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# CHOLINERGIC MECHANISMS IN THE RAT SOMATOSENSORY CEREBRAL CORTEX

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## SUMMARY

1. The responses of identified cells in the rat cerebral cortex to cholinomimetic and anticholinergic substances has been investigated.

2. Acetylcholine and muscarinic agonists have an excitatory action on 80% of pyramidal tract cells. This response is found especially on cells responding to specific thalamic stimulation and the burst of spikes evoked from this site can sometimes be blocked by the iontophoresis of atropine. This strongly suggests an excitatory transmitter function for acetylcholine in a specific thalamocortical pathway.

3. Experiments on non-pyramidal tract cells have detected a muscarinic depression of some cells, and a nicotinic excitation of some cells above a depth of 600 $\mu$ in the cortex.

4. It is suggested that the increased release of acetylcholine from the cortex produced by atropine administration may be due to an excess of muscarinic inhibitory over excitatory synapses in the cortex.

## INTRODUCTION

A great deal of evidence has accumulated suggesting that acetylcholine is a synaptic transmitter in the cerebral cortex (Feldberg, 1945). Acetylcholine itself is present in synaptic vesicles (Whittaker & Sheridan, 1965) and the enzymes responsible for its synthesis and degradation, choline acetylase and acetylcholinesterase are present in the cerebral cortex (Burgen & Chipman, 1951; Feldberg & Vogt, 1948). Also topically applied cholinomimetic and anticholinergic drugs alter evoked potentials (Malcolm, Saraiva & Spear, 1967), and acetylcholine is released from the surface of the cerebral cortex in anaesthetized animals, this release being increased by stimulation of corticopetal pathways (Collier & Mitchell, 1966; MacIntosh & Oborin, 1953; Mitchell, 1963).

Further, when applied by microiontophoresis to the vicinity of single cells in the cerebral cortex acetylcholine has been reported to produce

excitatory (Krjnević & Phillis, 1963*a*) and depressant (Phillis & York, 1967*a*; Randić, Siminoff & Straughan, 1964) responses. The former effect is apparently mediated by receptors of the muscarinic type (Dale, 1914) and is confined to pyramidal tract cells, that is cells whose axons form part of the pyramidal tract.

Apart from this very limited knowledge of the action of acetylcholine, however, little effort has been directed towards the more precise identification of the synapses or pathways at which acetylcholine could function as a neurotransmitter. Mitchell (1966) has suggested the existence of two cholinergic systems in the central nervous system, one being a non-specific pathway from the reticular formation, mediating arousal, and the other a specific thalamocortical system. Certainly the reduction of acetylcholine release and of cortical cholineacetylase and acetylcholinesterase following undercutting of the cerebral cortex supports the idea of a subcortical origin for much cortical acetylcholine (Hebb, Krnjević & Silver, 1963; MacIntosh & Oborin, 1953). Krnjević & Phillis (1963*b*) have also reported blocking the late repetitive discharges of cortical cells following thalamic stimulation, by systemic atropine administration.

In the present study an attempt has been made to define functionally the type of cell responding to acetylcholine in the cerebral cortex. Acetylcholine and some cholinomimetic and anticholinergic drugs have been applied by microiontophoresis and the cells characterized in terms of their responses to various forms of afferent stimulation. Cells were classified as pyramidal tract or non-pyramidal tract cells and the former were further classified depending on whether or not they responded to stimulation in the thalamus (n. ventralis posterolateralis), the contralateral cortex, or local (direct) cortical stimulation. Experiments have also been performed to try and block the evoked activity of cells by the microiontophoretic application of anticholinergic substances.

In addition, the pharmacology of the acetylcholine inhibitory response is discussed, and a nicotinic excitatory response to acetylcholine on some superficial cells is described. Some of these results have been communicated briefly (Stone, 1972*a*).

## METHODS

Eighty-four male hooded rats were anaesthetized with Dial (Ciba; allobarbitone and urethane) 1 ml./kg, intraperitoneally, or urethane (25% solution) 5 ml./kg intraperitoneally, or halothane (Fluothane, I.C.I. 1·5% in oxygen) administered by a tracheal cannula after preparation under ether anaesthesia.

The preparation of animals and the procedure involved in stimulation of the pyramidal tract and the identification of pyramidal tract cells is described elsewhere (Stone, 1972*c*). The area studied was the forepaw area of the somatosensory cortex since this could be readily and reproducibly delineated by recording the response evoked by stimulation of the contralateral forepaw with a pair of needle electrodes.

For stimulation of the specific somatosensory relay nucleus of the thalamus, n. ventralis posterolateralis, a monopolar electrode was inserted stereotaxically at co-ordinates AP 3·0; L 3·0; V 5·5 according to the atlas of Fifkova & Marsala (1967). The position of the electrode was checked by recording the response evoked by stimulation of the contralateral forepaw (Hunt & O'Leary, 1957), and, in some preparations, by subsequent histology.

For local cortical stimulation (Krnjević, Randić & Straughan, 1966) a silver wire electrode was placed in contact with the cortical surface 2–4 mm from the site of micro-electrode penetration and the cortex was stimulated with four cathodal square wave pulses of 0·1 msec duration at a frequency of 10/sec.

Transcallosal stimulation was effected by a silver wire electrode resting on the contralateral cortical surface at a point where the evoked response following stimulation of the opposite forepaw was maximal. Single cathodal square wave pulses of 0·1 msec duration were used. Stimulation at all these sites was achieved via a Devices Digitimer Unit and Devices Stimulus Isolators.

*Iontophoresis and recording.* Five-barrelled micropipettes with over-all tip diameters of 4–8 $\mu$ and individual barrel resistances when filled with 1 M-KCl of 2–12 MΩ were used for microiontophoresis. The centre barrel was used for recording single cell spike activity and contained 1 M potassium acetate. From this a silver wire electrode led to a cathode follower and preamplifier and thence to D 43 Telequipment oscilloscopes for observation of the spikes. The signal on the display beam was then led through an emission follower and pulse-shaping circuit into a Devices Pen Recorder. The type of record produced by this system has been described by Cross & Silver (1966).

One outer barrel of the pipette always contained 0·2 M sodium chloride or bitartrate to enable the effect of current alone to be tested on cells. Current balancing was practised routinely, that is, a current was passed through the sodium chloride containing-barrel which balanced the currents being passed through the drug-containing barrels. The algebraic sum of current at the electrode tip was therefore always zero.

The barrels were filled by allowing drug solutions to diffuse into the tips of the water-filled pipettes for 24 hr. During this time the electrodes were kept in a closed container protected from light, so as to minimize drug oxidation.

Braking currents of 15 nA were applied to the drug solutions to reduce spontaneous effusion of drugs during experiments (Bradley & Candy, 1970). Control experiments for pH effects were also carried out using sodium chloride solutions adjusted to the pH of the drug solution. Although it has been suggested recently (Frederickson, Jordan & Phillis, 1971) that this procedure is inadequate as a control of pH effects, the evidence for this assertion has been disputed (Stone, 1972*e*).

The following drugs were used:

Acetylcholine chloride (Koch-Light; Halewood Chemicals) 0·2 M, pH 4·0; acetyl-$\beta$-methylcholine bromide (Koch-Light) 0·2 M, pH 4·0; atropine sulphate (BDH) 0·2 M, pH 4·5; nicotine bitartrate (BDH) 0·2–0·5 M, pH 3·5; D-tubocurarine chloride (Burroughs Wellcome) concentrated (65 mM) pH 4·5; carbachol chloride (BDH) 0·2 M, pH 4·5; pilocarpine nitrate (Patterson) 0·2 M, pH 5·0; physostigmine sulphate (McFarlane Smith) 0·2 M, pH 5·0; sodium L-glutamate (BDH) 0·2 M, pH 8·0; strychnine nitrate (Paterson) 20 mM, pH 5·5.

The depths of cells in the cortex were obtained from a micrometer gauge attached to the micromanipulator holding the multibarrel pipette.

The experimental technique consisted of detecting a pyramidal tract cell and noting its depth and latency of antidromic activation (Stone, 1972*c*) and then determining the responses of the cell to several drugs. The responsiveness of the cell to

local, transcallosal, and thalamic stimulation as described above was then investigated, and finally the effects on those responses of any antagonist drugs being used was tested. Only a proportion of cells, of course, were held long enough to be subjected to the whole of this programme.

Some of the problems involved in studying the pharmacology of single cells in the cerebral cortex have been discussed previously (Johnson, Roberts, Sobieszek & Straughan, 1969). In particular the necessity of obtaining clear and reproducible responses to agonist agents and of using fairly long dose cycles to reduce the tendency of some agents to show tachyphylaxis is mentioned in that paper, and attention has been paid to these points in the results to be described. Drug applications have also been made regularly spaced in time to prevent variation of response due to drug movement caused by the braking current (Salmoiraghi & Stephanis, 1965).

*Histology.* The heads of some animals were placed in formol saline for 48 hr to harden the soft tissues, after which the brain was removed and embedded in paraffin wax. Sections were then cut at 15 $\mu$ and stained by the method of Klüver & Barrera (1953). Results from experiments in which the medullary electrode was not accurately localized in the pyramidal tract were discarded.

## RESULTS

### *Pyramidal tract cells*

1673 pyramidal tract (PT) cells have been identified in eighty-four rats, and the distribution of their latencies of antidromic activation described elsewhere (Stone, 1972*c*). The results from 446 of these cells are relevant to the present report. All the cells studied were spontaneously active.

*Acetylcholine.* Applications of acetylcholine with iontophoretic currents of 20–80 nA caused excitation of 310 of 388 PT cells (80 %). No depressant responses (reduction of firing) have been seen on these cells. A typical response to acetylcholine is seen in Fig. 1. The excitation had a latency of up to 18 sec and in many instances persisted for up to 25 sec after ending the acetylcholine ejection. Furthermore, provided a period of 10–15 sec elapsed between successive applications, little tachyphylaxis was apparent. This contrasts with the marked tachyphylaxis which tends to occur with excitatory responses to several other amines (Phillis & Tebēcis, 1967; Roberts & Straughan, 1967).

*Cholinomimetics.* Physostigmine sulphate was ejected with currents of 50 nA for 1–5 min. The response to acetylcholine was almost invariably enhanced by this procedure (Fig. 3). The excitation produced by L-glutamate was unaffected by a similar application of eserine. Methacholine (acetyl-$\beta$-methylcholine bromide) was found to excite all those PTcells (fifty-six) on which it was tested which were also excited by acetylcholine, but to have no effect on cells which were unaffected by acetylcholine. The excitation produced by this substance was closely similar in latency and duration to that produced by acetylcholine (Fig. 1).

Carbachol (60 nA) was applied to twenty PT cells which were excited

by acetylcholine. On seventeen of these a clear excitation was produced as seen in Fig. 3. This effect had a latency of some 10–20 sec and often lasted over 90 sec. The drug had no action on five cells unaffected by acetylcholine. The potent muscarinic alkaloid pilocarpine produced prolonged effects very similar to those of carbachol (Fig. 3) and the two substances proved to be approximately equipotent.

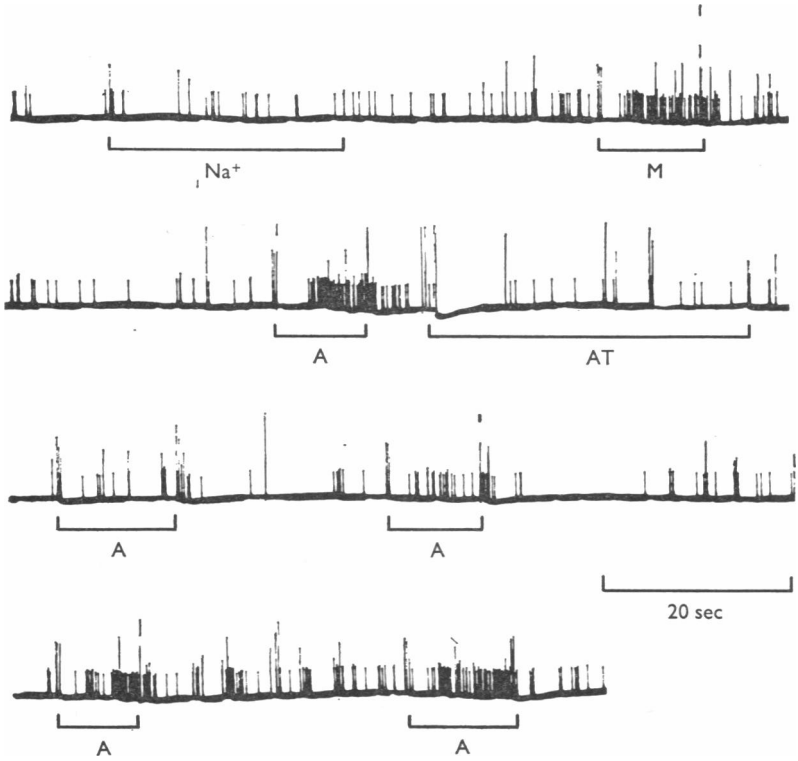


Fig. 1. Consecutive records showing excitatory responses of a pyramidal tract cell to 40 nA doses of acetylcholine (A) and methacholine (M) applied by microiontophoresis. Atropine, 40 nA (AT), blocks the acetylcholine response which then gradually reappears over the ensuing 90 sec. There is a slight reduction of firing rate produced by atropine (see Fig. 2). A control application of sodium ion ($Na^+$), also with a current of 40 nA, shows that the ejecting current itself has no effect on the cell.

Nicotine bitartrate had no effect on the PT cells studied (forty-eight) when ejected with currents varying from 15 to 150 nA.

*Anticholinergic agents.* D-tubocurarine (50–100 nA) had no readily interpretable effects itself on PT cells although slight and often transient changes of rate were occasionally seen, and it did not alter or block responses to any of the agonist agents described above.

Atropine (40–60 nA) or hyoscine (40 nA) readily blocked the excitant responses to acetylcholine or methacholine when applied for 30–90 sec. The resulting block lasted from 1 to 4 min; recovery of a normal acetylcholine response by the cell in Fig. 1, for example, took almost 90 sec. Continual oscilloscope monitoring of spike height and discharge was carried out to ensure that the effects of these drugs were not due to their direct depressant (local anaesthetic) actions as seen by previous workers (Krnjević & Phillis, 1963*b*) and as exemplified by the slight reduction of spontaneous firing rate following atropine in Fig. 1. The specificity of the

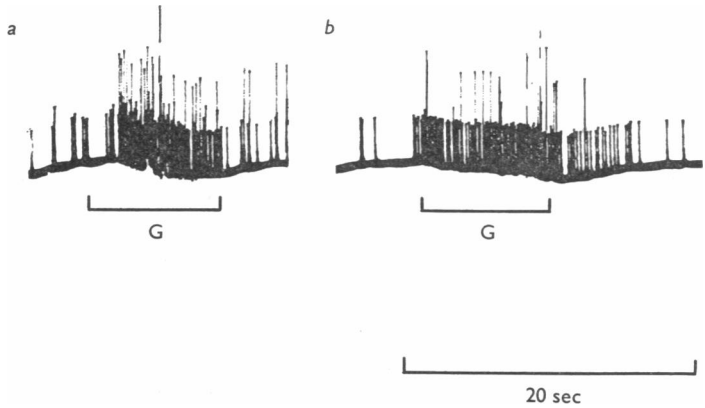


Fig. 2. Excitatory responses of a pyramidal tract cell to the iontophoresis of L-glutamate (G) with a current of 40 nA (*a*) before, and (*b*) immediately after the iontophoresis of atropine (40 nA) which causes a slight reduction of the spontaneous firing rate as in Fig. 1. The ability of the cell to respond to the excitant is unaffected.

block was also demonstrated by the absence of any effect of atropine on L-glutamate excitation (40 nA) while the spontaneous firing rate was reduced (Fig. 2) or on responses to noradrenaline, 5-hydroxytryptamine or dopamine (see Fig. 5 and Stone, 1972*d*) in doses which readily blocked the effects of acetylcholine and related agonists.

No changes of firing rate as marked as those of Krnjević & Phillis (1963*c*) were seen to result from atropine iontophoresis. This is probably due to the fact that much weaker solutions (0·2 M) were used in the present study, rather than the 3 M solutions of Krnjević & Phillis.

*Afferent stimulation.* In an attempt to define more precisely the type of PT cell responding to acetylcholine, the responses of these cells to local, transcallosal or thalamic stimulation was noted. There was no apparent correlation between the depression of cell firing induced by local cortical stimulation (Krnjević *et al.* 1966) or the cell firing evoked by transcallosal stimulation and the ability of cells to respond to acetylcholine. For

example, 74 % of PT cells depressed by local cortical stimulation, and 77 % of cells responding to transcallosal stimulation were excited by acetylcholine, compared with 80 % of the total PT cell population studied. By contrast almost all (96 %) of the PT cells excited by acetylcholine responded to specific thalamic stimulation (n. ventralis posterolateralis) with an early burst of action potentials (Fig. 4).

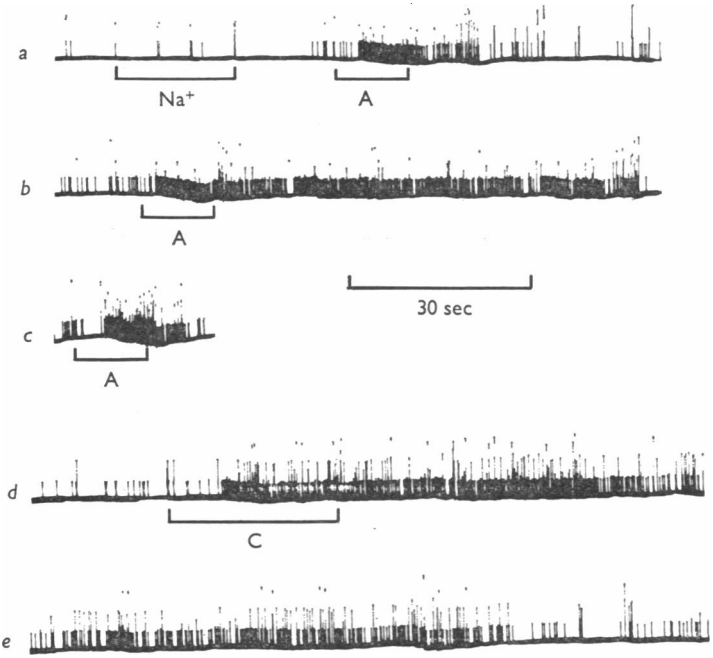


Fig. 3. (*a*). A 60 nA dose of acetylcholine (A) causes excitation of a pyramidal tract cell, whereas a control current of 60 nA is ineffective ($Na^+$).

(*b*) After the iontophoresis of eserine salicylate with a current of 40 nA for 2 min, the acetylcholine response is greatly potentiated in time.

(*c*) 5 min after *b*, the acetylcholine response has recovered almost to normal.

(*d* and *e*) are consecutive records showing an excitatory response to carbachol (60 nA). The effect lasted for several minutes on most cells.

Since the responses of PT cells are muscarinic in nature and are readily blocked by atropine as described earlier, several experiments were performed with the object of trying to block evoked responses of PT cells by the iontophoresis of atropine. Transcallosally evoked responses were unaffected, as were locally induced depressions of firing. Thalamically evoked bursts, however, were reversibly blocked on five of twenty-seven cells by atropine (60 nA) administered for 1 min (Fig. 4). Even such an apparently low incidence of success is encouraging, for the small size of the micro-

pipette tip compared with the relatively enormous expanse of PT cell surface area must make it unlikely that the atropine would reach those synapses activated during a particular form of synaptic stimulation. Only limited success was obtained also by Krnjević & Phillis (1963*b*) in blocking late thalamically evoked discharges of cells by iontophoretically applied atropine. It will be seen in Fig. 4 that the first two spikes are unaffected by

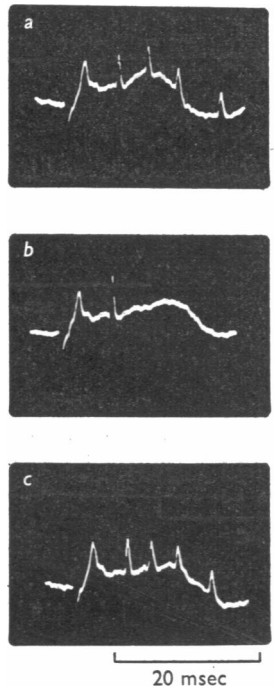


Fig. 4*a*. A burst of spikes, 450 $\mu$V in size, is elicited in a pyramidal tract cell by stimulation of the ventral posterolateral nucleus of the thalamus.

*b*. The latter part of this burst is blocked towards the end of a 30 sec application of atropine by microiontophoresis (current 60 nA).

*c*. Return of the whole burst 2 min after the end of the atropine application.

atropine. This may indicate that a transmitter other than acetylcholine is responsible for those spikes, or that the responsible synapses, if cholinergic, are situated at some distance from the electrode tip. Alternatively this phenomenon may be related to the presence of synaptic barriers preventing the efficient access of the blocking agent. Such barriers were proposed by Curtis & Eccles (1958) to explain the failure of cholinergic blocking agents to prevent the first few spikes evoked in a Renshaw cell following ventral root stimulation (Curtis & Eccles, 1958; Eccles, Fatt & Koketsu, 1954).

Nicotine and D-tubocurarine appeared to have no effect on PT cells' responses to afferent stimulation.

*Non-pyramidal tract cells*

Of 153 non-PT cells tested with cholinergic agents, 25 % were depressed by acetylcholine and the muscarinic agonists methacholine, carbachol and pilocarpine. The latencies of these actions were very similar to the latencies for excitation of PT cells. Acetylcholine and methacholine, for example,

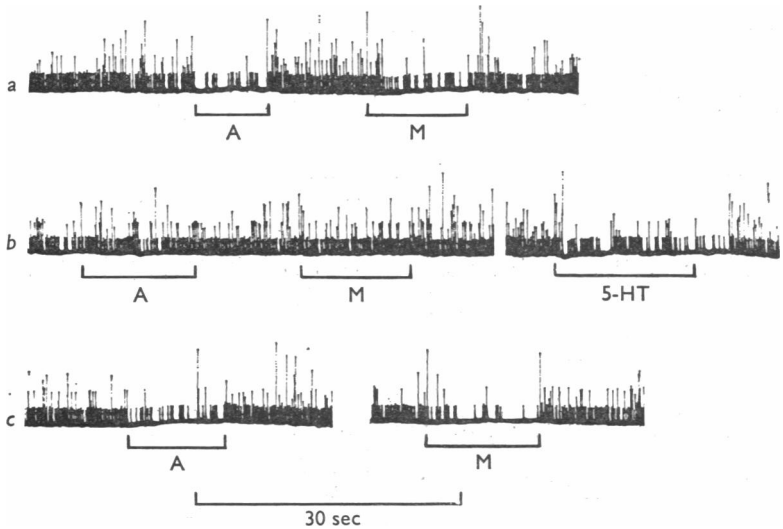


Fig. 5*a*. Depression of a non-pyramidal tract cell by acetylcholine 40 nA (A) and methacholine 40 nA (M). These responses are no longer obtainable in *b* after the iontophoresis of atropine (40 nA) for 30 sec, although a depressant response to 5-hydroxytryptamine, 40 nA (5-HT) is unaffected. Two minutes later, in *c*, the depressions are once more apparent.

produced depression of firing often within 10 sec and ending equally promptly (Fig. 5) whereas the effects of carbachol and pilocarpine often outlasted the iontophoretic ejection by several minutes. These depressant responses, which were usually encountered at cortical depths of 500–1600 $\mu$, could be reversibly antagonized by atropine or hyoscine (Fig. 5). Depression of the same cells by noradrenaline or 5-hydroxytryptamine was never affected by atropine (Fig. 5 and Stone, 1972*d*) indicating some specificity of blockade.

Although not a common anticholinergic agent, strychnine has been reported to block acetylcholine depressant responses in the cortex (Phillis & York, 1967*b*). When ejected with currents of up to 120 nA for 10 min from a solution containing 200 mM sodium chloride, strychnine has not

been observed to antagonize acetylcholine responses on the cells studied here. It is not likely, however, that this was due to a failure to eject the alkaloid from the pipette, since it has been shown to cause a block of some 5-hydroxytryptamine depressant responses (Stone, 1972*d*), sometimes on cells on which an acetylcholine depression was unaffected at the same time.

Acetylcholine also caused excitation of some superficial cells, above a depth of 600 $\mu$, the responsive cells accounting for 8% of all apparently cholinoceptive non-PT cells studied. The acetylcholine excitation was similar to that seen on PT cells, but it was mimicked by nicotine (60 nA)

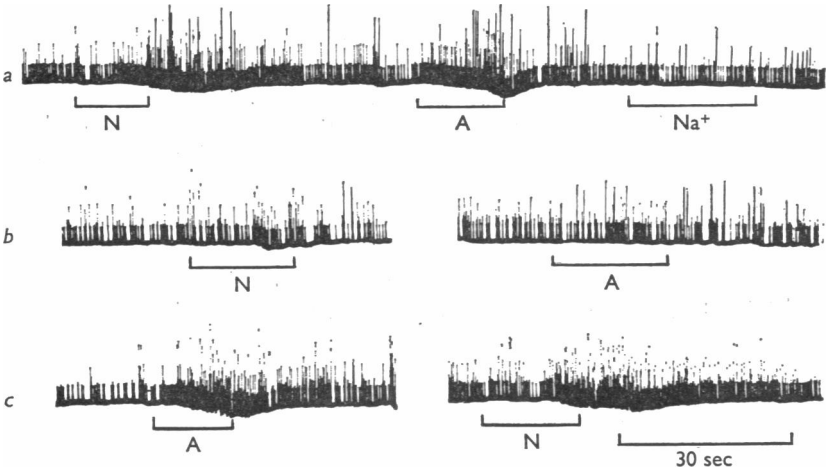


Fig. 6*a*. Excitatory responses of a non-pyramidal tract cell, depth 430 $\mu$ to acetylcholine 60 nA (A), and nicotine 60 nA (N). A control current of 60 nA is ineffective. In *b* the responses are blocked after the iontophoresis of D-tubocurarine, 60 nA for 1 min. Recovery of the response is seen 3 min later in *c*.

which excited the cells with a latency of 5–20 sec and had a more prolonged action (15–65 sec duration from a ten second application). This effect was blocked by D-tubocurarine, 60 nA applied for 1 min (Fig. 6). As with atropine L-glutamate excitation was usually unaffected at the time of acetylcholine blockade. In two instances this was not so, and some reduction of glutamate excitation was seen. These results were discarded.

## DISCUSSION

These results confirm previous reports of the existence of excitatory receptors on pyramidal tract (PT) cells in the cerebral cortex responding to the iontophoresis of acetylcholine, although hitherto the experiments

have been performed on cats (Crawford & Curtis, 1966; Krnjević & Phillis, 1963*a*, *b*). However, it is further shown that there is some correspondence of identity between those cells responding to acetylcholine and those responding to stimulation of a thalamic relay nucleus (n. ventralis posterolateralis) with a short latency burst of action potentials. This relationship is emphasized by the demonstration that this burst can be at least partly blocked by the iontophoresis of atropine at a time when responses to other drugs are unaffected. These observations, together with previous evidence that acetylcholine released from the cortex can be increased by specific thalamic stimulation (Collier & Mitchell, 1966), and the histochemical evidence of a dense cholinergic fibre system around deep pyramidal cells (Krnjević & Silver, 1965) suggests strongly that cholinergic muscarinic excitatory synapses on PT cells may be activated as a result of specific thalamocortical activity. These synapses could be part of the specific thalamocortical cholinergic system proposed by Mitchell (1966) and supported by experiments suggesting a subcortical origin for cortical acetylcholine (Hebb *et al.* 1963; MacIntosh & Oborin, 1953). This does not of course preclude the possibility that similar receptors could be involved in more complex pathways mediating the late repetitive discharge of cells and involved in cortical arousal (Mitchell, 1966).

There appears to be much to be gained by identifying in some way cells being studied by micro-iontophoresis. From the present experiments there seems to be a clear division between the muscarinic excitatory responses of acetylcholine, occurring only on PT cells, and inhibitory responses on non-PT cells.

Whilst it is impossible to know the precise relationship between the iontophoresis of a chemical and the resulting response of a neurone, it is unlikely that the responses to acetylcholine described here are due to actions on, for example, blood vessels as has been suggested for noradrenaline excitations (Stone, 1971, 1972*b*). The responses have a much shorter latency and offset that can reasonably be explained in terms of such an indirect action and, in particular, it would be difficult to reconcile such a non-neuronal mechanism of action with the remarkable homogeneity of response found on specified populations of cells such as PT cells.

The acetylcholine inhibitory response has been noted or postulated by other workers (Phillis & York, 1967*a*; Vazquez, Krip & Pinsky, 1969) although they were not detected in the original investigations of Krnjević & Phillis (1963*a*, *b*, *c*). The receptors mediating this inhibition have previously been reported to be intermediate between the classical muscarinic and nicotinic type (Phillis & York, 1968), but there are two possible explanations for the discrepancy between that study and the present finding that the inhibitory response is muscarinic only. One possibility is

that the difference is simply a species difference between the cat and the rat. This would be strange in view of the close similarity between the cholinergic muscarinic excitatory responses of PT cells in the two species. The other possible explanation may be that Phillis & York (1968) used 'half-saturated solutions' of drugs. This would mean that their solution of dihydro-$\beta$-erythroidine was somewhat greater than 1 M, and of atropine almost 2 M. In the present report a 0·2 M solution of atropine is shown to block satisfactorily both excitatory and depressant responses of acetylcholine, but the interfering potent non-specific depression noted by Krnjević & Phillis (1963*b*) and Phillis & York (1968) were not seen, presumably due to the use of a much weaker solution. The concentrated dihydro-$\beta$-erythroidine solution may also have apparently blocked acetylcholine depression due to a non-specific action, causing the false interpretation that a nicotinic component existed in the inhibitory receptor mechanism.

The presence of both muscarinic (Dale, 1914) excitatory and inhibitory responses to acetylcholine could be the pharmacological basis for the increased release of acetylcholine from the cerebral cortex *in vivo* which results from the administration of atropine (Mitchell, 1963). For example, the blockade by atropine of the excitatory synapses could reduce the activity of neurones which would normally be activated subsequently in the cortex and, since some of those neurones would presumably release acetylcholine, a reduction of acetylcholine release would result. Conversely, blockade of the inhibitory synapses could increase acetylcholine release from subsequent cholinergic neurones. Denisenko (1965) has previously suggested that a high ratio of muscarinic inhibitory to excitatory synapses might exist in the cerebral cortex, and if this is so, then the net effect of atropine would be to increase cortical acetylcholine release.

This suggestion is an extension of the original explanation of MacIntosh (1963), proposed at a time when the various types of acetylcholine response in the cortex were not known. MacIntosh's proposal was that atropine blocked acetylcholine acting as an inhibitory transmitter in a negative feed-back loop, thus indirectly increasing the activity of the cholinergic neurone. This hypothesis, however, does not consider the effect of atropine in blocking muscarinic excitatory synapses, and also postulates a specific functional role for acetylcholine for which there is little evidence.

The small proportion of nicotinic (Dale, 1914) excitatory effects on superficial cells has not been reported previously in the parietal cortex, but several papers have provided evidence of nicotinic effects in the cerebrum. Armitage, Hall & Sellers (1969), for example, discussed marked changes of acetylcholine release and of e.e.g. activation resulting from

nicotine administration, and D-tubocurarine has been shown to markedly alter electrocortical activity (Chang, 1953; Feldberg, Malcolm & Sherwood, 1956). Furthermore, Cairnie & Malcolm (1960) have attributed some of the changes of cortical evoked potentials to an action on the cortex itself rather than on distant structures. These changes could therefore be mediated by the nicotinic receptors demonstrated above. The discovery of these nicotinic responses is in keeping with other studies which have found nicotinic responses to iontophoretic acetylcholine in the brain stem (Bradley, Dhawan & Wolstencroft, 1966) and pyriform cortex (Legge, Randic & Straughan, 1966). These actions were excitatory, and of quite prolonged duration similar to the effects described above.

It is difficult to suggest a reason for these responses not having been detected earlier in the parietal cortex (Krnjević & Phillis, 1963*a*, *b*, *c*). One possibility, however, may be that Krnjević & Phillis used quite strong solutions of acetylcholine (3 M) in their experiments. In the present study all the cells on which the nicotinic response has been detected were found above a depth of 600 $\mu$ and probably, therefore, were quite small in size. A 3 M solution could have resulted in a sufficiently large spontaneous effusion of acetylcholine to swamp, and thereby inactivate, acetylcholine receptors on such superficial, small cells, even before the iontophoresis of acetylcholine was attempted.

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