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Adenosine-Sensitive Adenylate Cyclase in Rat Brain Homogenates: Kinetic Characteristics, Specificity, Topographical, Subcellular and Cellular Distribution

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SUMMARY

PRÉMONT, JOËL, MICHELLE PEREZ, GÉRARD BLANC, JEAN-POL TASSIN, ANNE-MARIE THIERRY, DENIS HERVÉ AND JOËL BOCKAERT: Adenosine-sensitive adenylate cyclase in rat brain homogenates: kinetic characteristics, specificity, topographical subcellular and cellular distribution. *Mol. Pharmacol.* **16**, 790–804 (1979).

Two adenosine receptor sites have been detected in striatal homogenates. One triggered the stimulation and the other the inhibition of the adenylate cyclase. They clearly had different chemical specificity toward adenosine analogues. In particular, N6 derivatives of adenosine were full agonists on the stimulatory site and had no affinity for the inhibitory site. Adenosine stimulation of the adenylate cyclase in striatal homogenates had characteristics similar to those of other neurotransmitter-sensitive adenylate cyclases: 1—Adenosine increased the maximal reaction velocity of adenylate cyclase without altering the K_m (18 μM) for its substrate Mg-ATP. 2—Increasing the concentration of free Mg^{2+} increased the adenylate cyclase activity with characteristics suggesting the existence of an allosteric site for Mg^{2+}. 3—Adenosine increased the apparent affinity of the Mg^{2+} allosteric site. 4—GTP was required in order to obtain optimal stimulation. 5—The highest total and specific adenosine-sensitive adenylate cyclase activities were observed in fractions enriched in nerve endings while the activity of this enzyme was very low in cytosol and myelin fractions. Adenosine-sensitive adenylate cyclase was found in homogenates of striatum, nucleus accumbens, globus pallidus, tuberculum olfactorium, olfactory bulb and posterior cerebellar cortex but not in homogenates of frontal and parietal cerebral cortex, anterior and central cerebellar cortex, substantia nigra, ventral tegmental area, hippocampus and hypothalamus. The topographical distribution of the adenosine sensitive adenylate cyclase was also heterogeneous within the striatum. On the contrary the specific [^{3}H]adenosine uptake was similar in brain areas whether or not they contained an adenosine-sensitive adenylate cyclase. The adenosine-sensitive adenylate cyclase did not disappear after a complete degeneration of presynaptic dopaminergic terminals. Furthermore, the near complete disappearance of the basal, dopamine and adenosine-sensitive adenylate cyclases, after kainic acid injection into striatum, indicated that these enzymes are not located on glial cells, axons or nerve terminals of extrinsic neurons but rather on neurons having their cell bodies within this structure.

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INTRODUCTION

Adenosine has been shown to stimulate cyclic AMP production in brain slices from virtually all species and regions studied (1). Evidence that stimulation is due to a direct interaction of adenosine with a specific extracellular receptor coupled with an adenylate cyclase has previously been provided. Thus when adenosine uptake is inhibited, the adenosine effect on cyclic AMP accumulation persisted (2). In addition, 2-chloroadenosine, a potent stimulant of cyclic AMP formation in brain slices, is not transformed into 2-chloro cyclic AMP (3). An adenosine sensitive adenylate cyclase has been demonstrated in several cell types including neuroblastoma cells (4–6). Finally, in brain we have recently reported that adenosine is able to stimulate an adenylate cyclase in rat striatal homogenates (7).

Since adenosine is structurally related to ATP, the substrate of the adenylate cyclase, we decided that it was important to analyze fully the specificity and characteristics of the adenosine-sensitive adenylate cyclase in striatal homogenates and to compare these biochemical properties with those of other neurotransmitters or hormone sensitive adenylate cyclases. Furthermore, in order to determine whether or not adenosine is able to stimulate all brain adenylate cyclases, we have studied its regional, subcellular and cellular distribution in rat brain.

MATERIALS AND METHODS

Homogenate preparation. Male Sprague-Dawley rats weighting 250–400 g were killed by decapitation at 2 p.m. Their brains were removed and their striata were dissected at 4° with glass manipulators. Tissues were homogenized using a glass teflon-homogenizer (five strokes) in 2 mM Tris-maleate pH 7.2, 2 mM EGTA[2] and 300 mM sucrose (usually 2 striata in 3 ml).

When the topographical distribution of adenosine-sensitive adenylate cyclase was examined, the brain was cut behind the striatum and the frontal and rostral parts were fixed with sodium chloride 0.9% on a Leitz-Wetzler microtome stage refrigerated at −7°C. They were cut into slices of 500 μm thickness. The localization of slices was determined according to the atlas of König and Klippel (8) as described by Tassin *et al.* (9). The first slice was between the 9700 μm × 9200 μm planes and was termed the 9700 μm slice. Discs of the different brain areas (1.4 mm diameter) were punched out with a cooled stainless steel tube. The discs were blown into 500 μl microfuge plastic tubes containing 60 μl of the homogenization medium described above. The samples were homogenized (5 strokes) using a piston made with dental cement (Stellon from Detrey).

Adenylate cyclase assay. Adenylate cyclase activity was measured by the conversion of [α^{32}P]ATP to cyclic [^{32}P]AMP. The reaction mixture (final volume, 100 μl) contained 25 mM Tris-maleate (pH 7.2), 0.5 mM ATP, 1 mM $MgSO_4$, 0.4 mM EGTA (added with the homogenate) 0.2 mg/ml of creatine kinase, 20 mM creatine phosphate, 0.1 mM papaverine[3] and 0.4 IU/ml of adenosine deaminase except when stated.

The reaction was initiated by adding the homogenate (20 μl). After 2 min at 30°, 1 μCi of [α^{32}P]ATP and 0.001 μCi of cyclic [^{3}H]AMP were added and the reaction was allowed to proceed for 5 min at 30°. The reaction was then stopped by addition of 100 μl of a solution containing 5 mM ATP, 5 mM cyclic AMP, 50 mM Tris-HCl (pH 7.4) and 1% sodium dodecyl sulfate. The cyclic [^{32}P]AMP formed and the cyclic [^{3}H]AMP added as a recovery marker were isolated according to Salomon *et al.* (10). The range of variation between independent determinations was less than 10%.

Determination of glutamic acid decarboxylase (GAD) and choline acetyltransferase (CAT) activities and dopamine content. Twenty-five microliters of homoge-

[2] The abbreviations used are: EGTA, ethylene glycol bis (-aminoethyl ether) N,N'-tetraacetic acid; GAD, glutamate decarboxylase; CAT, choline acetyltransferase; DA, dopamine; 6-OHDA, 6-hydroxydopamine; ADA, adenosine deaminase.

[3] Erratum: in the previous paper (*Mol. Pharmacol.* 13:662–670, 1977) we forgot to mention that papaverine (0.1 mM) was present in all the adenylate cyclase assays.

nate used for the adenylate cyclase assay were diluted twice with the same medium; 50 μl of a 20 mM sodium phosphate, 2 mM dithiothreitol, 0.4% triton × 100 solution were added. After sonication glutamic acid decarboxylase and choline acetyltransferase activities were determined on two 20 μl aliquots.

a) Estimation of glutamic acid decarboxylase activity. The glutamic acid decarboxylase activity was estimated on the first 20 μl aliquot (defined above) following the method of Moskal *et al.* (11) with slight modifications. The samples were incubated with *L*-[1-^{14}C]-glutamic acid at 37° for 20 minutes. $^{14}CO_2$ was trapped on a paper impregnated with hyamine. The reaction was linear for 30 minutes and the results are expressed in n moles of $^{14}CO_2$ produced per hour per mg protein.

b) Estimation of choline acetyltransferase activity. To the second 20 μl aliquots were added 30 μl of 50 mM sodium phosphate, 200 mM sodium chloride, 2 mM phenanthroline, pH 7.4 solution and the reaction was performed for 6 min at 37° on 5 μl of this mixture incubated with [1-^{14}C]-acetyl coenzyme A as described by Fonnum (12). In these conditions ^{14}C acetylcholine formation was linear for 10 min. Results are expressed in nmoles of ^{14}C acetylcholine formed per hour per mg protein.

Dopamine content was measured as previously described by Gauchy *et al.* (13).

[^{3}H]-adenosine uptake measurement. Two striata were dissected, homogenized in 300 mM sucrose with a glass teflon homogenizer, centrifuged at 10,000 × *g* during 15 min at 4°. The pellet was resuspended in 1 ml of 300 mM sucrose. Twenty microliters were diluted in 360 μl of an incubation medium containing 125 mM NaCl; 5 mM KCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$; 10 mM glucose; 50 mM Tris-HCl pH 7.4 and incubated for 5 min at 37°. Uptake was begun by the addition of 2.5 μM of ^{3}H adenosine in 20 μl and the reaction was stopped 10 min later by cooling the samples to 4°. Blank samples were performed without the incubation period. Synaptosomes were centrifuged at 20,000 × *g* for 5 min and washed three times with 500 μl of the incubation medium. The pellet was solubilized in 50 μl Triton × 100 (1%) and counted.

Chemicals. ATP (disodium salt), 5'AMP, ADP, guanosine, 2-chloro-adenosine, 3'-deoxyadenosine, adenosine, inosine, adenine papaverine, theophylline, kainic acid, were obtained from Sigma; dopamine, 2' deoxyadenosine from Calbiochem; creatine kinase, phosphocreatine, cyclic AMP and adenosine deaminase from Boehringer-Mannheim; 5' deoxyadenosine from P.L. Biochemicals; N6-methyladenosine, N6 dimethyladenosine from I.C.N.; N6-phenylisopropyladenosine was kindly donated by Boehringer-Mannheim.

Radiochemicals. [^{3}H]cyclic AMP (ammonium salt), 25 Ci/mmol; [α^{32}P]ATP (disodium salt), 10–20 Ci/mmole and 2-[^{3}H]-adenosine 12 Ci/mmole were purchased from New-England Nuclear. *L*-[1-^{14}C]glutamic acid, 55 mCi/mmole, [1-^{14}C]-acetyl coenzyme A, 59 mCi/mmole and [^{3}H]-S-adenosylmethionine, 10.2 Ci/mmole from Amersham.

RESULTS

1—Specificity of the Adenosine Receptor Implicated in Adenylate Cyclase Stimulation

Table 1 is a summary of results previously described (7) and new data on the chemical specificity of the adenosine receptor involved in adenylate stimulation. All results on the stimulation produced by the different analogues are expressed as percent of that obtained with adenosine. It is important to recall that in adenylate cyclase assay, adenosine concentration is high enough to produce a submaximal stimulation of the adenosine-sensitive adenylate cyclase (7). Therefore, the maximal stimulation of the adenylate cyclase by adenosine is given by the ratio between the adenylate cyclase activities measured in the presence of 10 μM of added adenosine and in the presence of adenosine deaminase (0.4 IU/ml). The phosphorylation on the 5' position give compounds which are neither agonist nor antagonist. The ADP was tested in the absence of an ATP regenerating system to avoid ATP synthesis which would introduce a change in the specific radioactivity of ATP. We had checked that adenosine

TABLE 1

Chemical specificity of adenosine receptors implicated in the stimulation of the striatal adenylate cyclase

This table is a summary of several experiments made in different adenylate cyclase assay conditions always in the presence of mM Mg^{2+} (see text and below). In each experiment the maximal stimulation by adenosine was measured as described in paragraph 1 of the text and taken as 100%. The maximal stimulations produced by each analogue expressed as percent of this stimulation ± SEM. The dose-response curves for agonists followed Michaelis-Menten type of kinetics.

	Maximal stimulation	K_a app[c] (μM)	K_I app[d] (μM)
Adenosine[b]	100	0.50	
5′ AMP	0	—	
ADP[a]	0	—	
ATP	0	—	
Inosine	0	—	
Guanosine	0	—	
2-chloro-adenosine	100 ± 2 (n = 7)	1.2 ± 0.5 (n = 4)	
N6-phenylisopropyl-adenosine	100 ± 6.5 (n = 5)	3.2 ± 1 (n = 3)	
N6-dimethyl-adenosine	100 ± 3.4 (n = 5)	28.0 ± 8 (n = 3)	
N6-methyl-adenosine	120 ± 4.5 (n = 5)	85.0 ± 16 (n = 3)	
6-mercapto-purine riboside	0	—	
5′-deoxyadenosine	36 ± 7 (n = 5)	9.0 ± 2 (n = 3)	
3′-deoxyadenosine[b]	non-competitive inhibition	—	
2′-deoxyadenosine[b]	non-competitive inhibition	—	
Theophylline	competitive inhibition		20.0

[a] This compound was tested in the absence of an ATP regenerating system.

[b] These compounds were tested in the absence of adenosine deaminase.

[c] K_a app: concentration giving half-maximal stimulation.

[d] K_I app: apparent inhibition constant of the adenosine sensitive adenylate cyclase.

Compounds devoid of agonist property were tested from 0.1 to 50 μM.

stimulation of the adenylate cyclase still occurs in these conditions. The stimulation of the adenosine sensitive adenylate cyclase by 2-chloro-adenosine did not decrease when increasing the ATP concentration (see Fig. 1). This indicates that ATP did not interact with the adenosine receptor. Since mercapto-purine riboside and inosine were inactive, it can be concluded that the nitrogen atom in the 6 position is essential for the interaction with the receptor. However, an important substitution on the amino group in 6 position is compatible with activation, i.e., N6-phenylisopropyl-adenosine; N6-methyl and dimethyladenosine are full agonists having rather large differences in their affinity for the receptor. Substitution by halogen in the 2 position of the purine group gives compounds such as 2-chloroadenosine which are full agonists not deaminated by ADA. 5′-deoxyadenosine is a partial agonist. We have previously shown (7) that the inhibitions of the adenylate cyclase by 2′ and 3′ deoxyadenosine were similar whatever the concentration of adenosine present in the assay indicating a non-competitive inhibition of the enzyme. However, since these compounds are deaminated by ADA it is impossible so far to know whether these compounds have some agonist activity in addition to their non-competitive inhibition. Theophylline was a competitive inhibitor of the stimulation by adenosine and 2 chloro-adenosine; its apparent K_I was 20 μM as previously reported (7). Using this value and the dose inhibition curves of the adenylate cyclase by theophylline determined in the presence of several concentrations of added adenosine (data not shown) it is possible to determine the apparent affinity of adenosine for its receptor (0.5 μM).

2—Effects of ATP and Mg^{2+}. Evidence for a Biphasic Stimulatory and Inhibitory Effect of Adenosine on Striatal Adenylate Cyclase

a) Substrate dependence of basal and adenosine sensitive adenylate cyclase. In the presence of a constant amount of Mg^{2+} (4 mM), the basal adenylate cyclase activity increased as a function of ATP concentration following Michaelis-Menten kinetics as shown by Hofstee plot of the data (right part of Fig. 1). The K_m for the substrate was 18 μM. 2-Chloro-adenosine increased the maximal velocity of the adenylate cyclase reaction without altering the apparent affinity for its substrate Mg-ATP. The K_m determined by the Hofstee plot was 20 μM (Fig. 1). In the presence of 2-chloro-adenosine the enzyme was less susceptible to inhibition at high ATP concentration (Fig. 1).

b) Mg^{2+} dependence of basal, 2-chloro-adenosine and dopamine sensitive adenylate cyclases. In the presence of low concentrations of ATP (10 μM), the adenylate cyclase activities measured in the absence or presence of dopamine or 2-chloro-adenosine increased as the function of Mg^{2+} concentrations (Fig. 2). Increasing the molar ratio of Mg^{2+} to ATP from 2 to 1000 decreased the fold stimulation by dopamine and 2-chloroadenosine. In the presence of 10 mM Mg^{2+} there was still a stimulation by dopamine while the stimulation by 2-chloro-adenosine disappeared.

c) Dose-dependent effects of adenosine and some adenosine analogues on adenylate cyclase at high Mg^{2+} concentration. At 1 mM Mg^{2+} concentration of adenosine, 2-chloro-adenosine and 5′-deoxyadenosine above 100 μM are inhibitory of the maximal stimulation produced by lower concentrations of these compounds (data not shown). At 10 mM Mg^{2+} the relative stimulations produced by these nucleosides are weak (Figs. 2 and 3) which allows them to reveal their inhibitory effect on the adenylate cyclase. Figure 3 shows that the inhibition by 2′-deoxyadenosine, 5′-deoxyadenosine and adenosine is dose-dependent and saturable.

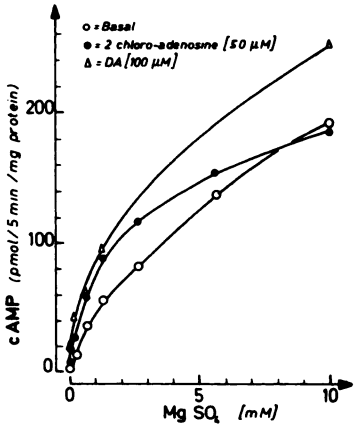

FIG. 2. *Effect of adding various concentration of Mg^{2+} in the presence of 10 μM added ATP on adenylate cyclase activity in the absence* (○) *and in the presence of dopamine* (△) *or 2-chloro-adenosine* (●)
The protein concentration was 0.49 mg/ml.

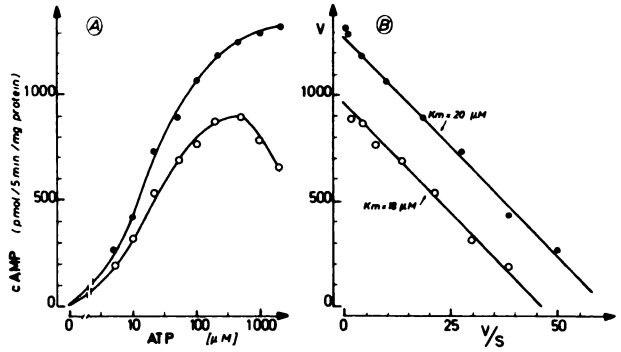

FIG. 1. *Effect of various concentrations of added ATP in the presence of 4 mM added Mg^{2+} on adenylate cyclase activity in the absence* (○) *or presence* (●) *of 10 μM 2-chloro-adenosine*
The right-hand part of the figure shows a Hofstee plot (14) of the data. In such a plot the slope of the line is equal to $-K_m$. The protein concentration was 0.44 mg/ml.

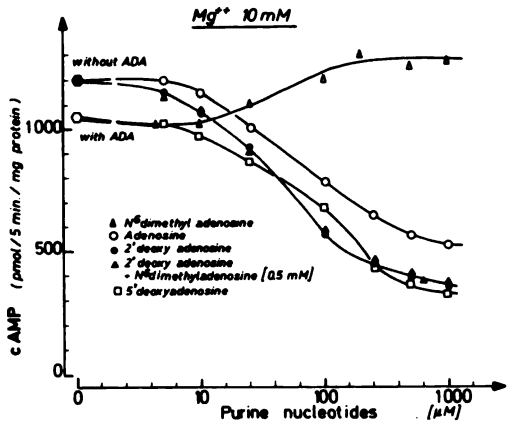

FIG. 3. *Effect of adenosine and some adenosine analogues on the striatal adenylate cyclase measured in presence of 10 mM Mg^{2+}*

The difference between the adenylate cyclase activities measured in the presence or in the absence of ADA represents the residual effect of adenosine on the stimulatory site detected in the presence of 10 mM Mg^{2+}. The effects of 5'-deoxyadenosine and N6-dimethyladenosine were tested in the presence of ADA because they are not deaminated by this enzyme. The affinity of N6-dimethyladenosine for the stimulation of the adenylate cyclase did not change when tested either at 1 or 10 mM Mg^{2+}. The protein concentration was 0.47 mg/ml.

The IC_{50} for inhibition was in the range of 50 μM which is 10 to 100 times higher than concentrations of these adenosine derivatives giving 50% of adenylate cyclase activation (Table 1). N6-dimethyl-adenosine like other N6-derivatives (data not shown) were always activators, even at 10 mM. The inhibition produced by 2'-deoxyadenosine was not affected by adding a concentration of N6-dimethyladenosine (0.5 mM) which saturated all the adenosine receptor sites implicated in adenylate cyclase stimulation. This could indicate that the nucleoside site implicated in the adenylate cyclase stimulation is different from that implicated in the adenylate cyclase inhibition.

3—Topographical Distribution of Adenosine Stimulated Adenylate Cyclase in Rat Brain and Striatum

In the presence of ADA a highly significant stimulation of basal adenylate cyclase activity by 2-chloro-adenosine was found in the posterior cerebellar cortex, olfactory bulb, tuberculum oflactorium, striatum, nucleus accumbens and globus pallidus (Table 2). No stimulation was found in cerebral cortex, anterior and central cerebellar cortex, substantia nigra, ventral tegmental area, hippocampus and hypothalamus. Similarly the adenylate cyclase present in homogenates of guinea-pig cerebral cortex was not stimulated by 2-chloro-adenosine (data not shown). The lack of response in this brain area was observed whatever the Mg^{2+} and ADA (up to 15 IU/ml) concentrations used in the adenylate cyclase assay either on fresh and frozen tissues. The distribution of adenosine-sensitive adenylate cyclase in rat striatum is shown in Fig. 4. There was a markedly heterogeneous dis-

TABLE 2

Regional distribution of adenosine sensitive adenylate cyclase in rat brain

The different brain areas were dissected from a series of frozen slices (−7°C) and localized according to the atlas of König and Klippel (8). Results are the mean ± SEM of three or four experiments.

	Adenylate cyclase activity (pmoles/5 min/mg protein)	
	Adenosine-deaminase	Adenosine-deaminase + 2-chloro-adenosine (10 μM)
Frontal cerebral cortex	277 ± 4	262 ± 11
Parietal cerebral cortex	300 ± 13	317 ± 13
Anterior cerebellar cortex	310 ± 20	338 ± 1
Central cerebellar cortex	398 ± 15	433 ± 9
Posterior cerebellar cortex	297 ± 4	352 ± 9*
Olfactory bulb	283 ± 4	334 ± 7*
Tuberculum olfactorium	137 ± 8	213 ± 6**
Substantia nigra (A_9)	260 ± 3	264 ± 8
Ventral tegmental area (A_{10})	135 ± 0.5	129 ± 10
Hippocampus	85 ± 10	91 ± 3
Hypothalamus	290 ± 5	241 ± 5
Rostral striatum	1030 ± 25	1965 ± 55**
Caudal striatum	845 ± 30	1385 ± 50**
Nucleus accumbens	580 ± 5	1335 ± 25**
Globus pallidus	208 ± 3	400 ± 3**

* $P < 0.01$.

** $P < 0.001$ (Student's t test).

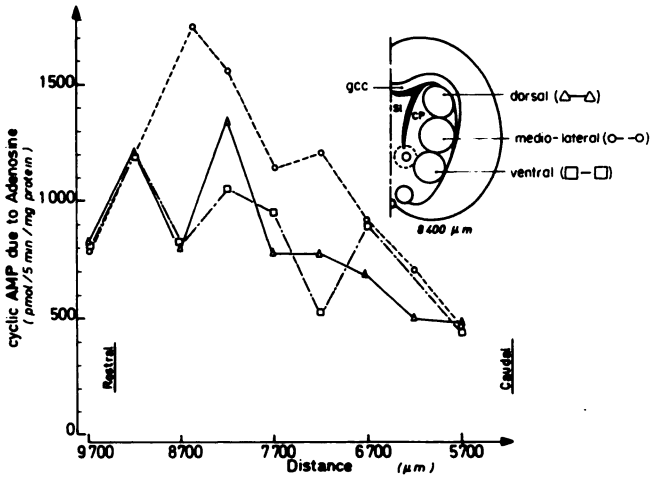

FIG. 4. *Topographic distribution of adenosine sensitive adenylate cyclase in the striatum*

The ordinate represents the difference between the amount of cyclic AMP produced in the presence and absence of 50 μM 2-chloro-adenosine. Discs were punched out on serial slices in the dorsal, mediolateral and ventral parts of the striatum. The abscissae indicated the rostrocaudal localization of discs according to the atlas of König and Klippel (8). Incubation medium (40 μl) contained between 20 and 25 μg protein. Values represent the mean of three independent experiments. For each experiment determinations were done in triplicate with an homogenate prepared from a single disc. SL: nucleus septi lateralis; cp: caudate putamen (striatum); gcc: genu corporis callosi.

tribution from the rostral to the caudal part of the striatum and also between the dorsal, mediolateral and ventral areas of the serial slices. The activity in the mediolateral discs was always the highest. We have previously shown that the topographical distribution of dopamine and serotonin-sensitive adenylate cyclases was, in rat brain, well correlated with the distribution of dopamine and serotonin-containing terminals, respectively (15). The distribution of these nerve terminals was studied either by measurement of monoamine content of the different brain structures or by measurement of specific uptake of the [^{3}H]monoamines (9). It was interesting to know whether there was a similar regional distribution of specific adenosine-sensitive adenylate cyclase and specific [^{3}H]adenosine uptake. [^{3}H]Adenosine uptake measured on synaptosomes was linear as a function of time for at least 15 min. Its apparent K_m for [^{3}H]adenosine was 9 μM, a figure which compared well with that reported in other systems (16). The specificity of the [^{3}H]adenosine uptake is shown in Table 3. The ability of adenosine and its phosphorylated derivatives to block [^{3}H]adenosine uptake decreased in the following order: adenosine, 5′ AMP, ADP and ATP. It is impossible to know if the phosphorylated derivatives were active by themselves or had to be dephosphorylated to adenosine in order to compete for the uptake process. Inosine was a very weak inhibitor of [^{3}H]adenosine uptake compared with adenosine. This was confirmed by the observation that [^{3}H]adenosine up-

TABLE 3

Chemical specificity of the [^{3}H]adenosine uptake

[^{3}H]-Adenosine uptake was performed as described in METHODS. Protein (60 μg) was added per sample. Each value is the mean of three determinations.

Compounds added	^{3}H adenosine uptake (pmoles/mg protein/10 min)	% Inhibition
None	60	0
ATP 10 μM	36	39
ADP 10 μM	30	50
5′ AMP 10 μM	21	65
Adenosine 10 μM	20	66
Inosine 10 μM	52	13
Papaverine 100 μM	9	85
ADA (1 IU/ml)	3	95
ADA (1 IU/ml) + Papaverine 100 μM	9	85

take was suppressed in the presence of ADA. Papaverine, a competitive inhibitor (17, 18) of the adenosine uptake, was indeed very effective in brain synaptosomes (Table 3). It was thus clear that we were measuring specific [^{3}H]adenosine uptake in brain synaptosomes. However, we found the same amount of [^{3}H]adenosine uptake in two areas which contained no adenosine-sensitive adenylate cyclase (cortex and hypothalamus: 65 and 77 pmoles/10 min/mg protein, respectively) and in striatum which had a high content of adenosine-sensitive adenylate cyclase (65 pmoles/10 min/mg protein). This indicates that, unfortunately, if there is an adenosine uptake in putative purinergic nerve terminals, which could be present in areas containing adenosine receptors, it is diluted by the adenosine uptake of other cells.

4—Subcellular Distribution of the Adenosine-Sensitive Adenylate Cyclase: Evidence for a GTP Requirement

Primary subfractionation of striatal homogenates showed that most of the adenosine-sensitive adenylate cyclase was present in the nuclear and the mitochondrial subfractions while the microsomal fraction had a very low content of this enzyme (Table 4). The high recovery in the nuclear pellet (1000 × *g*) was mainly due to the gentle homogenization procedure employed. Homogenization was performed by a motor driven (100 × rpm) Teflon glass homogenizer (4 strokes). More drastic homogenization resulted in a loss of adenosine stimulation. The mitochondrial fraction, containing nerve ending, was further subfractionated according to Jones and Matus (19). In the presence of GTP (5 μM), 65% of the total adenosine- and 72% of the total dopamine-sensitive adenylate cyclases which are recovered in the different fractions of the gradient were found in the F2 fraction (mainly composed of synaptic membranes) (Table 4). This fraction also contained the highest specific activity of basal, 2-chloro-adenosine and dopamine-sensitive adenylate cyclases. In this fraction enzyme stimulation by 2 chloroadenosine and dopamine were lower in the absence of GTP (15% and 39%, respectively) than in its presence (52% and 100%, respectively) (Table 4). Figure 5 shows another experiment indicating that in the F2 fraction the adenylate cyclase stimulation by 2-chloroadenosine and dopamine was apparent only in the presence of GTP. The difference in the importance of GTP requirement in the experiments reported in Table 4 and Figure 5 could be explained by a variable contamination by GTP in the F2 fraction. It is surprising that GTP is inhibitory in homogenates and stimulatory in washed particles. However, this could be due to a biphasic effect of GTP in the presence of EGTA, stimulatory at low concentrations and inhibitory at high concentrations as it has been reported by Yamamuna *et al.* (20). It is likely that in homogenates there is enough GTP to mask the stimulatory effect.

Effect of Degeneration of Presynaptic Dopaminergic Terminals on the Adenosine Sensitive Adenylate Cyclase in Striatal Homogenates

The topographical distribution of the adenosine-sensitive adenylate cyclase in striatum described above resembles that of the dopamine-sensitive adenylate cyclase and of endogenous dopamine content described previously (21). In order to test the hypothesis that the adenosine-sensitive adenylate cyclase could be on the dopaminergic terminals we destroyed them by injecting 6-hydroxydopamine into the substantia nigra. Figure 6 shows that the adenosine-sensitive adenylate cyclase was still present after complete degeneration of these fibers. Furthermore a slight increase in the maximal response was observed in the lesioned sides when compared to the response in the contralateral sides (Fig. 6).

Effect of Kainic Acid Injection on Adenosine- and Dopamine-Sensitive Adenylate Cyclases in Rat Striatum

One week after microinjection of kainic acid (14 nmoles), a rigid glutamate analogue, into rat striatum, the activities of basal, 2-chloradenosine and dopamine-sensitive adenylate cyclases, glutamate decarboxylase (GAD), choline acetyltransferase (CAT) and dopamine content (DA) were

TABLE 4

Distribution of adenosine sensitive adenylate cyclase activity in subcellular fractions of striatum

Fractionations were performed according to Jones and Matus (19) modified as follows. Initial homogenization of striatum was in 9 volumes of 0.30 M sucrose, 2 mM Tris-maleate (pH 7.2), 2 mM EGTA (pH 7.2) using a glass homogenizer with a Teflon pestle at 100 rpm (4 strokes). The crude mitochondrial pellet was lysed in 2 mM Tris-maleate (pH 7.2) for 30 min. The lysate was made up to 34% (w/w) sucrose by addition of the appropriate volume of 48% (w/w) sucrose and laid on the button of the tube. It was overlaid by two phases of 28.5% (w/w) and 10% (w/w) sucrose. Sucrose solutions were made up in 2 mM Tris-maleate (pH 7.2) 2 mM EGTA (pH 7.2). The sedimentation flotation density gradient was centrifuged at 60 000 × *g* for 110 min. F_1 was collected at 10%–28.5% interphase; F_2 at 34%–28.5% and F_3 was the pellet. The different fractions were collected, diluted in a large volume of 2 mM Tris-maleate (pH 7.2), 2 mM EGTA (pH 7.2) and centrifuged at 100,000 × *g* for 30 min and resuspended in the initial homogenization medium. The GTP 2 chloroadenosine and dopamine concentrations were 5 μM, 50 μM and 100 μM, respectively. Each value is a mean of triplicate determinations obtained within a single experiment. Two other experiments gave the same distribution.

	Protein (mg)	Adenylate cyclase activities (pmoles cyclic AMP/5 min/mg protein)						Total activities (% of homogenate)			
		Basal		+ 2-chloro-adenosine		+ dopamine		2-chloroadenosine sensitive AC		Dopamine sensitive AC	
		−GTP	+GTP	−GTP	+GTP	−GTP	+GTP	−GTP	+GTP	−GTP	+GTP
A) *Primary subfractions*											
Starting homogenate	37.4	640	466	1307	829	1528	1100	100	100	100	100
P1 (1000 × g pellet) nuclear	14.6	692	570	1367	1042	1546	1371	39	50	37	49
S1 (1000 × g supernatant)	24.7	323	295	815	535	1025	730	48	43	52	45
P2 (10 000 × g pellet) (mitochondrial)	13.2	433	553	913	961	1012	1250	25	39	22.3	38.3
S2 (10 000 × g supernatant) microsomal	10.5	76	81	135	134	148	140	2.4	4.3	2.0	2.2
B) *Subfractions of P2*											
F1 (myelin)	1.14	52	176	90	235	68	325	.18	.5	.45	.68
F2 (mainly synaptic plasma membranes)	4.02	832	1082	959	1646	1158	2175	2.0	16.4	3.8	18.0
F3 (mainly mitochondria)	4.56	286	341	638	603	639	694	6.2	8.6	4.7	6.5
Total Recovery (%)	93							49.8	79.8	48.0	76.4

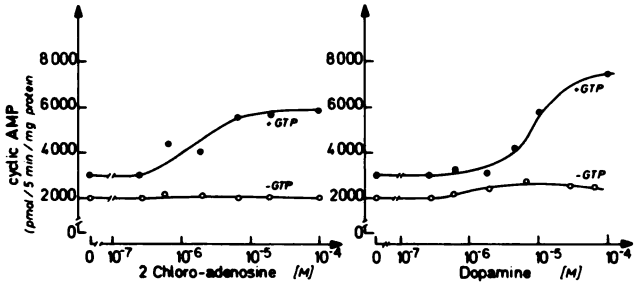

FIG. 5. *GTP requirement for adenylate cyclase stimulation by 2-chloroadenosine and dopamine in partially purified synaptic membranes*

F2 fraction was prepared as described in Table 4. The GTP and protein concentrations were 5 μM and 0.54 mg/ml, respectively. Each value is the mean of triplicate determinations of a single experiment. Two other experiments have given the same results.

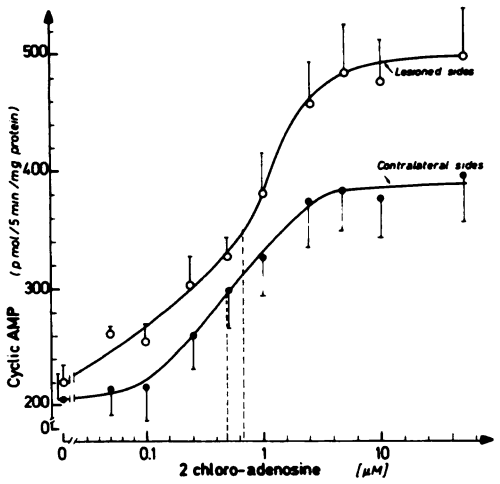

FIG. 6. *Effect of presynaptic dopaminergic terminal lesions on the adenosine sensitive adenylate cyclase of rat striatum*

6-OHDA (8 μg in 4 μl) was injected into right substantia nigra. The experiment was done three weeks later. The dopamine concentrations were 79.5 ± 4.8 and 2.1 ± 0.8 ng/mg protein in the contralateral and lesioned sides, respectively. The values are the mean ± SEM of three experiments determined in triplicate.

measured in intact and injected striata. Basal and dopamine-sensitive adenylate cyclases were also measured in the corresponding substantia nigra. The content of dopamine in control ipsilateral and contralateral striata were similar indicating that dopaminergic nerve terminals were not affected by this treatment (Table 5). The activity of GAD and CAT, enzymes contained mainly in neurons whose cell bodies are located within the striatum (22), were decreased by 95% and 93%, respectively. In substantia nigra, the activity of GAD decreased only by 34%. The greatest effect of kainic acid treatment on GAD and CAT activities observed in our experiments compared to the results of other authors (60–80% decrease) was not due to use of the discrete area of striatum where the microinjections were done (see legend to Table 5) since a control experiment in which the entire striatum was used gave the same results.

These results are in agreement with the general conclusion, drawn from experiments similar to those described above and cytological analysis, that kainic acid destroys neurons whose cell bodies are in the striatum, sparing axons and terminals of extrinsic neurons while glial cells undergo some proliferation (23, 24). The observation that dopamine sensitive adenylate cyclase disappeared in the substantia nigra of the ipsilateral side (Table 5) while the basal adenylate cyclase in this structure was unchanged, is in accordance with the previous report by Schwartz *et al.* (25) and further strengthened the proposition made by several authors that this adenylate cyclase is on terminals of neurons whose cell bodies are located in striatum (26–28). Kainic acid injection (14 nmoles) resulted in a dramatic decrease in basal adenylate cyclase activity by 94% and 92% when compared to the control and contralateral activities, respectively (Table 5). The increases in cyclic AMP production due to 2-chloroadenosine and dopamine were reduced by 99% by kainic treatment. However, there was a

TABLE 5

Effect of kainic acid injection into striatum on adenosine and dopamine adenylate cyclase

14 nmoles of kainic acid in 0.5 μl saline buffer with 10 mM Tris-base (final pH: 7.3) were injected into rat striatum (1.5 μl/min); coordinates were: 1.5 mm anterior to the bregma, 2.7 mm apart from the midline, 6 mm under the skull surface. These coordinates correspond to the 8800 μm slice according to the atlas of König and Klippel (8). One week later the brain was dissected, frozen to −7°C and cut into serial of frontal slices (500 μm thickness). Two slices adjacent to the injected site were selected. Eight discs (1.4 mm diameter) were punched out of them. One disc was used to determine dopamine content. The others were homogenized in 200 μl of a Tris-maleate 2 mM pH 7.2; EGTA 2 mM and 300 mM sucrose solution. This sample was used to determine adenylate cyclase, GAD and CAT activities. Substantia nigra was dissected and homogenized in 100 μl of same medium. The specific activity of GAD was 282 ± 13 nmoles/mg protein/hour and 870 ± 43 nmoles/mg protein/hour in control striata and substantia nigra, respectively. The specific activity of CAT in control striata was 122 ± 4 nmoles/mg protein/hour. The dopamine content of control striata was 89 ± 11 ng/mg protein.

Treatment	Adenylate cyclase activites (pmoles/5 min/mg protein)			GAD	CAT	DA
	Basal	+ DA (100 μM)	2-chloroadenosine (50 μM)	% of Control		
1) *Control*						
Striatum (n = 4)	1760 ± 85	3777 ± 244**	3058 ± 170**	100	100	100
Substantia nigra	176 ± 12	311 ± 17**		100		
2) *Kainic acid injection*						
Ipsilateral						
Striatum (n = 6)	105 ± 5	125 ± 5*	123 ± 4*	5 ± 1	6 ± 1	82.5 ± 6
Subst. nigra (n = 4)	133 ± 10	169 ± 17		66 ± 6		
Contralateral						
Striatum (n = 6)	1337 ± 56	3070 ± 128**	2378 ± 89**	110 ± 10	94 ± 4	111 ± 5
Subst. nigra (n = 4)	126 ± 5	250 ± 10**		115 ± 9		

*, $p < 0.5$, ** $p < 0.001$, when compared to basal adenylate cyclase activity.

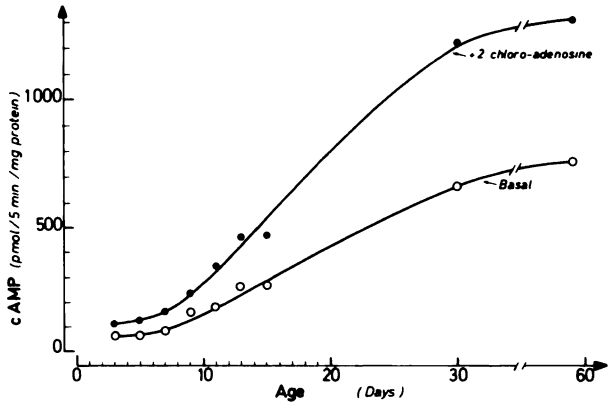

FIG. 7. *Ontogenic development of adenosine-sensitive adenylate cyclase in rat striatum*

The basal and adenosine-sensitive adenylate cyclase activities were determined on striatal homogenates of rats of different ages during the same experiment. Each value is a mean of triplicate determinations.

slight but significant stimulation of the basal adenylate cyclase activity by 2-chloroadenosine (17%) and by dopamine (19%) in the treated striata (Table 5). Injection of 6, 8, 10 and 12 nmoles kainic acid gave similar results (data not shown).

Ontogenic Development of the Adenosine-Sensitive Adenylate Cyclase in Rat Striatal Homogenates

Figure 7 shows that the stimulation of the basal adenylate cyclase by 2-chloro-

adenosine was already present at birth. The percent stimulation did not change during development. Thus the adenylate cyclase activity measured in the presence of 2 chloroadenosine followed a pattern of development similar to that of basal adenylate cyclase.

DISCUSSION

These results confirm and extend the previous report on the presence of an adenosine-sensitive adenylate cyclase in striatal homogenates (7). The chemical specificity of the adenosine receptor coupled with this adenylate cyclase in brain is very similar to the adenosine receptors involved in adenylate cyclase stimulation in peripheral systems such as Leydig cells (29, 30) and platelets (31) but also with adenosine receptor involved in the inhibition of lipolysis induced by catecholamine in fat cells (32). The most important characteristics are as follow: 1) When tested in the presence of ADA, phosphorylated derivatives of adenosine were inactive (Table 1) (30). 2) A nitrogen atom in the N6 position is absolutely required (29, 32–34); thus inosine and 6-mercapto-purine-riboside were ineffective (Table 2). 3) Large substitution in the N6 position resulted in active compounds (Table 1). Therefore, N6-phenyl-isopropyladenosine is a potent agonist in stimulating the adenosine receptor coupled with adenylate cyclase and on the fat cell system (32). 4) 2-Chloroadenosine is always active (29–32). 5) Theophylline is a competitive inhibitor of the adenylate cyclase stimulation by adenosine and on the fat cell system.

There is some uncertainty concerning the action of analogues modified on the ribose moiety. 2′ and 3′-Deoxyadenosine have been reported to be active on an adenylate cyclase of Leydig cells (29). However, it is difficult to eliminate the possibility of a contamination by adenosine since these compounds cannot be tested in the presence of ADA. 5′-Deoxyadenosine was found to be a partial agonist in our system (Table 1). Furthermore, the analysis of these ribose modified analogues is complicated by their inhibitory effects at concentrations above 10 μM. In striatal homogenates there is also another "adenosine receptor" which triggers an inhibition of the adenylate cyclase. This "inhibitory adenosine receptor" is likely to be different from the stimulatory one since: 1) its affinity for adenosine (50 μM) was 100-fold lower, 2) N6 derivatives such as N6 dimethyladenosine and N6 phenylisopropyladenosine (data not shown) are only stimulatory, 3) N6-dimethyladenosine did not modify the inhibition by 2′-deoxyadenosine (Fig. 3). Therefore the chemical specificities of the "stimulatory" and "inhibitory" adenosine receptor sites in rat striatal homogenates are very much like those described in peripheral tissues (29). A more detailed experimentation is necessary to clarify the mechanism of this inhibition.

A detailed analysis of the interaction of ATP and Mg^{2+} with any hormone-sensitive adenylate cyclase is complex. However, some general characteristics have arisen which are also found for the adenosine-sensitive adenylate cyclase in striatal homogenates. Adenosine increased the reaction velocity without altering the affinity of the enzyme for its substrate Mg-ATP (Fig. 1). In the presence of 4 mM Mg^{2+}, ATP concentrations above 0.5 mM are inhibitory on the basal adenylate cyclase. This is possibly due to two distinct factors first, an increase in free ATP known to be a competitive inhibitor with respect to Mg-ATP and, second, ATP could chelate a significant fraction of the added Mg^{2+}, thus lowering the concentration of free Mg^{2+} also known to act at an apparent stimulatory allosteric site (35, 36). Mg^{2+} increased the adenylate cyclase activity over a range of concentrations far in excess of those required to complex with most of the 10 μM added ATP (Fig. 2). Adenosine and dopamine increased the apparent affinity of the enzyme for the Mg^{2+} either by changing the characteristics of activation by free Mg^{2+} or by lowering the affinity for free ATP. Indeed, the enzyme was less susceptible to the inhibition by high ATP concentration in the presence of 2-chloroadenosine than in its absence (Fig. 1). Histamine and dopamine-sensitive adenylate cyclases in rat brain have been found to be mainly localized on synaptic membranes (36, 37). In this study, subcel-

lular distribution of adenosine-sensitive adenylate cyclase was found to be very similar to that of dopamine-sensitive adenylate cyclase, suggesting that this enzyme is also localized on synaptic membranes. An interesting observation is that, as for other hormone (38) or neurotransmitters sensitive adenylate cyclases (36, 37), GTP is required to obtain an optimal stimulation of the basal adenylate cyclase by adenosine in synaptic membranes (Table 4 and Fig. 5).

The most important discrepancy between the results obtained in brain slices as first reported by Sattin and Rall (39) and in brain homogenates is that in some areas such as cerebral cortex, there was no adenylate cyclase stimulation by adenosine whatever the adenylate cyclase assay conditions so far tested (Table 2). At least two hypotheses can be proposed to explain these different results: 1) we have not yet found the right conditions to measure the adenylate cyclase stimulation in cerebral cortex homogenates; 2) the cyclic AMP accumulation due to adenosine in brain slices is not due to a direct activation of an adenylate cyclase. Indeed it has been found by Minneman *et al.* (24) that after treatment of striatum with kainic acid the adenosine-induced cyclic AMP production in striatal slices was unchanged. Since we found that cyclic AMP production due to adenosine in striatal homogenates was reduced by 99% after kainic acid treatment, it seems clear that there is another way for adenosine to stimulate cyclic-AMP accumulation in brain slices than a direct stimulation of the adenylate cyclase. In addition to an effect on cyclic AMP accumulation adenosine has also been shown to modulate the release of acetylcholine (40) and noradrenaline (41) in peripheral nerve terminals. One cannot rule out the possibility that in brain there is another adenosine receptor not coupled with an adenylate cyclase, which is widely distributed and able to modify the release of unknown compounds that themselves are able to modulate cyclic AMP concentrations. The topographical distribution of the adenosine-sensitive adenylate cyclase in rat striatum (Fig. 4) resembles that of both dopamine-sensitive adenylate cyclase and dopamine content (12). This could suggest a functional relationship between these two neuromodulators. However, lesions of dopaminergic terminals in rat striatum by injection of 6-OHDA in substantia nigra did not destroy adenosine-sensitive adenylate cyclase. Kainic acid injection in rat striatum is known to destroy neurons whose cell bodies are localized in this brain area leaving intact nerve terminals or axons of extrinsic neurons and glial cells (22, 23, 42, 43). Since basal, dopamine and adenosine-sensitive adenylate cyclases are reduced by more than 90% by this treatment it can be concluded that these enzymes are mostly located on striatal interneurons or on cells bodies of striatal neurons projecting out of this structure. Consequently the glial population of the striatum contains a poorly active adenylate cyclase.

In conclusion, adenosine interacts with a specific receptor coupled with an adenylate cyclase of neurons of certain brain areas with characteristics similar to those of others neurotransmitter-sensitive adenylate cyclases.

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