rameters are necessary to confirm this assumption (Lerner & Torchia, 1986).

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Energetics of Reserpine Binding and Occlusion by the Chromaffin Granule Biogenic Amine Transporter†

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ABSTRACT: The energetics of reserpine binding to the bovine adrenal biogenic amine transporter suggest that H^+ ion translocation converts the transporter to a form which binds reserpine essentially irreversibly. Reserpine binding to bovine adrenal chromaffin granule membrane vesicles is accelerated by generation of a transmembrane pH difference (ΔpH) (interior acid) or electrical potential ($\Delta\psi$) (interior positive). Both components of the electrochemical H^+ potential ($\Delta\tilde{\mu}_{H^+}$) must be dissipated to block reserpine binding, and generation of either one stimulates the binding rate. Reserpine binding is less dependent than amine transport on the ΔpH, suggesting that translocation of fewer H^+ ions is required to expose the high-affinity site than are required for net transport. Bound reserpine dissociates very slowly, if at all, from the transporter. Binding is stable to 1% cholate, 1.5% Triton X-100, 1 M SCN^-, and 8 M urea, but sodium dodecyl sulfate (0.035%) and high temperatures (100 °C) released bound resperine, indicating that binding is noncovalent. The results raise the possibility that the transporter, by translocating one H^+ ion outward down its concentration gradient, is converted to a form that can either transport a neutral substrate molecule inward or occlude reserpine in a dead-end complex.

The biogenic amine transporter is responsible for accumulation of serotonin, dopamine, norepinephrine, epinephrine, and histamine within secretory vesicles in a variety of cells (Kanner & Schuldiner, 1987; Njus et al., 1986). The energy required for amine accumulation comes from an ATP-driven H^+ pump in the secretory vesicle membrane which acidifies the vesicle lumen (Rudnick, 1986a,b). The amine transporter exchanges intravesicular H^+ ions for cytoplasmic biogenic amines, thereby coupling the downhill flux of accumulated H^+ ions with uphill amine accumulation. Although the H^+/amine stoichiometry for this process has been known for many years (Knoth et al., 1981a; Johnson et al., 1981) and some kinetic aspects of the transport process have been described (Maron et al., 1983), many aspects of the mechanism have remained elusive. The ionic form of the substrate amine which binds to the transporter is not known. Although a variety of evidence suggests that each amine molecule exchanges in its uncharged form with one H^+ ion (Ramu et al., 1983; Scherman & Henry, 1981; Kobold et al., 1985), the possibility that the cationic species (which predominates at cytoplasmic pH) exchanges with two H^+ ions has never been ruled out (Knoth et al., 1981b; Daniels & Reinhard, 1988). Moreover, the mechanism by which H^+ and amine fluxes are coupled remains unknown.

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Reserpine is a competitive inhibitor of amine transport in vivo and in vitro (Stitzel, 1977). [^{3}H]Reserpine binding to chromaffin granule membranes has been found to require the presence of ATP (Weaver & Deupree, 1982; Scherman & Henry, 1984). This ATP dependence is thought to represent generation of an electrochemical H^+ potential ($\Delta\tilde{\mu}_{H^+}$)[1] by the granule ATPase, since protonophores block ATP-induced binding (Weaver & Deupree, 1982). Scherman and Henry (1984) have interpreted these data to mean that reserpine binding sites are unmasked in response to $\Delta\tilde{\mu}_{H^+}$. Although such an interpretation is attractive, other explanations have not been ruled out. For example, reserpine binding could be accelerated by the transmembrane pH difference (ΔpH) if it were concentrated intravesicularly as a weak base by nonionic diffusion. Reserpine association with chromaffin granule vesicles could represent not binding, but rather a balance between transporter-catalyzed influx and passive efflux. Furthermore, the binding site for [^{3}H]reserpine need not be identical with the site of transport inhibition.

In this paper, we demonstrate that reserpine binding is energized under conditions where the transmembrane electrical potential ($\Delta\psi$) represents the sole source of energy. Both the ΔpH and the $\Delta\psi$ generated by the ATPase energize transport, and each component of the $\Delta\tilde{\mu}_{H^+}$ is sufficient by itself to facilitate binding. Once bound, reserpine dissociates at a negligible rate, even in the presence of detergents or chaotropic agents and in the absence of electrochemical driving forces. The kinetics of reserpine binding and inhibition of transport are similar, and consistent with a single binding site mediating both phenomena. Furthermore, reserpine binding dependence on ΔpH and $\Delta\psi$ suggests that less than two H^+ ions are transported in a step which precedes substrate binding. The results are consistent with a model in which the energy invested in the H^+ translocation step can be released either by substrate translocation or by reserpine binding.

Experimental Procedures

Materials

Chromaffin granule membrane vesicles were prepared as described previously (Schuldiner et al., 1978) by repeated osmotic lysis of chromaffin granules isolated by differential sedimentation. Sodium cholate was recrystallized 3 times from ethanol. [^{3}H]Reserpine was obtained from New England Nuclear, [^{3}H]serotonin was purchased from Amersham, and all other reagents were of the highest quality commercially available.

Methods

Serotonin Transport. Membranes were diluted to a concentration of approximately 0.25 mg/mL in 0.3 M sucrose containing 10 mM K-Hepes, pH 8.5, 5 mM KCl, 2.5 mM $MgSO_4$, 5 mM Na_2ATP, and 0.275 μM [^{3}H]serotonin (unless indicated otherwise). Rates of serotonin transport were measured after incubating this suspension for 1 min, and steady-state levels were measured after 6 min. Reactions were stopped by dilution, filtration, and washing, and filtered vesicles were counted as described previously (Schuldiner et al., 1978).

Reserpine Binding. Membranes were diluted to a concentration of approximately 0.25 mg/mL in 0.3 M sucrose containing 10 mM K-Hepes, pH 8.5, 5 mM KCl, and 2.5 mM $MgSO_4$ in the presence or absence of 5 mM Na_2ATP and 1–8 nM [^{3}H]reserpine (unless indicated otherwise). Where reserpine binding rates were measured, the incubation time was 5 min at 37 °C. At the appropriate time, a 400-μL sample of the suspension is applied to a column of 2-mL Sephadex G-50 fine which had been packed in a 2-mL disposable syringe and precentrifuged at 100*g* for 10 s. The column with applied sample was centrifuged once more for 1 min at 225*g* and the effluent counted in 4 mL of 40% Lumax (Lumac, Landgraaf, The Netherlands) in toluene. All measurements of reserpine binding, unless noted otherwise, represent specific binding (total binding minus values from samples containing excess unlabeled reserpine). Reserpine samples were usually 30–50% of the total binding (see Figure 2 and Table I for examples).

Triton-solubilized samples were assayed by incubating 0.25 mL of solubilized membranes with 275 mg of washed Bio-Beads SM-2 (Bio-Rad) with agitation for 30 min at 4 °C. Bio-beads were prepared by washing 10 g with 10 mL of methanol overnight and then washed again with 100 mL of methanol followed by 1 L of water. They can be stored for long periods in water at 4 °C. Before use, excess water is removed by centrifuging in a syringe for 5 min at maximum speed in a table-top centrifuge. After incubation of the sample with Bio-Beads, the beads are sedimented by centrifugation, and a sample of the supernatant fluid is counted. Very little protein is lost in this stage. Essentially all of the free reserpine and some of the nonspecifically bound reserpine remain bound to the beads, while reserpine bound to solubilized protein remains in the supernatant fluid.

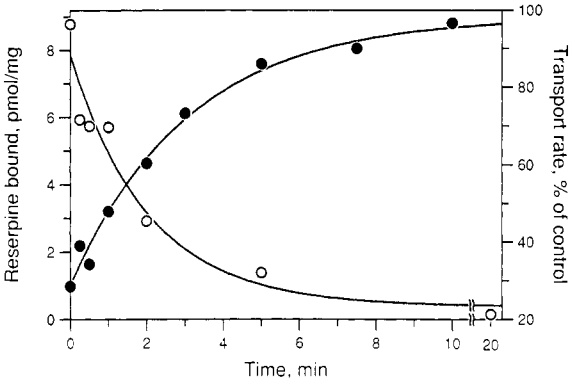

FIGURE 1: Time course of reserpine inhibition and binding. Membrane vesicles (0.044 mg) were added to 200 μL of 0.3 M sucrose containing 10 mM K-Hepes, pH 8.5, 5 mM KCl, 2.5 mM $MgSO_4$, 5 mM ATP, and 8 nM either ^{3}H-labeled or unlabeled reserpine at 30 °C. After incubation for the indicated times, reserpine binding (closed circles) was assayed by gel filtration as described under Experimental Procedures. Transport (open circles) was measured by addition of 0.275 μM [^{3}H]serotonin, further incubation for 1 min, dilution, filtration, and washing as described under Experimental Procedures. The 100% control rate in the absence of reserpine was 137 pmol mg^{-1} min^{-1}. The lines represent nonlinear regression fits of the data.

Results

Time Course of Reserpine Inhibition

The time course of reserpine binding has been reported to be relatively slow (Scherman & Henry, 1984; Weaver & Deupree, 1982). To determine if reserpine inhibited amine transport with a similar time course, we incubated chromaffin granule membrane vesicles with ATP in the presence or absence of 8 nM reserpine. At various time intervals, [^{3}H]-serotonin was added and transport measured over a time course of 1 min. Figure 1 demonstrates that inhibition by reserpine develops slowly at this concentration. Reserpine inhibition increases with time with a pseudo-first-order rate constant of 0.49 min^{-1}. Also shown in Figure 1 is the time course of

[1] Abbreviations: ΔpH, transmembrane pH difference; $\Delta\psi$, transmembrane electrical potential difference; $\Delta\tilde{\mu}_{H^+}$, transmembrane electrochemical potential difference for H^+ ions; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TBZ, tetrabenazine.

Table I: Retention of Bound Reserpine in Detergent Extracts[a]

cholate time (h)	pmol of reserpine bound/mg of membrane protein			Triton time (h)	pmol of reserpine bound/mg membrane protein		
	total	+reserp	specific		total	+reserp	specific
before detergent	1.15	0.533	0.622	before detergent	3.20	1.21	1.99
0	0.889	0.311	0.578	0	1.97	0.14	1.83
0.5	0.875	0.32	0.556	1	1.76	0.15	1.62
2	0.8	0.222	0.578	3.5	1.89	0.14	1.75
4	0.711	0.311	0.40	20	1.84	0.12	1.72
20	0.667	0.324	0.342				

[a] Membrane vesicles were diluted to a concentration of 0.5 mg/mL in 0.3 M sucrose containing 10 mM K-Hepes, pH 8.5, 5 mM KCl, 2.5 mM $MgSO_4$, 5 mM ATP, and either 1 nM (cholate experiment) or 2 nM (Triton experiment) [^{3}H]reserpine. Reserpine controls contained, in addition, 5 μM unlabeled reserpine. After 30 min at 37 °C, sodium cholate was added to a concentration of 1% (w/v), or in the case of Triton, the vesicles were collected by centrifugation at 34000g for 30 min and resuspended to a concentration of 3.3 mg/mL, and then Triton X-100 was added to a final concentration of 1.5% (w/v). At the indicated times after detergent addition, samples were taken and assayed as described under Experimental Procedures for cholate- and Triton-solubilized samples. Data represent the mean of triplicates for a representative experiment which has been repeated 6 times for cholate and 3 times for Triton. In this experiment, the standard deviation of the mean was typically 10% of the mean.

reserpine binding measured under the same conditions in a separate experiment. The binding data are fit best to a pseudo-first-order rate constant of 0.32 min^{-1}, in good agreement with the time course of inhibition. These results are consistent with the proposal that reserpine inhibits serotonin transport by binding to the site measured with [^{3}H]reserpine.

Reserpine Occlusion

Interaction with Other Ligands. As previously described by Weaver and Deupree (1982) and Scherman and Henry (1984), [^{3}H]reserpine becomes associated with chromaffin granule membranes in the presence of ATP. The data in Figure 2 illustrate this phenomenon and also demonstrate the ability of other substrates and ligands to block [^{3}H]reserpine binding. Although serotonin, tetrabenazine, carbonyl cyanide *m*-chlorophenylhydrazone, and epinephrine all inhibit [^{3}H]-reserpine binding, addition of none of these substrates causes bound reserpine to dissociate. Significantly, tetrabenazine, which is thought to bind to a pharmacologically distinct site (Scherman & Henry, 1984), also fails to displace bound reserpine. Finally, lowering the pH had no effect on reserpine dissociation (see Table II below). Under none of these experimental conditions could the dissociation constant be estimated, since less than 7% dissociates during an 8-h incubation at 37 °C.

Detergent Solubilization. To test the stability of the transporter–reserpine complex to detergent solubilization, we measured reserpine binding and dissociation in cholate and Triton X-100. The concentration of cholate necessary to solubilize the transporter is similar to that required for solubilization of reconstitutively active amine transporter (Maron et al., 1979) (data not shown). The stability of bound [^{3}H]reserpine after solubilization in cholate or Triton X-100 is shown in Table I. In this experiment, vesicles were incubated for 30 min with [^{3}H]reserpine in the prescence or absence of excess unlabeled reserpine, and then cholate or Triton X-100 was added to a concentration of 1% or 1.5%, respectively. Samples were taken to determine remaining bound [^{3}H]reserpine at the indicated times after detergent addition. The results indicate that much of the nonspecifically bound reserpine is dissociated from the membrane fraction in detergent while over 90% of the specifically bound ligand remained. If cholate is added prior to reserpine, the transporter loses its ability to subsequently bind reserpine (data not shown). Specifically bound [^{3}H]reserpine dissociated very slowly from membranes solubilized in either cholate or Triton (Table I). At low pH (below 4) in cholate, however, significant dissociation occurs without a corresponding loss of reconstitution activity (data not shown). The observation that reserpine remains specifically bound in detergent-solubilized membranes indicates that reserpine which associates with the membrane vesicles in the presence of ATP does not represent a balance between reserpine transport and passive efflux.

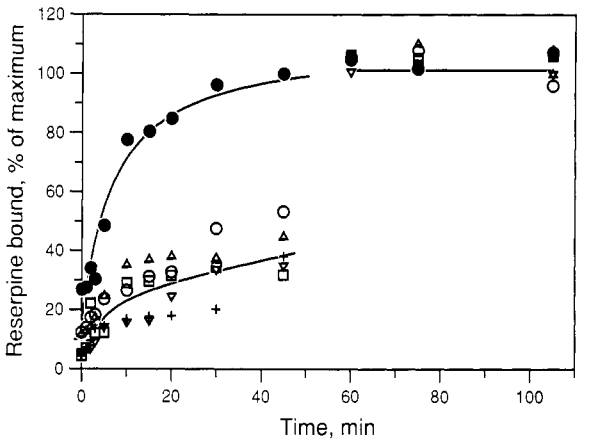

FIGURE 2: Effect of ligands and CCCP on reserpine binding and dissociation. Reserpine binding was measured in the presence of ATP at 30 °C as described under Experimental Procedures using 1 nM [^{3}H]reserpine in the presence and absence of 10 μM tetrabenazine (TBZ), 1 mM serotonin, 1 mM epinephrine, 5 μM unlabeled reserpine, or 3 μM CCCP. Inhibitors were added at zero time for the points up to 45 min. Dissociation of reserpine was measured by addition of the above agents to membranes which had bound [^{3}H]reserpine for 45 min in the presence of ATP with no inhibitors present. For the points at times greater than 45 min, inhibitors were added only at 45 min. Results are expressed as the percent of binding at 45 min.

Darchen et al. (1989) also recently observed persistent binding to chromaffin granule membranes solubilized with cholate. In contrast to the results shown in Table I, however, they reported that Triton X-100 caused bound reserpine to be released from the transporter. The reason for this discrepancy seems to lie with the method used to measure binding. Using the filtration assay of Darchen et al. (1989), we observed trapping of only a small fraction of the Triton-solubilized protein (data not shown).

The apparently irreversible inhibition of amine transport by reserpine observed in vivo and in vitro (Stitzel 1977) has been ascribed to the difficulty of removing reserpine associated with the lipid bilayer. Kanner et al. (1979) observed reveral of reserpine inhibition by washing vesicles with asolectin liposomes. If binding to lipid were the only reason for slow reserpine dissociation, solubilization of the transporter in detergent should lead to dissociation of bound [^{3}H]reserpine. The above results showing stable binding in detergent argue against this possibility. We cannot presently explain the reactivation of reserpine-treated vesicles by liposomes. A possible expla-

Table II: Effect of Chaotropic Agents, Acid, Heat, and SDS on Reserpine Dissociation[a]

treatment	time	dissociation (%)
1 M SCN^-	2 h	0
8 M urea	2 h	0
1 M SCN^- + 1% cholate	2 h	50
100 °C	5 min	100
0.03% SDS	30 min	0
0.05% SDS	30 min	76
0.075% SDS	30 min	95
pH 5.5	2 h	0

[a] Membranes were allowed to bind [^{3}H]reserpine (1 nM) for 30 min at 37 °C in the presence of ATP. Portions of the suspension were treated for the indicated times as described, and remaining bound reserpine was determined by gel filtration as described under Experimental Procedures. Results are expressed relative to the amount of reserpine bound before treatment in each case (2.0 pmol/mg).

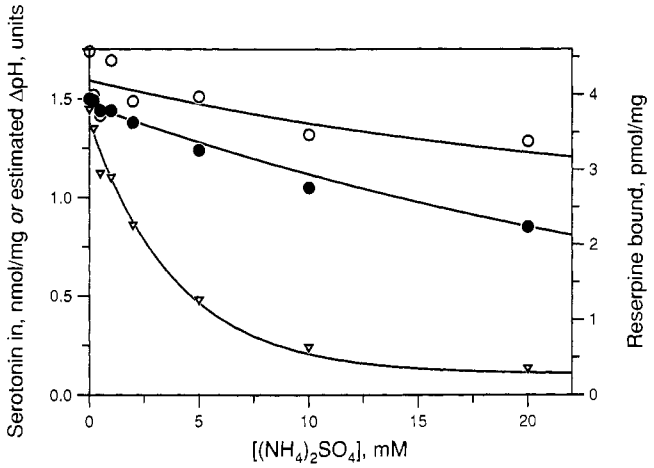

FIGURE 3: Inhibition by $(NH_4)_2SO_4$ of serotonin transport and reserpine binding. Steady-state transport (triangles) and binding rates (open circles) were measured at 37 °C as described under Experimental Procedures in the presence of the indicated concentration of $(NH_4)_2SO_4$. ΔpH (closed circles) was calculated from the accumulated serotonin gradient as described in the text.

nation is that some unidentified component of the liposome preparation stimulates reserpine dissociation.

Chaotropic Agents. Neither 1 M SCN^- nor 8 M urea increased the dissociation of bound [^{3}H]reserpine from intact membrane vesicles (Table II). Cholate-solubilized membranes were somewhat more sensitive to thiocyanate; 50% of bound label dissociated in 2 h from solubilized membranes in the presence of 1 M SCN^-. Complete release of bound [^{3}H]-reserpine was achieved only by boiling the preparation for 5 min or treatment with SDS. Table II also shows the dependence of [^{3}H]reserpine dissociation on SDS concentration.

Energetics of Reserpine Binding

Effect of Ammonium and Uncoupler. Since the final level of reserpine binding is similar in the presence and absence of ATP (Scherman & Henry, 1983), the effect of the electrochemical H^+ potential ($\Delta\tilde{\mu}_{H^+}$) in reserpine binding is expressed entirely in the binding rate, which must reflect a change in the state of the transporter. Moreover, binding is slow relative to transport, suggesting that the transporter is in equilibrium with the electrochemical H^+ gradient before binding occurs. We therefore measured the rate of binding in the presence of ATP and varied the components of $\Delta\tilde{\mu}H^+$ by addition of ammonium sulfate or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Figure 3 presents results with $(NH_4)_2SO_4$ addition, which is expected to dissipate the transmembrane pH difference (ΔpH) with little effect on the membrane potential ($\Delta\psi$). For comparison, the effect of $(NH_4)_2SO_4$ on the

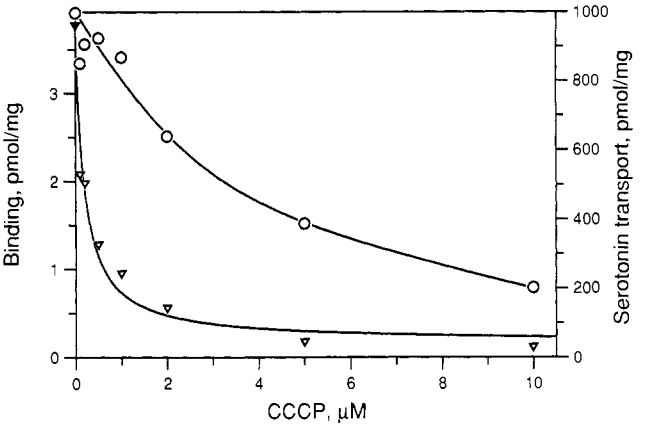

FIGURE 4: Inhibition by CCCP of serotonin transport and reserpine binding. Transport (triangles) and binding (circles) were determined as described in the legend to Figure 3 in the presence of the indicated concentrations of CCCP.

Table III: Effect of Ionophores on Reserpine Binding and Serotonin Transport in Cl^--Free Medium[a]

	serotonin transport (nmol/mg)	reserpine binding rate [pmol mg^{-1} $(5\ min)^{-1}$]
no addition	2.09 ± 0.18	4.37 ± 0.3
2 μM nigericin	0.24 ± 0.02	3.57 ± 0.3
5 μM valinomycin	1.39 ± 0.1	4.02 ± 0.25
valinomycin + nigericin	0.11 ± 0.02	1.00 ± 0.08

[a] Steady-state serotonin transport and reserpine binding rate were measured at 37 °C under the conditions listed above and as described under Experimental Procedures. The reaction medium consisted of 150 mM sodium isethionate containing 10 mM K-Hepes, pH 8.5, 2.5 mM $MgSO_4$ and 5 mM Na_2ATP with [^{3}H]reserpine or serotonin as describeds under Experimental Procedures. The resultss represent the averages of triplicates and are shown with standard deviations. This experiment was one of a series of three, each of whcih gave essentially identical results.

steady-state level of serotonin transport is also shown. Transport is known to proceed by translocation of two H^+ ions per molecule of substrate (Knoth et al., 1981a; Johnson et al., 1981). From the data in Figure 3, it is clear that transport is much more sensitive to dissipation of ΔpH than is reserpine binding. Also shown in Figure 3 is an estimate of ΔpH from the transport measurements, using the previously determined stoichiometry of 2 H^+/serotonin and an intravesicular volume of 5 μL/mg of protein. This calculation does not take into account transport driven by $\Delta\psi$, which we assume to be of minor importance in the presence of external Cl^-. While the absolute values of estimated ΔpH may not be precise, the decrease on addition of $(NH_4)_2SO_4$ should reflect proportionately the dissipation of ΔpH. It is noteworthy that the decrease in reserpine binding rate parallels the calculated decrease in ΔpH.

A similar experiment using CCCP is shown in Figure 4. Unlike $(NH_4)_2SO_4$, CCCP dissipates ΔpH by increasing the rheogenic H^+ leak. As in the case with $(NH_4)_2SO_4$, CCCP inhibits serotonin transport to a greater extent than reserpine binding. If the same number of H^+ ions were translocated in serotonin transport and reserpine binding, then we would expect agents like $(NH_4)_2SO_4$ and CCCP to inhibit both processes in parallel. The lower sensitivity observed for binding suggests that fewer H^+ ions must cross the membrane to generate the form of the transporter that binds reserpine than are exchanged for each molecule of serotonin.

Effect of Ionophores in Cl^--Free Medium. Generation of ΔpH by chromaffin granule membrane vesicles is thought to

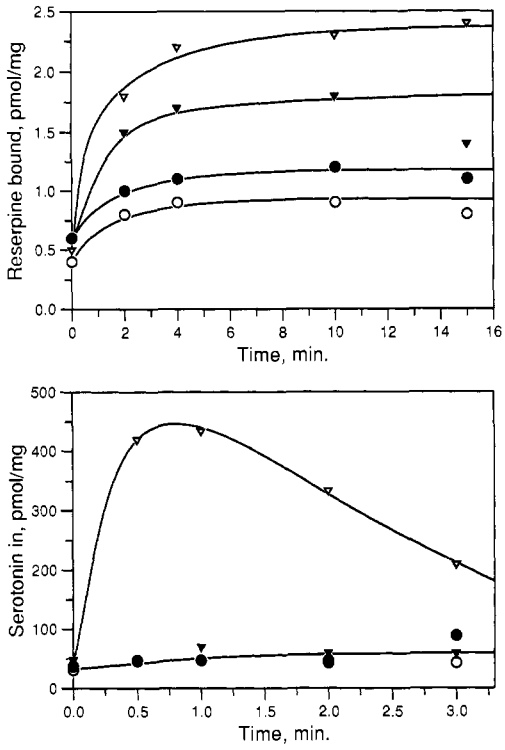

FIGURE 5: Stimulation of reserpine binding and serotonin transport by artificially imposed ΔpH. Membrane vesicles were equilibrated with 10 mM K-Hepes, pH 7.4, containing 25 mM KSCN and 125 mM KCl. (Upper panel) Binding was initiated by diluting 70 μg of the equilibrated membrane suspension 33-fold at 37 °C with one of the following media containing 0.6 nM [^{3}H]reserpine: (open triangles) 0.3 M sucrose containing 10 mM Tris–Hepes, pH 8.5, 2.5 mM $MgSO_4$, and 5 μM nigericin; (open circles) the same medium plus 1 μM unlabeled reserpine; (closed triangles) the same medium without nigericin; (closed circles) 150 mM KCl containing 10 mM Tris–Hepes, pH 8.5, 2.5 mM $MgSO_4$, and 5 μM nigericin. (Lower panel) Transport reactions were initiated by dilution of 20 μg of the membrane suspension 40-fold into the media indicated above containing 0.23 μM [^{3}H]serotonin.

depend on Cl^- influx which compensates for electrogenic H^+ pumping (Johnson & Scarpa, 1979). Table III presents data showing the effects of two ionophores, nigericin and valinomycin, on serotonin transport and reserpine binding in Cl^--free medium. Nigericin catalyzes the electroneutral exchange of K^+ and H^+ ions and can therefore dissipate the ΔpH without affecting Δψ. Valinomycin catalyzes rheogenic K^+ flux which will decrease Δψ generated by H^+ pumping.

From the effects of the two ionophores on transport, it is clear that the major driving force for serotonin accumulation is a ΔpH, even in Cl^--free medium. Transport is inhibited much less by valinomycin, which partially dissipates Δψ (as measured in separate experiments by SCN^- accumulation), than it is by nigericin, which is expected to dissipate ΔpH (Table III). Despite prevailing opinion to the contrary, removing external permeant anions is apparently not sufficient to prevent ΔpH generation. In contrast with transport, the reserpine binding rate was hardly affected by dissipating ΔpH with nigericin (Table III). Thus, the previous conclusion (Scherman & Henry, 1984) that Δψ alone could energize reserpine binding is correct, although it was based on assumptions about the effect of permeant anions which we now believe to be unjustified. Valinomycin also did not significantly inhibit reserpine binding. Only when both ionophores were added, and both components of the $\Delta\tilde{\mu}_{H^+}$ were dissipated, was binding substantially reduced. The observation that Δψ can serve as the sole driving force for reserpine binding indicates that the effect of ATP is not simply to concentrate reserpine inside the vesicles by ΔpH-driven nonionic diffusion.

Effect of Artificially Generated ΔpH. Stimulation of reserpine binding by artificial generation of a ΔpH in the absence of ATP strengthens the conclusion that this component of $\Delta\tilde{\mu}_{H^+}$ supports reserpine binding and that ATP stimulation of reserpine binding is accounted for entirely by $\Delta\tilde{\mu}_{H^+}$ generation. Membrane vesicles equilibrated with K^+ develop a ΔpH (acid in) when diluted into K^+-free medium in the presence of nigericin, an ionophore that catalyzes H^+ influx coupled to the downhill efflux of K^+. The data shown in Figure 5 demonstrate that this manipulation drives both biogenic amine transport [as previously established by Schuldiner et al. (1978)] and reserpine binding. These effects are not seen when the equilibration and dilution media are identical. When nigericin is absent, transport is not stimulated, but some increase in binding is observed relative to the controls.

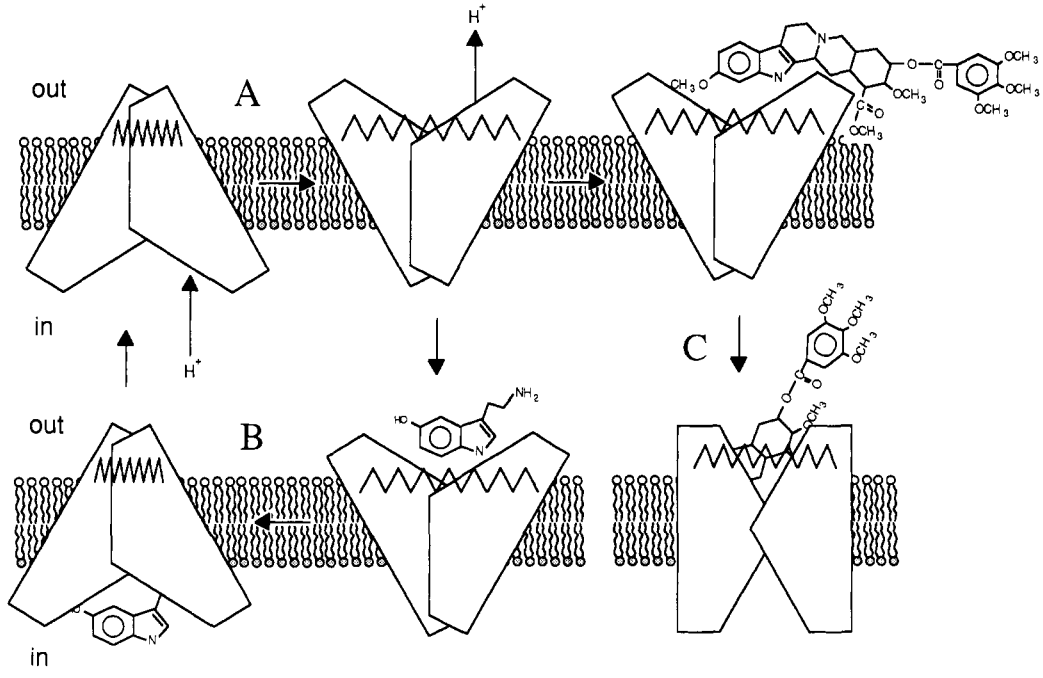

FIGURE 6: Model for serotonin transport and reserpine binding.

This increase is likely due to the jump in external pH induced by diluting vesicles equilibrated at pH 7.4 into sucrose medium at pH 8.5. Thus, both increasing external pH and decreasing internal pH increase the reserpine binding rate. The results of independent experiments (not shown) indicate that increased ΔpH and not decreased internal pH is responsible for the nigericin effect. In those experiments, external pH was varied in the presence of nigericin and high K^+ both internally and externally. Under these conditions, the ΔpH should remain clamped at zero, and the internal pH should follow external pH. No increase in reserpine binding rate was observed as the external pH was lowered from 8.5 to 6.5.

Discussion

The results presented here establish the usefulness of reserpine binding as a probe that measures an energy-dependent step in the catalytic cycle of the biogenic amine transporter. From the dependence of the reserpine binding rate on ΔpH and $\Delta\psi$, we conclude that the appearance of a high-affinity reserpine binding site on the transporter requires translocation of fewer H^+ ions than does the complete transport cycle. The overall stoichiometry for amine transport is well established. The equivalent of two H^+ ions and one positive charge cross the membrane in exchange for each protonated amine molecule (Knoth et al., 1981a, Johnson et al., 1981). The data presented in Figures 3–5 and Table III establish that the reserpine binding rate is less sensitive than transport to changes in the transmembrane pH difference (ΔpH) and that binding is stimulated at lower values of ΔpH and $\Delta\psi$.

These results suggest that fewer H^+ ions are translocated in the step which generates the high-affinity reserpine binding site than in the overall transport cycle. However, the reserpine binding rate, like amine transport, is stimulated by both the transmembrane electrical potential ($\Delta\psi$) (interior positive) and also the ΔpH (interior acid) which are generated by electrogenic H^+ pumping. In most cases, our experimental manipulation of $\Delta\psi$ and ΔpH affected neither the external pH nor the reserpine concentration. We therefore assume that changes in binding rate reflect changes in the availability of reserpine binding sites, and consider it likely that translocation of a single H^+ ion with its accompanying positive charge generates the reserpine binding form of the amine transporter.

Figure 6 schematically demonstrates a physical model to explain transport, reserpine binding, and reserpine occlusion by the biogenic amine transporter. In this model, the high-affinity form of the transporter is apparently also a higher energy form that requires H^+ translocation for its formation. The energy invested in the transporter by H^+ flux may be released by ligand binding and converted either into vectorial movement of a neutral substrate molecule across the membrane or directly into binding energy in the case of reserpine. According to the scheme in Figure 6, H^+ transport forces the transporter protein to undergo a conformational change (A) which reveals the high-affinity site. A second conformational change (B) is induced by neutral substrate or reserpine binding to this site. In the case of a substrate such as serotonin, the second conformational change results in the ligand binding site being exposed to the vesicle interior, where the substrate can dissociate and be protonated. Subsequent H^+ efflux regenerates the high-affinity state. The structure of reserpine, however (possibly the bulk of its side chain), restricts the conformational change triggered by reserpine binding so that instead of releasing the ligand on the interior, the complex becomes trapped (C) in a state from which reserpine cannot readily dissociate and which cannot translocate another H^+ ion to regenerate the high-affinity form.

Registry No. H, 12408-02-5; reserpine, 50-55-5; serotonin, 50-67-9.

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