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The effect of choline mustard on the rat superior cervical ganglia

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1 The effect of choline mustard aziridinium ion (ChM) on the isolated superior cervical ganglion of the rat was investigated.

2 In the presence of ChM (22.5 μM), stimulation at 1 Hz resulted in a slowly developing blockade of transmission, which did not occur at a stimulus frequency of 0.1 Hz.

3 Choline (0.1 mM) slowed the onset of the blockade produced by stimulation at 1 Hz in the presence of ChM.

4 The presence of excess thiosulphate ions prevented the action of ChM on the transmission in the superior cervical ganglion.

5 Treatment of the ganglion with ChM (22.5 μM) only slightly inhibited the depolarization produced by carbachol (dose ratio 1.3), suggesting the drug produced a small degree of receptor blockade.

6 [^{3}H]-choline accumulation in the rat superior cervical ganglia displays several components: (a) sodium-dependent high affinity uptake (SDHAU) that can be activated further by preincubation in a high concentration of K^+ ions; (b) sodium-dependent low affinity uptake (SILAU); (c) linear diffusional accumulation which does not saturate.

7 Hemicholinium-3 selectively inhibits the activated sodium-dependent high affinity uptake, but is a weak inhibitor of resting sodium-dependent high affinity uptake and sodium-independent low affinity uptake.

8 ChM inhibits both activated and resting sodium-dependent high affinity uptake, but is a very weak inhibitor of sodium-independent low affinity uptake. Homocholine shows similar selectivity.

9 ChM inhibition of activated sodium-dependent high affinity uptake is very much more persistent than that of hemicholinium-3.

10 Hemicholinium-3 and ChM both inhibit [^{3}H]-acetylcholine synthesis.

Introduction

Nitrogen mustards have been shown to block irreversibly postsynaptic receptor sites and thus have been extensively used as research tools in the study of the mode of action of drugs at these sites (Graham, 1957; Gill & Rang, 1966; Vulvuis & Victor, 1980). It has also been found (Clement & Colhoun, 1975) that haloalkylamine derivatives of choline, such as choline mustard (ChM;I) and acetylcholine mustard (II) are capable of blocking transmission at the neuromuscular junction by acting presynaptically and it has been suggested that this action occurs by inhibition of acetylcholine (ACh) synthesis. Like other haloalkylamines these compounds appear to act after cyclization to the aziridinium ion (Graham, 1957; Gill & Rang, 1966).

Subsequently, Rylett & Colhoun (1977) investigated the effects of ChM on choline uptake and ACh synthesis in rat brain synaptosomes, and showed that it caused alkylation of the high affinity choline carrier which is involved in ACh synthesis. Further work by these authors (Rylett & Colhoun, 1980) has shown that ChM can also alkylate choline acetyltransferase, but that this occurs only in intact synaptosomes and not when the enzyme is studied in solution. They suggest that ChM is transported by the high affinity choline carrier system, and that coupling of choline

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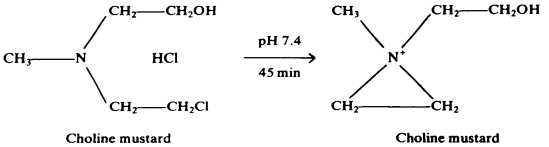
acetyl transferase to the carrier causes it to be alkylated by transported ChM much more readily than by free ChM.

Although a synaptosomal preparation can provide useful biochemical data it is unsuitable for comparing electrophysiological and biochemical changes in the same tissue. Thus we have investigated the biochemical and electrophysiological effects of choline mustard in the rat superior cervical ganglion, *in vitro*, and have found evidence that in this preparation choline mustard exerts a presynaptic action which is manifested by a reduction in the synthesis of ACh.

Methods

Male hooded rats (250–400 g) were anaesthetized with urethane (1.5 g kg^{-1}, i.p.) and the superior cervical ganglia with attached nerve trunks were desheathed and placed in Krebs solution maintained at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2 for 30 min. Each ganglion was subsequently placed in a triple chambered bath as described by Bowery & Tullet (1975). The preganglionic nerve was placed in contact with two platinum stimulating electrodes. Recording electrodes, consisting of non-polarizable Ag/AgCl in an agar bridge, were placed in the centre chamber (containing the ganglion body) and the third chamber (containing the postsynaptic nerve). The central chamber was perfused continuously with Krebs solution at 37°C, at a rate of 2 ml min^{-1}. Ganglionic action potentials elicited by supramaximal preganglionic nerve stimulation with 1 ms square pulses in the presence or absence of ChM were recorded with these electrodes and capacitor-coupled amplification (time constant 10 s). Depolarizing responses to agonists added to the solution flowing through the central chamber for 2 min, were recorded from the same electrodes, connected via a d.c. microelectrode amplifier to a chart recorder.

The composition of the Krebs solution was as follows (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11. The solution was bubbled with 95% O_2 and 5% CO_2 and its pH was 7.4. ChM, synthesized by Dr E.W. Gill, Department of Pharmacology, Oxford, was dissolved in Krebs solution at room temperature, 45 min before use, and then stored on ice. This allows cyclization to occur, thus forming the reactive aziridinium ion. The formation of the aziridinium ion was estimated by the method of Gill & Rang (1966).



Choline mustard

Choline mustard aziridinium ions (70% yield)

For metabolism and uptake studies, superior cervical ganglia were removed as described previously and preincubated for 30 min at 37°C in the following medium (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, HEPES 25, glucose 11, the pH was adjusted to 7.4 by adding NaOH (2M). If sodium-free medium was required NaCl was replaced with LiCl and the pH adjusted with 2M LiOH. These media were bubbled with O_2.

For choline uptake studies, ganglia were preincubated as described, and then incubated in the appropriate concentration of [^{3}H]-choline for 10 min unless otherwise stated. The ganglia were then washed for 1 min, blotted in filter paper and weighed 1 min later. The ganglia were dissolved overnight in 1 ml of NCS tissue solubilizer (Amersham) in plastic scintillation vials. The following day, the NCS was neutralized with 30 µl of glacial acetic acid to reduce chemiluminescence, and the amount of radioactivity in each sample was estimated by liquid scintillation counting, using the external standard method to correct for quenching. In some experiments the preincubation and/or the incubation medium contained added compounds.

For measurement of ACh synthesis, ganglia were removed and preincubated in the appropriate HEPES buffer for 30 min at 37°C as described previously, and then incubated in the appropriate concentration of [^{3}H]-choline for 10 min. The ganglia were then washed for 1 min, blotted on filter paper, and weighed 1 min later. After weighing, the ganglia were homogenized in 50 µl of formic acid: acetone (15:75 v/v), containing ACh (50 µg), and choline (50 µg) as markers. Subsequently 10 or 20 µl volumes of extract and 20 µg of phosphorylcholine (as marker), were subjected to high voltage paper electrophoresis (4 kV for 20 min) with acetic: formic acid buffer (1.5 M: 0.7 M, pH 2.0; Potter & Murphy, 1967). [^{3}H]-choline and its tritiated metabolites were stained with iodine vapour. The stained areas were marked, and cut from the paper and the stain was allowed to sublime. The radioactivity was eluted from the paper with water, and the amount of radioactivity present in each sample was estimated by liquid scintillation counting using the external standard method to correct for quenching.

Results

Effects of choline mustard on transmission

Prolonged exposure to ChM (22.5 M) caused a reduction in ganglionic transmission which was shown to be dependent on the frequency of indirect stimulation (Figure 1). As the frequency of stimulation was increased, the time required to obtain a given degree of

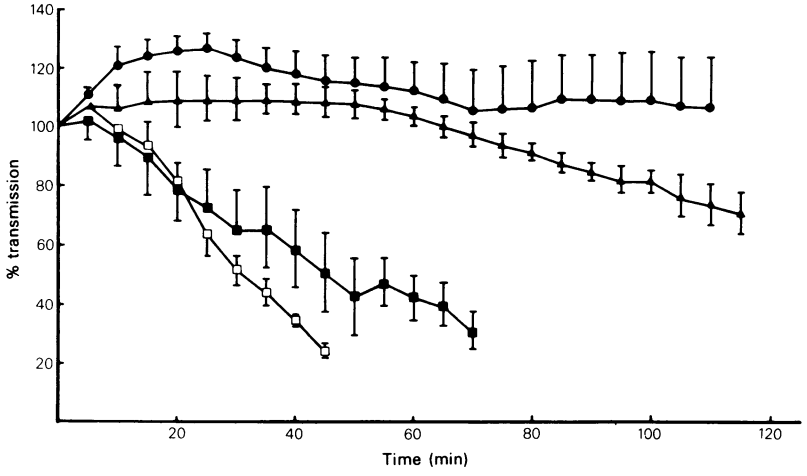


Figure 1 Effect of choline mustard (ChM, 22.5 μM) on the amplitude of the ganglionic action potential in response to supramaximal 2 Hz (□), 1 Hz (■), and 0.1 Hz (▲) preganglionic nerve stimulation. The effect of preganglionic nerve stimulation at 2 Hz(●) in the absence of ChM is also shown. Ordinate scale: % of initial action potential amplitude; Abscissa: time after adding the blocking agent. At least 4 ganglia were used to construct each curve. Vertical lines show s.e.mean.

ganglionic blockade was decreased. Stimulation at 1 Hz for 45 min resulted in a 50% reduction of the original action potential amplitude, whereas at 0.1 Hz no reduction occurred within 45 min and at 115 min there was only a 25% reduction in action potential height. Stimulating the ganglia at a frequency as high as 2 Hz for 110 min in the absence of ChM did not result in any significant reduction in action potential amplitude (Figure 1). When choline was included in the Krebs solution the blocking effect of ChM during stimulation at 1 Hz was retarded, though

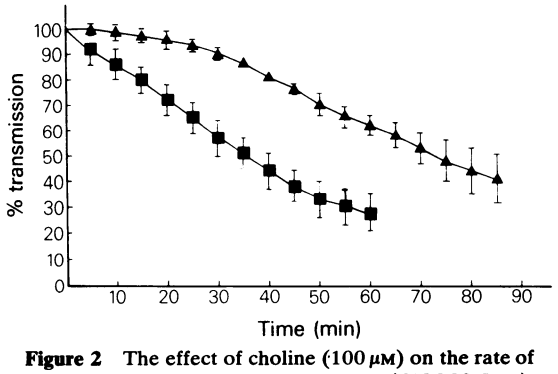


Figure 2 The effect of choline (100 μM) on the rate of blockade produced by choline mustard (ChM 22.5 μM). (■), ChM alone; (▲) ChM and choline. Stimulation frequency 1 Hz. Ordinate scale: % of initial action potential amplitude; abscissa scale: time after adding blocking agent. At least 4 ganglia were used to construct each curve. Vertical lines show s.e.mean. The times for the action potentials heights to reach 50% of their initial amplitude ($T_{\frac{1}{2}}$) were significantly different ($P < 0.02$).

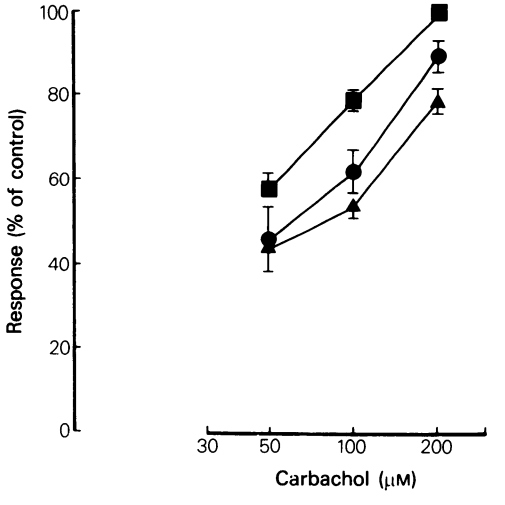


Figure 3 The effect of 30 min treatment of the ganglion with Krebs solution (●), and choline mustard (ChM (22.5 μM) (▲), on the control (■) log concentration-effect curves for carbachol. In all cases carbachol was applied for 2 min and the resulting depolarization was expressed as a percentage of the control response to 200 μM carbachol. Each point represents the mean of at least 4 determinations; vertical lines show s.e.mean.

not completely prevented (Figure 2). The blocking effect of ChM was also prevented by the addition of excess thiosulphate (× 10) to the stock choline mustard solution. Since thiosulphate reacts specifically with aziridinium ions, this result suggests that the blocking effect is produced by ChM itself and not by

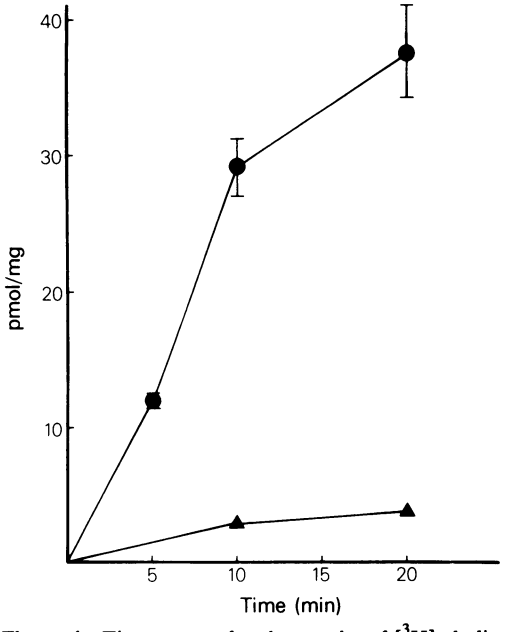


Figure 4 Time course for the uptake of [^{3}H]-choline (6.06 μM) by the resting rat superior cervical ganglia. (●) represents the total [^{3}H]-choline uptake at 37°C; (▲) represents the total [^{3}H]-choline uptake at 0°C. Each point is the mean of the uptake in at least 4 ganglia; vertical lines show s.e.mean.

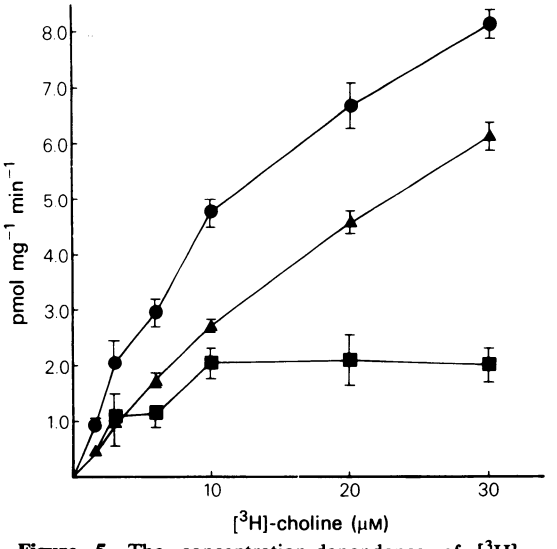


Figure 5 The concentration-dependence of [^{3}H]-choline uptake into resting unstimulated rat superior cervical ganglia: (●) represents uptake in the presence of the normal sodium ion concentration; (▲) represents uptake when all the sodium ions have been replaced by lithium ions; (■) represents the difference between the two uptakes. Each point is the mean of the uptake in at least 4 ganglia; vertical lines show s.e.mean. The ganglia were incubated for 10 min in the appropriate concentration of [^{3}H]-choline.

any of the other possible reaction products (Golumbic, Fruton & Bergmann, 1946). Once the blocking action of ChM had set in it was not reversed by the removal of ChM, by resting or by the addition of choline.

Effect of choline mustard on postsynaptic sensitivity

Dose-response curves to carbachol were shifted slightly to the right in a parallel manner (dose-ratio approximately 1.3) after treatment of the ganglia for 30 min with ChM (22.5 μM) (Figure 3). This very slight degree of postsynaptic block is insufficent to account for the effect of ChM on transmission, which is therefore assumed to depend on a presynaptic effect.

The uptake of [^{3}H]-choline by the superior cervical ganglia

Figure 4 shows the way in which the amount of [^{3}H]-choline taken up into isolated desheathed ganglia increased with incubation time. The uptake was measured at an external [^{3}H]-choline concentration of 6.06 μM. Uptake was approximately linear for 20 min.

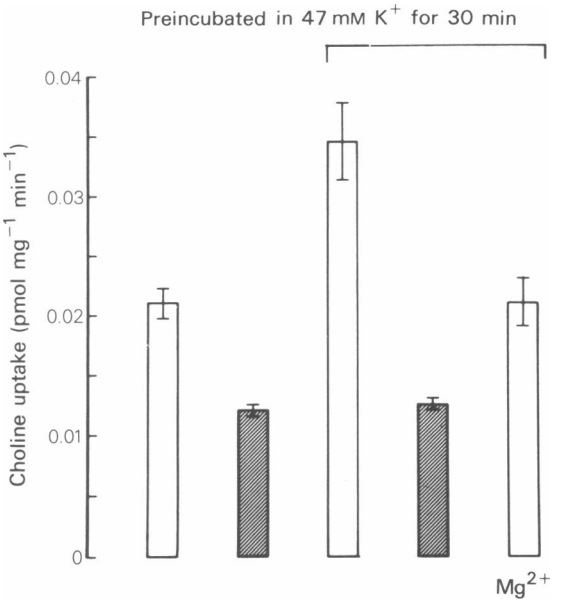


Figure 6 The effect of preincubation in a high potassium ion concentration on choline (0.03 μM) uptake. Some ganglia were preincubated and incubated in media where the sodium ions had been replaced by lithium ions (hatched columns). Other ganglia were preincubated in a medium where all the calcium ions had been replaced by magnesium ions and the potassium ion concentration raised to 47 mM (Mg^{2+}). Each column represents the mean ± s.e.mean of at least 5 determinations.

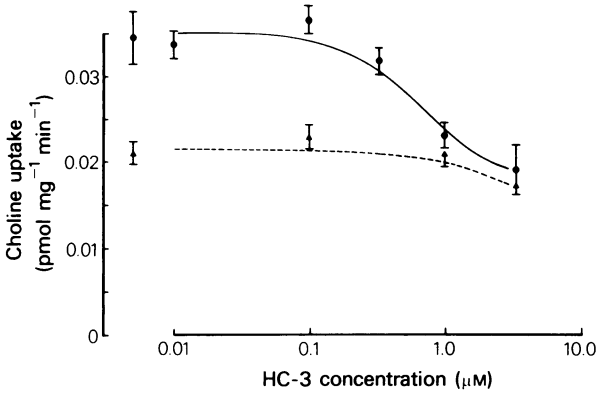


Figure 7 The concentration-dependence of the inhibition of the resting and potassium-activated high affinity choline (0.03 μM) uptake by hemicholinium-3. (▲) Inhibition of resting uptake; (●) inhibition of potassium-activated uptake. Sodium ions were present throughout the experiment. Each point represents the mean of the uptake in at least 4 ganglia; vertical lines show s.e.mean.

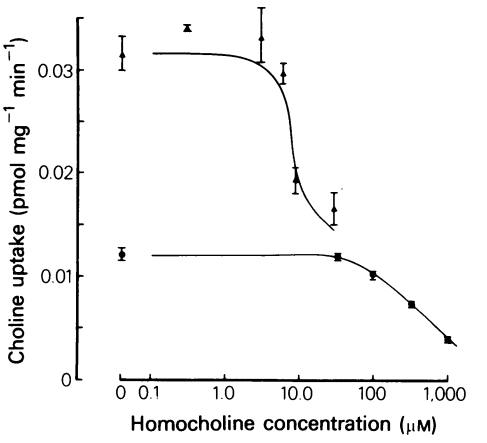


Figure 9 The concentration-dependence of the inhibition of SILAU and potassium-activated SDHAU by homocholine. (▲) Inhibition of potassium-activated SDHAU; (●) Inhibition of SILAU. The concentration of choline in the incubation medium was 0.03 μM. Each point is the mean of at least 4 determinations; vertical lines show s.e.mean.

In Figure 5 the two components, Na^+-dependent and Na^+-independent, are resolved. The lower curve which represents the difference between uptake in HEPES buffer containing Na^+ (upper curve) and the uptake in the absence of Na^+ (middle curve), shows the Na^+-dependent component as a function of [^{3}H]-choline concentration. This component saturates at approximately 10 μM, and at about 1 μM approximately 50% of the measured accumulation which has occurred reflects the sodium-dependent system. [^{3}H]-choline accumulation can be increased by preincubation for 30 min in HEPES buffer containing a high [K^+] concentration (47 mM). This procedure increases the [^{3}H]-choline uptake by approximately 70%. This increased accumulation does not occur in sodium-free medium, and is reduced in sodium-containing buffer if the Ca^{2+} ions are replaced by Mg^{2+} ions (Figure 6). The study of choline uptakes (10 μM–6 mM) in sodium-free buffer could be shown to contain two saturable components: a saturable process (K_t = 156 μM, V_{max} = 50.25 pmol mg^{-1} min^{-1}, sodium-independent low affinity uptake, SILAU) and a linear component.

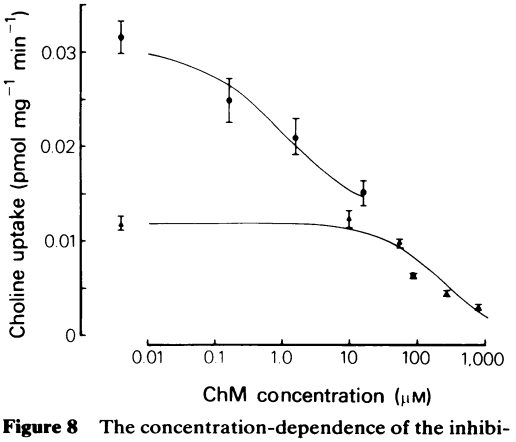


Figure 8 The concentration-dependence of the inhibition of SILAU and potassium activated SDHAU by choline mustard (ChM). (●) Inhibition of potassium-activated SDHAU; (▲) inhibition of SILAU. The choline concentration was 0.03 μM. Each point is the mean of at least 4 determinations; vertical lines show s.e.mean.

The effect of inhibitors on [^{3}H]-choline accumulation by the rat superior cervical ganglia

Hemicholinium-3 (HC-3) caused only very slight inhibition of resting choline uptake, whether or not sodium was present. However HC-3 showed considerably more potency against activated (preincubated in 47 mM [K^+] for 30 min before incubation) sodium-dependent high affinity uptake, (SDHAU), IC_{50} approximately 0.7 μM (Figure 7). This type of selective inhibition of activated (as opposed to resting) SDHAU by HC-3 has been demonstrated in rat brain slices by Polak, Molenaar & Van Gelder (1977). ChM was a potent inhibitor of both resting and activated SDHAU, IC_{50} = 1.0 μM, and thus lacked the discrimination of HC-3. In sodium-free medium ChM was a weak inhibitor of SILAU uptake, IC_{50} = 170 μM (Figure 8). Homocholine shows similar selectivity (Figure 9). The IC_{50} for homocholine against SDHAU is approximately 8 μM. In sodium-free medium homocholine was also a weak inhibitor of SILAU, IC_{50} = 380 μM. ChM blockade of activated SDHAU persists for at least 1 h whereas that of

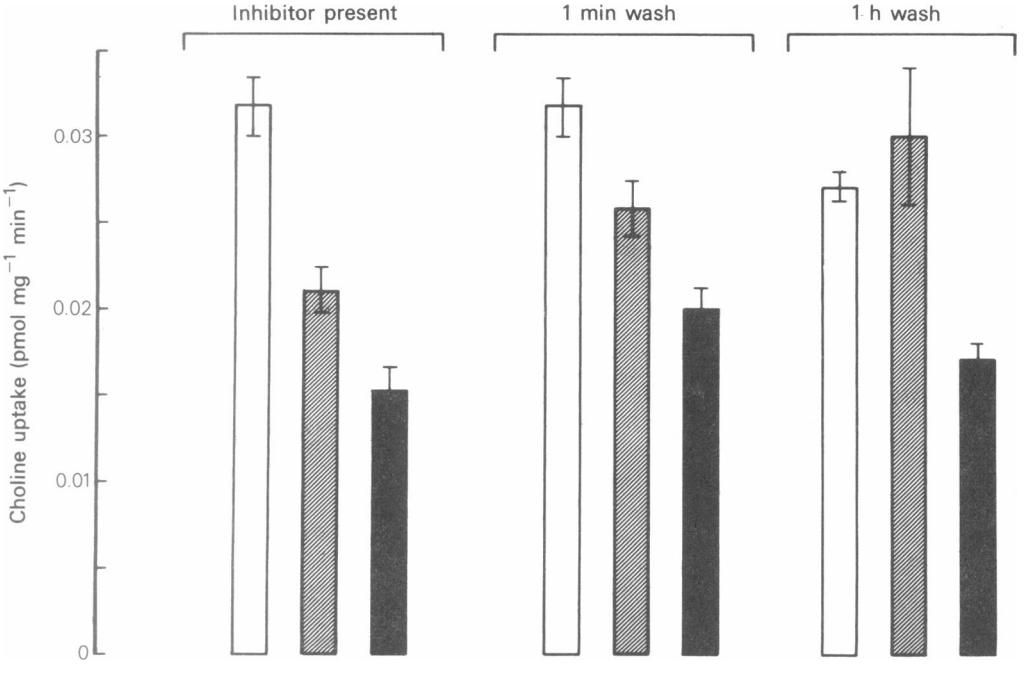


Figure 10 The reversibility of choline uptake inhibitors. Ganglia were preincubated in 47 mM potassium ions for 30 min (open columns); or preincubated in 47 mM potassium ions and HC-3 2 μM for 30 min (hatched columns); or preincubated in 47 mM potassium ions and choline mustard (ChM) 22.5 μM for 30 min (solid columns). Ganglia in the first three columns were incubated in normal fresh HEPES buffer containing the inhibitor that was present in the preincubation medium and [^{3}H]-choline (0.03 μM) for 10 min. Other ganglia were washed for various periods of time (1 min wash, or 1 h wash) to remove inhibitor, and then incubated in fresh normal HEPES buffer containing [^{3}H]-choline (0.03 μM) for 10 min. Each column is the mean of the uptake in at least 4 ganglia; vertical lines show s.e.mean.

HC-3 is rapidly reversed by washing the ganglia (Figure 10).

The effect of [^{3}H]-choline uptake inhibitors on [^{3}H]-choline metabolism.

Preincubation in high K^+ medium not only increases uptake but also increased [^{3}H]-acetylcholine synthesis. However, [^{3}H]-phosphorylcholine synthesis was reduced (Figure 11). HC-3, 1 μM, inhibited [^{3}H]-acetylcholine synthesis in K^+ activated ganglia, and also increased [^{3}H]-phosphorylcholine synthesis (Figure 11). ChM inhibited [^{3}H]-acetylcholine synthesis but unlike HC-3 did not increase the synthesis of [^{3}H]-phosphorylcholine.

The relationship between the transport of [^{3}H]-choline and its metabolites

It has been reported that a large proportion of the [^{3}H]-choline transported by the SDHAU is acetylated to [^{3}H]-acetylcholine (Yamamura & Snyder, 1973). However, in the ganglia, at choline concentrations that saturate the resting SDHAU (30 μM), only 34% of the [^{3}H]-choline transported by the resting SDHAU system is acetylated (Figure 12), and so there does not appear to be a strong coupling between resting SDHAU and ACh synthesis in the rat superior cervical ganglia.

Discussion

These results demonstrate that presynaptic stimulation of the rat superior cervical ganglion in the presence of choline mustard aziridinium ions causes a frequency-dependent blockade of transmission. These findings are qualitatively similar to those of Bowman & Rand (1961) for the actions of triethylcholine at the mammalian neuromuscular junction. However, ChM caused a more persistent block which was not reversed by the addition of choline once the blockade had set in, whereas the effects of triethylcholine were readily reversed by this procedure. However, addition of choline, during the period of stimulation markedly slowed the onset of blockade

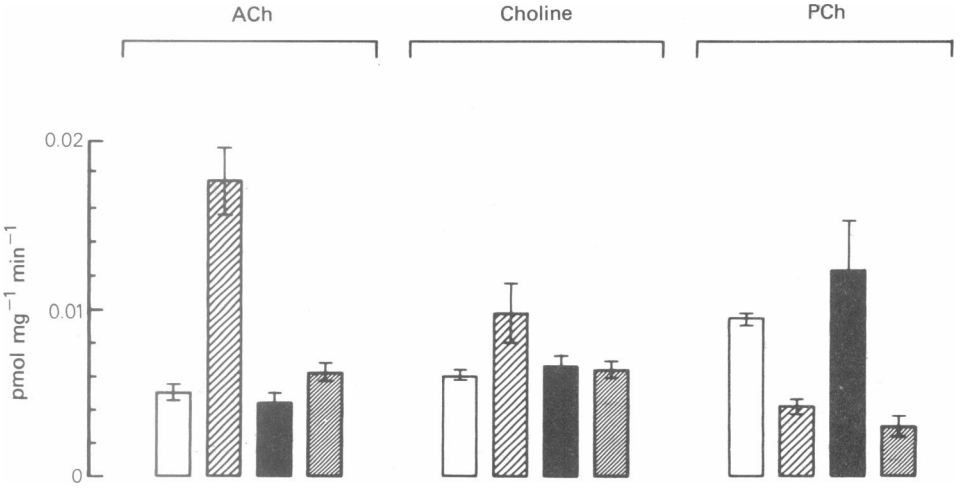


Figure 11 Ganglionic metabolism of [^{3}H]-choline (0.03 μM) under various conditions. For each metabolite: the first (open columns) represent the metabolism of [^{3}H]-choline by the resting unstimulated ganglia: the second (hatched columns) represent the metabolism of [^{3}H]-choline by ganglia that have been preincubated in 47 mM (30 min, 37°C) potassium ions and then transfered to normal sodium-containing HEPES buffer containing [^{3}H]-choline (0.03 μM) for 10 min: the third (solid column) represents the metabolism of [^{3}H]-choline by ganglia that have been preincubated in 47 mM potassium ions (30 min, 37°C) and then transfered to normal HEPES buffer containing [^{3}H]-choline (0.03 μM) and HC-3 1 μM for 10 min: the fourth (closely hatched column) represents the metabolism of [^{3}H]-choline by ganglia that have been preincubated in 47 mM potassium ions (30 min, 37°C) and transfered to normal HEPES buffer containing [^{3}H]-choline (0.03 μM) and ChM (16.2 μM). Each column is the mean of the uptake in at least 4 ganglia; vertical lines show s.e.mean.

by ChM. This latter observation is in agreement with the findings of Bowman & Rand (1961).

Studies by Clement & Colhoun (1975) of the effects of ChM on transmission in the rat phrenic nerve diaphragm preparation also demonstrated

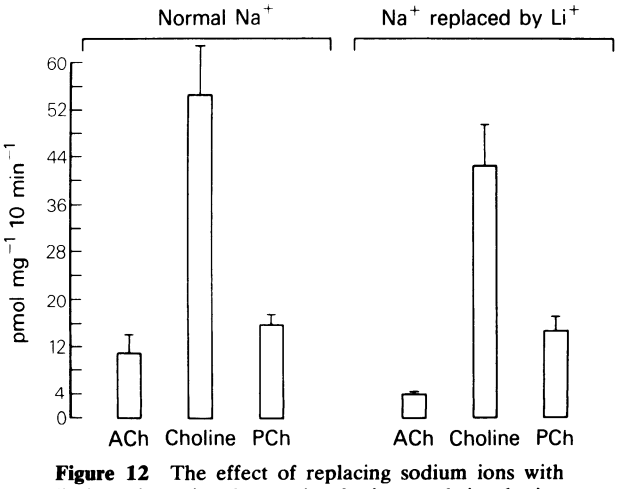


Figure 12 The effect of replacing sodium ions with lithium ions in the preincubation and incubation medium on the metabolism in the resting ganglia of [^{3}H]-choline (30.3 μM). Each column is the mean of at least 4 experiments; vertical lines show s.e.mean.

qualitively similar findings to those described here. Studies with carbachol demonstrate that postsynaptic receptor function in the ganglia which had been exposed to ChM appear to be virtually unimpaired and thus it is unlikely that the blockade of transmission produced by this substance is due to irreversible blockade of receptors or prolonged receptor desensitization. These results do not totally exclude the possibility that ChM may be blocking nerve conduction, though it is unlikely that such an effect would be influenced by choline concentration in the way that was found, so it is much more likely to be an effect on ACh release. The aziridinium ion appears to be the active chemical species as sodium thiosulphate (which reacts with aziridinium ions (Golumbic *et al.*, 1946) pretreatment of the mustard abolishes its ganglionic blocking activity. Transmission studies with ChM in the rat phrenic nerve diaphragm (Clement & Colhoun, 1975) and these studies in the superior cervical ganglion, showing a frequency-dependent blockade, which is antagonized by choline, suggest a presynaptic action, possibly via inhibition of ACh synthesis. Data obtained from studies on human erythrocytes (Clement & Colhoun, 1975) and synaptosomes from rat cerebral cortex (Rylett & Colhoun, 1980) and data presented in this paper support this view.

The inhibition of ACh synthesis from choline could

be due to the ChM inhibiting the high affinity transport of choline; an inhibition of choline acetyltransferase; or a decrease in release of ACh from nerve terminals. There is considerable evidence to suggest that ChM blocks the high affinity transport of choline into cholinergic nerve terminals. Clement & Colhoun (1975) showed that ChM could inhibit the influx of [^{3}H]-choline into red blood cells, and postulated that it may also inhibit the influx of [^{3}H]-choline into cholinergic nerve terminals. Subsequently, Rylett & Colhoun (1980) showed that ChM could inhibit the high affinity uptake of [^{3}H]-choline into rat cortical synaptosomes in a persistent manner ($K_i = 2.64\ \mu M$). The results described here show that ChM inhibits K^+-activated SDHAU into the rat ganglia, in a persistent manner ($IC_{50} = 1.0\ \mu M$). In fact, the blocking effect of ChM was not reversed by washing the ganglia for upto 1 h. Also, homocholine was found to be a good inhibitor of activated SDHAU ($IC_{50} = 8\ \mu M$) and this compares well with the value of 4.0 μM given by Simon, Mittag & Kuhar, 1975. These findings are important because SDHAU is thought to be closely associated with ACh synthesis in the cholinergic nerve terminals (Kuhar, Sethy, Roth & Aghajanian, 1973; Carroll & Buterbauch, 1975; Guyenet, Lesfrene, Rossier, Beaujouan & Glowinski, 1973 a,b; Sommachi & Kataoka, 1974).

Resting SDHAU in the rat superior cervical ganglia does not appear to be closely associated with ACh synthesis, because with a saturating concentration of [^{3}H]-choline (30.3 μM) only 34% of the sodium-dependent uptake is acetylated. This is at variance with what has been reported in synaptosomes (Yamamura & Synder, 1973). Also, in contrast to synaptosomes, this resting SDHAU is not very sensitive to HC-3 inhibition, although it is sensitive to ChM and homocholine inhibition. It would appear therefore that high affinity choline uptake in the ganglion does not reside in the nerve terminals and this may account for why denervation (Bowery & Neal, 1975) and HC-3 fail to affect it. However, K^+-activated SDHAU is highly associated with ACh synthesis as all the extra accumulated choline appears to be acetylated. Comparison of the K^+-activated increase in SDHAU (Figure 7) and K^+-activated increase in ACh synthesis in Figure 11, reveals that the increases are similar. K^+-activated high affinity uptake and the subsequent increase in [^{3}H]-acetylcholine synthesis, are also very sensitive to HC-3 and ChM inhibition. This is further evidence that the ganglion blocking activity of ChM is due to an inhibition of ACh synthesis.

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