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3-Hydroxy-*N*,*N*-dimethylpiperidinium: a precursor of a false cholinergic transmitter

B.A. Hemsworth, S.M. Shreeve* & G.B.A. Veitch**

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Sunderland Polytechnic, Sunderland, Tyne & Wear SR1 3SD, Department of Pharmacology*, The University of Texas, Health Science Centre at San Antonio, San Antonio, Texas 78284, U.S.A. and Department of Pharmacy**, University of Aston in Birmingham, Gosta Green, Birmingham B4 7ET

1 A cyclic choline analogue, 3-hydroxy-*N*,*N*-dimethylpiperidinium, has been shown to be transported into a crude preparation of synaptosomes by high and low affinity transport mechanisms. Under conditions favouring the high affinity system the synaptosomes metabolized approximately 50% of the accumulated analogue to 3-acetoxy-*N*,*N*-dimethylpiperidinium, which was detected by paper electrophoresis.

2 The phrenic nerve endplate region of a mouse hemidiaphragm accumulated 3-hydroxy-*N*,*N*-dimethylpiperidinium on nervous stimulation. This tissue metabolized approximately 60% of the accumulated analogue to the acetylated derivative which was released on nervous stimulation into the bathing medium.

3 Compared to acetylcholine, 3-acetoxy-*N*,*N*-dimethylpiperidinium was shown to be 57 times less potent an agonist at the nicotinic receptors of the frog rectus abdominis muscle and 162 times less potent an agonist at the muscarinic receptors of the guinea-pig ileum.

4 It is concluded that 3-hydroxy-*N*,*N*-dimethylpiperidinium is a precursor of a false cholinergic transmitter.

Introduction

A number of linear choline analogues have been shown to be precursors of false cholinergic transmitters (Collier *et al.*, 1976; 1977; Ilson *et al.*, 1977). Hemsworth *et al.* (1984) studied a series of cyclic choline analogues with regard to their action on cholinergic nerve terminals. 3-Hydroxy-*N*,*N*-dimethylpiperidinium (3-hydroxypiperidinium) was shown to cause a presynaptic block in the rat phrenic nerve-diaphragm preparation and was found to be acetylated *in vitro* by a partially purified extract of the enzyme acetyl coenzyme A: choline-*O*-acetyltransferase (E.C.2.3.1.6, choline acetyltransferase, ChAc) at a rate of 55%, compared to choline 100%. Of the cyclic analogues we had studied 3-hydroxypiperidinium showed the highest rate of acetylation and it was therefore thought of interest to investigate this compound further.

Methods

Preparation of 3-hydroxy-N-methyl-N-[^{14}C-methyl]-piperidinium iodide ([^{14}C]-3-hydroxypiperidinium)

3-Hydroxy-*N*-methylpiperidine (Aldrich Chemical Co., London, England) was methylated with [^{14}C-methyl] iodine, 55.6 mCi mmol^{-1} (Radiochemical Centre, Amersham, England). The product was purified by ion-exchange chromatography as described by Barker & Mittag (1975) and, as a result of this procedure, was in a solution containing 300 mM NaCl. [^{14}C]-3-hydroxypiperidinium iodide had a specific activity of 55.6 mCi mmol^{-1}. It was shown to be radiochemically and chemically pure by paper electrophoresis (Potter & Murphy, 1967) and t.l.c. (Barker & Mittag, 1975).

Preparation of 3-acetoxy-N,N-dimethylpiperidinium iodide (3-acetoxypiperidinium)

3-Hydroxy-*N*-methylpiperidine (Aldrich Chemical Co., London) was refluxed with acetyl chloride (BDH Chemicals, Ltd., Poole) in anhydrous ether in the presence of anhydrous pyridine. After cooling, the mixture was filtered and the precipitate was partitioned between chloroform and 10% sodium carbonate. The chloroform extract was dried over magnesium sulphate and the chloroform was then distilled off, leaving a brown oil (3-acetoxy-*N*-methylpiperidine). This residue was methylated by reacting with methyliodine (BDH Chemicals, Ltd., Pool) in anhydrous ether. The mixture was allowed to stand for two days. The precipitate was recrystallized once from ethanol and then twice from diacetone alcohol, to give colourless needles of 3-acetoxypiperidinium iodide ($C_9H_{18}INO_2$). Structure and purity were confirmed by i.r. and micro-analysis.

Transport of [^{14}C]-3-hydroxypiperidinium into synaptosomes

A crude preparation of synaptosomes (P_2 fraction of Gray & Whittaker, 1962) was prepared from rat forebrain. Synaptosomes from 30 mg of tissue were suspended in 1.0 ml of Krebs-Ringer phosphate buffer (KRP) (composition (mM) NaCl 175, KCl 4, glucose 10, $CaCl_2$ 1, $MgCl_2 6H_2O$ 2, sodium phosphate buffer 10, pH 7.4), containing [^{14}C]-3-hydroxypiperidinium (0.5 μCi) at the required concentration. The synaptosomes were incubated at 37°C (controls at 4°C), after which uptake was terminated by cooling the suspension in ice and subsequent centrifugation (2,000 *g* for 10 min). The supernatant was discarded and the resultant pellet was washed with KRP solution (5 ml) at room temperature. After a second centrifugation process, 200 μl of NaOH (1 mM) was added to the pellet which was then pipetted onto glass fibre papers. After drying at room temperature, any radioactivity on these papers was detected by using a toluene scintillation fluor and counting in a liquid scintillation spectrometer. In some experiments, choline and hemicholinium-3 (HC-3) were added to the KRP buffer and, in other experiments, the KRP buffer was modified such that NaCl was replaced by LiCl, on a molar basis.

In the experiments measuring the acetylation of 3-hydroxypiperidinium, the synaptosomes were incubated and uptake terminated, as previously described, but the pellet was washed with KRP buffer containing physostigmine sulphate (0.5 mM). After the final centrifugation the pellet was extracted, by following the general method of Barker & Mittag (1975), using 1 ml of 1:10 dilution of electrophoresis buffer containing physostigmine sulphate (0.5 mM).

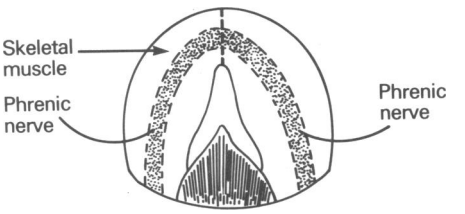

Figure 1 A diagramatic representation of a mouse diaphragm. Dots indicate the position of neuromuscular endplates seen with the aid of a light beneath the tissue. Dashed lines show where the diaphragm was cut.

The extract was freeze-dried and dissolved in 20 μl of methanol. The radioactively labelled compounds present in the extract were separated and identified by paper electrophoresis.

Mouse phrenic nerve-diaphragm preparation

The phrenic nerve endplate region was dissected out from a mouse hemidiaphragm (Figure 1) and was placed in a 10 ml glass beaker containing 5 ml of Krebs-physostigmine solution (composition (mM): NaCl 117.4, KCl 5.4, $MgSO_4 7H_2O$ 0.57, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.6, glucose 11.1, physostigmine sulphate 0.03). This solution was vigorously bubbled with 5% CO_2 in O_2 delivered from a fine glass pipette. The incubation medium was maintained at 37°C by mounting the beaker in a water bath. [^{14}C]-3-hydroxypiperidinium (55.6 μCi μmol^{-1}) was added to the beaker to make a final concentration of 36 μM. Due to the purification procedure, this compound is in a solution of 300 mM NaCl and therefore, the Krebs-physostigmine solution was modified so that the correct concentration of NaCl was maintained. The phrenic nerve was held just out of the incubation medium by a pair of fine platinum electrodes and the nerve was kept moist by the bubbling of the bathing fluid. Rectangular pulses of 0.1 ms duration were applied to the phrenic nerve at a voltage initially required for maximal muscle contraction. The nerve was stimulated at 1 Hz for 30 min in the presence of [^{14}C]-3-hydroxypiperidinium. The preparation was then washed three times with fresh Krebs-physostigmine solution and the tissue was finally incubated with 5 ml of this solution. The tissue was left at rest for 10 min and then stimulated, as described above, for 30 min. The tissue was then removed from the bath and extracted with a 1:10 dilution of electrophoresis buffer containing physostigmine sulphate (0.5 mM). This extract was freeze-dried. The final incubation medium was also freeze-dried. Sufficient methanol was added to the freeze-

dried residues and the radiolabelled compounds present in the extract were separated and identified by paper electrophoresis. Each experiment was conducted in duplicate.

Paper electrophoresis

Paper electrophoresis was used to identify the radiolabelled metabolites synthesized by synaptosomes and by the mouse phrenic nerve-diaphragm preparation. Non-radioactive 3-hydroxypiperidinium and 3-acetoxypiperidinium were added to the solutions containing the radiolabelled compounds to be separated and identified. Samples of such solutions were applied to the midline of the electrophoresis paper and allowed to dry. The compounds were then subjected to electrophoresis at 500 V for 2 h, on a V-type Durrum cell (Beckman), using 1.5 M acetic acid/0.75 M formic acid buffer, as described by Potter & Murphy (1967). After electrophoresis, the paper was stained by iodine vapour whilst barely damp and the position of any stained band was recorded. The iodine was allowed to evaporate off and, after drying, the electrophoresis paper was cut into 0.5 cm or 1 cm strips. The strips were placed in a scintillation vial containing toluene scintillation fluor, and any radioactivity on the strips was detected and estimated by liquid scintillation spectrometry.

Frog rectus abdominis muscle

The frog rectus abdominis muscle was suspended in frog-Ringer solution (composition (mM): NaCl 111, KCl 1.9, $CaCl_2$ 1.1, NaH_2PO_4 0.1, $NaHCO_3$ 4.8, glucose 11) which was equilibrated with air at room temperature (20°C). 3-Acetoxypiperidinium or acetylcholine chloride (ACh) was added at 4 min intervals and left in contact with the muscle for 30 s. The preparation was then washed twice with fresh frog-Ringer solution and allowed to relax to its baseline position. Contractions were recorded isometrically by means of a force displacement transducer and a Washington recorder.

Guinea-pig ileum

The terminal portion of the ileum, 2–4 cm in length, was removed and suspended in Tyrode solution (composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 5.6) which was equilibrated with air at 37°C. 3-Acetoxypiperidinium or ACh was added to the bath at 2 min intervals and left in contact with the tissue for 30 s. The preparation was then washed twice with fresh Tyrode solution. Contractions were recorded isotonically, using a Washington recorder.

Cholinesterase determination

The de-esterification of 3-acetoxypiperidinium by cholinesterase enzymes was estimated *in vitro* by a null-point titration method, using a Radiometer (Copenhagen) pH-Stat Unit (Burette Unit ABU12; Titration Assembly TTA60; Titrator TTT2; pH Meter PHM22 and Recorder SBR3) with a 25 ml water-jacketed reaction chamber for automatic titration of N/200 NaOH from the 25 ml Burette. The temperature was maintained at 37°C. Acetylcholinesterase (AChE, acetylcholine hydrolase, E.C. 3.1.1.7. obtained from bovine erythrocytes, 2.6 units mg^{-1} of solid) and cholinesterase (ChE, Type IV, obtained from horse serum, 21.3 units mg^{-1} of solid) were obtained from Sigma Chemical Co., London. Each enzyme was made up in distilled water to give a concentration of 1 mg protein ml^{-1} and 0.2 ml of one of these solutions was added to 6 ml of a modified Ringer solution (composition (mM): NaCl 153.7, KCl 4.02, $CaCl_2$ 3.31). Sufficient water was then added so that the final incubation volume (after the addition of the substrate) was 10 ml. The incibation system was left for 5 min to equilibrate to 37°C and the pH was adjusted to pH 7.4. 3-Acetoxypiperidinium was then added and the hydrolysis of the compound was followed for 10 min. Various substrate concentrations were used and blank values were obtained using denatured enzymes. Calculations concerning the absolute rates of hydrolysis were determined using the linear portion of the time/rate of hydrolysis curve.

Protein determination

Protein was determined by the method of Lowry, *et al.* (1951).

The piperidinium compounds are the iodide salts and are referred to as such throughout the text.

Results

Transport of [^{14}C]-3-hydroxypiperidinium into synaptosomes

The amount of 3-hydroxypiperidinium accumulated by the synaptosomes was calculated by taking into consideration the specific activity of the [^{14}C]-3-hydroxypiperidinium at the various concentrations used. The accumulation of 3-hydroxypiperidinium at 37°C was shown to be linear for 7 min for the range of concentrations chosen. The transport of [^{14}C]-3-hydroxypiperidinium into synaptosomes was subsequently expressed as μmol of [^{14}C]-analogue accumulated g^{-1} of protein 7 min^{-1}. The accumulation of 3-hydroxypiperidinium by synaptosomes at

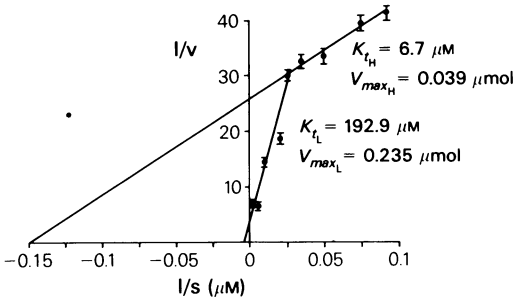

Figure 2 Double-reciprocal plot of the transport of [^{14}C]-3-hydroxypiperidinium into synaptosomes. Each point is the mean of at least four determinations (± s.e.mean). K_t is that concentration of substrate which provides one-half the maximum velocity uptake and was calculated for the high affinity (H) and low affinity (L) transport systems, using Michaelis-Menten kinetics. V_{max} is expressed as μmol of [^{14}C]-3-hydroxypiperidinium accumulated g^{-1} of protein in 7 min. Synaptosomes were incubated at 37°C for 7 min.

37°C was about three times that at 4°C. Values obtained at 4°C were subtracted from the values obtained at 37°C.

A Lineweaver-Burk (1934) plot was drawn (Figure 2). The curve could be resolved into two distinct components. Lower concentrations of 3-hydroxypiperidinium (11–33 μM) gave a straight line, with a correlation coefficient of 0.98, as calculated by regression analysis. This component presumably represents the high affinity transport of 3-hydroxypiperidinium into synaptosomes; the K_{tH} value (concentration of 3-hydroxypiperidinium which provides one-half the maximal rate of transport by the high affinity mechanism) was 6.7 μM and the V_{maxH} (maximum rate of transport by the high affinity mechanism) was 0.039 μmol g^{-1} of protein 7 min^{-1}. Higher concentrations (49–509 μM) gave the other component with a correlation coefficient of 0.99. This straight line represents the low affinity transport of 3-hydroxypiperidinium into synaptosomes; the K_{tL} value (concentration of 3-hydroxypiperidinium which provides one-half the maximal rate of transport of the low affinity mechanism) was 192.9 μM and the V_{maxL} (maximum rate of transport by the low affinity mechanism) was 0.235 μmol g^{-1} of protein 7 min^{-1}.

Studies with [^{3}H]-choline showed that its transport into a crude preparation of synaptosomes could also be resolved into high (K_{tH} = 3.5 μM, V_{maxH} = 0.13 μmol g^{-1} of protein 7 min^{-1}) and low (K_{tL} = 35 μM, V_{maxL} = 0.4 μmol g^{-1} of protein 7 min^{-1}) affinity components. These studies agree with the work of Yamamura & Snyder (1973).

The effects of HC-3 and choline on the proposed high and low affinity transport systems of 3-hydroxypiperidinium were determined at 11 μM and 49 μM, respectively (Table 1). HC-3 and choline both had a greater inhibitory action on the high affinity transport mechanism. The transport of 3-hydroxypiperidinium into synaptosomes was also studied at reduced sodium concentrations. The high affinity transport system (determined at 13 μM and 19 μM) was more sodium-dependent than the low affinity transport system (determined at 59 μM and 209 μM) (Table 2).

Identification of the radioactive materials accumulated by synaptosomes

After incubation of the synaptosomes with [^{14}C]-3-hydroxypiperidinium (11 μM), the synaptosomes were extracted and the compounds present in the extract were separated by paper electrophoresis. Two bands corresponding to 3-hydroxypiperidinium and 3-acetoxypiperidinium were visualized with iodine vapour. The electrophoresis paper was cut into 0.5 cm or 1 cm strips and any radioactivity on the strips was detected. Two peaks of radioactivity corresponding to the two bands previously visualized with iodine vapour were detected (Figure 3). It is concluded that [^{14}C]-3-hydroxypiperidinium is

Table 1 Inhibition of the transport of 3-hydroxypiperidinium into synaptosomes by choline and hemicholinium-3 (HC-3)

		[^{14}C]-3-hydroxypiperidinium accumulated at the following concentrations	
Inhibitor		11 μM	49 μM
Control (no inhibitor)		100%	100%
HC-3	2 μM	28%	60%
HC-3	100 μM	23%	25%
Choline	100 μM	0%	67%

Synaptosomes were incubated with [^{14}C]-3-hydroxypiperidinium at 37°C for 7 min. Values are the mean of four determinations, ± s.e.mean were less than 10%.

Table 2 Effect of sodium ion concentration on the transport of 3-hydroxypiperidinium into synaptosomes

Incubation conditions	*[^{14}C]-3-hydroxypiperidinium accumulated at the following concentrations*			
	13 μM	19 μM	59 μM	209 μM
NaCl (185 mM) (control)	100%	100%	100%	100%
NaCl (10 mM) and LiCl (175 mM)	53%	58%	89%	91%

Synaptosomes were incubated with [^{14}C]-3-hydroxypiperidinium at 37°C for 7 min. Values are the mean of four determinations, ± s.e.mean were less than 10%

metabolized to [^{14}C]-3-acetoxypiperidinium by synaptosomes. Apart from the acetylated derivative, no other metabolite was detected. Approximately 50% of the accumulated analogue was metabolized to the acetylated derivative.

Mouse phrenic nerve-diaphragm preparation

Potter (1970) showed that by incubating the rat phrenic nerve-diaphragm in a medium containing [^{14}C]-choline (30 μM) at 37°C, the synthesis, storage and release of [^{14}C]-ACh by this muscle could be studied. Since 3-hydroxypiperidinium is a choline analogue, it was thought of interest to incubate the phrenic nerve-diaphragm in a medium containing [^{14}C]-3-hydroxypiperidinium. In our laboratories, the mouse diaphragm is used routinely in electrophysiological experiments and, therefore, in order to be able to relate directly the biochemical data presented in this study with some proposed electrophysiological studies, it was decided to use the mouse phrenic nerve-diaphragm preparation in the present work, as opposed to the rat preparation used by Potter (1970). Potter (1970) showed that the diaphragm muscle accumulated choline from the incubation medium, and there is some evidence that ChAc is present in small amounts in skeletal muscle (Tucek, 1972; 1973). Therefore, to increase the likelihood that any acetylation of the 3-hydroxypiperidinium is affected by neuronal ChAc, only the nerve endplate region of the muscle was used.

The mouse phrenic nerve endplate region was preincubated for 30 min in Krebs-physostigmine solution containing [^{14}C]-3-hydroxypiperidinium and the nerve was electrically stimulated, as described in Methods. The tissue was then washed and placed in fresh Krebs-physostigmine solution and after a rest period of 10 min, the nerve was stimulated for a further 30 min. The Krebs-physostigmine medium

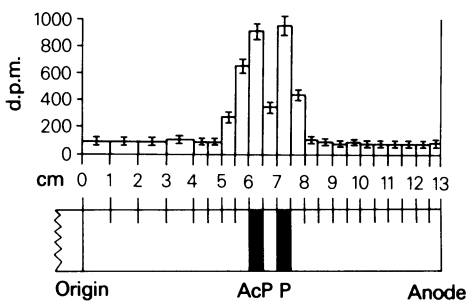

Figure 3 Paper electrophoresis of an extract from synaptosomes which had been previously incubated with [^{14}C]-3-hydroxypiperidinium (11 μM) at 37°C for 7 min. For each experiment, the contents of three incubating tubes were extracted and one-third of the pooled extracts were subjected to electrophoresis. The experiment was repeated three times. AcP and P show the position of nonlabelled 3-acetoxypiperidinium and 3-hydroxypiperidinium respectively on the electrophoresis paper and the bar graph above shows the radioactivity detected (± s.e.mean) on the corresponding paper strips cut from the electrophoresis paper.

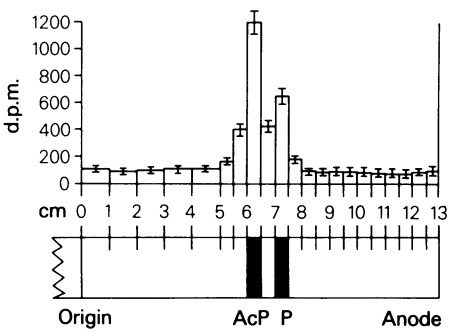

Figure 4 Paper electrophoresis of the Krebs-physostigmine solution which bathed the electrically stimulated mouse phrenic nerve-diaphragm preparation. This tissue had been pre-incubated with [^{14}C]-3-hydroxypiperidinium. One-third of the Krebs-physostigmine solution has been subjected to electrophoresis. The experiment was repeated six times. AcP and P show the position of the non-labelled 3-acetoxypiperidinium and 3-hydroxypiperidinium, respectively on the electrophoresis paper and the bar graph above shows the radioactivity detected (± s.e.mean) on the corresponding paper strips cut from the electrophoresis paper.

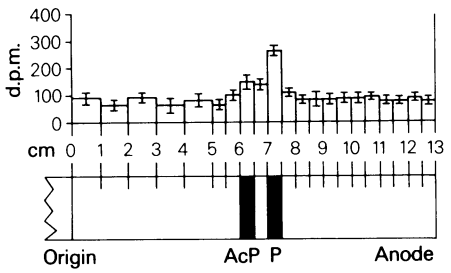

Figure 5 Paper electrophoresis of an extract from the electrically stimulated mouse phrenic nerve-diaphragm preparation which had been preincubated with [^{14}C]-3-hydroxypiperidinium. One-third of the extract has been subjected to electrophoresis. The experiment was repeated six times. AcP and P show the position of the non-labelled 3-acetoxypiperidinium and 3-hydroxypiperidinium, respectively on the electrophoresis paper and the bar graph above shows the radioactivity detected (±s.e.mean) on the corresponding paper strips cut from the electrophoresis paper.

bathing the diaphragm after this final period of nerve stimulation was found, by means of paper electrophoresis, to contain [^{14}C]-hydroxypiperidinium and [^{14}C]-3-acetoxypiperidinium (Figure 4). An extract of the diaphragm nerve-muscle tissue itself, after the final period of nerve stimulation, was also shown to contain both [^{14}C]-3-hydroxypiperidinium and [^{14}C]-3-acetoxypiperidinium (Figure 5). In both the tissue extract and the Krebs-physostigmine solution, there was no evidence of any other radiolabelled metabolite. Control experiments were conducted where the tissue was preincubated with [^{14}C]-3-hydroxypiperidinium and then incubated in fresh Krebs-physostigmine solution without any nervous stimulation. In these experiments no [^{14}C]-3-acetoxypiperidinium was detected in the Krebs solution bathing the preparation but [^{14}C]-3-hydroxypiperidinium was detected. The amount of [^{14}C]-3-hydroxypiperidinium released from the tissue was the same irrespective of whether the tissue was stimulated or not.

Figures 4 and 5 show the average results of six separate experiments, in which one phrenic nerve endplate region was dissected from a mouse hemidiaphragm, and at the end of the experiment, one-third of the tissue extract and one-third of the Krebs-physostigmine solution were subjected to electrophoresis. The specific activity of [^{14}C]-3-hydroxypiperidinium is 1 μCi 0.018 μmol^{-1}. Therefore, the total amount of both [^{14}C]-3-hydroxypiperidinium and [^{14}C]-acetoxypiperidinium in the tissue, as calculated from Figures 4 and 5, is 1.3 μmol g^{-1} tissue (wet weight). It can also be calculated from these figures that approximately 60% of the accumulated [^{14}C]-3-hydroxypiperidinium is acetylated, which is equal to 0.8 μmol g^{-1} tissue (wet weight). The amount of [^{14}C]-3-acetoxypiperidinium released from intracellular sites in the diaphragm, when there is no nervous stimulation, was zero, whereas the total amount of [^{14}C]-3-acetoxypiperidinium released from intracellular sites, when the phrenic nerve was stimulated, was 90% of the total [^{14}C]-acetoxypiperidinium present in the tissue.

Table 3 Hydrolysis of ACh and 3-acetoxypiperidinium by acetylcholinesterase (AChE) and choline esterase (ChE)

Substrate	K_m (μM)	V_{max}	*Enzyme*
ACh	0.56	9.09	AChE
	0.84	3.20	ChE
3-Acetoxypiperidinium	1.13	3.13	AChE
	0.90	2.50	ChE

Lineweaver-Burk (1934) plots of the reciprocal of the rate of hydrolysis against the reciprocal of the substrate concentration were calculated using regression analysis and K_m and V_{max} values were determined. V_{max} is the maximum rate of hydrolysis (number of mol of analogue hydrolysed by 1 mg protein in 1 min) at infinite substrate concentration

Pharmacological actions of 3-acetoxypiperidinium

3-Acetoxypiperidinium caused a contracture of the guinea-pig ileum and the frog rectus abdominis muscle. In both cases, the log dose-response curves were parallel to ACh. The equipotent molar ratios of this analogue, compared to ACh was 162 on the ileum and 57 on the rectus abdominis muscle. 3-Acetoxypiperidinium was a substrate for AChE and ChE, as shown in Table 3.

Discussion

Preliminary screening of 3-hydroxypiperidinium using the rat phrenic nerve-hemidiaphragm preparation (Hemsworth *et al.*, 1984) suggested that this choline analogue has a prejunctional blocking action at the neuromuscular junction. 3-Hydroxypiperidinium was shown to inhibit the high affinity transport of choline into synaptosomes and to act as a substrate for ChAc *in vitro*. Because of this *in vitro* acetylation, it was thought possible that 3-hydroxypiperidinium could be acetylated *in vivo* by the cholinergic neurone to 3-acetoxypiperidinium, which could then behave as a false transmitter. In

order for 3-hydroxypiperidinium to be acetylated *in vivo*, it must, like choline, be transported into the nerve terminal from the extracellular fluid to the intracellular sites of acetylation.

The results presented in this study show that radioactively labelled 3-hydroxypiperidinium was transported into synaptosomes. The transport could be resolved into both high and low affinity components. The high affinity transport was more dependent upon Na^+, and more sensitive to the inhibitory action of HC-3 than the low affinity transport. This transport process is similar to the transport of choline (Yamamura & Snyder, 1973) and of some of the other choline analogues which have been shown to be transported into synaptosomes (Barker & Mittag, 1975; Collier *et al.*, 1977). Collier *et al.* (1976) and Ilson *et al.* (1977) have shown that some N-ethyl analogues of choline, which have previously been extensively investigated for prejunctional blocking activity at cholinergic neurones (Bowman *et al.*, 1962; Hemsworth & Morris, 1964; Hemsworth & Smith, 1970; Luqmani & Richardson, 1982) are incorporated into the cat superior cervical ganglion and subsequently released as their acetylated derivatives.

Since choline and 3-hydroxypiperidinium antagonize each others transport into synaptosomes, it is possible that the high affinity mechanism for transporting 3-hydroxypiperidinium is the same mechanism as that responsible for the high affinity transport of choline into the cholinergic neurone. The apparent K_{tH} of the transport of 3-hydroxypiperidinium was about twice that of choline, and the maximum velocity of transport was 30% that of choline. Under conditions favouring the high affinity system the synaptosomes metabolized approximately 50% of the 3-hydroxypiperidinium accumulated to 3-acetoxypiperidinium.

The phrenic nerve endplate region of a mouse hemidiaphragm was shown to accumulate and release 3-hydroxypiperidinium. The rate of release of the parent choline analogue was not changed by nerve stimulation. This is similar to the efflux of choline from the rat phrenic nerve-diaphragm preparation, observed by Potter (1970).

We have also shown in the present experiments that the phrenic nerve endplate region of a mouse hemidiaphragm acetylated the accumulated 3-hydroxypiperidinium to 3-acetoxypiperidinium, which was then released upon electrical stimulation of the phrenic nerve as a false cholinergic transmitter.

Compared to ACh, 3-acetoxypiperidinium is a weak nicotinic agonist, and this inactivity would contribute to the presynaptic neuromuscular blocking properties of 3-hydroxypiperidinium. However, the primary reason for paralysis in the phrenic nerve-hemidiaphragm would be due to a lack of ACh in the neurones which would produce a reduced release of ACh on nerve stimulation rather than due to the presence of 3-acetoxypiperidinium in the nerve.

The present experiments illustrate that 3-hydroxypiperidinium fulfils the criteria for the compound to be a false inactive cholinergic neurotransmitter.

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