0026-895X/86/030252-06$02.00/0

MOLECULAR PHARMACOLOGY, 30:252-257

[^{3}H]Dihydrotetrabenazine Binding to Bovine Striatal Synaptic Vesicles

JOSEPH A. NEAR

Medical Sciences Program, Indiana University School of Medicine, Bloomington, Indiana 47405

Received January 17, 1986; Accepted June 12, 1986

SUMMARY

[2-^{3}H]Dihydrotetrabenazine (2-hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11bH-benzo[*a*]quinolizine) bound to a single class of binding sites in synaptic vesicles isolated from bovine corpus striatum, with an apparent dissociation constant (K_d) of 2.3 nM and a B_{max} of 15.1 pmol/mg of protein determined at equilibrium. Kinetic determination of the equilibrium dissociation constant yielded a value of 5.4 nM. ATP had no effect on the apparent K_d or B_{max}, nor did it alter the kinetics of association or dissociation. Dopamine, serotonin, and other substrates for transport into synaptic vesicles inhibited binding at concentrations that were several orders of magnitude higher than their K_m values for transport in the presence of ATP. The potent uptake blocker reserpine inhibited binding with a K_i of 340 nM in the absence of ATP, but biphasic inhibition, with K_i values of 3.2 and 345 nM, was observed in the presence of ATP. With incubation times of 24 hr, the potency of reserpine as an inhibitor of binding in the absence of ATP is increased by 1 to 2 orders of magnitude, implying a slow association rate for reserpine in the absence of nucleotide. These results suggest that dihydrotetrabenazine interacts with the catecholamine/serotonin porter in synaptic vesicles, although the binding site is probably not identical to that involved in active transport of substrate.

Synaptic vesicles sequester catecholamines and serotonin by means of an active transport system that is energized by a transmembrane proton electrochemical gradient generated by a proton translocating ATPase (1, 2). The catecholamine uptake system of adrenal medullary chromaffin granules has been studied more extensively and appears to possess similar characteristics. Uptake is dependent on the presence of a transmembrane proton electrochemical gradient and inhibited by low concentrations of reserpine (3–6). *In vivo*, reserpine causes depletion of catecholamine stores that persists until destruction of the granules (7). Tetrabenazine and DH-TBZ exhibit shorter durations of action with respect to catecholamine depletion (8), while also inhibiting catecholamine transport *in vitro* (9).

Binding of [^{3}H]reserpine to adrenal medullary chromaffin granules has been demonstrated *in vivo* (10) and *in vitro* (11, 12), and ATP profoundly affects the characteristics of the latter. Binding of [^{3}H]DH-TBZ to chromaffin granules has also been described, and ATP has no effect on either equilibrium binding constants or binding kinetics (13, 14). Because the central nervous system is a primary target for catecholamine-depleting agents, we have been engaged in the characterization of substrate transport and inhibitor binding by synaptic vesicles isolated from the catecholamine-rich corpus striatum of mammalian brain. [^{3}H]Reserpine binds with high affinity to a single class of sites, and this binding is both ATP dependent and inhibited by the proton ionophore CCCP (15). Transport substrates inhibit binding at concentrations that are comparable to their K_m values for transport. However, once bound, reserpine is not easily displaced by addition of a large excess of substrates, tetrabenazine, or unlabeled reserpine. This report describes binding of [^{3}H]DH-TBZ with high affinity to bovine striatal synaptic vesicles, provides evidence for the localization of binding in synaptic vesicles, and demonstrates that the potency of reserpine as an inhibitor of [^{3}H]DH-TBZ to a fraction of these sites is modulated by ATP.

Materials and Methods

Preparation of synaptic vesicles. Bovine brains were obtained from a local slaughterhouse within 10 min of death, and striata were homogenized immediately in ice-cold 0.32 M sucrose, 5 mM HEPES (pH 7.4), 0.3 mM phenylmethylsulfonyl fluoride; with 8 strokes by hand in a loose-fitting Dounce homogenizer before transportation to the laboratory. Synaptic vesicles and other fractions were prepared as described by Burke and DeLorenzo (16). Briefly, striata were homogenized in a glass homogenizer with a motor-driven Teflon pestle (10 strokes, 500 rpm) and centrifuged at 2,000 × g_{max} to give a nuclear pellet (P_1), and the supernatant was centrifuged at 30,000 × g_{max} for 30 min to yield a synaptosome-rich mitochondrial pellet (P_2). The supernatant was further centrifuged (180,000 × g_{max}, 60 min) to give a crude microsomal fraction (P_3). P_2 was subjected to hypotonic lysis in ice-

This work was supported by Grant NS 20784 awarded by the National Institute of Neurological and Communicative Disorders and Stroke.

ABBREVIATIONS: DH-TBZ, dihydrotetrabenazine; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ester)-*N,N,N′,N′*-tetraacetic acid.

cold distilled water containing 0.3 mM phenylmethylsulfonyl fluoride and homogenized (5 strokes), then diluted with 0.6 volume of buffer to give a final concentration of 160 mM KCl, 5 mM NaCl, and 10 mM Tris maleate (pH 6.5). Centrifugation at 20,000 × g_{max} for 20 min gave the lysed synaptosomal pellet (P_2L). The supernatant was centrifuged for 40 min at 64,000 × g_{max} to yield a coated vesicle-rich pellet (CV). $MgCl_2$ was added to the supernatant to a final concentration of 5 mM and this was centrifuged at 186,000 × g_{max} for 60 min to give a synaptic vesicle-rich pellet (SV). The final vesicle pellet was resuspended by hand homogenization in a loose-fitting Teflon-glass homogenizer in 50 mM potassium phosphate, pH 6,8, 50 mM KCl, 10 mM NaCl, 2 mM EGTA to a protein concentration of 1–2 mg/ml and stored at −80° for up to 3 weeks without appreciable loss of binding activity. The synaptic vesicle fraction has been characterized previously by means of marker enzyme analysis and electron microscopy (17, 18).

Preparation of DH-TBZ. DH-TBZ was prepared and purified by a variation of a procedure described previously (13). Tetrabenazine (Fluka Chemical) was treated with a 2-fold molar excess of $NaBH_4$ in dry methanol at 0° for 3 hr. The reaction was quenched by addition of an equal volume of 0.01 N HCl, methanol was evaporated under reduced pressure, and precipitated DH-TBZ was dried under vacuum. The dried residue was taken up in a small volume of ethyl acetate:acetone (1:1) and chromatographed on a column of silica gel 60 G (EM Reagents) using the same solvent to separate unreacted tetrabenazine and the DH-TBZ isomers. The appropriate fractions were evaporated and DH-TBZ was recrystallized once from methanol. The identity of products was confirmed by thin layer chromatography (silica gel 60, EM Reagents) using the solvent systems and visualizing agent described previously (19). In all studies described here, the faster running isomer I of dihydrotetrabenazine was employed, although isomer II exhibited similar properties with respect to inhibition of dopamine transport and reserpine and DH-TBZ binding.

Preparation of [^{3}H]DH-TBZ. [^{3}H]DH-TBZ (2.9 Ci/mmol) was prepared from tetrabenazine and [^{3}H]$NaBH_4$ (5–15 Ci/mmol, New England Nuclear) and purified by thin layer chromatography as described (13). The faster running isomer I was eluted from the chromatography plate with methanol and stored at −20°. Specific activity was determined by measurement of absorbance at 282 nm in 0.1 N HCl, employing a molar extinction coefficient of 3600. [^{3}H]DH-TBZ was repurified periodically when the amount of radioactivity that did not comigrate with authentic DH-TBZ rose above 2% of the total radioactivity applied to the chromatograph plate.

Dopamine transport and [^{3}H]DH-TBZ binding. Transport of [^{3}H]dopamine and [^{3}H]reserpine binding was determined as described previously (15, 20). IC_{50} values for inhibition of transport activity were obtained from probit plots. All such experiments were carried out under initial rate conditions for dopamine transport with a [^{3}H]dopamine concentration of 3 μM and a K_m for dopamine transport of 3 μM (20). Unless otherwise noted, [^{3}H]DH-TBZ binding was assayed in a total volume of 600 μl, 50 mM potassium phosphate (pH 7.4), 50 mM KCl, 10 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, and 30–45 μg of synaptic vesicle protein for 1 hr at 23°. Ice-cold buffer (4 ml) was then added to the mixture and it was filtered under reduced pressure (Millipore HAWP, 0.45-μm pore size). The filters were washed with two additional 4-ml aliquots of buffer and transferred to scintillation vials, and radioactivity was determined in 10 ml of Aquasol (New England Nuclear). In preliminary experiments binding was observed to be linearly dependent on protein concentration up to 45 μg of vesicle protein/600-μl assay volume at 3 nM [^{3}H]DH-TBZ. Under these conditions, total binding was 505 ± 5 cpm, nonspecific binding was 42 ± 7 cpm, and specific binding was 463 cpm when 32 μg of synaptic vesicle protein were employed. Apparent dissociation constants for [^{3}H]DH-TBZ binding and inhibitory constants for various other compounds were obtained by weighted nonlinear least squares analysis (21). Where necessary, drugs were first dissolved in dimethyl sulfoxide or methanol, diluted with buffer, then added to the mixture to give a final vehicle concentration of less than 0.1%, which had no effect on binding or transport activity. B_{max} values are all expressed in terms of mg of protein, determined by the method of Markwell *et al.* (22).

Results

Concentration dependence of [^{3}H]DH-TBZ binding. The concentration dependence of [^{3}H]DH-TBZ binding to bovine striatal synaptic vesicles is shown in Fig. 1 (*inset*). Nonspecific binding, defined as the amount of filter-bound radioactivity observed after incubation in the presence of 10 μM tetrabenazine, was linearly dependent on [^{3}H]DH-TBZ concentration and was generally less than 15% of total binding at 50 nM. Preliminary experiments showed that inclusion of 10 μM tetrabenazine in the wash buffer was only slightly beneficial in lowering nonspecific binding at all protein concentrations tested (data not shown). Because this might have compromised accurate determination of equilibrium binding constants, this technique was not deemed necessary or desirable.

A linear Scatchard plot of the data (Fig. 1) implies a single class of binding sites for [^{3}H]DH-TBZ in striatal synaptic vesicles. Nonlinear least squares curve fitting of the untransformed data yielded an apparent dissociation constant of 2.1 nM and a site density of 13.2 pmol/mg of protein in this experiment. In three independent experiments on fresh synaptic vesicles, an apparent dissociation constant of 2.3 ± 0.5 nM and a B_{max} of 15.1 ± 1.1 pmol/mg were observed. Storage of synaptic vesicles at −80° for 2 weeks had little effect on binding parameters (K_d^{app} = 3.3 ± 0.7 nM, B_{max} = 13.7 ± 1.3 pmol/mg, n = 4). In all determinations of binding parameters, attempts to resolve the data into two classes of sites by means of a nonlinear least squares curve-fitting method were unsuccessful, yielding a best fit to a single class of binding sites.

Identification of bound and unbound radioactivity. In order to ascertain the chemical form of bound radioactivity,

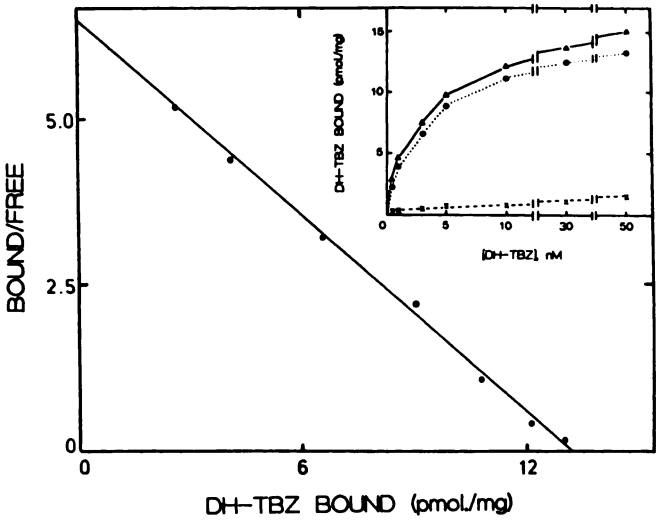

Fig. 1. Concentration dependence of [^{3}H]DH-TBZ binding. [^{3}H]DH-TBZ binding was determined in the absence of ATP as described in Materials and Methods. Each assay tube contained 32 μg of synaptic vesicle protein. The Scatchard plot shown was obtained by weighted nonlinear least squares analysis of the data, with the *line* indicating the best fit to the data. Each *point* is the mean of three determinations with standard deviation less than 11% of the indicated value. *Inset:* Nonspecific binding (×) was determined by inclusion of 10 μM unlabeled tetrabenazine in the assay. Specific binding (●) was calculated by subtraction of nonspecific binding from total binding (▲) determined in the absence of excess unlabeled tetrabenazine.

synaptic vesicles were incubated with 5 nM [^{3}H]DH-TBZ for 1 hr, then pelleted (130,000 × *g*, 60 min). The pellet was extracted with ethanol, and this extract was subjected to thin layer chromatography. Greater than 97% of the specifically bound radioactivity comigrated with authentic DH-TBZ. Similarly, 96% of the unbound radioactivity present in the supernatant was identified as DH-TBZ by thin layer chromatography.

[^{3}H]DH-TBZ is not transported. Because tetrabenazine and its derivative DH-TBZ are inhibitors of catecholamine and serotonin transport in synaptic vesicles, it is possible that [^{3}H]DH-TBZ may be a substrate for transport. In order to rule out this possibility, vesicles were exposed to the membrane-permeabilizing agent saponin prior to determination of [^{3}H]DH-TBZ binding. This treatment releases vesicle contents such as accumulated dopamine and completely inhibits [^{3}H]dopamine transport (15). However, in two experiments, saponin had little effect on [^{3}H]DH-TBZ binding (K_d^{app} = 2.0 ± 0.4 nM, B_{max} = 13.1 ± 0.8 pmol/mg), suggesting that vesicle-associated DH-TBZ was bound and not transported to an appreciable extent.

ATP dependence of [^{3}H]DH-TBZ binding. ATP is absolutely required for dopamine transport by synaptic vesicles. In addition, reserpine binding to synaptic vesicles (15) and to adrenal chromaffin granules (11) is markedly affected by the presence of ATP. However, ATP had no effect on the apparent dissociation constant of B_{max} for [^{3}H]DH-TBZ binding to striatal vesicles (K_d^{app} = 3.1 ± 0.9 nM, B_{max} = 14.0 ± 1.7 pmol/mg) in three experiments.

Kinetic analyses of [^{3}H]DH-TBZ binding. The apparent dissociation constant for [^{3}H]DH-TBZ binding was derived kinetically (23). The dissociation rate constant (6.54×10^{-4} S^{-1}) was obtained as the slope when $\ln([B]/[BT])$ was plotted as a function of time (Fig. 2, *top*), where $[B]$ is the concentration of bound ligand at time T, and $[BT]$ is the concentration of bound ligand at zero time. The association rate constant was obtained using the relation $\ln([B_{eq}]/([B_{eq}]-[B])) = ([L]k_1 + k_{-1})T$ where $[L]$ is the concentration of [^{3}H]DH-TBZ, $[B_{eq}]$ is the concentration of bound ligand at equilibrium, and $[B]$ is the concentration of bound ligand at time T. A slope of 2.8×10^{-3} S^{-1} was obtained from Fig. 2 (*bottom*), and an association rate constant of 1.21×10^6 M^{-1} S^{-1} was calculated. The equilibrium dissociation constant of 5.4×10^{-9} M was calculated from the relation $K_d = k_{-1}/k_1$, and this is in good agreement with values obtained from equilibrium binding studies. Inclusion of 3 mM ATP in assays had no effect on association or dissociation rates (data not shown).

Enrichment of [^{3}H]DH-TBZ binding in synaptic vesicles. Various fractions obtained from the vesicle preparation were examined for their ability to bind [^{3}H]DH-TBZ in order to ascertain the subsynaptic location of binding activity. The greatest enrichment was observed in the synaptic vesicle fraction, with a specific activity approximately 4-fold higher than that of the homogenate (Table 1). Appreciable enrichment was also seen in the coated vesicle-rich fraction as well as the microsomal and synaptosomal fractions. These findings imply that [^{3}H]DH-TBZ binding activity is associated with synaptic vesicles in preference to other subsynaptic structures or organelles.

Inhibition of binding by transport substrates. Tetrabenazine and DH-TBZ inhibited [^{3}H]DH-TBZ binding with IC_{50} equivalent to their IC_{50} values for inhibition of [^{3}H]dopamine transport (Table 2). In the absence of ATP, however,

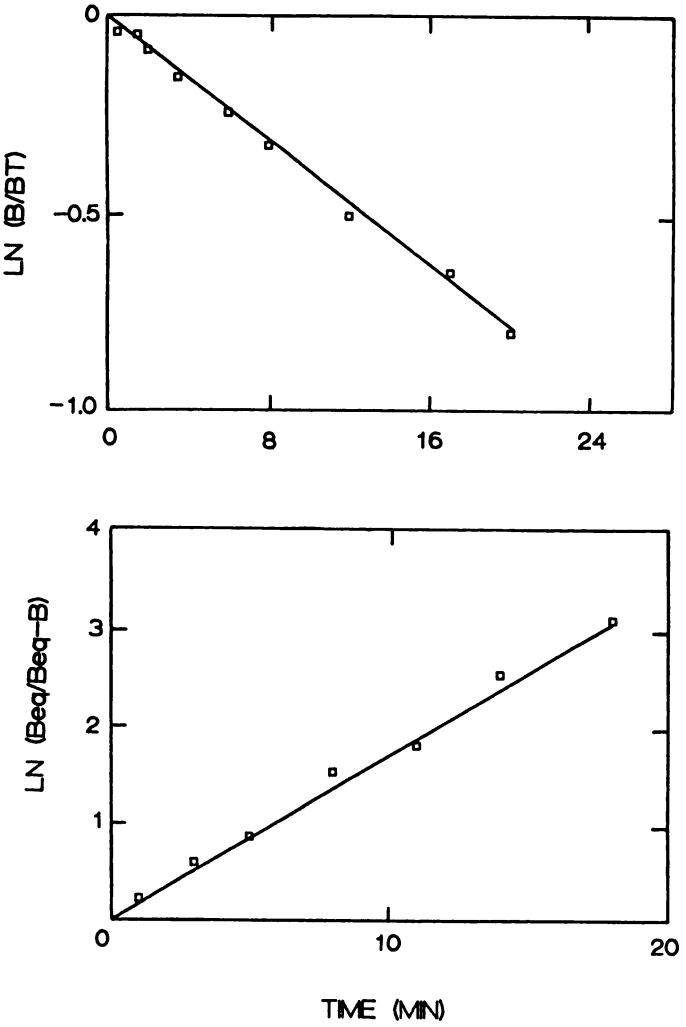

Fig. 2. Kinetics of [^{3}H]DH-TBZ binding. *Top:* Dissociation of [^{3}H]DH-TBZ. Synaptic vesicles (42 μg) were incubated with 10 nM [^{3}H]DH-TBZ for 2 hr, then nonradioactive DH-TBZ was added in a small volume to give a final concentration of 10 μM. Aliquots of the reaction mixture were filtered at various times for determination of the amount of remaining bound radioactivity. Nonspecific binding was constant throughout the period and was approximately 4% of the total binding at zero time. Each *point* is the mean of three determinations and was corrected for nonspecific binding. *Bottom:* Association of [^{3}H]DH-TBZ. Synaptic vesicles (42 μg) were incubated for various lengths of described in Materials and Methods. Each *point* is the mean of three determinations and was corrected for nonspecific binding. Nonspecific binding was constant throughout the period and was less than 5% of total binding. B_{eq} was determined after 2 hr of incubation and was taken as the equilibrium value.

transport substrates and reserpine exhibited IC_{50} values for inhibition of binding that are 2–3 orders of magnitude higher than those for inhibition of dopamine transport. This result is markedly different from that obtained for inhibition of reserpine binding reported previously for synaptic vesicles (15) and adrenal chromaffin granules (11), where a good agreement was obtained between inhibitory potencies for substrate transport and [^{3}H]reserpine binding when both were determined in the presence of ATP. However, [^{3}H]DH-TBZ binding did exhibit stereospecificity for transport substrates, as indicated by the 2-fold higher potency of (−)-norepinephrine when compared with (+)-norepinephrine. A similar ratio of potencies is observed for inhibition of [^{3}H]dopamine transport and [^{3}H]reserpine binding, although the absolute potencies of both stereoisomers are

TABLE 1

Binding of [^{3}H]DH-TBZ to subsynaptic fractions

Crude subsynaptic fractions were prepared from bovine corpus striatum as described in Materials and Methods. Specific binding of 1 nM [^{3}H]DH-TBZ was determined as described in Materials and Methods. Each assay tube contained 50 μg of protein in a total volume of 2 ml. Specific binding to the indicated fraction was divided by the specific binding to the homogenate in order to obtain relative binding. Each value is the mean ± standard deviation of six determinations on two separate preparations.

Fraction	Relative binding
SV	3.89 ± 0.55
CV	2.34 ± 0.31
P_3	1.69 ± 0.24
P_2L	1.12 ± 0.13
P_2	1.76 ± 0.47
P_1	0.48 ± 0.09
Homogenate	1.00 ± 0.12

TABLE 2

Inhibition of transport and binding by various agents

Indicated activities were determined in the presence of at least seven different concentrations of the indicated inhibitors, and IC_{50} values were obtained from probit analysis after correction for nonspecific binding as described in Materials and Methods. Protein concentrations ranged from 35 to 41 μg per assay.

Agent	IC_{50}		
	Dopamine uptake	Reserpine binding	DH-TBZ binding
		μM	
Reserpine	0.004	0.003	0.31
Tetrabenazine	0.007	0.020	0.0065
DH-TBZ	0.010	0.015	0.008
Serotonin	2.6	5.9	2200
Dopamine	3.7	6.6	5500
Epinephrine	3.6	8.4	8800
(−)-Norepinephrine	4.1	9.2	4600
(+)-Norepinephrine	8.8	17.7	9400
(+)-Butaclamol	>5	>5	>5
Spiperone	>10	>10	>10

much higher (15). It is unlikely that [^{3}H]DH-TBZ binding reflects labeling of dopamine receptors because the dopamine receptor antagonists (+)-butaclamol and spiperone had no effect on [^{3}H]DH-TBZ binding at concentrations below 5 and 10 μM, respectively.

Modulation of reserpine potency by time and ATP. The modulation of [^{3}H]reserpine binding to synaptic vesicles and chromaffin granules by ATP has been described previously (11, 15). In order to determine whether ATP has any effect on the ability of reserpine to inhibit [^{3}H]DH-TBZ binding, synaptic vesicles were incubated with various concentrations of unlabeled reserpine and 5 nM [^{3}H]DH-TBZ in the presence or absence of ATP for 60 min. In the absence of ATP a smooth monophasic inhibition curve was observed that was fitted to a single class of binding sites with K_i^{app} = 340 nM, assuming a K_d of 2.7 nM for DH-TBZ binding and competitive inhibition by reserpine (Fig. 3). Incubation in the presence of ATP resulted in a biphasic inhibition curve, indicating two classes of sites with apparent K_i values of 3.2 nM and 345 nM. Although similar K_i values were obtained in five different preparations, the fraction of binding sites with higher apparent affinity for reserpine was highest (66%) in freshly isolated synaptic vesicles and reduced considerably (31%) in vesicles subjected to freeze-thaw (Table 3). Inclusion of saponin caused a further reduction in the fraction of binding that was sensitive to low concentrations of reserpine. This effect was most likely due to dissipation of the ATPase-generated transmembrane electrochemical proton gradient that is required for high affinity reserpine binding.

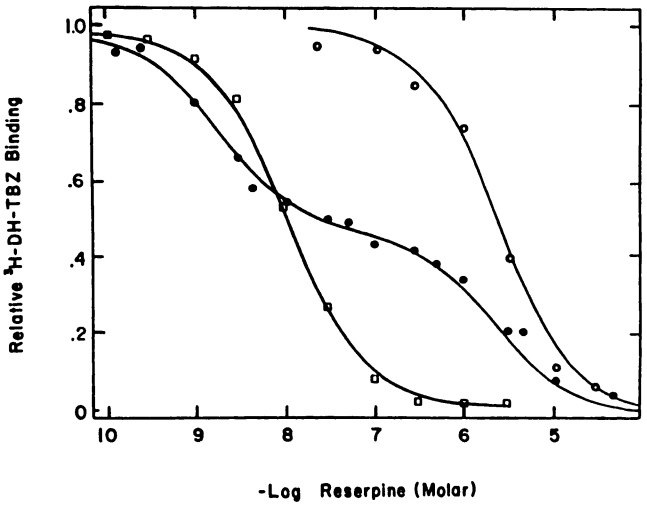

Fig. 3. Reserpine inhibition of [^{3}H]DH-TBZ binding. Synaptic vesicles were incubated with the indicated concentrations of unlabeled reserpine and 5 nM [^{3}H]DH-TBZ in the presence (●) and absence (○) of 2 mM ATP for 1 hr, or in the absence of ATP for 24 hr (□). Each *point* is the mean of two determinations with standard deviation of less than 6%, and was corrected for nonspecific binding. Nonspecific binding was unaffected by reserpine and was 3% of total binding in the absence of reserpine. The *curves* indicate the best fits to the data as determined by nonlinear least squares analysis, assuming competitive inhibition of [^{3}H]DH-TBZ by reserpine in all cases and a dissociation constant for [^{3}H]DH-TBZ binding of 2.3 nM. Protein concentrations were between 40 and 45 μg/650 μl.

TABLE 3

Effect of ATP on inhibitory potency of reserpine

Freshly isolated or freeze-thawed synaptic vesicles were incubated in duplicate with 5 nM [^{3}H]DH-TBZ for 1 hr with 12 concentrations of reserpine under conditions described in Materials and Methods. When present, ATP concentration was 3 mM and saponin was 0.1% (w/v). R_1 and R_2 represent B_{max} values for high affinity and low affinity reserpine inhibition, respectively, when data were subjected to nonlinear least squares curve fitting. Independent fits were performed for each condition, and a single K_d = 2.3 nM for DH-TBZ was assumed. Apparent K_i values for reserpine ranged from 0.2 to 1.1 nM for inhibition of binding to R_1 and 110 to 630 nM for inhibition of binding to R_2.

Preparation	Treatment	R_1	R_2
		pmol/mg	
Fresh	none	0.0[a]	12.6
	+ATP	8.1	4.0
	+ATP, saponin	1.7	12.5
	+ATP, CCCP	2.3	13.1
Freeze-thaw	none	0.0	13.4
	+ATP	4.2	9.5
	+ATP, saponin	1.1	12.6

[a] Best fit was to a single class of binding sites.

Although ATP did not increase the total number of [^{3}H]DH-TBZ-binding sites and had no effect on their affinity for DH-TBZ, it clearly did increase the sensitivity of a fraction of these sites to inhibition by reserpine.

Scherman and Henry (12) have shown that ATP serves to accelerate the binding of [^{3}H]reserpine to adrenal chromaffin granules. Because equilibration of reserpine in the absence of ATP may not be complete within 1 hr, the potency of reserpine as an inhibitor of [^{3}H]DH-TBZ binding was also assessed at longer incubation times. Under these conditions a monophasic inhibition curve was obtained with a dramatic increase in reserpine potency (K_i^{app} = 6.2 ± 5.1 nM, n = 3) when compared with the 60-min incubation (Fig. 3). Similar apparent inhibitory constants were obtained when the concentration of [^{3}H]DH-TBZ was 1 nM or 20 nM. Inclusion of ATP had little effect on

the potency of reserpine in assays carried out for 24 hr (not shown). Scatchard plots of [^{3}H]DH-TBZ binding were similar for incubation times of 1, 12, and 24 hr, although a decrease in B_{max} was observed when incubation times exceeded 24 hr. These findings are consistent with previous observations that the association of reserpine in the absence of ATP is very slow.

Discussion

Results described here imply a single class of binding sites for [^{3}H]DH-TBZ in preparations of synaptic vesicles from bovine corpus striatum. Binding was saturable and reversible, was unaffected by the presence of ATP, and the apparent dissociation constants obtained from both kinetic and equilibrium determinations agree well with the inhibitory potency of DH-TBZ determined in transport experiments described here, and with dissociation constants observed previously for [^{3}H] DH-TBZ binding to adrenal medullary chromaffin granules (14). Because binding was independent of ATP and was unaffected by the membrane-permeabilizing agent saponin, it is unlikely that [^{3}H]DH-TBZ is a substrate for transport. DH-TBZ had no effect on the proton translocating ATPase that energizes transport in synaptic vesicles,[1] hence binding and inhibition of substrate transport cannot be attributed to interaction with this enzyme. Although tetrabenazine has been previously shown to act as a dopamine receptor antagonist and can compete with the dopamine antagonist spiperone for binding to striatal dopamine receptors (24), the concentrations required (0.5–5 μM) are several orders of magnitude higher than those that inhibited [^{3}H]DH-TBZ binding to striatal synaptic vesicles. Finally, the highest specific activity for DH-TBZ binding was found in a synaptic vesicle fraction. These findings strongly suggest that [^{3}H]DH-TBZ-binding sites are associated with the catecholamine/serotonin transporter in synaptic vesicles from bovine corpus striatum.

However, a number of discrepancies are apparent upon closer examination of these results. One of these is the lack of agreement between the number of [^{3}H]DH-TBZ-binding sites detected here and the number of [^{3}H]reserpine-binding sites observed previously in the same vesicle preparation. [^{3}H]Reserpine labels less than one-quarter of the number of [^{3}H]DH-TBZ-binding sites in striatal vesicles (15). This may be explained in part by differences in the assay procedure. In order to minimize nonspecific binding, reserpine binding is performed using a gel filtration column to spearate bound from free ligand, and a filter assay was used for DH-TBZ binding. Whereas approximately 95% of protein applied to filters was retained, less than 65% of applied protein is eluted in the void volume from gel filtration columns. In addition, reserpine binding at short incubation times is ATP dependent and inhibited by agents that render membranes permeable to protons, indicating a requirement for the presence of a transmembrane proton electrochemical gradient. [^{3}H]DH-TBZ exhibited no such requirement and therefore may bind to leaky vesicles and vesicle fragments that cannot support such a gradient.

Although ATP had no effect on [^{3}H]DH-TBZ binding, the nucleotide did alter reserpine inhibition of [^{3}H]DH-TBZ binding to striatal vesicles. In the absence of ATP a monophasic inhibition curve indicated a single K_i^{app} in the range of 100–500 nM reserpine, whereas addition of ATP caused a shift in the potency of reserpine at a fraction of the sites to the nM range when binding was carried out for 1 hr. This ATP-dependent increase in reserpine potency was sensitive to membrane-permeabilizing agents and proton ionophores and, thus, may be related to the ATP dependence of high affinity [^{3}H]reserpine binding reported previously (11, 15). In addition, increasing the duration of the incubation to 24 hr enhanced the potency of reserpine as an inhibitor of [^{3}H]DH-TBZ binding, yielding monophasic inhibition curves with K_i values in the nM range in the presence and absence of ATP. It is therefore likely that, with short incubation times, ATP accelerates the association of reserpine with intact vesicles and, thereby, enhances its potency as an inhibitor of [^{3}H]DH-TBZ binding to such vesicles. ATP cannot similarly affect the association of reserpine with leaky vesicles that are unable to maintain an electrochemical gradient, and much longer incubation times are required before equilibrium of these vesicles with the inhibitor is achieved. This would account for the low potency of reserpine in the absence of ATP, biphasic inhibition curves in the presence of ATP, and uniformly high potency regardless of the presence of the nucleotide with long incubation times. Scherman and Henry (12) have observed two classes of [^{3}H]reserpine-binding sites in adrenal chromaffin granules. Although ATP had little or no effect on equilibrium binding constants, ATP did accelerate binding kinetics.

Another apparent discrepancy in results presented here involves the large differences between the potencies of transport substrates as inhibitors of dopamine transport and their potencies as inhibitors of [^{3}H]DH-TBZ binding. The same situation appears to prevail in chromaffin granules, where serotonin and norepinephrine are more potent inhibitors of transport than of [^{3}H]DH-TBZ binding by several orders of magnitude (14). Several explanations for this phenomenon are possible. First, the inhibitory potencies of transport substrates for inhibition of [^{3}H]DH-TBZ binding were determined in the absence of ATP, and those for inhibition of transport were necessarily determined in the presence of ATP. Thus, the IC_{50} for inhibition of [^{3}H]DH-TBZ binding may reflect the dissociation constant for binding to the substrate-binding site of the unenergized transporter. A second alternative is that DH-TBZ and transport substrates interact with physically distinct sites on the transporter. This possibility is supported by the observation that different molecular species are labeled by photoaffinity analogs of tetrabenazine and the substrate serotonin, implying that the catecholamine/serotonin porter comprises at least two nonidentical subunits with separate binding sites for substrate and tetrabenazine congeners (25, 26).

Examination of the type of inhibition exhibited by various substrates and inhibitors in binding and transport assays may be useful in understanding the roles of various inhibitor-binding sites. Although catecholamines are competitive inhibitors of [^{3}H]DH-TBZ binding in the absence of ATP (27) with K_i values in the mM range, tetrabenazine and DH-TBZ are not simple competitive inhibitors of dopamine transport in synaptic vesicles.[2] Because DH-TBZ does not act by binding exclusively to the same enzyme form as the substrate, agreement between the K_m for substrate transport and the K_i for inhibition of DH-TBZ binding by substrates is not to be expected. In contrast, reserpine has been shown to be a competitive inhibitor of

[1] J. Near, unpublished results.

[2] J. A. Near and S. D. Detwiler, manuscript in preparation.

substrate transport (4, 28), and its binding at nM concentrations in the presence of ATP is inhibited by substrates with K_i values equivalent to their K_m values for transport. Therefore, reserpine binding probably takes place at the substrate-binding site. Whether DH-TBZ or substrates are competitive inhibitors of [^{3}H]reserpine binding, and the type of inhibition exhibited by reserpine and substrates in [^{3}H]DH-TBZ binding assays with or without ATP, are subjects of current investigation.

References

1. Toll, L., C. B. Gundersen, Jr., and B. D. Howard. Energy utilization in the uptake of catecholamines by synaptic vesicles and adrenal chromaffin granules. *Brain Res.* **136**:59–66 (1977).
2. Seidler, F. J., D. F. Kirksey, C. Lau, W. L. Whitmore, and T. A. Slotkin. Uptake of (−)-^{3}H-norepinephrine by storage vesicles prepared from whole rat brain: properties of the uptake system and its inhibition by drugs. *Life Sci.* **21**:1075–1086 (1977).
3. Kirschner, N. Uptake of catecholamines by a particulate fraction of the adrenal medulla. *J. Biol. Chem.* **237**:2311–2317 (1962).
4. Phillips, J. H. Transport of catecholamines by resealed chromaffin-granule "ghosts." *Biochem. J.* **144**:319–325 (1974).
5. Schuldiner, S., H. Fishkes, and B. I. Kanner. Role of a transmembrane pH gradient in epinephrine transport by chromaffin granule membrane vesicles. *Proc. Natl. Acad. Sci. USA* **75**:3713–3716 (1978).
6. Carlsson, A., N. A. Hillarp, and B. Waldeck. Analysis of the Mg^{++}-ATP dependent storage mechanism in the amine granules of the adrenal medulla. *Acta Physiol. Scand.* **59**:1–38 (1963).
7. Lundborg, D., and R. E. Stitzel. Effects of reserpine and monoamine oxidase inhibitors on the uptake and subcellular distribution of ^{3}H-adrenaline. *Br. J. Pharmacol.* **33**:98–104 (1968).
8. Pletcher, A., A. Brossi, and K. F. Gey. Benzoquinolizine derivatives: a new class of monoamine decreasing drugs with psychotropic action. *Int. Rev. Neurobiol.* **4**: 275–290 (1962).
9. Scherman, D., and J.-P. Henry. Effect of drugs on the ATP-induced and pH gradient-driven monamine transport by bovine chromaffin granules. *Biochem. Pharmacol.* **29**:1883–1890 (1980).
10. Giachetti, A., R. Hollenbeck, and P. Shore. Localization and binding of reserpine in the membrane of adrenomedullary amine storage granules. *Naunyn-Schmiedebergs Arch. Pharmacol.* **283**:263–280 (1974).
11. Deupree, J. D., and J. A. Weaver. Identification and characterization of the catecholamine transporter in bovine chromaffin granules using [^{3}H]reserpine. *J. Biol. Chem.* **259**:10907–10912 (1984).
12. Scherman, D., and J.-P. Henry. Reserpine binding to bovine chromaffin granule membranes: characterization and comparison with dihydrotetrabenazine binding. *Mol. Pharmacol.* **25**:113–122 (1984).
13. Scherman, D., P. Jaudon, and J.-P. Henry. Liaison d'un derive de la tetrabenazine au transporteur des monoamines de la membrane des granules chromaffines. *C. R. Acad. Sci. Paris* **293**:221–224 (1981).
14. Scherman, D., P. Jaudon, and J. P. Henry. Characterization of the monoamine carrier of chromaffin granule membrane by binding of [2-^{3}H]dihydrotetrabenazine. *Proc. Natl. Acad. Sci. USA* **80**:584–588 (1982).
15. Near, J. A., and H. R. Mahler. Reserpine labels the catecholamine transporter in synaptic vesicles from bovine caudate nucleus. *FEBS Lett.* **158**:31–35 (1983).
16. Burke, B. E., and R. J. DeLorenzo. Calcium ion and calmodulin-dependent phosphorylation of endogenous synaptic vesicle tubulin by a vesicle-bound calmodulin kinase system. *J. Neurochem.* **38**:1205–1218 (1982).
17. DeLorenzo, R. J., and S. D. Freedman. Calcium-dependent phosphorylation of synaptic vesicle proteins and its possible role in mediating neurotransmitter release and vesicle function. *Biochem. Biophys. Res. Commun.* **77**:1036–1043 (1977).
18. Near, J. A., and H. R. Mahler. Properties of the bovine striatal synaptic vesicle ATPase. *Biochem. Int.* **8**:265–270 (1984).
19. Schwartz, D. E., H. Bruderer, J. Rieder, and A. Brossi. Metabolic studies of tetrabenazine, a psychotropic drug in animals and man. *Biochem. Pharmacol.* **15**:645–655 (1966).
20. Kuhr, W. G., A. G. Ewing, J. A. Near, and R. M. Wightman. Amphetamine attenuates the stimulated release of dopamine *in vivo*. *J. Pharmacol. Exp. Ther.* **232**:388–394 (1984).
21. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–239 (1980).
22. Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87**:206–210 (1978).
23. Kitabgi, P., R. Carraway, J. Van Rietchoten, C. Granier, J. L. Morgat, A. Menez, S. Leeman, and P. Freychet. Neurotensin: specific binding to synaptic membranes from rat brain. *Proc. Natl. Acad. Sci. USA* **74**:1846–1850 (1977).
24. Login, I. S., M. J. Cronin, and R. M. MacLeod. Tetrabenazine has properties of a dopamine receptor antagonist. *Ann. Neurol.* **12**:257–262 (1982).
25. Isambert, M.-F., and J.-P. Henry. Photoaffinity labeling of the tetrabenazine binding sites of bovine chromaffin granule membranes. *Biochemistry* **24**:3660–3667 (1985).
26. Gabizon, R., T. Yetinson, and S. Schuldiner. Photoinactivation and identification of the biogenic amine transporter in chromaffin granules from bovine adrenal medulla. *J. Biol. Chem.* **257**:15145–15150 (1982).
27. Scherman, D., and J.-P. Henry. The catecholamine carrier of bovine chromaffin granules form of the bound amine. *Mol. Pharmacol.* **23**:431–436 (1983).
28. Kanner, B. I., H. Fishkes, R. Maron, I. Sharon, and S. Schuldiner. Reserpine is a competitive and reversible inhibitor of the catecholamine transporter of bovine chromaffin granules. *FEBS Lett.* **100**:175–178 (1979).

Send reprint requests to: Dr. Joseph A. Near, Departments of Pharmacology and Medical Sciences, Myers Hall, Indiana University, Bloomington, IN 47403.