Reserpine labels the catecholamine transporter in synaptic vesicles from bovine caudate nucleus

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Tritiated reserpine binds to synaptic vesicles from bovine caudate with high affinity (K_d^{app} = 1.25 nM, B_{max} = 3.3 pmol/mg protein). This interaction is both ATP-dependent and sensitive to the protonophores CCCP and nigericin, suggesting that a proton electrochemical gradient is required for binding. Dopamine, epinephrine, norepinephrine and serotonin all inhibit reserpine binding at concentrations similar to those required for inhibition of dopamine uptake. Treatment with saponin to release vesicle contents results in complete loss of accumulated dopamine but retention of bound reserpine. These results indicate that reserpine binds to the catecholamine transport system of synaptic vesicles with high affinity and specificity.

Reserpine *Synaptic vesicle* *Dopamine uptake*

1. INTRODUCTION

A great deal of attention has been focused on the ATP-dependent uptake of catecholamines into synaptic vesicles and chromaffin granules, but little is known concerning the exact nature of the transport system itself. Reserpine is a specific competitive inhibitor of both systems [1–4] with K_i for inhibition of uptake over 1–10 nM. Although the in vivo binding of reserpine to chromaffin granules had been shown in [5], the specific interaction of radiolabeled reserpine with the adrenal chromaffin granule in vitro was reported in [6]. ATP and magnesium were required for binding and the uncoupler FCCP inhibited the interaction. We now describe the ability of this compound to bind with high affinity and selectivity to synaptic vesicles isolated from bovine caudate nucleus and present evidence that this binding reflects specific labeling of sites involved in catecholamine transport.

Abbreviations: APPNHP, β-imidoadenosine 5′-triphosphate; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; EGTA, ethyleneglycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PMSF, phenylmethyl sulfonyl fluoride

2. MATERIALS AND METHODS

[^{3}H]Dopamine (37 Ci/mmol) and [^{3}H]reserpine (27.9 Ci/mmol) were obtained from New England Nuclear (Boston MA). All other materials were of the highest quality available.

Bovine caudates were obtained within 10 min of death and homogenized immediately in ice cold 0.32 M sucrose, 0.3 mM PMSF with 4 strokes by hand in a loose-fitting Dounce homogenizer before transportation to the laboratory. The inclusion of PMSF was essential to inhibit protease activity. Synaptic vesicles were prepared as in [7], and their identity confirmed by electron microscopy and determination of neurotransmitter content [8]. They contained 50 ± 15 pmol dopamine/mg vesicle protein, and analysis of their protein composition by SDS–polyacrylamide gel electrophoresis under denaturing conditions demonstrated only very low levels of contamination by coated vesicles as judged by clathrin content. The final vesicle pellet was taken up in 50 mM KPO_4 (pH 7.4), 50 mM KCl, 10 mM NaCl, 2 mM EGTA at 1–2 mg protein/ml and stored at −80°C for up to 1 week before use. Vesicles stored in this way ex-

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hibited 70–80% of the dopamine uptake activity present in fresh vesicles.

The association of tritiated reserpine and dopamine with synaptic vesicles was determined by a modification of the method in [9]. A 100 μl solution containing 50 mM KPO_4 (pH 7.4), 50 mM KCl, 10 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, 0.02% ascorbate, 2 mM ATP, 5–40 μg protein and the appropriate concentration of radiolabeled reserpine or dopamine was incubated at 23°C for 10 min, then chilled rapidly in an ice bath; 50 μl of the same buffer without ATP or magnesium, containing 5 mg/ml blue dextran-2000 was added and 100 μl of the mixture placed on an 8 cm column of Sephadex G-50–80 in a Pasteur pipette and eluted with the same buffer. All fractions with blue color were collected in scintillation vials, 10 ml Aquasol (New England Nuclear) added and radioactivity determined. Specific binding of reserpine was taken as the difference between values obtained in the presence and absence of 2 μM non-radioactive reserpine. Similarly, specific dopamine uptake was defined as the difference between values obtained in the presence and absence of 100 nM cold reserpine. Non-specific binding was <25% of total binding for reserpine, and <15% for dopamine. Unless otherwise indicated, binding activities are expressed as pmol/mg protein, determined as in [10].

3. RESULTS

3.1. *The catecholamine transporter binds reserpine with high affinity*

A Scatchard plot of the binding of tritiated reserpine to synaptic vesicles from bovine caudate in the presence of ATP is shown in fig.1. The ligand binds with an app. K_d of 1.3 nM, and 3.3 pmol/mg protein are bound at saturation. This apparent affinity compares favorably with the IC_{50} for inhibition of dopamine uptake by reserpine determined under the same conditions (4 nM), whereas the extent of binding at saturation is several orders of magnitude lower than that of dopamine uptake (1–2 nmol/mg protein; unpublished). If reserpine interacts with the transporter responsible for uptake of catecholamines, dopamine and other members of this class of transmitters should act as inhibitors of reserpine binding at concentrations comparable to

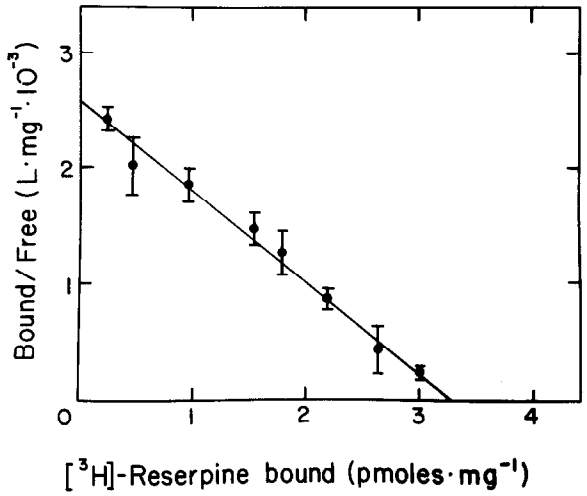

Fig.1. Scatchard plot of specific reserpine binding to bovine caudate synaptic vesicles in the presence of 2 mM ATP. Each point is the mean of 4 determinations ± SD. Non-specific binding was linearly dependent on the concentration of radioactive ligand, and was <25% of total binding at up to 12 nM.

those required for inhibition of dopamine uptake. All such candidates tested exhibit inhibitory potencies in the micromolar range (table 1), suggesting that amine uptake and reserpine binding occur by a common mechanism since these compounds also

Table 1

Inhibition of reserpine binding and dopamine uptake[a]

Compound	Dopamine uptake IC_{50} (μM)	Reserpine binding IC_{50} (μM)
Reserpine	0.004	0.003
Tetrabenazine	0.007	0.020
Harmaline	0.10	0.22
Dopamine	3.7	6.6
Epinephrine	3.6	8.4
(−)Norepinephrine	4.1	9.2
(±)Norepinephrine	8.8	17.7
Serotonin	2.6	5.9

[a] Assay mixtures contained 2 mM ATP and either 1.5 nM reserpine or 3 μM dopamine

IC_{50} was defined as the concentration of inhibitor required for 50% inhibition of specific binding. Non-specific binding in the presence of all agents tested was equal to non-specific binding in the absence of agent

inhibit dopamine uptake at similar concentrations. Inhibition of both dopamine uptake and reserpine binding by norepinephrine is also stereoselective (table 1). However, once reserpine is bound to vesicles, subsequent incubation with dopamine (10 mM), tetrabenazine (10 μM) or unlabeled reserpine (10 μM) for periods up to 2 h failed to displace bound radioactivity (not shown).

3.2. *Binding requires an electrochemical gradient generated by ATP hydrolysis*

Catecholamine uptake into synaptic vesicles and adrenal chromaffin granules depends on a transmembrane proton electrochemical gradient generated by a proton-translocating ATPase [11–13]. Reserpine binding was also ATP-dependent (table 2) and of the 3 ATP analogs tested, only α,β-methylene-ATP stimulated binding. This finding suggests that a hydrolysable terminal phosphate may be required for activity and is consistent with the postulate that ATP must be hydrolyzed by the proton-translocating ATPase to generate the electrochemical gradient required for reserpine binding and amine transport. Additional evidence for this is provided by results obtained by the addition of the uncouplers nigericin and CCCP. Both act by short-circuiting the transmembrane proton gradient, and both are potent inhibitors of ATP-dependent reserpine binding (table 2).

Table 2

Effects of uncouplers and nucleoside phosphates on reserpine binding

Addition	Rel. act.[a]
None	0.05 ± 0.01
ATP (2 mM)	1.00 ± 0.03
α,β-Methylene-ATP (2 mM)	0.31 ± 0.01
β,γ-Methylene-ATP (2 mM)	0.05 ± 0.02
AMPPNP (2 mM)	0.05 ± 0.01
ADP (2 mM)	0.38 ± 0.04
AMP (2 mM)	0.01 ± 0.03
CTP (2 mM)	0.52 ± 0.05
GTP (2 mM)	0.90 ± 0.03
ITP (2 mM)	0.89 ± 0.02
UTP (2 mM)	0.86 ± 0.01
CCCP (0.1 μM) + ATP (2 mM)	0.04 ± 0.02
Nigericin (0.1 μM) + ATP (2 mM)	0.06 ± 0.03

[a] Relative activity is defined as the ratio of specific binding under test condition to specific binding in the presence of ATP alone; reserpine was 5 nM; each value is the mean of 3 determinations ± SD

3.3. *Binding can be distinguished from catecholamine uptake*

To determine whether reserpine is also transported into vesicles, samples were first incubated under the standard conditions, then incubated a second time with various concentrations of saponin at 0°C to release vesicle contents. As shown in fig.2, >90% of vesicle-associated reserpine remained bound at up to 0.15% saponin, while almost all the accumulated dopamine was released by this detergent at 0.02%. Once bound, reserpine was also insensitive to subsequent treatment of the vesicles with CCCP alone or in combination with saponin. Conversely, prior incubation with CCCP or saponin, treatments which should prevent generation of a transmembrane gradient during the binding reaction, abolished all high-affinity binding.

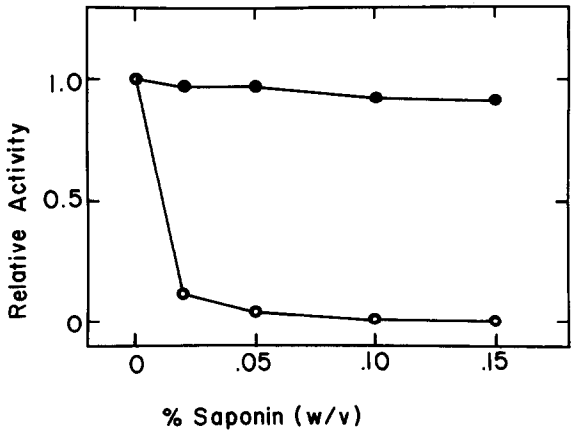

Fig.2. Effect of saponin treatment on accumulated dopamine and bound reserpine. Vesicles were incubated for 10 min at 23°C with either reserpine (●) or dopamine (○), cooled to 0°C, then incubated a second time in the presence of various concentrations of saponin (0°C, 10 min) before application to Sephadex columns. Relative activity was defined as the ratio of specific binding under the test condition to that observed in the absence of saponin. Each point is the mean of 4 determinations with SD < 8%.

4. DISCUSSION

Reserpine inhibits catecholamine uptake into adrenal chromaffin granules [1,3] both competitively with substrates for transport [2] and irreversibly under normal conditions [4]. Reserpine-sensitive, ATP-dependent transport has also been demonstrated in isolated platelet granules [14], synaptic vesicles from rat heart [15] and brain [9,11], and in each case the basic mechanism appears to be the same. Previous findings with chromaffin granules imply operationally separable species responsible for generation of proton electrochemical potential (ATPase), and actual uptake of catecholamines (transporter) [16,21]. The competitive nature of reserpine inhibition, and the insensitivity of granule and vesicle ATPases to reserpine, suggest that reserpine interacts with the transporter itself. Here, we have shown that reserpine binds to synaptic vesicles with an apparent affinity which is comparable to its potency as an inhibitor of dopamine uptake. Moreover, other catecholamines inhibit both reserpine binding and dopamine uptake at similar concentrations and, in the case of norepinephrine, in a stereoselective fashion.

Although the observation that the capacity of synaptic vesicles for reserpine is but a small fraction of that for dopamine may be an indication that dopamine is transported to the interior of the vesicle, while reserpine only binds to the transporter, such evidence does not constitute convincing proof. In studies on the lactose carrier protein [22,23] it was concluded that several compounds with high affinity for the carrier were actively transported, but a high rate of passive efflux resulted in low levels of net accumulation at equilibrium [24]. Dopamine is also known to be subject to both active uptake into and efflux from chromaffin granules [25]. In the case of reserpine binding, however, the possibility of uptake into the vesicle interior may be dismissed since saponin treatment, which releases accumulated dopamine by permeabilizing the vesicle membrane [26], does not release bound reserpine (fig.2).

The binding of several uptake inhibitors, including reserpine, to chromaffin granules has been described in [6,27,28]. Comparisons of affinities in the two systems may be significant, but the requisite information is not yet available to compare binding capacities: while adrenal chromaffin granules are relatively homogeneous with respect to the transmitters they contain, this is not true for synaptic vesicles from striatum, which, in addition to catecholamines and serotonin are also involved in the accumulation of other transmitters.

The ATP-dependence of reserpine binding described here most likely reflects a conformational change in the transporter induced by the proton electrochemical gradient. Changes in app. K_m for the lactose carrier system in response to an electrochemical gradient [23,29] and the asymmetric, mutually exclusive binding of bongkrekate-type and atractylate-type inhibitors to the mitochondrial ADP/ATP translocator [30] are but two examples of how alternate conformational states can affect the interaction of ligands with transport proteins. In the present instance, the gradient generated by ATP hydrolysis may affect the transporter by one or more of several different mechanisms. It may lead to the unmasking of a cryptic site on the outer surface of the vesicle, or increase the affinity of a site already available in the unenergized state. Another possibility is that such a gradient is required for irreversible movement of reserpine across the membrane to form a high-affinity complex with the transporter on its internal face, or within the bilayer itself. The latter possibility may well explain the reversal of reserpine inhibition in chromaffin granules by washing with phospholipid vesicles [4].

Whether reserpine binding to the outer surface takes place in the absence of ATP, or if this ligand binds to sites on the inner surface of synaptic vesicles under any conditions is not yet known. Studies of reserpine binding in the absence of ATP, and exploration of the nature of conformational changes in the transporter induced by the electrochemical gradient, are the subjects of on going investigations.

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