Sidedness and Chemical and Kinetic Properties of the Vesamicol Receptor of Cholinergic Synaptic Vesicles†

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ABSTRACT: Cholinergic synaptic vesicles isolated from *Torpedo* electric organ contain a receptor for the compound *l*-2-(4-phenylpiperidino)cyclohexanol (vesamicol, formerly AH5183), which when occupied blocks storage of acetylcholine (AcCh). The inside or outside orientation of the receptor and its chemical and ligand binding kinetics characteristics were studied. Binding of [^{3}H]vesamicol to the receptor is inhibited efficiently by the protein modification reagents 4-(chloromercuri)benzenesulfonate and *N,N'*-dicyclohexylcarbodiimide and by protease treatment of cholate-solubilized receptor. The receptor in native vesicles is resistant to irreversible inactivation by proteases, elevated temperature, or pH extremes. [^{3}H]Vesamicol binding depends on deprotonation of a group of $pK_{a_1} = 6.26 \pm 0.03$ and protonation of a group of $pK_{a_2} = 10.60 \pm 0.04$, which is probably the tertiary amine of the drug molecule itself. The membrane-impermeant zwitterionic vesamicol analogue *dl-trans*-4-oxo-4-[[5,6,7,8-tetrahydro-6-hydroxy-7-(4-phenyl-1-piperidinyl)-1-naphthalenyl]amino]butanoic acid (TPNB) is an effective inhibitor of AcCh active transport with an IC_{50} value of $(51 \pm 8) \times 10^{-9}$ M. At 23 °C, [^{3}H]vesamicol bound to the receptor at a rate of $(1.74 \pm 0.06) \times 10^5$ M^{-1} s^{-1}, and excess unlabeled vesamicol displaced a low concentration of bound [^{3}H]vesamicol at 0.29 ± 0.01 min^{-1}. At 0 °C, 10 μM unlabeled vesamicol displaced 36 ± 2% of a low concentration of bound [^{3}H]vesamicol at 0.16 ± 0.02 min^{-1} and 64 ± 2% at 0.013 ± 0.001 min^{-1}. One micromolar unlabeled vesamicol behaved similarly. In a different preparation of vesicles, 10 μM TPNB displaced 22 ± 2% at 0.28 ± 0.05 min^{-1} and 78 ± 1% at 0.013 ± 0.001 min^{-1}. Several types of receptor heterogeneity are consistent with the data. It is concluded that the vesamicol receptor is a stable protein often exhibiting heterogeneity, which faces the cytoplasmic compartment of the cholinergic nerve terminal. It probably contains a binding site carboxylate in a hydrophobic environment, which ion pairs with the protonated tertiary ammonium group of the drug. It also contains a cytoplasmically oriented sulfhydryl group, which is linked to but not part of the binding site.

Synaptic vesicles isolated from the electric organ of *Torpedo* exhibit active transport of exogenous acetylcholine (AcCh).[1] The transport system is composed of at least three components: namely, an ATPase thought to pump protons into the vesicle; an AcCh transporter, which draws on the protonmotive force to drive secondary active transport of AcCh; and a receptor for the drug *l*-2-(4-phenylpiperidino)cyclohexanol (vesamicol, formerly AH5183) (Bahr & Parsons, 1986a; Marshall & Parsons, 1987). When the vesamicol receptor is occupied, AcCh active transport is blocked noncompetitively (Anderson et al., 1983; Bahr & Parsons, 1986b).

Vesamicol originally was discovered as a neuromuscular blocking agent with unusual characteristics of action (Marshall, 1970). The drug inhibits the quantal release of AcCh from every cholinergic nerve terminal preparation that has been examined, presumably as a secondary effect of storage block (Lupa et al., 1986; Suszkiw & Toth, 1986; Collier & Řičný, 1986; Řičný & Collier, 1986; Jenden et al., 1987; Michaelson et al., 1986; Jope & Johnson, 1986; Collier et al., 1986; van der Kloot, 1986; Carroll, 1985; Otero et al., 1985; Welner & Collier, 1985; Melega & Howard, 1984). The drug also inhibits nonquantal release of AcCh from the motoneural terminal, which suggests that nonquantal release is mediated by synaptic vesicle membrane incorporated into the cytoplasmic membrane as a result of exocytosis (Vyskočil, 1985; Edwards et al., 1985). Quantitative autoradiography has shown that the vesamicol receptor is distributed heterogeneously in brain in a manner highly correlated with other cholinergic terminal markers (Marien et al., 1987). It now is clear that the vesamicol receptor is a new major component of the cholinergic terminal, but its function in vivo is uncertain.

Biochemical characterization of the vesamicol receptor system is just beginning. We would like to know whether the receptor is a protein, and if so, what its gross chemical properties are, and whether it faces the cytoplasmic compartment or the inside of the synaptic vesicle. The sidedness of the receptor cannot be deduced a priori because vesamicol is a tertiary amine. The small fraction of the drug that is unprotonated at neutral pH could mediate nonspecific permeation of the drug to the inside of the vesicle where it could reprotonate and bind to an internal receptor. We report here the results of experiments designed to answer these questions.

Materials and Methods

VP_1 synaptic vesicles were isolated from the electric organ of *Torpedo californica* as described by Anderson et al. (1982).

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[1] Abbreviations: AcCh, acetylcholine; vesamicol (formerly AH5183), 2-(4-phenylpiperidino)cyclohexanol; TPNB, *dl-trans*-4-oxo-4-[[5,6,7,8-tetrahydro-6-hydroxy-7-(4-phenyl-1-piperidinyl)-1-naphthalenyl]-amino]butanoic acid; MBS, 4-(chloromercuri)benzenesulfonate; DCC, *N,N'*-dicyclohexylcarbodiimide; IC_{50}, concentration required to inhibit half of vesamicol binding or AcCh active transport; EDTA, ethylenediamine-*N,N,N',N'*-tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid.

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Briefly stated, this involves differential sedimentation velocity pelleting, equilibrium buoyant density centrifugation, and controlled-pore glass bead filtration of vesicles in isosmotic glycine (ammonia free; Calbiochem)–sucrose solutions. Many experiments were performed in buffer A composed of 0.70 M glycine, 0.10 M *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes), 1 mM ethylenediamine-*N,N,N',N'*-tetraacetic acid (EDTA), and 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), titrated to pH 7.8 with 0.80 N KOH. [^{3}H]AcCh (100 mCi/mmol) was from Amersham, and [^{3}H]vesamicol (32.7 Ci/mmol) was synthesized as described (Bahr & Parsons, 1986a). *dl-trans*-4-Oxo-4-[[5,6,7,8-tetrahydro-6-hydroxy-7-(4-phenyl-1-piperidinyl)-1-naphthalenyl]amino]butanoic acid (TPNB) was a gift from Dr. Gary A. Rogers, Department of Chemistry, University of California, Santa Barbara, CA. Its synthesis will be described elsewhere. 4-(Bromomethyl)benzoic acid was a gift from Dr. Rogers. All other reagents were from usual commercial sources.

[^{3}H]Vesamicol binding was studied by using either the centrifugation–gel filtration method (Bahr & Parsons, 1986a) or the glass fiber filter method. For centrifugation–gel filtration, 200 μL of sample typically was applied to a 2.0-mL precentrifuged column of Sephadex G-50–150 (Sigma Chemical Co.) equilibrated in the sample buffer and held in a disposable syringe barrel at 4 °C. The sample was centrifuged at 250*g* for 4 min, and the void volume solution was assayed for radioactivity by liquid scintillation spectroscopy in 5 mL of Hydrofluor (National Diagnostics, Inc.) and for protein content. Glass fiber filters (Whatman, 2.4 cm GF/F) were coated for 2 h with 0.5% poly(ethylenimine). Typically, 120 μL of vesicles incubated under the stated conditions and concentrations of [^{3}H]vesamicol was filtered rapidly with vacuum suction through immediately prerinsed filters and washed rapidly in like manner 3 times with 2.5-mL portions of ice-cold sample buffer. Wet filters were incubated for 1 h in a scintillation vial containing 7 mL of Hydrofluor before determination of radioactivity. Protein was determined as described with a bovine serum albumin standard (Bradford, 1976). Nonspecific binding is defined as [^{3}H]vesamicol binding in the presence of 100-fold excess nonradioactive *l*-vesamicol. Specifically bound [^{3}H]vesamicol is the difference between the total bound and nonspecifically bound [^{3}H]vesamicol.

All data reported are the averages of duplicates or triplicates, unless otherwise stated, which typically varied from each other by less than 10%. When equations modeling drug interactions with the vesamicol receptor were fit to the data, the best parameters were determined by regression analysis on a microcomputer. One standard error is quoted to indicate the accuracy of the estimates.

RESULTS

Chemical Modification and Proteolysis of the Receptor. In order to determine if the vesamicol receptor is a protein, purified *Torpedo* electric organ synaptic vesicles were incubated with a series of protein modification reagents, after which the effects on [^{3}H]vesamicol binding were determined by utilizing a saturating concentration of the drug (Table I). Binding was blocked completely by 1 mM concentrations of the lipid-insoluble sulfhydryl-group modifier 4-(chloromercuri)benzenesulfonate (MBS) and the lipid-soluble carboxyl-group modifier *N,N'*-dicyclohexylcarbodiimide (DCC) but not completely by even 10 mM concentrations of reagents that react with other groups such as the amino, guanidino, phenolic, sulfide, or disulfide groups. The moderate inhibition produced by dithiothreitol is of note because this is a very mild reagent that is highly specific for disulfide bonds characteristic of proteins. The amount of receptor inactivation caused by MBS and DCC was studied at lower concentrations of each reagent. As shown in Figure 1, the mercurial was quite potent, with an IC_{50} value of 1×10^{-6} M. The IC_{50} value for DCC was higher at 7×10^{-5} M. In contrast to DCC, the water-soluble carboxyl-group modifier *N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide was a poor inhibitor (Table I). Several reagents, notably trinitrobenzenesulfonate, which reacts with nucleophiles, *N*-ethyl-5-phenylisoxazolium-3'-sulfonate, which is a water-soluble carboxyl-group reagent, and iodoacetate, which reacts with sulfhydryls, actually caused an increase in vesamicol binding. Thus, typical protein

Table I: Inhibition of Vesamicol Binding by Protein Modification Reagents

reagent[a]	concn 1 mM	concn 10 mM
4-(chloromercuri)benzenesulfonate	100	100
trinitrobenzenesulfonate	−5	−35
N-ethyl-5-phenylisoxazolium-3'-sulfonate	−20	−8
iodoacetate	−21	11
dl-dithiothreitol	14	26
N,N'-dicyclohexylcarbodiimide	99	99
N-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide	44	56
phenylglyoxal	37	31
4-chloro-7-nitrobenzoxadiazole	69	94
glutaraldehyde	32	56
4-(bromomethyl)benzoate	32	42

	time 2 h	time 4 h	time 24 h
proteinase K[b]	0		58
proteinase K, cholate[b]		90	100

[a] A separate 188-μL portion of pure synaptic vesicles (0.11 mg/mL) in 100 mM Hepes titrated to pH 7.80 with 0.80 M KOH and made isosmotic (800 mosM, determined on a Wescor 5100 C vapor pressure osmometer) with sucrose was incubated in the presence of the indicated concentration of a chemical reagent for 1 h at 23 °C. Water-soluble stable reagents were dissolved just prior to use in the above buffer; ethanol-soluble reagents were taken to dryness in the reaction vial in a Savant Speed Vac concentrator, and then the vesicle–buffer solution was added and vortexed. The sample was made 500 nM in [^{3}H]vesamicol and incubated for 60 min at 23 °C. Bound [^{3}H]vesamicol was determined by centrifugation–gel filtration. Results are expressed as the percent binding relative to the unreacted control. Negative percent inhibition means that drug binding was enhanced. [b] Separate 165-μL portions of pure synaptic vesicles (0.11 mg/mL) in isosmotic buffer A, or separate 165-μL portions of pure synaptic vesicles (0.070 mg/mL) solubilized in 0.5% sodium cholate in buffer composed of 200 mM glycine, 10 mM Hepes, 10 mM EDTA, and 1 mM EGTA, adjusted to pH 7.4 with 0.80 M KOH, were mixed with 10 μL of proteinase K (3 mg/mL, dissolved in ice-cold water). They were incubated at 23 °C for the indicated times after which [^{3}H]vesamicol binding was assayed as above.

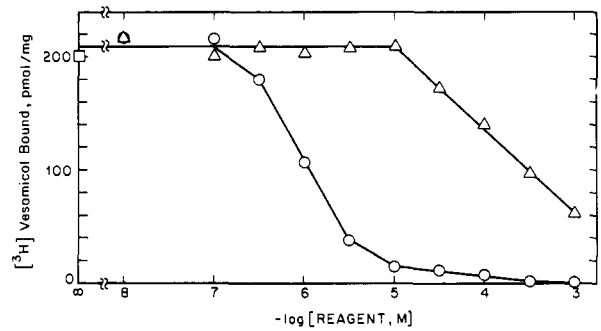


FIGURE 1: Effect of DCC and MBS on binding of [^{3}H]vesamicol. Receptor was treated with the indicated concentration of DCC (△) or MBS (○) and assayed for [^{3}H]vesamicol binding as in Table I. The lines connecting the data were drawn manually.

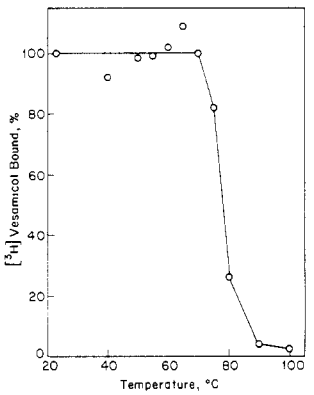


FIGURE 2: Heat denaturation of the vesamicol receptor. A separate 175-μL portion of pure synaptic vesicles (0.12 mg/mL) in buffer A contained in a thin-walled glass test tube was immersed in a water bath at the indicated temperature for 15 min, removed, and allowed to cool to 23 °C. [^{3}H]Vesamicol binding activity was assayed as in Table I. Binding is expressed relative to the 250 nmol of specifically bound [^{3}H]vesamicol/mg of vesicle protein that was observed in control vesicles at 23 °C. Similar results were obtained with one other preparation of vesicles.

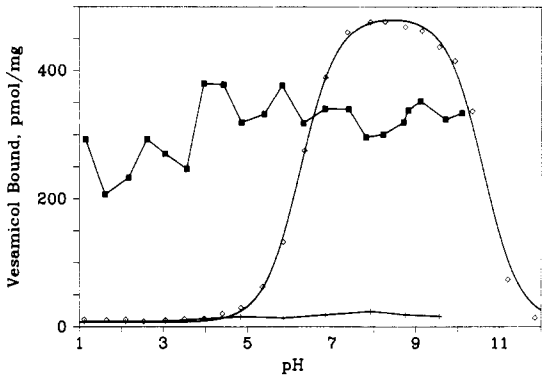


FIGURE 3: pH dependence of vesamicol binding and receptor stability. To study receptor stability (■), a separate 20-μL portion of pure synaptic vesicles (0.50 mg/mL) resuspended in unbuffered 0.4 M NaCl was mixed with 80 μL of wide-range buffer composed of 28.6 mM citric acid, 28.6 mM KH_2PO_4, 28.6 mM H_3BO_3, and 28.6 mM diethylbarbituric acid that was adjusted to an acidic or basic pH with HCl or NaOH. The pH value shown is the actual resulting value. After incubation for 60 min at 23 °C, the pH was then readjusted to approximately neutrality by addition of 100 μL of 0.4 M Hepes adjusted to pH 7.5 with KOH. [^{3}H]Vesamicol added in 0.4 M NaCl was used to assay binding as in Table I. Each point represents a single datum. To study the pH dependence of [^{3}H]vesamicol binding, a separate 10-μL portion of pure synaptic vesicles (1.0 mg/mL) resuspended in unbuffered 0.4 M NaCl was mixed with 98 μL of the wide-range buffer. After incubation at the indicated pH for 30 min at 23 °C, [^{3}H]vesamicol binding was assayed as above for the amount of total bound (◇) and nonspecifically bound (+) [^{3}H]vesamicol by the glass fiber filter method. The equation for the bell-shaped curve is $y = c + B_{max}/(1 + [H^+]/K_{a_1} + K_{a_2}/[H^+])$, where y is the amount of bound [^{3}H]vesamicol, B_{max} is the maximum bound [^{3}H]vesamicol, c is the nonspecifically bound [^{3}H]vesamicol, K_{a_1} is the acid dissociation constant for the group that must be unprotonated for drug binding, and K_{a_2} is the acid dissociation constant for the group that must be protonated. Similar results were obtained with one other preparation of vesicles for both experiments.

modification reagents affected the vesamicol receptor in both inhibitory and stimulatory senses.

Intact vesicles were incubated with a high concentration of proteinase K, which is a nonspecific protease able to degrade most proteins. Vesamicol binding remained intact for 2 h, and only part of the binding activity was lost after 24-h incubation (Table I). A control experiment using bovine serum albumin as substrate and the assay procedure given in the Boehringer Mannheim catalogue demonstrated that the protease indeed was highly active under the incubation conditions. The receptor can be solubilized in cholate detergent with full retention of the vesamicol binding activity and good stability. The solubilized receptor was more susceptible to proteolysis since most of the vesamicol binding activity was lost after 4 h (Table I). This is typical behavior for membrane-associated proteins.

Heat Inactivation of the Receptor. Intact vesicles were incubated at elevated temperatures. Vesamicol binding remained intact up to 70 °C, above which a highly cooperative inactivation occurred with a midpoint at about 77 °C (Figure 2). This is typical heat denaturation behavior for a protein.

pH Dependence of Receptor Stability and Vesamicol Binding. Most protein–ligand binding interactions exhibit substantial dependence on the pH. This can arise from irreversible denaturation of the protein or from reversible changes in the affinity of the ligand due to changes in the protonation state of specific groups in the binding site or the drug. Possible irreversible pH-dependent denaturation of the vesamicol receptor was tested by incubating vesicles at various pH values and returning them to neutrality, after which the amount of vesamicol binding was determined. Most of the binding was retained even for vesicles incubated at the pH extremes of 1.1 and 10.2 (Figure 3). The data scatter in this experiment was somewhat large since it was difficult to readjust the pH to exactly the same value for all samples for the binding measurement, but it was deemed not critical to improve the experimental protocol since it is clear that the vesamicol receptor is extremely resistant to pH–induced irreversible denaturation.

This result allowed us to determine the effect of pH on drug binding per se. The binding was strongly pH dependent in that it fell at both low and high pH values (Figure 3). The data were described well by a two-titration equation, from which it was deduced that a group of $pK_{a_1} = 6.26 \pm 0.03$ must be unprotonated and a group of $pK_{a_2} = 10.60 \pm 0.04$ must be protonated for optimal binding. These titratable groups can be in the receptor or the drug with equivalent effects.

Inhibition of AcCh Active Transport by a Lipid-Insoluble Analogue of Vesamicol. One approach to determining the sidedness of the vesamicol binding site would be to determine whether a lipid-insoluble analogue of the drug can inhibit AcCh active transport. *dl-trans*-4-Oxo-4-[[5,6,7,8-tetrahydro-6-hydroxy-7-(4-phenyl-1-piperidinyl)-1-naphthalenyl]-amino]butanoic acid (TPNB), the structure of which is shown in Figure 4, is a candidate analogue. It contains a carboxyl group, making it a zwitterion that is unlikely to penetrate the vesicle membrane. Nevertheless, it had an IC_{50} value for inhibition of AcCh active transport of $(51 \pm 8) \times 10^{-9}$ M, which is nearly equipotent with vesamicol itself. Thus, TPNB is a good analogue of vesamicol.

Time Dependencies for Association and Dissociation of Vesamicol. Another approach to determining whether the vesamicol binding site resides on the outside of the vesicle membrane is to determine the rate of association of [^{3}H]-vesamicol with its receptor and the rates of displacement of bound [^{3}H]vesamicol by nonradioactive vesamicol or TPNB. If a membrane permeation step is involved in receptor binding, we expect to see slow binding of [^{3}H]vesamicol and much slower displacement of bound [^{3}H]vesamicol by the polar TPNB than by vesamicol itself.

We had failed in a previous attempt to quantitate the rate of association of vesamicol with its receptor because it is rapid and the centrifugation–gel filtration assay method available at the time is relatively slow (Bahr & Parsons, 1986a). The newly developed glass fiber filter assay allows more rapid sampling, and with it we now have measured the association rate behavior at 23 °C (Figure 5). In this experiment, a low

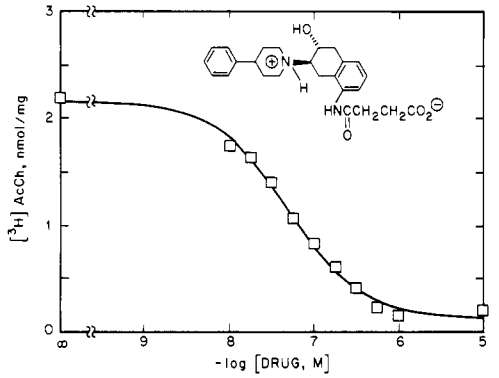


FIGURE 4: Inhibition of AcCh active transport by a lipid-insoluble analogue of vesamicol. Pure synaptic vesicles resuspended in buffer A were incubated in 0.15 mM diethyl *p*-nitrophenyl phosphate (Sigma Chemical Co.) for 60 min at 23 °C to inhibit AcCh esterase. To this solution was added concentrated components in buffer A, resulting in 10 mM MgATP and an ATP regeneration system as described (Bahr & Parsons, 1986b). Uptake was initiated immediately by the addition of a 20-μL portion of the vesicle (1.0 mg of protein/mL)–ATP solution to 60 μL of buffer A containing TPNB (the structure is shown in the figure) and [^{3}H]AcCh (final assay concentration of 49 μM). Samples were incubated for 60 min at 23 °C. Uptake was stopped by the addition of 170 μL of ice-cold buffer A containing 0.30 mM nonradioactive vesamicol. The amount of [^{3}H]AcCh taken up by vesicles was determined by centrifugation–gel filtration. A hyperbolic titration curve was fit to the data by nonlinear regression analysis to obtain an IC_{50} value of 51 ± 8 nM. Similar results were obtained with two other preparations of vesicles.

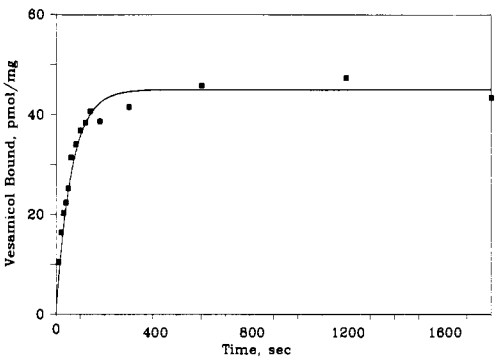


FIGURE 5: Time dependence for association of vesamicol. A separate 108-μL portion of pure synaptic vesicles (0.13 mg/mL) in buffer A was mixed at 23 °C with 12 μL of 100 nM [^{3}H]vesamicol in buffer A. The amount of bound [^{3}H]vesamicol was determined at the indicated time by the glass fiber filter assay method. The second-order rate equation for a reversible reaction that was used to fit the data by regression analysis was $\ln [C(LR_{f_0} - CR_{b_t})/LR_{f_0}(C - R_{b_t})] = [(LR_{f_0} - C^2)/C]k_2t$, where C is the amount of [^{3}H]vesamicol bound at equilibrium, R_{b_t} is the amount of [^{3}H]vesamicol bound at time t, L and R_{f_0} are the initial concentrations at time 0 for ligand and receptor, respectively, and k_2 is the second-order association rate constant. R_{f_0} was measured separately with an equilibrated saturating concentration (1 μM) of [^{3}H]vesamicol to be 420 pmol/mg of protein. Similar results were obtained with one other preparation of vesicles.

concentration of [^{3}H]vesamicol was utilized in order to slow the association rate. This resulted in binding to about 10% of the available receptors. A second-order rate equation fit the data well, with $k_2 = (1.74 \pm 0.06) \times 10^5$ M^{-1} s^{-1}. Thus, association obeys simple bimolecular kinetics, which is expected for a direct encounter between external drug and the receptor.

The dissociation of bound [^{3}H]vesamicol induced by addition of excess nonradioactive vesamicol was studied at 23 °C. A single first-order dissociation rate constant fit the data with a value of 0.29 ± 0.01 min^{-1} (not shown). This is similar to the value measured previously (Bahr & Parsons 1986a). The dissociation induced by addition of TPNB in a separate preparation of vesicles also was studied at 23 °C. In this case,

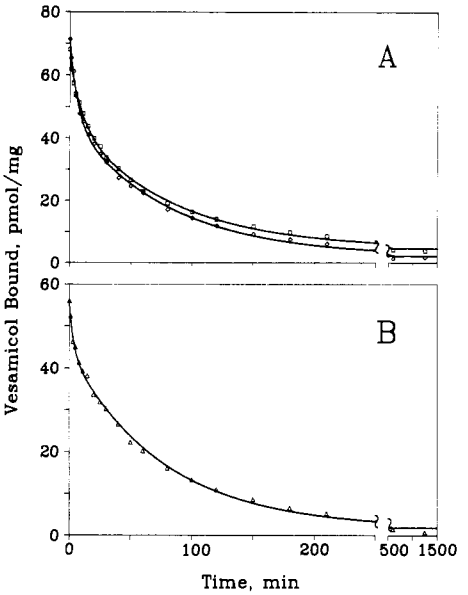


FIGURE 6: Time dependence for displacement of bound [^{3}H]vesamicol at 0 °C. (A) Displacement by nonradioactive vesamicol. Pure synaptic vesicles in buffer A were mixed with [^{3}H]vesamicol to yield a final vesicle and [^{3}H]vesamicol concentration of 0.10 mg of protein/mL and 11 nM, respectively, in a total volume of 7.0 mL. This solution was equilibrated in an ice bath at 0 °C for 2 h. At time 0, 20 μL of nonradioactive vesamicol in buffer A at 0 °C was added to the vesicle suspension to yield a final nonradioactive vesamicol concentration of 1 μM (□) or 10 μM (◇). The amount of bound [^{3}H]-vesamicol in 100-μL portions was determined periodically by the glass fiber filter method. The equation fit to the data by regression analysis was $R_{b_t} = R_{0_1}e^{-k_{1_1}t} + R_{0_2}e^{-k_{1_2}t} + C$, where R_{b_t} is the amount of [^{3}H]vesamicol bound at time t, R_{0_1} and R_{0_2} are the amounts of [^{3}H]vesamicol bound to sites 1 and 2, respectively, at time 0, k_{-1_1} and k_{-1_2} are the first-order dissociation rate constants for sites 1 and 2, respectively, and C is the residual amount of [^{3}H]vesamicol bound after the new equilibrium was reached. (B) Displacement by TPNB. Pure synaptic vesicles in buffer A were mixed with [^{3}H]vesamicol to yield a final vesicle and [^{3}H]vesamicol concentration of 0.09 mg of protein/mL and 10 nM, respectively, in a total volume of 6.5 mL. This solution was equilibrated in an ice bath at 0 °C for 2 h. Dissociation induced by 10 μM added TPNB was studied as above.

two parallel first-order dissociation rate constants were required to fit the data where 43 ± 9% of the bound [^{3}H]vesamicol dissociated at 0.42 ± 0.01 min^{-1} and 57 ± 6% at 0.079 ± 0.024 min^{-1} (not shown). Thus, some of the [^{3}H]vesamicol dissociated faster from this preparation and was effectively prevented from recombining by the excess TPNB.

Even though the above results present no indication that transmembrane permeation is required to reach the vesamicol receptor, a more rigorous test of its outside orientation was carried out by studying the displacement process at 0 °C at two different concentrations of unlabeled vesamicol and one of TPNB. Due to restricted diffusion in the membrane at the lower temperature, the zwitterionic TPNB surely could not penetrate very effectively. Polar compounds found to penetrate the erythrocyte membrane at elevated temperature do not do so at 0 °C (Staros & Richards, 1974). The results of the low-temperature experiment are shown in Figure 6. As before, two parallel first-order rate constants were required to fit the data. With 1 μM vesamicol 32 ± 4% of the bound [^{3}H]vesamicol dissociated with an apparent rate constant of 0.10 ± 0.02 min^{-1} and 68 ± 4% with one of 0.013 ± 0.001 min^{-1}. With 10 μM vesamicol 36 ± 2% dissociated at 0.16 ± 0.02 min^{-1} and 64 ± 2% at 0.013 ± 0.001 min^{-1}. With 10 μM TPNB in a different preparation of vesicles 22 ± 2% dissociated at 0.28 ± 0.05 min^{-1} and 78 ± 1% at 0.013 ± 0.001 min^{-1}. Thus, in all cases at 0 °C a small fraction of the bound

[^{3}H]vesamicol dissociated relatively rapidly and the majority dissociated more slowly.

Discussion

These results clearly indicate the protein nature of the vesamicol receptor, since some types of protein modification reagents (including high temperature) and a protease are able to inactivate it and other protein modification reagents increase the amount of drug binding. Since a saturating concentration of [^{3}H]vesamicol was utilized in this study, the increased binding suggests that some receptor was unavailable in the untreated vesicles. This is being investigated further. Indeed, in work to be reported elsewhere we have found that the vesamicol receptor system is very complex, and for this reason we have purposely limited the investigation reported here to a first-order analysis of the title subjects. For example, we see no profit in a detailed analysis of the binding or kinetic characteristics of chemically modified receptor when we have reason to believe that substantial endogenous heterogeneity is present.

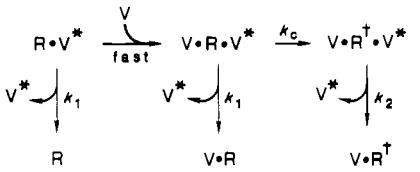
With the knowledge that the receptor is a protein, one can expect a priori that the drug binding site will contain a carboxylate amino acid side chain to ion pair with the protonated tertiary ammonium group of the drug. The critical titratable group of $pK_{a_1} = 6.26$ is a candidate for this role. The group must be in the receptor since the drug contains no group that could have such a dissociation constant. Furthermore, if the $pK_{a_1} = 6.26$ group is a carboxyl, it likely resides in a hydrophobic environment since a normal aspartic or glutamic acid pK_a is about 4.0–4.7. This conclusion is consistent with the observation that DCC, which is hydrophobic, can inactivate the receptor whereas a water-soluble carbodiimide cannot. Thus, a simple self-consistent model of the drug binding site emerges from these data where the $pK_{a_1} = 6.26$ group is a carboxyl group that serves to ion pair with vesamicol in a hydrophobic binding site.

The ability of MBS to inactivate the receptor suggests that a cysteine residue is linked to the drug binding site. It probably is not part of the binding site per se since iodoacetate, which also modifies sulfhydryl groups, did not inactivate the receptor. This could occur if only the larger MBS interferes with drug binding. Since MBS is membrane impermeant the critical modified sulfhydryl group must be on the outside of the vesicle membrane.

The titratable group of $pK_{a_2} = 10.60$, which must be protonated for drug binding, could be the sulfhydryl detected by MBS, but it seems more likely that this group is the tertiary amine of the drug itself. This is because deprotonation of the drug surely does occur with a pK_a of about this value, and there is no evidence from the pH-binding profile that more than one titratable group is important in this pH range. Thus, all the chemical modification and pH-binding profile data are consistent with each other and are readily interpreted.

In many but not all preparations of vesicles two parallel first-order rate constants were required to obtain a satisfactory fit to [^{3}H]vesamicol dissociation data. This suggests that at least two forms of the receptor exist. However, several qualifying statements must be made. First, variability in the quantitative properties of the vesamicol receptor system in different vesicle preparations is the norm, and the origin of the variability is not understood. It currently is under study. Thus, changes in the number and relative contributions of dissociation processes and in the values of the rate constants must be interpreted cautiously when results from different vesicle preparations are compared. Second, the observation made here that two exponential terms usually were required cannot be interpreted uniquely. A common interpretation is that two classes of noninteracting receptor–ligand complex exist before addition of the unlabeled competing drug and the two exponential terms reflect different dissociation rates of the complexes. This might at first analysis be thought likely to result in apparent negative cooperativity in equilibrium binding curves due to site heterogeneity. However, an alternative explanation for dissociation rate constant heterogeneity also is consistent. The accompanying paper demonstrates that [^{3}H]vesamicol binding can be positively cooperative (Gracz et al., 1988). This strongly suggests that the receptor is oligomeric and that it undergoes a conformational change generating tighter binding at higher extents of saturation. In the dissociation rate studies [^{3}H]vesamicol was equilibrated with the vesicles at a low fraction of receptor occupancy, typically about 10%. Addition of a large excess of unlabeled competitor lead to rapid occupation of all available receptor sites. If this resulted in conversion of all of the receptor–drug complexes to a higher affinity state at a rate comparable to the rate of dissociation of the low-affinity state, the *transient* site heterogeneity would be expressed as two different dissociation rates. This is shown in Scheme I, for which the time course of the bound radioactivity is given by eq 1. Here V* and V are

Scheme I



$$dpm_t = \left(\frac{k_1 - k_2}{k_1 + k_c - k_2}\right) dpm_0 e^{-(k_1+k_c)t} + \left(\frac{k_c}{k_1 + k_c - k_2}\right) dpm_0 e^{-k_2 t} + C \quad (1)$$

labeled and unlabeled vesamicol (or analogue), respectively, R is a multisubunit receptor in the low-affinity conformation and R† is in the high-affinity conformation, k_1 and k_2 are first-order dissociation rate constants, k_c is the first-order conformational change rate constant, dpm_0 and dpm_t are the amounts of bound V* at time 0 and at the time t after addition of unlabled competitor, respectively, and C is the residual binding at time infinity. The mathematical form of eq 1 is identical with that describing the dissociation behavior of preexisting noninteracting complexes. The regression values given under Results determine the parameters of Scheme I. Thus, for competition by 1 μM unlabeled vesamicol at 0 °C, k_1, k_c, and k_2 would be 0.042, 0.061, and 0.013 min^{-1}, respectively. For 10 μM unlabeled vesamicol they would be 0.065, 0.090, and 0.013 min^{-1}, respectively. For 10 μM TPNB they would be 0.070, 0.21, and 0.013 min^{-1}, respectively. If Scheme I obtains, these numbers indicate a 3–5-fold slower dissociation rate for drug bound at high receptor occupancy compared with low occupancy. In sum, dissociation rate constant heterogeneity observed by the method utilized here is compatible with either positively *or* negatively cooperative equilibrium binding curves. Substantial additional kinetic and equilibrium studies will be required to distinguish among all possibilities. As stated before, in view of the likelihood that important coupled factors are not yet known and under control, such detailed studies seem unwarranted at this time.

Nevertheless, within the context of the goals of this study, the rate constant data clearly do not suggest that the vesicle membrane must be penetrated to gain access to the vesamicol

receptor. This conclusion is independent of the detailed mechanistic interpretation of the origin of receptor heterogeneity. Thus, the zwitterionic analogue TPNB is a good inhibitor of AcCh active transport and a rapid and effective competitor for the vesamicol receptor, even at 0 °C. If the membrane were a significant barrier, penetration through it would be expected to be rate limiting. Yet, TPNB displaced [^{3}H]vesamicol at nearly the same rate as did the same concentration of unlabeled vesamicol, and a 10-fold lower concentration of unlabeled vesamicol was also nearly as effective. Thus, dissociation of the radiolabeled complex per se was rate limiting for loss of bound radioactivity. Furthermore, the ratio of the dissociation and association rate constants at 23 °C, for preparations that did not exhibit heterogeneity, predicts an equilibrium dissociation constant of 28 nM. This compares well with measured values ranging from 19 to 34 nM (Gracz et al., 1988; Bahr & Parsons, 1986a). Thus, all of the data are consistent with an outward orientation of a stable protein receptor that often exhibits heterogeneity. In vivo, the drug apparently reaches its receptor by diffusion through the cytoplasmic membrane, and once inside the nerve terminal it is free to interact. This orientation suggests that the vesamicol receptor could be subject to interaction with endogenous cytoplasmic factors.

Although there is no evidence for permeation of the vesicle membrane by vesamicol, the value of the association rate constant that was observed is low. Typical soluble enzyme–substrate interaction rates are 10–100-fold higher (Fersht, 1985). Slow binding can be caused by factors such as multiple-step mechanisms, the requirement for extensive desolvation, and a partially buried binding site. The possibility that a carboxyl group in a hydrophobic environment ion pairs with the bound drug suggests that the binding site might be partially buried in the membrane.

Finally, these results demonstrate again that the vesamicol receptor and the AcCh active transport system are not identical. Several of the chemical modification reagents, which have little inhibitory and even have stimulatory effects on the binding of vesamicol to its receptor, are able to inactivate the active transport of AcCh (Parsons & Koenigsberger, 1980). These are trinitrobenzenesulfonate, phenylglyoxal, and 4-chloro-7-nitrobenzoxadiazole. As with the receptor, active transport is sensitive to a mercurial and DCC (Parsons & Koenigsberger, 1980). The greater sensitivity of the active transport is not surprising since it presumably requires several functioning components (namely, the ATPase and the transporter), whereas the receptor function (as studied here) requires only an intact binding site.

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