Reserpine Binding to a Vesicular Amine Transporter Expressed in Chinese Hamster Ovary Fibroblasts*

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(Received for publication, August 20, 1992)

The potent antihypertensive drug reserpine inhibits the transport of biogenic amines into adrenal chromaffin granules and synaptic vesicles. Reserpine acts by binding almost irreversibly to the vesicular amine transporter, and this interaction has been used both to study the mechanism of transport and to purify the protein responsible. Recent isolation of a cDNA for the rat chromaffin granule amine transporter (CGAT) by selection in the neurotoxin 1-methyl-4-phenylpyridinium now permits an analysis of the interaction with reserpine at a molecular level. Using membranes from stable transformants expressing the transporter, we show that reserpine binds specifically and quantitatively to CGAT. As with the native protein in bovine chromaffin granules, a pH gradient accelerates reserpine binding, and amine substrates compete for binding with reserpine. However, 1-methyl-4-phenylpyridinium and tetrabenazine, the other principal inhibitor of vesicular amine transport, compete very poorly with reserpine for binding, suggesting that they interact with CGAT at distinct sites.

The drug reserpine prevents the transport of biogenic amines into chromaffin granules and synaptic vesicles. As a result, reserpine depletes amine stores and has provided considerable information on the physiological role of biogenic amines in the nervous system. Reserpine potently reduces blood pressure, presumably by depleting amines in adrenal chromaffin granules. However, it frequently produces a disabling side effect of lethargy that resembles depression and has limited its clinical utility (Fritz, 1954). This observation has given rise to the amine hypothesis of affective disorders which in modified form still provides a useful framework for considering this group of major psychiatric disorders. Reserpine has also proven useful to study the mechanism of neurotransmitter transport into synaptic vesicles.

Neurotransmitters are stored in synaptic vesicles so that their release can be regulated by neural activity. For classical neurotransmitters such as the monoamines, vesicular storage involves transport from the cytoplasm where the transmitters accumulate after synthesis or removal from the synapse. Recently, vesicular transport activities have been described for acetylcholine, glutamate, γ-aminobutyric acid, and glycine (Anderson et al., 1982; Naito and Ueda, 1983; Maycox et al., 1988; Carlson et al., 1989; Fykse and Fonnum, 1988; Hell et al., 1988; Kish et al., 1989; Christensen, 1990; Burger et al., 1991). However, the transport of biogenic amines has served as the paradigm to understand this transport process (Njus et al., 1986; Johnson, 1988; Kanner and Schuldiner, 1987). Similar if not identical vesicular amine transport systems have been identified in the adrenal medulla, central aminegic neurons, platelets, and mast cells. Using bovine adrenal chromaffin granules as an abundant source of material with robust transport activity, vesicular transport has been found to depend on a pH gradient generated by the vesicular H⁺-ATPase and involve the exchange of lumenal protons for cytoplasmic amine (Njus et al., 1986; Johnson, 1988; Kanner and Schuldiner, 1987). In contrast, plasma membrane neurotransmitter transporters terminate the action of the transmitter by reuptake from the synapse and act by cotransport with Na⁺ (Kanner and Schuldiner, 1987). Further, plasma membrane amine transport shows considerable selectivity for the different amines whereas the chromaffin granule transporter recognizes dopamine, norepinephrine, epinephrine, serotonin, and histamine with approximately similar affinity (Njus et al., 1986; Johnson, 1988). The plasma membrane and vesicular transport systems also differ in their interaction with various pharmacologic agents. Whereas cocaine and a variety of clinically useful anti-depressant drugs inhibit plasma membrane amine transport, reserpine and tetrabenazine are the two principal agents that inhibit vesicular amine transport.

Reserpine and tetrabenazine interact with the vesicular amine transporter in distinct ways. Reserpine binds at the site of amine recognition and the imposition of a proton gradient accelerates reserpine binding, suggesting that the interaction may require mechanisms also involved in transport (Weaver and Deupree, 1982; Scherman and Henry, 1984). In contrast, tetrabenazine binds at a site in the transport complex remote from amine recognition and is unaffected by a pH gradient (Scherman and Henry, 1984). Tetrabenazine also dissociates from the transporter much more rapidly than reserpine (Scherman and Henry, 1984; Rudnick et al., 1990).

Reserpine dissociates very slowly if at all from the chromaffin granule amine transporter, even after solubilization (Rudnick et al., 1990). Thus, it has been used to label the transporter and follow its separation through a variety of procedures (Stern-Bach et al., 1990). Purification of the material labeled in this way has yielded two proteins that differ in pi. Reconstitution in proteoliposomes has shown both to catalyze amine transport with the expected properties. The more acidic isoform (pi ~ 3.5) is a glycoprotein of 80 kDa and has been used to determine partial peptide sequence for the chromaffin granule transporter.

Using the approach of cloning by functional expression, we have recently isolated a cDNA from rat pheochromocytoma PC12 cells whose sequence confirms the identity of the puri-
fied protein as the chromaffin granule transporter. The cDNA was isolated as a gene that suppresses the toxicity of 1-methyl-4-phenylpyridinium (MPP\(^+\))\(^1\), an agent that causes Parkinsonism in model systems (Liu et al., 1992a). MPP\(^+\) is the active metabolite of the parent toxin N-methyl-1,2,3,6-tetrahydropyridine and enters aminergic populations through a plasma membrane amine transport system (Javitch et al., 1985). Inside the cell, MPP\(^+\) inhibits oxidative phosphorylation, resulting in cell death. The chromator transporter protects against the toxicity of this compound by sequestering it within a vesicular compartment. Membranes prepared from stable transformants expressing the cloned cDNA show amine transport activity as measured by the uptake of \([^{3}H]\)dopamine. The transport shows the expected dependence on a proton gradient, affinity for amine transmitters, and pharmacology, although the sensitivity to tetrabenazine is slightly reduced (Liu et al., 1992b). Since mRNA transcripts for these sequences were found only in the adrenal gland, we have termed this gene the chromaffin granule amine transporter (CGAT).

A distinct, but related, vesicular amine transporter is expressed in central aminergic populations, and we term this the synaptic vesicle amine transporter.

We now report that the rat CGAT protein expressed in CHO fibroblasts binds to reserpine with properties similar to those previously reported from bovine chromaffin granules. The binding is quantitative and linear over the range investigated, correlating with CGAT mRNA expression in a number of different CHO transformants. Imposition of a pH gradient accelerates but is not required for binding, and amine substrates compete with reserpine for binding at the expected affinities. Interestingly, MPP\(^+\) and tetrabenazine compete relatively poorly with reserpine for binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—\([^{3}H]\)Reserpine was obtained from the Nuclear Research Centre (Negev, Israel). Sephadex LH-20 was from Pharmacia LKB Biotechnology Inc. Cells—Stable MPP\(^+\)-resistant CHO cells were obtained as described (Liu et al., 1992b), by transfection with the CDMS expression vector containing CGAT followed by selection in 1 mM MPP\(^+\). For at least 1 week prior to the experiment, cells were grown in the absence of MPP\(^+\).

**Preparation of Membrane Vesicles**—Cells were disrupted in a water bath sonicator (Laboratory Supplies Co., Inc., Hicksville, NY) in cold 0.15 NaCl and 0.01 M K-Hepes, pH 7.4, containing 5 mM MgSO\(_4\), 5 mM Na-EGTA, 1 μg/ml leupeptin, and 0.2 mM diisopropylfluorophosphate (1 ml of solution/10° cells), and the cell debris removed by centrifugation at 3,500 \(\times\) g for 5 min. Membranes were collected by centrifugation in a Beckman TL ultracentrifuge (213,000 \(\times\) g, 40 min), resuspended in 0.32 M sucrose, 10 mM Hepes-KOH, pH 7.4 (SH) containing 5 mM MgSO\(_4\) and used within 1 h. Cell disruption by homogenization at 0.01-mm clearance yielded binding that was essentially identical to that observed in membranes prepared by sonication.

**Reserpine Binding**—Measurement of reserpine binding was performed as described by Rudnick et al. (1990). Membranes were diluted to a protein concentration of approximately 0.25 mg/ml in a solution containing 320 mM sucrose, 10 mM K-Hepes, pH 7.4, 4 mM KCl, 5 mM ATP, and 4 mM MgSO\(_4\). \([^{3}H]\)Reserpine (20 Ci/mmol) was added to the final concentration indicated in the text, and the mixture was incubated at 32 °C. After incubation for the time indicated, a 200-μl sample of the suspension was applied to a 3-ml column of Sephadex LH-20 (prepacked in a disposable syringe by centrifugation for 90 s in a clinical centrifuge), centrifuged for 2 min, and the effluent assayed for radioactivity. Where indicated, parallel reaction mixtures containing 2 μM reserpine were used to subtract nonspecific binding which was typically less than 10% of the binding to membranes from wild-type CHO cells.

**Dot-blot Analysis of RNA Levels**—Amounts of RNA varying from 2.5–20 μg were adsorbed onto nylon (Hybond, Amersham Corp.), prehybridized in 50% formamide, 5 × SSC, 5 × Denhardt’s solution, 0.5% SDS, 200 μg/ml denatured salmon sperm DNA for 4 h at 42 °C, hybridized in the same solution with the CGAT cDNA insert labeled by random priming (Feinberg and Vogelstein, 1983) at 42 °C for 16 h, washed twice in 2 × SSC, 0.1% SDS for 1 h at 50 °C, 0.1 × SSC, 0.1% SDS for 1 h at 60 °C, and submitted to autoradiography.

**RESULTS**

**Reserpine Binds Quantitatively to CGAT Expressed in CHO Fibroblasts**—Although reserpine potently inhibits vesicular amine transport, its binding to the transporter has been difficult to assay because the drug is extremely lipophilic and adheres nonspecifically to a variety of surfaces. To circumvent this problem, a binding assay was developed in which the free and bound drug are separated by gel filtration chromatography (Rudnick et al., 1990). We have now used this assay to measure reserpine binding to CHO transformants expressing CGAT. \([^{3}H]\)Reserpine binds in a time-dependent manner to membranes from stable transformants that have been selected in MPP\(^+\) and shown to express substantial amine transport activity (Liu et al., 1992b) (Fig. 1). At 0.5 nM reserpine, binding increases monotonically for approximately the first 20 min of incubation then gradually stops increasing and remains stable for at least 150 min. An excess of ligand (2 μM reserpine) eliminates the observed interaction and membranes from wild-type CHO cells, and cells transformed with the plasmid vector alone show no significant binding (data not shown). Thus, reserpine binds specifically to the CGAT expressed in this fibroblast cell line. The transporter does not require any modifications or accessory proteins specific for a neuroendocrine cell to bind reserpine.

A comparison of different CHO transformants indicates that reserpine binds quantitatively to the chromaffin granule transporter. Transformants selected in MPP\(^+\) express an extraordinarily high level of the transporter, perhaps due to gene amplification following prolonged exposure to the toxin, a phenomenon observed with several genes encoding drug resistance (Endicott and Ling, 1989; Schimke, 1986). On the other hand, cotransformation with a selectable marker for drug resistance to neomycin yields cells that express much lower levels of transport activity as judged by an assay in which the cell is loaded with dopamine and its distribution visualized by glyoxylic acid-induced fluorescence (Liu et al., 1992b). We have now correlated the level of reserpine binding by these

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\(^1\) The abbreviations used are: MPP\(^+\), 1-methyl-4-phenylpyridinium; CGAT, chromaffin granule amine transporter; CHO, Chinese hamster ovary; CCCP, carbonyl cyanide m-chlorophenylhydrazone; NEM, N-ethylmaleimide.
Reserpine Binding by the Chromaffin Granule Amine Transporter

Reserpine binds significantly slower to membranes from the MPP+-resistant transformants (Fig. 3). However, the rate of binding is still somewhat higher than in the presence of CCCP. The discrepant effects of a proton ionophore and ATP removal, which are both considered to reduce amine transport and reserpine binding by eliminating the pH gradient, may derive from the presence of residual endogenous ATP in the membrane preparation which serves to generate some electrochemical gradient. To further address this possibility and to determine the type of ATPase involved, we examined the rate of binding in membranes pretreated with the sulfhydryl reagent N-ethylmaleimide (NEM) that inhibits the vacuolar (V)-type ATPase at low concentrations (Rudnick, 1986). Fig. 3 shows that 10 μM NEM slows reserpine binding to rates comparable with those achieved in the presence of CCCP. This supports the role of the V-ATPase in the formation of ΔpH across the membrane and the contribution of this gradient to reserpine binding. Higher concentrations of NEM such as 20 and 30 μM had no further effect on the rate of binding (data not shown). However, binding in the presence of either CCCP or NEM was always significantly higher than binding in the presence of excess cold ligand. This result is consistent with previous observations that ΔpH accelerates but is not required for binding to reserpine.

Reserpine Binding to CGAT with Two Components of Distinct Affinity—[3H]Reserpine binding to chromaffin granule membranes and to the purified transporter reconstituted in proteoliposomes demonstrates two distinct high affinity components. The molecular basis for the observed differences in affinity may derive from differences in the environment or state of the transporter. To determine whether expression in CHO cells confers both components of reserpine binding, we have measured equilibrium binding at various concentrations of reserpine to membranes from the MPP+-selected stable transformants. Analysis by Scatchard plot indicates two binding constants of 0.9 and 5 nM (Fig. 4). The membranes express 3.5 pmol/mg protein of the high affinity sites and 12.3 pmol/mg protein of the low affinity sites. The total number of

Fig. 3. Binding of reserpine is accelerated by a proton electrochemical gradient. Membrane vesicles from the CHO stable MPP+-resistant transformants (85 μg of protein) were incubated at 32 °C for the indicated time periods, and the reaction was terminated by filtration on Sephadex LH-20 columns. All the reactions were carried out in the presence of ATP except for (C). When added, CCCP concentration was 5 μM (■), reserpine was 2 μM (○). For treatment with NEM (□), membranes were incubated in 25 μl of a suspension identical in composition to the reaction medium except that it did not contain ATP and it included NEM (10 μM). After 40 min at room temperature, the treated membranes were assayed as described. A control not treated with NEM but incubated for the same period of time showed activity indistinguishable from that of the membranes that were assayed without any incubation (not shown). Increasing the NEM concentration to 20 and 30 μM did not cause further inhibition (not shown).

 CHO transformants, we have first examined the dependence of such binding on ATP. In the absence of ATP, reserpine binds significantly slower to membranes from the MPP+-selected CHO transformants (Fig. 3). However, the rate of binding is still somewhat higher than in the presence of CCCP. The discrepant effects of a proton ionophore and ATP removal, which are both considered to reduce amine transport and reserpine binding by eliminating the pH gradient, may derive from the presence of residual endogenous ATP in the membrane preparation which serves to generate some electrochemical gradient. To further address this possibility and to determine the type of ATPase involved, we examined the rate of binding in membranes pretreated with the sulfhydryl reagent N-ethylmaleimide (NEM) that inhibits the vacuolar (V)-type ATPase at low concentrations (Rudnick, 1986). Fig. 3 shows that 10 μM NEM slows reserpine binding to rates comparable with those achieved in the presence of CCCP. This supports the role of the V-ATPase in the formation of ΔpH across the membrane and the contribution of this gradient to reserpine binding. Higher concentrations of NEM such as 20 and 30 μM had no further effect on the rate of binding (data not shown). However, binding in the presence of either CCCP or NEM was always significantly higher than binding in the presence of excess cold ligand. This result is consistent with previous observations that ΔpH accelerates but is not required for binding to reserpine.

Reserpine Binds to CGAT with Two Components of Distinct Affinity—[3H]Reserpine binding to chromaffin granule membrane vesicles and to the purified transporter reconstituted in proteoliposomes demonstrates two distinct high affinity components. The molecular basis for the observed differences in affinity may derive from differences in the environment or state of the transporter. To determine whether expression in CHO cells confers both components of reserpine binding, we have measured equilibrium binding at various concentrations of reserpine to membranes from the MPP+-selected stable transformants. Analysis by Scatchard plot indicates two binding constants of 0.9 and 5 nM (Fig. 4). The membranes express 3.5 pmol/mg protein of the high affinity sites and 12.3 pmol/mg protein of the low affinity sites. The total number of

Fig. 2. Comparison of reserpine binding and CGAT RNA levels. Membranes from the various sources were prepared as described under "Experimental Procedures." □, clone selected in G418; ○ and ■, clones 9 and 13 selected in MPP+ for 5 days; and □, clone exposed to MPP+ for 3 weeks. Binding was assayed for 30 min at various protein concentrations (10–300 μg) at 3.75 nM reserpine. In each case, the values given are from duplicates at concentrations in which binding was linear with the protein. Nonspecific binding is subtracted in all cases. For estimation of RNA levels, poly(A)+ RNA from the different sources was dot-blotted (2.5–20 μg of RNA) and probed with labeled CGAT. The relative amounts were estimated by densitometry.

ΔμH+ Accelerates Reserpine Binding to the CHO Transformants—In bovine chromaffin granule membranes, the imposition of a proton electrochemical gradient stimulates binding of reserpine to the amine transporter. Previous studies have suggested that ΔμH+ accelerates binding by shifting the equilibrium between two forms of the transporter. In one form, the reserpine binding site is unavailable to the ligand. In the other, ΔpH acts to recruit binding sites for reserpine (Rudnick et al., 1990). The transition between these two states may also mediate the transport of amines since this depends on ΔpH as well. To determine whether the presence of a pH gradient had a similar effect on the rat CGAT protein expressed in CHO cells, we measured the binding of [3H]reserpine to membranes from the stable transformants in the presence and absence of the proton ionophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), which dissipates any ΔμH+ across the membranes. As shown in Fig. 3, 5 μM CCCP markedly inhibits reserpine binding in the CHO transformants. However, after 150 min, binding reaches 50% of the equilibrium values achieved in the absence of CCCP (data not shown). Thus, expression of CGAT in CHO cells reconstitutes the acceleration of reserpine binding by a pH gradient.

In chromaffin granules and synaptic vesicles, an ATPase of the vacuolar type is responsible for generating ΔμH+ (Forc gas, 1989; Rudnick, 1986; Stone et al., 1989). To determine whether a similar ATPase is also responsible for the electrochemical gradient that accelerates reserpine binding in the

different transformants with the expression of CGAT mRNA. Fig. 2 shows that [3H]reserpine binding varies as a linear function of CGAT transcripts over a wide range of expression. Clones selected in G418 show low but measurable levels of [3H]reserpine binding and CGAT mRNA whereas two clones selected in MPP+ for 5 days have 10-20-fold more binding sites and transcripts. Exposure to MPP+ for 3 weeks further increases both binding and CGAT mRNA 80-100-fold. Thus, reserpine binding provides an estimate of transporter number. The availability of a quantitative assay for reserpine binding to the vesicular amine transporter expressed in CHO cells now also provides an experimental system to characterize the interaction at a molecular level.
Reserpine Binding by the Chromaffin Granule Amine Transporter

The binding sites approximate those found in chromaffin granules, confirming the very high levels of CGAT expression in this heterologous system that were suggested by Northern analysis (Liu et al., 1992b). The proportion of low and high affinity sites also resembles the proportion found in bovine chromaffin granules, indicating that CHO cells do not lack a component required for binding at either affinity.

Pharmacology of Reserpine Binding to CGAT—The monoamine neurotransmitters compete with reserpine for binding to chromaffin granules. To determine whether they also compete for binding to the rat transporter expressed in CHO cells, we have assessed the rate of binding by 2 nM [3H]reserpine after a 5-min incubation of the CHO membranes with the various transmitters (Fig. 5). Serotonin inhibits reserpine binding with greater potency than dopamine, consistent with previous reports from chromaffin granules (Johnson, 1988) and with the observed relative affinity for transport (Liu et al., 1992b). However, the actual IC50 values are consistently somewhat higher (2.5 and 18 μM for serotonin and dopamine, respectively) than the affinities for transport (0.6 and 2.3 μM). This discrepancy may derive at least in part from the use of relatively high reserpine concentrations and the essential irreversibility of reserpine binding (Rudnick et al., 1990). Interestingly, the toxin MPP+ competes extremely poorly with reserpine for binding to the transporter, with an IC50 of 500 μM.

We have also examined the interaction between tetrabenazine and reserpine for binding to the transporter. Previous studies have shown that tetrabenazine inhibits amine transport into bovine chromaffin granules with a potency in the low nanomolar range but that considerably more is required to inhibit reserpine binding (Scherman and Henry, 1984). In contrast, we have found that tetrabenazine inhibits the transport activity of rat CGAT with an IC50 of 4 μM (Liu et al., 1992b). It was thus of considerable interest to determine whether tetrabenazine was correspondingly less potent in the inhibition of reserpine binding. The results indicate an IC50 of 40 μM for inhibition of reserpine binding by tetrabenazine, consistent with both its reduced potency for inhibition of transport and its apparently indirect interaction with the site of reserpine binding.

FIG. 4. Scatchard plot of reserpine binding to CGAT. Membrane vesicles (40 μg of protein) were incubated at 32 °C in different reserpine concentrations (0.1-51 nM). At each concentration the nonspecific binding was estimated from controls to which 2 μM cold reserpine was added. The reactions were terminated after 40 min of incubation by filtration on Sephadex LH-20 columns.

FIG. 5. Effect of various inhibitors on the rate of reserpine binding. Membrane vesicles (80 μg) were incubated with 2 nM [3H]reserpine and the following inhibitors: serotonin (●), dopamine (○), tetrabenazine (□), and MPP+ (□) at the concentrations indicated. After 5 min the reaction was terminated as described. Control values were 2.3 ± 0.1 pmol/mg protein.

DISCUSSION

The CGAT cDNA has been isolated as a gene conferring resistance to MPP+ in CHO cells (Liu et al., 1992a). The sequence predicts a novel mammalian protein with 12 transmembrane domains, supporting its role as a transporter. In addition, partial amino acid sequences obtained from purified bovine adrenal vesicular amine transporter are highly homologous to sequences predicted from the DNA sequence of CGAT (Stern-Bach et al., 1992). Furthermore, transport activity detected in homogenates of CGAT transformants shows characteristics similar to the ones described in bovine adrenal chromaffin granules (Liu et al., 1992b). The results described here extend the previous analysis and strongly support the contention that the CGAT cDNA encodes one form of a vesicular amine transporter. In addition, expression of the transporter in heterologous cells in a form able to transport amines and bind reserpine permits a more detailed characterization of both properties.

The results indicate that rat CGAT expressed in CHO fibroblasts binds quantitatively to reserpine. We can detect binding to reserpine with as little as 40 pg of membrane protein from 106 cells that have been selected in G418. MPP+-selected transformants express considerably more CGAT, and membranes from these cells bind correspondingly more reserpine. Interestingly, the amount of binding expressed by MPP+-selected cells is substantially higher than expressed by PC12 cells which served as the source for the cDNA library, and even higher than the maximal levels reported for chromaffin granules prepared from bovine adrenal medulla. Further, reserpine binding varies as a linear function of CGAT mRNA levels and thus provides an excellent tool to quantitate the levels of protein. Since a preparation of purified bovine transporter reconstituted in proteoliposomes contains approximately 12,500 pmol of binding sites/mg of protein (Stern-Bach et al., 1990), we estimate that the CGAT expressed in MPP+-resistant CHO cells accounts for about 0.1% of the total cell membrane protein. Calculation based on the predicted molecular mass of the protein and the total number of binding sites as determined in Fig. 4 yields a very similar estimate (0.06%).

The binding activity is apparently targeted to intracellular organelles in which an ATPase, most likely of the vacuolar type, generates a ΔΨ across the membrane (Stone et al., 1989). The data do not exclude the presence of CGAT in other membranes. Nonetheless, the observation that CCCP reduces the extent of reserpine binding to 50-60% of the total (after 3 h of incubation) suggests that most or all of the CGAT occurs in membranes that exhibit a pH gradient. The proportion of low and high affinity sites resembles that observed in chromaffin granules, supporting the expression of the protein in a physiological environment within CHO cells. The similar affinity at both sites to results in chromaffin granules also supports this conclusion. In addition, ΔΨ accelerates but is not required for reserpine binding to rat CGAT expressed in this system. This may reflect a transition between two states.
of the protein that are also involved in transport. In one model, reorientation of the amine recognition site to the cytoplasmic face of the membrane after delivery of the amine to the vesicle lumen uses the energy derived from a pH gradient. Such reorientation may also be required for reserpine binding and the ability of amines to compete with reser- pine for binding at affinities similar to those for transport supports this hypothesis. In addition, the existence of two binding components differing in affinity for reserpine may similarly reflect two different states of the transporter. Using this heterologous expression system, it will now be possible to explore the relationship between these two sites and their significance for transport.

Amine transmitters compete with reserpine for binding to the bovine chromaffin granule amine transporter at concentrations very close to those at which they inhibit transport. The amines compete at similar affinity for binding to CGAT expressed in CHO cells. Thus, expression in a heterologous cell system reconstitutes the native interaction with reserpine in considerable detail. Reserpine binding can now be used to dissect the molecular basis for ligand recognition.

The ability of CGAT to protect against MPP⁺ toxicity presumably derives from sequestration of the toxin within intracellular vesicles, away from its primary site of action in mitochondria. Studies using bovine chromaffin granules have shown transport of MPP⁺ that is dependent on a pH gradient and potently inhibited by both reserpine and tetrabenazine (Daniels and Reinhard, 1987; Scherman et al., 1988). In one study, the affinity for transport of the toxin appeared similar to the affinity for transport of monoamine transmitters, but the $K_v$ values observed for all the compounds exceeded those reported elsewhere by a factor of approximately 10-fold (Daniels and Reinhard, 1987). Our results now show that MPP⁺ competes very poorly with reserpine for binding to rat CGAT expressed in CHO cells. The clear discrepancy with amine transmitters is very surprising since the same site presumably recognizes all of these compounds, and reserpine is nonetheless very potent in inhibiting transport of MPP⁺. One possible explanation for the findings is that MPP⁺ may contact different amino acid residues in the recognition site than the monoamines. Indeed, it has a distinctly different structure. Reserpine contacts the same residues as the amines and presumably inhibits transport regardless of the substrate and in particular, regardless of whether they compete for binding. Alternatively, CGAT may transport MPP⁺ with reduced affinity, yet still protect against its toxicity. Since MPP⁺ permeates the plasma membrane as a cation, it will probably equilibrate with the transmembrane electrical potential and reach extremely high levels in the cytoplasm (>1 mM) which are still well within the observed affinity of CGAT for MPP⁺, at least as determined by competition for binding with reserpine. Differences in the affinity for transmitters and toxins may also account for the selective vulnerability of specific neuronal populations.

Pharmacologic studies have suggested that the vesicular amine transporters expressed by different tissues are identical. However, purification of the transporter from bovine adrenal chromaffin granules has yielded two putative isoforms (Stern-Bach et al., 1990). The existence of different isoforms is now further supported by the identification of a cDNA in the rat brain (synaptic vesicular amine transporter) that is distinct from but highly related to CGAT. The existence of at least two different vesicular amine transporters also raises the possibility that they differ in function. Surprisingly, the adrenal transporter from rat is significantly less sensitive to tetrabenazine than the adrenal transporter from the cow.