LOW AFFINITY, ADENOSINE 5'-TRIPHOSPHATE-DEPENDENT [³H]DOPAMINE BINDING IN SYNAPTIC MEMBRANES FROM BOVINE CAUDATE IS UNRELATED TO DOPAMINE-STIMULATED ADENYLATE CYCLASE¹

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Abstract

[³H]Dopamine binds to synaptic plasma membranes from bovine caudate nucleus with an apparent dissociation constant $K_d^{app} = 0.78 \mu\text{M}$ and a capacity $\beta_{max} = 102 \text{ pmol mg}^{-1}$ of protein. NaCl or KCl at 100 mM causes a doubling of the binding capacity while reducing the affinity for dopamine by half. ATP at millimolar concentrations is required for binding in all of these cases, and linear Scatchard plots are obtained both in the absence and presence of salts. Binding is insensitive to low concentrations of inhibitors of synaptosomal uptake but is extremely sensitive to inhibition by reserpine, an agent believed to be specific for uptake by synaptic vesicles. The two stereoisomers of butaclamol are approximately equipotent as inhibitors of dopamine binding. At $\leq 10 \mu\text{M}$ concentrations, neither ouabain nor inhibitors specific for the mitochondrial electron transport-linked ATPase, such as oligomycin, efrapeptin, and antimycin A, show any inhibition of binding, while orthovanadate ($50 \mu\text{M}$), dicyclohexyl carbodi-imide ($50 \mu\text{M}$), and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole ($10 \mu\text{M}$) are all effective inhibitors. Inhibition also is observed with ammonium chloride and extremely low concentrations of carbonyl cyanide *m*-chlorophenyl hydrazone (50 nM) and nigericin (3 nM) plus potassium. In other systems, these three agents act as inhibitors of proton-translocating ATPases by dissipating a transmembrane proton gradient. Concentrations of vanadate, carbonyl cyanide *m*-chlorophenyl hydrazone, and nigericin plus potassium that completely abolish ATP-dependent dopamine binding have no inhibitory effect on dopamine stimulation of adenylate cyclase measured under identical conditions, implying that the two activities are unrelated. The similarity between the properties of low affinity dopamine binding and those of uptake systems in synaptic vesicles and adrenal chromaffin granules suggests the possibility that the apparent binding reaction is, in fact, due to the transport of dopamine into vesiculated membrane fragments.

Since the demonstration of dopamine-dependent adenylate cyclase activity in the central nervous system (Kebabian et al., 1972; Brown and Makman, 1972), there has been a great deal of interest in identifying compounds which specifically label the receptor responsible for stimulation of the enzyme. This task has been complicated by the presence of multiple receptors for dopaminergic agonists and antagonists (Titeler et al., 1978; Creese and

Sibley, 1979; Snyder and Goodman, 1980; Iversen et al., 1980; Creese et al., 1981). Kebabian and Calne (1979) proposed that D1 receptors are linked to adenylate cyclase, while D2 receptors are not. Titeler et al. (1979) have expanded this classification by suggesting that receptors that exhibit high affinity for dopamine agonists be designated D3 receptors and that they may function as presynaptic autoreceptors (Roth, 1979).

Although there is some evidence that [³H]spiperone, [³H]apomorphine, and [³H]ADTN³ bind with high affin-

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³ The abbreviations used are: ADTN, 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; $\alpha,\beta\text{-CH}_2\text{-ATP}$, α,β -methylene adenosine 5'-triphosphate; $\beta,\gamma\text{-CH}_2\text{-ATP}$, β,γ -methylene adenosine 5'-triphosphate; $\beta,\gamma\text{-NH-ATP}$, β,γ -imidoadenosine 5'-triphosphate; $\gamma\text{-S-ATP}$, adenosine

ity to a receptor linked to a GTP-modulated adenylate cyclase (Creese et al., 1978, 1979; Creese and Snyder, 1978; Zahniser and Molinoff, 1978; Sibley and Creese, 1979), these compounds also label D2 or D3 receptors. One antagonist of dopamine-dependent adenylate cyclase which may label the D1 receptor specifically is *cis*-[³H]flupenthixol (Hyttel, 1978; Cross and Owen, 1980). However, specific agonists of the D1 receptor that bind to it with high affinity have been lacking. Sano and Maeno (1976) reported that [³H]dopamine binds to a synaptic membrane fraction from canine caudate nucleus at micromolar concentrations and suggested that this binding may reflect the reaction with the receptor responsible for dopamine stimulation of adenylate cyclase. However, binding was stimulated by ATP at millimolar concentrations, while GTP at micromolar concentrations had no effect on the reaction (Sano et al., 1976b). In addition, several compounds which are potent antagonists of the dopamine-dependent enzyme were less effective as inhibitors of [³H]dopamine binding.

In the following report, we describe the characteristics of the low affinity binding reaction in membranes of bovine caudate as well as results of experiments with inhibitors and uncouplers of proton-translocating ATPases designed to clarify its requirements for ATP. This and other evidence suggest to us that low affinity binding and binding to the dopamine (D1) receptor linked to adenylate cyclase are independent and unrelated.

Materials and Methods

Materials. [α -³²P]ATP (10 to 30 Ci/mmol) and [³H]dopamine (10 to 40 Ci/mmol) were from New England Nuclear, Boston, MA; dopamine HCl, ATP, ADP, AMP, ITP, GTP, UTP, α,β -CH₂-ATP, β,γ -CH₂-ATP, β,γ -NH-ATP, adenosine, cAMP, serotonin, ouabain, chlorpromazine, reserpine, pargyline, L-epinephrine, DL-isoproterenol HCl, saponin, NAD, NADH, NADP, NADPH, antimycin A, valinomycin, oligomycin, and amphetamine sulfate were from Sigma, St. Louis, MO; ADTN and CCCP were from Calbiochem, La Jolla, CA; γ -S-ATP was from Boehringer Mannheim, Indianapolis, IN; NBD-Cl was from ICN, Cleveland, OH; and DCCD was from J. T. Baker, Philipsburg, NJ.

Nigericin and efraeptin were generous gifts of Eli Lilly Co., Indianapolis, IN and (+)- and (-)-butaclamol were supplied by Ayerst, Montreal, Quebec. Fluphenazine HCl was kindly provided by Squibb, Princeton, NJ, apomorphine by Merck, Sharp and Dohme, Rahway, NJ,

and spiperone by Janssen Pharmaceutical, Beerse, Belgium.

Preparation of SPM. Fresh adult bovine brains were obtained from a local slaughterhouse and kept cooled on ice during transportation to the laboratory. Caudates were removed within 2 hr of death, weighed, and minced with scissors in 6 vol of 0.32 M sucrose, 5 mM sodium HEPES, pH 7.4, 1 mM EGTA. The material was homogenized in a glass homogenizer with a motor-driven Teflon pestle (15 strokes, 500 rpm). Synaptic plasma membranes (SPMs) were prepared essentially as described by Salvaterra and Matthews (1980) with the exception that all solutions contained 5 mM sodium HEPES, pH 7.4. SPMs were stored frozen at -80°C for periods up to 1 month without loss of binding activity.

[³H]Dopamine binding assay. [³H]Dopamine binding was determined in a manner similar to that described by Sano et al. (1979a). The incubation mixture contained, in a total volume of 300 μ l, 80 mM Tris/maleate, pH 7.4, 0.6 mM EGTA, 0.02% ascorbic acid, 8 mM MgSO₄, 1 mM ATP, 0.5 μ Ci of [³H]dopamine diluted with nonradioactive dopamine to the appropriate concentration, and 100 to 500 μ g of SPM. The reaction was initiated by the addition of SPM, allowed to proceed for 5 min at 30°C, and then terminated by adding 4 ml of ice cold 80 mM Tris/maleate, pH 7.4, 8 mM MgSO₄, 0.02% ascorbate, 0.6 mM EGTA containing 500 μ M nonradioactive dopamine and filtering over a glass fiber filter (GF/C, Whatman, Clifton, NJ) under reduced pressure. The filter was washed with two additional 4-ml aliquots of the above buffer and placed in scintillation vials, and 8 ml of Aquasol (New England Nuclear, Boston, MA) was added. After the filters were allowed to clarify overnight, radioactivity was determined by liquid scintillation spectrometry with an efficiency of 33 to 36%.

Specific binding was calculated by subtracting the radioactivity bound in the presence of 500 μ M nonradioactive dopamine (nonspecific binding) from that observed in the absence of excess cold dopamine (total binding). Nonspecific binding ranged from 5 to 40% of the total binding, depending upon the concentration of dopamine present in the reaction mixture. When binding activity was to be compared with adenylate cyclase activity, both assays were carried out in the presence of 10 mM theophylline and 10 μ M GTP, although neither compound had any effect on binding activity at these concentrations.

Adenylate cyclase. The assay for dopamine-dependent adenylate cyclase was carried out as described by Sano et al. (1979a) using [γ -³²P]ATP as the substrate. The reaction was allowed to proceed for 5 min at 30°C. The reaction was terminated and the amount of [³²P]cAMP formed was determined as described by Salomon et al. (1974).

Other methods. Protein was determined by the method of Markwell et al. (1978). Some inhibitors were dissolved in ethanol or dimethyl sulfoxide and diluted with assay buffer before use. The final concentration of solvent in the assay was always less than 0.5%, and the solvent alone did not affect either dopamine binding or adenylate cyclase activity.

5'-O-(3-thiotriphosphate; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazine; DCCD, dicyclohexyl carbodi-imide; dopamine, 3,4-dihydroxyphenylethylamine; EGTA, ethylene glycol bis(β -aminoethyl ether)-N,N,N',N'-tetra-acetic acid; GTP, guanosine 5'-triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; IC₅₀, concentration required for 50% inhibition of specific binding; ITP, inosine 5'-triphosphate; NAD, NADH, nicotinamide-adenine dinucleotide and its reduced form; NADP, NADPH, nicotinamide-adenine dinucleotide phosphate and its reduced form; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; serotonin, 5-hydroxytryptamine; NEM, N-ethylmaleimide; SPM, synaptic plasma membrane; Tris, tris(hydroxymethyl)amino-methane; UTP, uridine 5'-triphosphate.

Results

Binding parameters. The characteristics of [^3H]dopamine binding to bovine caudate SPM in the presence of ATP are shown in Figure 1, yielding a linear Scatchard plot with $K_d^{app} = 0.6 \mu\text{M}$ and $\beta_{max} = 102 \text{ pmol mg}^{-1}$. Nonspecific binding, defined as binding in the presence of $500 \mu\text{M}$ nonradioactive dopamine is linearly dependent on the dopamine concentration and, at $10 \mu\text{M}$ dopamine, accounts for 20 to 30% of the total binding. Specific binding was abolished by trypsin treatment, incubation at 80°C for 5 min, or omission of ATP in the binding assay (data not shown) as reported previously (Sano et al., 1979a). However, we have been unable to observe the negatively cooperative binding in the absence of ATP described by Sano and Maeno (1976) who used membranes from canine caudate. Although dopamine does bind to bovine membranes with very low affinity in the absence of ATP ($K_d^{app} \approx 10^{-4} \text{ M}$), this form of binding also can be detected in the presence of ATP.

Regional distribution. We also have examined ATP-dependent binding activity in membranes from bovine cortex and cerebellum. Dissociation constants were similar to that observed in striatum, but the total number of sites was much lower for both cerebellum ($\beta_{max} \approx 9 \text{ pmol mg}^{-1}$) and cortex ($\beta_{max} \approx 6 \text{ pmol mg}^{-1}$).

Effects of GTP and salts on dopamine binding. Dopamine stimulation of adenylate cyclase in cell-free homogenates of caudate nucleus exhibits a strict requirement for guanine nucleotides (Kebabian et al., 1979), and in other adenylate cyclase systems, GTP, or analogs closely related to it, causes a decrease in receptor affinity for agonists (Limbird, 1981). However, in the present

instance, $50 \mu\text{M}$ GTP caused no appreciable change in either receptor affinity or capacity (Table I).

Because earlier work had shown that cations affect the properties of a dopamine receptor labeled by [^3H]spiperone (Usdin et al., 1980), we examined the effects of NaCl and KCl on the low affinity binding of [^3H]dopamine. Both KCl and NaCl at 100 mM cause a doubling of the capacity while reducing the affinity for dopamine by half (Table I; Fig. 1). The dependence on the NaCl concentration is shown in Figure 2, where increasing the salt concentration up to approximately 100 mM leads to enhanced binding. Increasing the salt concentration beyond 150 mM results in a gradual decline in binding. Similar results were obtained with KCl, KBr, and LiCl (data not shown). CaCl_2 also elicited similar effects at approximately half the concentration of the monovalent ions, suggesting that the observed effects are due to nonspecific alterations in ionic strength.

pH dependence of binding. ATP-dependent dopamine binding is also pH dependent as shown in Figure 3. In the absence of added salts, the pH optimum lies between 6.8 and 7.0. The addition of 100 mM NaCl or KCl shifts this optimum to a higher pH (6.9 to 7.3).

Inhibition by dopamine-related compounds. A variety of compounds have been shown previously to affect systems capable of interacting with catecholamines. The

Figure 1. Scatchard plot of [^3H]dopamine binding to synaptic plasma membranes. Binding activity was determined as described under "Materials and Methods" at pH 7.4 with 1 mM ATP in the absence (●) and presence (○) of 100 mM NaCl. Specific binding was calculated by subtracting nonspecific binding in the presence of $500 \mu\text{M}$ cold dopamine from total binding in the absence of excess cold dopamine. Each point is the mean of three determinations $\pm$ SD. L, liter.

TABLE I

[^3H]Dopamine binding to synaptic membranes
[^3H]Dopamine binding was determined as for Figure 1. All parameters were obtained from Scatchard plots.

Addition	K_d^{app} μM	β_{max} $\text{pmol mg}^{-1} \text{ protein}$
None	0.78	102
GTP ($50 \mu\text{M}$)	0.65	104
NaCl (100 mM)	1.3	201
KCl (100 mM)	1.15	204

Figure 2. Dependence of [^3H]dopamine binding on NaCl concentration. Binding assay was performed as described under "Materials and Methods" with $3 \mu\text{M}$ [^3H]dopamine and 1 mM ATP. Activity is presented as the ratio of specific binding to the highest specific binding attained. Each point is the mean of three determinations with SD less than 10%.

Figure 3. pH dependence of [^3H]dopamine binding in the presence and absence of 100 mM NaCl. The assay was performed as described under "Materials and Methods" with 3 μM [^3H]dopamine and 1 mM ATP in the presence (○) and absence (●) of 100 mM NaCl. Each point is the mean of three determinations with SD less than 10%. Data are presented as the ratio of specific binding to the highest specific binding attained.

effects produced by some of them on the present reaction are shown in Table II. Amphetamine and benztropine, both effective and selective inhibitors of the synaptosomal uptake system (Coyle and Snyder, 1969; Wong et al., 1980), are relatively ineffective inhibitors of binding. In contrast, reserpine, a potent inhibitor of uptake into synaptic vesicles (Toll et al., 1977; Toll and Howard, 1978), inhibits low affinity dopamine binding at nanomolar concentrations.

The tritiated forms of spiperone, dopamine, ADTN, and apomorphine all bind to SPM with affinities in the nanomolar range (Near and Mahler, 1981). It is highly unlikely that these receptors are responsible for low affinity dopamine binding since their capacities are lower than those described in Table I by several orders of magnitude and, in addition, none of these four ligands inhibits low affinity dopamine binding with an IC_{50} of less than 0.9 μM (Table II).

ATP analogs. In order to explore the role of ATP on low affinity dopamine binding, we examined the ability of several related compounds to either replace ATP or inhibit its stimulatory effect. Although several compounds are able to antagonize ATP stimulation, only GTP can replace ATP fully at the concentrations tested (Table III). Half-maximal stimulation of [^3H]dopamine binding was obtained with 80 μM ATP or 300 μM GTP.

ATPase inhibitors and uncouplers. ATP-dependent dopamine binding is relatively resistant to inhibition by ouabain (Table IV), an agent specific for the (Na^+ , K^+)-ATPase. Binding is also unaffected by the addition of the mitochondrial ATPase inhibitors, oligomycin or efrapetin, or of antimycin A, which inhibits electron transport. However, uncouplers which act by dissipating a transmembrane electrochemical proton gradient are very efficient inhibitors of binding: CCCP, a protonophore, and nigericin, which catalyzes a K^+ - H^+ exchange (Ashton and Steinrauf, 1970), both are effective. As expected, the latter compound is most potent in the presence of 10 mM KCl, less potent with 10 mM NaCl, and least effective in the absence of monovalent cations.

Valinomycin plus CCCP should mimic the effect of nigericin, allowing the electrically neutral exchange of K^+ and H^+ when potassium is present (Toll and Howard, 1978). As predicted, the potency of CCCP is enhanced

TABLE II
Effect of ligands related to dopamine on [^3H]dopamine binding

Compound	IC_{50}^a
	μM
Reserpine	0.002
Fluphenazine	0.08
Trifluoperazine	0.1
Haloperidol	0.1
Spiperone	0.9
Chlorpromazine	2.0
Dopamine	2.8
ADTN	3
Serotonin	5
(+)-Butaclamol	6
(-)-Butaclamol	8
Amphetamine	10
Epinephrine	17
Norepinephrine	21
Apomorphine	30
Isoproterenol	34
Benztropine	50
Pargyline	>50

^a Concentration of inhibitor giving 50% inhibition of specific [^3H]dopamine binding. The [^3H]dopamine concentration was 1 μM . Nonspecific binding in the presence of all tested agents was equal to nonspecific binding in the absence of drug. Total binding was 10,520 cpm in the absence of 500 μM unlabeled dopamine and 2,760 cpm in its presence.

TABLE III
Effect of ATP-related compounds on [^3H]dopamine binding

Compound	Concentration	Relative [^3H]Dopamine Binding ^a	
		-ATP	+ATP (1 mM)
	mM		
None	1	0.15 $\pm$ 0.05	1.0 $\pm$ 0.06
GTP	1	0.95 $\pm$ 0.07	0.98 $\pm$ 0.07
$\alpha,\beta\text{-CH}_2\text{-ATP}$	1	0.13 $\pm$ 0.10	0.86 $\pm$ 0.06
$\beta,\gamma\text{-CH}_2\text{-ATP}$	1	0.12 $\pm$ 0.07	0.94 $\pm$ 0.03
$\beta,\gamma\text{-NH-ATP}$	1	0.14 $\pm$ 0.05	0.45 $\pm$ 0.05
$\gamma\text{-S-ATP}$	2	0.20 $\pm$ 0.08	0.20 $\pm$ 0.07
ADP	2	0.20 $\pm$ 0.07	0.68 $\pm$ 0.05
	10	0.10 $\pm$ 0.08	0.15 $\pm$ 0.06
AMP	2	0.14 $\pm$ 0.05	0.73 $\pm$ 0.03
	10	0.15 $\pm$ 0.06	0.49 $\pm$ 0.10
Adenosine	2	0.15 $\pm$ 0.05	0.74 $\pm$ 0.07
cAMP	2	0.17 $\pm$ 0	0.62 $\pm$ 0.05
UTP	2	0.22 $\pm$ 0.07	0.83 $\pm$ 0.06
	10	0.28 $\pm$ 0.07	0.42 $\pm$ 0.03
ITP	2	0.24 $\pm$ 0.03	0.90 $\pm$ 0.05
NADH	2	0.16 $\pm$ 0.10	0.90 $\pm$ 0.05
	10	0.18 $\pm$ 0.08	0.81 $\pm$ 0.06
NAD	2	0.15 $\pm$ 0.07	0.77 $\pm$ 0.05
	10	0.10 $\pm$ 0.05	0.20 $\pm$ 0.06
NADPH	2	0.14 $\pm$ 0.06	0.87 $\pm$ 0.10
	10	0.15 $\pm$ 0.06	0.83 $\pm$ 0.08
NADP	2	0.14 $\pm$ 0.05	0.79 $\pm$ 0.06
	10	0.14 $\pm$ 0.09	0.64 $\pm$ 0.07

^a Binding activity relative to that found in the presence of 1 mM ATP. The [^3H]dopamine concentration was 3 μM . Each value is the mean of three determinations $\pm$ SD.

synergistically by the addition of 1 μM valinomycin and 10 mM KCl to the reaction mixture, resulting in an IC_{50} of 7 nM for the inhibition of dopamine binding (Table IV). Ammonium ion is another agent which has been shown to dissipate transmembrane proton gradients by alkalization of the acidic interior of chromaffin granules and other organelles (Johnson et al., 1978; Pollard et al., 1979). Half-maximal inhibition on dopamine binding is observed with 7 mM NH_4Cl . Taken together, the results of these experiments suggest the possibility that ATP-dependent [^3H]dopamine binding requires the maintenance of a transmembrane proton gradient, possibly across the surface of vesiculated membranes.

Orthovanadate acts as an inhibitor for a wide spectrum of enzymes, including several ATPases (Macara, 1980; Sorensen and Mahler, 1981), and is also a potent inhibitor of low affinity dopamine binding (Table IV). Since vanadate can act as an oxidant, control incubations were run in parallel using nonradioactive dopamine, and the mixtures were analyzed for dopamine by liquid chromatography with electrochemical detection (Dayton et al., 1979). There was no significant decrease in the amount of authentic dopamine after incubation with 50 μM sodium orthovanadate. Separate experiments involving

preincubation of membranes with 50 μM vanadate, 3 μM nonradioactive dopamine, and 1 mM ATP followed by washing and incubation with ATP and [^3H]dopamine in the standard assay mixture revealed that there was no effect on binding; therefore, vanadate inhibition is reversible (data not shown). Several other compounds (NEM, NBD-Cl, and DCCD) which covalently modify proteins and are potent inhibitors of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase of the SPM (Sorensen and Mahler, 1981) also inhibit dopamine binding.

Effect of selected inhibitors on adenylate cyclase. The observation that several compounds that usually are not considered to act as dopaminergic antagonists are potent inhibitors of low affinity dopamine binding suggested that it might prove fruitful to determine whether they also would act as inhibitors of the dopamine-dependent adenylate cyclase. As shown in Table V, nigericin plus KCl, CCCP, and sodium orthovanadate at appropriate concentrations completely inhibit [^3H]dopamine binding to SPM, while exerting little or no effect on dopamine-dependent adenylate cyclase activity. Saponin, which can permeabilize cell membranes and membrane vesicles (Endo et al., 1977), reduces [^3H]dopamine binding by 90% while leaving dopamine stimulation of adenylate cyclase unaffected. These agents or treatments operationally dissociate the low affinity binding of [^3H]dopamine from the binding to the receptor responsible for the stimulation of adenylate cyclase. It is therefore highly unlikely that the two entities are identical.

TABLE IV
Inhibitors of [^3H]dopamine binding

Compound	IC_{50}^a μM
Nigericin	>3
Nigericin + 10 mM NaCl	0.02
Nigericin + 10 mM KCl	0.003
CCCP	0.05
CCCP + 10 mM KCl + 1 μM valinomycin	0.007
Sodium orthovanadate	8.0
NaN_3	700
NH_4Cl	7,000
DCCD	50
NEM	200
NBD-Cl	11
Valinomycin + 10 mM KCl	5
Antimycin A	>5
Oligomycin	>5
Efrapreptin	>100
Ouabain	>20,000

^a Concentration giving 50% inhibition of [^3H]dopamine binding. The concentrations of dopamine and ATP were 1.5 μM and 1 mM, respectively.

Discussion

Characteristics of binding reactions. [^3H]Dopamine binds to synaptic plasma membranes from bovine caudate nucleus with a dissociation constant of 0.78 μM and a capacity of 102 pmol mg^{-1} of protein at pH 7.4 in the absence of salt. This binding is completely dependent on the presence of ATP. These characteristics are similar to those of the low affinity dopamine binding in membranes from canine caudate nucleus described by Sano and Maeno (1976), who reported capacities between 16 and 43 pmol mg^{-1} of protein in a crude membrane preparation and 178 pmol mg^{-1} of protein in a purified membrane fraction (Sano et al., 1976b). The ATP-independent negatively cooperative dopamine binding observed in crude membranes from canine caudate (Sano and Maeno, 1976) is absent from SPM of bovine caudate. This may reflect a difference in species or tissue preparation. The results

TABLE V
Inhibitors of [^3H]dopamine binding do not inhibit dopamine-stimulated adenylate cyclase

[^3H]Dopamine binding and adenylate cyclase activation were carried out under the same conditions as described under "Materials and Methods" using 10 μM [^3H]dopamine.

Methods using 10 μM [^3H]dopamine.		Adenylate Cyclase		
Inhibitor	Relative [^3H]Dopamine Binding	–Dopamine	+Dopamine	Dopamine-dependent
		<i>pmol mg⁻¹ protein min⁻¹ $\pm$ SD</i>		
None	1.0 $\pm$ 0.05	88 $\pm$ 4	138 $\pm$ 7	50
Vanadate (50 μM)	N.D. ^a	114 $\pm$ 5	178 $\pm$ 10	65
Nigericin (1 μM) + 100 mM KCl	N.D.	110 $\pm$ 2	150 $\pm$ 7	40
CCCP (10 μM)	N.D.	105 $\pm$ 3	155 $\pm$ 5	50
Saponin (0.02%)	0.09 $\pm$ 0.04	427 $\pm$ 4	474 $\pm$ 11	47

^a N.D., not detectable (<5% of control).

of studies with the inhibitors described here confirm that low affinity dopamine binding does not involve the receptors showing high affinities for either spiperone or dopamine described previously (Near and Mahler, 1981). In addition, there is no detectable low affinity dopamine binding to a subfraction from bovine caudate enriched in postsynaptic membranes (J. A. Near and H. R. Mahler, unpublished results).

Dopamine binding and the dopamine-dependent adenylate cyclase. In agreement with results reported previously (Sano et al., 1979b), GTP at low concentrations shows no effect on [^3H]dopamine binding. Guanine nucleotides are required for the stimulation of adenylate cyclase by hormones or other ligands in many other systems (Limbird, 1981; Ross and Gilman, 1980) as well as in the dopamine-dependent system found in the caudate nucleus (Clement-Cormier et al., 1975; Roufogalis et al., 1976; Kebabian et al., 1979). In other systems, additional effects of guanine nucleotides on agonist binding also have been described (Rodbell et al., 1971; Ross et al., 1977; Kent et al., 1980). In addition, there have been several reports of GTP effects on high affinity binding by dopamine agonists or on the displacement of bound [^3H]spiperone by dopamine (Creese et al., 1978, 1979; Creese and Snyder, 1978; Zahniser and Molinoff, 1978; Sibley and Creese, 1979; Usdin et al., 1980). Therefore, it is not unreasonable to expect that, if low affinity binding is related to activation of adenylate cyclase, GTP would alter the characteristics of binding of dopamine as it does in other systems. Such is clearly not the case.

Additional evidence that fails to support the identification of low affinity dopamine binding with the receptor responsible for stimulation of adenylate cyclase is provided by results with certain inhibitors. The fact that chlorpromazine and fluphenazine are poor inhibitors of dopamine binding but potent inhibitors of its stimulation of adenylate cyclase has been noted previously (Sano et al., 1979b). However, a similar situation holds true for the stereoisomers of butaclamol. The K_i for inhibition of dopamine-dependent adenylate cyclase by (+)-butaclamol is in the low nanomolar range (Iversen, 1975; Hyttel, 1978), while that for its stereoisomer (–)-butaclamol is approximately three orders of magnitude higher. This stereospecific difference in potency is not reflected in the effects of the butaclamol isomers on dopamine binding reported here; both stereoisomers exhibit similar potencies as inhibitors of binding at micromolar concentrations.

The strongest evidence against the involvement of ATP-dependent binding of dopamine on the activation of adenylate cyclase comes from the studies that examine the effects of several inhibitors on both phenomena under identical assay conditions. Their results clearly demonstrate that inhibition of dopamine binding by CCCP, nigericin plus KCl, or vanadate is in no way correlated with inhibition of dopamine-dependent adenylate cyclase and force us to conclude that the two activities are not related.

There exists yet another possible way in which the requirement for ATP may be rationalized in terms of an effect on the cyclase, namely by serving as a phosphate donor in a protein kinase/protein phosphatase system (Richard et al., 1981). Recent evidence suggests that such

a system is present in membranes from rat cerebral cortex, where both ATP-dependent activation of adenylate cyclase and the calcium-dependent phosphorylation of a single protein are inhibited by (–)-isoproterenol (Whittemore et al., 1981). However, the fact that γ -S-ATP does not stimulate dopamine binding to striatal membranes (Table III) rules out the possibility that a similar phosphorylation/dephosphorylation system is involved in the ATP-dependent dopamine binding described here, since this ATP analog can replace ATP in protein phosphorylation reactions (Gratecos and Fischer, 1974; Ehrlich, 1982).

Nature of the system responsible for dopamine binding. If low affinity dopamine binding cannot be explained as a property of an adenylate cyclase-coupled receptor, its requirement for ATP may suggest an alternative hypothesis. The most likely one is that ATP serves as substrate for an ATPase, since nonhydrolyzable analogs of the nucleotide do not stimulate binding. The fact that uncouplers and ionophores, which can dissipate a transmembrane electrochemical gradient, also interfere with this ATP-dependent dopamine binding points to the possibility that the system is coupled to a proton-translocating ATPase and that hydrolysis of ATP is required to generate a pH gradient across the membrane. Such an ATPase is present in the membrane of chromaffin granules (Apps and Glover, 1978; Konings and DePotter, 1980; Flatmark and Ingebretsen, 1977) and probably performs an important function in the uptake of catecholamines by maintaining the interior acidity of the granules (Johnson et al., 1979; Johnson and Scarpa, 1979; Toll and Howard, 1978). In chromaffin granule ghosts, the ATP requirement for catecholamine uptake disappears when an artificial pH gradient (acid inside) is imposed (Schuldiner et al., 1978; Ingebretsen and Flatmark, 1979), and phospholipid vesicles formed in the presence of a cholate extract of the particles will accumulate catecholamines in a reserpine-sensitive manner in response to an artificially imposed pH gradient (Maron et al., 1979). Although the uptake system in synaptic vesicles has not been characterized to the same extent, its mechanism is thought to be similar (Toll and Howard, 1978, 1980).

ATP-dependent dopamine binding exhibits many properties that are similar to those of the catecholamine transport system. Both are sensitive to inhibition by proton ionophores, including valinomycin plus K^+ in the presence of CCCP (Schuldiner et al., 1978; Toll and Howard, 1978, 1980; Apps et al., 1980), and insensitive to inhibitors of mitochondrial ATPase and valinomycin alone (Toll and Howard, 1980; Apps et al., 1980). Nonhydrolyzable ATP analogs cannot replace ATP in either system (Phillips, 1974; Hoffman et al., 1975; Toll et al., 1977) and reserpine is a very potent inhibitor of both (Kirshner, 1962). Finally, orthovanadate, which at micromolar concentrations abolishes ATP-dependent dopamine binding to SPM, also partially inhibits uptake by chromaffin granule ghosts (Apps et al., 1980).

Preliminary experiments with a synaptic vesicle fraction isolated from bovine caudate confirm the presence of an ATP-dependent, reserpine-sensitive, uptake system ($K_d^{\text{app}} = 1.3 \mu\text{M}$; $\beta_{\text{max}} = 45 \text{ pmol mg}^{-1}$). However, comparisons of specific activities based upon the total amount

of dopamine associated with membranes or vesicles at saturation have little meaning in the case of an uptake system. The amount of associated dopamine also will depend on such factors as the enclosed volume of the synaptic or membrane vesicle and the continued integrity of the membrane during the assay. A more accurate determination of the specific activity will require a kinetic analysis of the uptake reaction.

If a similar uptake system is present in the SPM preparation studied here, one obvious requirement is that an interior space be present into which protons and dopamine can accumulate. Such a space can be generated by vesiculation of membrane fragments. An abundance of closed vesicles is found in a similar preparation from rat cortex (Salvaterra and Matthews, 1980). Whether apparent [^3H]dopamine binding reflects binding to a receptor, uptake into membrane vesicles, or both remains unresolved. We have attempted to address this question by examining the effects of saponin, an agent which can permeabilize cells and vesicles (McGraw et al., 1980; Endo et al., 1977). Saponin treatment does inhibit apparent binding, perhaps by allowing free exchange of protons and dopamine between the intravesicular space and the external medium. However, this single result is not sufficient to permit a firm conclusion regarding the nature of apparent dopamine binding. We also have sought to obtain ATP-independent uptake of dopamine into SPM by imposing an artificial transmembrane pH gradient. These attempts were unsuccessful, possibly due to the low buffering capacity of the internal space and the inaccessibility of this space to manipulation from the outside. In analogous experiments by others, chromaffin granule ghosts were resealed or vesicles were formed in the presence of high concentrations of salt or buffer (Ingebrechtsen and Flatmark, 1979; Schuldiner et al., 1978; Maron et al., 1979).

It is also unknown whether apparent dopamine binding activity is normally present in the synaptic plasma membrane *in vivo* or if it arises from contamination by or fusion with other organelles. We have been unable to isolate a fraction enriched in binding activity with a bouyant density significantly different from that of the bulk of the SPM fraction by centrifugation on continuous gradients of Percoll. However, the binding activity described here probably does not reflect the major route by which dopamine is retrieved from the synaptic cleft after release, since synaptosomal dopamine uptake is inhibited by ouabain, amphetamine, and benztropine and specifically requires sodium ion (Coyle and Snyder, 1969; Kuhar, 1973; Wong et al., 1980). The cation effects described here are nonspecific and none of these selective inhibitors are effective in their characteristic concentration range (Tables III and IV). It is also unlikely that dopamine is being taken up in response to a pH gradient generated in a nonspecific manner by a membrane-bound proton-translocating ATPase. Although artificial vesicles will accumulate catecholamines upon imposition of a transbilayer pH gradient (Nichols and Deamer, 1977), this uptake is not saturable and is not susceptible to reserpine inhibition (Maron et al., 1979). Rather, the apparent [^3H]dopamine binding described here most clearly resembles the catecholamine uptake systems of synaptic vesicles and adrenal chromaffin granules. Confirmation that this

activity is indeed correlated with uptake into vesiculated synaptic membranes and explanation of the presence of this activity in such a membrane fraction must await further study.

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