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PHARMACOLOGICAL STUDIES ON FELINE BETZ CELLS

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SUMMARY

1. A study has been made of the sensitivity of single neurones in the pericruciate cortex of anaesthetized and unanaesthetized cats to cholinomimetics administered electrophoretically from multibarrel micropipettes.

2. A high proportion of deep pyramidal cells, including Betz cells, were excited by these substances, and the receptors involved have muscarinic characteristics.

3. Atropine specifically reduced the sensitivity of cortical neurones to acetylcholine, but no such action could be demonstrated for dihydro-β-erythroidine, gallamine or general anaesthetics.

4. The significance of these results is discussed in relation to the possible synaptic or non-synaptic action of acetylcholine upon cortical neurones.

INTRODUCTION

The considerable amount of indirect evidence which favoured a role of acetylcholine (ACh) as an excitatory transmitter substance in the mammalian cerebral cortex (Feldberg, 1945, 1957; Hebb, 1957) has recently gained support from the observations that electrophoretically administered ACh excites certain cortical neurones (Krnjević & Phillis, 1963*a*, *b*; Spehlmann, 1963; Stefanis, 1964*a*). These results, taken in conjunction with studies of cortical acetylcholinesterase and acetyltransferase, have led to the proposal that deep pyramidal cells of the cortex are innervated by cholinergic corticopetal fibres, the origins of which remain to be identified (Krnjević, 1964; Krnjević & Phillis, 1963*a*).

However, in addition to excitation, electrophoretically administered acetylcholine depresses cortical neurones (Randić, Siminoff & Straughan, 1964). Such dual effects have also been observed with spinal interneurones (Curtis, Ryall & Watkins, 1966) and Renshaw cells (Curtis & Ryall, 1966*a*, *b*, *c*), and are not necessarily related to the operation of acetylcholine as a synaptic transmitter upon such spinal cells. Accordingly, the present study of the acetylcholine sensitivity of Betz and other neurones

of the feline pericruciate cerebral cortex was carried out in order to confirm and extend the earlier pharmacological investigations. In addition, an attempt was made to establish the most suitable type of preparation, particularly with regard to anaesthesia, as a preliminary to the comparison of the pharmacology of synaptic and acetylcholine-induced responses of Betz cells.

METHODS

Preliminary experiments were performed upon cats anaesthetized with intraperitoneal diallylbarbituric acid (Dial, Ciba Ltd., 40–50 mg/kg), Dial compound (diallylbarbituric acid 100 mg/ml. with urethane 400 mg/ml., Ciba Ltd.; 0·4–0·5 ml./kg) or pentobarbitone sodium (Nembutal, Abbott Laboratories; 35–40 mg/kg). The medullary pyramids were exposed from the ventral aspect and the left pre- and post-cruciate cortical areas were exposed by partial removal of the calvarium, extending forward to include the posterior wall of the left frontal sinus (Crawford & Curtis, 1964). After incision of the dura the cortex was covered with polyethylene sheeting, leaving a small exposed area for insertion of the micro-electrode. The whole area was continuously flushed with a warmed mammalian Ringer solution (at 38° C and saturated with 95 % O_2 and 5 % CO_2), and fine forceps were used to remove the pia from suitable small avascular areas. The head of the animal was fixed in a modified Horsley–Clarke head frame which allowed the pericruciate cortex to be orientated in an approximately horizontal plane, and a small pressor plate, mounted upon a micromanipulator, was used to control cortical pulsation.

In a second series of experiments, unanaesthetized *cerveau isolé* cats were used. The animals were prepared under halothane anaesthesia (Fluothane, I.C.I. Ltd., 1½–3 % vapour mixtures in air on 'open circuit') by either supra- or immediately subtentorial approaches using a blunt knife to sever the brain stem, or by a subtentorial approach using a radiofrequency electrocoagulating current passed between needles inserted stereotaxically. This latter technique was preferred, as (*a*) the occipital poles of the cerebral hemispheres were undisturbed, (*b*) insertion of the rack of coagulating electrodes did little or no damage to the great cerebral vein, and (*c*) the incidence of cerebral swelling was somewhat reduced. The rack consisted of four stainless-steel needles held parallel to each other in a transverse plane, each 2 mm apart and each separately insulated with 'Araldite' coating resin except for 2 mm at the tip. The assembly was carried on a small micromanipulator and was inserted stereotaxically at an angle of some 30° to the Horsley–Clark vertical plane, just posterior to the tentorium. A current of 30–40 mA (500,000 c/s) was passed for 15–20 sec between adjacent pairs of needles (Wyss Coagulator, Type OC 60; J. Monti, Geneva), the first lesions being made 0–2 mm anterior and 10 mm below the Horsley–Clark zero co-ordinate and straddling the mid line. The needles were then withdrawn in steps of 2 mm and lesions made at five or six levels. After the pericruciate cortex had been exposed as described above, the anaesthesia was discontinued. Clinical decerebrate rigidity supervened within 2–5 min, and the pupils which were previously dilated became constricted and failed to react to painful stimuli to the paws, body or face. Reflexes were active, in some cats to such a degree that paralysis with gallamine triethiodide (Flaxedil, May & Baker, Ltd) and artificial ventilation were required. A period of at least an hour elapsed between the cessation of halothane administration and the recording of action potentials from cortical neurones. Post-mortem section of the brains of these preparations revealed gross tissue destruction for some 4–5 mm on either side of the mid line in an oblique plane 1–2 mm thick passing dorso-posteriorly from the pyramidal tracts and involving the pontine nuclei, medial lemniscus and part of the pontine reticular formation, the medial longitudinal bundle and the brachium conjunctivum.

In all animals, extracellular negative–positive spike potentials (0·1–0·5 mV in amplitude)

were recorded by means of the central barrel (4 M-NaCl) of five-barrel micropipettes of over-all tip diameter 4–6 μ (see Curtis, 1964). The outer four barrels contained aqueous solutions of the substances to be tested by electrophoretic ejection, and the pipettes were filled by centrifugation. The concentrations of the solutions were usually 1 M, unless otherwise specified in the text. Each pipette was re-examined microscopically after use, and in no case was there more than 1–2 μ difference between the levels of the various orifices. In many instances accumulated tissue debris was present (Anderson & Curtis, 1964*a*), and this may have interfered with the ejection of drugs. The cortical surface was observed by means of a microscope (magnification × 40) and estimations of the depths of neurones were based upon the positions at which extracellularly recorded action potentials were of maximum amplitude. The micropipettes were introduced normally to the cortical surface, the position of which was measured both as the pipette entered cortical tissue, and as it was withdrawn. An error of at least 50 μ in the estimates of cell depth was probably introduced by the removal previously of the pia. Whenever dimpling of the cortical surface occurred, indicating some obstruction to free penetration by the micropipette, investigation in that particular area was discontinued.

Betz cells (in the anaesthetized preparations only) were identified by a constant latency antidromic response to electrical stimulation of the ipsilateral medullary pyramid (latency within the range 0·5–5 msec), the response to two stimuli at an interval of 1 msec, and by the ability to follow tetanic antidromic volleys (see Phillips, 1956, 1959, 1961). These cells were generally located at depths greater than 0·5 mm beneath the cortical surface. In the *cerveau isolé* preparations, however, no such absolute identification of Betz cells was made, and the depth below the cortical surface, the pattern of spontaneous activity, and the firing of each cell induced by ACh and other cholinomimetics was compared with those of antidromically identified cells in anaesthetized cats.

As in an earlier investigation (Crawford & Curtis, 1964), the intermittent ejection of DL-homocysteic acid (0·2 M solution, pH 8, sodium salt) was used to locate otherwise quiescent cells. A firing-frequency indicating system, which gave a direct record of drug-induced firing frequency upon a paper recorder, was used as previously described (Andersen & Curtis, 1964*a*). In addition, amplified spike responses could be photographed from an oscilloscope.

In all experiments the systemic blood pressure was monitored with a strain gauge pressure transducer.

RESULTS

Excitation by acetylcholine

All of the pericruciate neurones studied (a total of 231 cells in fourteen anaesthetized animals, and 102 in nine *cerveau isolé* preparations) were readily excited by either L-glutamic acid or DL-homocysteic acid (see Crawford & Curtis, 1964; Krnjević, 1964). However, as was also found by Krnjević & Phillis (1963*b*), there was wide variation between one preparation and another in the proportion of cells excited by acetylcholine. This variation cannot have been due simply to differences in the depth of anaesthesia, as in three preparations lightly anaesthetized with diallylbarbituric acid every Betz cell identified was found to be excited by ACh, whereas in one unanaesthetized *cerveau isolé* cat no acetylcholine-sensitive cells were found despite an apparently normal sensitivity of neurones to excitant amino acids, and normal cortical circulation. Furthermore, in many anaesthetized animals acetylcholine-sensitive and insensitive Betz cells were located close to each other and within short intervals of time.

In anaesthetized cats 75 % of Betz cells, located at depths of 0·4–1·5 mm, and 17 % of the cells which failed to respond antidromically to pyramidal tract volleys (located between 0·15–1·5 mm beneath the cortical surface) were fired by acetylcholine. Usually electrophoretic currents of 40–80 nA were required for appreciable excitation, and thus the extracellular acetylcholine concentrations were presumably considerably greater than those required to excite Renshaw cells. The uncontrolled diffusional efflux of acetylcholine from micropipettes containing 1 M ACh bromide is often adequate to fire these spinal neurones, although the demonstration of 'muscarinic' receptors necessitates ejection with currents of the order of 20–40 nA (Curtis & Ryall, 1966*a*). Cortical neurones with a moderate to high level of irregular spontaneous activity (5–20 spikes/sec) appeared to be more readily excited by acetylcholine than were quiescent cells, or cells

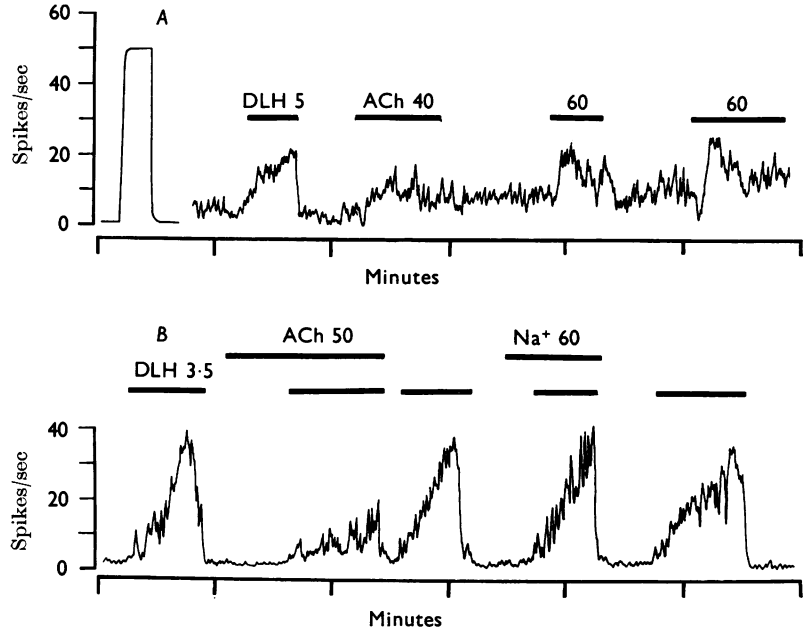

Fig. 1. Patterns of firing of two cortical neurones in a cat anaesthetized with Dial-urethane. This and Figs. 2 and 3 are tracings of the original records which were plotted by means of a rectilinear ink-writing paper recorder. The inset tracing shows the response of the recording system to a 15 sec train of impulses at 50 c/s. *Ordinates*—spikes/sec. *Abscissae*—time. Electrophoretic ejections are indicated by horizontal markers. *A*. Firing of a Betz cell by DL-homocysteic acid (DLH, 5 nA) and acetylcholine (ACh, 40 and 60 nA). *B*. Depression of the DLH-induced firing of another Betz cell by the simultaneous administration of acetylcholine (ACh, 50 nA) from another barrel of the micropipette. DLH was ejected with a current of 3·5 nA. The ejection of sodium ions (Na^+) with a current of 60 nA did not reduce the sensitivity of this neurone to the amino acid.

firing in 'spindles' after barbiturate anaesthesia (Dempsey & Morison, 1943; Verzeano & Calma, 1954). As has been reported by Krnjević & Phillis (1963*b*), it was not possible to convert ACh-insensitive quiescent cells into ACh-sensitive units merely by creating an artificial 'background' depolarization with the ejection of an acidic amino acid.

The excitation of both Betz and unidentified cortical neurones by acetylcholine was characteristically of slow onset, with latencies varying between 5 and 15 sec. The rate of firing of many cells was transiently depressed at the start of the ejection of ACh (Fig. 1*A*), and occasionally there was no apparent excitation of the cell until the ejecting current had been terminated. Following the end of the ejecting current, the firing usually declined over a period of 15–30 sec, but occasionally some excitation persisted for as much as 1 min after ACh administration (see Fig. 3). Although some difficulty was occasionally experienced in determining whether depression of neurone activity by acetylcholine was not merely due to the passage of a cationic (outward) current from the micropipette, with a consequent anodal depressant effect on cells located in the vicinity of the orifice, acetylcholine clearly depressed eleven cells, six of which were identified as Betz cells, in the absence of previous or subsequent excitation.

Results from one such neurone are illustrated in Fig. 1*B*. This cell was readily fired by DL-homocysteic acid (DLH, 3·5 nA) and when the frequency exceeded approximately 35 spikes/sec, the spikes changed shape and firing ceased because of excessive depolarization (see Crawford & Curtis, 1964). However, when DLH was ejected during the simultaneous administration of acetylcholine (ACh, 50 nA) the maximal firing frequency was reduced to about 15 spikes/sec. The response to the amino acid had recovered fully when next tested 12 sec after the end of the acetylcholine ejection, and was not reduced by the simultaneous administration of Na^+ ions using an even larger cationic current of 60 nA passed through a barrel containing NaCl solution. The ejection of sodium ions with a current of 80 nA also failed to reduce the amino acid sensitivity of this neurone. A similar direct depressant action of ACh probably accounts in part for the transient fall in firing rate which often preceded the eventual excitation of many other cells. This initial depression had a latency of the order of 100 msec and as a rule was only partially reproduced by the passage of Na^+ ions. The degree of depression, however, was not as profound as that observed in an investigation of the acetylcholine sensitivity of Purkinje cells (Crawford, Curtis, Voorhoeve & Wilson, 1966).

In the series of unanaesthetized *cerveau isolé* preparations, 102 consecutive cells were examined for spontaneous activity and sensitivity to electrophoretically ejected ACh or carbamylcholine. Superficial cells (0·1–0·6 mm depth) usually lacked spontaneous activity and failed to

respond to cholinomimetics, but at greater depths (0·7–1·2 mm) the majority (84 %) of spontaneously active cells were excited by ACh. The distribution in depth (aggregated in the main between 0·8 and 1·1 mm), the patterns of excitation and the relative potencies of a number of choline esters were the same as those found with identified Betz cells in the earlier experiments. In agreement with the proposal of Krnjević & Phillis (1963*b*), it appears that the acetylcholine-sensitive units at these depths represent members of the deep pyramidal cell population. It should be stressed, however, that the use of micropipettes of tip diameter 4–5 μ introduces into this investigation a considerable sampling bias towards large neurones (see Amassian, 1961). As a consequence it cannot be concluded that deep pyramidal cells are the only acetylcholine-sensitive neurones in the cortex.

Excitation by other cholinomimetics

In general, the results of the present experiments confirm earlier reports (Krnjević & Phillis, 1963*c*) that many choline esters are effective excitants of cortical neurones, usually in proportion to their muscarinic potency.

As with the estimation of relative potency of excitant amino acids (see Curtis & Watkins, 1963; Crawford & Curtis, 1964), care was taken to eject each drug for a sufficient time to ensure that maximal effects were observed for the electrophoretic current used, and to compare the extracellular concentrations (assumed to be approximately linearly related to the magnitudes of ejecting currents) of each excitant needed to produce comparable firing rates of the same cell. The comparisons were made upon several cells in different cats, and wherever possible were repeated at different levels of evoked firing frequency. In this manner the effects of differences in the time course of excitation upon the estimate of relative potency were minimized.

Carbamylcholine and acetyl-β-methylcholine were as potent as, or slightly more effective than, acetylcholine and had more prolonged effects after the ejections had been terminated, as illustrated in Fig. 2. Excitation by these choline esters was often preceded by transient depression of the spontaneous firing of cortical neurones. Propionylcholine was $\frac{1}{4}$–$\frac{1}{2}$ as active as acetylcholine, and *n*-butyrylcholine was apparently inactive upon the few cells tested using electrophoretic currents of the order of 80–100 nA.

(±)-Muscarine excited all seven cells (previously found to be sensitive to ACh) upon which it was tried. The potency of this substance was found to vary over a comparatively wide range, from 1 to 4 times as powerful as acetylcholine, and the offset of firing after the ejection of muscarine had been terminated was usually much longer than that following excitation

by ACh. On one occasion, firing persisted for 5 min after the ejection of muscarine with a current of 80 nA. This appears to be an extreme example of the tendency of Betz cells to fire for prolonged periods after chemical excitation with many of the cholinomimetics.

Oxo-tremorine (1-(2-oxopyrrolidino)-4-pyrrolidino-butyne-2) excited two of three cells tested in a *cerveau isolé* preparation. These neurones were also fired by acetylcholine, and non-cholinoceptive cells were found to be insensitive to oxotremorine.

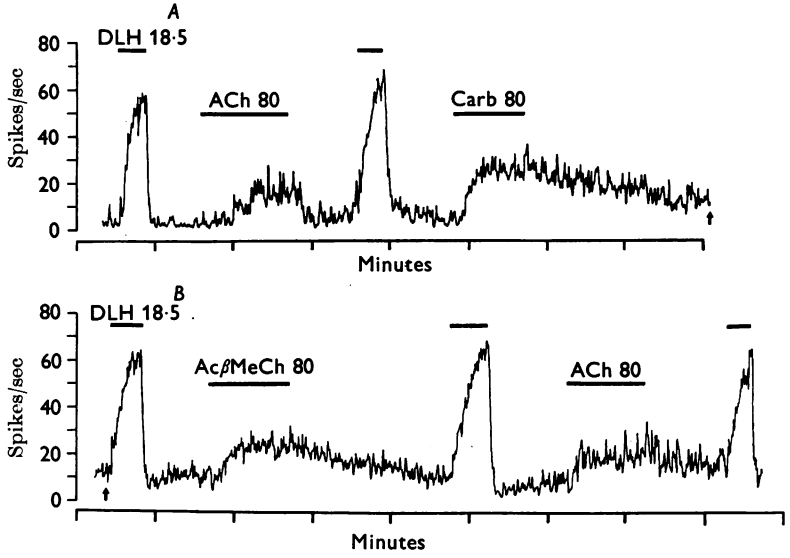

Fig. 2. A comparison of the excitant action of cholinomimetics upon an unidentified cortical neurone (Dial-urethane preparation.) The tracings *A*, *B* are continuous at the point marked by the arrows. Electrophoretic currents of 80 nA were used to eject acetylcholine (ACh), carbamylcholine (Carb) and acetyl-β-methylcholine (AcβMeCh) and the sensitivity of the cell to DL-homocysteic acid (DLH, 18·5 nA) was checked on five occasions.

In contrast with the earlier findings of Krnjević & Phillis (1963*a*, *c*), nicotine did not excite cells which were insensitive to acetylcholine. However, maximum electrophoretic currents were of the order of 80–100 nA and under these conditions nicotine was found to be roughly $\frac{1}{2}$–$\frac{1}{4}$ as potent as acetylcholine. Since the electrophoretic currents used to assess the acetylcholine sensitivity of many neurones rarely exceeded 100 nA, it is possible that had larger currents been used to eject nicotine, more nicotine-sensitive neurones would have been found. The latency of onset of excitation by nicotine was not significantly longer than that for acetylcholine.

Acetylcholine antagonists

Atropine. In the light of the relative sensitivity of cortical cells to cholinomimetics it was of interest to use atropine as an antagonist as a means of establishing the possible muscarinic nature of the acetylcholine-receptor (Dale, 1914). When injected intravenously in doses of 0·1–2·0 mg/kg, atropine sulphate reduced the sensitivity of cells to ACh, but neither altered that to the acidic amino acid, nor reduced the 'background' synaptic firing of the units tested.

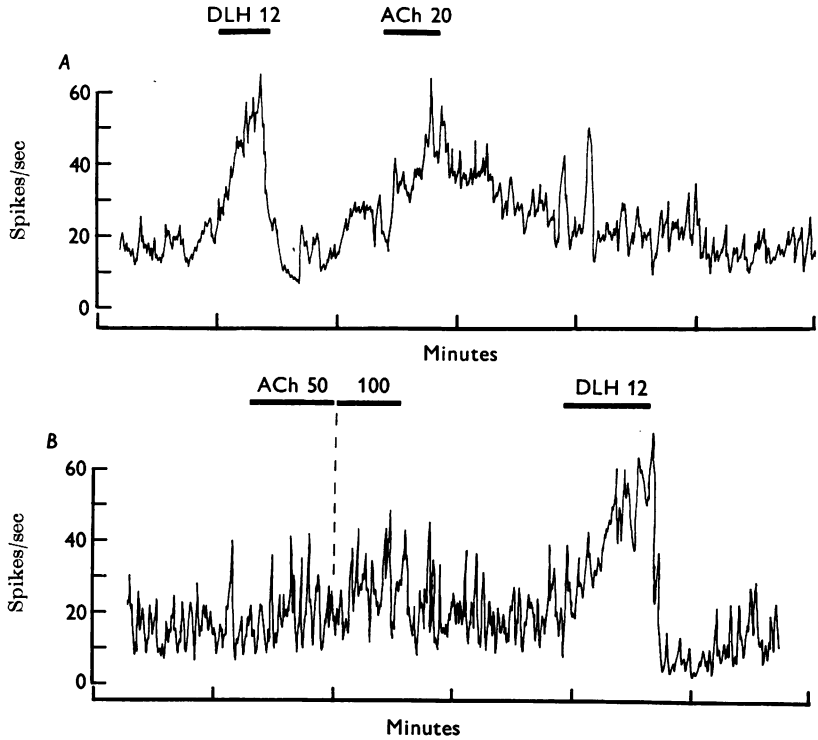

Fig. 3. The effect of intravenous atropine sulphate upon the sensitivity of a Betz cell to DL-homocysteic acid (DLH, 12 nA) and acetylcholine (ACh, 20, 50 and 100 nA). *A*—control series. *B*—7 min after atropine sulphate 1·2 mg/kg intravenously. *Cerveau isolé* preparation.

These features of atropine action are illustrated in Fig. 3 for a neurone in the pericruciate cortex of a *cerveau isolé* preparation. This cell was firing spontaneously in an irregular fashion, with an average frequency of the order of 15–20 spikes/sec. DL-Homocysteic acid, ejected with a current of 12 nA, increased the firing rate of 55–60 spikes/sec, and a similar increase in frequency was produced by acetylcholine, using a current of 20 nA.

This cell was thus rather more sensitive to acetylcholine than many other cortical neurones (see Figs. 1*A*, 2, 4 and 5). Following the intravenous injection of 0·2 mg/kg of atropine sulphate, the spontaneous discharge rate was unaltered, and the acetylcholine sensitivity was reduced slightly. However, when observations were made 7 min after an additional dose of 1·0 mg/kg, acetylcholine ejected with currents of 50 and 100 nA increased the firing rate merely to some 25–30 spikes/sec, although the mean spontaneous firing was still virtually identical with that during the control period, and DL-homocysteic acid (which had also been tested at intervals between the atropine injections) still fired the cell at approximately 65 spikes/sec (Fig. 3*B*).

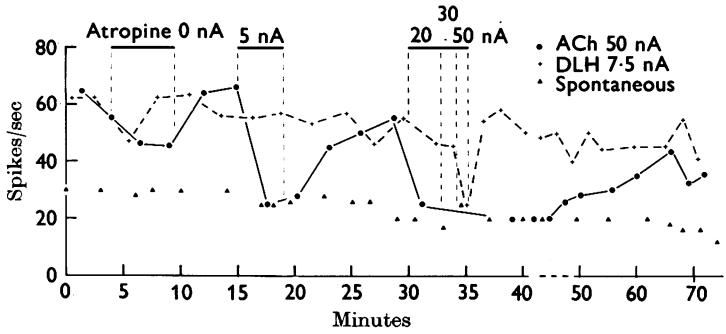

Fig. 4. The effect of electrophoretically administered atropine upon the sensitivity of a Betz cell to alternate electrophoretic ejections of acetylcholine (ACh, 50 nA, ●) and DL-homocysteic acid (DLH, 7·5 nA, +). The background spontaneous firing rate of the cell is plotted by the filled triangles. Atropine was allowed to diffuse (0 nA) from a micropipette containing 0·1 M atropine sulphate, and was later ejected with currents of, 5, 20, 30 and 50 nA. Note change in time scale between 40 and 50 min. This animal was anaesthetized with Dial.

When administered electrophoretically atropine also specifically reduced the acetylcholine sensitivity of cortical neurones. The results plotted in Fig. 4 are from a Betz cell, which responded antidromically with a latency of 3·5 msec after stimulation of the pyramids, in a cat anaesthetized with diallylbarbituric acid. The filled circles and crosses plot respectively the maximal firing frequencies produced by alternate ejections of acetylcholine (ACh, 50 nA, 30 sec) and DL-homocysteic acid (DLH, 7·5 nA, 30 sec), the magnitudes of the ejecting currents having been adjusted to produce control responses to both excitants of approximately equal frequency. The spontaneous firing of this Betz cell was irregular throughout the 74 min of observation, and the mean value is indicated by the filled triangles. When atropine diffused from a 0·1 M solution of atropine sulphate in one barrel of the five-barrel micropipette (Atropine, 0 nA) the acetylcholine sensitivity of the cell decreased slightly, but there was no

sustained depression of the amino acid sensitivity. The active ejection of atropine with an outward current of 5 nA abolished the action of acetylcholine with little or no change in the response induced by DL-homocysteic acid. The acetylcholine sensitivity recovered within 10 min but was again abolished by the further administration of atropine with a current of 20 nA. Again, this depression was not accompanied by much reduction in the amino acid induced response until the current used to eject atropine was increased to 50 nA. The subsequent reduction in the amino acid sensitivity recovered within 2 min of the cessation of atropine administration, but, in contrast, the acetylcholine recovery was incomplete after 35 min. In other experiments the recovery time for acetylcholine sensitivity after abolition by such doses of atropine varied from 25 to 40 min. It is noteworthy that in this and other similar experiments the reduction in acetylcholine sensitivity was not associated with any marked depression of the spontaneous neurone activity.

Dihydro-β-erythroidine was tested upon fifteen cells using currents of 10–60 nA. The acetylcholine sensitivity of two cells was depressed without a reduction in either the spontaneous firing or the response to DL-homocysteic acid. The acetylcholine responses of the other thirteen cells were not reduced by dihydro-β-erythroidine, and five cells were actually excited, as indicated by an increased background firing rate and an increased sensitivity to DL-homocysteic acid. The action of this curariform agent upon cortical neurones is thus clearly different from the very specific antagonism towards acetylcholine which can be demonstrated with Renshaw cells (see Curtis & Ryall, 1966*b*).

Gallamine triethiodide was tested electrophoretically upon eleven cells in three animals, one of which was a *cerveau isolé* preparation. Ten cells were excited in a characteristic fashion, groups of high frequency discharges occurring in bursts, as has previously been described for medullary neurones (Salmoiraghi & Steiner, 1963), thalamic neurones (Andersen & Curtis, 1964*b*), spinal interneurones and Renshaw cells (Curtis & Ryall, 1966*b*). This excitation was apparently independent of acetylcholine sensitivity, but, as gallamine was possibly a more potent excitant of cortical neurones than acetylcholine, such a conclusion may be unwarranted in view of the upper limitation of approximately 100 nA which was placed upon currents used to eject acetylcholine. The remaining neurone showed a decrease in acetylcholine sensitivity during the administration of gallamine which was, however, accompanied by a decrease both in the rate of spontaneous discharge and in the sensitivity to DL-homocysteic acid. Thus the specific antagonism of gallamine towards acetylcholine which has been reported by Krnjević & Phillis (1963*c*) has not been confirmed.

In view of the frequent use of gallamine as a muscle relaxant in this series of experiments it was necessary to establish whether or not intravenous administration altered the sensitivity of cortical neurones towards acetylcholine. Following doses of up to 4 mg/kg no effect was observed upon the spontaneous activity of single neurones or the sensitivity to either acetylcholine or DL-homocysteic acid. In addition, there was no significant difference in the ratio of acetylcholine-sensitive units to the total number of cells encountered in *cerveau isolé* preparations before and after the use of gallamine. Accordingly, it was considered that the intravenous administration of gallamine in the usual doses of 2 mg/kg at approximately half hourly intervals had little or no effect upon the behaviour of cortical neurones in these experiments.

Other compounds

5-*Hydroxytryptamine* was found to depress both the spontaneous and amino acid induced excitation of Betz and unidentified pericruciate neurones when ejected with currents of 20–100 nA from saturated aqueous solutions of the creatinine sulphate complex. This depression had a short latency of both onset and offset (1–2 sec), and was exerted upon ACh-sensitive and non-sensitive cells alike. In agreement with Krnjević & Phillis (1963*d*), 3-hydroxytyramine had a similar depressant action, and was approximately equipotent with 5-hydroxytryptamine. These results are thus similar to those described for ventrobasal thalamic neurones (Andersen & Curtis, 1964*b*), but clearly differ from those obtained in the lateral geniculate nucleus (Curtis & Davis, 1962), where 3-hydroxytyramine was much less potent as a neuronal depressant that 5-hydroxytryptamine, and the depression by both substances did not involve a reduction in amino acid sensitivity.

Anaesthetics

When small doses of barbiturates are administered intravenously in previously unanaesthetized animals, there is usually a change in the spontaneous firing pattern of single pericruciate neurones from a predominantly irregular discharge (projection activity) to one in which the spikes occur in spindles or bursts (see Dempsey & Morison, 1943). Barbiturates also reduce the chemical sensitivity of such cells, and it has been reported that the sensitivity to acetylcholine is reduced to a greater extent than that to an excitant amino acid (Krnjević & Phillis, 1963*b*, *c*). If the action of barbiturates were thus to some extent specific, a partial explanation would be provided for the low incidence of acetylcholine-sensitive cells in some anaesthetized cats.

A simple method of estimating the effect of systemically administered

anaesthetics has been to observe the peak firing rates evoked during alternate ejections of acetylcholine and an excitant amino acid before, during and after administration of the anaesthetic agent. Electrophoretic currents were chosen which produced submaximal firing rates for the cell, and the actual rates evoked by each excitant were adjusted to be as similar as possible. The control period was always at least 5–10 min in duration to ensure that recording conditions were stable. The extracellular spike shape and size were carefully monitored on the oscilloscope screen, and results were rejected if changes occurred in the spike concurrently with alteration in arterial blood pressure or respiration. In most experiments the animals were paralysed and artificially respired in order to limit the relative movement between the micropipette and a neurone because of alterations in respiration.

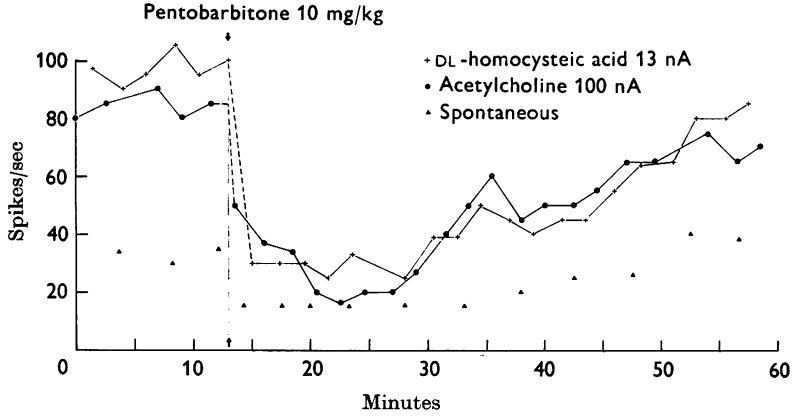

Fig. 5. The effect of pentobarbitone (10 mg/kg, intravenously) on the sensitivity of a deep pyramidal cell in a *cerveau isolé* cat to acetylcholine (100 nA, ●) and DL-homocysteic acid (13 nA, +). The spontaneous firing rate is plotted by the filled triangles.

Under these conditions, it has been difficult to show any preferential effect of anaesthesia upon the acetylcholine sensitivity of pericruciate neurones. Results from a typical experiment in which 10 mg/kg pentobarbitone sodium (Nembutal) was administered to an unanaesthetized *cerveau isolé* cat are shown in Fig. 5. A rapid decline in the sensitivity of this cortical neurone to both excitants followed the injection. A maximal effect was reached in 7–10 min, and thereafter recovery occurred slowly over more than 1 hr (full recovery is not shown in the figure). Before the administration of pentobarbitone the irregular spontaneous firing of this neurone had a mean frequency of 25–30 spikes/sec. Within 30 sec of the intravenous injection spindles were observed, and the mean discharge rate was of the order of 12–15/sec. It is noteworthy that the depression

and recovery of chemical sensitivity to both excitants, and of the mean spontaneous firing frequency, occurred with the same time course. There was no associated change of spike shape or size, nor was the fall in chemical sensitivity concurrent with the slight fall in mean blood pressure which followed within 30–60 sec of the injection of pentobarbitone.

It might be proposed that the observed reduction in chemical sensitivity by pentobarbitone was associated with the removal of the background synaptic barrage, which previously held the cell in a partially depolarized state. However, when small currents were used to eject DL-homocysteic acid continuously in an attempt to restore this background firing, superimposed acetylcholine or DL-homocysteic acid failed to evoke responses of the same frequency as before the barbiturate. Furthermore, if the background synaptic barrage had made a large contribution to the depolarization of the cell by chemical excitants, its removal would be expected to depress low frequency firing (in response to just-suprathreshold concentrations of the excitants) more than high frequency firing. However, anaesthesia did not seem to reduce low rates of induced firing to any greater extent that it did intermediate and higher frequencies. Thus the removal of the normal background depolarization by anaesthesia is not the only mechanism whereby the efficacy of chemical excitation is reduced. On the contrary, the decline in spontaneous firing after barbiturates appears rather to reflect decreased post-synaptic sensitivity to all chemical excitants, the synaptic transmitters included, although alterations in the activity of other neurones responsible for the spontaneous firing of the neurone under observation cannot be excluded.

Essentially similar results were obtained in other unanaesthetized *cerveau isolé* preparations with doses of 2·5–10 mg/kg pentobarbitone, 5–10 mg/kg of diallylbarbituric acid (Dial, Ciba Ltd.) and 5–30 mg/kg of sodium methylthioethyl-2-pentyl-thiobarbiturate (Thiogenal, Merck, A. G.). The time course of the depression and recovery of chemical sensitivity was shorter after the latter than after the other barbiturates. Ethyl carbamate (Urethane, B.D.H., Ltd; 50–200 mg/kg) and α-chloralose (B.D.H. Ltd, 30–35 mg/kg) also depressed the spontaneous and chemically induced activity of pericruciate neurones, again without any special effect on the firing produced by acetylcholine. Whereas reversible depression of cortical neurones followed the administration of even small doses of all of the barbiturates tested, the amount of urethane which was required approximated the dose necessary for clinical anaesthesia.

Halothane vapour (Fluothane, I.C.I. Ltd, $1\frac{1}{2}$–3 %, v/v, in air) and nitrous oxide-oxygen mixtures (60–90 % nitrous oxide) were also tested by respiring the animals with these mixtures. Considerable difficulty was experienced in retaining stable recording conditions, presumably because

of fluctuations in blood pressure, but again no evidence was obtained for a selective reduction of acetylcholine sensitivity. Furthermore, the results suggested that of all the anaesthetic agents tested, fluothane and nitrous oxide had the least depressant effect on the sensitivity of neurones to acetylcholine and DL-homocysteic acid.

In several experiments, pentobarbitone was ejected electrophoretically as an anion from freshly prepared solutions of the sodium salt. The spontaneous firing rate of cortical neurones was diminished, together with the response to acetylcholine and DL-homocysteic acid. Although the acetylcholine sensitivity of several neurones appeared to be more resistant to the barbiturate ejected with currents of the order of 20 nA, this was not always the case. Furthermore, both the acetylcholine and amino acid responses were reduced when larger currents (60 nA) were used to eject pentobarbitone. As there was no evidence of 'specificity' of action against either excitant when the anaesthetic was administered systemically, the resistance of the acetylcholine sensitivity presumably indicates that the activated amino acid receptors were exposed to a higher average concentration of electrophoretically administered barbiturate than were the acetylcholine receptors. This explanation, which would be in accordance with the amplitudes of the electrophoretic currents used to administer the various drugs from what must be virtually a point source, lends support to the proposal that the acetylcholine receptors are widely dispersed over the nerve cell membrane.

DISCUSSION

The most striking feature of this and previous investigations of the sensitivity of deep pyramidal cells to cholinomimetics (Krnjević & Phillis, 1963*b*, *c*) is the marked similarities between the muscarinic receptors of these neurones and those of Renshaw cells (Curtis & Ryall, 1966*a*, *b*). Thus similar, and comparatively high, electrophoretic currents are required to produce above-threshold concentrations of acetylcholine; the relative potencies of cholinomimetics active at peripheral muscarinic receptors are much the same, and a specific antagonism can be demonstrated between atropine and acetylcholine. As there is no reason to suppose that the comparatively slow time course of the 'muscarinic' action of acetylcholine upon Renshaw cells results from the diffusion of the excitant to receptors located at some distance from the micropipette orifices, and thus from the neurone soma, it is probable also that the kinetics of the acetylcholine-receptor interaction, and possibly also those of the consequent alteration of membrane conductance, account in part for the observed time course of acetylcholine action upon deep pyramidal cells of the cortex. Since the depths at which extracellular spikes were recorded from these cells indicate that the orifices of the multibarrel

micropipettes were located close to the somas and bases of the apical dendrites, some of the muscarinic receptors are presumably located in the vicinity of these portions of the neurones. Thus the simplest explanation of the presence of such muscarinic sites is that proposed by Krnjević & Phillis (1963*b*; see also Spehlmann, 1963), namely that they are located beneath the terminals of cholinergic fibres. This proposal is fully in accordance with the presence of acetylcholine within nerve endings isolated from cortical tissue (de Robertis, 1964; Whittaker, 1964), the release of acetylcholine from the cortical surface in amounts which correlate with the state of cortical 'activity' (MacIntosh & Oborin, 1953; Mitchell, 1963), the presence of choline acetylase and cholinesterase in the cerebral cortex (Hebb, 1957), and particularly with the recent demonstration that acetylcholinesterase is located within nerve terminals impinging upon the bodies of deep pyramidal cells of the cat (Krnjević & Silver, 1965).

However, in view of the recent failure to demonstrate any necessary connexion between the presence of muscarinic receptors on Renshaw cells (Curtis & Ryall, 1966*c*), spinal interneurones (Curtis *et al.* 1966) and cerebellar Purkinje cells (Curtis & Crawford, 1965; Crawford, Curtis, Voorhoeve & Wilson, 1966) and the operation of acetylcholine as an excitatory transmitter upon these neurones, an alternative explanation may be offered for the acetylcholine sensitivity of deep pyramidal cells. Similar apparently muscarinic receptors have now been detected upon many neurones throughout the feline central nervous system (see Curtis *et al.* 1966), the sensitivity of these cells to acetylcholine is much lower than that of Renshaw cells, and in many instances depression precedes excitation. It may be proposed that muscarinic 'receptors' form part of the external surface of non-synaptic, and possibly merely structural, membrane of mammalian nerve cells: a mechanism whereby choline-containing molecules could form an acetylcholine 'receptor' has recently been outlined by Watkins (1965). Such receptors may function in the normal exchange of ions across cell membranes, although not necessarily under the control of acetylcholine, but are so altered by the presence of comparatively high concentrations of acetylcholine that an ionic movement is initiated which presumably eventually involves a predominantly inward flux of sodium ions (see Watkins, 1965; Curtis *et al.* 1966). A consequence of this hypothesis is that large neurones, particularly those having a complicated dendritic system and hence an extensive surface area, would be more likely to be sensitive to acetylcholine than small neurones. It is thus noteworthy that a high proportion of the large cortical efferent cells, Betz cells, Purkinje cells (Crawford *et al.* 1966), and hippocampal pyramidal cells (Biscoe & Straughan, 1965; Stefanis, 1964*b*) are sensitive to cholinomimetics.

When pentobarbitone was administered electrophoretically, the sensitivity of Betz cells to DLH was occasionally reduced to a greater extent than that to ACh. This observation, together with the relatively high electrophoretic currents required to produce above-threshold concentrations of acetylcholine, suggest that 'muscarinic' receptor sites are widely, and possibly fairly uniformly but sparsely, dispersed over the nerve cell membrane. As it is necessary to position the recording and drug-ejecting micropipettes close to the cell body in order to record extracellular action potentials, the diffusion of cholinomimetics to distant membrane areas may be restricted in a particular instance by the arrangement of glial cells around the neurone. This limitation in the number of 'muscarinic' receptors influenced by acetylcholine may explain in part the extreme variations found in the sensitivity of deep pyramidal cells to acetylcholine, in contrast to the invariable finding that synaptically identified Renshaw cells are excited by electrophoretically administered acetylcholine. Other factors would include differing degrees of tissue hypoxia which would predispose to excitation by weak depolarizing agents, and the known stabilizing effect of general anaesthetics upon membranes. The apparently 'specific' effect of atropine could arise merely from differences in the number of muscarinic and amino acid receptors actuated by the particular excitants, and does not necessarily indicate the functional role of the acetylcholine-sensitive sites. The action of atropine may thus be considered as a stabilization of the membrane, an effect not incompatible with the depressant action of higher concentrations on the amino acid sensitivity of nerve cells (see also Curtis & Phillis, 1960; Krnjević & Phillis, 1963*a*, *c*), rather than as a specific antagonism at muscarinic transmitter receptor sites.

These two explanations of the action of acetylcholine upon deep pyramidal cells, synaptic and non-synaptic, are of course not mutually exclusive, and it is possible that effects involving subsynaptic transmitter receptors are obscured to some extent by effects on non-synaptic structures. The available evidence, particularly the histological demonstration of acetylcholinesterase (Krnjević & Silver, 1965) favours a synaptic role of acetylcholine in the cerebral cortex of the cat, if indeed the presence of this enzyme *within* nerve terminals indicates their cholinergic nature. However, a detailed neurophysiological and pharmacological analysis is required to establish the origin and course of a cholinergic pathway terminating upon deep pyramidal, including Betz, cells, and in such an analysis the effect of atropine upon synaptic responses may be of critical importance.

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