0026-895X/92/020352-08$03.00/0

MOLECULAR PHARMACOLOGY, 41:352-359

A_{2A} Adenosine Receptors from Rat Striatum and Rat Pheochromocytoma PC12 Cells: Characterization with Radioligand Binding and by Activation of Adenylate Cyclase

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Received January 28, 1991; Accepted November 15, 1991

SUMMARY

Binding assays and assays of activation of adenylate cyclase with the agonists 5′-*N*-ethylcarboxyamidoadenosine (NECA) and CGS21680 have been used to compare adenosine receptors in rat pheochromocytoma PC12 cells and in rat striatum. The [^{3}H]NECA binding showed two components, whereas [^{3}H]CGS21680 bound to one component in both tissues. The K_d value for the high affinity site labeled with [^{3}H]NECA in PC12 cell membranes (2.3 nM) was lower than that in striatum (6.5 nM). The [^{3}H]CGS21680 binding site showed a K_d value of 6.7 nM and 11.3 nM in PC12 cells and striatum, respectively. In the presence of GTP the K_D values of [^{3}H]NECA and [^{3}H]CGS21680 for the high affinity site were increased severalfold, whereas the low affinity sites for [^{3}H]NECA were no longer detected with filtration assays. A comparison of the ability of a series of agonists and antagonists to inhibit high affinity binding of [^{3}H]NECA to A_2 receptors in PC12 cell and striatal membranes indicated that agonists had higher affinities and antagonists had lower affinities in PC12 cells, compared with affinities in striatal membranes. Analysis of activation of adenylate cyclase in PC12 cell membranes suggested that the dose-dependent stimulation by NECA involved two components, whereas CGS21680 stimulated via one component. The maximal stimulation by NECA significantly exceeded that caused by CGS21680. In intact PC12 cells, NECA caused a greater accumulation of AMP than did CGS21680, as was the case in membranes. In striatal membranes, NECA and CGS21680 showed similar maximal stimulations of adenylate cyclase. Both NECA and CGS21680 were more potent in PC12 cell membranes than in striatal membranes, in agreement with binding data. However, in contrast to binding data, antagonists were not less potent versus stimulation of adenylate cyclase by NECA or CGS21680 in PC12 cell membranes, compared with striatal membranes. *In toto*, the results suggest that A_{2A} receptors in striatum are virtually identical to the A_{2A} receptors in PC12 cells. But, in addition to an A_{2A} receptor, it appears that a lower affinity functional receptor, probably an A_{2B} receptor, is present in PC12 cells and PC12 cell membranes, whereas such a functional low affinity receptor is not detectable in striatal membrane.

Adenosine receptors have been subdivided into two major subtypes, termed A_1 and A_2 receptors, on the basis of inhibition or stimulation of adenylate cyclase, respectively (1). Subsequently, it was demonstrated that brain A_2 receptors could be further subdivided into two classes; one receptor had a high affinity for adenosine (EC_{50}, 0.1–1 μM) and occurred mainly in striatum, whereas the other had a low affinity for adenosine (EC_{50}, 5–10 μM) and occurred throughout the brain (2). Bruns *et al.* (3) proposed that high affinity A_2 receptors be designated A_{2A} and low affinity receptors be designated A_{2B}.

Structure-activity relationships for agents acting as agonists or antagonists at A_{2A} receptors have been based on binding data for A_{2A} receptors in striatal membranes (3) and data on stimulation of adenylate cyclase via A_{2A} receptors in PC12 cell and platelet membranes (4–6). Analysis of such data indicates a lack of correspondence between profiles for agonists/antagonists at A_{2A} receptors of rat PC12 cells and A_{2A} receptors of human platelets, suggestive of the existence of different subtypes of the high affinity A_{2A} adenosine receptors. Furthermore, K_i values for the A_{2A} binding assay in rat striatal membranes do not always correspond to K_B values for inhibition of the A_{2A} receptor-mediated activation of adenylate cyclase in rat PC12 cell membranes (7), again suggestive of different subtypes of the A_{2A} receptors.

In order to assess whether the A_{2A} receptors of rat striatum and the A_{2A} receptors of rat pheochromocytoma cells are iden-

I.H. was supported by a grant from the International Life Sciences Institute (Washington, DC).

ABBREVIATIONS: NECA, 5′-*N*-ethylcarboxyamidoadenosine; APEC, 2-[2-aminoethylamino)carbonylethylphenylethylamino]-5′-*N*-ethylcarboxyamidoadenosine; CGS21680, 2-(p-2-carboxyethyl)phenylethylamino)-5′-*N*-ethylcarboxyamidoadenosine; CHA, N^6-cyclohexyladenosine; CPA, N^6-cyclopentyladenosine; *R*-PIA, N^6-[(*R*)-1-methyl-2-phenylethyl]adenosine; 8-pSPT, 8-*p*-sulfophenyltheophylline; XAC, xanthine amine congener, 1,3-dipropyl-8-4-(2-aminoethyl)aminocarbonylmethyloxyphenylxanthine; DMSO, dimethylsulfoxide; 8-pSP-1,3-dipropylxanthine, 8-*p*-sulfophenyl-1,3-dipropylxanthine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

tical or different, data on binding assays and on activation of adenylate cyclase for both striatum and PC12 cells have been compared using two agonists, NECA and CGS21680. NECA is widely used as a potent A_2 agonist, both for binding assays and for activation of adenylate cyclase (2–6). CGS21680 is proving to be a nearly specific A_{2A} agonist for radioligand binding assays and for activation of adenylate cyclase (8, 9). CGS21680 is a potent agonist at A_{2A} receptors but very weak agonist at A_{2B} receptors (10). NECA is potent at both A_{2A} and A_{2B} receptors.

The present analysis of binding and adenylate cyclase data for PC12 cells and striatum indicates that the binding sites in both systems correspond to the receptors coupled to adenylate cyclase and suggests that the A_{2A} receptors in both preparations are virtually identical. However, there is evidence for two components in NECA-activated adenylate cyclase in PC12 cells, suggesting the presence of both an A_{2A} receptor and a functional low affinity adenosine receptor, presumably of the A_{2B} subclass. This low affinity receptor responds to NECA but not to CGS21680.

Experimental Procedures

Materials. [^{3}H]NECA (specific activity, 18 Ci/mmol), [^{3}H]CGS21680 (48.1 Ci/mmol), and [α-^{32}P]ATP were from New England Nuclear (Boston, MA). NECA, *R*-PIA, CHA, CPA, 2-chloroadenosine, 8-pSPT, and 8-pSP-1,3-dipropylxanthine were from Research Biochemicals, Inc. (Wayland, MA). CGS21680 was kindly provided by Dr. M. Williams (formerly of Ciba-Geigy Corp., Summit, NJ). APEC (11), XAC (12), and 3,7-dimethyl-1-propargylxanthine (13) were synthesized as previously described. Caffeine, adenosine deaminase, creatine phosphate, and creatine kinase were from Sigma (St. Louis, MO). Rolipram was provided by Schering AG (Berlin). Other agents and reagents were from standard commercial sources.

Cell culture. PC12 cells, derived from a pheochromocytoma tumor of the rat adrenal medulla, were provided by Dr. G. Guroff (National Institutes of Health, Bethesda, MD). Cells were grown in Dulbecco's modified Eagle's medium with a 6% fetal calf serum, 6% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin and were kept at 37° in an atmosphere enriched in CO_2. For the present study, cells in weeks 8–17 of passage were used. At higher passages, the magnitude of the NECA-elicited activation of adenylate cyclase was reduced, apparently due to the increase in basal activity of adenylate cyclase.

Membrane preparation. Striatal tissue from male Sprague-Dawley rats (body weight, 126–150 g) was homogenized in 20 volumes of ice-cold 50 mM Tris·HCl, pH 7.7, using a Polytron (setting 6, 10 sec). The homogenate was centrifuged at 48,000 × *g* for 15 min at 4°. The resulting pellet was resuspended in buffer and recentrifuged at 48,000 × *g* for 15 min. The final pellet was frozen until the time of binding assay. For assay of adenylate cyclase, pellets were freshly prepared from the striatal homogenates.

PC12 cells were washed from flasks with 50 mM Tris·HCl buffer, pH 7.4, containing 1 mM EDTA and 150 mM NaCl, twice, and were homogenized in 5 mM Tris·HCl buffer, pH 7.4, containing 1 mM EDTA, using a Polytron (setting 5, 10 min). The homogenate was centrifuged at 1000 × *g* for 10 min. The supernatant was recentrifuged at 48,000 × *g* for 20 min, and the resultant pellet was resuspended in the same buffer and recentrifuged at 48,000 × *g* for 20 min. The final pellet was frozen until the time of binding or adenylate cyclase assay.

Radioligand binding assay. Binding of [^{3}H]NECA to rat striatal and PC12 cell membranes was measured by a modification of the method described by Bruns *et al.* (3). Incubations were for 60 min at 25°, in glass tubes containing 1 ml of Tris·HCl buffer, pH 7.7, with striatal or PC12 cell membranes (protein concentration, 0.15–0.2 mg/ml), 4 nM [^{3}H]NECA, 10 mM $MgCl_2$, and 0.1 unit/ml adenosine deaminase. For striatal membranes, 50 nM CPA was added, to eliminate A_1 receptor binding. Because only the A_2 subtype of adenosine receptors is expressed in PC12 cells, CPA was not used for assays with PC12 membranes. CPA had no effect on binding of [^{3}H]NECA to PC12 cell membranes (data not shown). Nonspecific binding was defined in the presence of 30 μM *R*-PIA. Binding of [^{3}H]CGS21680 was examined under the same conditions, except that CPA was not used, because [^{3}H]CGS21680 does not bind to A_1 receptors (9). Nonspecific binding of CGS21680 was defined in the presence of 30 μM *R*-PIA. For saturation studies, membranes were incubated with nine different concentrations of [^{3}H]NECA and [^{3}H]CGS21680, ranging from 0.5 to 128 nM. Binding reactions were terminated by filtration, under reduced pressure, through Whatman GF/B filters (pretreated with 0.3% polyethyleneimine), using a Brandel cell harvester (Gaithersburg, MD). Filters were washed three times with ice-cold buffer (5 ml) and placed in scintillation vials, and bound radioactivity was determined using liquid scintillation counting. Protein concentrations were determined with a protein assay kit from Bio-Rad (Richmond, CA), using bovine serum albumin as a standard.

Adenylate cyclase assay. Adenylate cyclase assays were conducted in a total volume of 250 μl of 50 mM Tris·HCl, pH 7.4, containing 0.1 mM [α-^{32}P]ATP (0.9 μCi/tube), 10 μM GTP, 1 unit of adenosine deaminase, 5 mM creatine phosphate, and 0.3 mg/tube creatinine kinase. For assays with PC12 cell membranes, 0.1 mM cAMP, 0.1 mM rolipram, and 30 μg/tube bovine serum albumin were included (5). For assays with striatal membranes, 1 mM dithiothreitol and 100 μM papaverine were included (2). The concentration of $MgCl_2$ was 5 mM for PC12 cells and 2 mM for striatum. Agonists and antagonists were added from stock solutions in water or DMSO. Appropriate controls were included. Incubations were conducted for 10 min at 37° and were initiated by the addition of PC12 cell membranes (about 10 μg) or striatal membranes (about 30 μg) to reaction mixtures that had been preincubated for 10 min at 37°. Reactions were stopped by addition of 0.5 ml of trichloroacetic acid containing 0.25 ml of 1 mM cAMP and [^{3}H]cAMP. cAMP was isolated by a two-step chromatographic procedure, using Dowex 50 and alumina columns, as described (14). The loss of [^{32}P]cAMP on the columns was corrected for by comparison with the losses of [^{3}H]cAMP standard.

In the presence of 4% DMSO, both basal and stimulated activities of adenylate cyclase were significantly increased in striatal membranes (~30%) but not in PC12 cell membranes (data not shown). At 2% DMSO, the increase was not significant. At 8% DMSO, there was a 47% stimulation in striatal membranes, whereas adenylate cyclase activity was decreased by 15% in PC12 cell membranes. The basis for such selective effects of DMSO is not known. DMSO was used at 2–4% with some compounds in adenylate cyclase assays.

Assay of cAMP level. PC12 cells were scraped from the walls of the flasks, washed with buffer (108 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 10 mM glucose, 20 mM HEPES, pH 7.4), suspended (5 × 10^6 cells/ml) in the same buffer containing 3 units/ml adenosine deaminase, and then preincubated for 10 min at 37°. The phosphodiesterase inhibitor rolipram (final concentration, 30 μM) was added, and incubation was continued for 10 min at 37°. Assays were initiated by transferring PC12 cells (1 × 10^6 cells/tube, 0.5 ml) into buffer containing different concentrations of NECA or CGS21680, at 37°. Incubations were for 15 min. Assays were terminated by heating at 95° for 2 min. After centrifugation, cAMP content in the supernatant was measured using a cAMP assay kit from Amersham (Arlington Heights, IL).

Data analysis. The EC_{50} and IC_{50} values were obtained from concentration-response curves by computer analysis, using the GraphPAD computer program (GraphPAD Software Inc., San Diego, CA). K_i values for binding were obtained from IC_{50} values by the Cheng-Prusoff equation (15), using the K_d values of [^{3}H]NECA binding to high affinity sites. K_B values for inhibition of adenylate cyclase activation were calculated using the Schild equation (16). The LIGAND program (17) was used for analysis of Scatchard data for one and two sites.

Results

Binding of [^{3}H]NECA and [^{3}H]CGS21680 in PC12 cell and striatal membranes. Two classes of [^{3}H]NECA binding sites were revealed by a nonlinear regression analysis in PC12 cell membranes (Fig. 1). The high affinity binding site exhibited a K_d of 2.31 nM and a B_{max} of 200 fmol/mg of protein, whereas the low affinity site exhibited a K_d of 43 nM and a B_{max} of 171 fmol/mg of protein (Table 1). [^{3}H]NECA binding also showed two components in striatal membranes (Fig. 1; Table 1). The K_d values in PC12 cell membranes (2.3 nM and 43 nM) were severalfold lower than those in striatal membranes (6.5 nM and 286 nM). The density of sites was severalfold greater in striatal membrane than in PC12 membranes. In the presence of 100 μM GTP, the affinity of [^{3}H]NECA decreased in both PC12 cell membranes and striatal membranes (data not shown). In both membranes, the lower affinity binding sites were no longer detectable, and the K_D for the high affinity site was reduced severalfold (Table 1).

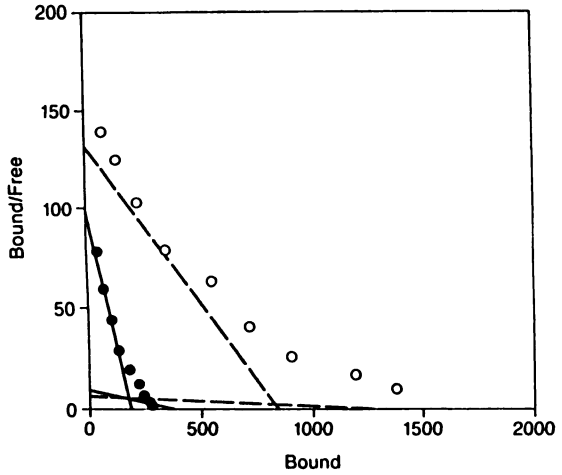

Fig. 1. Comparison of Scatchard plot of saturation binding data for [^{3}H]NECA to membranes from rat PC12 cells (●) and striatum (○). Membranes were incubated with concentrations of [^{3}H]NECA ranging from 0.5 to 128 nM. Nonspecific binding was measured in the presence of 30 μM *R*-PIA. Each value represents the mean of three experiments. *Lines* are based on results of computer analyses with the LIGAND program (17). For K_D and B_{max} values, see Table 1.

TABLE 1

Parameters of [^{3}H]NECA and [^{3}H]CGS21680 binding to membranes from rat PC12 cells and striatum

[^{3}H]NECA binding in striatal membranes was carried out in the presence of 50 nM CPA to eliminate A_1 binding. Binding of [^{3}H]NECA and [^{3}H]CGS21680 was measured over the concentration range 0.05–128 nM, in the absence or presence of 100 μM GTP, as described in Experimental Procedures. Nonspecific binding was measured in the presence of 30 μM *R*-PIA. Values are means ± standard errors from three experiments or are from a single experiment.

Ligand and membrane	GTP	High affinity		Low affinity	
		K_D	B_{max}	K_d	B_{max}
		nM	*fmol/mg of protein*	*nM*	*fmol/mg of protein*
[^{3}H]NECA					
PC12 cells	–	2.31 ± 0.30	200 ± 30	42.5 ± 9.9	171 ± 12
	+	25.5	240	ND[a]	ND
Striatum	–	6.56 ± 0.42	862 ± 140	286 ± 56	171 ± 147
	+	45.9 ± 16	993 ± 320	ND	ND
[^{3}H]CGS21680					
PC12 cells	–	6.71 ± 0.14	196 ± 12		
	+	36.5	166		
Striatum	–	11.3 ± 0.5	915 ± 137		
	+	25.8 ± 5.2	744 ± 115		

[a] ND, not detected by the filtration assay.

Binding of [^{3}H]CGS21680 to membranes from rat PC12 cells and striatum, using *R*-PIA to define nonspecific binding, was analyzed by a Scatchard plot (Fig. 2; Table 1). [^{3}H]CGS21680 bound to only one apparent site in both preparations (PC12 cells: K_d, 6.7 nM; B_{max}, 196 pmol/mg of protein; striatum: K_d, 11.3 nM; B_{max}, 915 fmol/mg of protein) (Table 1). As was the case for [^{3}H]NECA, the K_d value in PC12 cells (6.5 nM) was significantly lower than that in striatum (11.3 nM). In the presence of 100 μM GTP, the affinity of [^{3}H]CGS21680 decreased severalfold in both PC12 cell membranes and striatal membranes (Table 1). The B_{max} values for high affinity sites in PC12 or striatal membranes were in good agreement, regardless of whether [^{3}H]NECA or [^{3}H]CGS21680 was used as the ligand (Table 1).

Activity of adenylate cyclase in PC12 cell and striatal membranes. Activation of adenylate cyclase in PC12 and striatal membranes by the A_2 agonists NECA, CGS21680, and APEC was compared. NECA is a potent A_2 receptor agonist that activates both A_{2A} and A_{2B} receptors coupled to adenylate cyclase (2, 3). CGS21680 is a potent and selective agonist for A_{2A} receptors (10). APEC is a potent and selective A_2 agonist that was synthesized from CGS21680 and is closely related in structure to CGS21680 (11). Dose-response curves for the effects of the agonists on adenylate cyclase in PC12 cell membranes are shown in Fig. 3. NECA produced larger maximal activation than the other agonists and showed a shallow curve with Hill coefficients significantly lower than unity, with n_H ranging from 0.64 to 0.72, suggesting that NECA activated adenylate cyclase through interaction with more than one site. Nonlinear regression analysis for two sites provided an apparent EC_{50} value for the high affinity site of 0.61 ± 0.004 μM and an apparent EC_{50} value for the low affinity site of 1.82 ± 0.36 μM (Table 2). Dose-response curves for CGS21680 and APEC were more consonant with stimulation via one site, because the Hill coefficients were only slightly less than unity (n_H = 0.87 and 0.82, respectively). The maximal stimulation was significantly less than with NECA and rather closely matched the

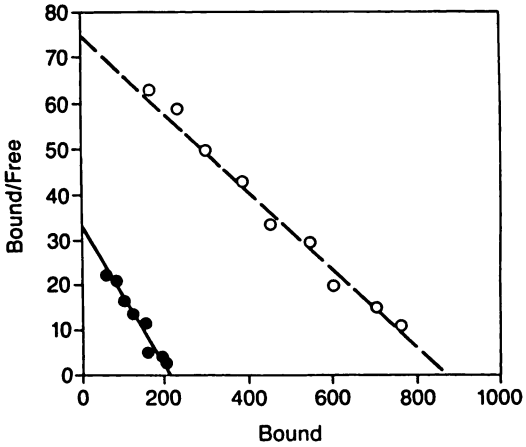

Fig. 2. Comparison of Scatchard plot of saturation binding data for [^{3}H]CGS21680 to membranes from rat PC12 cells (●) and striatum (○). Membranes were incubated with concentrations of [^{3}H]CGS21680 ranging from 0.5 to 128 nM. Nonspecific binding was measured in the presence of 30 μM *R*-PIA. Values from one of three experiments are depicted. For K_D values, see Table 1.

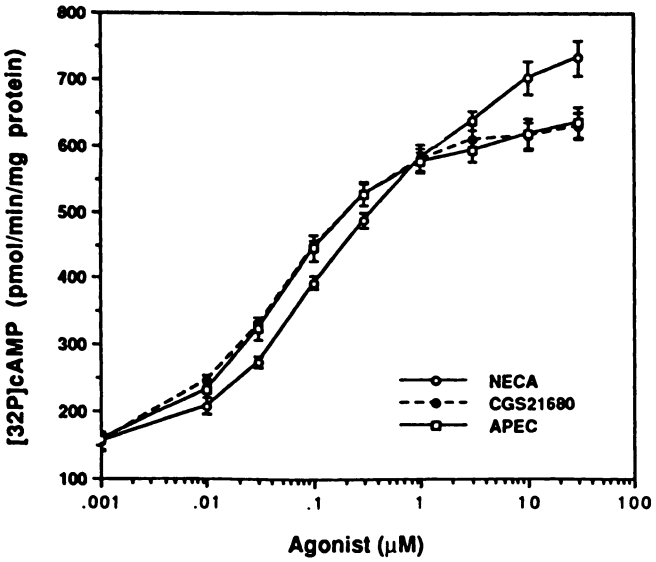

Fig. 3. Effects of adenosine receptor agonists on adenylate cyclase in membranes from rat PC12 cells. PC12 cell membranes were incubated with NECA (○), CGS21680 (●), or APEC (□), and adenylate cyclase activity was determined as described in Experimental Procedures. Data were analyzed using a nonlinear regression program. Values are means ± standard errors of three experiments. For EC_{50} values, see Table 2.

TABLE 2

Effects of adenosine receptor agonists on adenylate cyclase activity in membranes from rat PC12 cells and striatum

Membranes were incubated with NECA, CGS21680, or APEC, and adenylate cyclase activity was determined as described in Experimental Procedures. Data were analyzed using nonlinear regression analysis (GraphPAD). Values are means ± standard errors from three to six experiments. NECA activation curves were also analyzed for two components, and results are presented as high affinity and low affinity components, with the apparent percentage contribution of each to maximal formation given in parentheses.

	EC_{50}	Hill coefficient	cAMP maximal formation
	μM		*pmol/min/mg of protein*
PC12 cells			
NECA	0.211 ± 0.067	0.67 ± 0.01	745 ± 29
High affinity	0.061 ± 0.004 (61%)		
Low affinity	1.82 ± 0.36 (39%)		
CGS21680	0.072 ± 0.006	0.87 ± 0.08	633 ± 35
APEC	0.057 ± 0.001	0.82 ± 0.04	624 ± 26
Striatum			
NECA	0.156 ± 0.021	0.94 ± 0.04	405 ± 85
CGS21680	0.306 ± 0.038	0.97 ± 0.04	425 ± 11
APEC	0.088 ± 0.015	1.05 ± 0.02	414 ± 12

maximal stimulation due to the high affinity component for NECA.

In the striatum, all three agonists appeared to stimulate adenylate cyclase via only a high affinity receptor (Hill coefficient approximately 1.0), and the maximal activation by each of the three agonists was the same (Fig. 4; Table 3). Each of the three agonists was more potent in PC12 cells than in striatum as an activator of the high affinity receptor (NECA, 2.6-fold; CGS21680, 5.4-fold; APEC, 1.5-fold).

Accumulation of cAMP in PC12 cells. NECA also induced accumulation of cAMP in intact PC12 cells in a biphasic manner (Fig. 5). The EC_{50} values for high and low affinity components were similar to the EC_{50} values for NECA-stimulated adenylate cyclase in PC12 cell membranes. In contrast, CGS21680 stimulated an accumulation of cAMP in a monophasic manner, leading to a maximal accumulation less than that elicited by NECA (Fig. 5). The EC_{50} value of 0.064 μM for CGS21680 in intact cells was similar to the EC_{50} of 0.072 μM for CGS21680 for stimulation of adenylate cyclase in PC12 membranes.

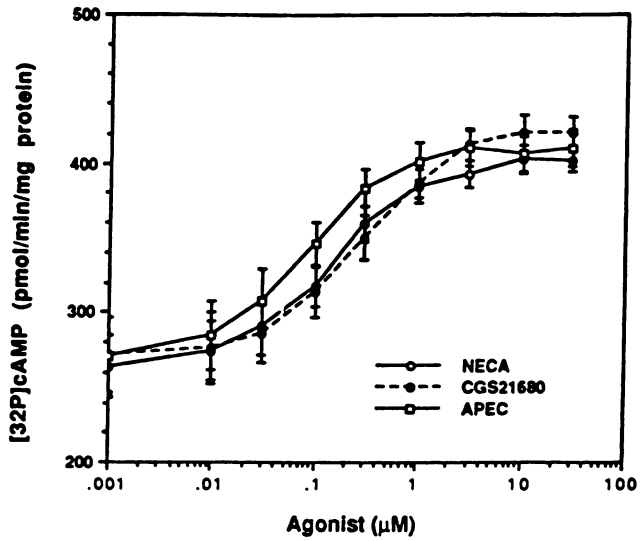

Fig. 4. Effects of adenosine receptor agonists on adenylate cyclase in membrane from rat striatum. Striatal membranes were incubated with NECA (○), CGS21680 (●), or APEC (□), and adenylate cyclase activity was determined as described in Experimental Procedures. Data were analyzed using a nonlinear regression program. Values are means ± standard errors of three experiments. For EC_{50} values, see Table 2.

TABLE 3

Effects of agonists and antagonists on [^{3}H]NECA binding to membranes from rat PC12 cells and striatum

Binding of [^{3}H]NECA was assayed at a final concentration of 4 nM. At this concentration of [^{3}H]NECA, there is virtually no binding to the low affinity component. Nonspecific binding was determined in the presence of 30 μM *R*-PIA. K_i values were obtained using the equation of Cheng and Prusoff (15), from IC_{50} values determined by log-logit analysis. Values are means ± standard errors, with the number of experiments given in parentheses.

	K_i			
	PC12 cells		Striatum	
	μM			
Agonists				
R-PIA	0.030 ± 0.007	(3)	0.141 ± 0.012	(3)
2-Chloroadenosine	0.017 ± 0.004	(3)	0.076 ± 0.012	(3)
CHA	0.158 ± 0.024	(3)	0.463 ± 0.039	(3)
NECA	0.0039 ± 0.0005	(3)	0.0097 ± 0.0013	(3)
CGS21680	0.0059 ± 0.0010	(3)	0.0166 ± 0.0007	(3)
APEC	0.0029 ± 0.0004	(3)	0.0138 ± 0.0020	(3)
Antagonists				
Theophylline	31.0 ± 3.6	(3)	22.4 ± 3.4	(3)
8-pSPT	9.53 ± 1.59	(3)	6.13 ± 0.65	(3)
8-pSP-1,3-dipropylxanthine	1.62 ± 0.44	(4)	1.29 ± 0.11	(3)
Caffeine	95.7 ± 11.7	(3)	40.6 ± 6.0	(3)
3,7-Dimethyl-1-propargylxanthine	45.1 ± 7.6	(3)	16.0 ± 4.3	(5)
XAC	0.033 ± 0.001	(3)	0.023 ± 0.001	(3)

Effects of agonists and antagonists on [^{3}H]NECA binding in PC12 cell and striatal membranes. The displacement by *R*-PIA and 8-pSPT of specific binding of 4 nM [^{3}H]NECA to membranes from rat striatum and PC12 cell was examined (Fig. 6). The IC_{50} value for the agonist *R*-PIA in PC12 cell membranes (0.082 ± 0.020 μM) was smaller than that in striatal membranes (0.21 ± 0.17 μM). In contrast, the IC_{50}

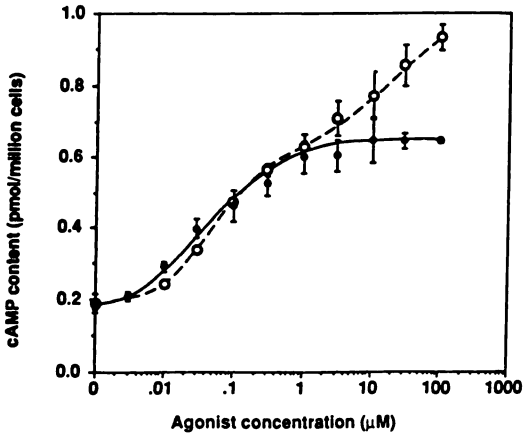

Fig. 5. Effects of NECA and CGS21680 on cAMP content in intact PC12 cells. PC12 cells were incubated with different concentrations of NECA (○) or CGS21680 (●). cAMP content was determined as described in Experimental Procedures. Values are means ± standard errors of three experiments. NECA produced a biphasic dose-response curve, and two EC_{50} values were calculated (0.066 ± 0.010 μM for the high affinity site and an apparent 7.5 ± 2.1 μM for the low affinity site, assuming that the response at 100 μM is near-maximal). CGS21680 produced a monophasic curve (EC_{50} value, 0.064 ± 0.0136 μM).

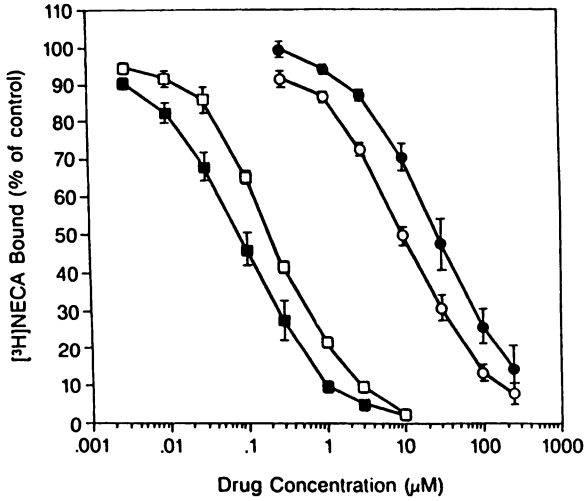

Fig. 6. Inhibition of [^{3}H]NECA binding to membranes from rat PC12 cells and striatum. Membranes were incubated with 4 nM [^{3}H]NECA in the absence or presence of varying concentrations of *R*-PIA (*squares*) or 8-pSPT (*circles*). *Closed symbols*, PC12 cell membranes; *open symbols*, striatal membranes. Values are means ± standard errors of three experiments. For K_i values derived from this and similar experiments with other agonists and antagonists, see Table 3.

value for the antagonist 8-pSPT in PC12 cell membranes (26 ± 4 μM) was larger than that in striatal membranes (9.9 ± 1.1 μM). In order to explore the generality of such differences, inhibition of [^{3}H]NECA binding by several other agonists and antagonists was examined, and K_i values were calculated by the Cheng-Prusoff equation, using a K_d value of 2.3 nM in PC12 cell membranes and a K_d value of 6.6 nM for striatal membranes. Each of the agonists examined was more potent in PC12 cells than in striatum, whereas nearly all the antagonists examined were less potent in PC12 cells than in striatum (Table 3).

Effects of antagonists on stimulation of adenylate cyclase by NECA and CGS21680 in PC12 cells and striatal membranes. The effect of the antagonist 8-pSPT on NECA-stimulated adenylate cyclase activity in membranes from PC12 cells and striatum was examined. In PC12 cell membranes, 8-pSPT shifted the dose-response curve for NECA to the right (Fig. 7). The Hill coefficient appeared to be slightly increased (n_H = 0.80). The maximal response to NECA was still greater than that with CGS21680. A K_B value for 8-pSPT of 3.9 ± 1.0 μM was obtained by Schild analysis of the data. However, such analyses are not valid for a response consisting of more than one component. Therefore, the experiment was repeated with CGS21680, which gave a Hill coefficient close to unity in the absence (n_H = 0.90) and presence (n_H = 0.95) of 8-pSPT (data not shown). Schild analysis provided a K_B of 6.7 ± 1.6 μM for 8-pSPT antagonism of the CGS21680 response.

In striatal membranes, 8-pSPT shifted the dose-response curve of NECA and of CGS21680 to the right (data not shown), and Schild analysis afforded K_B values of 6.1 μM and 5.5 μM, respectively (Table 4). The Hill coefficients in these striatal experiments were near 1.0 both in the absence and in the presence of 8-pSPT. There is extensive literature on K_B values for antagonists of NECA-stimulated adenylate cyclase in PC12 cell membranes (see Refs. 4, 5, 6, and 18 and references cited therein). In order to ascertain the significance of such values, the apparent K_B values versus NECA stimulation of adenylate cyclase for theophylline, 8-pSPT, 8-pSP-1,3-dipropylxanthine, caffeine, 3,7-dimethyl-1-propargylxanthine, and XAC, in PC12 cell membranes, were derived by Schild analysis and were compared with true K_B values for the same xanthines versus CGS21680 stimulation of adenylate cyclase (Table 4). In all cases, the apparent K_B values versus NECA were only somewhat lower than true values versus CGS21680. A similar comparison was made for K_B values versus NECA and K_B values versus CGS21680 in striatal membranes. In this comparison the K_B values were not significantly different versus NECA or versus CGS21680 for each xanthine antagonist, except for caffeine, which was slightly more potent versus CGS21680 than versus NECA (Table 4). A comparison of K_B values versus CGS21680 in PC12 cell membranes and striatal membranes

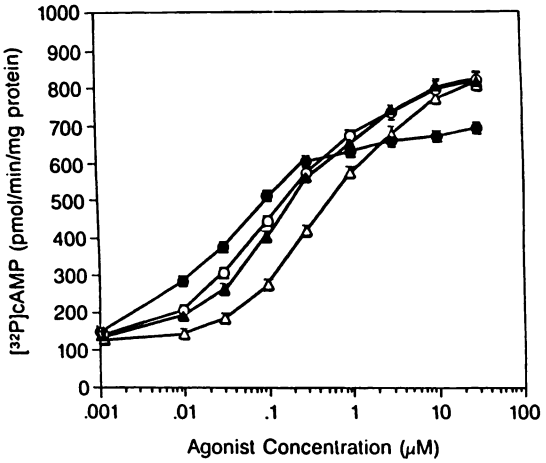

Fig. 7. Effects of 8-pSPT on NECA-stimulated adenylate cyclase in membranes from PC12 cells. PC12 cell membranes were incubated with different concentrations of NECA in the absence (○) or presence (▲, 1 μM; △, 10 μM) of the xanthine antagonist 8-pSPT. Adenylate cyclase activity was determined as described in Experimental Procedures. Data were analyzed using a nonlinear regression program. Values are means ± standard errors of three experiments. The response to CGS21680 (●) is shown for comparison. For K_B values from this experiment and experiments with other antagonists versus NECA and CGS21680, see Table 4.

TABLE 4

Effects of antagonists on activation of adenylate cyclase by NECA or CGS21680 in membranes from PC12 cells and striatum

Membranes were incubated with varying concentrations of NECA in the absence or presence of the xanthine, as described in Experimental Procedures. The EC_{50} values for NECA or CGS21680 were used to calculate K_B values, using the Schild equation. Each value represents the mean ± standard error of three experiments.

Antagonist	K_B			
	PC12 cells		*Striatum*	
	NECA	*CGS21680*	*NECA*	*CGS21680*
	μM			
Theophylline	13.7 ± 0.4	20.3 ± 1.8	23 ± 2	18 ± 5
8-pSPT	3.9 ± 1.0	6.7 ± 1.6	6.1 ± 0.7	5.5 ± 1.1
8-pSP-1,3-dipropylxanthine	0.70 ± 0.05[a]	1.1 ± 0.1	1.5 ± 0.23	2.0 ± 0.5
Caffeine	36 ± 4	44 ± 4	70 ± 7	43 ± 13
3,7-Dimethyl-1-propargylxanthine	8.6 ± 0.4	11.8 ± 0.2	13 ± 4	10 ± 2
XAC	0.019 ± 0.0011[a]	0.038 ± 0.004	0.025 ± 0.0041	0.019 ± 0.002

[a] These values are significantly lower than values previously reported in Ref. 5.

(Table 4) indicated comparable potencies in most cases, in contrast to the results of binding assays (Table 3), where, with the exception of 8-pSPT and 8-pSP-1,3-dipropylxanthine, all of these same antagonists were somewhat more potent versus [^{3}H]NECA binding in striatal membranes than in PC12 cell membranes.

Discussion

The high affinity A_{2A} receptors from bovine striatum have been shown to be glycoproteins with apparent molecular weights of about 45,000, separable from the coexisting A_1 receptors (19, 20). The A_{2A} receptors of rat PC12 cells and of rabbit striatum also have apparent molecular weights of about 45,000 (21). An A_2 receptor of a smooth muscle DDT_1 MF-2 cell line had an apparent molecular weight of 42,000. A canine A_{2A} receptor has been cloned and sequenced (22, 23).

Biochemical evidence for distinct subclasses of A_{2A} receptors has not been obtained as yet, although there is pharmacological evidence for such subclasses. For example, potencies of agonists/antagonists at A_{2A} receptors of rat PC12 cells and at A_{2A} receptors of human platelets, as assessed with adenylate cyclase assays, in many instances do not correspond (4–6). Furthermore, potencies of agonists/antagonists in adenylate cyclase assays for A_{2A} receptors of either rat PC12 cells or human platelets (4–7, 18) do not always correspond to potencies in binding assays for A_{2A} receptors of rat striatum (3, 7). Such differences between potencies of agents in adenylate cyclase assays and potencies of the same agents in binding assays might reflect either merely assay-dependent differences or the existence of pharmacologically distinct subtypes of A_{2A} receptors. In the present study, both binding data and adenylate cyclase data for PC12 cells and striatal membranes have been compared for several agonists and antagonists.

The PC12 cell has been a useful model for the characterization of A_{2A} receptors (4–6, 18, 24, 25). Binding of [^{3}H]NECA to sites in PC12 cell membranes having properties of A_{2A} receptors has been reported (26). The reported K_D value of 4.7 nM and the B_{max} of 260 fmol/mg of protein are consonant with the present results at the high affinity site. The assay conditions differ to some extent from the present conditions. Major differences were in the use of *R*-PIA in the present study, rather than 2-chloroadenosine (26), to define nonspecific binding, a temperature of 25° in the present study, rather than 37° (26), and, finally, the use of a lower maximal concentration of [^{3}H]NECA for Scatchard analysis in the former study (26). One other study on [^{3}H]NECA binding to PC12 cell membranes (27) focused on binding at 0°, under which conditions binding seemed to be mainly to a membrane constituent that resembles an A_2 receptor but is not (see Ref. 28). Binding of [^{3}H]CGS21680 to PC12 membranes has previously not been reported, although photoaffinity labeling of an A_{2A} receptor in PC12 membranes using a derivative of CGS21680 has been attained (21). Neither A_1 receptor-mediated inhibition of adenylate cyclase activity nor [^{3}H]CHA or [^{3}H]CPA binding to A_1 receptors was detected for PC12 cell membranes (26).

In contrast to PC12 cells, the striatum is a heterogeneous tissue and membranes from striatum are heterogeneous, containing both A_1 and A_{2A} receptors (29, 30). Striatum also contains A_{2B} receptors, whose functional activation of adenylate cyclase is readily detected in slice preparations but, remarkably, not in membranes (2). In contrast, the A_{2B} receptor of fibroblast cells activates adenylate cyclase both in intact cells (3, 31) and in membranes (32). It should be noted that [^{3}H]CGS21680 is nearly inactive at A_{2B} receptors of brain (10) or fibroblasts.[1] Striatal A_{2A} receptors from different species differ in radioligand binding properties (33), suggestive of subtypes of such brain receptors. Comparisons of activation of adenylate cyclase by A_{2A} receptors in striatal membranes with binding of radioligands to striatal A_{2A} receptors have not been reported previously.

Scatchard analysis suggested that [^{3}H]NECA binds to a high affinity site (K_D, 2.3 nM) and a low affinity site (K_D, 43 nM) in PC12 cell membranes (Fig. 1; Table 1). In the presence of GTP, only one site, with a K_D of 26 nM, was detected. In a prior study, [^{3}H]NECA appeared to bind to a single class of high affinity sites in PC12 cell membranes, although the lack of detection of the low affinity sites by Scatchard analysis might have been due to low concentrations of [^{3}H]NECA (maximum, 20 nM) used in that study (26). Scatchard analysis suggested that [^{3}H]NECA also binds to two sites in striatal membranes. Each of these [^{3}H]NECA binding sites in striatal membranes showed lower affinity for [^{3}H]NECA than did the corresponding sites in PC12 membranes (Table 1). Two explanations for the lower affinity component of [^{3}H]NECA binding may be advanced. *A priori*, it would be proposed that this represents a low affinity state of the A_{2A} receptor. The presence of both high and low affinity states of receptors, existing in a GTP-dependent equilibrium, is well known. However, a second possibility

[1] L. E. Brackett and J. W. Daly, unpublished observations.

is that [^{3}H]NECA binds both to the A_{2A} receptor, which is responsible for most of the activation at adenylate cyclase in PC12 cells, and to an A_{2B} receptor that appears to activate adenylate cyclase in PC12 membranes (Fig. 3) and is known to be present in striatal slices (2). The EC_{50} for NECA to activate cAMP accumulation via A_{2B} receptors is 2.6 μM in fibroblasts (3) and about 3 μM in striatal slices (see Ref. 2).

Studies with GTP did not provide an unambiguous answer. In the presence of GTP, the high affinity site for NECA was absent in PC12 cell membranes, and a site with similar B_{max} but an 11-fold lower affinity (K_D, ~26 nM) was detected, indicative of GTP-dependent conversion of the high affinity state of the A_{2A} receptor to a lower affinity state. The low affinity state (K_D, ~45 nM) in PC12 cell membranes was no longer detected. This result could be interpreted as conversion of the lower affinity site to a state that has too low an affinity to be detected by a filtration assay. The B_{max} values (total specific binding sites in the absence of GTP, 371 fmol/mg of protein; total specific binding in the presence of GTP, 240 fmol/mg of protein) are consonant with this interpretation. However, with the low levels of specific binding, the possibility that the low affinity state exists even in the absence of exogenous GTP cannot be ruled out. In striatum the situation was similar, with the high affinity site (K_D, 6.6 nM) being replaced by a 7-fold lower affinity site (Table 1). In this case, the new lower affinity site (K_d, 46 nM) does not correspond to a much lower affinity site (K_d, 286 nM) detected in the absence but not in the presence of GTP.

In contrast to NECA, [^{3}H]CGS21680 apparently bound to only one site in both PC12 cell and striatal membranes (Fig. 3). The B_{max} values for [^{3}H]CGS21680 were comparable to the B_{max} value for the high affinity component of [^{3}H]NECA binding. The K_d value for [^{3}H]CGS21680 was 2-fold greater than that for the high affinity site of [^{3}H]NECA binding. The presence of GTP increased the K_D of [^{3}H]CGS21680 by 5.4-fold and 2.3-fold, respectively, in PC12 cell membranes and striatal membranes (Table 1). The lack of a lower affinity binding site for [^{3}H]CGS21680 is consonant with one interpretation of the [^{3}H]NECA binding data, namely that this represents binding to an A_{2B} receptor. CGS21680 would bind only to the A_{2A} receptor, because it is a very weak agonist at A_{2B} receptors (10). However, in view of the relatively small change of K_D values on addition of GTP, the presence of a small amount of a lower affinity state might not have been detected by computer-assisted analysis of the data. Recently, [^{3}H]CGS21680 was reported to bind to one high affinity, low density site and to one low affinity, high density site in rat striatal membranes (34). Remarkably, binding of APEC-derivatives to A_{2A} receptors in bovine and rabbit striatal membranes was little affected by guanine nucleotides (19, 20).

Activation of adenylate cyclase elicited by NECA in PC12 cell membranes appeared to involve two components, because the Hill coefficient was significantly less than unity. Furthermore, the stimulation by NECA significantly exceeded that caused by CGS21680 or by APEC, both of which appeared to stimulate mainly via one site, based on Hill coefficients closer to unity. Analysis of the dose-response curve for NECA suggested that the major component of the response had an EC_{50} value of 0.06 μM, whereas the minor component had an EC_{50} of 1.8 μM. The former value is consonant with a high affinity A_{2A} receptor, whereas the latter is consonant with a low affinity A_{2B} receptor. The dose-response curves for CGS21680 and APEC were consonant with a single component, with EC_{50} values, respectively, of 0.074 and 0.057 μM.

Accumulations of cAMP elicited by NECA and CGS21680 in intact PC12 cells also differed, with NECA again causing a greater response than CGS21680 (Fig. 5). Because NECA can stimulate adenylate cyclase via both A_{2A} and A_{2B} receptors, whereas CGS21680 and probably its derivative APEC are active only at A_{2A} receptors (10), the results in both membranes and intact cells suggest that stimulation of adenylate cyclase by NECA is due to activation of both A_{2A} and A_{2B} receptors. Whether such putative A_{2B} receptors in PC12 cells are similar to those of human fibroblasts is not known.

A comparison of the ability of a series of agonists and antagonists to inhibit binding of [^{3}H]NECA to A_2 receptors in PC12 cell and striatal membranes indicates clear differences (Table 3). Such binding studies were conducted with 4 nM [^{3}H]NECA, where there would be little contribution from the low affinity binding sites. All agonists were more potent in PC12 cell membranes. The xanthine antagonists were more potent in striatal membranes than in PC12 cell membranes, although the difference was significant ($p < 0.05$) only for theophylline, caffeine, 3,7-dimethyl-1-propargylxanthine, and XAC. A comparison of the ability of a series of antagonists to inhibit NECA-stimulated adenylate cyclase in PC12 cell and striatal membranes showed only marginal differences (Table 4). However, in contrast to the results with binding assays (Table 3), certain of the xanthine antagonists in the adenylate cyclase assay were slightly more potent in PC12 cell membranes than in striatal membranes (Table 4). However, the difference between potencies in PC12 cell membranes and striatal membranes was significant ($p < 0.05$) for only three (theophylline, caffeine, and 3,7-dimethyl-1-propargylxanthine) of the six xanthines examined (Table 4). Because the stimulation of adenylate cyclase in PC12 membranes appears to involve two components, proposed to represent a major A_{2A} component and a minor A_{2B} component, Schild analyses of the rightward shifts of dose-response curves by antagonists, as reported for PC12 cells in many publications (4–7, 18), are not strictly valid. Therefore, Schild analyses of the effects of the six antagonist xanthines were repeated using CGS21680, an agonist that stimulates only A_{2A} receptors. In PC12 cells the K_B values versus all six xanthines were slightly greater versus CGS21680 than versus NECA. In striatum there was a good agreement for K_B values of the six antagonists versus either NECA or CGS21680 (Table 4). If the K_B values for the six antagonists versus CGS21680 are compared for PC12 cell membranes and striatal membranes, there is a good correlation (Table 4). This suggests that most of the apparent differences previously suggested for A_{2A} receptors of PC12 cells and striatum are due to the use of NECA, which we propose activates both an A_{2A} and an A_{2B} receptor coupled to adenylate cyclase in PC12 cell membranes but only an A_{2A} receptor in striatal membranes. The use of CGS21680, which activates only A_{2A} receptors, allows for a more valid comparison of the PC12 cell and striatal systems.

The present comparisons with CGS21680 indicates that the A_{2A} receptors of striatum and PC12 cells are virtually identical. Furthermore, the data indicate that NECA should be used with great caution in binding assays and for activation of adenylate cyclase, the latter because of possible involvement of both A_{2A} and A_{2B} adenosine receptors.

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