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Kinetic and Equilibrium Characterization of Vesamicol Receptor-Ligand Complexes with Picomolar Dissociation Constants

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SUMMARY

Previous studies from this laboratory characterized 83 analogs of vesamicol by their potencies for inhibition of acetylcholine active transport by synaptic vesicles isolated from *Torpedo* electric organ. Examination of the more potent of these compounds, plus five new analogs, by kinetic and equilibrium measurements on complexes with the vesamicol receptor (VR) revealed nine analogs that are significantly more potent than vesamicol. Equilibrium measurements were performed at very low protein concentrations and extended incubation times, which allowed the characterization of very high affinity analogs. Better understanding of the structural binding requirements of the VR has resulted, and a spatial map of allowed hydrophobicity has been clearly established. Three analogs were resolved, and they displayed enantioselectivity ratios as high as 260 for binding to the VR (10-times higher than that of vesamicol). The most potent analog, 4-aminobenzovesamicol (ABV), was synthesized in tritiated form and shown to dissociate from the VR with a half-life of about 14 hr at 20°. The estimated dissociation constant is ≤6.5 ± 0.5 pM. By reciprocal kinetic experiments with vesamicol and ABV, coincidence of the two binding sites on vesicles was established. The high affinity and enantioselectivity of ABV and other similar analogs, coupled with good chemical and radiochemical stability, make these ligands attractive for the study of the VR in complex tissues. The observed difference between the equilibrium dissociation constant for the vesamicol-VR complex as estimated by titration with [^{3}H]vesamicol (7.6 nM) and by displacement of subsaturating [^{3}H]vesamicol by nonlabeled vesamicol (1.0 nM) suggests that high and low affinity populations of the VR exist.

The discovery of vesamicol (formerly AH5183) as a selective inhibitor of ACh storage by synaptic vesicles in motor terminals (1) led to extensive investigations that established the existence of an allosteric VR (2, 3) and defined the pharmacophores essential for inhibition of ACh active transport (4). The latter structure-activity study has generated attempts by us and other investigators to develop vesamicol analogs for *in vivo* imaging of central and peripheral cholinergic nerve terminals using single-photon emission computed tomography and PET (5–9). Successful efforts will enable both qualitative (single-photon emission computed tomography and PET) and quantitative (PET) evaluation of the extent of degeneration of cholinergic nerve terminals in disease processes such as Alzheimer's disease, perhaps at a time before clinical symptoms are manifest.

In consideration of the goal described above, the need to reassess the potencies of some of the previously studied vesamicol analogs became apparent. The earlier work was based on a functional assay, namely inhibition of the active transport of ACh into *Torpedo* synaptic vesicles. Because of the low sensitivity of the assay, it was necessary to work at a relatively high vesicle protein concentration, and therefore all assays were performed at a protein concentration of about 0.2 mg/ml. In retrospect, that protein concentration yielded VR concentrations typically in the range of 20–100 nM. The range occurs because different preparations of vesicles express different amounts of VR (10). These concentrations of the VR made it impossible to detect analogs with affinities significantly greater than that of vesamicol.

An additional complication occurs because vesamicol analogs inhibit ACh active transport at concentrations that only partially occupy the VR (11). The ratio of the concentration of drug necessary to inhibit transport to that required to occupy the corresponding proportion of the VR is structure dependent and ranges from 0.7 to 0.04. Thus, even the rank orderings of potencies for transport inhibition and receptor binding are not the same.

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ABBREVIATIONS: ACh, acetylcholine; VR, vesamicol receptor; PET, positron emission tomography; DeHVes, dehydrovesamicol; ABV, 4-aminobenzovesamicol; GlyABV, *N*-glycyl-4-aminobenzovesamicol; NEFA, 4-*N*-ethylfluoroacetamidobenzovesamicol; AcABV, 4-acetamidobenzovesamicol; *t*-BuVes, 4-*t*-butylvesamicol; transDec, (*trans*)-cyclohexovesamicol; cisDec, (*cis*)-cyclohexovesamicol; BV, benzovesamicol; PEI, polyethyleneimine; IC_{50}, concentration required for 50% inhibition; K_V, dissociation constant for the vesamicol-vesamicol receptor complex; K_I, dissociation constant for inhibitors of vesamicol binding to the vesamicol receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

TABLE 1
Equilibrium and kinetic constants for dissociation of vesamicol analog-VR complexes

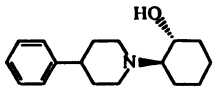
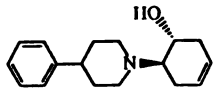
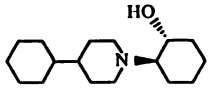
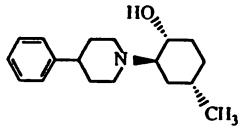
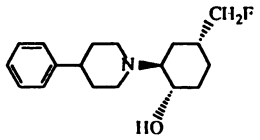
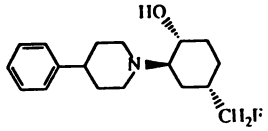
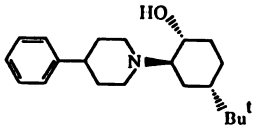
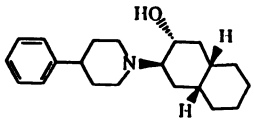
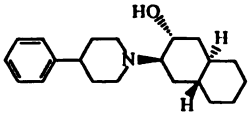
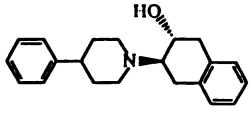
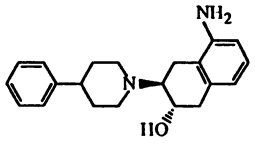
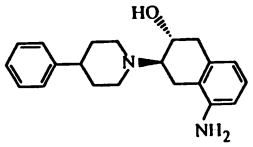
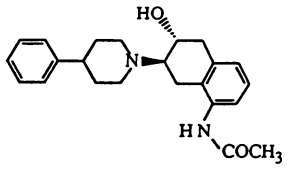
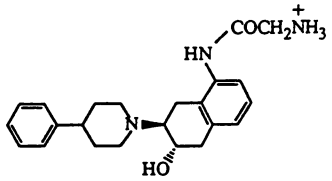
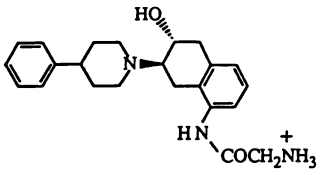
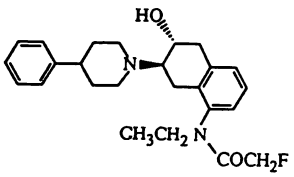
Compound[a]		$k_{-1} \times 10^{3}$[b]	K_i[c]
		min^{-1}	nM
	Vesamicol	167 ± 5[d]	1.0 ± 0.5[e] 0.7 ± 0.2[f]
	DeHVes	>90	0.34 ± 0.04
	H_6Ves	60 ± 20	ND[g]
	MeVes	ND	0.63 ± 0.04
	(+)-FMV	>3000	140 ± 10
	(−)-FMV	61 ± 15	0.70 ± 0.06[h]
	*t*-BuVes[i]	0.7 ± 0.1	0.068 ± 0.025
	cisDec	29 ± 4	ND
	transDec[j]	0.67 ± 0.06	0.009 ± 0.002
	BV	1.9 ± 0.3	0.055 ± 0.010

TABLE 1-*Continued*

Equilibrium and kinetic constants for dissociation of vesamicol analog-VR complexes

Compound[a]		$k_{-1} \times 10^{3}$[b]	K_i[c]
	(+)-ABV	160 ± 10[d]	1.7 ± 0.3[f]
	(−)-ABV	1.02 ± 0.06	0.0065 ± 0.0005[f]
		0.84 ± 0.05[d]	0.0084 ± 0.0009[f]
	AcABV	3.2 ± 0.7	0.18 ± 0.04
	(+)-GlyABV	ND	≥5.6 ± 0.3[k]
	(−)-GlyABV	3.5 ± 0.4	0.15 ± 0.02
	NEFA	4.4 ± 0.3	0.32 ± 0.04

[a] Structures of vesamicol analogs and the abbreviations that are used throughout the text. Some of the analogs are described by Rogers *et al.* (4) as analogs 1 [hexahydrovesamicol (H_6Ves)], 52 (*t*-BuVes), 65 (transDec), 66 (cisDec), 69 (BV), 72 (ABV), and 74 (AcABV). MeVes, 4-methylvesamicol; FMV, 4-fluoromethylvesamicol.

[b] All rate constants were determined at 22°, unless stated otherwise. For the following analogs, the rates of dissociation of the (+)-enantiomers were inferred from the association kinetics of the racemates, as described in Materials and Methods (all values are $min^{-1} \times 10^3$): *t*-BuVes, 160 ± 20; cisDec, 1000 ± 200; transDec, 340 ± 60; analog 67 in Rogers *et al.* (4), 1500 ± 120; BV, 200 ± 60; ABV, 540 ± 100 (40 ± 8 at 0°); AcABV, 1800 ± 200; and GlyABV, 1060 ± 140.

[c] All K_i values were determined by competition of racemic analogs against bound [^{3}H]vesamicol under equilibrium conditions at 22°, unless stated otherwise. Vesamicol was the pure (−)-enantiomer. K_i values are for the (−)-enantiomers except where isolated (+)-enantiomers were tested.

[d] 20°.

[e] This value differs from that obtained from Fig. 1B because the competition assay used for this determination does not sample the entire receptor population as does the equilibrium titration.

[f] Ratio of rate constants (k_{-1}/k_1) for dissociation (k_{-1}) and association (k_1) determined at 20°.

[g] ND, not determined.

[h] The value for (−)-(*trans*)-5-fluoromethylvesamicol is 50 ± 4 nM.

[i] Although in these two-dimensional drawings the relationships between the amino and alcohol groups of vesamicol and *t*-BuVes or transDec appear identical, the diequatorial and diaxial conformations that they assume are very different if the three-dimensional shapes are considered.

[j] Determined by equilibrium binding of (−)-[^{3}H]ABV to vesicles at 22°.

[k] (+)-GlyABV was not enantiomerically pure.

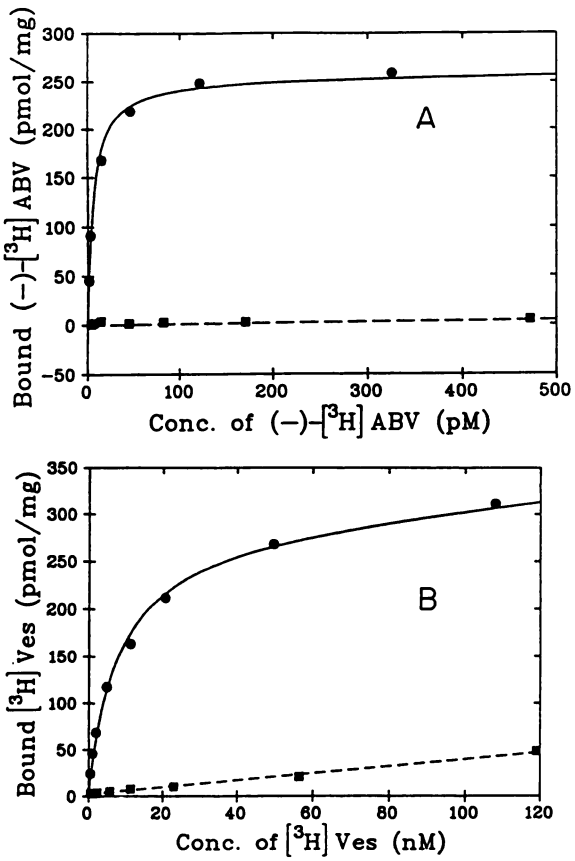


Fig. 1. A, Equilibrium binding of (−)-[^{3}H]ABV at 22° to synaptic vesicles isolated from electric organ, in the presence (■) and absence (●) of a 200-fold excess of (−)-ABV. Protein concentration was 0.09 μg/ml and the incubation time was 24 hr. Bound (−)-[^{3}H]ABV was determined as described in Materials and Methods and the free concentration was calculated by subtracting the bound concentration from the total solution concentration. Plotted values are the averages of duplicate data that exhibited a mean deviation for bound (−)-[^{3}H]ABV of 6.9%. Nonlinear regression analysis yielded a K_d value of 6.5 ± 0.5 pM. The experiment was repeated one other time under the same conditions, with similar results. B, Equilibrium binding of [^{3}H]vesamicol (*Ves*) at 22° to synaptic vesicles, in the presence (■) and absence (●) of a 200-fold excess of vesamicol. Protein concentration was 0.24 μg/ml and the incubation time was 26 hr. Bound and free ligand were determined as stated for A. Plotted values are the averages of duplicate data that possessed a mean deviation for bound [^{3}H]vesamicol of 4.6%. Nonlinear regression analysis yielded a K_d value of 7.6 ± 0.5 nM.

For the reasons detailed above we undertook in this study reevaluation of the potencies of virtually all vesamicol analogs that inhibited ACh transport with IC_{50} values in the range of 100 nM or lower. This paper reports kinetic and equilibrium measurements on the analog-VR complexes in synaptic vesicles obtained from *Torpedo* electric organ under conditions that attempt to ensure reliable determination of the relevant constants. In addition, the results for several new vesamicol analogs of potential use in probing the *in vivo* state of the VR are reported.

Materials and Methods

Synthesis of vesamicol analogs. DeHVes was synthesized by condensation in ethanol of 4-phenylpiperidine with the monoepoxide derived from 1,4-cyclohexadiene. 4-Methylvesamicol was synthesized by a similar condensation of 4-phenylpiperidine with the epoxides derived from 4-methylcyclohexene. The product was separated from the 5-positional isomer via silica gel chromatography and crystallization from acetonitrile. Racemic [^{3}H]ABV was prepared from the styryl precursor by DuPont NEN, in a manner similar to that used to produce tritiated vesamicol (4). The specific activity was 17.6 Ci/mmol. Optical resolution of [^{3}H]ABV and ABV was accomplished using a Chiralpak AD column (Daicel Chemical Industries) with 20% isopropanol/hexane as the eluent. GlyABV was synthesized from ABV and *N-tert*-butoxycarbonylglycine *p*-nitrophenyl ester (Sigma Chemical Co.) in acetic acid. The condensation product was deblocked in 50% trifluoroacetic acid/CH_2Cl_2. Neutral GlyABV was purified by silica gel chromatography and crystallized from CH_2Cl_2/CCl_4 (m.p., 178.5–180.5°). The Chiralpak AD column was used for optical resolution. NEFA was synthesized from AcABV via reduction with lithium aluminum hydride and acylation with fluoroacetyl chloride. The crude mixture was treated with methanolic KOH to destroy the ester that arises from diacylation. NEFA was purified on silica gel and crystallized from isopropanol/hexane. The synthesis and optical resolution of 4-fluoromethylvesamicol were reported elsewhere (12). All other analogs were described previously (4). Vesamicol is the resolved (−)-enantiomer.

Synaptic vesicle preparation. Reserve synaptic vesicles (VP_1) were isolated from the electric organ of *Torpedo californica* as described (13), with the modification that concentration of the vesicles after size exclusion chromatography on Sephacryl 1000 was carried out using Centriprep-30 centrifugal ultrafiltration concentrators (Amicon Corp.) at 4°. After concentration of the vesicles, the pH of the buffer (7.0) was adjusted to 7.8 by addition of the required amount of buffer A (100 mM HEPES, 700 mM glycine, 1 mM EDTA, 1 mM EGTA) at pH 8.2 (adjusted with KOH). Protein concentration was determined by the method of Bradford (14), using bovine serum albumin as a standard.

Equilibrium binding to synaptic vesicles. For the studies that involved equilibrium competition between [^{3}H]vesamicol and analogs, vesicles were diluted to 0.22 μg/ml protein with buffer A, pH 7.8, containing 4.4 nM [^{3}H]vesamicol. The vesicle solution was dispensed into Eppendorf tubes, to which duplicate samples of various concentrations of analogs were added to yield volumes of 1.0 ml each. Final concentrations of vesicle protein and [^{3}H]vesamicol were 0.20 μg/ml and 4.0 nM, respectively. Incubation at 22° was for 24 hr, during which time the free VR has been shown to retain ≥90% of its [^{3}H]vesamicol-binding capacity. Bound [^{3}H]vesamicol was assayed by rapid filtration of 980 μl from each sample through PEI-treated glass fiber filters as described (15). Tritium bound to the filters was assayed by liquid scintillation counting. Equilibrium dissociation constants were calculated by fitting the following pair of equations to the data sets:

$$V_B = R_T \cdot V_F \cdot K_I / (K_I \cdot V_F + K_V \cdot I_F + K_V \cdot K_I)$$

$$I_F = I_T \cdot V_F \cdot K_I / (K_I \cdot V_F + K_V \cdot V_B)$$

where V_B and V_F are the concentrations of bound and free [^{3}H] vesamicol, respectively, R_T is the total concentration of receptor, I_T and I_F are the total and free concentrations of analog, respectively, and K_V and K_I are the equilibrium dissociation constants for vesamicol and analog complexes, respectively. The equations were derived from the multiple equilibria and mass conservation for the VR. For these calculations, a value of 1.0 nM was used for K_V. Parameters were determined by nonlinear regression of a least-squares fit of the model to the data using the program MINSQ (MicroMath Scientific Software, Salt Lake City, UT). The same program was used for all other calculations in this study by applying the appropriate model to the data. All parameter values are quoted with error limits of 1 SD.

In addition to competition assays, direct binding experiments were performed using [^{3}H]vesamicol and (−)-[^{3}H]ABV, with protein concentrations of 0.24 and 0.09 μg/ml, respectively. In both cases, the incubation time was 24 hr.

Kinetics of association and dissociation. The association rate constants for tritiated ligands were determined under pseudo-first-order conditions; that is, the ligand concentration exceeded the receptor concentration by at least 15-fold. Association kinetics of each ligand were studied at more than one concentration to confirm that the slow

step in binding is a bimolecular process involving the ligand. The second-order rate constant for each ligand was calculated by dividing the pseudo-first-order rate constant by the ligand concentration. For [^{3}H]vesamicol and (+)-[^{3}H]ABV, association was monitored by rapid filtration of a sample of the vesicles through PEI-treated glass fiber filters, as described (15). For (−)-[^{3}H]ABV, which dissociates very slowly from vesicles, two different techniques were used to quench the association of ligand with receptor at precise time intervals. The first technique was a 1/10 dilution of 200-μl samples into ice-cold buffer followed by filtration as described above. For this method association was initiated in a solution with final protein and (−)-[^{3}H]ABV concentrations of 2.0 μg/ml and 17.7 nM, respectively. In the second technique, the final (−)-[^{3}H]ABV concentration was 14.0 nM and quenching was performed by dilution of 200-μl aliquots with 150 μl of buffer A containing 11 μM (−)-[^{1}H]ABV. The entire sample volume was then transferred (with washes) to filters as described above. A monoexponential equation was used to model the data. The rate of association of (−)-[^{3}H]ABV also was measured in the presence of 2 mM $MgCl_2$ plus 2 mM MgATP.

Rates of dissociation of tritiated ligands were measured by following their replacement by a large excess of the same nonlabeled ligand or a nonlabeled analog. [^{3}H]Vesamicol (10 nM) was displaced by 10 μM vesamicol or 1 μM (±)-ABV using vesicles from the same preparation, and (−)-[^{3}H]ABV (4.5 or 14 nM) was displaced by 10 μM vesamicol or 200 nM (−)-ABV in different preparations of vesicles. A monoexponential equation was used to model these data.

Rates of dissociation of nonlabeled ligands from vesicles were measured by a previously described procedure (16) herein referred to as "back-titration." The technique takes advantage of the very slow dissociation rate of most of the analogs in Table 1. Briefly, vesicles were allowed to equilibrate with ligand for 1 hr such that only the more potent (−)-enantiomer was bound to the vesicles. Dissociation was initiated by the addition of a large excess of nonlabeled vesamicol and, at suitable time intervals, samples of vesicles were applied to PEI-treated glass fiber filters with vacuum assistance. After application to the filters, the vesicles were washed and incubated with buffer A for sufficient time (two 10-min incubations at room temperature) to remove most of the nonlabeled vesamicol but little of the more slowly dissociating analog. Subsequent flooding and incubation (for 10 min) of the filter with an excess of [^{3}H]vesamicol allowed quantitation of receptor not occupied by a high affinity analog at the time of application to the filters. Control experiments (data not shown) demonstrated no loss of vesicles from the filters as a result of washing with copious volumes of buffer.

Results

In the present paper, we studied vesamicol analogs with IC_{50} values for inhibition of ACh active transport of ≤100 nM, by determining the affinities for the coupled VR by both equilibrium and kinetic methods. The analogs that are not discussed here did not bind with high affinity to the VR (the K_I values for the (−)-enantiomers were >1 nM). In addition, five new analogs of interest for conformational analysis, for derivatization, or as PET ligands were synthesized and their affinities were evaluated.

Equilibria. Competition between [^{3}H]vesamicol and nonlabeled analogs was performed under conditions that yielded equilibrium data, from which dissociation constants were calculated as described in Materials and Methods. An incubation time of 24 hr was required to approach equilibrium at the very low concentrations of vesicles and analogs that are necessary to characterize analogs with picomolar dissociation constants. Calculations based on the measured association rate constant of the analog ABV (see below) indicate that the approach to equilibrium would have been ≥90% for all analogs that were studied by displacement of [^{3}H]vesamicol. The dissociation constants obtained under these conditions are listed in Table 1. Except where explicitly noted, values were obtained for racemates, and the assumption was made that the values for the more potent (−)-enantiomers are one half those of the racemates. This assumption is valid if the (+)-enantiomers are significantly less potent than the (−)-enantiomers, which has been verified for vesamicol and four analogs. Where values are listed for both enantiomers, the resolved enantiomers were used for the determinations.

The synthesis of [^{3}H]ABV permitted attempts to directly determine the equilibrium dissociation constant for this analog. Shown in Fig. 1A are the averages of duplicate data at various concentrations of (−)-[^{3}H]ABV with and without a 200-fold excess of (−)-ABV. The K_I value derived from a regression fit was 6.5 ± 0.5 pM. A similar experiment with [^{3}H]vesamicol (Fig. 1B) gave a value of 7.6 ± 0.5 nM. Calculations (based on the respective rate constants for association reported below) indicate that equilibrium for solutions of vesamicol at all relevant concentrations were ≥99% complete at the end of the incubation time, but those of ABV were not. Therefore, the true dissociation constant for (−)-ABV must be <6.5 pM.

Kinetics. To compare equilibrium-derived and kinetically derived dissociation constants, association rate constants for vesamicol and ABV were determined under pseudo-first-order conditions using the tritiated ligands. The second-order nature of the binding phenomenon was confirmed by demonstrating that the values of the pseudo-first-order rate constants are directly proportional to the concentrations of the ligands. Shown in Fig. 2 are the results for (−)-[^{3}H]ABV, whereby association was quenched at the indicated times by two different techniques, i.e., dilution into ice-cold buffer or buffer containing excess nonlabeled ABV, followed by filtration on PEI-treated glass fiber filters to separate bound from free radioligand. For both experiments the calculated second-order rate constant is 1.0×10^8 M^{-1} min^{-1}. Incubating vesicles with

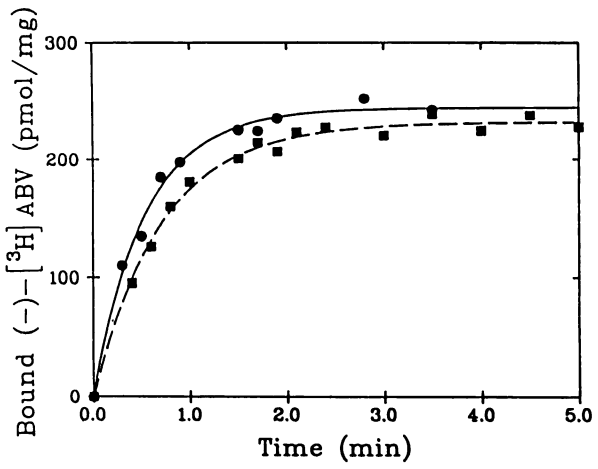


Fig. 2. Kinetics of association of (−)-[^{3}H]ABV with synaptic vesicles. Association of either 17.7 or 14.0 nM (−)-[^{3}H]ABV at 20° was quenched by dilution into ice-cold buffer (●) or buffer that contained a large excess of (−)-ABV (■), respectively. Protein concentration was 2.0 μg/ml. Although nonspecific binding to vesicles was negligible, binding to the filters contributed about 7% to the total binding. This contribution should be time independent and was subtracted from all values. A monoexponential equation was used to fit curves to the data and yielded calculated second-order rate constants of 1.02 ± 0.07 and 1.00 ± 0.05 × 10^8 M^{-1} min^{-1}, respectively.

MgATP had no effect on the association kinetics. Similar experiments were performed with [^{3}H]vesamicol and (+)-[^{3}H]ABV, using rapid filtration on PEI-treated glass fiber filters to separate bound from free radioligand. The second-order association rate constants for vesamicol and (+)-ABV are 2.3 ± 0.6 and 0.92 ± 0.11 × 10^8 M^{-1} min^{-1}, respectively (data not shown).

The dissociation of [^{3}H]vesamicol from synaptic vesicles was promoted by the addition of high concentrations of vesamicol, ABV, or AcABV. Data for replacement by vesamicol and ABV obtained with the same preparation of vesicles are shown in Fig. 3A. Monoexponential equations fit the data well, and the average value for the rate of dissociation that was derived from the three experiments is 0.167 ± 0.005 min^{-1} ($t_{1/2}$ = 4.15 min). Equivalent experiments in which (−)-[^{3}H]ABV was dissociated from vesicles by high concentrations of ABV or vesamicol are shown in Fig. 3B. The rate of dissociation by replacement with ABV is 8.4 ± 0.5 × 10^{-4} min^{-1} ($t_{1/2}$ = 14 hr) and by vesamicol is 7.1 ± 0.4 × 10^{-4} min^{-1}. The rate constant for the dissociation of (+)-[^{3}H]ABV from vesicles promoted by (±)-ABV is 0.16 ± 0.01 min^{-1} (data not shown).

For high affinity analogs it is possible to measure dissociation rates of the nonradiolabeled ligands by replacement with nonradiolabeled vesamicol, removal of nonbound ligand, and exchange of the nonradiolabeled vesamicol with [^{3}H]vesamicol. This we call the back-titration protocol, and it is described in Materials and Methods. An example is shown for (−)-ABV in Fig. 3B, and the values for all of the analogs thus derived are listed in Table 1. The value of 1.02 ± 0.06 × 10^{-3} min^{-1} for dissociation of ABV obtained from the rate of recovery of the VR measured by back-titration at 22° agrees favorably with the value of 0.84 ± 0.05 × 10^{-3} min^{-1} obtained from dissociation of (−)-[^{3}H]ABV at 20°.

Rates of dissociation of the less potent (+)-enantiomers were inferred from the kinetics of binding of the (−)-enantiomers in the racemic mixtures. Under conditions of high concentrations of racemic analog (300 nM), the rate of disappearance of the VR as a result of occupancy by the (−)-enantiomer (assayed by the back-titration technique) is biphasic, with loss of one half of the receptors being very rapid. Because the concentrations of the two enantiomers are equal, as are their association rate constants (within experimental error), the (−)-enantiomer is blocked by the (+)-enantiomer from binding rapidly to one half of the receptors. As the (+)-enantiomer dissociates, it is replaced by the (−)-enantiomer at a rate equal to one half the rate of dissociation of the (+)-enantiomer. This is because each enantiomer has an approximately equal chance of replacing the dissociated (+)-enantiomer, which slows the replacement process by a factor of 2. Therefore, the rate of replacement is one half the true rate of dissociation. The values for the dissociation rate constants for the nonradiolabeled (+)-enantiomers thus derived are included as a footnote to Table 1. When the back-titration assay was performed using (−)-ABV rather than the racemate, loss of 100% of titratable VR was rapid, as would be predicted (data not shown).

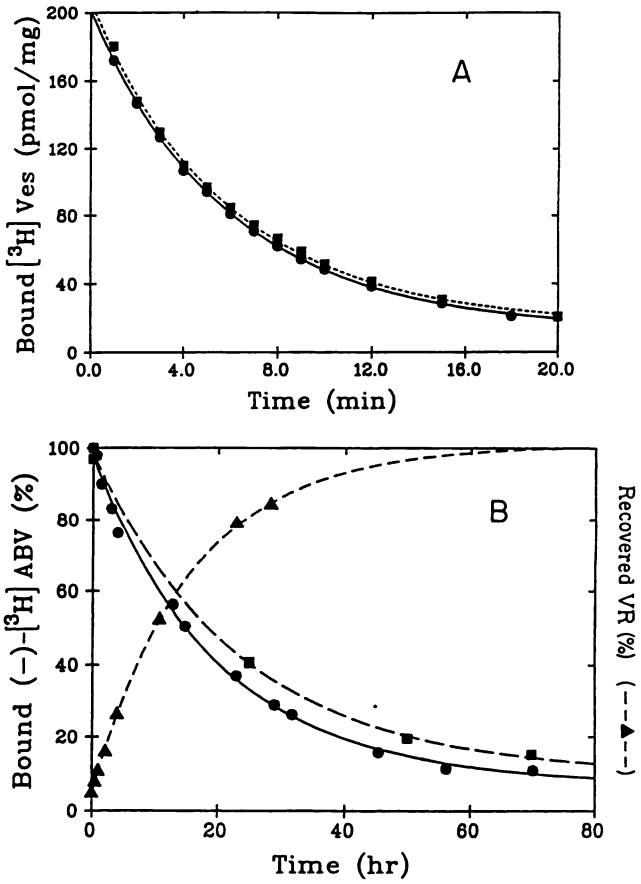


Fig. 3. A, Kinetics of dissociation of [^{3}H]vesamicol from synaptic vesicles. [^{3}H]Vesamicol (10 nM) was replaced at 20° by either 10 μM vesamicol (●) or 1 μM ABV (■). Synaptic vesicles from the same preparation were used at a protein concentration of 40 μg/ml. The total amount of bound [^{3}H]vesamicol was plotted, and therefore nonspecific binding can be estimated at about 10%. A monoexponential equation was used to fit curves to the data and yielded rate constants of 0.169 ± 0.002 min^{-1} and 0.169 ± 0.006 min^{-1}, respectively. B, Kinetics of dissociation of (−)-ABV from synaptic vesicles. At 20° (−)-[^{3}H]ABV (14 nM) was replaced by 200 nM (−)ABV (●) or (−)-[^{3}H]ABV (4.5 nM) was replaced by 10 μM vesamicol (■), and the progress of the reaction was monitored by determining the amount of tritium bound to vesicles at the indicated times. The total amount of bound [^{3}H]ABV was plotted, and therefore nonspecific binding was about 10%. At 22° nonradiolabeled (±)-ABV (300 nM) was replaced by 300 μM vesamicol (▲), and the progress of the dissociation was monitored by recovery of the VR by the back-titration protocol using [^{3}H]vesamicol, as described in Materials and Methods. Vesicles from different preparations and with different specific binding activities were used for the three experiments, and therefore quantitation was normalized to 100% of the maximum signal for graphical presentation. Monoexponential equations were used to fit curves to the data and yielded rate constants for replacement of (−)-[^{3}H]ABV by ABV or vesamicol of 0.84 ± 0.05 × 10^{-3} min^{-1} and 0.71 ± 0.04 × 10^{-3} min^{-1}, respectively, and for replacement of (−)-ABV by vesamicol of 1.02 ± 0.06 × 10^{-3} min^{-1}.

Discussion

The affinities of selected vesamicol analogs for the VR of synaptic vesicles isolated from electric organ were reassessed in this study by a variety of complementary methods. Equilibrium competition of nonlabeled analogs against subsaturating [^{3}H]vesamicol concentrations allowed calculation of the dissociation constants of the nonlabeled analogs (K_I) with the equations presented in Materials and Methods. The accuracy of the calculated K_I is dependent on accurate knowledge of both the concentration of free [^{3}H]vesamicol and the dissociation constant of the vesamicol-VR complex (K_V). If the VR is represented by a heterogeneous population, as suggested by the report of Kaufman *et al.* (11), then a value for K_V that is derived from a binding isotherm would not be appropriate. In a typical competition experiment, only a small fraction of the total receptor population is occupied by the radioligand and this

would be the subpopulation with highest affinity for the ligand. With regard to vesamicol and the VR, a comparison of the apparent value of K_V obtained by titration of the entire receptor population (7.6 nM) (Fig. 1B) with that obtained from a self-replacement experiment (1.0 nM) (Table 1) illustrates the point. The disagreement suggests that the value of 7.6 nM is likely an average value for a heterogeneous population. For this reason we have used the value of 1 nM as the K_V for vesamicol in the equations given above, to determine the K_I values for analogs (Table 1). It is reassuring that the kinetically derived K_V of 0.7 nM is within experimental error of this latter value, because the dissociation rate constant was measured by replacement of [^{3}H]vesamicol under conditions of subsaturation that sampled the putative higher affinity population.

The new estimates of K_I values together with the synthesis of new analogs of vesamicol permit a better understanding of the nature of the vesamicol binding site in the ACh transporter-VR complex. In an attempt to isolate and quantitate the individual contributions of hydrophobicity and conformation to binding affinities, we synthesized DeHVes. This structural change produces a conformation of the amino group, alcohol group, and cyclohexene ring similar to that in the BV series of compounds. Because of the double bond, the energy difference between the diequatorial and diaxial conformations of the amino and alcohol substituents will be less than that for vesamicol (17). The 3-fold higher affinity that is observed for DeHVes suggests that the lowest energy conformation of vesamicol is not optimal for binding to the VR. The larger 18-fold increase in affinity observed for BV suggests that the hydrophobic contribution provided by the additional ring of BV provides a 6-fold increase in affinity.

Other hydrophobic analogs are *t*-BuVes, cisDec, and transDec, with increased affinities of about 40-, 6-, and 110-fold, respectively. The 6-fold increase observed for cisDec (based on dissociation rate constants), which shares a diequatorial conformational preference with vesamicol, appears attributable to increased hydrophobicity provided by the additional ring. However, the much higher affinities of *t*-BuVes and transDec appear too large to be explained by a similar hydrophobic interaction. As discussed below, the K_I for *t*-BuVes is probably an overestimate of the true value and therefore the 40-fold increase in affinity is an underestimate of the contribution of the *t*-butyl substituent to increased affinity. Therefore, it is likely that the diaxial conformation of the amino and alcohol substituents, which is the lowest energy state for *t*-BuVes and transDec, is the preferred conformation bound by the VR. However, the receptor also binds analogs that exist overwhelmingly in the diequatorial conformation (e.g., vesamicol and cisDec), suggesting that the VR can bind both diaxial and diequatorial conformations of the amino and alcohol groups. An alternative explanation for these binding phenomena is that the *t*-butyl group of *t*-BuVes and the four additional methylene groups of transDec make additional and nearly equally favorable van der Waals interactions with the receptor that are not available to cisDec and BV. We think this unlikely.

Another noteworthy observation is the nearly 10-fold increase in affinity provided by the addition of the amino group to the 4-position of BV, to give ABV. With a K_I value of ≤6.5 pM, it is the most potent analog. This increased affinity may result from hydrogen bonding of the 4-amino group with the receptor. Substitution of either an acetyl (AcABV) or glycyl (GlyABV) residue on the amino group of ABV causes nearly equal decreases in ligand affinities (>20-fold). This decrease could be due to disruption of the hydrogen-bonding ability of the anilino nitrogen or to steric hindrance in this area. For NEFA, in which the anilino nitrogen has been both acylated and alkylated, an additional small decrease in affinity is observed, compared with AcABV. Although not included in Table 1, succinyl-ABV (analog 78 of Ref. 4) was also examined. The rate of dissociation was identical to that for (−)-ABV, and therefore we conclude that the succinyl group is at least partially labile under the conditions of our analysis. Because succinyl-ABV is unable to compete effectively against ABV, it is likely that a negative charge in the region of the 4-position lowers affinity dramatically. In contrast, an additional positive charge may be permitted (compare GlyABV with AcABV).

Because of the very high affinity of ABV and the significant structural variation from vesamicol, we thought it necessary to assess whether the vesamicol and ABV binding sites are coincident. The fact that they are is demonstrated in Fig. 3. Fig. 3A shows that (±)-ABV replaces all of the specifically bound [^{3}H]vesamicol at the same rate that it is replaced by vesamicol. The identical rates make it unlikely that ABV displaces vesamicol by binding to an alternative site. Replacement by (±)-AcABV (data not shown) gave the same result. Fig. 3B shows the inverse experiment, in which replacement of (−)-[^{3}H]ABV by either vesamicol or (−)-ABV was shown to proceed at the same rate. It is clear from the reciprocity of these results that vesamicol and ABV bind to the same sites.

One difficulty that arises in determining the K_I value for a potent nonradiolabeled drug that is hydrophobic is the uncertainty of the solution concentration of the drug. Although none of the vesamicol analogs appear to be as hydrophobic as reserpine, for example, we have experienced difficulty with aqueous solubilization of some of the analogs, namely *t*-BuVes, transDec, and BV. If a significant amount of ligand is lost to surfaces, then the determined K_I values are overestimates. A possible solution to this problem is to compare equilibrium-derived dissociation constants with rate constants for dissociation. The measurement of dissociation rates for receptor-ligand complexes can be made without accurate knowledge of the concentration of free ligand. In a family of drugs that are structurally similar, variations in equilibrium dissociation constants are expected to result mainly from differences in rates of dissociation rather than association. This is observed for vesamicol and (−)-ABV, where it was determined that a difference in equilibrium binding affinity of a thousandfold is coupled to only a 2-fold difference in association rate constants. For (+)- and (−)-ABV the rate constants of association are identical, despite a large difference in K_I values.

Thus, to have a more robust measure of analog potencies, the dissociation rate constants for this set of vesamicol analogs were determined by a back-titration method. A scatter plot that compares K_I values with dissociation rate constants for a number of the nonlabeled analogs is shown in Fig. 4. The values establish two lines of different slopes, and thus the plot has instructive value. Data for ligands with K_I values of >300 pM define a line with a slope of about 0.3, whereas data for ligands with K_I values of <300 pM define a slope of about 2. It is clear that the dissociation rate constant is not the only factor controlling the K_I value, because a slope of 1 would result in that case. Specifically, the K_I values for (−)-AcABV, (−)-GlyABV,

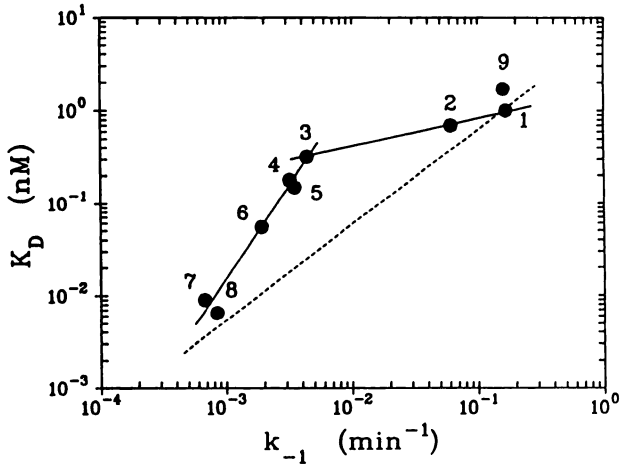


Fig. 4. Scatter plot correlating equilibrium and kinetic dissociation constants for vesamicol and analogs. Data are from Table 1, and the *lines* with slopes of about 0.3 and 2 were hand-drawn through the *points*. The *dashed line* defines a slope of 1 and passes through the datum for vesamicol (*1*). The other analogs are (−)-4-fluoromethylvesamicol (*2*), (−)-NEFA (*3*), (−)-AcABV (*4*), (−)-GlyABV (*5*), (−)-BV (*6*), (−)-transDec (*7*), (−)-ABV (*8*), and (+)-ABV (*9*).

and (−)-NEFA are 6–12-fold higher than predicted from their rates of dissociation from the VR. Loss of ligand to vessel surfaces is an unlikely explanation for the relatively low affinities, because these compounds are quite hydrophilic, relative to two of the other analogs that show greater apparent affinities. Thus, on the assumption that the solution concentrations were as expected, we conclude that the association rate constants for these analogs must be unexpectedly low.

Also in Fig. 4, if a line of slope 1 is drawn through vesamicol, then (−)-transDec lies only slightly above the line. This means that (−)-transDec associates only about 3-fold more slowly with the VR than does vesamicol. This is surprising because of the different conformations of the amino and alcohol groups in these compounds. One explanation would argue that the dissociation rate of a ligand from the VR binding site is controlled by short range (e.g., van der Waals, hydrogen bonding, or salt bridge) interactions that are very sensitive to conformation, whereas the association rate is dominated by a long range electrostatic attraction in which all vesamicol analogs appear much the same. Alternatively, the mechanism for the association process may be multistep, with interaction of the phenyl group with the binding site occurring first. Again, all vesamicol analogs tested here would appear very similar in this regard.

Comparison of the dissociation constants of the members of enantiomeric pairs also is informative. Although the enantiomeric selectivity of the VR for vesamicol was reported to be about 25-fold in favor of the (−)-enantiomer (4), much larger differences in enantiomeric affinities are seen for analogs. Even the simple addition of the fluoromethyl group to the 4-position of vesamicol results in a significant increase in enantioselectivity, to 200-fold. A 200-fold difference in affinities also is computed from the kinetically derived dissociation constants for (+)- and (−)-ABV. An apparent reduction in enantioselectivity due to addition of the glycyl residue to ABV to give GlyABV arises because the (+)-enantiomer of GlyABV eluted second from the chiral chromatography column used for resolution and so was contaminated by a small amount of the more potent (−)-enantiomer.

In an earlier communication that described the potency of ABV (16), use of the racemate for kinetic measurements resulted in misinterpretation of the significance of the slow binding of (±)-ABV to approximately 50% of the VR. The synthesis and optical resolution of (±)-[^{3}H]ABV have allowed us to reinterpret those earlier kinetic results as being due to the requirement that the (+)-enantiomer must dissociate from one half of the receptor molecules before the (−)-enantiomer can bind. The dissociation rate constants derived from this phenomenon for a number of the (+)-enantiomers are given in a footnote to Table 1. They are not listed in a prominent way because we see no utility in the values but recognize that others may.

As the cholinergic network of the human brain comes under increased scrutiny because of pathological changes such as those that occur in Alzheimer's disease, we expect some of these potent vesamicol analogs to have increased utility in diagnostic studies. Also, the availability of the nearly irreversible analog (−)-ABV, in nonlabeled and tritiated forms, will permit experimentation not possible with vesamicol. The extremely slow rate of dissociation exhibited by ABV might produce cholinergic hypofunction by blocking vesicular storage of ACh for a usefully long time period. Overall, it is clear that ABV, among currently known analogs of vesamicol, possesses an optimal combination of very high affinity, good chemical stability, and only moderate hydrophobicity. Analogs based on ABV show promise as imaging agents for cholinergic nerve terminals *in vivo*.

Acknowledgments

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