0026-895X/89/030452-07$02.00/0

MOLECULAR PHARMACOLOGY, 36:452-458

Fractional Vesamicol Receptor Occupancy and Acetylcholine Active Transport Inhibition in Synaptic Vesicles

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Received December 16, 1988; Accepted June 6, 1989

SUMMARY

Vesamicol [(−)-(*trans*)-2-(4-phenylpiperidino)cyclohexanol] receptor binding and inhibition of acetylcholine (AcCh) active transport by cholinergic synaptic vesicles that were isolated from *Torpedo* electric organ were studied for 23 vesamicol enantiomers, analogues, and other drugs. Use of trace [^{3}H]vesamicol and [^{14}C]AcCh allowed simultaneous determination of the concentrations of enantiomer, analogue, or drug required to half-saturate the vesamicol receptor (K_i) and to half-inhibit transport (IC_{50}), respectively. Throughout a wide range of potencies for different compounds, the K_i/IC_{50} ratios varied from 1.5 to 24. Compounds representative of the diverse structures studied, namely deoxyvesamicol, chloroquine, and levorphanol, were competitive inhibitors of vesamicol binding. It is concluded that many drugs can bind to the vesamicol receptor and binding to only a small fraction of the receptors can result in AcCh active transport inhibition. Possible mechanisms for this effect are discussed.

AcCh active transport that is carried out by synaptic vesicles purified from the electric organ of *Torpedo* is inhibited potently by the compound vesamicol [formerly called AH5183 (1)]. As a result of the block of the hypothesized AcCh transporter, vesamicol inhibits evoked and nonquantal release of AcCh from a wide range of intact cholinergic preparations (reviewed in Ref. 2). A saturable enantioselective receptor for vesamicol, which exhibits a dissociation constant of about 20–90 nM in different preparations of vesicles, is present on the cytoplasmic surface of the vesicles (3, 4). There is no transport of vesamicol by the vesicles, and inhibition of AcCh active transport is noncompetitive (5). This suggests that the vesamicol site and the AcCh site involved in transport are not identical.

There currently are two models for the mechanism of vesamicol-mediated inhibition (2). In model 1, vesamicol binds to an allosteric site in the AcCh transporter. In model 2, vesamicol binds to a separate protein that acts on AcCh active transport by an unknown mechanism. Recent data (6) have demonstrated that the vesamicol receptor is linked to a low affinity AcCh binding site (dissociation constant = 18 mM), which, however, probably is not the same as the binding site involved in AcCh active transport (Michaelis transport dissociation constant = 0.3 mM). The relationship between the two types of AcCh binding site is unknown, in part because binding of AcCh to the transporter or other sites cannot be observed directly. This presumably is due to fast dissociation of externally bound AcCh.

In an effort to distinguish between models 1 and 2, the work reported here determined whether vesamicol binding to the receptor corresponds directly to AcCh transport inhibition. As a result of an extensive structure-activity study, many new analogues of vesamicol now are available (7). A number of these were characterized in order to test whether the linkage between vesamicol receptor binding and transport inhibition depends on the structure of the bound drug.

Compounds unrelated to vesamicol also inhibit AcCh active transport by purified synaptic vesicles, albeit more weakly than vesamicol (1). Because the structures of many of these compounds, such as chloroquine and levorphanol, are so different from vesamicol and each other, it seemed possible that they do not bind to the same site. Rather, the possibility that there are multiple sites or receptors coupled to AcCh active transport had to be considered. Thus, a number of these compounds were tested for coincidence of transport inhibition and vesamicol receptor binding. Finally, compounds representative of structurally diverse drugs were competed against different vesamicol concentrations to determine whether they are competitive or noncompetitive inhibitors of vesamicol binding.

Materials and Methods

VP_1 synaptic vesicles were isolated from the electric organ of *Torpedo californica* as described (6). [^{14}C]AcCh (50 mCi/mmol) was from Amer-

This research was supported by Grant NS15047 from the National Institute of Neurological and Communicative Disorders and Stroke, United States Public Health Service, and by a grant from the Muscular Dystrophy Association.

ABBREVIATIONS: AcCh, acetylcholine; vesamicol, (−)-(*trans*)-2-(4-phenylpiperidino)cyclohexanol (formerly AH5183); K_i, drug concentration displacing half of bound [^{3}H]vesamicol; IC_{50}, drug concentration giving half-inhibition of AcCh active transport; K_d, the true equilibrium drug dissociation constant; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

sham or ICN Radiochemicals, Inc. [^{3}H]Vesamicol (32.7 Ci/mmol) was synthesized as described (7). It is the optically pure (−)-isomer. Diethyl *p*-nitrophenyl phosphate (paraoxon), potassium phosphoenolpyruvate, and pyruvate kinase (type III) were from Sigma Chemical Co. (St. Louis, MO). All commercially available drugs were obtained from Sigma except for the following. Quinacrine dihydrochloride hydrate was from Aldrich Chemical Co. (Milwaukee, WI), *N*-hydroxyethyl-4-(1-napthylvinyl)pyridinium bromide was from Calbiochem (San Diego, CA), and (+)- and (−)-enantiomers of vesamicol and the (±)-vesamicol analogues were synthesized as described (7). Haloperidol was a gift from Dr. Remi Quirion, Douglas Hospital (Montreal, Canada).

Drug binding and AcCh active transport by synaptic vesicles were studied using the following dual-label technique. Isosmotic buffer consisted of 0.7 M glycine, 0.10 M HEPES, 1 mM EDTA, and 1 mM EGTA, adjusted to pH 7.8 with 0.80 M KOH (buffer A). Concentrated synaptic vesicles (0.4 mg of protein/ml) were incubated 1 hr in 0.15 mM paraoxon at 23° to inhibit hydrolysis of AcCh by trace AcCh esterase. Vesicles were added last to a mixture of all other components in order to initiate AcCh uptake and drug binding. Drugs that were soluble as concentrated solutions only in ethanol were brought to dryness with a stream of nitrogen gas before addition of other components to the thin film of deposited drug. The final concentrations were 50 μM [^{14}C]AcCh, 10 nM [^{3}H]vesamicol, the stated concentration of unlabeled drug, 10 mM MgATP, 10 mM potassium phosphoenolpyruvate, 2 mM $MgCl_2$, 0.1 mg of vesicle protein/ml, and 10 units of pyruvate kinase/ml. The assay was terminated after a 30-min incubation at 23° by vacuum-assisted filtration of a 110-μl volume through a prerinsed 2.5-cm Whatman GF/F filter that was precoated with 0.5% polyethylenimine (8). The filter was immediately washed with four 3-ml volumes of ice-cold buffer A, and the wet filters were incubated 1 hr in scintillation vials containing 10 ml of Hydrofluor (National Diagnostics) and 0.5 ml of water. The amounts of ^{3}H and ^{14}C retained by the filters were determined by liquid scintillation counting using a standard two-channel counting technique (9). The data reported are the averages of duplicates, which typically exhibited a relative range of less than 5%. Competition of unlabeled drugs with [^{3}H]vesamicol was studied similarly, except that no [^{14}C]AcCh was present and four [^{3}H]vesamicol concentrations were utilized. Nonlinear regression analysis was carried out with MINSQ (MicroMath Scientific Software, Salt Lake City, UT). Protein was determined by the method of Bradford (10), using a bovine serum albumin standard.

Results

The correspondence between active transport inhibition and drug binding was studied by utilizing [^{14}C]AcCh and, in the same samples, a concentration of [^{3}H]vesamicol too low to give much transport inhibition. Addition of nonradioactive drug led to further transport inhibition and displacement of bound vesamicol. The ^{3}H and ^{14}C dpm bound to vesicles were determined by double-channel liquid scintillation counting. Regression analyses of the separated data then allowed determination of the IC_{50} value for transport inhibition and the apparent K_i value for inhibition of vesamicol binding to the receptor due to the unlabeled drug. The total amount of vesamicol receptor present also was estimated in some experiments, using a concentration of [^{3}H]vesamicol sufficient to nearly saturate the available receptors.

Vesamicol. When nonradioactive (−)-vesamicol, the pharmacologically more active enantiomer, was utilized as the inhibitor, the two sets of data in Fig. 1A were obtained. Hyperbolic titration curves fit both sets of data well. The IC_{50} value for AcCh active transport inhibition was 22 ± 4 nM, whereas the K_i for receptor binding was 86 ± 16 nM, or 3.9 ± 1.0-fold higher (Table 1). Thus, transport inhibition and receptor binding were not coincident (*t* test, $p < 0.001$). The amount of receptor in this preparation of vesicles was greater than 420 pmol/mg of protein.

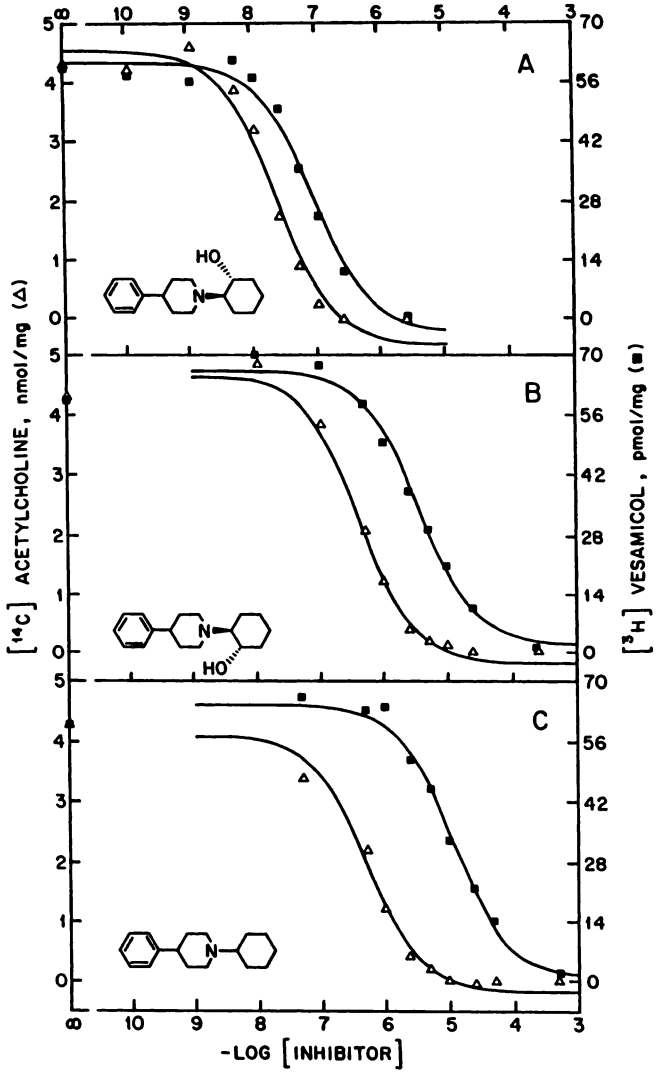


Fig. 1. Inhibition of [^{14}C]AcCh active transport and [^{3}H]vesamicol binding by unlabeled drugs. (−)-Vesamicol (A), (+)-vesamicol (B), and deoxyvesamicol (C) were studied. The assay was done in duplicate, as described in Materials and Methods, and the data were averaged. The typical relative range of the duplicate data was less than 5%. The concentrations of inhibitor are given as the negative logarithm base 10 of molarity. Hyperbolic titration curves were fitted to the averaged data by nonlinear regression analysis to give the following IC_{50} and K_i values, in nM, ±1 SE, respectively: A, 22 ± 4 and 86 ± 16; B, 430 ± 60 and 3,600 ± 700; and C, 510 ± 80 and 12,000 ± 2,000. In all three cases, $p < 0.001$ by Student's *t* test for corresponding IC_{50} and K_i values. The amount of receptor detected with 500 nM of [^{3}H]vesamicol was 420 pmol/mg of protein.

Effect of the hydroxyl group on the K_i/IC_{50} ratio. The hydroxyl group of (−)-vesamicol is important to the potency of the drug (7). To investigate the role of the hydroxyl group, the above experiment was repeated with (+)-vesamicol and deoxyvesamicol. Vesicles from the same preparation were utilized (Fig. 1, B and C). As with (−)-vesamicol, hyperbolic titration curves fit the data well, and AcCh transport inhibition occurred at lower concentrations of the drugs than those required to saturate the vesamicol receptor (Table 1). The order of drug analogue potencies measured against active transport was as expected, with (−)-vesamicol being more potent than (+)-

TABLE 1

Inhibition by vesamicol analogues

The experimental protocol and data analysis were the same as given in Materials and Methods and Fig. 1. Eleven to 13 drug concentrations were utilized in duplicate for each titration, and the fitted IC_{50} and K_i values are quoted ± 1 SE. The amount of receptor measured with 500 nM [^{3}H]vesamicol was obtained for the vesicle preparations utilized for the following analogues: analogues **1** and **2** [(−)- and (+)-vesamicol, respectively] and analogue **15** (deoxyvesamicol), 420 pmol/mg; analogues **3** and **4**, 727 pmol/mg; analogue **13**, 230 pmol/mg; analogue **14**, 225 pmol/mg; analogue **16**, 396 pmol/mg. The chemical names of the analogues and [compound designation in Ref. 7] are as follows: number **1** is (−)-(*trans*)-2-(4-phenylpiperidino)cyclohexanol [*R,R*]; **2** is (+)-(*trans*)-2-(4-phenylpiperidino)cyclohexanol [*S,S*]; **3** is (±)-(*cis*)-2-(4-phenylpiperidinomethylene)cyclohexanol [**59** (*cis*)]; **4** is (±)-(*trans*)-2-(4-phenylpiperidinomethylene)cyclohexanol [**60** (*trans*)]; **5** is (±)-(±)-(*trans*)-(*trans*)-*N,N′*-bis-(2-hydroxycyclohexyl)-4,4′-bipiperidine [**11**]; **6** is (±)-(±)-(*trans*)-(*trans*)-*N,N′*-bis-(2-hydroxycyclohexyl-4,4′-ethylenedipiperidine [**12**]; **7** is (±)-(±)-(*trans*)-(*trans*)-*N,N′*-bis-(2-hydroxycyclohexyl)-4,4′-trimethylenedipiperidine [**13**]; **8** is (±)-(*trans*)-2-piperidinocyclohexanol [**15**]; **9** is (±)-(*trans*)-2-[*N*-(3-phenylpropyl)-*N*-methylamino]cyclohexanol [**23**]; **10** is (±)-(*trans*)-2-[*N*-(2-phenethyl)-*N*-methylamino]cyclohexanol [**22**]; **11** is (±)-(*trans*)-2-(*N*-benzyl-*N*-methylamino)cyclohexanol [**21**]; **12** is (±)-*N*-(*trans*-2-hydroxycyclohexyl)-*N*-methyl-4-phenylpiperidinium iodide [**37** (*E,Z*)]; **13** is (±)-(*trans*)-2-hydroxy-3-(4-phenylpiperidino)-(*trans*)-decalin [**65**]; **14** is (±)-(*trans*)-5-acetamido-2-hydroxy-3-(4-phenylpiperidino)tetralin [**74**]; **15** is *N*-cyclohexyl-4-phenylpiperidine [**50**]; **16** is (±)-(±)-(*trans*)-(*trans*)-*N,N′*-bis[4-oxo-4-[[5,6,7,8-tetrahydro-6-hydroxy-7-(4-phenylpiperidino)-1-naphthalenyl]amino] butanoyl]hydrazine [**79**].

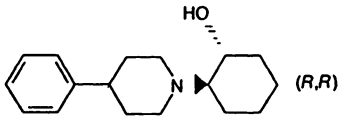
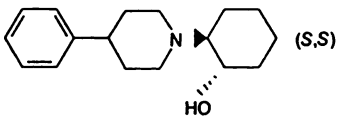
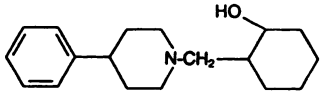
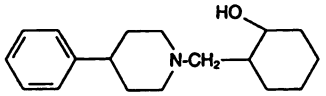
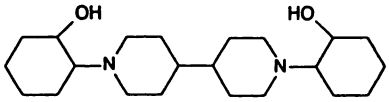
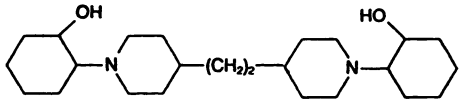
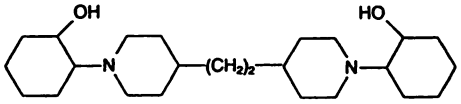
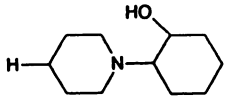
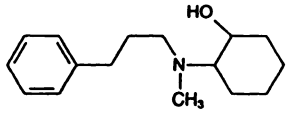
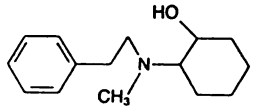
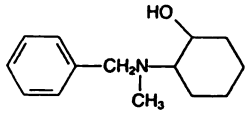
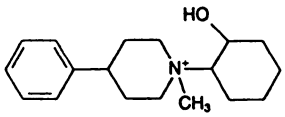
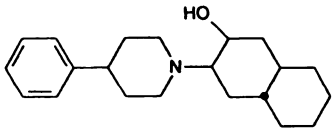
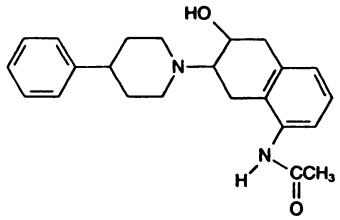
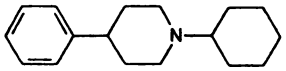
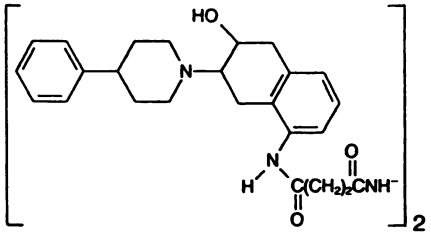
Analogue number	Structure	IC_{50}	K_i	K_i/IC_{50}
		nM	nM	
1		22 ± 4	86 ± 16	3.9
2		430 ± 60	3,600 ± 700	8.4
3		2,100 ± 360	13,000 ± 3,500	6.2
4		1,000 ± 250	16,000 ± 5,000	16
5		550 ± 100	1,700 ± 100	3.1
6		5,400 ± 2,200	39,000 ± 6,000	7.2
7		1,200 ± 560	2,100 ± 240	1.8
8		(0.94 ± 0.16) × 10^5	(17 ± 4) × 10^5	18
9		2,000 ± 600	2,900 ± 870	1.5
10		20,000 ± 9,600	66,000 ± 24,000	3.3
11		14,000 ± 4,000	52,000 ± 41,000	3.7

TABLE 1—Continued

Analogue number	Structure	IC_{50}	K_i	K_i/IC_{50}
		nM	nM	
12		4,600 ± 1,100	26,000 ± 8,000	5.7
13		11 ± 3	23 ± 4	2.1
14		46 ± 6	71 ± 11	1.5
15		510 ± 80	12,000 ± 2,000	24
16		1,000 ± 300	1,900 ± 540	1.9

vesamicol, which was more potent than deoxyvesamicol (7). The interesting new result is that the ratio K_i/IC_{50} was 8.4 ± 2.0 for (+)-vesamicol and 24 ± 5 for deoxyvesamicol. The K_i/IC_{50} ratios for the three drugs in Fig. 1 are all significantly different from each other (*t* test, $p < 0.05$). Thus, the extent of noncoincidence of receptor binding and transport inhibition depends on the analogue structure, even in a closely related series of compounds that differ only in the absence or presence and configuration of the hydroxyl group.

Independence of results on time and [^{3}H]vesamicol concentration. The titrations for (−)-vesamicol were apparently time independent, because incubation of vesicles for 20 min in the presence of all the other components before the addition of [^{14}C]AcCh to initiate transport resulted in no change in the transport inhibition and receptor binding curves (data not shown). In another experiment, it was shown that displacement of a 20-fold lower concentration of [^{3}H]vesamicol with nonlabeled vesamicol occurred in a nearly coincident manner (data not shown). Thus, there was no evidence that the particular concentration of [^{3}H]vesamicol or the incubation time chosen had affected the outcome of the experiments.

Other vesamicol analogues. The double-isotope study was carried out on a total of 16 vesamicol analogues or enantiomers, with the results summarized in Table 1. The K_i/IC_{50} ratios were between 1.5 and 24 in all cases. As in Fig. 1, two closely related series of compounds, namely numbers **3** and **4** and **5** through **7** exhibited clear differences among their members in the K_i/IC_{50} ratios. Compounds **3** and **4** were studied with the same preparation of vesicles. Overall, although a wide range of potencies was observed for the analogues, including one that is about 4-fold more potent than vesamicol itself when enantiomeric purity is taken into account (analogue **13**), the K_i/IC_{50} ratios consistently exhibited values comparable to or greater than 1. In no case was the ratio less than 1.

Structurally diverse drugs. The study was extended to seven drugs from a wide variety of cholinergic and noncholinergic pharmacologic categories (Table 2). The results were similar to those in Table 1, with the K_i/IC_{50} ratios falling between 1.7 and 11 in all cases. There is no evidence from these data, then, that these other drugs act by a fundamentally different mechanism to inhibit AcCh active transport.

Competition of analogues and drugs with vesamicol. To test whether different receptors are present, the binding of low to high concentrations of [^{3}H]vesamicol was studied in the presence of three drugs that are representative of the diverse structures that were studied in the double-isotope protocol. Deoxyvesamicol, chloroquine, and levorphanol all exhibited competitive inhibition of [^{3}H]vesamicol binding, rather than noncompetitive inhibition that would be indicative of another receptor site (Fig. 2). The K_d values found here were similar to

TABLE 2

Inhibition by other drugs

The experimental protocol and data analysis were the same as given in Materials and Methods and Fig. 1. Eleven to 13 drug concentrations were utilized in duplicate for each titration, and the fitted IC_{50} and K_i values are quoted ± 1 SE. The amount of receptor measured with 500 nM [^{3}H]vesamicol was obtained for the vesicle preparations utilized for the following drugs: haloperidol, 874 pmol/mg; quinacrine and chloroquine, 437 pmol/mg.

Drug	IC_{50}	K_i	K_i/IC_{50}
	μM	μM	
Haloperidol	0.4 ± 0.1	1.7 ± 0.3	4.3
Quinacrine	3 ± 1	13 ± 3	4.3
Chloroquine	1 ± 0.1	4 ± 0.4	4
Imipramine	9 ± 2	86 ± 31	10
Pyrilamine	10 ± 2	17 ± 3	1.7
Levorphanol	9 ± 1	99 ± 17	11
N-Hydroxyethyl-4-(1-napthylvinyl)pyridinium	25 ± 8	87 ± 13	3.5

or less than the corresponding K_i values observed in the double-isotope study. The differences probably were a result of the different vesicle preparations utilized.

Discussion

Both the [^{3}H]vesamicol and [^{14}C]AcCh concentrations utilized in this study were substantially subsaturating. Only 15% or less of the receptor or transporter was occupied by radiolabeled ligand in all cases. Thus, only small corrections for ligand competition would be required to obtain true K_d values (see Appendix and Ref. 11). The receptor concentrations present were comparable to the IC_{50} and K_i values for a few of the drugs (numbers **1** [(−)-vesamicol], **13**, and **14**). This was required because AcCh active transport cannot be monitored at lower vesicle concentrations. The observed IC_{50} and K_i values probably are somewhat elevated for these drugs, due to nonnegligible binding of drug to the vesicles. However, the errors are at least partially canceled in the K_i/IC_{50} ratio. The corrections can be ignored without risk to the conclusions, especially in view of the significant variability in the AcCh transport and receptor binding properties of different preparations of vesicles (3). Some of this variation appears to depend on the internal contents and length of storage of the vesicles after isolation (6) and, thus, it is difficult to control.

A striking pattern was observed in this study. In all cases, AcCh active transport was half-inhibited at lower concentrations of drug than those required to half-saturate the available receptors. These ranged from 1.5-fold for compounds **9** and **14** to 24-fold for deoxyvesamicol (compound **15**). Non-receptor-mediated inhibition of AcCh active transport at these low drug concentrations seems unlikely. The only readily conceptualized non-receptor-mediated mechanism is that uptake of the free base form of a drug could neutralize the ATPase-generated proton gradient that drives AcCh active transport (2). However, there is no evidence that vesamicol itself is transported, analogue **12** contains no basic group, and much higher, millimolar, concentrations of the ammonium ion are required to block AcCh transport by this mechanism (12).

Because representative compounds were competitive with vesamicol for binding to the vesamicol receptor, there is no evidence that other receptors account for the transport-inhibitory effects of the structurally diverse drugs. Rather, it seems probable that the vesamicol receptor can account for the transport inhibition caused by all of the drugs tested here. The observations can be summarized as follows. Inhibition of AcCh active transport by diverse compounds can occur by binding to a relatively small fraction of the vesamicol receptors, and the required fraction depends on the structure of the compound. The variable fraction cannot be solely the result of possible differences in the properties of different vesicle preparations, because large, structure-dependent differences in the K_i/IC_{50} ratio were seen using the same vesicle preparation.

The relationship between the fraction of the available receptors occupied at the IC_{50} value by either [^{3}H]vesamicol or the unlabeled analogue and the measured IC_{50} and K_i values is readily derived (see Appendix) and is given by Eq. 1.

$$\text{Occupied}_{IC_{50}} = \frac{(IC_{50} + [V^*]K_i/K_i^v)}{(IC_{50} + K_i)} \quad (1)$$

Here K_i^v is the K_i value for (−)-vesamicol (86 nM), which is the same as [^{3}H]vesamicol, and V^* is the free [^{3}H]vesamicol concentration, which was about 8 nM in these experiments.

It is instructive to consider in detail the results presented in Fig. 1 by utilizing Eq. 1. The calculation of $\text{occupied}_{IC_{50}}$ for (−)-

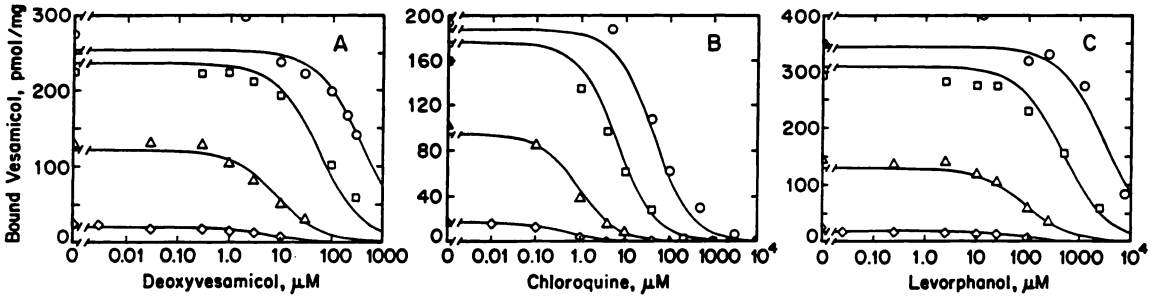


Fig. 2. Competition of unlabeled drugs with different concentrations of [^{3}H]vesamicol. Deoxyvesamicol (A), chloroquine (B), and levorphanol (C) were studied. The assay was done as described in Materials and Methods, except that a single datum was obtained at each competing nonlabeled drug concentration utilizing 2×10^{-9} M (◇), 2×10^{-8} M (△), 2.6×10^{-7} M (□), or 1.86×10^{-6} M (○) [^{3}H]vesamicol. Nonspecific binding, determined in the presence of a 100-fold excess of (−)-vesamicol relative to [^{3}H]vesamicol, was subtracted to obtain the specific binding reported. The *curves* shown were obtained by nonlinear regression analysis of simultaneous equations for noncompetitive inhibition of binding of the form $B = B_{max}([V^*]/K_d^v + [V^*]\cdot[A]/(K_d^v \cdot K_d^{va}))/(1+[V^*]/K_d^v+[A]/K_d^a+[V^*]\cdot[A]/(K_d^v \cdot K_d^{va}))$, where B is the amount of bound vesamicol in pmol/mg, B_{max} is the maximal amount of vesamicol binding, $[V^*]$ is the free [^{3}H]vesamicol, K_d^v is the vesamicol-receptor dissociation constant, $[A]$ is the unlabeled analogue concentration in μM, K_d^a is the analogue-receptor dissociation constant, and K_d^{va} is the dissociation constant for unlabeled analogue dissociating from the ternary complex. Errors are quoted ± 95% confidence limit. In all cases, the K_d^{va} value obtained was very large relative to K_d^a, thus reducing the equation to the competitive form. A, $B_{max} = 257 \pm 12$ pmol/mg, $K_d^v = 21 \pm 6$ nM, and $K_d^a = 4.5 \pm 1.5$ μM; B, $B_{max} = 189 \pm 12$ pmol/mg, $K_d^v = 19 \pm 6$ nM, and $K_d^a = 0.45 \pm 0.16$ μM; C, $B_{max} = 349 \pm 18$ pmol/mg, $K_d^v = 33 \pm 9$ nM, and $K_d^a = 57 \pm 21$ μM.

vesamicol gives 0.28, for (+)-vesamicol 0.19, and for deoxyvesamicol 0.13. Furthermore, at the respective IC_{50} drug concentrations, only a fraction of 0.11 of the receptors were occupied by (+)-vesamicol (another 0.08 of the sites being occupied by [^{3}H]vesamicol; see Appendix), and only 0.04 of the receptors were occupied by deoxyvesamicol (another 0.09 of the sites being occupied by [^{3}H]vesamicol). Because [^{3}H]vesamicol occupied a significant fraction of the sites relative to the fractions occupied by (+)-vesamicol and deoxyvesamicol at their IC_{50} values, these apparent IC_{50} values may not exclusively reflect inhibition by the analogues. Nevertheless, it is clear that, whereas deoxyvesamicol has the least affinity for the receptor, when bound it has the greatest efficacy for transport inhibition.

The total concentration of receptors expressed by this preparation of vesicles was at least 420 pmol/mg of protein. Because it has been estimated that 49 pmol of receptor/mg of protein corresponds to 1 receptor/vesicle (13), the occupancies computed above correspond to at least 2.4, 1.6, and 1.3 receptor/vesicle bound to a mixture of [^{3}H]vesamicol and the unlabeled analogue when (−)-vesamicol, (+)-vesamicol, or deoxyvesamicol, respectively, caused half-inhibition of AcCh active transport. In all cases, at least 1 receptor/vesicle was occupied when AcCh transport was half-inhibited.

The presumed receptor-mediated mechanism for inhibition of AcCh active transport is not apparent. If the system is homogeneous and the vesamicol receptor is linked stoichiometrically to the AcCh transporter, as for an allosteric site in the transporter, one would expect transport inhibition to correspond directly to receptor occupancy. This is contrary to observation. However, an *amplified* inhibition could occur if drug that bound to an allosteric site in the AcCh transporter induced a proton leak. Active AcCh transporter presumably normally couples proton efflux with AcCh uptake (2), and it is not difficult to envision uncoupling of these processes. This would dissipate the driving force for all the transporters. Furthermore, the magnitude of such a proton leak could depend on the drug structure if the leak depended on an induced conformation that varied with the drug structure. In this mechanism, at least 1 receptor/vesicle would have to be occupied to achieve complete inhibition of transport, and the observations *are* consistent with this requirement. However, vesamicol and deoxyvesamicol do not affect the vesicle ATPase activity, which suggests that the proton gradient is unaltered (1).

Another possibility for amplification of inhibition in a homogeneous transporter-receptor system is activation of an enzyme or enzyme-like activity. The value for the K_i/IC_{50} ratio of 24 obtained for deoxyvesamicol is consistent with agonist-like activity arising from an efficacious drug, where about 96% of the receptors are "spare" with respect to half-inhibition of AcCh active transport. However, no enzyme is known to be linked to the receptor, and the apparent time independence of the K_i/IC_{50} ratio for (−)-vesamicol suggests that such an activity would have to be rapid and self-limiting, for example, in a steady state. This seems unlikely in the simplified system of purified synaptic vesicles, because a potential intermediate substrate and coupling enzymes would all have to be present. Moreover, the agonist concept is not easily reconciled with the observations that a number of apparently structurally unrelated compounds produced K_i/IC_{50} ratios of 10 or greater, which suggests that a specific induced conformation of the receptor might not be critical, and that no compound with antagonist-like behavior has been found, that is, a compound that binds to the receptor without inhibiting AcCh active transport. Thus, we are unable to construct an attractive model for amplification in a homogeneous transporter-receptor system.

On the other hand, if the transporter-receptor system is heterogeneous, other possibilities arise. For example, only a small fraction of the vesamicol receptors might be linked to competent AcCh transporters, and only transporters linked to a receptor might be competent. A large majority of receptors that are unlinked or linked to noncompetent transporters might bind drugs more weakly. The K_i/IC_{50} ratio could depend on the drug structure if the conformation of the receptor binding site depended on its transporter environment. Because no large deviation from a hyberbola was apparent in the [^{3}H]vesamicol displacement data, the hypothetical high affinity receptors would have to be not easily detectable in such studies due to their small number. The possible existence of noncompetent transporter is supported by the existence of low affinity AcCh binding, although another possibility is that this arises from inwardly oriented transporter (6). An upper limit for the fraction of high affinity receptors is set by the largest K_i/IC_{50} value observed, that for deoxyvesamicol, at about 8% of the total receptors. The actual fraction could be significantly less than this. No molecular basis for such heterogeneity currently is known.

The apparent amplification observed in these experiments is consistent with a previous indirect deduction that only a few receptors per synaptic vesicle need to be occupied by vesamicol to achieve complete inhibition of AcCh active transport (13). Moreover, addition of vesamicol to vesicles *after* they actively transport [^{3}H]AcCh leads to rapid efflux of newly accumulated [^{3}H]AcCh in an amount at least 10 times greater than the amount of bound vesamicol. Endogenous unlabeled AcCh in vesicles is unaffected by vesamicol, however (13). The ability of vesamicol to distinguish between immigrant and endogenous AcCh, the substantial variability in the AcCh active transport capability and receptor binding properties of different vesicle preparations, and the possible presence of regulation linked to different metabolic states of synaptic vesicles (14) are all consistent with the existence of transporter heterogeneity in the purified vesicle system studied here. Apparent heterogeneity in the sensitivity of AcCh storage to the presence of vesamicol also has been observed in intact preparations of cholinergic nerve terminals (15). Thus, although only one drug receptor probably affects AcCh active transport, it might act in a complex and heterogeneous manner.

Acknowledgments

We thank Barry Hicks for determining that deoxyvesamicol has no effect on vesicle ATPase activity.

Appendix: Correction of K_i Values and Derivation of Occupancy Eq. 1

These derivations can be understood most readily by reference to Fig. 1. In Fig. 1A, both radiolabeled and nonlabeled (−)-vesamicol were present. The amount of receptor occupied by [^{3}H]vesamicol, $R \cdot V^*$, can be written by inspection using a distribution coefficient for the ratio of radiolabeled complex compared with all complexes,

$$R \cdot V^* = B_{max} \left(\frac{[V^*]}{[V] + [V^*] + K_d^v} \right) \qquad \text{(a)}$$

where $[V^*]$ and $[V]$ are the free radiolabeled and nonlabeled (−)-vesamicol concentrations, respectively, K_d^v is the vesamicol dissociation constant, and B_{max} is the total amount of receptor sites. The ratio of Eq. a at $[V] = K_i^v$ [the apparent K_i value for (−)-vesamicol, 86 ± 16 nM] to $[V] = 0$ is equal to 0.5 and gives the correction converting K_i^v to K_d^v,

$$K_d^v = K_i^v - [V^*] \quad \text{(b)}$$

Because $[V^*]$ was approximately 8 nM, the correction is small.

In Fig. 1, B and C, [^{3}H]vesamicol and an analogue were present. We again can write a distribution coefficient giving the fraction of receptor occupied by [^{3}H]vesamicol,

$$R \cdot V^* = B_{max}\left(\frac{[V^*]K_d^a}{[A]K_d^v + [V^*]K_d^a + K_d^aK_d^v}\right) \quad \text{(c)}$$

where $[A]$ is the free analogue concentration and K_d^a is the analogue dissociation constant. The ratio of Eq. c at $[A] = K_i^a$ [the apparent K_i value for the analogue; namely 3,600 ± 700 for (+)-vesamicol and 13,000 ± 3,500 for deoxyvesamicol] to $[A] = 0$ is equal to 0.5 and gives the correction converting K_i^a to K_d^a,

$$K_d^a = K_i^a\ K_d^v/([V^*]+K_d^v) \quad \text{(d)}$$

Substituting Eq. b yields the correction stated in observable quantities,

$$K_d^a = K_i^a(K_i^v - [V^*])/K_i^v \quad \text{(e)}$$

The expression for the amount of receptor occupied by analog, $(R \cdot A)$, is given by,

$$R \cdot A = B_{max}\left(\frac{[A]K_d^v}{[A]K_d^v + [V^*]K_d^a + K_d^aK_d^v}\right) \quad \text{(f)}$$

Eqs. b and e can be substituted into Eqs. c and f to compute occupancy of the receptor by [^{3}H]vesamicol or analogue, respectively, in terms of the observables. Summation of substituted Eqs. c and f yields Eq. 1 of the text.

References

1. Anderson, D. C., S. C. King, and S. M. Parsons. Pharmacological characterization of the acetylcholine transport system in purified *Torpedo* electric organ synaptic vesicles. *Mol. Pharmacol.* **24**:48–54 (1983).
2. Marshall, I. G., and S. M. Parsons. The vesicular acetylcholine transport system. *Trends Neurosci.* **10**:174–177 (1987).
3. Bahr, B. A., and S. M. Parsons. Demonstration of a receptor in *Torpedo* synaptic vesicles for the acetylcholine storage blocker L-*trans*-2-(4-phenyl[3,4-^{3}H]piperidino)cyclohexanol. *Proc. Natl. Acad. Sci. USA* **83**:2267–2270 (1986).
4. Kornreich, W. D., and S. M. Parsons. Sidedness and chemical and kinetic properties of the vesamicol (AH5183) receptor of cholinergic synaptic vesicles. *Biochemistry* **27**:5262–5267 (1988).
5. Bahr, B. A., and S. M. Parsons. Acetylcholine transport and drug inhibition kinetics in *Torpedo* synaptic vesicles. *J. Neurochem.* **46**:1214–1218 (1986).
6. Noremberg, K., and S. M. Parsons. Regulation of the vesamicol receptor in cholinergic synaptic vesicles by acetylcholine and an endogenous factor. *J. Neurochem.* **52**:913–920 (1989).
7. Rogers, G. A., S. M. Parsons, D. C. Anderson, L. M. Nilsson, B. A. Bahr, W. D. Kornreich, R. Kaufman, R. S. Jacobs, and B. Kirtman. Synthesis, acetylcholine storage-blocking activities and biological properties of derivatives and analogues of *trans*-2-(4-phenylpiperidino)cyclohexanol (vesamicol; AH5183). *J. Med. Chem.* **32**:1217–1230 (1989).
8. Bruns, R. F., K. Lawson-Wendling, and T. A. Pugsley. A rapid filtration assay for soluble receptors using polyethylenimine-treated filters. *Anal. Biochem.* **132**:74–81 (1983).
9. Hendee, W. R. *Radioactive Isotopes in Biological Research.* Wiley, New York, 215 (1973).
10. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
11. Bennett, B. P., Jr., in *Neurotransmitter Receptor Binding* (H. I. Yamamura, S. J. Enna, and M. J. Kuhar, eds.). Raven Press, New York, 57–90 (1978).
12. Anderson, D. C., S. C. King, and S. M. Parsons. Proton gradient linkage to active uptake of [^{3}H]acetylcholine by *Torpedo* electric organ synaptic vesicles. *Biochemistry* **21**:3037–3043 (1982).
13. Anderson, D. C., B. A. Bahr, and S. M. Parsons. Stoichiometries of acetylcholine uptake, release, and drug inhibition in *Torpedo* synaptic vesicles: heterogeneity in acetylcholine transport and storage. *J. Neurochem.* **46**:1207–1213 (1986).
14. Gracz, L. M., W.-C. Wang, and S. M. Parsons. Cholinergic synaptic vesicle heterogeneity: evidence for regulation of acetylcholine transport. *Biochemistry* **27**:5268–5274 (1988).
15. Cabeza, R., and B. Collier. Acetylcholine mobilization in a sympathetic ganglion in the presence and absence of 2-(4-phenylpiperidino)cyclohexanol (AH5183). *J. Neurochem.* **50**:112–121 (1988).

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