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Pharmacological Characterization of the Acetylcholine Transport System in Purified *Torpedo* Electric Organ Synaptic Vesicles

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SUMMARY

A wide variety of pharmacologically active compounds was surveyed for effects on active transport of [^{3}H]acetylcholine by synaptic vesicles isolated from the electric organ of *Torpedo californica*. In over 80 compounds tested, inhibitors of a wide range of potencies were found. The most potent inhibitor was 2-(4-phenylpiperidino)cyclohexanol (AH5183), which half-inhibited transport at 40 nM. This compound had been predicted by Marshall [Br. J. Pharmacol. **38**:503–516 (1970)] to block acetylcholine storage by vesicles *in vivo*. The synaptic vesicle active transport system is shown to be pharmacologically distinct from other cholinergic systems. The site of action of AH5183 and other potent inhibitors is not certain, but the possibility of trivial action on the vesicle ATPase or a vesicle proton gradient was eliminated. The results constitute new evidence supporting vesicle exocytosis as the source of evoked acetylcholine release by nerve terminals. AH5183 appears to be the prototype for a new family of anticholinergics. The possibility that some drugs that exhibit secondary anticholinergic effects act in part by antagonizing acetylcholine storage is discussed.

INTRODUCTION

Nerve terminals innervating the electroplax of *Torpedo* are purely cholinergic and are closely similar to motoneuron terminals. The electric organ thus provides an advantageous and often-studied model system for the mammalian cholinergic junction. The majority of AcCh[3] in electric organ is stored in synaptic vesicles (1). Continuous neural stimulation results in repetitive emptying and refilling of the vesicles with newly synthesized AcCh of cytoplasmic origin (2). The AcCh arises from extracellular Ch which is brought into the nerve terminal by the sodium-dependent, high-affinity uptake system (3) and converted to AcCh by Ch acetyltransferase (4). Compared with the rest of AcCh metabolism, relatively little is known about the storage of AcCh by synaptic vesicles.

Some mechanistic elements of this process have been recently elucidated. Highly purified electric organ synaptic vesicles contain a bicarbonate-stimulated Ca^{2+}- or Mg^{2+}-ATPase which drives active uptake of [^{3}H]AcCh (5). This appears to be linked to an internally acidic proton gradient generated by the ATPase (6). Uptake thus is inhibited by mitochondrial uncouplers and diffusible bases such as ammonia. Active transport of [^{3}H]AcCh fulfills a number of criteria for a specific carrier-mediated process. It is saturable, selective for AcCh as compared with Ch, osmotically labile, and inhibited by cold and protein modification reagents. Because [^{3}H]AcCh transport can be uncoupled from the ATPase, can be inhibited by mercurials without affecting the ATPase and also occurs (to a lesser extent) under passive conditions in the absence of ATP (7–10), it is likely that a transporter for AcCh exists which is different from the ATPase.

In order to define better the molecular aspects of AcCh transport, it is important to find drugs that exhibit specificity for different components of the system. The possibility that known drugs can act directly on vesicular AcCh storage in intact preparations has not been studied extensively. Among the few workers in this area is Marshall (11–14). Early work by Brittain *et al.* (15, 16) had shown that AH5183 caused neuromuscular blockade in anesthetized cats with characteristics suggesting presynaptic action. Because blockade was slow in onset; was enhanced by increased release of AcCh; was not antagonized by Ch, neostigmine, or tetraethylammonium; and was prevented by quaternization of the drug, Marshall hypothesized that AH5183 inhibits storage of newly synthesized AcCh by synaptic vesicles (11, 12). Gandiha and

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[3] The abbreviations used are: AcCh, acetylcholine; Ch, choline; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AH5183, 2-(4-phenylpiperidino)cyclohexanol; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone.

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Marshall (13) demonstrated that the nerve terminal content and evoked release of AcCh were reduced up to 88% after long-term stimulation in the presence of AH5183. They then used AH5183 to demonstrate that nicotinic agonists do not act indirectly on the end-plate by stimulating AcCh release (14). Toll and Howard (17) studied the effect of AH5183 on AcCh metabolism in PC12 cells. They found marked inhibition of the granular storage but not of the synthesis of AcCh.

In this paper we examine Marshall's prediction using our biochemical assay for active transport of [^{3}H]AcCh by highly purified *Torpedo* synaptic vesicles. We also survey a wide variety of cholinergic and other centrally and peripherally acting drugs in order to construct a pharmacological profile of AcCh storage. For those drugs exhibiting potent effects, possible modes of action were preliminarily investigated. An accompanying paper continues the pharmacological characterization in a study of the effects of some unusual anions (18).

MATERIALS AND METHODS

Torpedo californica electric organ synaptic vesicles were isolated as described (6). Briefly stated, this involves differential sedimentation velocity pelleting, equilibrium buoyant density centrifugation, and controlled-pore glass-bead filtration of vesicles in isosmotic glycine sucrose solutions. All commercially available drugs were obtained from Sigma Chemical Company (St. Louis, Mo.), except for the following. Quinacrine dihydrochloride hydrate, hemicholinium-3 and -15, 6,9-diamino-2-ethoxyacridine lactate monohydrate, oxotremorine sesquifumarate, 3,4-diaminopyridine, and tetraethylammonium chloride were obtained from Aldrich Chemical Company (Milwaukee, Wisc.). Cetylpyridinium chloride, *N*-methyl-, and *N*-hydroxyethyl-4-(1-naphthylvinyl)pyridinium iodide and bromide, respectively, were from Calbiochem (San Diego, Calif.). Tetramethylammonium chloride was from Eastman Kodak (Rochester, N. Y.) and was recrystallized from ethanol. Tetraphenylphosphonium chloride and thiocholine iodide were from Pfaltz & Bauer (Flushing, N. Y.). Flurazepam dihydrochloride and diazepam were from Applied Science Laboratories (Waltham, Mass.). Trifluoperazine dihydrochloride was from Smith Kline & French (Philadelphia, Pa.), gallamine triethiodide was from K & K Laboratories (Plainview, N. Y.), bretylium tosylate was from Chemical Dynamics Corporation (South Plainfield, N. J.), 4-aminopyridine was from Merck (Rahway, N. J.), and tetraethylpyrophosphate (technical grade) was from Chemicals Procurement Labs (College Point, N. Y.). The phencyclidine derivatives 4-(4′-hydroxy-piperidino)-4-phenylcyclohexanol, 1-(1-*m*-hydroxyphenyl)cyclohexyl-piperidine hydrochloride, 1-(1-phenylcyclohexyl)-4-hydroxypiperidine, and 4-phenyl-4-piperidinocyclohexanol were from the Research Technology Branch, Division of Research, National Institute on Drug Abuse. AH5183 was a generous gift from Dr. R. T. Brittain, Glaxo Group Research, Ltd. (Ware, England). Thioridazine hydrochloride and clozapine were generously donated by Dr. Ross Lane, of the University of Oregon, and fluphenazine was a generous gift from the Squibb Institute for Medical Research (Princeton, N. J.).

[^{3}H]AcCh transport experiments were carried out as described at pH 7.4 and at 23°, using about 0.5 mg of vesicle protein per milliliter (6). Active uptake of [^{3}H]AcCh was enhanced with an ATP-regenerating system and was conducted in the presence of 40 mM $KHCO_3$ to avoid stimulatory effects from anions associated with the tested inhibitors. [^{14}C]Mannitol was pre-equilibrated with vesicle suspensions, and the [^{3}H]AcCh uptake ratio was determined from the ratio of disintegrations per minute of [^{3}H]AcCh to disintegrations per minute of [^{14}C]mannitol inside synaptic vesicles as compared with the ratio outside as described (7). The uptake ratio gives the concentration of [^{3}H]AcCh inside the vesicles as compared with its concentration outside. Inhibitors were dissolved with no organic solvent in isosmotic buffer [0.60 M glycine, 0.20 M Hepes, 1 mM each of EDTA and EGTA, 0.02% (w/v) KN_3, titrated to pH 7.4 with 0.80 N KOH]. The total final inhibitor concentration is given. Uptake was terminated after 30 min by centrifugation-gel filtration at 4°. Residual active uptake of [^{3}H]AcCh is presented as the percentage of maximal uptake beyond the passive amount in order to compare different vesicle preparations, and is given by the percentage of active uptake = (uptake ratio with inhibitor−passive uptake ratio) × 100/(uptake ratio with no inhibitor−passive uptake ratio). Drug titrations were replicated with comparable results, except as noted. Typical experimental measurements for each point consisted of 500–1,500 cpm of [^{14}C]mannitol and 2,000–20,000 cpm of [^{3}H]AcCh inside the synaptic vesicles. ATPase assays were conducted with the coupled pyruvate kinase-lactate dehydrogenase method as described (6) when the tested drug did not absorb strongly at 340 nm, or by determining ^{32}P-phosphate released from [γ-^{32}P]ATP when it did (19). Vacuum filtration through 0.45-μm Millipore filters rapidly separated $^{32}P_i$ from acidic charcoal. Commercial [γ-^{32}P]ATP was purified on DBAE-cellulose (Collaborative Research) columns (20) equilibrated with 0.1 M $MgCl_2$, 0.25 M NaCl, 0.02% NaN_3, 0.01 M 2-(*N*-cyclohexylamino)ethanesulfonic acid, pH 9.0 (adjusted with 0.8 M NaOH), and eluted with 0.1 M $MgCl_2$, 0.4 M glycine, 0.02% NaN_3, and 0.1 M Hepes adjusted to pH 7.4 with 0.8 M NaOH.

RESULTS

Effect of AH5183 on [^{3}H]AcCh uptake by vesicles. Figure 1 gives the structure of AH5183. Figure 2 shows that AH5183 indeed exhibits concentration-dependent inhibition of [^{3}H]AcCh active transport by purified synaptic vesicles, with half-inhibition occurring at about 40 nM. Uptake was reduced to about the passive level by micromolar drug, where inhibition appeared to be essentially saturated. Thus, AH5183 is a potent inhibitor of AcCh active transport by cholinergic synaptic vesicles, confirming Marshall's hypothesis.

AcCh receptor effectors. It is of interest to determine whether a pharmacological similarity between the AcCh porter and other AcCh- or Ch-binding proteins exists. Table 1 lists nicotinic and muscarinic receptor agonists and antagonists which were screened for effects on active

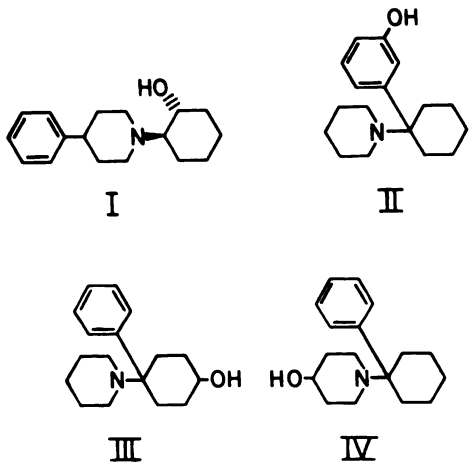

FIG. 1. *Structures of AH5183 and the isomeric hydroxylated phencyclidines*

I, AH5183; *II*, 1-(1-*m*-hydroxyphenyl)cyclohexylpiperidine; *III*, 4-phenyl-4-piperidinocyclohexanol; *IV*, 1-(1-phenylcyclohexyl)-4-hydroxypiperidine. Note that the same ring systems are present in all of the structures, which have a molecular formula $C_{17}H_{25}NO$.

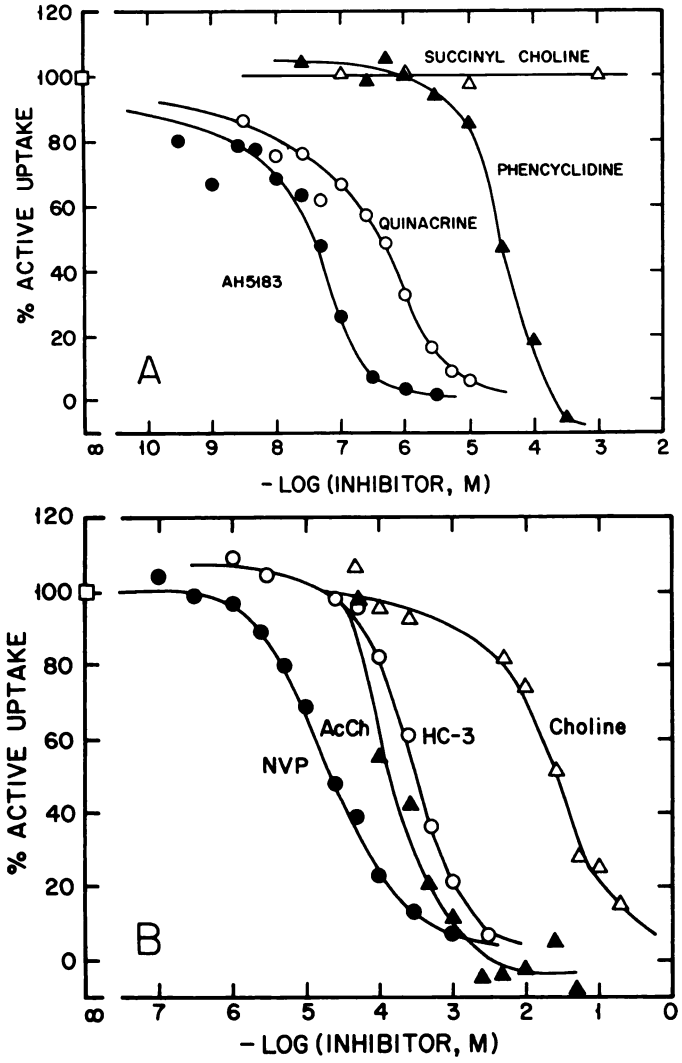

FIG. 2. *Inhibition of [^{3}H]AcCh active uptake by selected drugs*

Uptake in the absence of drugs for all curves is normalized to 100% and is indicated by □ on the ordinate. All drugs except AcCh and succinylcholine were incubated with the vesicles 1 hr before the start of [^{3}H]AcCh uptake. The [^{3}H]AcCh uptake ratio for each titration in the absence of the specified drug and the approximate IC_{50} value (in parentheses) for that drug were as follows: A, 26.5 for AH5183 (●, 40 nM), 26.5 for quinacrine (○, 0.4 μM), 14.4 for phencyclidine (▲, 30 μM), and 14.0 for succinylcholine (△, >10 mM); B, 18.0 for *N*-methyl-4-(1-naphthylvinyl)pyridinium (*NVP*) (●, 30 μM), 14.4 for nonradioactive AcCh (▲, 0.2 mM), 18.0 for hemicholinium-3 (*HC-3*) (○, 0.3 mM), and 14.9 for Ch (△, 30 mM).

transport of [^{3}H]AcCh. For each, an approximate IC_{50} is given for receptor interaction which was taken from the literature. Some of the drugs tested were found to inhibit active transport of [^{3}H]AcCh by the vesicles, and representative titrations for several of them are shown in Fig. 2.

Among nicotinic effectors, quinacrine was the most potent inhibitor, having an IC_{50} of 0.4 μM. This is about 4-fold more potent than its action on the nicotinic receptor channel, where it acts in a manner similar to that of local anesthetics (21). Curiously, the local anesthetic procaine also was about 10-fold more potent in acting on vesicle transport as compared with its nicotinic receptor channel binding. The other eight nicotinic drugs listed in Table 1 were from 1 to >5 orders of magnitude less potent here. In particular, numerous classic nicotinic drugs had no measureable effect.

TABLE 1

Inhibition of active transport by AcCh receptor drugs

Drug	[^{3}H]AcCh uptake (IC_{50})	Receptor binding[a] (IC_{50})
	μM	μM
Nicotinic effectors		
Quinacrine	0.4	1.5 (21)
d-Tubocurarine	10	0.1–0.2 (22, 23)
Phencyclidine	30	0.1–3 (24)
Procaine	100	1,000 (23)
Decamethonium	200	0.7–2 (22, 23)
Nicotine	300	80 (22)
Hexamethonium	>1,000	2–60 (21, 22, 23)
Gallamine	>10,000	0.2–8 (22, 23)
Carbamylcholine	>10,000	0.4–45 (21, 22, 23)
Succinylcholine	>10,000	
Muscarinic effectors		
Atropine	10	0.002 (25)
Oxotremorine	10	0.5 (25)
Pilocarpine	10	7 (25)
Bethanechol	>10,000	200 (25, 26)

[a] Literature references for receptor-drug dissociation constants are in parentheses.

Among muscarinic effectors, most were found to inhibit active transport of [^{3}H]AcCh, but all were less potent. Atropine was about 10^4-fold less potent. Furthermore, [^{3}H]AcCh transport was not differentially inhibited by antagonists or agonists. Thus, the active transport system for AcCh appears to be pharmacologically distinguishable from both the muscarinic and nicotinic receptors.

Other cholinergic protein effectors. The effects of drugs which are thought to be specific for other proteins important to cholinergic transmission were studied. A number were inhibitory. The results, compared with the known potencies for action on their primary targets, are shown in Table 2, and representative titrations are shown in Fig. 2.

TABLE 2

Inhibition of active transport by other cholinergic drugs

Drug	[^{3}H]AcCh uptake (IC_{50})	Primary activity[a] (IC_{50})
	μM	μM
Choline acetyltransferase inhibitors and substrates		
N-Hydroxyethyl-4-(1-napthylvinyl)pyridinium	5	0.6 (27)
N-Methyl-4-(1-naphthylvinyl)pyridinium	30	0.5 (27)
Acetylcholine	200	
S-Acetylcoenzyme A	>1,000	47 (28)
Coenzyme A	>1,000	75 (28)
Choline	20,000	1,900 (28)
High-affinity choline uptake inhibitors		
Hemicholinium-15	100	7.7 (29)[b]
Hemicholinium-3	300	0.06 (3)
Acetylcholinesterase inhibitors		
Physostigmine	300	0.06 (30)
Tetraethylpyrophosphate	>10,000	0.01 (30)

[a] Literature references are in parentheses.
[b] Value is for 40% inhibition.

The acetylcholinesterase inhibitors physostigmine and tetraethylpyrophosphate were 10^4- and at least 10^6-fold less potent in inhibiting [^{3}H]AcCh active transport, respectively. The sodium-dependent, high-affinity Ch uptake inhibitor hemicholinium-3 was nearly 10^4-fold less potent here, whereas hemicholinum-15, which is a very weak inhibitor of the high-affinity system, was more potent. Additional differences from high-affinity uptake are that the vesicle system strongly prefers AcCh as compared with Ch as the transported substrate and is not sodium-dependent (5). Several Ch acetyltransferase inhibitors, namely the naphthylvinylpyridines, were of equal or 10-fold less potency in acting on the vesicle transport system. However, the enzyme clearly is distinguishable, since it binds Ch better than AcCh (4), and the other substrate-product pair, *S*-acetylcoenzyme A and coenzyme A, had no significant effect here. Thus, all of the known proteins of the cholinergic synapse can be distinguished from the vesicular uptake system for AcCh.

Other pharmacological categories. Other drugs and compounds representing a wide variety of pharmacological categories were screened for effects on active transport of [^{3}H]AcCh as listed in Table 3. Of these, 19 inhibited active transport with IC_{50} values in the micromolar range. Thirteen had no significant effect. Many others had intermediate inhibitory potencies. Several apparent structural and charge factors affecting inhibitory potency can be deduced from these and the preceding data. Small hydrophilic molecules containing two or more charges, such as two positive or one positive and one negative charge, are completely ineffective. These include succinylcholine, hexamethonium, gallamine, γ-aminobutyric acid, betaine, and paraquat. Larger hydrophobic molecules containing two localized positive charges have moderate inhibitory potency. These include *d*-tubocurarine, decamethonium, and hemicholinium-3.

A number of hydrophobic molecules containing one or more positive charge, one of which is delocalized, and two or three aromatic rings are more potent inhibitors, with IC_{50} values of about micromolar. These include quinacrine, pyrilamine, chloroquine, 6,9-diamino-2-ethoxyacridine, and 9-aminoacridine. In general, no correlation was found between rank order and potencies of inhibition of [^{3}H]AcCh active transport for the tested drugs and their efficacies of action on their known primary targets. Rather, the correlations crossed pharmacological categories and were based on the gross hydrophobicity and charge characteristics outlined above.

However, hydrophobicity and charge characteristics only partially determine inhibitory potency. The most potent drug, AH5183, is a structural isomer of hydroxylated phencyclidine. Several hydroxylated phencyclidines are products of *in vivo* metabolism and are psychoactive (31). Available hydroxylated derivatives (Fig. 1) were screened for effects on [^{3}H]AcCh active transport. Table 3 shows that three monohydroxylated derivatives, namely 1-(1-*m*-hydroxyphenyl)cyclohexylpiperidine, 4-phenyl-4-piperidinocyclohexanol, and 1-(1-phenylcyclohexyl)-4-hydroxypiperidine, inhibited nearly 10^3- to 10^4-fold less potently than AH5183. The parent phencyclidine also was nearly 10^3-fold less potent. The dihydroxylated derivative 4-(4′-hydroxypiperidino)-4-phenylcyclohexanol was much less potent. The isomeric specificity for potent inhibition by AH5183 demonstrates that the particular 3-dimensional structure is critical.

Target site for inhibition. There are at least three ways a drug could inhibit active transport of [^{3}H]AcCh. It could inhibit the ATPase activity. It could uncouple the ATPase, with concomitant stimulation of the ATPase, by acting as a protonophore or a base inside of the vesicles. Or, most interestingly, it could bind to the hypothesized AcCh porter directly to inhibit translocation of AcCh. The first two possibilities were tested by examining whether the most potent drugs have an effect on the ATPase activity.

Indeed, 6,9-diamino-2-ethoxyacridine at 0.1 mM stimulated the ATPase from 40% to 90% in different preparations of vesicles, indicating uncoupling, but the drug had no significant effect on the ATPase in the micromolar concentration range, where it inhibits active transport of [^{3}H]AcCh. The other drugs that were tested also had no significant effect on the ATPase activity in the respective concentration ranges giving inhibition of [^{3}H]AcCh active transport. These drugs did slightly inhibit the ATPase at high concentrations as follows. AH5183 inhibited 5% at 0.3 mM; quinacrine, 37% at 0.3 mM; trifluoperazine, 12% at 0.1 mM; hemicholinium-3, 13% at 0.3 mM; *N*-methyl-4-(1-napthylvinyl)pyridinium, 10% at 0.3 mM; and chloroquine, 7% at 0.3 mM. This behavior is consistent with the absence of "trivial" inhibitory or uncoupling mechanisms associated with the ATPase activity.

Another test for the site of action of these drugs is available by examining their effects on passive uptake of [^{3}H]AcCh. In this situation, ATPase activity is irrelevant to [^{3}H]AcCh transport. Any effects of the drugs would be consistent with binding to the AcCh porter, since this probably is the only component required for passive transport. Figure 3 shows the effects of AH5183, quinacrine, hemicholinum-3, and D-amphetamine. Data scatter is increased as compared with the effects on active transport because much less total [^{3}H]AcCh uptake was measured. All of the drugs exhibited saturable partial inhibition, with IC_{50} values listed in the legend to Fig. 3. The residual uptake of [^{3}H]AcCh at high drug concentrations is not from binding to contaminating nicotinic AcCh receptor, since the results were essentially unchanged when receptor was blocked with a 2-fold equivalent of α-bungarotoxin (7). Also shown in Fig. 3 is the effect of the protonophore CCCP. In contrast to the other drugs, this authentic uncoupler had no significant effect. Other considerations also suggest that the drugs are not uncouplers. Even in vesicles previously treated with CCCP, they partially inhibit the passive uptake of [^{3}H]AcCh, giving results very similar to those shown in Fig. 3. Also, hemicholinium-3 is a bisquaternary amine which is unlikely to be membrane-soluble and which contains no dissociable proton binding site needed to create an uncoupler or to generate a base. In summary, it is clear that the potent inhibitory drugs are not acting as ATPase inhibitors, as protonophores or as internal bases.

DISCUSSION

We have surveyed a wide variety of pharmacologically active compounds for effects on active transport of [^{3}H]AcCh by purified *Torpedo* electric organ synaptic vesi-

TABLE 3

Inhibition of active transport by drugs from other pharmacological categories

Drug category[a]	IC_{50}	Drug Category	IC_{50}
	μM		μM
Cholinergic side effects		Antidepressants	
Strychnine	4	Imipramine	5
Bretylium tosylate	5	Clozapine	20
Quinine	20	Anti-anxiety	
Benactyzine	30	Flurazepam	3
1-(1-*m*-Hydroxyphenyl)cyclohexylpiperidine	30	Diazepam	100
1-(1-Phenylcyclohexyl)-4-hydroxypiperidine	100	Other compounds	
Arecoline	100	Chloroquine	0.5
4-Phenyl-4-piperidinocyclohexanol	300	6,9-Diamino-2-ethoxyacridine[c]	0.1–1
4-(4′-Hydroxypiperidino)-4-phenylcyclohexanol	1,000	9-Aminoacridine	3
Streptomycin	>1,000	Neutral red	6
Tobramycin	>1,000	Reserpine	8
Neomycin	>1,000	Thiamine	20
Histaminergics		Propidium	30
Pyrilamine	2	Hydralazine	40
Histamine	1,000	Acetylthiocholine	50
Antipsychotics		Thiocholine	100
Chlorpromazine	3	3,4-Diaminopyridine	100
Thioridazine	3	Cytochalasin D	>100
Fluphenazine	5	4-Aminopyridine	300
Trifluoperazine	10	FAD	>1,000
Narcotics		FMN	>1,000
Levorphanol	9	Atractyloside	>1,000
D,L-Amphetamine[b]	10	Colchicine	>1,000
Dextrorphan	20	Tris(hydroxymethyl)aminomethane	10,000
Morphine	40	Thiamine monophosphate	>10,000
Ketamine	100	Thiamine pyrophosphate	>10,000
Naloxone	200	Betaine	>10,000
Phenobarbital	10,000	Paraquat	>10,000
Other neurotransmitters		Li^+	>10,000
Serotonin	100	Tetraethylammonium	20,000
Epinephrine	100	Tetramethylammonium	20,000
Dopamine	100		
γ-Aminobutyric acid	>10,000		

[a] Categorization is somewhat arbitrary in cases of multiple actions.

[b] From two separate vesicle preparations, D-amphetamine had IC_{50} values from 13 to 25 μM, L-amphetamine from 5 to 20 μM.

[c] Data are from two separate vesicle preparations.

cles. Many drugs from different pharmacological categories were found to be inhibitory, with IC_{50} values as low as several micromolar. Only a few compounds were more potent. Among more than 80 drugs screened, the most potent was AH5183, which is precisely the one hypothesized by Marshall (11–12) to act on AcCh storage by vesicles *in vivo*. That this is a special effect is suggested by its dependence on the particular structural isomer. Several hydroxylated phencyclidines that are isomeric and should have very similar hydrophobicity and charge characteristics were 10^3- to 10^4-fold less potent.

The second most potent inhibitor was the antimalarial quinacrine, which is structurally similar to a number of other potent inhibitors found here which form an apparent family containing a delocalized positive charge similar to 4-aminoquinoline. Because quinacrine often is an inhibitor of flavin-dependent enzymes (32), FMN and FAD were tested for effects on [^{3}H]AcCh active uptake and for antagonism of quinacrine inhibition, but no effects were found. However, quinacrine is a nonspecific drug which acts on the nicotinic receptor channel (21), mammalian axonal membranes, phospholipase A_2, and energized submitochondrial particles, among other systems. Thus, the significance of inhibition by the 4-aminoquinoline-like family is not known.

The active transport system for AcCh storage is pharmacologically quite distinct from other cholinergic proteins in most cases. The closest similarity was to Ch acetyltransferase, since both systems are inhibited by the naphthylvinylpyridines. However, they are distinguishable by the lack of similarity in interactions with the substrates and products of the enzyme. Thus there is no reason to postulate an intimate role for Ch acetyltransferase in vesicular storage of AcCh, as has occasionally been suggested. However, since both systems are located in the nerve terminal cytoplasm and presumably would be exposed to the same concentration of drug, it is likely that the naphthylvinylpyridine family is not very specific in intact preparations.

The rather common occurrence of moderately potent inhibition by drugs of other pharmacological categories raises the possibility that some of them might inhibit AcCh storage in intact preparations under certain circumstances. By extrapolating from AH5183, we would expect that those which are membrane-permeant would

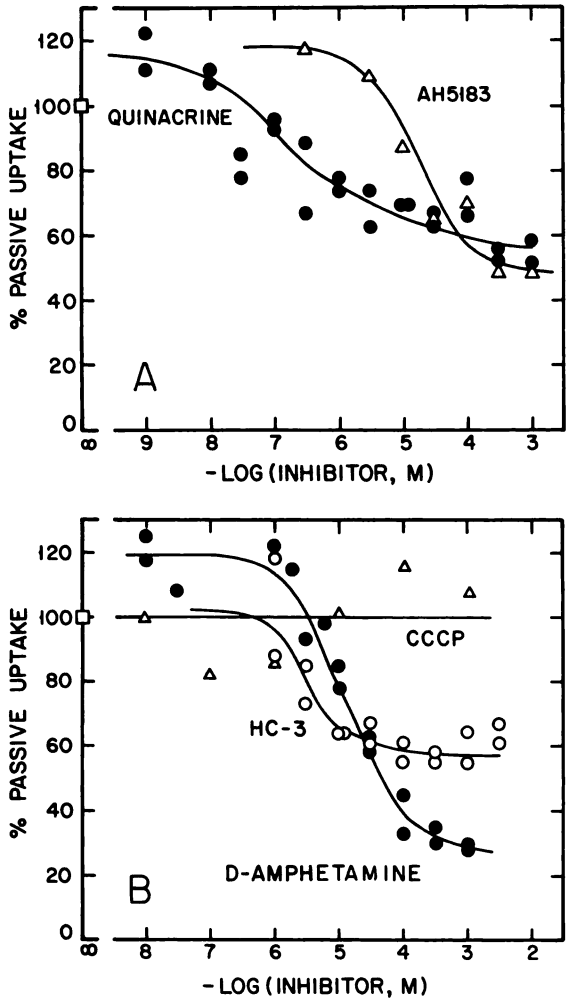

FIG. 3. *Inhibition of [^{3}H]AcCh passive uptake by selected drugs*

Uptake in the absence of drugs for all curves is normalized to 100% and is indicated by □ on the ordinate. The [^{3}H]AcCh uptake ratio for each titration in the absence of the specified drug and the approximate IC_{50} value (in parentheses) for that drug were as follows: A, 3.7 for quinacrine (●, 0.3 μM) and 2.8 for AH5183 (△, 20 μM). The titration for AH5183 was quite variable in different vesicle preparations, with the IC_{50} ranging from 0.3 μM to 20 μM in four preparations. Three different preparations gave the same IC_{50} for quinacrine. B, 3.3 for hemicholinium-3 (*HC-3*) (○, 3 μM); 4.0 for D-amphetamine (●, 10 μM); and 3.9 for CCCP (△, average of duplicate points, no effect).

be most likely at high or chronic doses to be effective at the vesicle level, especially after repetitive stimulation. A potential example of this is found in D-amphetamine. In addition to its adrenergic actions, high concentrations inhibit neuromuscular transmission, in part as an antagonist of the nicotinic receptor. However, Snider and Gerald (33) recently found that 1 mM D-amphetamine also reduces evoked release of AcCh from rat phenic nerve by 50% and concluded that several mechanisms contribute to the neuromuscular blocking properties. Since we found D-amphetamine to be an effective inhibitor of AcCh storage at 100-fold lower concentrations with purified vesicles, it seems quite possible that the presynaptic action is due in part to this mechanism. Many other drugs also exhibit cholinolytic side effects *in vivo* which are not well characterized. Examples are found in the phencyclidine, antihistamine, antipsychotic, antidepressant, and anxiolytic families (34–37). The possibility that some of these effects arise from direct action on AcCh storage seems open.

A contrasting example is found in the aminoglycoside family. These antibiotics act presynaptically to inhibit neuromuscular transmission, and the favored model is that they are calcium antagonists (38). Our observation that streptomycin, tobramycin, and neomycin have no effect on vesicle uptake of [^{3}H]AcCh is consistent with the above model for the presynaptic action.

The nature of the target site in the vesicles in which AH5183 and other potent inhibitors act was restricted in this study by the elimination of effects on the ATPase or a proton gradient. An obvious potential site of binding is to the AcCh transport site itself. Because the structures of the potent drugs are so different from each other and from AcCh, the possibility that several types of binding site are present, including regulatory sites, should also be considered. A full kinetic analysis of inhibitory behaviors could be informative. That the drugs themselves might be transported cannot be eliminated with current data. However, such transport would have to be quite nonspecific, since most of the inhibitors do not resemble AcCh. This seems unlikely under active conditions, since good specificity for AcCh is exhibited as compared with Ch (Fig. 2 and ref. 6).

There are several reasons to think that the AcCh porter has substantially different properties when comparing active and passive transport conditions. First, some of the drugs studied here exhibit large increases or decreases in potency when comparing inhibition of active and passive transport. AH5183 inhibited active uptake 8- to 500-fold more potently than passive uptake. The great variability in the ratio was caused by variability in inhibiting the passive uptake by different synaptic vesicle preparations, and is not understood. Quinacrine and D-amphetamine inhibit both processes about equally. Hemicholinium-3 inhibits active uptake 100-fold less potently than passive uptake. Second, AcCh and Ch are transported about equally well under passive conditions (6), but AcCh is highly preferrred under active conditions. Third, passive transport of [^{3}H]AcCh is relatively insensitive to the external pH, whereas active transport increases greatly at higher pH (6). Fourth, passive transport of AcCh is favored by an internally negatively charged vesicle (7), whereas active transport occurs into a presumptively internally positively charged vesicle (6). The significance of the apparent differences under the two conditions is not understood, but the drugs should be useful in their study.

The great potency of active transport inhibition by AH5183 allows an additional conclusion. Since the IC_{50} of 40 nM was approximately the same as the molarity of synaptic vesicles calculated as described (6), there was one or less binding target per synaptic vesicle. Similar results have been obtained using the uncoupler nigericin (6). If less than one target per vesicle is present, the vesicles must be structurally heterogeneous, and AH5183 must interact preferentially with an actively transporting subpopulation. Physiological heterogeneity in cholinergic vesicles has in fact been observed (2).

In conclusion, AH5183 appears to be the prototype for a new family of anticholinergics. Previous physiological

work suggested that it does not act on acetylcholinesterase, the nicotinic receptor, or high-affinity Ch uptake systems (11, 12, 15). It might, however, have other actions as a local anesthetic and α-adrenergic blocker (11). Toll and Howard (17) reported that at a single high concentration it partially blocked uptake of dopamine into PC12 cells. Additional work is required to delimit the pharmacological specificity of this drug *in vivo*. Nevertheless, our biochemical confirmation of the physiological prediction about its vesicular action is encouraging, and suggests that a pharmacology of AcCh storage is accessible. If AH5183 is in fact specific at the neuromuscular junction, the results reported here also constitute indirect evidence that the source of evoked AcCh release is vesicular and not cytoplasmic, as some workers have held. AH5183, acting as the "reserpine" of the cholinergic nerve terminal, should be of use in the elucidation of presynaptic cholinergic mechanisms.

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