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DOPAMINE RECEPTORS AND DOPAMINE-SENSITIVE ADENYLATE CYCLASE IN CANINE CAUDATE NUCLEUS

CHARACTERIZATION AND SOLUBILIZATION

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Abstract—The activities of dopamine-sensitive adenylate cyclase and [^{3}H]dopamine binding showed a similar subcellular distribution in the canine caudate nucleus, and were present primarily in the synaptic membrane fractions. Binding of [^{3}H]dopamine to the crude synaptic membranes was rapid, saturable and reversible in the presence of 2 mM ATP with a rate constant of 2.5×10^5 M^{-1} min^{-1} and 0.63 min^{-1} for the forward and reverse reactions respectively. The equilibrium dissociation constant (K_d) for the binding was about 1.5 μM, almost identical to the K_a of adenylate cyclase for dopamine to stimulate half-maximally. Binding of [^{3}H]dopamine in the absence of ATP exhibited a negative cooperativity, with two K_d values (0.11 μM and 8.1 μM), which was abolished by addition of 2 mM ATP. The major role of ATP appears to enhance the association of [^{3}H]dopamine to the membranes. [^{3}H]Dopamine binding in the presence of 2 mM ATP and dopamine-sensitive adenylate cyclase in the membranes were affected in a similar manner by catecholamines, some antidepressants and a variety of neuroleptics, with the exception of some phenothiazine derivatives such as chlorpromazine and fluphenazine. Propranolol and cocaine, an uptake inhibitor at neuronal membranes, did not inhibit either activity. Dopamine sensitivity of the particulate adenylate cyclase tended to be increased by the addition of 0.001% Lubrol PX in the incubation but was greatly impaired by more than 0.005% Lubrol PX. The synaptic membranes were solubilized with 2% Lubrol PX in the presence of NaF from the particulate fractions. This solubilization procedure preserved well not only fluoride sensitivity but also dopamine responsiveness of adenylate cyclase in the supernatant fluid.

Multiple classes of dopamine receptors have recently been demonstrated in the mammalian central nervous system [1–6]. Kebabian [3] has proposed two classes of dopamine mechanisms according to their association with, or independence of, dopamine-sensitive adenylate cyclase (EC 4.6.1.1). Meanwhile, attempts to label dopamine receptors with [^{3}H]dopamine have been made by several investigators [1, 2, 7–11], and the affinity of [^{3}H]dopamine to the receptor sites in their preparations has been found to be far greater than the ability of dopamine to stimulate dopamine-sensitive adenylate cyclase [12, 13]. This suggests that the [^{3}H]dopamine-binding component is distinct from the dopamine receptor unit of dopamine-sensitive adenylate cyclase. On the other hand, our previous brief report [14] demonstrated that binding of [^{3}H]dopamine to the synaptic membranes in canine caudate nucleus is increased allosterically at least several-fold by a concentration of ATP such as 2 mM, and that under our experimental conditions the dissociation constant (K_d) for dopamine in binding is almost identical to the K_a, the concentration of dopamine resulting in half-maximal stimulation of dopamine-sensitive adenylate cyclase. The present paper provides further evidence for a close correlation of [^{3}H]dopamine binding with dopamine stimulation of adenylate cyclase in canine caudate nucleus.

Our interests have also been directed to an elucidation of the mechanism for functional association of adenylate cyclase with dopamine receptors. One means of study is to solubilize dopamine-sensitive adenylate cyclase in order to dissociate it into unit components, and to reconstitute a dopamine-sensitive form from its unit components. Considerable effort has been made by a number of workers to solubilize the enzyme complex in a hormone-sensitive form from a variety of tissues, using non-ionic detergents; only a few groups have reported successful solubilization [15–18]. Some of these reports [15–17] appear to be ambiguous in their conclusions or in the reproducibility of the experimental results. In some papers an effort has been made to determine the enzyme activity in the absence of carried-in detergents [15–17] in order to obtain the hormone sensitivity of solubilized preparations. On the other hand, the Lubrol PX-solubilized adenylate cyclase of porcine kidney displays greater calcitonin sensitivity, in the presence of the carried-in detergent in the incubation, than does the membrane preparation [15]. Levey [16, 17] has demonstrated the importance of certain phospholipids for epinephrine and glucagon sensitivity of Lubrol PX-solubilized heart preparations, while Neer [18] has reported vasopressin sensitivity of Lubrol PX-solubilized preparations from the rat kidney in the absence of added phospholipids. Very recently, Drummond and Dunham [19] reported the inability of phospholipids to cause hormone sensitivity of Lubrol PX-solubilized heart preparations, in contrast to the reports by Levey [16, 17]. Thus, all the results so far reported by these workers are different and confusing, perhaps due to differences in tissues, animals and incubation conditions. The present paper also describes solubilization of dopamine-sensitive adenylate cyclase with Lubrol PX in a dopamine-sensitive form.

MATERIALS AND METHODS

Materials. Dopamine hydrochloride, pargyline hydrochloride, (−)isoproterenol hydrochloride, Lubrol PX and theophylline were purchased from Sigma, St. Louis, MO, U.S.A. ATP, GTP, Gpp(NH)p,* cyclic AMP and cyclic AMP phosphodiesterase were obtained from Boehringer-Mannheim/Yamanouchi, Tokyo, Japan. Imipramine, desmethylimipramine and sulpirirde were from Fujisawa, Osaka, Japan. [Ethyl-^{3}H(N)]dopamine (8.96 to 21.4 Ci/mmole) and [8-^{3}H]cyclic AMP (27.6 Ci/mmole) were from New England Nuclear, Boston, MA, U.S.A. and the Radiochemical Centre, Amersham, U.K. respectively. The following materials were also commercially obtained: perphenazine (Schering, Bloomfield, IL, U.S.A.), haloperidol (Dainihon, Osaka, Japan), methylperidol (Cilag-Chemie, Schaffhausen, Switzerland), (−)norepinephrine (Nakarai, Kyoto, Japan), (−)epinephrine (Tokyo Kasei, Tokyo, Japan), fluphenazine (Yoshitomi, Osaka, Japan), promethazine (Shionogi, Osaka, Japan), cocaine (Tanabe, Osaka, Japan), reserpine (Yamanouchi, Tokyo, Japan), and Whatman GF/B filter (Whatman, Kent, U.K.). Chlorpromazine, (±)propranolol hydrochloride, and clozapine were prepared in our laboratories.

Membrane preparations. Subcellular fractionation of the canine caudate nucleus was performed at 4° according to DeRobertis *et al.* [20]. Freshly isolated canine caudate nucleus was minced with scissors and homogenized in a Teflon homogenizer at 900 rev/min for 2 min in 25 vol. of 2 mM Tris–maleate buffer, pH 7.4, containing 2 mM EGTA and 0.32 M sucrose. The homogenate was centrifuged at 900 *g* for 10 min to obtain the nuclear fraction and the supernatant fluid was further centrifuged at 11,500 *g* for 20 min to obtain the mitochondrial fraction. The post-mitochondrial fraction was then centrifuged at 105,000 *g* for 60 min to obtain the microsomes and cytosol. After washing once with the Tris–sucrose buffer, the crude mitochondrial pellet was subjected to hypotonic exposure by homogenizing in 0.032 M sucrose containing 2 mM Tris–maleate buffer, pH 7.4. The suspension was centrifuged at 20,000 *g* for 30 min and M_1 (precipitable by this centrifugation), M_2 (the post-M_1 precipitable at 105,000 *g* for 60 min) and M_3 (the soluble fraction) were obtained by differential centrifugation. The M_1 fraction was further subdivided by centrifugation in a discontinuous sucrose gradient consisting of successive layers of 1.4, 1.2, 1.0, 0.9 and 0.8 M sucrose.

Solubilization. Solubilization of M_1 was performed at 4° with Lubrol PX. M_1 of about 10 mg protein was suspended in 1 ml of 100 mM glycylglycine buffer, pH 7.4, containing 0.25 M sucrose, 5 mM $MgSO_4$, 1 mM EDTA, 3 mM dithiothreitol, 5 mM NaF and 2% (v/v) Lubrol PX, homogenized with a Teflon homogenizer for 2 min at 900 rev/min, and then centrifuged in a Hitachi 80 P at 105,000 *g* for 60 min. The clear supernatant fluid thus obtained was diluted 100 times with 2 mM Tris–maleate buffer, pH 7.4, containing 2 mM EGTA and then used for further experiments.

Determination of adenylate cyclase. Adenylate cyclase was determined in the presence and absence of dopamine by the method of Clement-Cormier *et al.* [12] except for the presence of 2 mM ATP instead of 0.5 mM ATP. The standard assay mixture contained, in a final volume of 0.5 ml, 80 mM Tris–maleate, pH 7.4, 2 mM ATP, 8 mM $MgSO_4$, 10 mM theophylline, 0.6 mM EGTA, 10 μM GTP, 0.02% ascorbic acid and an appropriate amount of enzyme. The reaction was initiated by addition of ATP, carried out for 4 min at 30° with the particulate enzyme or for 6 min at 25° with the solubilized enzyme with constant shaking, and terminated by placing the assay tubes in a boiling water bath for 3 min. The mixture was centrifuged and the amount of cyclic AMP in the supernatant fraction was directly determined by the protein binding assay as described by Brown *et al.* [21, 22]. Under the incubation conditions, the activity of adenylate cyclase was proportional to either incubation time or protein concentration within the tested range.

Validity for a direct application to the supernatant fluid of the protein binding method of Brown *et al.* [21, 22] for cyclic AMP determination was specifically verified in those experiments which studied the dopamine sensitivity of membranous adenylate cyclase. First, treatment of the supernatant fluid with cyclic AMP phosphodiesterase resulted in a complete disappearance of cyclic AMP, as determined by the protein binding method. Second, a 0.5-ml aliquot of the supernatant fluid with added [^{3}H]cyclic AMP (11,000 dis./min) was applied to a neutral alumina column (0.4 × 2.5 cm) which had been equilibriated previously with 0.05 M ammonium formate buffer, pH 7.4. After the column was washed with 0.5 ml of the same equilibriation buffer, 1.5 ml of the buffer was applied and the resulting eluate was collected. The amount of cyclic AMP in the eluate was determined by the protein binding method. Correction for the loss of cyclic AMP during the process was made by measuring the recovery (80–90 per cent) of [^{3}H]cyclic AMP. The amounts of cyclic AMP obtained by a direct application to the supernatant fluid of the binding method were essentially the same as those after isolation of cyclic AMP by the column chromatography prior to the binding assay.

Determination of [^{3}H]dopamine binding. The ability of M_1 to bind [^{3}H]dopamine was determined by the filtration method as described previously [14]. The incubation mixture contained, in a total volume of 2.5 ml, 10 mM theophylline, 8 mM $MgSO_4$, 0.6 mM EGTA, 0.5 μM [^{3}H]dopamine (0.15 to 0.45 μCi), 10 μM GTP, 0.02% ascorbic acid, 80 mM Tris–maleate, pH 7.4, and an appropriate protein amount of M_1. After 20 min of preincubation at 0°, the binding reaction was initiated by raising the temperature rapidly to 30° with concomitant addition of 2 min ATP, maintained for 4 min, and terminated by filtering through a Whatman GF/B filter disk under vacuum. After quickly washing twice within 10 sec with 15 ml each of cold 50 mM Tris–maleate buffer, pH 7.4, the filter disk was dried at 70° for 1 hr and the radioactivity was determined in 10 ml of

* Abbreviations used are: Gpp(NH)p, guanylyl imidodiphosphate; cyclic AMP, adenosine 3′:5′-monophosphate; and EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*, *N*′-tetraacetic acid.

Bray's solution [23] in a Packard liquid scintillation counter (model 3390). [^{3}H]Dopamine binding to M_1 in the absence and presence of 500 μM nonradioactive dopamine was referred to as total binding and nonspecific binding of [^{3}H]dopamine respectively. Thus, specifically membrane-bound radioactivity of [^{3}H]dopamine defined in the present experiments was the excess over nonspecifically bound radioactivity, that is the difference between total binding and nonspecific binding. Under these conditions, specifically bound radioactivity which was verified to be dopamine as described before [14] ranged from 65 to 75 per cent of total binding. The word "binding" simply described in the present paper means specific binding unless otherwise stated.

Succinic dehydrogenase (*EC* 1.3.99.1). Succinic dehydrogenase activity was assayed by following the decrease in optical density at 600 nm of 2,6-dichloroindophenol at room temperature under aerobic conditions as described before [24]. The reaction mixture, with a total volume of 1.0 ml, contained 100 mM sodium phosphate buffer, pH 7.2, 10 mM sodium cyanide, 0.14 mM 2,6-dichloroindophenol, 26 mM sodium succinate and the proper amount of enzyme fractions (17–530 μg protein).

Acetylcholine esterase (*EC* 3.1.1.7). Acetylcholine esterase was assayed by following the increase in optical density of the reaction product of thiocholine and dithiobisnitrobenzoate at 412 nm at room temperature using the method of Ellman *et al.* [25]. The reaction mixture (total volume 3.12 ml) contained 95 mM sodium phosphate buffer, pH 8.0, 0.32 mM dithiobisnitrobenzoate, 0.48 mM acetylthiocholine and the proper amounts of enzyme fractions (4–120 μg protein).

Protein. Protein determinations were carried out by the method of Lowry *et al.* [26], using bovine serum albumin as a standard.

RESULTS

Subcellular distribution of dopamine-sensitive adenylate cyclase and [^{3}H]*dopamine binding protein.* Of the four primary fractions, the mitochondrial fraction, as marked by succinic dehydrogenase activity, exhibited the highest total activity as well as the highest specific activity of both dopamine-sensitive adenylate cyclase and [^{3}H]dopamine binding (Table 1). Most of both activities were also found in osmotically shocked crude mitochondria (M_1). Of six subfractions prepared from M_1 by a discontinuous sucrose gradient centrifugation, synaptic membrane-enriched fractions M_1 (0.9) and M_1 (1.0), as marked by acetylcholine esterase, showed the highest total amounts of both activities and the highest specific activity as well. The enzyme and binding activities were lowest in the mitochondrial pellet. It is of interest to note that M_1 fractions of canine cerebellum exhibited neither activity of dopamine-sensitive adenylate cyclase nor binding of [^{3}H]dopamine.

Stimulation of particulate adenylate cyclase by dopamine. Adenylate cyclase activity of M_1 proceeded almost linearly for at least 10 min in a dose-dependent manner with respect to the protein amount up to 200 μg under the standard conditions in the presence and absence of 100 μM dopamine. Maximal stimulation of adenylate cyclase by dopamine usually varied from 1.6- to 2.7-fold, depending upon the preparations, though the reason for such a preparation-dependent variation was unclear. The relative potencies of catecholamines in stimulating the particulate adenylate cyclase were compared. At low concentrations dopamine was more effective than either (−)norepinephrine or (−)epinephrine, as shown in Fig. 1. (−)Isoproterenol, a β-adrenergic agonist, was completely inactive even at a concentration as high as 1 mM. The maximal response of adenylate cyclase to dopamine was about 300 pmoles/min/mg of protein, almost identical to the response to either (−)norepinephrine or (−)epinephrine, and a half-maximal increase in cyclic AMP formation occurred in the presence of 2.8 μM dopamine (Table 2). On the contrary, the apparent K_a values for (−)norepinephrine and (−)epinephrine were 21 and 17 μM respectively. As already shown in the preliminary report [14], the dopamine response of adenylate cyclase was strongly inhibited by neuroleptics such as fluphenazine (K_i, 3.0×10^{-9} M), haloperidol (K_i, 3.3×10^{-8} M), chlorpromazine (K_i, 6.5×10^{-8} M) and reserpine (K_i, 2.0×10^{-8} M), but not by cocaine, an inhibitor of reuptake at neuronal membranes [27], or by (±)propranolol, a β-adrenergic antagonist (Table 2). NaF at 10 mM was also able to stimulate adenylate cyclase to a somewhat greater extent than that attained maximally by dopamine.

The effects of various concentrations of GTP on stimulation by 100 μM dopamine of adenylate cyclase in the M_1 fraction were studied. Though GTP alone exhibited no effect on the adenylate cyclase activity, Fig. 2 obviously indicates that GTP together with dopamine resulted in a dose-dependent increase in the activity with the apparent K_a of about 4.5 μM, the concentration to cause half-maximal stimulation.

Binding of [^{3}H]*dopamine to M_1 membranes.* Binding of [^{3}H]dopamine to M_1 increased linearly as a function of the protein amount up to 1 mg under the standard conditions. Heat treatment at 80° for 5 min completely inactivated the ability of M_1 to bind [^{3}H]dopamine. In agreement with our preliminary report [14], the amount of [^{3}H]dopamine specifically bound to M_1 membranes was saturable in the presence or the absence of 2 mM ATP, as shown in Fig. 3. Scatchard analysis showed the apparent dissociation constant (K_d) of 0.11 μM (B_{max} = maximal binding, 1.0 pmole/mg of protein) and 8.1 μM (B_{max}, 10.5 pmoles/mg of protein) in the absence of 2 mM ATP and 1.5 μM (B_{max}, 15.0 pmoles/mg of protein) in its presence. Hill analysis also gave a similar K_d value of 1.4 μM in the presence of 2 mM ATP. The Hill coefficient was computed to be 1.04 in the presence of 2 mM ATP and 0.70 in its absence, indicating that ATP was apparently an allosteric effector. It was noteworthy that the affinity of dopamine to the synaptic vesicle fraction M_2 (K_d = 0.11 μM) was more than ten times greater than the affinity to the synaptic membrane fraction M_1 (K_d = 1.5 μM), suggesting that the binding to both fractions was distinct.

In the presence of 2 mM ATP, binding of [^{3}H]dopamine was rapid and reached an equilibrium state within several minutes (Fig. 4). Removal of 10 μM GTP from the standard incubation mixture did not alter significantly binding of [^{3}H]dopamine.

Table 1. Distribution of dopamine-sensitive adenylate cyclase and [^{3}H]dopamine binding activities of the canine caudate nucleus*

Fraction	Protein (mg)	Adenylate cyclase activity (pmoles/min/mg protein)		[^{3}H]Dopamine binding (pmoles/mg protein)	RSA of succinic dehydrogenase†	RSA of acetylcholine esterase
		−Dopamine	+Dopamine	B_{max}		
A. Starting material (homogenate)	172	110	198	19.8	1.00	1.00
B. Primary subfractions						
Nuclear	32.5	101	189	20.6	1.09	0.70
Mitochondrial	89.0	99.3	195	21.3	1.51	1.15
Microsomal	24.1	29.5	57.7	2.53	ND‡	0.49
Cell sap	25.5	ND‡	ND‡	ND‡	ND‡	0.54
Recovery (%)	99.4	67.8	73.1	77.2		
C. Mitochondrial subfractions						
M_1	47.9	181	334	43.4	2.78	1.67
M_2	5.02	50.0	103	1.43	ND‡	ND‡
M_3	18.1	64.1	99.5	ND‡	ND‡	ND‡
Recovery (%)	79.8	115	100	113		
D. Subfractions of M_1						
M_1 (0.8)	8.60	945	166	22.4	ND‡	2.46
M_1 (0.9)	5.39	335	1090	178	ND‡	3.92
M_1 (1.0)	4.27	356	744	138	1.28	3.07
M_1 (1.2)	9.80	117	219	31.4	3.37	1.16
M_1 (1.4)	12.8	31.5	60.3	10.3	6.43	0.57
Pellet	1.90	22.8	45.7	77.9	6.52	0.86
Recovery (%)	89.3	70.2	89.2	120		

* Adenylate cyclase activity was determined in the presence and absence of 100 μM dopamine under standard assay conditions. [^{3}H]Dopamine binding activity was determined under the standard assay conditions except for the presence of various concentrations of [^{3}H]dopamine for the measurement of total binding. Maximal amounts of [^{3}H]dopamine binding (B_{max}) were calculated by Scatchard analysis. Succinic dehydrogenase and acetylcholine esterase activities were determined as described in Materials and Methods.

† RSA (relative specific activity) = percent recovered activity/percent recovered protein.

‡ Not detectable.

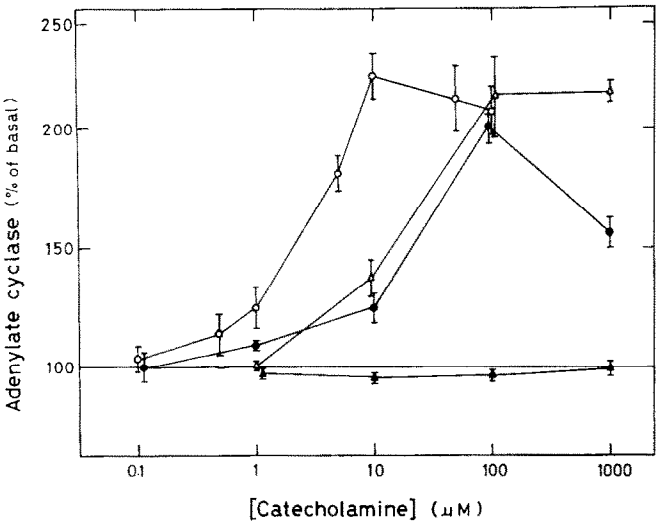

Fig. 1. Effects of catecholamines on adenylate cyclase of canine caudate nucleus. Adenylate cyclase of M_1 with 150 μg protein was determined under standard conditions in the presence of various concentrations of dopamine (○), (−)norepinephrine (●), (−)epinephrine (△) or (−)isoproterenol (▲). The results are the means of four determinations with the standard errors indicated by vertical bars. The basal activity of adenylate cyclase was 133 ± 21.6 pmoles of cyclic AMP formed/min/mg of protein.

Table 2. Effects of catecholamines and catecholamine antagonists on adenylate cyclase and [^{3}H]dopamine binding activities of M_1 membranes*

	Adenylate cyclase		[^{3}H]Dopamine binding
Compounds	K_a (M)	K_i (M)	K_i (M)
Dopamine	2.8×10^{-6}		1.6×10^{-6}
(−)Epinephrine	1.7×10^{-5}		2.7×10^{-5}
(−)Norepinephrine	2.1×10^{-5}		3.2×10^{-5}
(−)Isoproterenol	$> 10^{-3}$	†	4.4×10^{-5}
Promethazine	$> 10^{-4}$	2.2×10^{-6}	7.7×10^{-5}
Clozapine	$> 10^{-5}$	4.3×10^{-7}	1.2×10^{-6}
Chlorpromazine	$> 10^{-5}$	6.5×10^{-8}	1.3×10^{-6}
Perphenazine	$> 10^{-5}$	1.7×10^{-7}	4.0×10^{-7}
Methylperidol	$> 10^{-5}$	9.2×10^{-8}	8.0×10^{-8}
Haloperidol	$> 10^{-5}$	3.3×10^{-8}	6.7×10^{-8}
Fluphenazine	$> 10^{-5}$	3.0×10^{-9}	4.9×10^{-8}
Sulpiride	$> 10^{-4}$	$> 10^{-5}$	$> 10^{-4}$
Metoclopramide	$> 10^{-4}$	‡	8.0×10^{-5}
(±)Propranolol	$> 10^{-5}$	$> 10^{-6}$	$> 10^{-5}$
Pargyline	$> 10^{-5}$	$> 10^{-6}$	§
Cocaine	$>10^{-4}$	$> 10^{-5}$	$> 10^{-4}$
Imipramine	$> 10^{-4}$	1.1×10^{-6}	3.5×10^{-6}
Desmethylimipramine	$> 10^{-4}$	1.5×10^{-6}	4.2×10^{-6}
Reserpine	$> 10^{-5}$	2.0×10^{-8}	9.3×10^{-8}

* Binding of [^{3}H]dopamine and the dopamine-stimulated activity of adenylate cyclase were determined under the standard conditions. The inhibition constant (K_i) was obtained according to the equation, $K_i = IC_{50}/(1 + C/K)$, where C is the concentration of [^{3}H]dopamine in the binding assay or of dopamine in the assay of adenylate cyclase. K represents the concentration (K_d) of [^{3}H]dopamine (1.5 μM) required to give half-maximal binding to M_1 membranes or of dopamine (2.8 μM) to give half-maximal stimulation of the adenylate cyclase, and IC_{50} represents the concentration of the antagonists which reduced by 50 per cent the binding of [^{3}H]dopamine or the 40 μM dopamine-stimulated activity of adenylate cyclase. The K_a for adenylate cyclase and the K_i for [^{3}H]dopamine binding by catecholamines were calculated from Figs. 1 and 6 respectively.

† Although (−)isoproterenol at 10^{-4} M inhibited by 40 per cent the dopamine-stimulated activity, the inhibitory effect of the compound at more than 10^{-4} was not carried out.

‡ Although metoclopramide at 10^{-4} M inhibited by 16 per cent the dopamine-stimulated activity, the inhibitory effect of the compound at more than 10^{-4} M was not carried out.

§ Although pargyline at 10^{-5} M inhibited by 15 per cent the binding of [^{3}H]dopamine, the inhibitory effect of the compound at more than 10^{-5} M was not carried out.

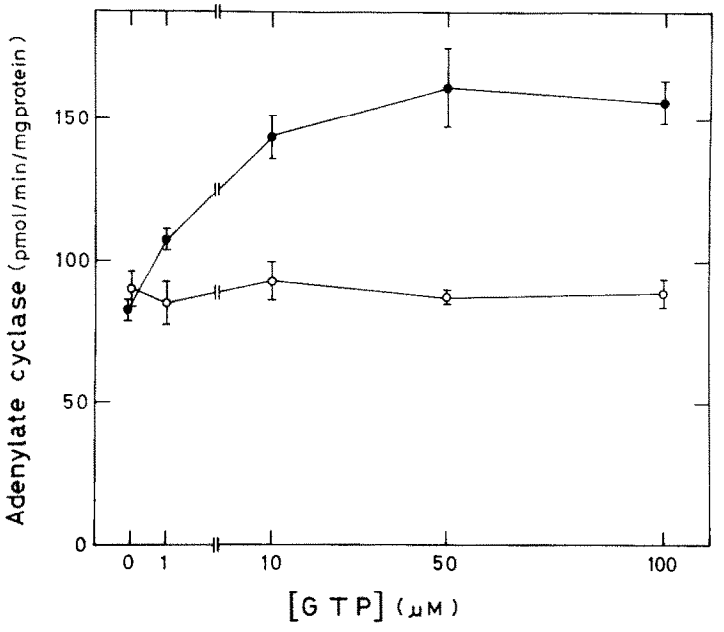

Fig. 2. Effects of various concentrations of GTP on dopamine stimulation of adenylate cyclase of M_1 membranes from canine caudate nucleus. Adenylate cyclase of M_1 with 76.5 μg protein was determined under standard conditions except for the presence of the indicated concentrations of GTP with (●) or without (○) 100 μM dopamine. The results are the means of four determinations with the standard errors indicated by vertical bars.

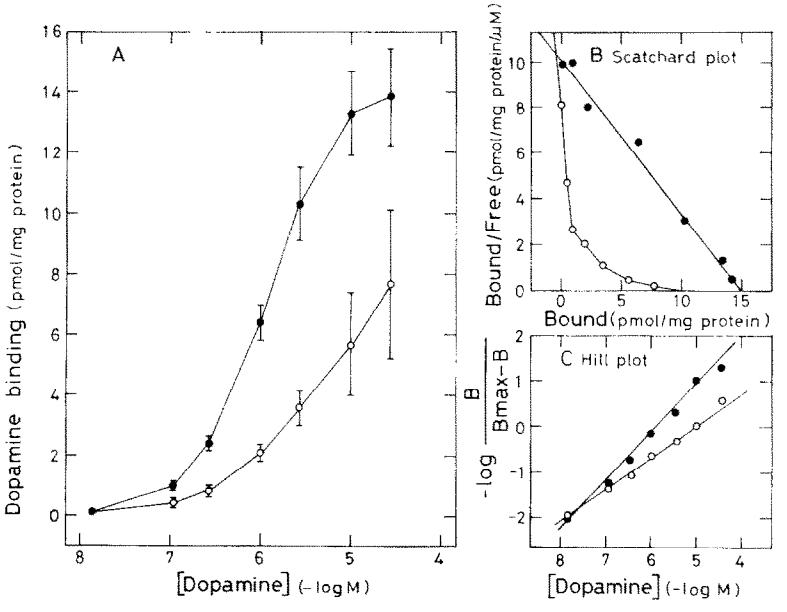

Fig. 3. Specific binding of dopamine to M_1 membrane preparations as a function of dopamine concentration. Total binding of 14 nM [^{3}H]dopamine plus indicated concentrations of nonradioactive dopamine to M_1 of 412 μg protein was determined under the standard assay conditions. Specific binding of dopamine was the difference between total binding at the indicated concentrations of dopamine and nonspecific binding in the presence of additional 500 μM nonradioactive dopamine. The results are the means of four determinations in the presence (●) and absence (○) of 2 mM ATP and 10 μM GTP. B_{max} (15.0 and 10.5 pmoles/mg of protein in the presence and absence of the nucleotides respectively) and B indicate the maximal binding and binding at the indicated concentration of dopamine respectively.

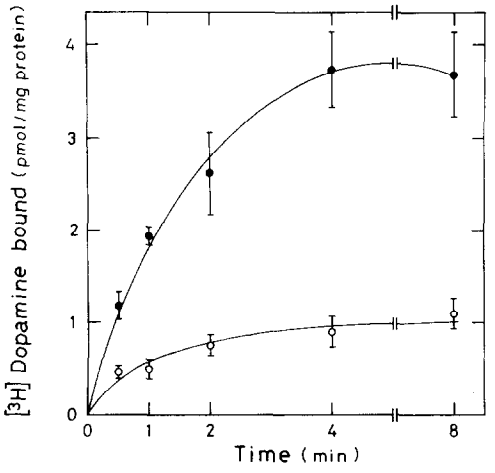

Fig. 4. Binding of [^{3}H]dopamine to M_1 membranes as a function of time. Binding of [^{3}H]dopamine to M_1 membranes with 410 μg protein was determined under the standard conditions in the presence (●) and absence (○) of 2 mM ATP except for no preincubation of 0° for 20 min. The results are the means of triplicate determinations with standard errors indicated by vertical bars.

The observed rate constant (K_{ob}) was calculated to be 0.75 min^{-1}. For the analysis of the dissociation reaction, M_1 was incubated with [^{3}H]dopamine for 6 min followed by the addition of a large amount of nonradioactive dopamine, and the displacement of [^{3}H]dopamine was then measured with time. The dissociation constant (K_2 = 0.63 min^{-1}) and the half-life time (1.1 min) were calculated from the slope of the dissociation curve at 30°, as illustrated in Fig. 5. When the dissociation was carried out at 0°, the release of [^{3}H]dopamine from M_1 was too slow to obtain the K_2 value. The second order rate constant (K_1) was calculated to be 2.5 × 10^5 M^{-1} min^{-1} according to the formula where DA is the concentration (0.5 μM) of [^{3}H]dopamine in the incubation mixture.

$$K_1 = \frac{K_{ob} - K_2}{DA}$$

Thus, the calculated K_d (K_2/K_1) was 2.6 μM, in fairly good agreement with the K_d of 1.5 μM which was obtained from the equilibrium studies in Fig. 3.

In the absence of ATP in the incubation mixture, binding of [^{3}H]dopamine to M_1 was apparently biphasic; a rapid phase in the first 30 sec was followed by a slow phase (Fig. 4). However, binding under these conditions was not high enough to allow a reliable kinetic analysis. As indicated in Fig. 5, the rate of displacement in the absence of ATP was as rapid as in its presence for the first 30 sec and thereafter slowed down considerably.

The relative potencies of nonradioactive catecholamines in competing with the specific binding of [^{3}H]dopamine to M_1 were also compared in the presence of 2 mM ATP (Fig. 6). Nonradioactive dopamine reduced binding of 0.5 μM [^{3}H]dopamine half-maximally at 1.9 μM, in good agreement with the results in Fig. 3. Other catecholamines were much less potent in competing with [^{3}H]dopamine binding than was nonradioactive dopamine. Half-maximal inhibition of [^{3}H]dopamine binding by (−)norepinephrine, (−)isoproterenol and (−)epinephrine occurred at about 32, 44 and 27 μM respectively. The potency of several neuroleptics in the inhibition of [^{3}H]dopamine binding to M_1 was very similar to that of dopamine-sensitive adenylate cyclase except for fluphenazine and chlorpromazine, both of which exhibited, particularly a greater inhibition of adenylate cyclase. Reserpine (K_i, 9.3 × 10^{-8} M) also strongly inhibited binding. Imipramine (K_i, 3.5 × 10^{-6} M) and desmethylimipramine (K_i, 4.2 × 10^{-6} M) inhibited [^{3}H]dopamine binding, whereas

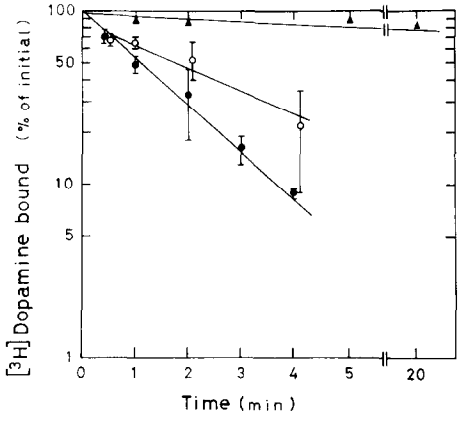

Fig. 5. Dissociation of [^{3}H]dopamine from M_1 membranes. Binding of [^{3}H]dopamine to M_1 membranes with 480 μg protein was carried out in the presence (●, ▲) and absence (○) of 2 mM ATP under standard assay conditions except for no preincubation at 0° for 20 min and an incubation time of 6 min. Subsequently a large excess of nonradioactive dopamine (1 mM) was added and immediately continued to incubate at 30° (●, ○) or 0° (▲). At the indicated time, the amount of [^{3}H]dopamine still specifically bound was determined. The results are the means of triplicate determinations with standard errors indicated by vertical bars.

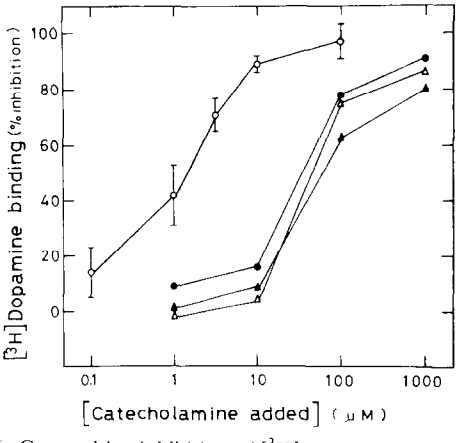

Fig. 6. Competitive inhibition of [^{3}H]dopamine binding to M_1 membranes by catecholamines. Binding of 0.5 μM [^{3}H]dopamine to M_1 with 400 μg protein was determined in the presence of various concentrations of dopamine (○), (−)epinephrine (●), (−)norepinephrine (△) or (−)isoproterenol (▲) under standard conditions. The amount of [^{3}H]dopamine bound to the membranes in the control experiments was 6.61 ± 0.73 pmoles/mg of protein. The results are the means of four determinations with standard errors indicated by vertical bars.

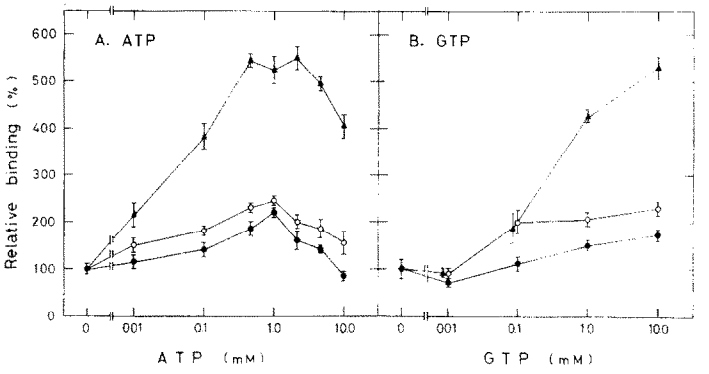

Fig. 7. Effects of various concentrations of ATP (A) and GTP (B) on binding of [^{3}H]dopamine to M_1 membranes. The procedure for assaying [^{3}H]dopamine binding was as described under Materials and Methods. In panel A taken from the previous report [14] for comparison, the amounts of dopamine bound to M_1 with 280 μg protein in the presence of 4.5×10^{-9} M (●), 1.05×10^{-7} M (○) and 1.00×10^{-6} M (▲) dopamine in the incubation mixtures lacking ATP were 0.067 ± 0.007, 1.11 ± 0.10 and 2.21 ± 0.79 pmoles/mg of protein respectively. In panel B, the amounts of dopamine bound to M_1 with 340 μg in the presence of 4.81×10^{-9} M (●), 1.05×10^{-7} (○) and 1.00×10^{-6} M (▲) dopamine in the incubation mixture lacking GTP were 0.082 ±0.004, 0.714 ± 0.044 and 3.13 ± 0.69 pmoles/mg of protein respectively. The results are the means of triplicate determinations with standard errors indicated by vertical bars.

promethazine, pargyline, (±)propranolol and cocaine failed to inhibit the binding.

In agreement with our preliminary report [14], dopamine receptors bound [^{3}H]dopamine in a negatively cooperative manner in the absence of ATP, and the addition of ATP abolished this cooperativity at relatively high concentrations such as 2 mM (Figs. 3 and 7). Since GTP was absolutely required for stimulation of the particulate adenylate cyclase by dopamine, as indicated in Fig. 2, it appeared to be of great interest to see whether GTP was able to increase [^{3}H]dopamine binding even at such low concentrations as to cause a considerable increase in the adenylate cyclase activity. As illustrated in Fig. 7, 10 μM GTP failed to affect binding of three different concentrations of [^{3}H]dopamine (4.8×10^{-9}, 1.1×10^{-7} and 1.0×10^{-6} M), whereas GTP, at higher concentrations than 0.1 mM, increased dose-dependently the binding of [^{3}H]dopamine and was particularly effective on binding at the highest concentration of [^{3}H]dopamine tested.

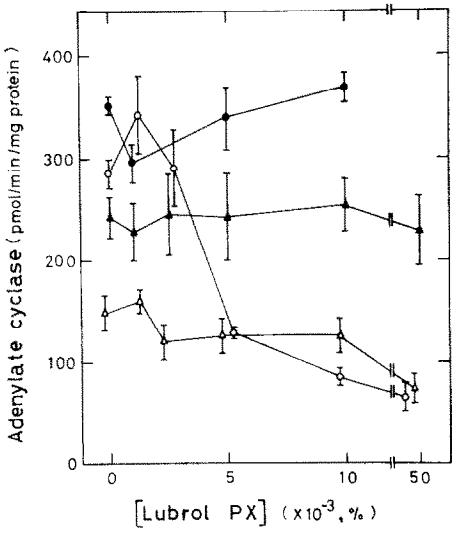

Fig. 8. Effects of increasing Lubrol PX concentration on adenylate cyclase of M_1 in the presence of 10 μM GTP (△), 100 μM Gpp(NH)p (▲), 10 μM GTP plus 10 mM NaF (●), or 10 μM GTP plus 100 μM dopamine (○). Adenylate cyclase activity of 96.7 μg protein was determined under the standard conditions except for the replacement of GTP by the indicated compounds. The results are the means of four determinations with standard errors indicated by vertical bars.

Stimulation by dopamine of adenylate cyclase in the Lubrol PX-solubilized preparation. The effect of varied concentrations of Lubrol PX on dopamine sensitivity of the particulate adenylate cyclase was studied in order to find the inhibitory concentration of Lubrol PX (Fig. 8). More than 0.005% of Lubrol PX in the reaction mixture completely eliminated dopamine sensitivity, while the addition of 0.001% Lubrol PX resulted in a slight increase in dopamine sensitivity without substantial alteration of basal activity. Stimulation of adenylate cyclase by either NaF or Gpp(NH)p, on the contrary, was virtually unaffected by Lubrol PX up to 0.01%.

Dopamine sensitivity of adenylate cyclase in the 105,000 *g* supernatant fluid after treatment of M_1 with 2% Lubrol PX was studied at a final concentration of less than 0.005% Lubrol PX as a carrier-over from the solubilization procedure. Cyclic AMP formation, in the absence and presence of 50 μM dopamine in the incubation, increased almost linearly with time up to 10 min, as indicated in Fig. 9. As a criterion for solubilization of the enzyme, it must be noted that further centrifugation of the solubilized preparations at 225,000 *g* for 1 hr did not alter the dopamine sensitivity of adenylate cyclase. Adenylate cyclase activity of the solubilized supernatant fluid was specifically stimulated by dopamine in a dose-dependent manner with an apparent K_a of 1.5 μM, as shown in Fig. 10. Other catecholamines such as (−)norepinephrine, (−)epinephrine and

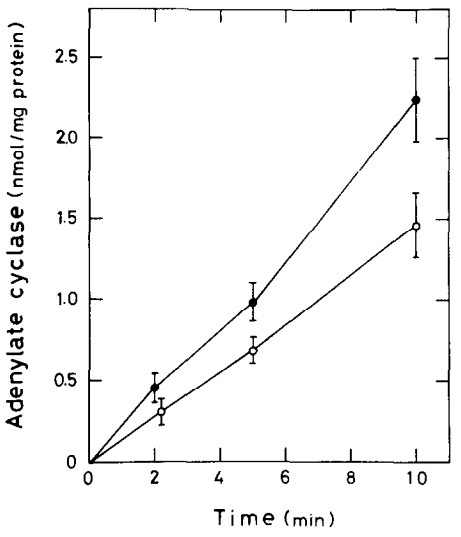

Fig. 9. Time course of dopamine-sensitive adenylate cyclase in the 105,000 *g* supernatant fluid. Adenylate cyclase activity with 5.4 μg protein was determined in the presence (●) and absence (○) of 50 μM dopamine under the standard conditions. The concentration of Lubrol PX in the reaction mixture was 0.004%. The results are the means of four determinations with standard errors indicated by vertical bars.

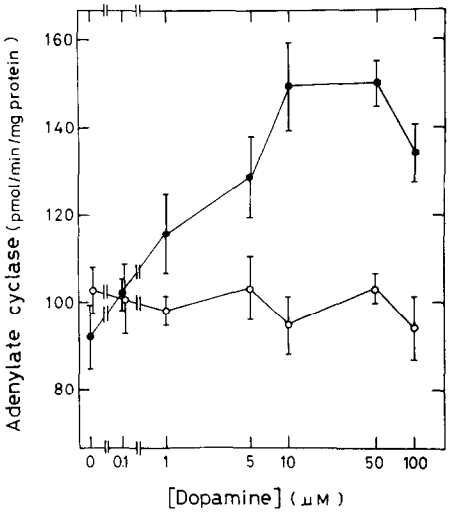

Fig. 10. Effects of various concentrations of dopamine on adenylate cyclase of the 105,000 *g* supernatant fluid. Adenylate cyclase activity with 3.7 μg protein was determined in the presence of indicated concentrations of dopamine under the standard conditions except for the presence (●) or absence (○) of 10 μM GTP. The concentration of Lubrol PX in the reaction mixture was 0.004%. The results are the means of four determinations with standard errors indicated by vertical bars.

(−)isoproterenol were virtually ineffective at 10 μM (Table 3). GTP was absolutely required for stimulation of the solubilized adenylate cyclase by dopamine as in the case of the particulate preparations. Stimulation of adenylate cyclase by dopamine in the 105,000 *g* supernatant fluid generally varied from 1.4- to 1.9-fold, so long as the membrane preparations used as the starting materials for solubilization were effective in stimulating adenylate cyclase by dopamine. This dopamine stimulation was almost completely blocked by either chlorpromazine or haloperidol at 10 μM (Table 3). NaF exhibited marked stimulation of adenylate cyclase of the 105,000 *g* supernatant fluid in a dose-dependent manner under the standard assay conditions as seen with the M_1 membranes. Removal of GTP from the standard incubation mixture did not alter significantly the stimulatory effect of NaF.

Table 3. Effects of catecholamine, Gpp(NH)p, NaF and neuroleptics on the solubilized adenylate cyclase*

Addition	Adenylate cyclase (pmoles/min/mg protein)
Expt. 1	
None	103 ± 14.6
10 μM dopamine	158 ± 5.6
− GTP	116 ± 16.0
− GTP + 50 μM Gpp(NH)p	336 ± 25.0
− GTP + 10 mM NaF	180 ± 33.8
Expt. 2	
None	147 ± 13.2
10 μM Dopamine	221 ± 18.4
10 μM (−)Norepinephrine	160 ± 22.1
10 μM (−)Epinephrine	150 ± 22.1
10 μM (−)Isoproterenol	162 ± 18.6
Expt. 3	
None	95 ± 12.5
10 μM Chlorpromazine	103 ± 9.8
10 μM Haloperidol	91 ± 6.0
40 μM Dopamine	161 ± 15.8
40 μM Dopamine + 10 μM chlorpromazine	112 ± 12.9
40 μM Dopamine + 10 μM haloperidol	95 ± 4.9

* Adenylate cyclase activity was determined under the standard conditions in the presence of the indicated compounds. The protein amounts of the 105,000 *g* supernatant fluid in the incubation mixtures were 3.9 μg in Expt. 1, 2.9 μg in Expt. 2, and 2.9 μg in Expt. 3.

DISCUSSION

The present studies have led us to conclude that a [^{3}H]dopamine binding component, which is characterized under incubation conditions similar to the assay system for dopamine-sensitive adenylate cyclase, is the dopamine receptor unit of the adenylate cyclase. The main supporting evidence is: (1) the similarity in subcellular distribution between dopamine-sensitive adenylate cyclase activity and [^{3}H]dopamine binding, (2) the similarity between the K_d of the binding component for [^{3}H]dopamine (about 1 μM) and the K_a of dopamine-sensitive adenylate cyclase for dopamine (2.8 μM), and (3) the similarity in the affinity to catecholamines and neuroleptics between [^{3}H]dopamine binding and dopamine-sensitive adenylate cyclase. The affinity to the [^{3}H]dopamine binding sites of neuroleptics which are well known to be dopamine antagonists is closely correlated to their potency in inhibiting dopamine-sensitive adenylate cyclase, except for chlorpromazine and fluphenazine. The K_i values of these phenothiazine derivatives for dopamine-sensitive adenylate cyclase are considerably smaller than the K_d values of the drugs in binding to [^{3}H]dopamine binding sites. This may be partly due to an inhibition of the coupling process of adenylate cyclase with the receptor by chlorpromazine and fluphenazine in addition to their occupation of the receptor. Furthermore, we have recently reported the dissociation of dopamine-sensitive adenylate cyclase into a catalytic unit and a [^{3}H]dopamine binding unit, and the reconstitution of them into a dopamine-sensitive complex [28, 29], indicating conclusively the physiological significance of [^{3}H]dopamine binding sites.

Recent investigations have demonstrated the existence of multiple dopamine receptors [1–6]. It appears that a class of dopamine receptors independent of dopamine-sensitive adenylate cyclase has a relatively higher affinity to dopamine at a nanomolar level whereas the other class of the receptors which is associated with adenylate cyclase has a lower affinity at a micromolar level [2–5]. Utilization of very low concentrations of [^{3}H]dopamine in the incubation for the total binding, together with the fact that relatively low concentrations of nonradioactive dopamine are used for the nonspecific binding, enables one to label virtually only dopamine receptors with the high affinity, independently of dopamine-sensitive adenylate cyclase. In fact, our preliminary studies using 1 μM dopamine for the nonspecific binding indicate that the high affinity receptor for [^{3}H]dopamine in the canine caudate membranes possesses a K_d of 12.0 nM, with a maximal binding of about 160 fmoles/mg of protein in the absence of ATP. The physiological significance of this receptor is yet to be elucidated. Thus, the high-affinity type of dopamine receptors appears to occupy only a very minor population in the canine caudate membranes.

[^{3}H]Dopamine binding in the absence of ATP under our present conditions appears to be negatively cooperative and shows two K_d values of 0.11 μM and 8.8 μM. ATP stimulates greatly the binding of [^{3}H]dopamine as an apparently positive allosteric effector, thereby abolishing the negative cooperativity. The role of ATP appears to enhance the association of [^{3}H]dopamine to the receptor of dopamine-sensitive adenylate cyclase which is located in the post-synaptic membranes of the caudate nucleus as demonstrated by pre-synaptic degeneration experiments with 6-hydroxydopamine [30]. This ATP-dependent enhancement of [^{3}H]dopamine binding is of great interest in view of the fact that ATP is concomitantly released with some neurotransmitters from the nerve ending through exocytosis [31–34].

Another possibility also exists that ATP-dependent binding of dopamine is associated with dopamine uptake at either pre-synaptic membranes or synaptic vesicles. However, the possibility of association with dopamine uptake at neuronal membranes can be ruled out by the fact that cocaine, a potent inhibitor of dopamine uptake [27], inhibits neither [^{3}H]dopamine binding to M_1 nor dopamine-sensitive adenylate cyclase. The present experiments also indicate that the affinity of dopamine to the synaptic vesicle fraction M_2 (K_d = 0.11 μM) is more than ten times greater than that to the synaptic membrane fraction M_1 (K_d = 1.5 μM). Philippu and Beyer [35] have shown that synaptic vesicles from the caudate nucleus of the pig do not clearly distinguish dopamine from norepinephrine in the ATP-dependent uptake reaction, in contrast to our system. These results suggest that dopamine binding to M_1 under our experimental conditions is distinct from dopamine transport into the synaptic vesicles.

In order to clarify the mechanism for coupling the dopamine receptor with the catalytic unit of adenylate cyclase, we have attempted at first to obtain dopamine-sensitive adenylate cyclase in a solubilized form free from the membrane structure. Since dopamine-sensitive activity of adenylate cyclase is considerably inhibited by 0.005% Lubrol PX, it is very important to measure dopamine sensitivity of adenylate cyclase in the solubilized preparations in the presence of less than 0.005% Lubrol PX in the assay mixture. Furthermore, the presence of NaF in the solubilization process is essential for the stability of dopamine sensitivity, and does not abolish a stimulatory effect of NaF on the solubilized adenylate cyclase. Since Lubrol PX exists in a form of micelle at a concentration higher than 0.005% at 4°, the micelle formation of Lubrol PX may be at least partly responsible for the impairment of dopamine sensitivity. The properties of solubilized dopamine-sensitive adenylate cyclase are virtually identical to those of the particulate form of enzyme.

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