not shown). The presence of NaCl during the NEM-pretreatment procedure did appear to increase the inactivation of high affinity binding to R_i sites without causing a deleterious effect on high affinity R_a site binding. In three experiments the residual binding of [^{3}H]CHA to aliquots of preparations of striatum treated with NEM (3 mM, 37°C, 15 min) in the presence of 100 mM NaCl was 49 ± 13% of that present in aliquots treated with NEM in the absence of NaCl; the [^{3}H]NECA − [^{3}H]CHA difference in the NaCl group was 91 ± 5% of the minus NaCl group indicating that the presence of this concentration of NaCl did not increase the inactivation of R_a site binding. All succeeding experiments involving NEM used this protocol (3 mM NEM, 100 mM NaCl, 37° C, 15 min).

Figure 4 summarizes an experiment in which CHA competition curves of [^{3}H]NECA binding to preparations of hippocampus and to control and NEM-pretreated aliquots of a preparation of striatum were determined. It should be noted that a relatively high concentration of [^{3}H]NECA was used and that each curve is normalized with respect to the specific binding in the absence of added CHA. It is clear that the competition curve in the control aliquot of striatum is very shallow ($n_H = 0.43$) and to the right of the curve for hippocampus and that NEM-pretreatment shifts the inhibition curve to the right and increases its steepness ($n_H = 0.77$). These results are in accord with what would be expected if [^{3}H]NECA bound to both R_a and R_i sites in striatum and NEM pretreatment selectively blocked R_i site binding. IC_{50} values for CHA, L-PIA, D-PIA and NECA determined in similar experiments on control preparations of hippocampus and NEM-pretreated preparations of striatum are summarized in Table 1. The n_H values for the inhibition curves are also shown. The lower IC_{50} values for L-PIA and CHA in hippocampus as compared to NEM-pretreated striatum and the greater degree of stereospecificity for phenylisopropyl-

Table 1. IC_{50} values for the inhibition of [^{3}H] 5′-N-ethylcarboxamide adenosine binding to hippocampus and NEM-pretreated striatum

Inhibitor	NEM-pretreated striatum	Hippocampus
N^6-(D-phenyliso-propyl)adenosine	20,800 ± 10,200 (0.96 ± 0.05)	524 ± 71 (0.82 ± 0.12)
N^6-(L-phenyliso-propyl)adenosine	1,600 ± 470 (0.93 ± 0.08)	18 ± 4 (0.79 ± 0.01)
N^6-Cyclohexyladenosine	2,070 ± 540 (0.72 ± 0.06)	22 ± 6 (0.83 ± 0.0)
5′-N-Ethylcarboxamide adenosine	386 ± 66 (0.69 ± 0.13)	502 ± 230 (0.77 ± 0.15)

IC_{50} values are in nM. $N = 3$ in all groups. n_H values ± S.E. are shown in parentheses. The range of [^{3}H]NECA concentrations used in these experiments was 60–85 nM. The IC_{50} N^6-(D-phenylisopropyl)adenosine/IC_{50} N^6-(L-phenylisopropyl)adenosine ratios in striatum and hippocampus were 11.9 ± 2.5 and 28.8 ± 4.6, respectively. These values are statistically different, $P < 0.05$

Table 2. Effects of various compounds on [^{3}H]NECA binding to NEM-pretreated striatum[a]

A[b]. Compound	Percent specific binding with 0.1 mM competitor
2′,5′-Dideoxyadenosine	61 ± 6
Inosine	92 ± 5
Adenine	66 ± 1
Tubercidin	86 ± 7

B[c]. Compound	IC_{50}, μM	n_H
2-Chloroadenosine	1.3 ± 0.1	0.87 ± 0.01
Theophylline	60.5 ± 19.5	0.61 ± 0.04
8-Phenyltheophylline	3.3 ± 0.8	0.82 ± 0.02
Diethylphenylxanthine	1.8 ± 0.5	0.83 ± 0.10

[a] 0.3 μM CHA was present in all tubes to minimize the influence of residual R_i receptors

[b] $N = 3$ in all groups. The concentration of [^{3}H]NECA used was 71–88 nM

[c] $N = 2$. 71 and 88 nM [^{3}H]NECA were in the experiments on 2-chloroadenosine, theophylline and 8-phenyltheophylline. 29 and 62 nM [^{3}H]NECA were used in the experiments on diethylphenylxanthine

adenosine in hippocampus strongly support the hypothesis that the [^{3}H]NECA binding in hippocampus is to R_i sites while the [^{3}H]NECA binding in NEM-pretreated striatum is primarily to R_a sites (Londos et al. 1981; Daly 1982). The greater potency of NECA compared to L-PIA and CHA in NEM-pretreated striatum and the lesser potency of NECA compared to L-PIA and CHA in hippocampus further support this conclusion. The abilities of several other adenosine analogs and of xanthine derivatives to inhibit [^{3}H]NECA binding to preparations of NEM-pretreated striatum were also determined. These experiments were performed in the presence of 0.3 μM CHA to minimize [^{3}H]NECA binding to residual R_i receptors and are summarized in Table 2. Either the percent specific binding remaining in the presence of 0.1 mM concentrations of the inhibitors or the IC_{50} (and n_H) values are given. The relative greater potency of 8-phenyltheophylline and DPX as compared to theophylline along with the very low potencies of 2′,5′-dideoxyadenosine, inosine, adenine and tubercidin are indicative of binding to R type adenosine receptors (Londos et al. 1981; Daly 1982). The IC_{50} of 2-chloroadenosine is indicative of binding to R_a rather than R_i adenosine receptors (Londos et al. 1981; Daly 1982).

Initial attempts to determine the parameters that define [^{3}H]NECA binding to NEM-pretreated preparations of striatum were unsuccessful. Scatchard plots of binding data obtained using a wide range of [^{3}H]NECA concentrations were curvilinear. While the LIGAND program resolved the plots into two components, the parameters defining binding to the lower affinity component were extremely erratic and in many cases unreasonably large (data not shown). This no doubt was at least partially due to the relatively high concentrations of [^{3}H]NECA necessary for these experiments and the consequently low percent specific binding. We therefore tried to resolve the situation using kinetic experiments. We did not attempt to measure the rate (k_{ob}) because it was fast and reached equilibrium within 2 min when 60 nM [^{3}H]NECA was studied. The dissociation of [^{3}H]NECA from NEM-pretreated striatum initiated by the addition of 100 μM L-PIA could be followed with reasonable accuracy. The dissociation was biphasic (Fig. 5); the faster dissociating

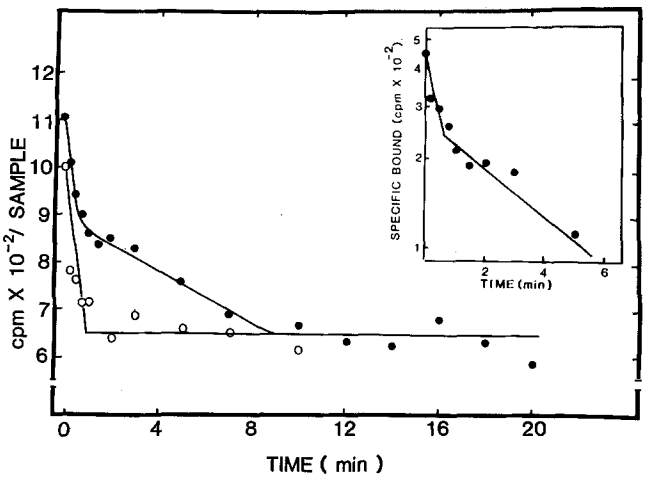

Fig. 5. Dissociation of [^{3}H]NECA from NEM-pretreated striatum after the addition of 100 μM L-PIA. Control (●——●). 100 Gpp(NH)p present during the entire incubation (○——○). All samples were incubated with 62 nM [^{3}H]NECA for 15 min prior to the addition of L-PIA. The *asymptote* approximates nonspecific binding as determined by the presence of 100 μM L-PIA during the entire incubation. *Ordinate:* Total bound radioactivity (cpm × 10^{-2}/ sample). The inset is the logarithmic plot showing the biphasic nature of the dissociation of [^{3}H]NECA in the absence of Gpp(NH)p

component had a $t_{1/2}$ of < 1 min while the slower dissociating component had a $t_{1/2}$ of 4–5 min. In the presence of Gpp(NH)p only the faster component was present (Fig. 5). Dissociation of [^{3}H]NECA from the slower dissociating component ($t_{1/2} = 4-5$ min) was faster than the dissociation of [^{3}H]NECA from preparations of hippocampus ($t_{1/2} = 11$ min) and the amount of [^{3}H]NECA bound to this component was greater than could be accounted for by residual R_i sites (estimated with a high concentration of [^{3}H]CHA). We therefore tentatively concluded that these two

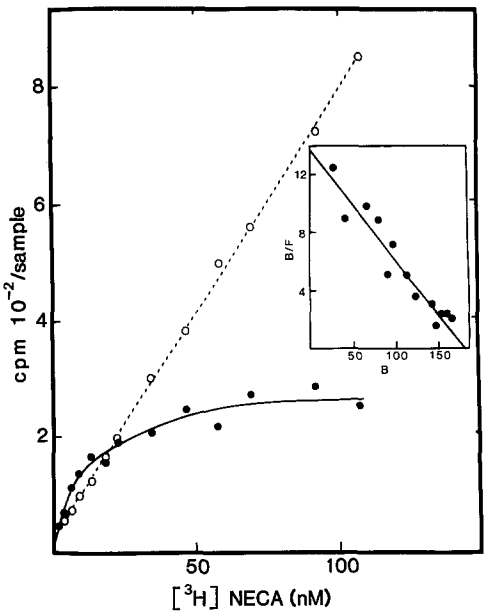

Fig. 6. Relationship between the [^{3}H]NECA concentration and nonspecific and specific binding to NEM-pretreated striatum. 100 μM L-Pia was added 1 min before filtration and the difference between the radioactivity bound in these samples minus that bound in samples that contained 100 μM L-PIA during the entire incubation was taken as specifically bound radioactivity. *Ordinate*: cpm × 10^{-2}/sample. *Abscissa*: [^{3}H]NECA concentration. Specifically bound radioactivity (●——●); nonspecifically bound radioactivity (○----○. The inset shows the Scatchard plot of specific binding converted to fmol/mg protein. *Ordinate:* Bound/Free (pmol/mg·μM) *Abscissa* = Bound (fmol/mg protein). $K_D = 12$ nM. B_{max} 173 fmol/mg protein

components were primarily associated with high and low affinity states of the R_a receptor.

Figure 6 shows a Scatchard plot of [^{3}H]NECA binding to the slower dissociating component present in NEM-pretreated preparations of striatum. This and similar experiments were performed by allowing the binding of [^{3}H]NECA to reach equilibrium (15 min), adding 100 μM L-PIA (final concentration) and filtering the sample 1 min later. This and two similar experiments gave $K_D = 16.9 \pm 3.6$ nM and $B_{max} = 188 \pm 13$ fmol/mg protein. In all cases, analysis with LIGAND showed that the fit of the data was not improved by a 2 site model. Total specific [^{3}H]NECA binding (no dissociation period) in the presence of a wide range of [^{3}H]NECA concentrations was determined in the presence of 100 μM Gpp(NH)p. Scatchard plots of data from these experiments were linear with $K_D = 83 \pm 18$ nM and $B_{max} = 343 \pm 33$ fmol/mg protein ($N = 4$). This B_{max} probably underestimated the true B_{max} by approximately 10% due to dissociation during the filtration procedure.

If [^{3}H]CHA binds specifically to R_i receptors and [^{3}H]NECA binds to both R_a and R_i receptors, the amount of [^{3}H]NECA bound should exceed the amount of [^{3}H]CHA bound ([^{3}H]NECA/[^{3}H]CHA > 1) only in preparations of tissues that possess R_a receptors. Table 3 summarizes a group of experiments in which [^{3}H]NECA (30–40 nM) and [^{3}H]CHA (11–26 nM) binding was determined in preparations of various brain areas. Both [^{3}H]NECA binding and the [^{3}H]NECA/[^{3}H]CHA ratio were highest in striatum. The [^{3}H]NECA/[^{3}H]CHA ratio was greater than one in only two

Table 3. Regional distribution of CHA and NECA binding sites in rat brain

	NECA[a]	CHA[a]	NECA/CHA[b]	*N*
Cortex	298 ± 23	372 ± 39	0.80 ± 0.05	3
Cerebellum	335 ± 20	417 ± 10	0.81 ± 0.06	3
Hippocampus	401 ± 35	409 ± 12	0.98 ± 0.08	3
Striatum	539 ± 14	337 ± 10	1.61 ± 0.05*	3
N. accumbens	356 ± 17	283 ± 12	1.26 ± 0.08**	4
Thalamus	217 ± 39	227 ± 52	0.98 ± 0.04	3

[a] Ligand bound (fmol/mg/protein)
[b] Ratio of amounts of NECA and CHA bound in each preparation
* Significantly greater than 1, $P < 0.001$
** Significantly greater than 1, $P < 0.05$

brain areas, the striatum and the nucleus accumbens. Of the areas studied only these two areas possess adenylate cyclase activity that is stimulated by adenosine analogs (Premont et al. 1979).

Discussion

It is well established that preparations of rat striatum contain adenylate cyclase activity that is stimulated by adenosine analogs (Premont et al. 1977; Anand-Srivastava and Johnson 1980) and thus by definition contain R_a adenosine receptors (Londos et al. 1980). While ligand binding studies have suggested that R_i receptors are also present in the striatum (Murray and Cheney 1982; Patel et al. 1982; Williams and Risley 1980), inhibitory effects of adenosine analogs on adenylate cyclase in striatal membranes have not been reported. We have demonstrated that CHA and NECA inhibit the adenylate cyclase activity in preparations of striatal synaptic membranes when appropriate assay conditions are employed. This inhibition is antagonized by 8-phenyltheophylline and is mediated by R_i receptors rather than by "*P* sites" (Londos and Wolff 1977). Although we have confirmed that the adenylate cyclase activity in the same striatal membranes can be stimulated by adenosine analogs under different assay conditions, we have not attempted to optimize the conditions to study this stimulation further. We have confirmed our earlier observation (Yeung and Green 1983) that the adenylate cyclase activity in hippocampal membranes is inhibited but not stimulated by adenosine analogs, i.e., the hippocampus contains only R_i receptors.

Our initial studies on the binding of NECA to striatal membranes suggested that [^{3}H]NECA binds to both R_a and R_i adenosine receptors in the striatum. We therefore characterized the binding of [^{3}H]NECA to hippocampal membranes which contain only R_i receptors and showed that this ligand does bind to R_i receptors with high affinity.

The finding that [^{3}H]NECA binds to R_i receptors in hippocampus with high affinity suggested that part of the binding of this ligand to striatal membranes was to R_i receptors and that this binding would have to be minimized in order to study the binding of the remaining site(s). As there was convincing evidence that inhibitory or negatively coupled systems are more susceptable to inactivation by NEM than are stimulatory or positively coupled systems (Stadel and Lefkowitz 1979; Kilpatrick et al. 1982; Harden et al. 1982; Jakobs et al. 1982) and we had previously showed that CHA

binding to hippocampal membranes is blocked by NEM (Yeung and Green 1983), we determined the effects of NEM-pretreatment on [^{3}H]NECA binding to striatal membranes (Fig. 3). It was clear from these studies that the striatal membranes contain an NEM-resistant [^{3}H]NECA binding site that is not present in hippocampal membranes. The pharmacological characteristics of this site as defined by the relative potencies of different adenosine analogs to antagonize this binding and comparisons with similar experiments performed on hippocampal membranes (Tables 1 and 2) support the hypothesis that this binding is to R_a adenosine receptors. This conclusion is further supported by the observation that Gpp(NH)p decreases the affinity of [^{3}H]NECA for this site.

While our studies were in progress Schütz et al. (1982) reported on the binding of [^{3}H]NECA to rat liver plasma membranes. Their methods differed from ours in two important aspects: (1) they studied binding at 4° C and (2) they used cold NECA to correct for nonspecific binding. They concluded that [^{3}H]NECA binds to a high affinity/low capacity site which they interpreted to be the R_a receptor and to a second low affinity/high capacity site of unknown function. It would appear that the low affinity/high capacity site included in their assay is the same as the low affinity/high capacity site excluded from our analyses by using L-PIA to correct for nonspecific binding. Direct comparisons with the high affinity binding that we measured with that measured by Schütz et al (1982) are difficult to make because of the different temperatures used. The work of Schütz et al. (1982) along with the results presented herein suggest that [^{3}H]NECA can be used to study binding to R_a adenosine receptors if appropriate precautions are taken.

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