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Some pharmacological actions of acetylsecohemicholinium

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The effects of the acetylated derivative of HC-3 (acetylsecohemicholinium; AcHC-3) have been studied at cholinergic nerve terminals and compared with the effects of the parent compound. AcHC-3 blocked neuromuscular transmission in nerve-muscle preparations; it was shown to be less effective than HC-3 in producing a pre-junctional block in the rat diaphragm but was more effective than HC-3 in eliciting a post-junctional blocking effect in the chick biventer muscle. On the frog rectus abdominis muscle AcHC-3 caused a substantial potentiation of the contractures elicited by acetylcholine but did not by itself cause a contracture of the muscle. AcHC-3 inhibited the synthesis of acetylcholine by cholinergic nerve ending particles and inhibited the uptake of [^{14}C]choline into brain synaptosomal fractions to a similar extent to HC-3. AcHC-3 was shown to be a substrate for cholinesterase enzymes although the rate of hydrolysis was much less than the rate of hydrolysis of acetylcholine. It is concluded that AcHC-3 is effective in inhibiting cholinergic transmission and this action is exerted by the open chain (seco) compound and is not due to the hydrolysis of the AcHC-3 by cholinesterases to form the active HC-3 molecule.

The hemicholinium compounds have been shown to interfere with transmission at cholinergic synapses and hemicholinium No. 3 (HC-3) is the most active of these compounds, which were first studied by Long & Schueler in 1954. The primary mechanism by which HC-3 inhibits cholinergic transmission is thought to be by preventing the transport of choline from the extracellular fluid to the intraneural sites where it is acetylated (MacIntosh, Birks & Sastry, 1956; Potter, 1968).

HC-3 has been shown to exist in aqueous solution as a cyclic hemiacetal structure (Maggio, 1968; di Augustine & Haarstad, 1970), although the original structural formula of the compound was reported to be an open chain structure (Long & Schueler, 1954); acetylsecohemicholinium No. 3 (AcHC-3) has an open ring (seco) structure (Schueler, 1955). Maggio (1968) reported that AcHC-3 was comparable to HC-3 with respect to toxicity in mice and potentiation of the cardiovascular responses to the catecholamines; and de Balbian Verster, Haarstad & White (1968) showed that AcHC-3 inhibited partially purified choline acetyltransferase (ChAc) from rat brain.

Domino, Mohrman & others (1973) and Maggio-Cavaliere (1976) have reported on some of the pharmacological actions of AcHC-3. The present study further investigates its pharmacological activity on cholinergic transmission and compares the effects of pre-junctional and post-junctional blockade with those of the parent compound HC-3. The hydrolysis of AcHC-3 by cholinesterase enzymes has not previously been investigated and in the present work experiments were performed to determine any hydrolysis of AcHC-3. These results should be of assistance in determining whether the pharmacological effects of AcHC-3 are due to its hydrolysis to the active parent compound HC-3.

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MATERIALS AND METHODS

Nerve-muscle preparations

(a) *Rat phrenic nerve-diaphragm.* Experiments were performed on the isolated nerve-diaphragm preparation of the rat (Bülbring, 1946) mounted in McEwen (1956) solution at 37°. The nerve was stimulated by supramaximal rectangular pulses of 0·1 ms duration. In some experiments two hemidiaphragms were mounted in the same bath; one nerve was stimulated at 1 Hz and the other at 0·1 Hz. The muscle contractions were recorded isometrically by means of a Grass FT ·03C force displacement transducer on a Beckman dynograph.

(b) *Chick biventer cervicis muscle.* Isolated biventer cervicis muscles from 5–10 day old chicks were mounted in Krebs-Henseleit solution (NaCl, 6·95; KCl, 0·34; $CaCl_2$, 0·28; KH_2PO_4, 0·162; $MgSO_4$, 0·294; $NaHCO_3$, 2·1 and dextrose, 2·0 g litre^{-1}) at 32° and stimulated indirectly with rectangular pulses of 0·1 ms duration (Ginsborg & Warriner, 1960). In some experiments two muscles were mounted in the same bath and one muscle stimulated at 1 Hz

and the other muscle at 0·1 Hz. Muscle contractions were recorded by means of a Grass force displacement transducer on a Beckman dynograph. At intervals acetylcholine was added to the bath and left in contact with the muscle for 30 s during which the electrical stimulation was stopped.

(c) *Frog rectus abdominis muscle.* The frog rectus abdominis muscle was suspended in frog-Ringer solution (NaCl 115·3; KCl 4·29; $CaCl_2$, 1·19; and $NaHCO_3$, 1·43 mM). Acetylcholine in submaximal doses was added at 4 min intervals and left in contact with the muscle for 45 s. The preparation was then washed twice and allowed to relax to its baseline position. The hemicholinium compound was added to the bath 1 min before the next dose of acetylcholine, which was given in the presence of HC-3 or AcHC-3. Contractions were recorded isometrically on a Beckman dynograph.

Measurement of choline uptake

Synaptosomal fractions of guinea-pig brain were prepared by the method of Whittaker, Michaelson & Kirkland (1964). The experiments were performed immediately after fractionation and isolation, which was at 0–4°. Choline uptake was determined according to Kuhar, Sethy & others (1973). The fraction was resuspended in Krebs-Henseleit solution and 200 μl incubated at 37° for 5 min with 100 μl [^{14}C]choline (5·2 mCi mmol^{-1}, 2·27 μCi ml^{-1}), with 1·5 ml Krebs-Henseleit solution and with either 0·2 ml of the hemicholinium solution or with 0·2 ml of distilled water. All assays were in duplicate. Incubations were terminated by transferring the incubation tubes to an ice bath before centrifuging in a refrigerated centrifuge at 2000 *g* for 5 min. The supernatant was removed and the pellet resuspended in 5 ml ice-cold Krebs-Henseleit solution and recentrifuged. The supernatant was again removed and the synaptosomal particles were dissolved in 0·2 ml of M NaOH, plated on a glass fibre filter and counted in a liquid scintillation counter (Bosmann & Hemsworth, 1970).

Acetylcholine synthesis

Two types of synaptosomal fractions were used as described previously by Hemsworth (1971a). One fraction was suspended in Krebs solution (NaCl 6·92, KCl 0·34, $CaCl_2$ 0·296, K_2HPO_4 0·162, $MgSO_4$ 0·294, $NaHCO_3$ 2·1, dextrose 2·0 g litre^{-1}) and 2 μl of this suspension was used in the incubation. A second fraction was suspended in 0·1% Triton X-100 and homogenized, 30 strokes, with a Ten Broeck homogenizer; 2 μl of this suspension was used for incubation. Each fraction contained 10–30 mg protein ml^{-1}.

To obtain partially purified ChAc, fresh rat or guinea-pig cerebral cortices were homogenized in 0·1% Triton X-100 containing 200 mM KCl (Potter, Glover & Saelens, 1968) in a Virtis homogenizer. The homogenate was centrifuged at 45 000 *g* for 1 h and the supernatant fractionated with ammonium sulphate. The fraction precipitating between 20 and 30% (w/v) ammonium sulphate was collected by centrifugation, dissolved in 0·1% Triton X-100 containing 200 mM KCl and dialysed to remove the ammonium sulphate. The enzyme prepared in this manner was used immediately or was stored at −20°. Before use the stored enzyme was passed through a Sephadex G-25 column to remove any choline. ChAc activity was determined by a modification of the procedure of McCaman & Hunt (1965) and the acetylated material isolated by the reineckate precipitation procedure described by Hemsworth (1971a).

Cholinesterase determinations

Bovine erythrocytes made up in distilled water to a concentration of 0·5 mg protein ml^{-1} were used as a source of acetylcholinesterase, AChE (E.C. 3.1.1.7). Horse serum was made up in a similar way to the same concentration and used as a source of cholinesterase, ChE (E.C. 3.1.1.8).

Null-point titration method

Enzymic hydrolysis of acetylcholine and AcHC-3 was determined by a null-point titration method using a Radiometer (Copenhagen) pH-Stat unit as described by Hemsworth (1971b). The incubation mixture contained 50 μl ChE, 0·95 ml water, 1·8 ml of a salt solution containing (mM): NaCl, 153·7; KCl, 4·02; $CaCl_2$, 3·31 (Stavinoha, Ryan & Endecott, 1969), and 0·2 ml substrate (acetylcholine or AcHC-3, $3{\cdot}0 \times 10^{-2}$ M) at 37°. The acid released upon hydrolysis of acetylcholine or AcHC-3 was titrated with 0·01 M NaOH and the rate of hydrolysis determined as described by Jensen-Holm, Lausen & others (1959).

Protein

Total protein was determined by the method of Lowry, Rosebrough & others (1951). Crystalline bovine serum albumin was used as standard.

Materials

Acetylhemicholinium No. 3 (AcHC-3), 4,4′-biphenylene)-bis(2-oxoethylene)-bis(2-acetoxyethyldimethyl-

ammonium bromide), (mol. wt 657·64) was prepared by Eastman Organic Chemicals, Rochester, New York. HC-3 (hemicholinium-3), 2,2′-(4,4′-biphenylene)bis-(2-hydroxy-4,4-dimethylmorpholinium bromide), (mol. wt 574·36) was obtained from Aldrich Chemical Co. and recrystallized from methanol–ether. Choline [^{14}C]methyl chloride (52 mCi mmol^{-1}) and acetyl-[1-^{14}C]coenzyme A (53·6 mCi mmol^{-1}) were purchased from The Radiochemical Centre, Amersham.

RESULTS

Nerve muscle preparations

(a) *Rat phrenic-nerve diaphragm.* Fig. 1 shows the blocking effect of AcHC-3 150 μg ml^{-1} (0·23 mM) on the preparation, stimulated directly at 1 Hz. This concentration had no effect on a muscle stimulated at 0·1 Hz. Neostigmine, 1 μg ml^{-1} (3·2μM) added to the bath when the muscle contractions had been reduced by approximately 50% by AcHC-3 did not reverse the neuromuscular block. When the drug was washed out contractions returned slowly towards normal (Fig. 1).

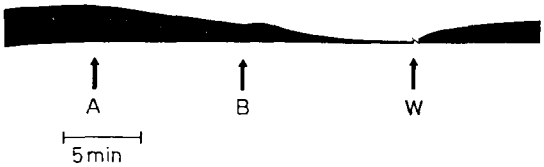

FIG. 1. Contractions of the rat hemi-diaphragm muscle elicited by stimulation of the phrenic nerve at a frequency of 1·0 Hz. At A, 150 μg ml^{-1} AcHC-3 and at B, 1 μg ml^{-1} of neostigmine were added to muscle bath. At W the drugs were removed from the organ bath and replaced by fresh bathing solution.

Choline 200 μg ml^{-1} (1·43mM), added to the preparation in the continued presence of AcHC-3 did not reverse the block; however, if choline was added after removal of the drug from the bath, the twitches rapidly returned to the control value, an effect similar to that observed with HC-3 (Chang & Rand, 1960; Hemsworth, 1971b).

At 500 μg ml^{-1}, (0·76mM), AcHC-3 blocked both the slowly (0·1 Hz) and the more rapidly (1 Hz) stimulated muscle to a similar degree; this effect was antagonized by neostigmine, suggesting a curare-like block. This response is similar to the effect of HC-3 (Chang & Rand, 1960; Bowman, Hemsworth & Rand, 1967).

(b) *Chick biventer cervicis muscle.* AcHC-3 from 1 μg ml^{-1} (1·5 μM) to 1 mg ml^{-1} (1·5 mM) did not cause a contracture of the preparation, indicating a lack of

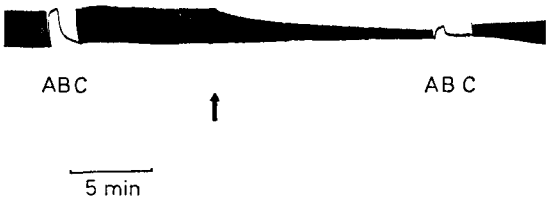

FIG. 2. Contractions of the biventer cervicis muscle of the chick in response to indirect stimulation at a frequency of 1 Hz. At the arrow 5 μg ml^{-1} AcHC-3 was added to the bath. At A, electrical stimulation was stopped and, 5 μg ml^{-1} acetylcholine was added to the bath. At B, the acetylcholine was removed from the bath and replaced with fresh Krebs-Henseleit solution. At C, electrical stimulation of the nerve was recommenced.

appreciable depolarizing activity. Fig. 2 shows the effect of 5 μg ml^{-1} (7·6 μM) of AcHC-3 on the contractions of the biventer muscle in response to nerve stimulation; 15 μg ml^{-1} (26 μM) of HC-3 was necessary to produce a similar effect (see also Marshall, 1969). The block produced by AcHC-3 appeared to be post-junctional and non-depolarizing since, when the indirectly elicited twitch was reduced in size by 75%, the acetylcholine contracture was reduced to a similar extent.

(c) *Frog rectus abdominis muscle.* AcHC-3, from 1 μg ml^{-1} (1·5 μM) to 1 mg ml^{-1} (1·5 mM) did not cause a contracture of the preparation; however, 10 μg ml^{-1} (15 μM) AcHC-3 potentiated the responses of the muscle to acetylcholine by 40%. Higher concentrations of AcHC-3 blocked the responses of the muscle to acetylcholine. An inhibition by 50% of the responses of the frog muscle to 0·4 μg ml^{-1} (2·2 μM) acetylcholine was produced by 75 μg ml^{-1} (114 μM) AcHC-3; a similar amount of inhibition was also produced by 30 μg ml^{-1} (52 μM) HC-3 and by 0·4 μg ml^{-1} (0·6 μM) (+)-tubocurarine. HC-3 did not itself cause a contracture of the muscle and the maximum potentiation of the responses to acetylcholine by HC-3 was 5% which was produced in the presence of 2 μg ml^{-1} (3·5μM) HC-3.

Choline uptake

The pre-junctional blocking action of HC-3 is thought to be due to its ability to prevent the transport of choline from the extracellular fluid to the intraneuronal sites of its acetylation (MacIntosh & others, 1956; Gardiner, 1961).

HC-3 is known to inhibit the uptake of choline into nerve ending particles (synaptosomes) *in vitro* (Hemsworth, Darmer & Bosmann, 1971; Simon, Mittag & Kuhar, 1975) and a comparison was made of the inhibition of the uptake of [^{14}C]choline into

synaptosomal fractions from rat brain by HC-3 and AcHC-3 at a final concentration of 10^{-5}M. AcHC-3 was shown to be as effective as HC-3 itself in this respect. AcHC-3, 10^{-5} M, inhibited choline uptake by 83%, compared with 80% inhibition by 10^{-5} M HC-3.

Inhibition of choline acetyltransferase (ChAc)

HC-3 has been shown to inhibit the synthesis of acetylcholine by organised nerve tissue (MacIntosh & others, 1956; Gardiner, 1961). In the present study AcHC-3 $2{\cdot}5 \times 10^{-3}$ M inhibited by 28% the synthesis of acetylcholine by mitochondrial fractions of guinea-pig brain. HC-3, at the same concentration caused the same % inhibition of acetylcholine synthesis. When the soluble ChAc enzyme extracted in Triton X-100 was used for the incubation with both HC-3 and with AcHC-3, no inhibition of ACh synthesis was obtained. These experiments suggest that the AcHC-3 is acting in a similar manner to HC-3 itself and preventing the transport of choline to the intracellular sites of its acetylation.

Effects on cholinesterase

AcHC-3 was examined as a possible substrate for ChE and AChE. Table 1 shows the results obtained using a null-point titration method for determining the amount of acid liberated on hydrolysis of either AcHC-3 or acetylcholine by the enzymes. AcHC-3 was hydrolysed to a small extent by both ChE and AChE but very much more slowly than acetylcholine.

DISCUSSION

The results demonstrate that AcHC-3 has an action of its own on neuromuscular transmission and this effect is not due to hydrolysis of the compound by cholinesterase to form the active HC-3 molecule.

Table 1. *Hydrolysis of acetylcholine and AcHC-3 by bovine erythrocyte AChE and horse serum ChE.* Determinations were made using a pH-stat titration procedure as described in Methods. The final concentration of substrate, acetylcholine or AcHC-3, in the incubation system was 10^{-2} M. Bovine erythrocyte AChE and horse serum ChE were both made up in a concentration of 0·5 mg ml^{-1}. The figures show the μmol of substrate hydrolysed min^{-1} ml^{-1} enzyme.

Enzyme	Acetylcholine	AcHC-3
Bovine erythrocyte	56·4	2·8
Horse serum	95·5	0·9

AcHC-3 was shown to affect neuromuscular transmission by an action at both the pre- and the post-junctional site.

Bowman & others (1967) proposed a number of tests for examining the pre- and post-junctional blocking action of a drug at the neuromuscular junction. The rat phrenic nerve-diaphragm muscle is one such preparation. On this preparation, AcHC-3 was shown to produce a pre-junctional blocking action, but it was less effective than HC-3 (see Bowman & others, 1967). Because it has been shown that HC-3 has multiple sites of action at the neuromuscular junction of the rat diaphragm (Tagaki, Kojima & others, 1970), other preparations must be used to confirm the extent of pre- and post-junctional block. Marshall (1969) demonstrated that the chick biventer cervicis muscle was more sensitive than mammalian muscle to any post-junctional (curare-like) blocking action. This preparation illustrated that AcHC-3 was more effective than HC-3 in its post-junctional blocking effect.

The frog rectus abdominis muscle is also a useful preparation for determining the competitive or the depolarizing post-junctional activity of a drug. AcHC-3 did not show any marked depolarizing activity and did not elicit a contracture of the muscle, however, the responses to acetylcholine were potentiated by low doses of AcHC-3. The potentiation of the responses to acetylcholine by AcHC-3 were much greater than the effect of HC-3 which has been shown to be due to its anticholinesterase activity (Hemsworth, 1971b).

HC-3 affects acetylcholine synthesis and release by inhibiting the transport of choline from the extracellular fluid to the intraneuronal site of its acetylation (MacIntosh & others, 1956; Gardiner, 1961). In the present experiments, AcHC-3 was as effective in this respect as HC-3, demonstrating that the addition of two acetyl groups does not reduce the affinity of the drug for the choline transport site. Hemsworth & others (1971) studied the affinity of the choline uptake system and found that the transport of choline into synaptosomes could be influenced by many compounds which affect cholinergic transmission, although there did not appear to be any specific structural requirement for inhibition of choline incorporation. However, from the work of Snyder, Yamamura & others (1973), the incubation time used by Hemsworth & others (1971) was too long and it is highly likely that rupture of the synaptosomes occurred during their incubation process.

de Balbian Verster & others (1968) reported that

AcHC-3 was an inhibitor of ChAc which was non-competitive for choline. More recently Domino & others (1973) found AcHC-3 to inhibit their crude ChAc enzyme preparation by a mixed inhibition process (Webb, 1963) although they found HC-3 to be ineffective at the same concentrations. Also, these investigators stated that their results should be regarded as preliminary because of their use of unpurified acetyl CoA.

The present results showed that although AcHC-3 inhibited acetylcholine synthesis by synaptosomal fractions of guinea-pig brain, the compound had no inhibitory action on the soluble ChAc enzyme under the conditions used. This action of AcHC-3 is similar to those of other bis-quaternary compounds (Hemsworth, 1971a) and is similar to the effects of the triethyl analogue of choline on ether and non-ether treated fractions of rabbit brain (Bull & Hemsworth, 1965). Some differences in the specificity of ChAc towards different substrates and inhibitors have previously been reported (Burgen, Burke & Desbarats-Schonbaum, 1956; Dauterman & Mehrotra, 1963; Hemsworth & Morris, 1964) and it has been suggested that these effects may be caused by species differences, varying degrees of enzyme purification, different incubation conditions and varying substrate concentrations (Hemsworth & Smith, 1970). Bradshaw & Hemsworth (1976) have recently shown that the method of isolation of the products of enzymic acetylation produces differences in the values reported for the specificity of ChAc. It is highly likely that one or more of these factors accounts for the difference between the present results and those of de Balbian Verster & others (1968), and Domino & others (1973).

Schueler (1955) originally showed that the hemicholinium-like molecules formed a cyclic keto-hemiacetal structure; this cyclization was the basis for the term hemicholinium. Schueler also showed that minor changes in the cationic heads of HC-3-like molecules markedly reduced or abolished hemicholinium-like activity. Since that time, many workers have investigated the structure-activity relations for hemicholinium-like activity by altering both the cationic head and the biphenyl nucleus (Marshall & Long, 1959; Powers, Kruger & Schueler, 1962; Thampi, Domer & others, 1966; Benz & Long, 1967; Long, Evans & Wong, 1967; di Augustine & Haarstad, 1970). Different interpretations have been put forward resulting from the type of experiments performed, but the general conclusions were that the hemi-acetal structure is necessary for a potent hemcholinium-like action. di Augustine & Haarstad (1970) did observe some pharmacological activity with an open chain (seco) HC-3-like compound, but this activity was 200 times less than the corresponding closed-ring hemi-acetal compound.

The present data demonstrate the opposite of this conclusion in that the open-ring (seco) form of AcHC-3 is as active as HC-3 itself. This action of AcHC-3 is not due to its hydrolysis to HC-3 because the rate of hydrolysis by cholinesterase (Table 1) is so slow that the effect of the hydrolysis product in the action of AcHC-3 on cholinergic transmission must be insignificant.

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