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Is an acetylcholine transport system responsible for nonquantal release of acetylcholine at the rodent myoneural junction?

(neuromuscular junction/transmitter release)

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ABSTRACT **Experiments were performed to investigate the effects on the spontaneous, nonquantal release of acetylcholine (AcCho) from motor nerve terminals of substances known to inhibit the AcCho transport system present in cholinergic synaptic vesicles. In mouse diaphragms, the hyperpolarization normally produced by *d*-tubocurarine in the endplate area of muscle fibers that had been treated by an anticholinesterase was partly or completely blocked by 2-(4-phenylpiperidino)cyclohexanol (AH5183, 0.1–1 μM), quinacrine (0.1 μM), and tetraphenylborate (1 μM). Since the sensitivity of the subsynaptic area to AcCho was not changed, the block of the hyperpolarizing action of *d*-tubocurarine indicated an inhibition of the spontaneous, nonquantal release of AcCho. This was confirmed in experiments on rat diaphragm using direct radioenzymatic measurement of the AcCho released into the incubation medium. The release of AcCho from the innervated diaphragm was decreased by about 50% in the presence of AH5183 (0.01–1 μM) and by 42% in the presence of quinacrine (0.1–1 μM). The AcCho released was presumably neural, since the release of AcCho from 4-day denervated diaphragms was not diminished by either AH5183 or quinacrine. The results indicate that the spontaneous release of AcCho from the motor nerve terminals is highly sensitive to low concentrations of specific inhibitors and is probably mediated by a carrier. It is proposed that spontaneous release is due to the incorporation into the membrane of the nerve terminal during exocytosis of the vesicular transport system responsible for moving AcCho into the vesicle.**

Acetylcholine (AcCho) is released from the nerve terminal of the neuromuscular junction in several ways. There is electrophysiological evidence for the random release of quanta of AcCho, which gives rise to small, transient postsynaptic depolarizations (miniature endplate potentials or MEPPs), and for the stimulation evoked multiquantal release, which gives rise to endplate potentials (1).

In addition to the quantal release, there are several lines of evidence that AcCho may escape from the intramuscular axon branches by another route, the so-called nonquantal release. In incubation experiments the AcCho released into the bathing medium by MEPPs can account for only about 1–4% of the total AcCho released (2–4). In electrophysiological experiments, the local application of the AcCho receptor blocking agent *d*-tubocurarine (*d*-TC) to the endplate area of muscles previously treated with an anticholinesterase produces a small hyperpolarization presumably due to block of the depolarization produced by the nonquantally released AcCho ("H effect") (5–8). The hyperpolarization is present only after treatment with an anticholinesterase, which likely increases the local concentration of AcCho to a level sufficiently high to cause a slight depolarization.

The mechanism underlying the nonquantal release of AcCho is unknown. Synaptic vesicles isolated from the electric organ of *Torpedo* have been shown to take up AcCho by an active process (9). If the transport system responsible for this is present in vesicles in the nerve terminal of the neuromuscular junction, and if it retains its orientation following the incorporation of the vesicle membrane into the axon membrane during exocytosis, then it would move AcCho from the axoplasm into the extracellular space. We have investigated the effects on nonquantal release of several agents that block the transport of AcCho by vesicles (10, 11) to determine whether the release may be due to this transport system. Nonquantal release has been measured electrophysiologically and by direct analysis of bath AcCho.

METHODS

Electrophysiological Experiments. The experiments were performed on hemidiaphragms dissected from decapitated female white specific-pathogen-free mice. The thoracic side of the diaphragm was carefully cleaned of pleura, which often covers the endplate zone of the muscle. The diaphragms were washed several times and then pinned to small discs of Silgard, which were placed into an oxygenated (95% O_2/5% CO_2) solution containing (mM) NaCl, 137; KCl, 5.0; $CaCl_2$, 2.0; $MgCl_2$, 1.0; $NaHCO_3$, 11.0; NaH_2PO_4, 1.0; and glucose, 11.0 (pH = 7.4) unless otherwise stated. The preparations were treated with an irreversible anticholinesterase, 0.01 mM diethoxy-*p*-nitrophenyl phosphate (armin), for 30 min and then rinsed several times before the measurements were made (12).

The muscles were placed in a small, round polyvinyl chloride chamber that contained 1 ml of solution and was kept at about 20°C. The tissue was about 1 mm below the surface of the solution, which was oxygenated by continuous blowing of the gas mixture upon the surface. The small volume chamber without perfusion gave a larger hyperpolarization than found previously (6, 7, 13, 14). The reasons for this are not known.

The resting membrane potentials (RMPs) and MEPPs were recorded from muscle fibers in the most superficial layers with conventional glass micropipettes with resistances of 15–30 MΩ. The RMPs were usually measured in the endplate zone, indicated visually by the ends of intramuscular branches of the phrenic nerve, and occasionally also in the endplate free area either 1 mm from the central tendon or

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Abbreviations: AcCho, acetylcholine; *d*-TC, *d*-tubocurarine; MEPP, miniature endplate potential; RMP, resting membrane potential.

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from the rib arch. The measurements were usually started 15 min after immersing the muscle in the bath. For the RMP measurements 12–34 fibers were impaled before and after treatment with *d*-TC (0.01 mM, Burroughs Welcome, Research Triangle Park, NC). Each group of impalements was usually completed within 5–10 min, and so the overall duration of the experiments, including curarization, was usually <1 hr. In some experiments, one half of the diaphragm was used as a control and the other was treated with a drug; in others both parts were treated with the agents. For the MEPP studies, the amplitudes and other parameters were measured from photographic records.

The drug 2-(4-phenylpiperidino)cyclohexanol (AH5183) was dissolved in 1 ml of ethanol and then 9 ml of the above described saline (without Ca^{2+} and glucose) was added to form the stock solution (0.1 mM). Quinacrine (Sigma) and sodium tetraphenylborate (Aldrich) were dissolved in distilled water; they were added to the muscles together with anticholinesterase and were also present in the bath during the electrophysiological measurements.

Measurements of AcCho Release, Content, and Synthesis. The experiments were performed with isolated hemidiaphragms of rats (Wistar, males, 180–220 g of body weight) as described by Doležal and Tuček (15). Left hemidiaphragms were incubated at 38°C for 120 min in an incubation medium containing (mM) NaCl, 123; KCl, 5; $CaCl_2$, 2.5; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; choline chloride, 0.01; paraoxon (Sigma), 0.058; and glucose, 5. The medium was under an atmosphere of 95% O_2/5% CO_2. The contents of AcCho in the diaphragms and in the incubation medium (the latter corresponding to the release of AcCho during the incubation) were measured radioenzymatically by using the method of Goldberg and McCaman (16) as used by Doležal and Tuček (15). The synthesis of AcCho occurring during the incubation period was calculated as the sum of the contents of AcCho in the tissue and in the medium at the end of incubation minus the average content of AcCho in the tissue at the beginning of incubation [measured in separate experiments; it was 1.12 ± 0.07 nmol/g of wet weight (mean ± SEM)]. In the denervation experiments, the diaphragms were removed from animals 4 days after an operation in which, under ether anesthesia, about 5 mm of the left phrenic nerve was removed through an incision to the sixth intercostal space. The average AcCho content (mean ± SEM) of the denervated muscles before the incubation was 0.40 ± 0.04 mmol/g of wet weight.

The activity of choline acetyltransferase was measured in homogenates of the diaphragms as described by Tuček (17). The portion of the total AcCho synthesis that was inhibited by 2 μM bromoacetylcholine was taken as a measure of the choline acetyltransferase activity; the rest was probably due to the activity of carnitine acetyltransferase (see ref. 17).

RESULTS

Electrophysiological Measurements. *Hyperpolarizing effect.* The nonquantal release was determined electrophysiologically by measuring the change in membrane potential produced by the addition of 0.01 mM *d*-TC. The average hyperpolarization (mean ± SD) for nine muscles (controls, Tables 1 and 2) was 8.1 ± 2.6 mV. This is similar to the 9.2 mV reported previously (12).

AH5183 has been found to be the most potent inhibitor of AcCho transport in *Torpedo* synaptic vesicles of the >80 compounds investigated by Anderson *et al.* (10). The concentration producing 50% block of uptake (IC_{50}) is 40 nM. Nonquantal release as demonstrated by the loss of the hyperpolarizing effect was inhibited by AH5183; 0.01 μM produced little or no effect, 0.1 μM significantly reduced the hyperpolarization, and 1 μM seemed to block it completely (Table 1).

Table 1. Effects of AH5183 on the hyperpolarizing effect

Treatment	Concentration, μM	Membrane potential, mV		H effect, mV
		Control	With *d*-TC	
Control*		−69.3 ± 5.2 (20)	−78.0 ± 5.7 (26)	8.7
AH5183	0.01	−71.1 ± 5.3 (26)	−77.8 ± 5.4 (26)	6.7
Control*		−71.9 ± 4.2 (28)	−75.2 ± 5.2 (25)	3.3
AH5183	0.01	−70.3 ± 3.8 (25)	−71.0 ± 4.6 (26)	0.7
Control*		−66.2 ± 3.9 (16)	−78.1 ± 3.9 (25)	11.9
AH5183	0.1	−72.9 ± 4.3 (26)	−77.4 ± 4.1 (25)	4.5
Control†		−70.0 ± 3.2 (20)		
AH5183	0.1	−78.1 ± 2.8 (21)	−78.7 ± 3.2 (21)	0.6
AH5183	0.1	−76.7 ± 2.3 (25)	−77.6 ± 3.4 (26)	0.9
AH5183	0.1	−78.8 ± 3.2 (33)	−78.3 ± 0.8 (27)	−0.5
Control*		−63.2 ± 3.0 (24)	−75.6 ± 4.7 (20)	12.4
AH5183	1	−73.7 ± 3.8 (21)	−74.6 ± 4.3 (21)	0.9
AH5183	1	−79.2 ± 2.9 (20)	−79.1 ± 3.8 (26)	−0.1
AH5183	1	−78.8 ± 3.2 (33)	−78.3 ± 2.8 (27)	−0.5

Values give the average RMP (±SD), with the number of potentials measured shown in parentheses.
*Muscles from the same diaphragm used for control and experimental preparations.
†Same muscle used for control, AH5183, and AH5183 + *d*-TC.

The time course of the onset of block in 1 μM AH5183 was examined (Fig. 1). The RMPs of 10 or more muscle fibers were measured at each time. The membrane became hyperpolarized within 10 min after addition of the drug. The hyperpolarization suggests that the nonquantal release was blocked within 10 min. The drug was washed out by rinsing

Table 2. Effects of various treatments on the hyperpolarizing effect

Treatment	Concentration, μM	Membrane potential, mV		H effect, mV
		Control	With *d*-TC	
Control*		−72.2 ± 3.9 (28)	−80.5 ± 3.7 (26)	8.3
Quinacrine	0.01	−73.7 ± 3.9 (28)	−78.2 ± 3.6 (31)	4.5
Quinacrine	0.1	−74.9 ± 4.0 (30)	−75.0 ± 3.4 (30)	0.1
	0.1	−78.5 ± 3.8 (12)	−80.3 ± 5.4 (15)	1.8
	0.1	−74.4 ± 5.6 (32)	−77.4 ± 4.1 (26)	3.0
Control*		−71.3 ± 4.2 (27)	−79.2 ± 4.6 (27)	7.9
Ph_4B	1	−63.7 ± 5.1 (31)	−77.2 ± 3.8 (22)	13.5
Control*		−72.7 ± 4.7 (30)	−80.6 ± 3.7 (33)	7.9
Ph_4B	1	−75.4 ± 5.2 (29)	−79.1 ± 4.2 (23)	3.7
Control*		−73.5 ± 4.0 (36)	−79.6 ± 4.7 (34)	6.1
Ph_4B	1	−76.1 ± 5.1 (30)	−76.9 ± 3.4 (28)	0.8
Control*		−74.3 ± 3.9 (20)	−80.8 ± 2.9 (20)	6.5
Ph_4B	1	−76.0 ± 3.7 (19)	−79.9 ± 3.9 (21)	3.9
Ph_4B	1	−70.1 ± 4.1 (32)	−70.7 ± 4.5 (24)	0.6

Values give average RMP (±SD), with the number of potentials measured shown in parentheses.
*Muscles from same diaphragm used for control and experimental preparations.

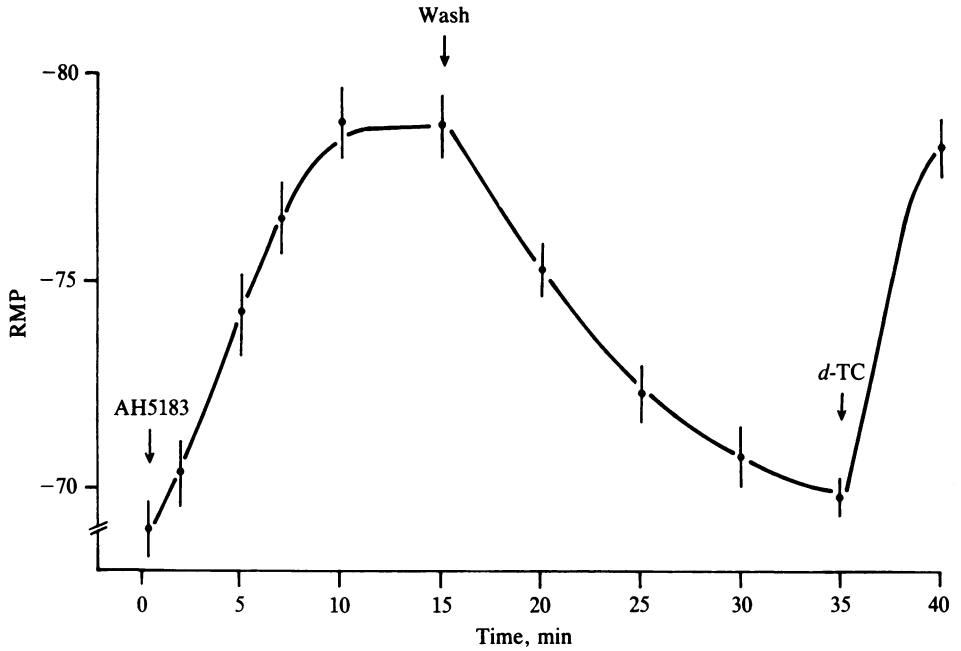

FIG. 1. Time course of the potential change following addition and washout of 1 μM AH5183. Each point is the average (±SEM) of 10 measurements of the RMP in the junctional region.

the chamber several times with fresh bath solution, and the membrane potential returned to the control level within about 20 min.

Two other very potent blocking agents described by Anderson *et al.* (10, 11) were quinacrine (IC_{50} = 0.4 μM) and tetraphenylborate (IC_{50} = 0.3 μM). Both agents blocked the hyperpolarization produced by *d*-TC (Table 2). Quinacrine at 0.01 μM may have had a small effect, but at 0.1 μM, the hyperpolarization was much reduced. Higher concentrations were not investigated because quinacrine has been found to block nicotinic junctions in the *Torpedo* electric organ (18). Tetraphenylborate at 1 μM reduced the hyperpolarization produced by *d*-TC in four experiments of five; in the fifth there was a depolarization in its presence that was unexplained.

The transport of AcCho into the vesicle appears to be linked to a proton gradient across the vesicle (19). If the nonquantal release is due to the presence of this transport system in the membrane of the nerve terminal, then an increase in the pH of the bath should inhibit the release by setting up a large pH gradient in the opposite direction. In a series of experiments in glycine buffer, at pH 6.4 and 7.4, the addition of *d*-TC produced the usual hyperpolarization; at pH 8.4 and 9.4, *d*-TC produced little or no change in membrane potential (Table 3). In one muscle in glycine buffer at pH 8.4 or 9.4, the RMP became hyperpolarized by about 8 mV within 5 min and reached the same level found after addition of *d*-TC (Table 3, lower part). The effect was reversed when the original solution (pH 7.4) was restored, and the RMP was the same in a more acid, bicarbonate-buffered, solution (pH 6.4).

Effects on MEPP. The reduction of the hyperpolarization induced by *d*-TC could be due to a direct curare-like action on the AcCho receptors by the compounds investigated. To exclude this possibility, the effects on the size of the spontaneous MEPPs were examined. No substantial changes in MEPP size, frequency, or time course were found after AH5183 at 1 μM (Table 4). A similar absence of the effect was observed with 0.5 μM quinacrine (data not shown).

Measurements of AcCho Release, Content, and Synthesis. In these experiments, the AcCho released into the bath was measured directly. In innervated diaphragms the release of AcCho was inhibited by both AH5183 and quinacrine (Table

Table 3. Effect of pH on RMP

pH	Buffer	Membrane potential, mV		H effect, mV
		Control	*d*-TC	
6.4	Glycine	−71.3 ± 3.5 (20)	−78.4 ± 3.0 (20)	7.1
		−69.5 ± 4.0 (20)	−75.6 ± 3.2 (15)	6.1
7.4	Glycine	−73.5 ± 2.8 (20)	−80.1 ± 4.1 (20)	6.6
		−71.6 ± 2.9 (20)	−78.9 ± 3.0 (17)	7.3
8.4	Glycine	−77.9 ± 2.8 (20)	−78.3 ± 3.3 (20)	0.4
		−78.4 ± 3.0 (20)	−76.9 ± 4.0 (20)	−1.5
9.4	Glycine	−77.6 ± 3.1 (20)	−78.2 ± 2.0 (20)	0.6
		−77.0 ± 2.1 (20)	−79.0 ± 3.2 (23)	2.0
6.4*	Bicarbonate	−68.7 ± 4.1 (38)		
9.4*	Glycine	−79.6 ± 2.2 (32)		
7.4*	Phosphate	−70.6 ± 4.8 (33)		
9.4*	Glycine	−78.4 ± 3.1 (27)		
8.4*	Glycine	−78.0 ± 3.3 (35)		
9.4*	Glycine	−79.8 ± 3.6 (32)	−80.0 ± 3.3 (25)	

Values give average RMP (±SD), with the number of potentials measured shown in parentheses.
*Same muscle used for all measurements.

Table 4. Effects of AH5183 on various MEPP parameters

Parameter	Control	Time in AH5183	
		10 min	30 min
Amplitude, mV	1.1 ± 0.1 (25)	1.1 ± 0.1 (30)	0.99 ± 0.11 (32)
Frequency, Hz	1.3 ± 0.4 (10)		1.49 ± 0.5 (10)
Rise time, sec	0.89 ± 0.04 (21)	0.9 ± 0.5 (30)	0.84 ± 0.06 (24)
Half-decay time, sec	3.4 ± 0.2 (19)	3.25 ± 0.06 (30)	3.1 ± 0.1 (24)

Values give average MEPP parameters (±SD), with the number shown in parentheses.

5). It appears that the inhibition observed with 0.01 μM AH5183 (the lowest concentration tested) was already maximal; the release of AcCho was reduced by 46%. At 1 μM, AH5183 diminished the release of AcCho by about 50%. In the presence of 0.01 μM quinacrine, the release of AcCho was reduced by 22%, and the release was inhibited by about 42% with 0.1 and 1 μM quinacrine.

The AcCho content of the innervated diaphragms at the end of the incubation was diminished in the presence of 0.01 and 0.1 μM AH5183 and of 0.01, 0.1, and 1 μM quinacrine but not in the presence of 1 μM AH5183. The synthesis of AcCho was reduced at all concentrations of AH5183 and quinacrine tested.

In diaphragms denervated for 4 days, the release of AcCho, which was presumably from the muscles, was not affected by either AH5183 (0.01 and 1 μM) or quinacrine (0.1 μM) (Table 6). The AcCho content of the tissue at the end of the incubation was reduced in the presence of 1 μM AH5183. The synthesis of AcCho was not changed by either AH5183 or quinacrine.

The activity of choline acetyltransferase was not significantly altered in the presence of AH5183. The bromoacetylcholine-sensitive synthesis of AcCho in the homogenates of the diaphragm was diminished by about 4.6% at 0.1 μM and by about 6% at 1 μM.

DISCUSSION

The mechanism underlying the nonquantal release of AcCho from the nerve terminal is unknown. It seems likely that the axon membrane is relatively impermeable to a cation the size of AcCho. A vesicular transport system of the type described by Anderson *et al.* (10, 11) could be responsible, especially since the orientation of this transport system following exocytosis could move AcCho out from the nerve terminal cytoplasm into the synaptic cleft.

The accumulation of a high concentration of AcCho in intact vesicles requires a pH gradient in which the vesicle interior is acidic compared to the axoplasm (19). Presumably the transport system exchanges protons for AcCho, moving AcCho into the vesicles and protons out. At the usual bath pH (7.4) the pH of the synaptic cleft may be a little less acidic than the axoplasm (however, see below), and so the gradient across the vesicle membrane may not be the same as across the nerve terminal. However, it is possible that the AcCho transport system incorporated in the membrane of the nerve terminal membrane may still be able to mediate an exchange of AcCho and protons. The concentration of AcCho in the cytoplasm of cholinergic neurons has been estimated to be about 170 μM (20), and the initial bath concentration is zero. At 38°C, with a membrane potential of −75 mV, $[AcCho]_{ext}$ would be expected by the Nernst equation to be about 1/16th of $[AcCho]_{int}$, or about 10 μM. The bath concentration measured after a 2-hr incubation was in the range of 1 μM (Table 5), which is considerably less.

Although the simplest assumption for the value of the pH in the synaptic gap is to equate it to the pH in the bath, this may not be true. It has been reported that the calcium level in the synaptic cleft is higher than in the extracellular bath medium (21). One possible explanation for this is the presence of fixed charges in the cleft, which would also alter the pH in the cleft. If true, this would also explain why block of nonquantal release required a bath pH of 8.4.

Some of the effect of alkaline pH on the hyperpolarizing effect is likely due to a direct effect on the depolarization produced by AcCho. At the frog neuromuscular junction, a change in pH from 7.4 to 9.4 has no effect on the size of the MEPPs but reduces the depolarization produced by iontophoretic application of AcCho by about 40% (22). However, the complete block of the hyperpolarizing effect at pH 9.4 requires an additional explanation, which we propose may be the block of the transport enzyme by the pH gradient.

The evidence presented herein is consistent with the idea that nonquantal release of AcCho is produced by the AcCho transport system found in synaptic vesicles. It is interesting that tetraphenylborate has been shown to block transmission at the neuromuscular junction. It decreases the amplitude of the spontaneous MEPPs, and the magnitude of the reduction increases with time (23). In concentrations of 50 μM or higher, it has no effect on the depolarization produced by carbachol applied by microperfusion (24). It was suggested that tetraphenylborate may reduce the concentration of AcCho in the vesicles (23).

Isolated muscles incubated *in vitro* release AcCho and the

Table 5. Effect of AH5183 and quinacrine on the content of AcCho in the tissue, the release of AcCho into the incubation medium, and the synthesis of AcCho in experiments with 2-hr incubations of isolated rat hemidiaphragms

Inhibitor	AcCho content in the tissue		AcCho release into the medium		AcCho synthesis	
	nmol/g	% of control	nmol/g in 2 hr	% of control	nmol/g in 2 hr	% of control
None (control)	3.89 ± 0.50	100	7.61 ± 0.72	100	10.38 ± 1.01	100
AH5183, μM						
0.01	2.96 ± 0.15	76	4.13 ± 0.14	54	5.98 ± 0.05	58
0.1	2.90	75	4.43	58	6.21	60
1	4.13 ± 0.21	106	3.79 ± 0.66	50	6.80 ± 0.80	66
Quinacrine, μM						
0.01	3.12 ± 0.02	80	5.92 ± 0.76	78	7.92 ± 0.76	76
0.1	3.15 ± 0.27	81	4.39 ± 0.58	57	6.42 ± 0.75	62
1	2.82 ± 0.41	72	4.36 ± 0.60	57	6.06 ± 0.97	58

Values are means ± SEM of three experiments with the exception of incubations with 0.1 μM AH5183, which are means of two values, and of controls, which are means of six values.

Table 6. Effect of AH5183 and quinacrine on the content of AcCho in the tissue, the release of AcCho into the incubation medium, and the synthesis of AcCho in experiments with 2-hr incubations of isolated denervated rat hemidiaphragms

Inhibitor	AcCho content in the tissue		AcCho release into the medium		AcCho synthesis	
	nmol/g	% of control	nmol/g in 2 hr	% of control	nmol/g in 2 hr	% of control
None (control)	2.85 ± 0.29	100	2.14 ± 0.27	100	4.59 ± 0.49	100
AH5183, μM						
0.01	2.95 ± 0.28	104	2.54 ± 0.09	119	5.09 ± 0.44	111
0.1	1.87 ± 0.35	66	2.92 ± 1.13	136	4.39 ± 1.03	96
Quinacrine, 0.1 μM	2.88 ± 0.93	101	2.77 ± 0.44	129	5.27 ± 0.64	115

Values are means ± SEM of three experiments with the exception of incubations with 0.01 μM AH5183, which are means of four values, and of controls, which are means of five values.

amount of released AcCho can be measured at the end of the incubation provided that the activity of cholinesterase in the tissue is inhibited. Paraoxon was used as a cholinesterase inhibitor in the present experiments. There are two sources for the AcCho released into the medium: (*i*) the intramuscular nerve branches and terminals; (*ii*) the muscle fibers (15, 17). Under the conditions used in the present experiments, the release from the neural tissue represents between 30% (25) and 53% (15) of total release. Therefore, if AH5183 and quinacrine inhibit the release of AcCho from the nerves [as can be judged from electrophysiological experiments, since the hyperpolarization has been shown to be caused by the release of neural rather than muscular AcCho (25)], complete inhibition of neural AcCho release would be expected to diminish the total AcCho release by one-third to one-half. The inhibitory effects observed with 0.01–1 μM AH1583 (42–50%) and with 0.1–1 μM quinacrine (42–43%) thus apparently reflect total or very nearly total inhibition of neural AcCho release. The fact that AH5183 (0.01 and 1 μM) and quinacrine (0.1 μM) did not diminish the release of AcCho from denervated muscles (Table 6) supports the interpretation that the inhibitors of the AcCho transport system reduced or blocked the release of the neural rather than the muscular AcCho.

The decreases in AcCho synthesis observed in the innervated muscles were probably a secondary consequence of the decrease of AcCho release, in agreement with known relations between AcCho synthesis and release (20, 26). The diminished content of AcCho in the tissue at the end of incubations in the presence of 0.01 and 0.1 μM AH5183 and of 0.01–1 μM quinacrine can be explained by the block of AcCho accumulation in synaptic vesicles. It is difficult to explain, however, why the content of AcCho in the tissue appeared unaffected after the incubations with 1 μM AH5183. Note also that AH5183 has been shown to act only on the AcCho transport system and not on choline acetyltransferase (27).

Nonquantal release has been found by electrophysiological techniques to be inhibited by botulinum toxin (BTX) [mouse (12) and rat (25); note, however, that Stanley and Drachman (13) reported that botulinum toxin was without effect on the mouse junction]. The effects of BTX on AcCho release as analyzed directly are also consistent with a block of nonquantal release (25, 28). This suggests that BTX may act by blocking the postulated vesicular AcCho transport system. The blocking of the filling of vesicles is consistent with the use-dependent block produced by BTX and has indeed been proposed as a possible mechanism of action of BTX (29).

Today the weight of evidence appears to support the notion of vesicular release of transmitter at the neuromuscular junction. However, another view is that the release is due to a structure that is ". . . a mechanism that binds AcCho to saturation . . ." that ". . . must be charged from the cytoplasmic pool and must release in a nonelectrogenic manner. Calcium remains naturally (primarily or secondarily) the trigger of such release performed by some carrierlike mechanism or saturable gate." This structure has been termed a vesigate by Tauc (30). An AcCho transport system in the nerve terminal membrane, as proposed herein, has some of these proposed properties, but there is no evidence that this enzyme participates in AcCho release evoked by nerve activity (in fact, we found the compounds blocking the enzyme to be without effect on neuromuscular transmission at the concentrations used herein).

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