

**THE ACTION OF CHOLINOMIMETIC SUBSTANCES ON IMPULSE
CONDUCTION IN THE HABENULOINTERPEDUNCULAR PATHWAY
OF THE RAT *IN VITRO***

BY D. A. BROWN, R. J. DOCHERTY* AND J. V. HALLIWELL

*From the Medical Research Council Neuropharmacology Research Group,
Department of Pharmacology, The School of Pharmacy, University of London,
29/39 Brunswick Square, London WC1N 1AX*

(Received 3 January 1984)

SUMMARY

1. The effects of some cholinomimetic substances and their antagonists on the peak height of compound action potentials recorded from the terminal region of the habenulointerpeduncular pathway have been studied using a rat brain slice preparation.

2. Carbachol and acetylcholine (ACh) depressed the peak height of the compound action potential and increased the latency to peak.

3. The nicotinic agonists nicotine and dimethylphenylpiperazinium depressed the peak height of the compound action potential while muscarine and glutamate had no effect.

4. The depressant effect of carbachol was blocked by the nicotinic antagonists hexamethonium, mecamylamine and *d*-tubocurarine but not by atropine.

5. Physostigmine enhanced the effects of ACh and, to a lesser extent, carbachol. In the presence of physostigmine, carbachol or ACh initiated a spontaneous oscillation of the amplitude of the compound action potential which was Ca²⁺ dependent and was blocked by mecamylamine.

6. It is concluded that depression of the amplitude of the compound action potential is due to activation of presynaptic nicotinic receptors. The results are discussed with reference to possible cholinergic mechanisms in the habenulointerpeduncular pathway.

INTRODUCTION

We have recently described the responses of neurones in the interpeduncular nucleus (i.p.n.) to stimulation of their afferent input in the fasciculus retroflexus of Meynert recorded in a transverse slice preparation of the rat brain (Brown, Docherty & Halliwell, 1983). These neurones were readily excited by nicotinic cholinomimetic drugs, applied either ionophoretically or by bath perfusion. During the course of these experiments we also noted that these drugs strikingly depressed the presynaptic action potential recorded within the i.p.n. We now report the results of some experiments on this presynaptic effect.

* To whom correspondence should be sent.

METHODS

Albino rats (150–500 g) of either sex were killed by cervical dislocation and their brains rapidly removed. A thin slice (400–600 μm) of brain containing the i.p.n. and all or some of the fasciculus retroflexus of Meynert was taken, excess cortical tissue was trimmed off, and the slice incubated in Krebs solution for at least 2 h at room temperature before being transferred to the recording chamber. The slice was kept totally submerged in a chamber constantly perfused with oxygenated Krebs solution at 2–5 ml min^{-1} at $30 \pm 1^\circ\text{C}$. (This temperature ensures adequate oxygenation of the Krebs solution to satisfy the metabolic demands of brain slices of this thickness: Harvey, Scholfield & Brown, 1974.) The Krebs solution used in both the recovery and the recording chambers had the following composition (in mequiv l^{-1}): Na^+ , 145; K^+ , 3; Ca^{2+} , 2.5; Mg^{2+} , 1.2; Cl^- , 125; HCO_3^- , 25; H_2PO_4^- , 1; D-glucose, 11 (pH = 7.4 when bubbled with 95% O_2 /5% CO_2). In some experiments Ca^{2+} in the Krebs solution was replaced with a high concentration of Mg^{2+} in order to reduce synaptic transmission. The composition of this 'Ca²⁺-free' Krebs solution was as above except (in mequiv l^{-1}): Ca^{2+} , 0; Mg^{2+} , 7; Cl^- , 137. Full details of the methods and equipment used for preparation and maintenance of the slice used in the present experiments have appeared elsewhere (Brown & Halliwell, 1981).

Electrical stimuli were delivered to afferent fibres in the fasciculus retroflexus using Teflon-coated platinum–iridium wire electrodes (over-all diameter = 35 μm) positioned on one or other fasciculus under visual control. Stimulation was monopolar, tip negative, constant-current stimuli (1–500 μA , 50–200 μs) being supplied by an optically coupled isolated stimulator (Neurolog, Digitimer Ltd., U.K.). Compound action potentials were recorded extracellularly using conventional glass micro-electrodes (d.c. resistance $\leq 5 \text{ M}\Omega$ when filled with 10% (w/v) NaCl), amplified (Neurolog, Digitimer Ltd., U.K.) and displayed on an oscilloscope (Tektronix, U.S.A.). Signals were further processed using a peak-height detector (Courtice, 1977) coupled to a chart recorder to give a continuous record of compound action potential amplitude.

Drugs were administered by addition to the Krebs solution perfusing the recording chamber or by iontophoresis. For bath application the perfusion was switched from a reservoir containing Krebs solution to one containing an identical solution plus the appropriate drug for a set period; usually 60 s for agonists. The 'dead-time' between the reservoir and the recording chamber was about 30 s. For iontophoresis a seven-barrelled micropipette (over-all tip diameter, 5–15 μm) was glued to the recording electrode with the tip of the recording electrode projecting 20–40 μm beyond the tip of the iontophoresis electrodes. Solutions for iontophoresis were made up in distilled water to a concentration of 0.1 M and the pH of the solutions adjusted to ~ 7 by adding NaOH. Drugs were administered by passing constant current down one or more barrels of the iontophoresis electrode, using automatic current balancing to minimize unwanted current effects. Capacitance in each barrel was neutralized by negative capacitance neutralization circuits.

Drugs used were as follows: acetylcholine chloride, atropine sulphate, carbachol chloride, L-glutamate, hexamethonium bromide, D,L-muscarine chloride, methacholine chloride, *α*-tubocurarine chloride, physostigmine (all Sigma), mecamlamine (MSD). α -Bungarotoxin was a generous gift from Dr J. O. Dolly, Department of Biochemistry, Imperial College, University of London.

RESULTS

Some of the features of afferent fibre volleys recorded in the i.p.n. have been described previously (Brown & Halliwell, 1981). In brief, unilateral stimulation of the fasciculus retroflexus evoked a predominantly negative, di- or tri-phasic extracellular field in the i.p.n. Sometimes two negative peaks could be recorded within the nucleus, as the incoming action potential traversed the nucleus back and forth, following the complex looping pathway of the afferent terminals within the nucleus. The conduction velocity in these non-myelinated terminal fibres is 0.2–0.3 m s^{-1} (Brown & Halliwell, 1981).

In the present experiments, the recording electrode was placed at a point within

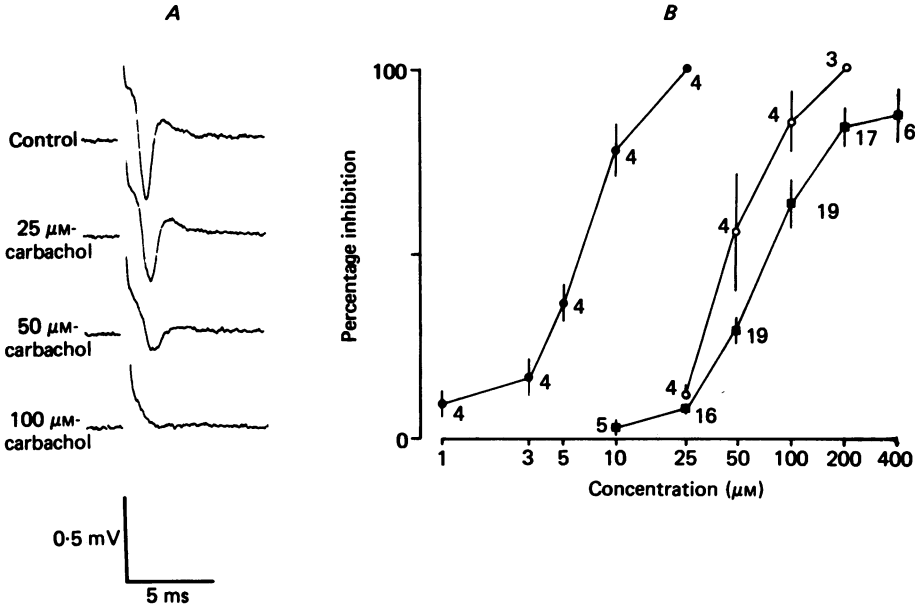


Fig. 1. *A*, compound action potentials recorded from the i.p.n. in normal Krebs solution (control) and then after adding 25, 50 and 100 μM-carbachol. *B*, mean dose-response curves for the depression of compound action potentials by carbachol (■—■), DMPP (○—○) and nicotine (●—●). The number of values averaged are indicated next to the appropriate points.

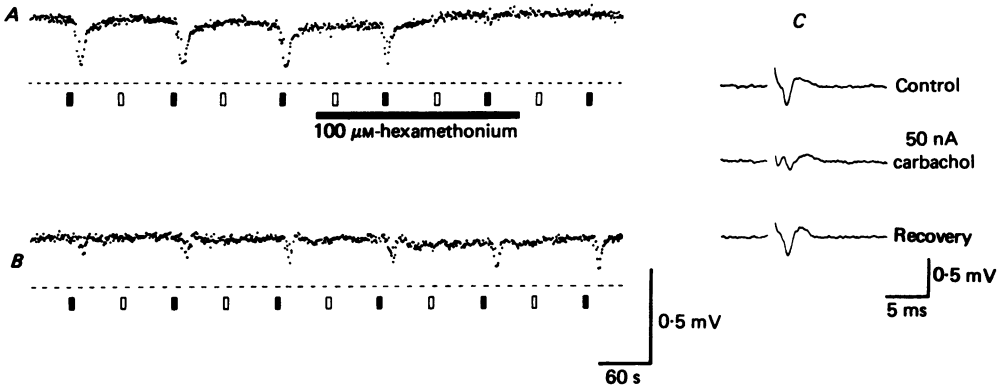


Fig. 2. *A* and *B* show a continuous record of the amplitude of compound action potentials evoked at a frequency of 0.2 Hz and recorded with a peak-height detector. (There is a 6 min break between traces *A* and *B*.) The dashed line indicates zero. Carbachol (50 nA; ■) and methacholine (200 nA; □) were injected ionophoretically from adjacent barrels of a micropipette for 10 s at the times indicated. Hexamethonium (100 μM) was added to the superfusate during the period indicated by the horizontal black bar. *C* shows typical individual compound action potentials from the same experiment recorded before (top), during (middle) and after (bottom) administration of carbachol.

the nucleus where only one negative peak in the compound action potential could be recorded to simplify analysis (see Fig. 1). Under control conditions compound action potentials evoked by low-frequency (≤ 2 Hz) stimulation of the fibre tract could be recorded at constant latency and amplitude for several hours.

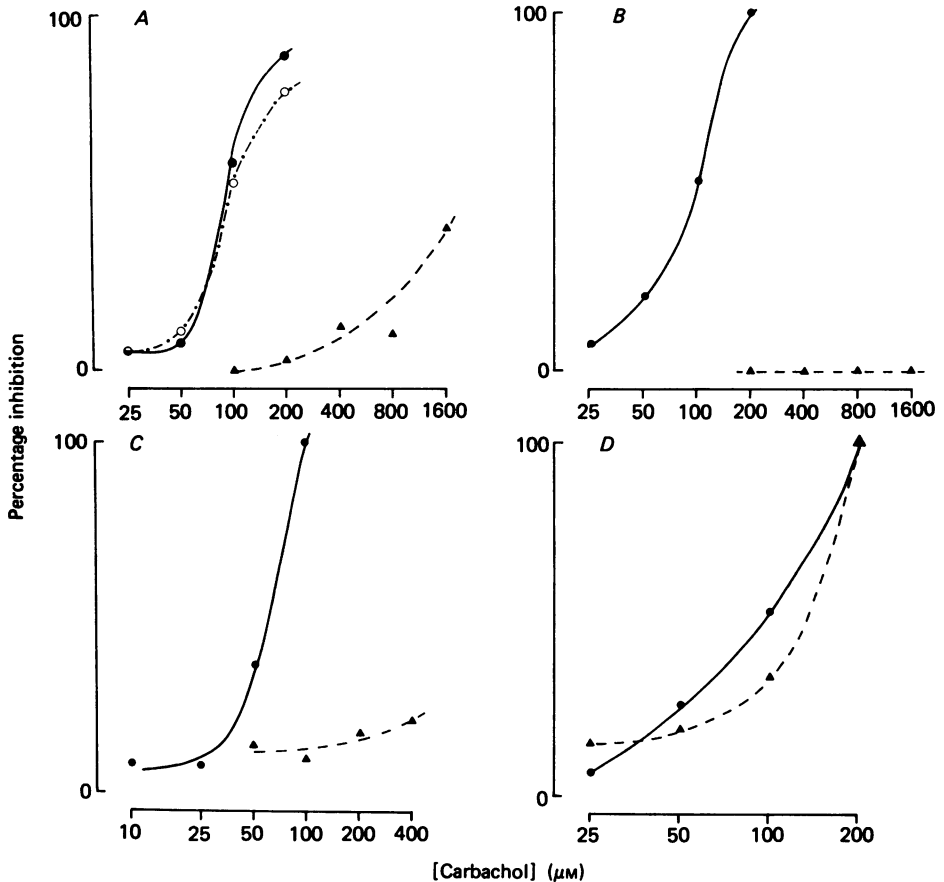


Fig. 3. Dose-response curves for the inhibition of compound action potentials by carbachol, plotted as in Fig. 1, before (●—●), during (▲---▲) and after (○-.-.-○) administration of *A*, hexamethonium ($5 \mu\text{M}$); *B*, mecamylamine ($1 \mu\text{M}$); *C*, *d*-tubocurarine ($100 \mu\text{M}$) and *D*, atropine ($0.1 \mu\text{M}$). Data are from four separate experiments.

Fig. 1 *A* illustrates the effect of *carbachol*, applied via the perfusing fluid, on this evoked presynaptic response. Carbachol produced a rapid (≤ 60 s), reversible and concentration-dependent reduction in the peak amplitude of the compound action potential and an increased latency to peak. The compound action potential was depressed by half at about $80 \mu\text{M}$ (Fig. 1 *B*).

In contrast to this striking effect on the non-myelinated terminal spike, carbachol produced minimal depression ($\leq 10\%$, $n = 3$, at concentrations up to $200 \mu\text{M}$) of the compound action potential recorded from the myelinated part of the fasciculus retroflexus before the fibres enter the i.p.n. This indicates that the reduced terminal

spike was not due to a reduced excitability of the fascicular fibres at the point of stimulation. In accord with this, carbachol also reduced the terminal compound action potential when applied ionophoretically (50–280 nA) into the i.p.n. itself, away from the myelinated part of the fibre tract (Fig. 2).

Since carbachol also excites i.p.n. neurones at comparable concentrations (Brown *et al.* 1983) depression of the afferent fibre spike might result from some form of

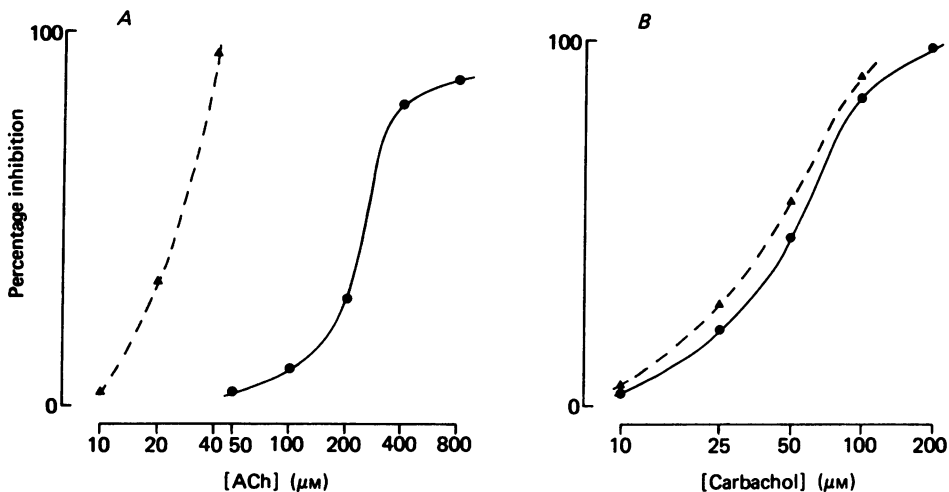


Fig. 4. Effect of physostigmine (1 μM) on the depression of the compound action potential by A, ACh and B, carbachol (two separate experiments). Graphs show dose-response curves for percentage compound action potential depression before (●—●) and after (▲---▲) adding physostigmine (1 μM).

chemically transmitted feed-back. This can be excluded by two observations. *First*, carbachol still reduced the afferent spike when transmission from fascicular fibres to i.p.n. neurones was blocked with a Ca^{2+} -free, 7 mM- Mg^{2+} solution (cf. Brown *et al.* 1983). *Secondly*, glutamate, which also excites i.p.n. neurones (Brown *et al.* 1983), did not depress the afferent spike at concentrations up to 10 mM.

The effect of carbachol was replicated by the nicotinic agonists *nicotine* (1–50 μM ; median effective dose (ED_{50}) 7 μM , $n = 4$) and *dimethylphenylpiperazinium* (DMPP, 25–200 μM ; ED_{50} 50 μM , $n = 4$; see Fig. 1B). In contrast, the muscarinic agonist, *muscarine* had no clear effect at concentrations up to 200 μM ($n = 2$). In the absence of anticholinesterase drugs, *acetylcholine* (ACh) produced a variable depressant effect at relatively high concentrations (≤ 0.1 mM; see Fig. 4). Physostigmine strongly potentiated the action of ACh (see below).

Elevation of external $[\text{K}^+]$ also reduced the terminal action potential amplitude. The action potential was abolished at 12 mM-external K^+ , i.e. four times the normal level.

ACh antagonists

The depressant action of carbachol was blocked by the ganglion-blocking drugs *hexamethonium* (1–100 μM ; $n = 5$; see Fig. 3A), *d-tubocurarine* (100 μM , $n = 3$; Fig. 3C) and *mecamylamine* (1–100 μM , $n = 5$; Fig. 3B). The effect of hexamethonium

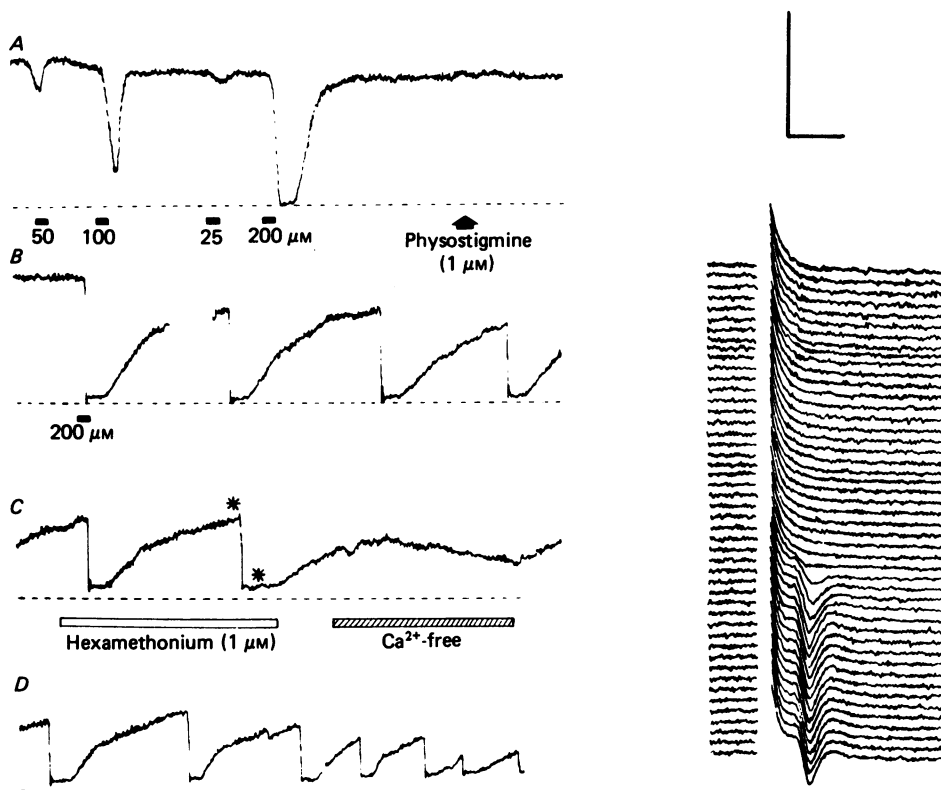


Fig. 5. The left-hand side of the Figure shows the output of a peak-height detector recording the amplitude of compound action potentials evoked at 0.2 Hz. Traces A–D are consecutive. The dashed line represents zero. Carbachol (25–200 μM) was administered at the horizontal black bars. Physostigmine (1 μM) was administered at the arrow in A. Hexamethonium (1 μM) and 'Ca²⁺-free' Krebs solution were administered at the open bar and hatched bar respectively. The right-hand side of the Figure is a raster display (starting at the bottom) of compound action potentials recorded every 5 s between the points marked with asterisks in C showing development of spontaneous loss of the compound action potential. The calibration bars (top right) represent 2 mV and 4 min for A–D and 1.5 mV and 2 ms for the raster display.

was readily reversible. The action of mecamylamine was more persistent in that no recovery could be detected after 90 min washing.

In contrast *atropine* had little effect at low (muscarine receptor blocking) concentrations ($\leq 0.1 \mu\text{M}$, $n = 3$); concentrations $\geq 0.1 \mu\text{M}$ appeared to produce some modest antagonism (Fig. 3D), which may reflect effects on nicotinic receptors.

α -*Bungarotoxin* (0.15 μM) had no effect when tested in one experiment (in which hexamethonium (10 μM) was effective).

Anticholinesterase effects

Physostigmine (1 μM) strongly potentiated the response to ACh (Fig. 4). This is to be expected since the i.p.n. contains high levels of cholinesterase (Lewis, Shute & Silver, 1967). However, two additional effects of physostigmine were noted.

First, it produced a small, but clear, potentiation of the action of carbachol. Secondly, a high dose of ACh or carbachol applied in the presence of physostigmine frequently initiated spontaneous 'oscillations' in spike amplitude with a cycle time of several minutes, in the manner shown in Fig. 5. This behaviour was prevented by replacing Ca^{2+} in the superfusate with 7 mM- Mg^{2+} (Fig. 5) or by adding mecamlamine (100 μM). Neither hexamethonium (100 μM) nor atropine (1 μM) appeared to modify this phenomenon.

DISCUSSION

The present experiments show that the non-myelinated terminals of the fasciculus retroflexus of Meynert in the interpeduncular nucleus possess ACh receptors, whose effect is to slow conduction and depress the amplitude of the compound (multifibre) action potential recorded within this nucleus. These receptors are clearly nicotinic, since they are activated by such nicotinic agonists as nicotine and DMPP, and are blocked by the nicotinic antagonists tubocurarine, hexamethonium and mecamlamine. This spectrum of drug sensitivity suggests a closer resemblance to the receptors in autonomic ganglia than to those at muscle end-plates, a conclusion supported by the apparent ineffectiveness of α -bungarotoxin, which is also ineffective on ganglion cells (see Brown, 1979).

These effects are reminiscent of previous observations on peripheral non-myelinated axons (Armett & Ritchie, 1960, 1961; Brown & Marsh, 1978) and peripheral sensory nerve endings (Brown & Gray, 1948; Douglas & Gray, 1953; Diamond, 1955), in which the effect of ACh is to depolarize the fibres. Depolarization may also underly the action potential block in the i.p.n. although we have no direct evidence for this; depolarization by modest concentrations of K^+ strongly reduced spike amplitude. However, there is one noteworthy difference from the response of peripheral fibres: physostigmine strongly potentiated the action of ACh on the i.p.n., instead of blocking it (cf. Armett & Ritchie, 1961).

The fascicular fibres proximal to the i.p.n. itself were relatively insensitive to the nicotinic agonists. This may, of course, relate to the fact that the afferent axons are myelinated, since peripheral myelinated axons are not blocked by such agents (Brown & Marsh, 1978). Nevertheless, not all non-myelinated terminals in the central nervous system are sensitive to ACh: for example, action potentials recorded from the terminal region of the lateral olfactory tract or Schaeffer collateral pathways in the hippocampus appear not to be depressed by quite high (≤ 0.5 mM) concentrations of carbachol (J. V. Halliwell & R. J. Docherty, unpublished observations). In this respect, therefore, the fascicular fibre terminals appear to be rather unusual.

Although direct transmission from fasciculus retroflexus fibres to interpeduncular neurones in this slice preparation may be mediated by an aspartate-like substance (Brown *et al.* 1983), biochemical and histological evidence suggests a strong cholinergic component to the fasciculus retroflexus (for references see Brown *et al.* 1983) and ACh release following activation of fascicular fibres (by stimulation of the habenular nuclei) has been demonstrated (Sastry, Ziolkowske, Hansen, Kavanagh & Evoy, 1979). This raises the question as to whether the nicotinic cholinergic receptors on the fibre terminals might be activated by ACh released from fascicular fibres when they are stimulated thereby producing a degree of feed-back inhibition. Some

evidence for this is yielded by our previous observation (Brown *et al.* 1983) that hexamethonium tended to enhance synaptic responses of interpeduncular neurones to stimulation of the fasciculus retroflexus, an effect which we previously regarded as 'non-specific', but which now could be interpreted as resulting from effects on presynaptic ACh receptors.

The present data suggest that inhibition of the compound action potential is due entirely to a presynaptic action of nicotinic cholinomimetics. However, previous work has shown that there is no clear interaction between the fibres of one fasciculus and those of the contralateral fasciculus at the presynaptic level (Brown & Halliwell, 1981), nor is there evidence for axo-axonic synapses in the i.p.n. One possible speculation we are left with would be an endogenous mechanism whereby ACh is released from fascicular fibres and acts on the fibres from which it is released (presumably causing depolarization) or by diffusion on neighbouring fibres. The importance of such a system in the normal function of the i.p.n. is difficult to assess but the biochemical evidence for an important physiological role for ACh in the i.p.n. is compelling. The peculiar oscillations in the amplitude of the action potential observed in the presence of physostigmine, which were blocked by mecamlamine, might then be regarded as a distortion of such an endogenous mechanism. None of this evidence is particularly conclusive, but suggests that further tests may be warranted.

On a more practical point: the sensitivity of the presynaptic fibres to nicotinic agonists was surprisingly high, at least as great as that of the i.p.n. neurones themselves (even when transmitted effects to the latter were blocked with Mg). This suggests some caution in attributing nicotinic effects in the central nervous system solely to the activation of post-synaptic receptors even when drugs are administered by iontophoresis.

Supported by a grant from the Medical Research Council.

REFERENCES

- ARMETT, C. J. & RITCHIE, J. M. (1960). The action of acetylcholine on conduction in mammalian non-myelinated fibres and its prevention by an anticholinesterase. *J. Physiol.* **152**, 141-158.
- ARMETT, C. J. & RITCHIE, J. M. (1961). The action of acetylcholine and some related substances on conduction in mammalian non-myelinated nerve fibres. *J. Physiol.* **155**, 372-384.
- BROWN, D. A. (1979). Neurotoxins and the ganglionic (C_6) type of nicotinic receptor. In *Advances in Cytopharmacology*, vol. 3, ed. CECCARELLI, B. & CLEMENTI, F. New York: Raven Press.
- BROWN, D. A., DOCHERTY, R. J. & HALLIWELL, J. V. (1983). Chemical transmission in the rat interpeduncular nucleus *in vitro*. *J. Physiol.* **341**, 655-670.
- BROWN, D. A. & HALLIWELL, J. V. (1981). An *in vitro* preparation of the diencephalic interpeduncular nucleus. In *Electrophysiology of Isolated CNS Preparations*, ed. KERKUT, G. A. & WHEAL, H. V., pp. 285-308. London: Academic Press.
- BROWN, D. A. & MARSH, S. (1978). Axonal GABA-receptors in mammalian peripheral nerve trunks. *Brain Res.* **156**, 187-191.
- BROWN, G. L. & GRAY, J. A. B. (1948). Some effects of nicotine-like substances and their relation to sensory nerve endings. *J. Physiol.* **107**, 306-317.
- COURTICE, C. (1977). A circuit for recording evoked action potential amplitudes. *J. Physiol.* **268**, 1-2P.
- DIAMOND, J. (1955). Observations on the excitation by acetylcholine and by pressure of sensory receptors in the cat's carotid sinus. *J. Physiol.* **130**, 513-532.

- DOUGLAS, W. W. & GRAY, J. A. B. (1953). The excitant action of acetylcholine and other substances on cutaneous sensory pathways and its prevention by hexamethonium and tubocurarine. *J. Physiol.* **118**, 373-383.
- HARVEY, J. A., SCHOLFIELD, C. N. & BROWN, D. A. (1974). Evoked surface positive potentials in isolated mammalian olfactory cortex. *Brain Res.* **76**, 235-245.
- LEWIS, P. R., SHUTE, C. C. D. & SILVER, A. (1967). Confirmation from choline acetylase analysis of a massive cholinergic innervation to the rat hippocampus. *J. Physiol.* **191**, 215-224.
- SASTRY, B. R., ZIALKOWSKE, S. E., HANSEN, L. M., KAVANAGH, J. P. & EVOY, E. M. (1979). Acetylcholine release in interpeduncular nucleus following stimulation of habenula. *Brain Res.* **164**, 334-337.