STIMULATION BY ATP OF [^{3}H]DOPAMINE BINDING TO SYNAPTIC MEMBRANE FRACTIONS OF CANINE CAUDATE NUCLEUS

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SUMMARY : The rate of [^{3}H]dopamine binding to crude synaptic membranes from canine caudate nucleus was considerably increased by 2 mM ATP, 5'-adenylyl-imidodiphosphate and GTP or by 1 mM 5'-guanylyl-imidodiphosphate, while strongly inhibited by 2 mM ADP and GDP. Half maximal concentrations of [^{3}H]dopamine to bind to the membranes were 1.11 x 10^{-7} M and 8.75 x 10^{-6} M in the absence of 4 mM ATP, indicating a negative cooperativity of the dopamine receptor, and 9.25 x 10^{-7} M in its presence. Hill coefficient was increased from 0.70 to 1.04 by addition of 4 mM ATP. The optimal concentration of ATP for [^{3}H]dopamine binding was in the range of 0.5 to 5 mM.

INTRODUCTION : In mammalian caudate nucleus rich in dopaminergic neuronal terminals, dopamine has been demonstrated to function as a neurotransmitter in association with the postsynaptic receptor[1-5]. Although neuroleptics inhibit both dopamine-stimulated activity of adenylate cyclase and [^{3}H]-dopamine binding to brain membranes in fairly good parallel correlation, some discrepancies exist as to whether the dopamine binding subunit of adenylate cyclase is identical to [^{3}H]dopamine binding protein[6-8]. For example, chlorpromazine is more active in inhibition of dopamine-stimulated activity of adenylate cyclase than haloperidol, whereas the relation in the inhibition of [^{3}H]dopamine binding between the two drugs is reverse[6,7,9,10]. The discrepancies may be attributable at least partly to the difference in incubation conditions, mainly a lack of addition of ATP to the [^{3}H]dopamine binding assay system. The present report will describe a stimulation by ATP of [^{3}H]dopamine binding to brain synaptic membranes and a negative cooperativity of the dopamine receptor.

MATERIALS AND METHODS : [^{3}H]Dopamine(ethyl-1-^{3}H(N)) was purchased from New England Nuclear. ATP, GTP, AMPPNP, GMPPNP, ADP, GDP, AMP, GMP, adenosine, guanosine and cyclic AMP were obtained from Boehringer-Yamanouchi. Cyclic GMP, dopamine hydrochloride and Whatman GF/B filter papers were from Yamasa Shoyu, Sigma Chemical and W&R Balston, respectively. Perphenazine,

The abbreviations used are : cyclic AMP, adenosine 3':5'-monophosphate; cyclic GMP, guanosine 3':5'-monophosphate; AMPPNP, 5'-adenylyl-imido-diphosphate; GMPPNP, 5'-guanylyl-imidodiphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.

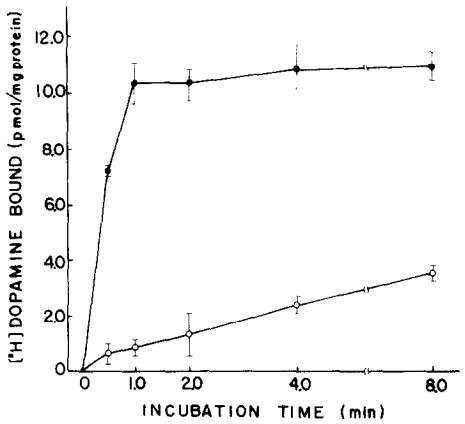

Fig. 1 : Time course of [^{3}H]dopamine binding to M_1 membranes in the presence (•) and absence (o) of 4 mM ATP. The incubation condition in the presence of 2.5 x 10^{-6} M [^{3}H]dopamine and the procedure for assaying the binding of [^{3}H]dopamine are described under "MATERIALS AND METHODS". Vertical bars indicate the standard error of three experiments.

methylperidol and haloperidol were from Schering, Cilag-Chemie and Dainihon, respectively. Chlorpromazine and clozapine were prepared in our laboratories.

Crude synaptic membranes were prepared from caudate nucleus of dogs according to DeRobertis et al[11]. Freshly isolated caudate nucleus was homogenized in 25 volumes of 0.32 M sucrose containing 2 mM EGTA and 2 mM Tris-maleate buffer, pH 7.4 in a Teflon homogenizer at 900 rev/min for 2 min. The homogenate was centrifuged at 900 g for 10 min and the supernatant was further centrifuged at 11,500 g for 20 min. After washing once with the sucrose-Tris buffer described above, the pellet was subjected to hypotonic exposure by homogenizing in 0.032 M sucrose containing 2 mM EGTA and 2 mM Tris-maleate buffer pH 7.4 for 2 min. The suspension was centrifuged at 20,000 g for 30 min and the resulting pellet, referred to as M_1, was stored at -80° until use.

The binding of [^{3}H]dopamine was measured as follows. The incubation mixture with a total volume of 2.5 ml contained 10 mM theophylline, 16 mM $MgSO_4$, 0.6 mM EGTA, an indicated amount of [^{3}H]dopamine, 80 mM Tris-maleate buffer, pH 7.4 and about 250 μg protein of M_1 membranes. After 20 min preincubation at 0°, the binding reaction was initiated by raising rapidly the temperature to 30° with concomitant addition of 4 mM ATP, carried out for 4 min and terminated by filtering through a Whatman GF/B filter paper, followed by washing quickly twice with 15 ml of cold 50 mM Tris-maleate buffer, pH 7.7. The washed filter paper was dried at 70° for 1 hr and the radioactivity was determined in 10 ml of Bray's solution[12] in a Packard liquid scintillation counter, Model 3390. The binding of [^{3}H]dopamine to M_1 in the presence of 10^{-3} M nonradioactive dopamine was referred to as nonspecific binding of [^{3}H]dopamine. Thus, the membrane-bound radioactivities of [^{3}H]dopamine shown in the present results were the excess over the nonspecifically bound radioactivities. Under the standard assay conditions in the presence of 2.5 x 10^{-6} M [^{3}H]dopamine, the specifically bound radioactivity ranged from 47 to 59% of the total bound radioactivity. The specifically bound radioactive compound was verified to be dopamine as follows. The bound radioactivity (4.19 x 10^5 dpm) was extracted from the filter paper with ethanol containing 0.05% nonradioactive dopamine and 1%

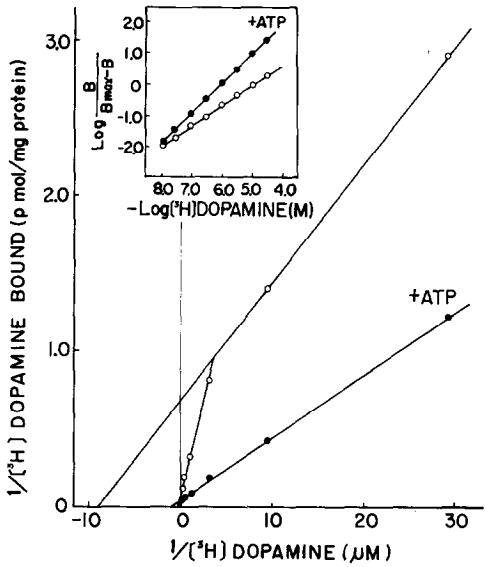

Fig. 2 : Liveweaver-Burk plot of [^{3}H]dopamine binding to M_1 membranes in the presence (●) and absence (o) of 4 mM ATP. The incubation condition and the procedure for assaying [^{3}H]dopamine binding are described under "MATERIALS AND METHODS". Results are the average of three experiments. The inset indicates the Hill plot of [^{3}H]dopamine binding to M_1 membrane fractions. B represents the amount of bound [^{3}H]dopamine at various concentrations of added [^{3}H]dopamine. Bmax represents the maximal amount of [^{3}H]dopamine bound to membranes and was derived by the Lineweaver-Burk plot. The values of Bmax were 16.1 pmol/mg protein in the absence of 4 mM ATP and 20.8 pmol/mg protein in its presence.

ascorbic acid. After concentration under reduced pressure in N_2 gas, the residue was subjected to thin layer chromatography in the solvent system of n-butanol:ethanol:1 M acetic acid (3:3:1). Only one radioactive spot was found at the dopamine position with a Rf value of 0.40. The activity of dopamine-sensitive adenylate cyclase of M_1 was determined according to Clement-Cormier et al[11] except for utilization of 4 mM ATP instead of 0.5 mM. The reaction was carried out for 4 min at 30°. Protein was determined by the method of Lowry et al[13], with bovine serum albumin as a standard.

RESULTS : In a complete system, the rate of [^{3}H]dopamine binding to M_1 membranes was very rapid and the amount of bound [^{3}H]dopamine reached a plateau level within 60 sec. as illustrated in Fig. 1. When ATP was omitted from the incubation mixture, the binding rate diminished greatly and, even 8 min after the onset of reaction, the amount of bound [^{3}H]dopamine was only less than one third of the saturated level which reached in the presence of ATP. GTP at 10 μM concentration, which was generally used for the assay of dopamine-sensitive adenylate cyclase[11], did not influence

Table 1 : Inhibition by neuroleptics of [^{3}H]dopamine binding and dopamine-sensitive adenylate cyclase. The binding of [^{3}H]dopamine at 5 x 10^{-7} M in the presence of 4 mM ATP and the dopamine-stimulated activity of adenylate cyclase were determined as described under "MATERIALS AND METHODS". Apparent inhibition constant Ki was obtained by the equation, $Ki=IC_{50}/(1+C/Ka)$, where C is the concentration of [^{3}H]dopamine in the binding assay or of dopamine in the assay of adenylate cyclase. Ka represents half the concentration of [^{3}H]dopamine (1.45 x 10^{-6} M) to give the maximal binding to M_1 membranes or of dopamine (2.5 x 10^{-6} M) to give the maximal stimulation of the adenylate cyclase. IC_{50} represents the concentration of neuroleptics to reduce by 50% the binding of [^{3}H]dopamine at 5 x 10^{-7} M or the adenylate cyclase activity which was stimulated by 4 x 10^{-5} M dopamine.

Neuroleptics	Ki(M)	
	[^{3}H]Dopamine binding	Dopamine-sensitive adenylate cyclase
Clozapine	6.96 x 10^{-7}	3.18 x 10^{-7}
Chlorpromazine	5.26 x 10^{-7}	3.29 x 10^{-8}
Perphenazine	1.48 x 10^{-7}	1.77 x 10^{-7}
Methylperidol	7.56 x 10^{-8}	6.12 x 10^{-8}
Haloperidol	2.30 x 10^{-8}	2.47 x 10^{-8}

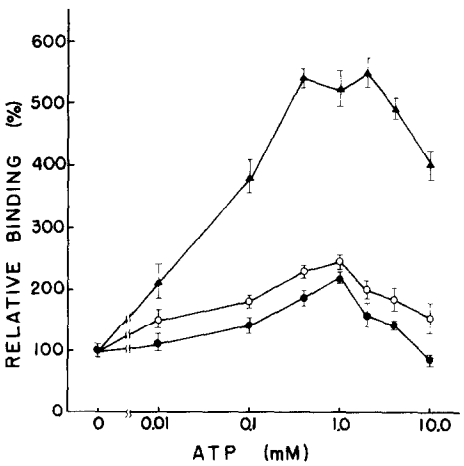

Fig. 3 : Effects of varying concentrations of ATP on [^{3}H]dopamine binding to M_1 membranes. The incubation condition and the procedure for assaying [^{3}H]dopamine binding are described under "MATERIALS AND METHODS". The amounts of bound [^{3}H]dopamine in the presence of 4.50 x 10^{-9} M (●), 1.05 x 10^{-7} M (o) and 1.00 x 10^{-6} M (▲) [^{3}H]dopamine in the incubation mixtures were respectively, (6.67 ± 0.668) x 10^{-2}, 1.11 ± 0.099 and 2.21 ± 0.786 pmol/mg protein in the control experiment lacking ATP. Vertical bars indicate the standard error of three experiments.

[^{3}H]dopamine binding. When the reciprocal of [^{3}H]dopamine binding to M_1 was plotted as a function of the reciprocal of dopamine concentration according to the Lineweaver-Burk plot in the absence of 4 mM ATP, the receptor exhibited a negative cooperativity with two association constants (Ka), 1.11 x 10^{-7} M and 8.75 x 10^{-6} M, as illustrated in Fig. 2. In the presence of ATP, only one Ka value (9.25 x 10^{-7} M) was observed. The total capacity of binding [^{3}H]dopamine (Bmax) was slightly altered from 16.1 to 20.8 pmol/mg protein by addition of ATP. The inset in Fig. 2 indicates an increase of the Hill coefficient from 0.70 to 1.04 by addition of 4 mM ATP.

Under the standard assay conditions, the binding of [^{3}H]dopamine at 5 x 10^{-7} M was proportional to the amount of M_1 up to 500 μg protein. Several neuroleptics tested exhibited a strong inhibition of [^{3}H]dopamine binding to M_1 (Table 1). Similar inhibition by the neuroleptics was observed with dopamine-stimulated activities of adenylate cyclase except for chlorpromazine, in agreement with the reports by others[5,6,8], which was 16 times less active for the binding of [^{3}H]dopamine than for the adenylate cyclase. The optimal concentration of ATP to stimulate [^{3}H] dopamine binding was 0.5 to 5 mM, as shown in Fig. 3. The effects of

Table 2 : Effect of various nucleosides and nucleotides on [^{3}H]dopamine binding to M_1 membranes. The incubation condition and the procedure for assaying the binding of [^{3}H]dopamine are described under "MATERIALS AND METHODS". The concentration of nucleosides and nucleotides was 2 mM except for GMPPNP whose concentration was 1 mM. The results are the average ± standard error in three experiments. The absolute amounts of bound dopamine at three different concentrations of [^{3}H]dopamine, 4.57 x 10^{-9}, 1.05 x 10^{-7} and 1.00 x 10^{-6} M in the absence of the test compounds were (7.07 ± 0.443) x 10^{-2}, (8.03 ± 0.699) x 10^{-1} and 2.38 ± 0.314 pmol/mg protein, respectively.

Compound	Relative binding (%)		
	[^{3}H]Dopamine concentration		
	4.57 x 10^{-9} M	1.05 x 10^{-7} M	1.00 x 10^{-6} M
None	100 ± 6.0	100 ± 8.7	100 ± 13.2
ATP	170 ± 8.6	348 ± 15.8	618 ± 69.3
AMPPNP	153 ± 10.3	288 ± 10.2	218 ± 9.2
ADP	87 ± 2.7	61 ± 10.5	25 ± 7.7
AMP	97 ± 6.4	106 ± 1.0	102 ± 7.7
Adenosine	102 ± 4.0	95 ± 4.3	77 ± 12.3
Cyclic AMP	97 ± 5.2	97 ± 10.9	91 ± 14.2
GTP	131 ± 0.2	240 ± 24.5	329 ± 85.7
GMPPNP	86 ± 4.3	118 ± 11.2	183 ± 19.1
GDP	64 ± 5.0	95 ± 12.9	41 ± 2.7
GMP	75 ± 4.0	76 ± 14.0	94 ± 13.8
Guanosine	69 ± 5.7	88 ± 7.6	76 ± 17.1
Cyclic GMP	153 ± 9.5	167 ± 10.8	218 ± 25.5

other nucleotides and nucleosides were compared with those of ATP at 2 mM, except for GMPPNP whose concentration was 1 mM, (Table 2). AMPPNP, GTP, GMPPNP and cyclic GMP increased [^{3}H]dopamine binding although less considerably than ATP, whereas ADP and GDP lowered dopamine binding. The effects of those nucleotides were noticeably marked at higher concentrations of dopamine regardless the stimulation or inhibition.

DISCUSSION : The present studies demonstrate that ATP elevated the rate of dopamine binding to the receptor protein in the postsynaptic membranes without substantial alteration of the total binding capacity (Fig. 2). Since either AMPPNP, GTP or cyclic AMP in addition to ATP increased the binding of [^{3}H]dopamine, it is very unlikely that the stimulatory activity of ATP involves the hydrolysis of itself or the phosphorylation of protein. The possibility that the specific binding of [^{3}H]dopamine to M_1 under the present conditions may be due to the coprecipitation of membranes with complexes of ATP, dopamine and a trace of heavy metal can be ruled out by the following facts. First, the boiled preparation of M_1 did not bind [^{3}H]dopamine. Second, the binding of [^{3}H]dopamine was highly selective to synaptic membrane fractions rich in dopamine receptors of the caudate nucleus, whereas M_1 fractions from canine cerebellum which lacks dopaminergic neurons exhibited little binding of [^{3}H]dopamine under the same conditions (Data are not shown). Third, the binding of [^{3}H]dopamine was rather specifically reduced by nonradioactive dopamine than by norepinephrine. Fourth, the neuroleptics at the concentration less than a micromolar level inhibited markedly the binding of [^{3}H]dopamine.

It has been reported[5,6,8] that several neuroleptics inhibited competitively the stimulation of the adenylate cyclase by dopamine as well as [^{3}H]dopamine binding to brain membranes in a fairly good parallel relation, except for some neuroleptics such as chlorpromazine by which the inhibition of [^{3}H]dopamine binding was much less than that of dopamine-sensitive adenylate cyclase. Such difference in inhibitions of the two activities by certain neuroleptics can not be ascribed to the difference of the incubation conditions, since our experiments demonstrated that under the identical conditions chlorpromazine inhibited dopamine-sensitive adenylate cyclase 16 times as greatly as [^{3}H]dopamine binding. Accordingly, it appears premature to conclude that the regulatory subunit of adenylate cyclase is identical to [^{3}H]dopamine receptor.

Although neurotransmitters were reported by some workers[14-17] to be released along with ATP from nerve endings by a process of exocytosis,

the physiological significance of released ATP has yet been unclear. However, the present results may suggest that the role of released ATP is to elevate the rate of dopamine binding to the receptor protein.

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