ACETYLCHOLINE RELEASE FROM THE CEREBRAL CORTEX

RATE OF ACETYLCHOLINE RELEASE FROM THE CEREBRAL CORTEX IN RELATION TO ITS PHYSIOLOGICAL ACTIVITY

by

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I INTRODUCTION

Since the demonstration of the presence of acetylcholine in the animal body by Dale and Dudley in 1929 (20) an extensive and complex amount of experimental work has been done to determine the role of ACh in neurophysiological processes.

In 1933 Dale (19) coined the terms cholinergic and adrenergic to describe nerve fibres which act by release of either ACh or noradrenaline and adrenaline. He suggested using the terms cholinoceptive and adrenoceptive to denote sensitivity to the two transmitter substances. Chang and Gaddum in 1933 (10) demonstrated the presence of ACh in the superior cervical ganglion. Dale, Feldberg and Vogt (21) in 1936 demonstrated that ACh is the chemical transmitter at the motor end plate.

While the chemical transmission mediated by ACh in the autonomous nervous system and at the motor end plate has been unquestionably established (19,21,34), the role of ACh in the central nervous system has been less clear. This is due in part to the anatomical complexities of the brain and in part because of technical difficulties. However, as J. Crossland stated in 1960 (13), among chemical transmitter

substances, ACh is the more likely to "hold the key to our final understanding of transmission in the brain."

From the time of the demonstration by Dikshit in 1934 (26) of the presence of ACh in the brain to the present, there has been an impressive amount of indirect evidence (5,12,16,22,31,32,35,50,54,61,65,68,76,84) to show that ACh is a central nervous system transmitter for certain synapses that are as yet not identified.

The only synapse in the central nervous system whose operation can, with assurance, be ascribed to ACh is at the Renshaw cell level. The Renshaw cells of the anterior horn of the spinal cord receive innervation from collateral branches of the motor axons. Eccles and his associates (14,18,27,28,29) demonstrated that Renshaw cells are excited by intra-arterial or iontophoretic application of ACh. Their response to synaptic stimulation is increased and their threshold for ACh reduced by anticholinesterase drugs, and it is depressed by cholinergic blocking agents.

There have been several comparable investigations of the cholinoceptive pharmacology of neurones in various cortical regions (16,17,54,75,79,81) but the evidence obtained has been less definite. In the attempt to identify cholinergic transmission in the central nervous system, an

important step has been the demonstration by MacIntosh and Oborin (61), and Mitchell (65), of the release of ACh from the surface of the cerebral cortex and that this release was related to the physiological activity of the brain. A similar technique of continuous cortical perfusion has been used in the present work to study the effect of stimulation of various nervous structures on the cortical liberation of ACh, and to attempt to assess its significance.

II REVIEW OF LITERATURE

Most of the evidence regarding cholinergic transmission in the central nervous system is indirect. One of the first methods employed was the study of the determinations of the various aspects of the acetylcholine system on the cerebral cortex of mammals (12,13,35,36,41,50,51,58,71,76,90).

ACh content in the CNS

It has been shown by Richter and Crossland (76) and Elliott, Swank and Henderson (31) that the content of ACh of rat and cat brains varies inversely with the level of functional activity, being highest during deep barbiturate anaesthesia and lowest in animal convulsed by drugs or by electrical stimulation. Mantegazzini and Pepeu (63) studied the content of cortical ACh in cats after midbrain hemisection and obtained interesting results. They found that the cortical areas of the "sleeping" hemisphere showed an ACh content markedly higher than those of the "waking" hemisphere. From these data it is inferred that the nervous tissue contains an inactive material, the "bound" or stored ACh, and that during functional activity free ACh is liberated. Free ACh is then destroyed by cholinesterase and the supply of ACh is replenished by the cholinoacetylase system. It

is also suggested that ACh may be involved in the synaptic transmission in the central nervous system and that when cholinergic pathways are activated, there is a release and breakdown of ACh with a temporary fall of the level of stored ACh.

Cholinoacetylase and cholinesterase distribution

Hebb and Silver (44), and Feldberg and Vogt (35) determined the distribution of cholinoacetylase activity of some regions of the CNS and found high concentrations in the cortex, basal ganglia, geniculate bodies and thalamus. This distribution reasonably corresponds with the concentration of ACh (24,33,82). A histochemical survey of the cholinesterases of brains of rats and cats has disclosed the presence of considerable amounts of cholinesterase in the reticular formation, basal ganglia, thalamus and also the neocortex (36,50,51,53,57). Pope (71,72,73), in his studies of the biochemical architecture of rat and man cortex, examined the quantitative intralaminar distribution of acetylcholinesterase. He found that AChE activity is especially great in zones which are relatively acellular and contain high density of fibrillary plexuses of axons and dendrites. Gerebtzoff (40) confirmed the results of Pope and found that the AChE activity in the cerebral cortex is especially intense in the first layer and in the third layer. Hebb, Krnjevic

and Silver (43) studied the effect of undercutting the cortex on the AChE activity in the cat's brain. They undercut slabs of cortex in the suprasylvian and pericruciate areas of cats and examined the state of postulated cholinergic fibres after 1 to 5 weeks. In all cases, the cholinesterase staining was much reduced and only few fibres were visible and unevenly stained. It is reasonable to conclude that these estimates of the enzymatic activity of the cortex provide an indication of the presence and distribution of cholinergic fibres in the CNS. Furthermore, the studies of Hebb et al (43) seem to indicate that cholinergic fibres, which probably innervate cortical cells, "travel into the cortex from deep nuclei or distant cortical regions."

Centrifugation

Recently the intracellular distribution of the various components of the ACh system has been determined by homogenizing the nervous tissue and separating the different fractions by gravity centrifugation. Biochemical analysis of the fractions is then carried out and electron-microscopy employed to identify the particulated material in the various fractions. ACh and cholinoacetylase are both found in the fraction which contains mitochondria. This fraction can further be subdivided by centrifugation in a

gradient of sucrose solutions and ACh and cholinacetylase can be separated from the mitochondria. In the subfraction which contains them there is a large number of particles that can be recognized as detached nerve endings or "nerve ending particles," containing synaptic vesicles (22,23,24, 42,77,78,92,93,94). A further subdivision was carried out by using a technique of hyposmotic shock (22,94) which ruptured the external membrane of the nerve ending particles. Density gradient fractionation of the disrupted material resolved into several subfractions; soluble cytoplasm, isolated synaptic vesicles, microsomes, membrane fragments and mitochondria. ACh and cholinoacetylase became highly concentrated in the fraction containing isolated synaptic vesicles (22,94). AChE is not sharply localized in any one fraction but it seems present in the highest amount in the P_3 fraction of Wittaker or in the M_1 fraction of De Robertis which contain membrane fragments of nerve endings.

Action of ACh and anticholinesterases on the CNS

The action of ACh in the CNS has been studied either by applying the drug itself, or by applying other drugs which are known to modify its action. Small doses of ACh applied directly to the cortex cause an activation, i.e.

desynchronization and fast low amplitude activity in the EEG (1,5,11,33,37,38,64). Larger doses of ACh cause epileptiform spike discharges. These effects are potentiated by eserine or prostigmine. The direct application of anticholinesterases on the cortex (1,5,11,33,64,68) produced alerting reaction and epileptiform activity similar to the one produced by ACh. The arousing effect on the EEG of the anticholinesterases is not associated with a correlated aroused behaviour in the cat (33). Atropine applied locally on the cortex or injected intravenously is an antagonist of many, but not all, actions of ACh and anticholinesterases. The EEG pattern of arousal and the high voltage spike activity are abolished by atropine (1,13,33,64). Atropine, injected alone, produces an EEG pattern similar to sleep, therefore the opposite effect of ACh and antiacetylcholinesterase. However, here again we have an intriguing dissociation of behaviour and electrical activity of the brain. Funderburk et al (39), Wikler (95) and Bradley (3) showed that an animal which received an injection of atropine, although showing an EEG pattern similar to sleep, is behaviourally awakened; furthermore, after atropine it is not possible to produce the EEG pattern of arousal reaction with the usual stimuli.

A more elegant technique of studying the interaction of drugs on synapses in the CNS is by iontophoretic injection of them through multibarrel micropipettes near individual neurones. There have been several extensive investigations of neurones in various brain nuclei by this technique. Neurones fired by electrophoretic application of ACh are found in the inferior colliculi, mesencephalic reticular formation, lateral geniculate bodies, ventrobasal complex of the thalamus and the cerebral cortex (14,15,16,17,75,79,81). Krnjevic and Phillis (54) have demonstrated that cholinoceptive neurons are found in all cortex (about 15% of all cells studied) but particularly on the sensory motor areas. These ACh-sensitive cells were especially common at the depth of 0.8-1.3 mm. and probably they were deep pyramidal cells. These cells tended to occur in clusters. Excitation by ACh was prevented by atropine and hyoscine while tubocurarine was ineffective. Nicotine had no specific action on these neurons while muscarine had an excitatory effect on them. Krnjevic and Phillis concluded that cortical ACh receptors were of a muscarinic type. In these respects the ACh action on these cortical neurones differs from its action on Renshaw cells or sympathetic ganglion cells, being muscarinic in

type rather than nicotinic. Randic, Siminoff and Straughan (75) confirmed these results. They also found that on 5.5% of cells ACh had a depressant action on spontaneous firing or on the firing induced by iontophoretic application of L-glutamate. They suggested that this depressant effect on cortical neurones might be brought about in three ways; (a) ACh may depress the excitability of the neurones, (b) ACh may have a direct inhibitory effect on the cells, (c) the inhibition may be due to excitation of an intermediate neurone which has inhibitory synaptic connections with the unit under study.

ACh release

Another indirect method to study the role of ACh in the CNS is the quantitative determination of the amount of ACh released from the nervous tissue during various physiological activities. Since 1936 Feldberg and his associates (2,9,32,33) have been studying the release of ACh in the cerebrospinal fluid from the cisterna magna or into perfused ventricles in cats and dogs. They developed a method for perfusing different parts of the cat's cerebral ventricles. They found that ACh, which appears in the perfusing fluid containing anticholinesterase, enters the

perfusate in the anterior and inferior horn of the lateral ventricles as well as in the dorsal and ventral part of the 3rd ventricle, but in varying amounts. The greatest amount of ACh was found to come from the structures lining the anterior horn of the lateral ventricle, namely the caudate nucleus, the olfactory grey matter and the septum. The smallest amount of ACh came from the structures lining the ventral half of the 3rd ventricle, the wall of which contains hypothalamic nuclei.

In 1953 MacIntosh and Oborin (61) showed that ACh continually diffuses from the surface of the cortex into an eserinized saline solution contained in a small cup of lucite, and that the amount of ACh which enters the pool diminishes when the depth of anaesthesia is increased. They also showed that undercutting of the cortex entirely abolishes the release of ACh. Mitchell (65) used the same technique of obtaining samples from a lucite cylinder placed on the cortex and containing 1 ml. of eserinized Ringer's solution which was left in contact with the cortex for about 15'. In all experiments Mitchell utilized atropine because he found that this substance increased the output of ACh to a considerable degree. He demonstrated that the rate of liberation of ACh is directly related to

the electrical activity of the underlying cortex. The release of ACh from the surface of the cerebral cortex has also been studied by Szerb (83,84) and by Jasper et al (49). Szerb found that the average ACh output from the cortex in non-anaesthetized cats was of 0.99 ng/min/cm^2 of cortex (SD 0.51) and that the intravenous administration of atropine (1 mg/kg) increased the ACh output threefold with an average of 3.33 ng/min/cm^2 of cortex (SD 1.11). Jasper et al obtained an average cortical release of ACh of 1.25 ng/min/cm^2 of cortex in intact cats. They also studied the ACh output in encéphale and cerveau isolé preparations. They found that the cortex of the "sleeping" brain showed a lower ACh output than the cortex of the "waking" brain.

ACh and epilepsy

The production of convulsions by local application of ACh on the cortex aroused interest in the possibility that seizures might occur because of a faulty mechanism in the ACh system. Forster (37) pointed out that ACh acts as a true convulsant in producing focal seizures and that it is unique among known convulsants in being normally present in the CNS. The problem is indeed rather complex.

There is no doubt that under experimental conditions direct cortical application of ACh, as well as inhibition of acetylcholinesterase and consequent accumulation of free ACh in the brain, leads to epileptic activity. Furthermore, seizures produced by pentylenetetrazol (Metrazol) or by electroshocks determine an increase in the output of ACh from the cortex (65) and a decrease of ACh content of the brain (76). Another line of evidence which implicates ACh as the convulsant agent, is the demonstration by Pope et al (73) that cholinesterase activity in human epileptigenic areas and in epileptogenic aluminum-cream produced foci of monkeys is significantly higher than in normal control areas. This was also confirmed by Tower and Elliott (87,88). In keeping with these data are the findings of detectable levels of ACh in the cerebrospinal fluid of patients with epilepsy (89), and the results of Purpura (74) who reported selective activation of epileptogenic foci in two epileptic patients by topical application to the cortex of 2% ACh solution.

The study of the ACh metabolism of epileptogenic cortex produced conflicting results (see Tower 1960). Tower and Elliott (88) reported that slices of epileptogenic human cortex, incubated aerobically in glucose and saline

medium containing eserine, produced free ACh at the same rate as normal human cortex but their contents of "bound" ACh failed to increase during incubation, as is the case in normal cortex. The same results were obtained with the cortex of cats which were undergoing convulsions because of previous administration of methionine sulfoximide. However, in 1958 Pappius and Elliott (67), using a similar technique, were unable to confirm or reproduce the same results. The effect of ACh antagonists, such as atropine and hyoscine, has also been thoroughly investigated. Atropine, applied either locally or injected intravenously, blocks the epileptic activity induced by ACh or antiacetylcholinesterases (1,13,33,37,39,64,84). However, atropine does not prevent similar epileptic discharges elicited by electrical cortical stimulation or by pentylenetetrazol. This would suggest that epileptic activity is not necessarily caused by a faulty ACh mechanism, and that more than one mechanism is most likely at play.

III METHODS

Cats weighing 2.0-3.5 kg were used. All surgical procedures were conducted under ether anaesthesia. After the completion of the operation, all cut surfaces were locally anaesthetized by infiltration with 1% novocain and by a generous application of 2% xylocaine ointment; then ether anaesthesia was discontinued. Additional local anaesthesia was given as required, to maintain the animal comfortable and free of pain. The animals were curarized throughout the experiment with gallamine triethiodide (Flaxedil), 1 mg/kg, injected through the femoral vein. They were kept on artificial respiration. In 8 experiments, the cortex was exposed on the two sides and 2 "Polypenco" nylon chambers were gently applied over the surface of the exposed cortex (Fig. 1); in the remaining experiments only one chamber was used. To avoid leakage of fluid from the cup, the external junction between the brain and the cup was sealed by application of warm agar jelly. The chamber was then fixed to the skull with dental cement. The chamber was subsequently filled with Elliott's solution (30) containing prostigmine bromide 7.5×10^{-5} g./ml., and a continuous perfusion was carried out with a flow of

approximately 4 ml per hour. Each cup was covering 1.1 cm^2 of cortex. The fluid inside the chamber was stirred by a stream of oxygen bubbles. The cups were always applied over the primary somatosensory cortex. The solutions were kept in a thermos at a constant temperature of 37°, ± 0.5°; they reached the cortex through a calibrated perfusion set and were then collected in a graduated tube maintained by ice at 0 centigrade. Collection of samples was started only 30 minutes after the beginning of the perfusion and samples were collected every 15 minutes. To avoid entry of cerebrospinal fluid in the chamber, and to balance the CSF pressure against the fluid in the cup, the cisterna magna was connected to an external reservoir through a cannula.

Assay and identification of ACh

The ACh content of the specimens was assayed on the prostigminized frog rectus abdominis and/or on the prostigminized fundal strip of rat (10,62,91). The frog's rectus abdominis muscle was set up in a 5 ml bath of Ringer frog heart solution, containing 7.5×10^{-5} g./ml. of prostigmine bromide, and was continuously oxygenated through a bubbler. The muscle contractions were recorded by means of a force displacement transducer Grass FT-03, connected to an Offner

DC amplifier and transcripted with an Esterline Angus graphic ammeter (Fig. 2 and Fig. 3). The muscle was left for 30' in the prostigminized Ringer solution which was frequently changed to allow maximal sensitization. The muscle was then tested several times with a small amount of standard solutions of ACh chloride to ensure that its sensitivity was constant. If these conditions were fulfilled, the assay of the samples was started. The preparation would regularly detect as little as 20 ng of ACh in the 5 ml bath. The action of ACh in this preparation was a slow one (10,62), therefore the ACh or the samples were left in contact with the preparation for three minutes. The assay was repeated every 10'. All results are expressed in terms of ACh chloride. Each sample from the chamber had a volume between 1 to 2 ml. Therefore, it was impossible to prepare standard ACh solutions in inactivated chamber fluid, as suggested by Feldberg (32). However, in the course of each series of samples from one experiment pooled cup samples were treated with 1/10th volume of N-NaOH, allowed to stand at room temperature for about 30' and then neutralized with N-HCl. These samples were inactive when tested on the frog rectus. Similarly, when ACh chloride, in known amount was added, to these inactivated samples

and then assayed against standard ACh, the results were comparable. This suggests that interfering substances were not present in significant amounts. Few samples were assayed on a strip of fundus from a rat stomach suspended in a 9 ml bath of prostigminized Tyrode solution at 37° (91). Methysergide bimaleate (Sansert) was added to the bathing fluid, to render the muscle insensitive to 5-hydroxytryptamine. The sensitivity of this preparation was of 5 to 10 ng of ACh. There was a fairly good agreement (±15%) in terms of ACh between the 2 methods.

Electrical stimulation and recording

Bipolar surface silver electrodes were used to record the activity of the cortex. In all experiments, electrocorticograms were obtained from the sigmoid gyrus inside the chamber, as well as from the posterior suprasylvian gyrus outside the cup, and were then displayed on paper with an 8 channel Offner dynograph. Evoked potentials were recorded with monopolar silver electrodes on a Dumont dual-beam cathode-ray oscilloscope whose sweep was synchronized with the stimulus and photographed with a Grass camera. Direct electrical stimulation of central nervous tissue was applied by bipolar steel electrodes, insulated except at the very tip, and with the two tips 1 mm apart. Bipolar depth

electrodes were inserted stereotaxically in the mesencephalic reticular formation, according to the coordinates of Jasper and Ajmone-Marsan (48). The stimulation of the superficial radial nerve was carried out with a bipolar Harvard steel electrode and it was delivered with a Grass model S4 stimulator through a radio-frequency isolation unit. The stimulus current strength was measured throughout the experiment by inserting a monitoring resistance of 100 ohms, in series with the stimulating circuit, between the electrode and the isolation unit (Fig. 4). The voltage across the 100 ohms resistor was measured on an oscilloscope with a differential input and converted to current by Ohm's law.

IV RESULTS

Mitchell, studying the time course of the liberation of ACh from the cortex into the chamber's solution, concluded that ACh leaves the cortex by simple diffusion and that an equilibrium concentration is reached and maintained at 15-20 minutes. Mitchell also demonstrated a similar exchange in the opposite direction (65). We repeated the same type of experiment in one locally anaesthetized cat, and collected samples left in contact with the cortex for periods of 5, 10, 15, 20 and 25 minutes. The release of ACh was proportional to the time for the first 10-15 minutes, and the equilibrium was reached at 10 minutes. In the same animal we then collected samples by continuous perfusion of the cortex. As clearly shown in Figure 5, the amount of released ACh is considerably higher here than in specimens obtained by the previous method. This is probably due to the fact that continuous cortical perfusion maintains a low concentration of ACh in the cup's solution and thus reduces the rediffusion of ACh into the cortex. This method of local perfusion of the cortex was therefore employed throughout our experiments.

ACh release in the normal waking state

As is shown in Table I, the variations in ACh output from any single cat under local anaesthesia were small; even smaller were the differences between the outputs from the two sides of the cortex. However, large variations occurred from animal to animal. These variations may have been due to different stages of alertness and/or to individual variations. The average output of ACh in the normal waking state, based on 33 samples from 6 cats, was 3.06 ng/min/cm^2 of cortex with a SD of 1.05.

Factors influencing the rate of ACh release

The release of ACh from the surface of the cortex was approximately proportional to the functional activity of the brain. Thus, under deep barbiturate anaesthesia the ACh output was rather low, ranging from 0.3 to 0.8 ng/min/cm^2 of cortex. On the other hand, paroxysmal epileptic activity produced by pentylenetetrazol (100 mg/kg) increased the ACh output by about 115-210% above the resting level.

Our locally anaesthetized preparations, immobilized with gallamine triethiodide, were often drowsy or sleepy when not aroused by gentle stroking of their backs. During this light natural sleep, the animal's pupils were constricted and the ECoG showed spindle bursts. The average ACh output

Table 1

ACh Release (ng/min/cm^2 cortex) in the Normal Waking State

Animal	No. of samples	Mean	Standard Deviation
64–153	5	4.01	0.48
64–294	5	2.46	0.42
64–302	7	2.74	0.42
64–324	5	1.76	0.17
64–450	6	4.35	0.32
64–455	5	1.85	0.04

from the cortex was 2.02 ng/min/cm^2 of cortex, with a SD of 0.49. In light-sleeping animals the perfusion of prostigmine caused the appearance of fast asynchronous activity, which was limited to the perfused area. The remaining cortex presented slow waves and sleep spindles, well in keeping with the cat's drowsiness on the ECoG. In 5 out of 10 cats this asynchronous activity was later substituted by high voltage spiking, again limited to the region perfused. The spiking was similar to the one described by Miller et al (64), Brenner and Merrit (5), and others (37,38,84). The time of onset of this epileptic activity, as well as the shape of the spikes, varied considerably. Both these effects were abolished by atropine. One minute after the injection of atropine sulfate 1 mg/kg intravenously, the fast low-amplitude asynchronous activity was blocked and slow waves and sleep spindles reappeared over the perfused area (Fig. 6). The epileptic spike discharges were similarly suppressed. The correlation between this focal cortical activity and the amount of ACh released from the cortex, yielded interesting results. The presence of local desynchronization, or of high voltage spiking, did not change the ACh output in any appreciable way (Fig. 6 and 7). However, the administration of atropine

always increased the liberation of ACh by at least 100%, both in the unanaesthetized and the anaesthetized cortex (Fig. 6,7,8,10).

Moruzzi and Magoun (66) demonstrated in 1949 that the stimulation of the brain stem reticular formation in sleeping animals causes activation of the EEG and arousal. In the present work the high frequency stimulation (100-200 stimuli/sec.) of the mesencephalic reticular formation, as shown in Figure 8, produced an arousal which was accompanied by a 70-80% increase in the liberation of ACh from the surface of the cerebral cortex. Intravenous administration of atropine (1 mg/kg) prevented this activation. However, while the stimulation of the mesencephalic reticular formation did not produce an arousal in the ECoG, it produced an increase of 50-60% in the release of ACh from the cortex.

We studied then the effect of direct cortical and transcallosal stimulation on the release of ACh from the surface of the cortex. The cortical stimulation was carried out with bipolar steel electrodes, with a frequency of 40 stimuli/sec. and pulses of 5 msec. duration. The current-strength used varied from 0.5 to 0.8 milliamperes. Direct stimulation of the somatosensory cortex in locally anaesthetized animals did not change the ACh output of the cortex. Also the

contralateral cortex, which was often simultaneously stimulated via the transcallosal pathways, did not show any change in the ACh output (Fig. 9). We also tested the influence of atropine upon after-discharge elicited by repetitive cortical stimulation. We have shown previously that atropine blocks the epileptic activity produced by anti-cholinesterase yet the administration of atropine has no effect on the after-discharge, except on the level of ACh output. In an atropinized cat, direct cortical stimulation increases the ACh release by 50-150% (Fig. 10).

The effect of peripheral afferent stimulation on the release of ACh was studied by slow stimulation (1 stimulus/sec. or 1 stimulus/5 sec.) of the superficial radial nerve in cats, under local and general anaesthesia. In either case this excitation caused an increase of 40-50% in ACh output from the contralateral cortex.

ACh and epileptic activity

In our attempt to analyze the correlation between epilepsy and ACh, we produced experimental epileptic discharges by means of electrical stimulation and convulsant drugs. The criteria for considering a paroxysmal discharge as epileptic were those outlined by Penfield and Jasper (69), "not only an excessive activation of group of neurones in

the central nervous system, but an abnormal paroxysmal discharge usually hypersynchronous as well as excessive, and usually self-sustained, once precipitated by various agents."

A sustained local after-discharge evoked by electrical stimulation of the cerebral cortex, as already described, increased the release of ACh only in atropinized cats. Epileptic activity produced by cortical perfusion with prostigmine bromide did not influence the liberation of ACh. The intravenous injection of 100 mg/kg of pentylenetetrazol (Metrazol) elicited diffuse epileptiform discharges and increased the ACh release from the cortex by 115-210%. Atropine injection did not prevent the convulsive effects of the drug or the increase of ACh output. The application of a small (2 x 2 mm) filter paper, soaked with 1% solution of strychnine, on the surface of the cerebral cortex produced rhythmic repetitive spikes after 3-10 minutes. The ACh output during this paroxysmal activity was either normal or increased by 50-60%. The increase in ACh release occurred only when the epileptic activity was widely spread to diffuse cortical, and probably subcortical, areas.

V DISCUSSION

Before discussing the problem of ACh release from the cerebral cortex, we should evaluate the methods of measurement of ACh. We should consider the possibility that the substance present in the brain perfusate, which contracts the eserinized rectus abdominis of the frog, may not be ACh but a related substance.

Recently Ryall, Stone and Watkins (78) investigated this possibility. They studied the cholinomimetic activity of crude mitochondrial fraction obtained by differential centrifugation of brain homogenates. The crude mitochondrial preparation was used to increase the likelihood that any cholinomimetic substance detected may be derived from nerve terminals. The extracts were subjected to parallel biological assays, before and after purification by electrophoresis and chromatography. They found that in all tests the cholinomimetic activity was qualitatively and quantitatively similar to that of ACh. They concluded that they had provided "substantial evidence that the cholinomimetic activity was due to ACh and not to a related substance." The same opinion is held by MacIntosh "I know of no convincing evidence that substance other than ACh contribute importantly to the measurement in the case

of mammalian nervous tissue" (60). We may, therefore, be reasonably certain that the cholinomimetic activity of our brain perfusates was due to ACh. However, the necessity of still using a biological assay and the fact that we are dealing with ultramicro quantity of ACh represent a situation far from perfect. It is evident that because of the limitations of this measuring system, small differences in ACh output during different physiological activities, which may be of significance, will not be detected. On the other hand, until a sensitive reproducible chemical method can be devised, it is unwise to rely on small variations in ACh output and caution has to be inherent in the conclusions.

The present experiments show that the amount of ACh released from the surface of the cerebral cortex is roughly proportional to the physiological activity of the brain. Thus, the ACh output during barbiturate anaesthesia was 0.3-0.8 ng/min/cm^2 of cortex. During light natural sleep, as judged by the ECog and pupillary constriction, the average rate of release was 2.02 ng/min/cm^2 of cortical surface. In the normal waking state the average rate was about 3.06 ng/min/cm^2. These results agree fairly well with the data of MacIntosh and Oborin (61) and Mitchell (65).

However, the values of ACh released from the cortex in our experiments are higher than the ones obtained by these authors. This is most likely due to differences in the technique of collecting ACh. Mitchell studied the process of transfer of ACh from the cortex to the cup solution; he demonstrated that ACh leaves the cortex by simple diffusion and that an equilibrium concentration between cortex and perfusate solution is reached and maintained for 15-20 minutes. He also demonstrated a similar exchange of ACh in the opposite direction, from the perfusate to the cortex. It is not surprising, therefore, that the technique of collecting the perfusate after it has been left in contact with the cortex for 10-15 minutes, gave lower values of ACh than our method of continuous perfusion of the cortex. We know from the first law of diffusion that the flow is proportional to the gradient of concentration and, therefore, the rate of diffusion of ACh in the first few minutes is higher than in the last minutes. With our method of continuous local perfusion we maintained a constant low concentration of ACh in the perfusate, facilitating the diffusion from the cortex to the fluid and reducing the transfer of ACh in the opposite direction.

It is interesting to try to correlate the cortical output of ACh with the changes in the ACh content of the cortex. There seems to be an inverse relationship between the stored ACh contained in the brain and the free ACh liberated from the cortex. For instance, the amount of ACh present in the brain increases during anaesthesia while the ACh output of the cortical surface decreases. This indeed supports the idea that the increase of ACh in the brain during anaesthesia is due to a decrease in liberation of free ACh from the nervous tissue containing it, because of lessening of afferent stimuli reaching the cortical level. The inverse is also true; during some states of enhanced cortical activity, i.e. Metrazol seizures, the content of ACh decreases while the liberation of ACh augments (31,76,85,86).

Krnjevic and Miledi (55) and Krnjevic and Mitchell (56) studied the release of ACh from the rat hemidiaphragm by stimulation of the phrenic nerve. They found that the maximal release of ACh resulted from activating approximately 10,000 cholinergic nerve endings at 2 stimuli/sec. and it was 1.8×10^{-15} g/stimulus/nerve ending. Expressed in time, the maximal release was 216×10^{-15} g/minute/nerve ending.

If we now assume that a cholinergic ending in the cortex, when activated, will release a similar amount of ACh as at the neuromuscular junction, we may estimate the expected amount of ACh released by 1 mm^3 of cortex. The average neurone density of the cat cerebral cortex calculated by direct histological cell counting by Tower and Elliott (86), as well as by the chemical method of Heller and Elliott (45), has been estimated at about 30,000/mm^3. If we suppose that each cortical neurone has a cholinergic synapse, the maximum release of ACh to be expected would be 6.48 x 10^{-9} g/min/mm^3 of cortex. In the present experiments, the maximum amount of ACh released during metrazol induced seizures in atropinized animal, was of 13.8 x 10^{-9} g/min/cm^2 of cortex. The ACh entering the chamber covering 1 cm^2 of cortex is supposed to originate from a volume of cortical tissue of 0.2 cm^3. Then our maximum ACh output was 69 x 10^{-12} g/min/mm^3 of cortex. This estimate is perhaps too low because it assumes that all the ACh liberated is efficiently collected, and that during our maximum ACh output all cholinergic synapses are activated. Nevertheless, it seems clear that only a small proportion of cortical synapses are cholinergic. These values are in agreement with Mitchell who estimated that only 255 cortical nerve endings/mm^3 liberate ACh in

response to a single peripheral afferent volley. In either case, the cholinergic endings have been estimated at about 1% of all existing cortical terminals. Perhaps a more realistic figure for the number of cholinergic terminals in the cerebral cortex can be deducted from the results of iontophoretic application of ACh. Krnjevic and Phillis (54) found that in the sensory motor areas of the cat's cerebral cortex only 15% of all cells studied were cholinoceptive. It is reasonable to assume that each cholinoceptive cell receives cholinergic endings. Therefore, this value of 15% will also apply to the number of cholinergic synapses.

There is an increasing amount of evidence that the ACh released at the cortical surface is not generated by intracortical synaptic endings, but mainly by cortical terminals of fibres from subcortical structures. The anatomical work of Krnjevic and Silver (57) and of Hebb et al (43), as well as the physiological work of MacIntosh and Oborin (61), are in favor of this hypothesis. In the attempts to identify specific cholinergic pathways to the cortex, some evidence has been accumulated which indicates that cortical activation in normal states of arousal from natural sleep may be mediated by ACh liberated from cortical

afferents, probably of reticular origin. Small doses of ACh and anticholinesterases, applied locally on the cortex of drowsy animals, cause the appearance of fast asynchronous activity on the ECoG (5,33,37,38). The same alerting reaction is also observed with intracarotid or intravenous injection of anticholinesterases (25,33). This arousal reaction is easily blocked by administration of atropine (1,3,33,39,83,84). Jasper et al (49) compared the output of ACh from the cortical surface of encéphale isolé and cerveau isolé preparations and demonstrated a higher release of ACh from the cortex of encéphale isolé cats. Mantegazzini and Pepeu, in a similar experiment, studied the content of ACh in the cortex of cats after midbrain hemisection (63,70). They demonstrated a higher content of ACh in the "sleeping" hemisphere. Our own data showed that the rate of ACh released during light natural sleep is significantly less than during a normal waking state. We have also shown that the stimulation of the mesencephalic reticular formation increases the ACh output from the cortical surface, as well as arouses the cortex. The studies of Magoun, Moruzzi, Jasper and their associates (47,59,66,80) have clearly demonstrated that ascending influences from the unspecific thalamocortical projection system upon the cerebral cortex,

are important in initiating and regulating such states as arousal and wakefulness, as well as orientation and attention. Sharpless and Jasper (80) have distinguished tonic and phasic function in the non-specific ascending system. The tonic activation is derived from hypothalamus and midbrain, and it is of slow onset and prolonged duration. The phasic activation is of rapid onset and brief duration, and arises from the thalamic reticular system.

It is, therefore, concluded that the reticular activating system activates cortical cholinoceptive cells through cholinergic neurones. These cholinoceptive cells, as shown by Krnjevic and Phillis (54), are muscarinic in type, and ACh applied to them produces a very slow reaction. It now seems clear that the slow reaction of these muscarinic type receptors permits them to have a background facilitating function, which in turn will modulate the various states of alertness.

The fact that atropine administered intravenously, while preventing the activation of the ECoG by electrical stimulation of the mesencephalic reticular formation, did not prevent the increase of ACh output is of some interest. It suggests that atropine probably blocks the postsynaptic receptor sites of cholinoceptive cortical neurones, without

essentially interfering with the presynaptic terminals.

It is rather unlikely that the cortical afferent terminals from the activating reticular system are the only cortical cholinergic endings. Feldberg (32,35) suggested a possible alternation of the cholinergic and non-cholinergic neurones in the long pathways of the CNS. He reached this conclusion by studying the distribution of enzyme, or enzyme system, which synthesizes ACh over forty separate regions of the CNS of dog. He found that motor and sensory pathways consist of chains of neurones which are alternately cholinergic and non-cholinergic in character.

No significant number of cholinergic interneurones seems to be present in the cortex. Direct cortical and transcallosal stimulation in locally anaesthetized preparations does not increase the liberation of ACh. Paroxysmal activity of the cortical surface, elicited by local application of anticholinesterase, does not change the release of ACh. However, direct cortical stimulation in atropinized animals increases the ACh output. In the latter case, the atropinization of the animal significantly modifies the situation. Actually, as MacIntosh says, "it would appear that the administration of atropine may be the most specific way of increasing the

ACh discharge in the cortex." The reasons of this increase in ACh output are not altogether clear. Atropine appears to block post-synaptic receptor sites of cholinoceptive cortical neurones, permitting more ACh to be released in the superfusing solution. MacIntosh (60) suggests that some neuronal loops contain inhibitory interneurones under cholinergic control, and that the cholinergic blockade in reducing the activity of these elements would allow the whole loop to fire at higher frequency. Therefore, it is reasonable to expect that direct cortical stimulation of the atropinized cortex may involve neuronal loops and reverberatory circuits which under normal conditions would not be stimulated.

So far our discussion has been limited to the classical concept of the action of a chemical transmitter, in particular to the well-accepted theory that a presynaptic impulse liberates the transmitter, in our case ACh, which would act at the postsynaptic level, producing a depolarization of the post-synaptic membrane; if sufficient ACh is liberated, the depolarization reaches a critical level and a spike discharge occurs. However, some authors found this theory unsatisfactory (4,25,52). Koelle (52,53) proposed that ACh mobilized by a conducted pre-synaptic impulse may act

at two sites: post-synaptically, as classically accepted, and prior to that at the pre-synaptic level itself. The ACh acting at the pre-synaptic level will bring about the release of sufficient additional ACh, or some other neurohumoral agent, from the terminal, to initiate the post-synaptic effect. This hypothesis was formulated in an attempt to clarify a few unexplained data. These were, in particular, the presence of cholinesterase in neurones which are considered non-cholinergic such as the dorsal spinal root ganglia and the stellate ganglia (50,51,52); further, the findings of Burn et al (6,7,8) that after administration of sufficient atropine to block the effects of injected ACh, a dose of this substance produces sympathomimetic action. The evidence for Koelle's theory is derived mainly from studies of peripheral synapses, and to extrapolate from the peripheral to the central nervous system may be of questionable value. Until more clear evidence has been gathered in favor of a pre-synaptic action of a transmitter, there is no reason to modify our classical concept of synaptic transmission. Another theory which should be cited to complete the picture is the hypothesis of Desmedt and La Grutta (25) and Bremer (4), of a "local hormone" action of ACh on the cerebral cortex. Desmedt and La Grutta

studied the role of true cholinesterase and pseudo-cholinesterase in the cat's brain. They produced an arousal reaction in sleepy animals by intra-arterial administration of selective inhibitors of true cholinesterase and selective antagonists of pseudo-cholinesterase. In each case a definite threshold dose could be defined, and it was found that pseudo-cholinesterase inhibitors were more effective in producing arousal. They concluded that the pharmacological arousal, produced by anticholinesterase drugs, was related to the inactivation of brain pseudo-cholinesterase rather than to inactivation of brain true cholinesterase. They also pointed out that brain pseudo-cholinesterase is predominantly located in neuroglial cells (Koelle 1954). They proposed that the choline esters responsible for arousal might be secreted like "local hormone" by neuroglial cells, or some neurones. However, the selectivity of the cholinesterase inhibitors was only relative, the doses injected intra-arterially were rather high and no attempt was made to evaluate the effect of these inhibitors by direct local application on the cortex. Furthermore, the evidence presented was insufficient to establish their claims with any certainty.

In conclusion, from the evidence gathered from the literature and from our own data it appears that there are

indeed cholinergic synapses in the cerebral cortex. These synapses represent only a small percentage of the total cortical synapses but they have some interesting features. The receptor sites of these cholinoceptive neurones differ from Renshaw cells or sympathetic ganglion cells in being muscarinic in type rather than nicotinic. Their cholinergic terminals seem to originate from non-specific ascending fibres rather than from specific projections, and they play an important role in the arousal phenomenon.

In our study of the relationship between ACh and epilepsy we found that topical application of ACh or anti-cholinesterases on the cortex produced epileptiform spike discharges, probably by direct activation of cholinoceptive neurones. This epileptic activity is blocked by atropine. However, atropine does not prevent epileptic discharges produced by topic application of strychnine or direct electrical stimulation or pentylenetetrazol administration. Cortical epileptic activity, regardless of how it has been produced, does not determine an increase in the ACh liberation from the cortex. On the other hand, convulsive activity produced by pentylenetetrazol brings about a significant increase in ACh output. These data clearly show that ACh can trigger epileptic activity but an epileptic discharge

may as easily be produced by a different mechanism. If epileptic discharge is thought to be due to an abnormal accumulation of an excitatory substance, this substance does not seem to be ACh.

The possibility that some form of seizures might arise because of a faulty mechanism in the ACh system cannot be entirely discarded. Pope et al (73), for instance, found that cholinesterase activity in human epileptogenic areas, and in experimentally produced epileptogenic foci of monkeys, was higher than in normal control areas. Similar results were obtained by Tower and Elliott (87,88). Another aspect which should be kept in mind is the fact that a change in the ACh system may be the effect and not the cause of seizures. Thus, the administration of pentylenetetrazol which produces convulsive activity in an atropinized animal, with an increase of ACh output from the cortex, indicates that the drug directly activates cholinergic neurones, and that the paroxysmal firing of these neurones is the cause of the increased liberation of ACh.

SUMMARY

The rate of release of ACh from the pial surface of the cerebral cortex, in Flaxedilized cats under local anaesthesia, was determined by means of a special perfusion chamber fixed in the skull, through which Elliott's solution with prostigmine bromide was circulated at the rate of 4 ml. per hour.

During light natural sleep, as judged by the ECoG and pupillary constriction, the average rate of release was about 2 ng/min/cm^2 of cortical surface. In the normal waking state the average rate was about 3 ng/min/cm^2.

Application of prostigmine to the cortical surface caused local desynchronized activation of the ECoG, followed within 1 to 2 hours by epileptiform discharge. The rate of ACh released was not affected by activation or by the presence of epileptiform discharge.

Intravenous atropine sulfate (1 mg/kg) abolished the ECoG activation and epileptiform discharge, while increasing the rate of ACh release by at least 100%.

Direct cortical or transcallosal stimulation increased release rate only in the atropinized preparation. Some increase (40-50%) was produced by peripheral nerve stimulation in non-atropinized preparations.

Stimulation of the mesencephalic reticular formation produced arousal and a 70-80% increase in the liberation of ACh from the cerebral cortex. Intravenous administration of atropine during stimulation of the mesencephalic reticular formation prevented the ECoG activation, but the increase in ACh output persisted.

It is concluded that cortical activation in normal states of arousal from natural sleep may be mediated by ACh liberated from cortical afferent terminals, probably of reticular origin.

Atropine appears to block post-synaptic receptor sites of cholinoceptive cortical neurones, permitting more ACh to be released in the superfusing solution.

ACh may cause epileptic activity but is not the sole mechanism by which an epileptic discharge may be initiated.

Figure 1

Diagram of perfusion chamber in cross section.

S, stimulating electrodes; R, recording electrodes.

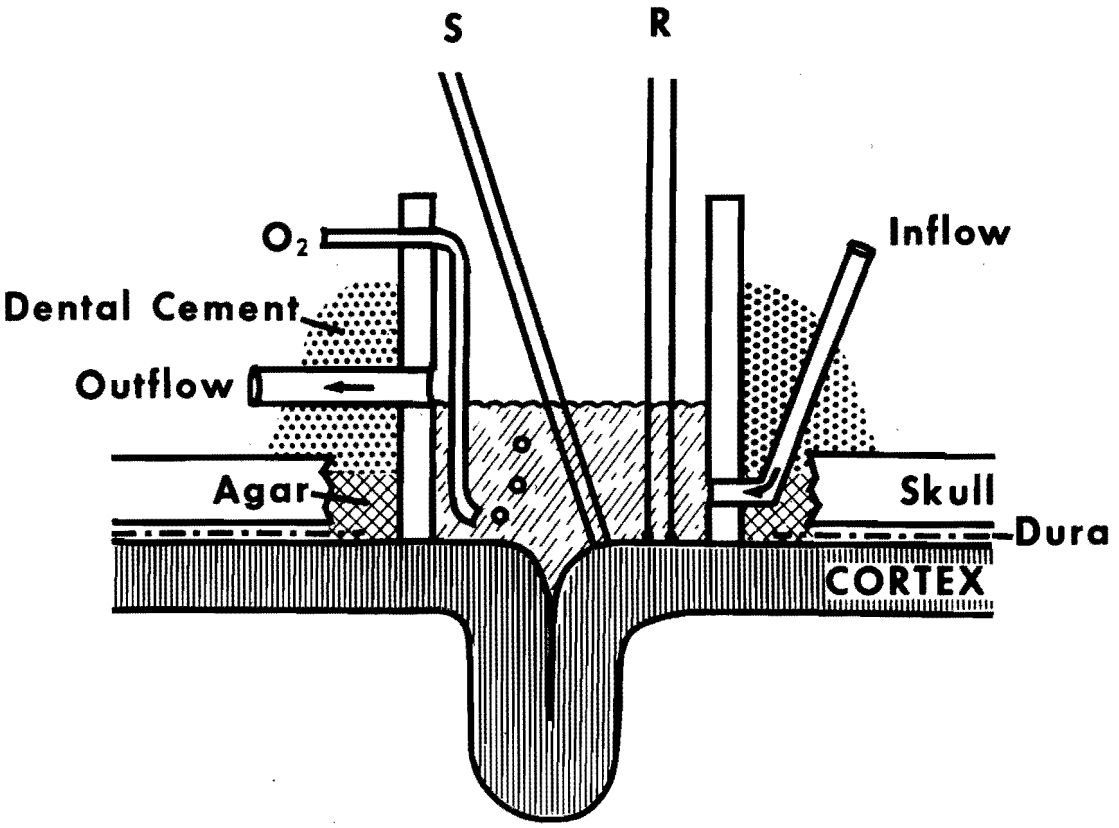

Figure 1

Figure 2

Experimental set-up for the bioassay of ACh in the frog's rectus abdominis.

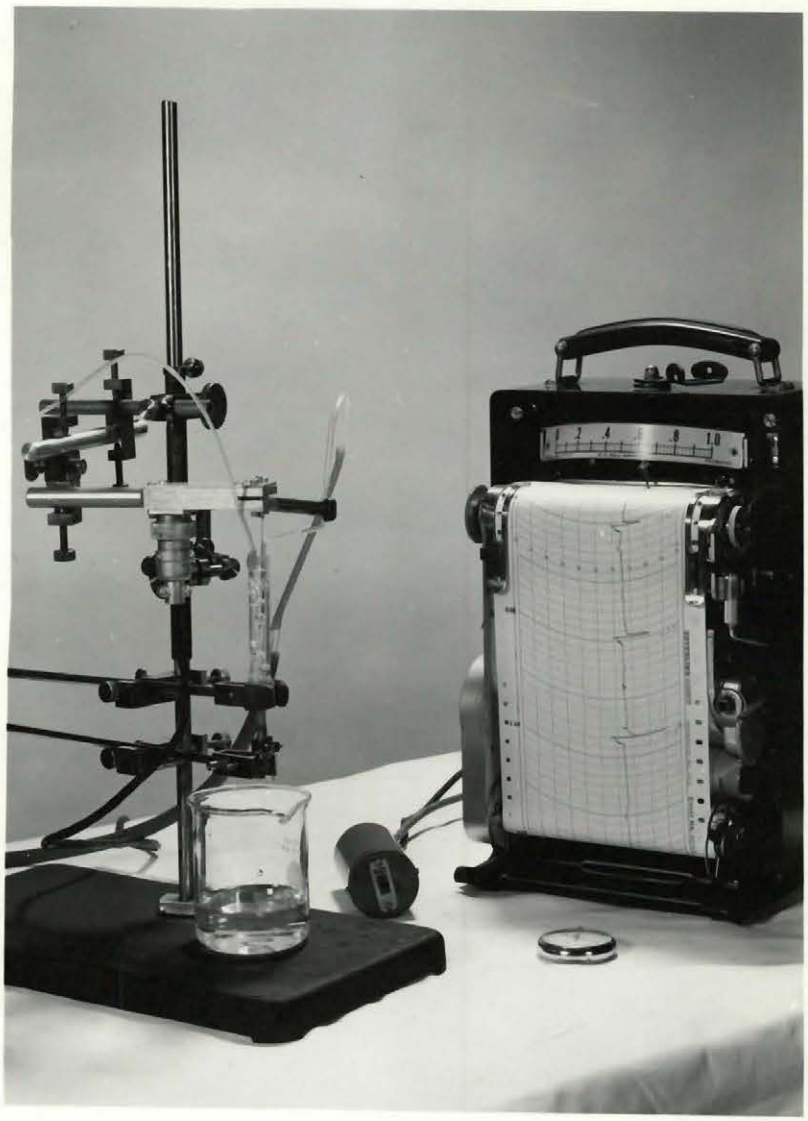

Figure 2

Figure 3

Close-up showing the bath containing the frog's rectus abdominis and the force displacement transducer.

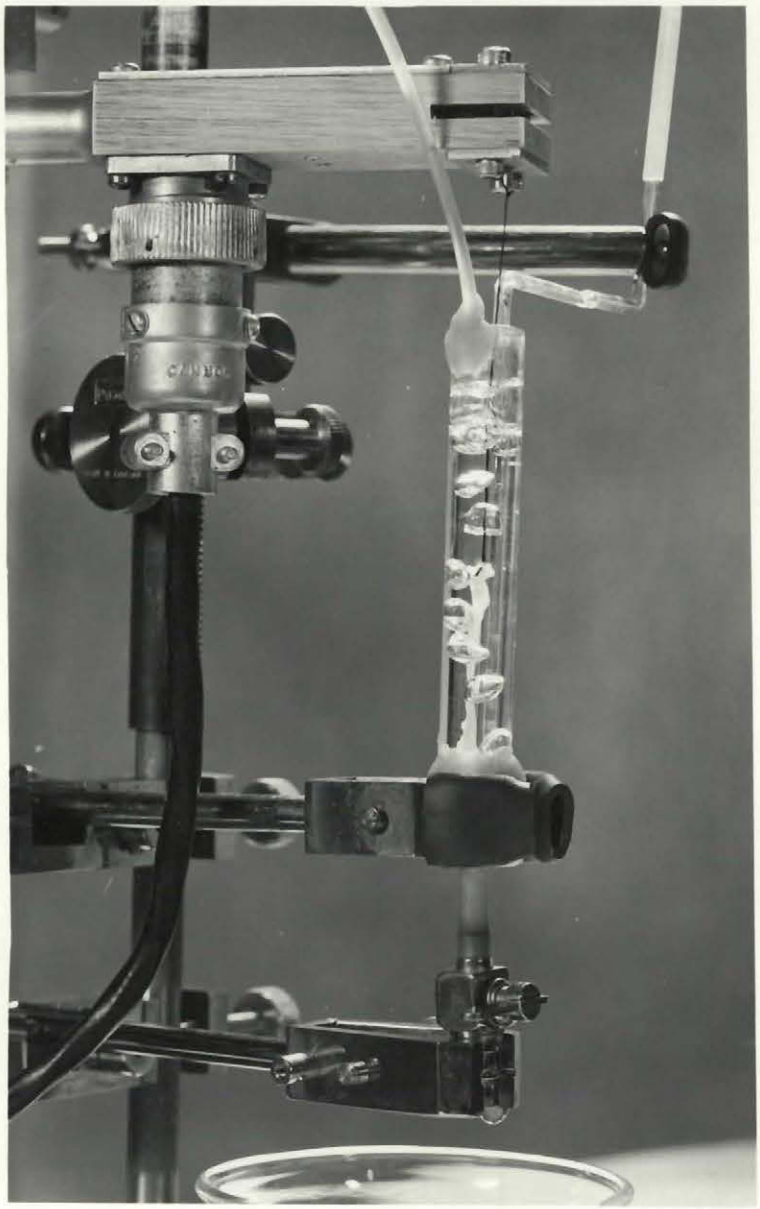

Figure 3

Figure 4

Diagram of the circuit used to measure the stimulus current strength.

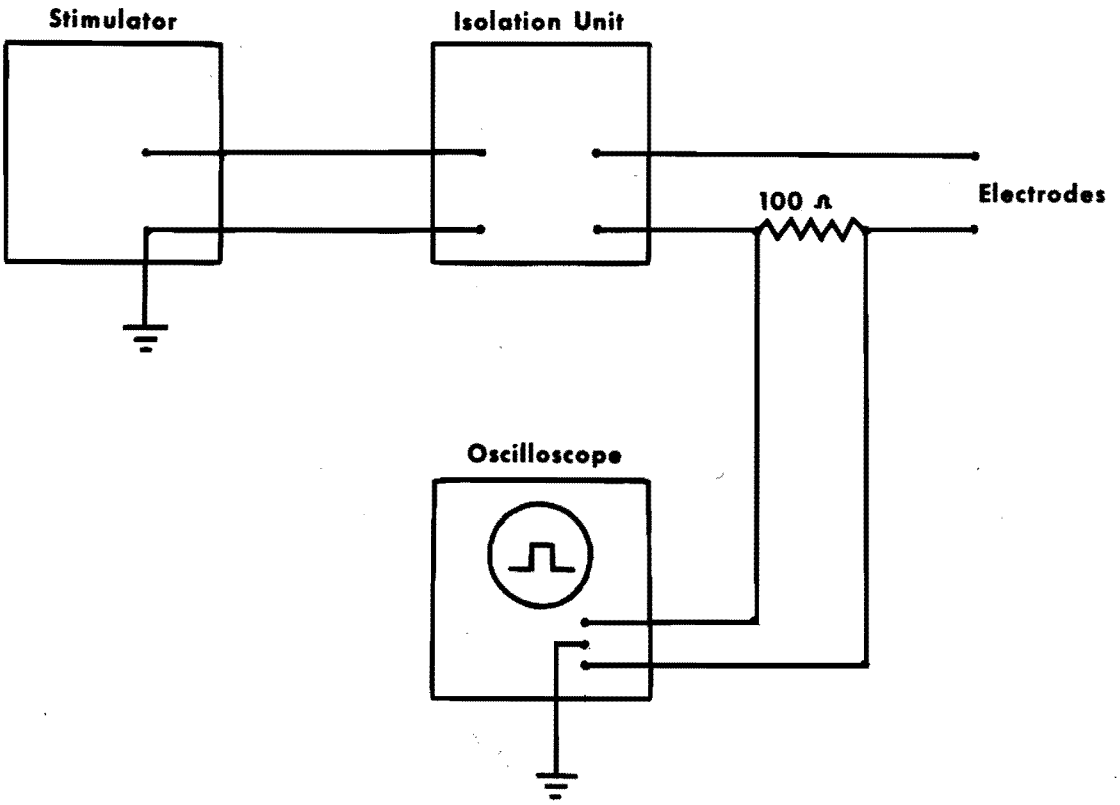

Figure 4

Figure 5

Spontaneous release of ACh from the primary somato-sensory cortex of the cat. The release of ACh from right and left cortex of a solution left in contact with the cortex for 15 minutes (A) is compared with output of the same right and left cortex from a solution which perfused the cortex for 15 minutes (B).

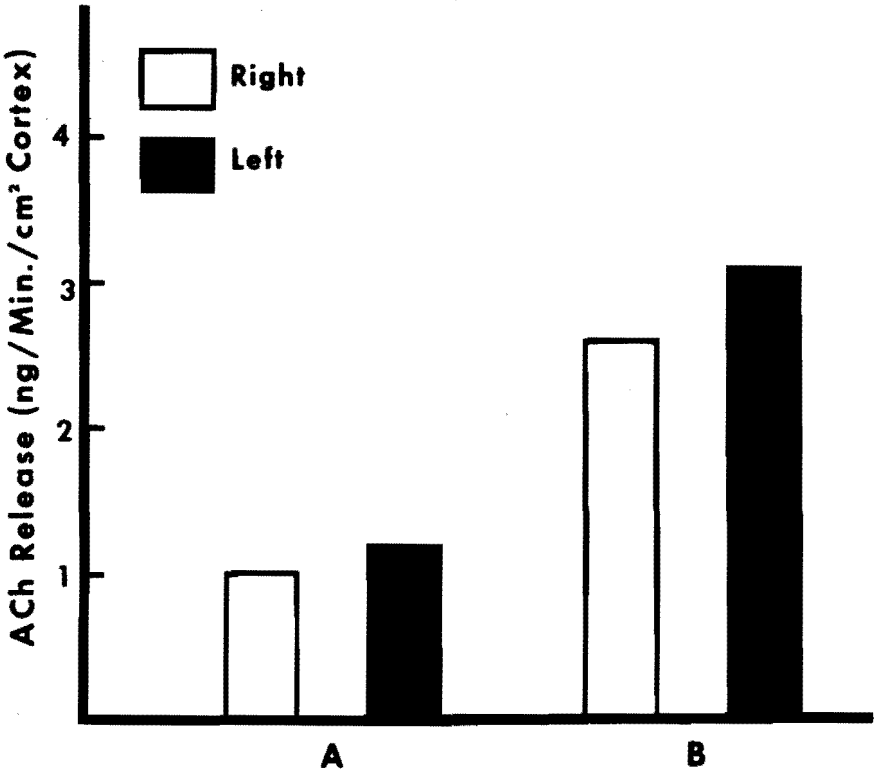

Figure 5

Figure 6

Correlation of electrical activity and ACh release from the cortex.

A, animal in normal drowsy state, sleep spindles are present over all the cortical areas.

B, 60 minutes after the cortical area perfused by prostigmine presents fast asynchronous activity, the ACh output is unchanged.

C, a few minutes after the administration of atropine 1 mg/kg i.v. the cortical activation is blocked and slow waves and sleep spindles reappear over the perfused area, the ACh output is considerably increased.

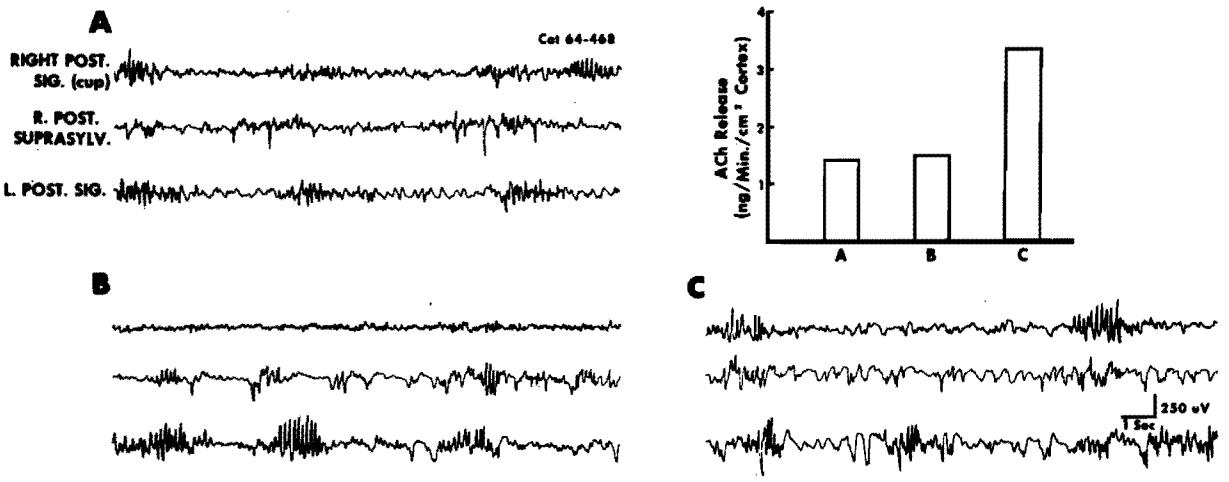
A
Cat 64-468
RIGHT POST. SIG. (cup)
R. POST. SUPRASYLV.
L. POST. SIG.
ACh Release (ng/Min./cm² Cortex)
A
B
C
B
C
250 uV
1 Sec

Figure 6

Figure 7

Correlation of electrical activity and ACh release from the cortex. The collection periods were consecutive.

A, animal in normal waking state.

B, 20 minutes after, continuous high voltage spiking is present over the cortex perfused by prostigmine, however, the ACh output is unchanged.

C, a few minutes after the administration of atropine 1 mg/kg i.v. the epileptic activity is suppressed and the ACh output is increased by 160%.

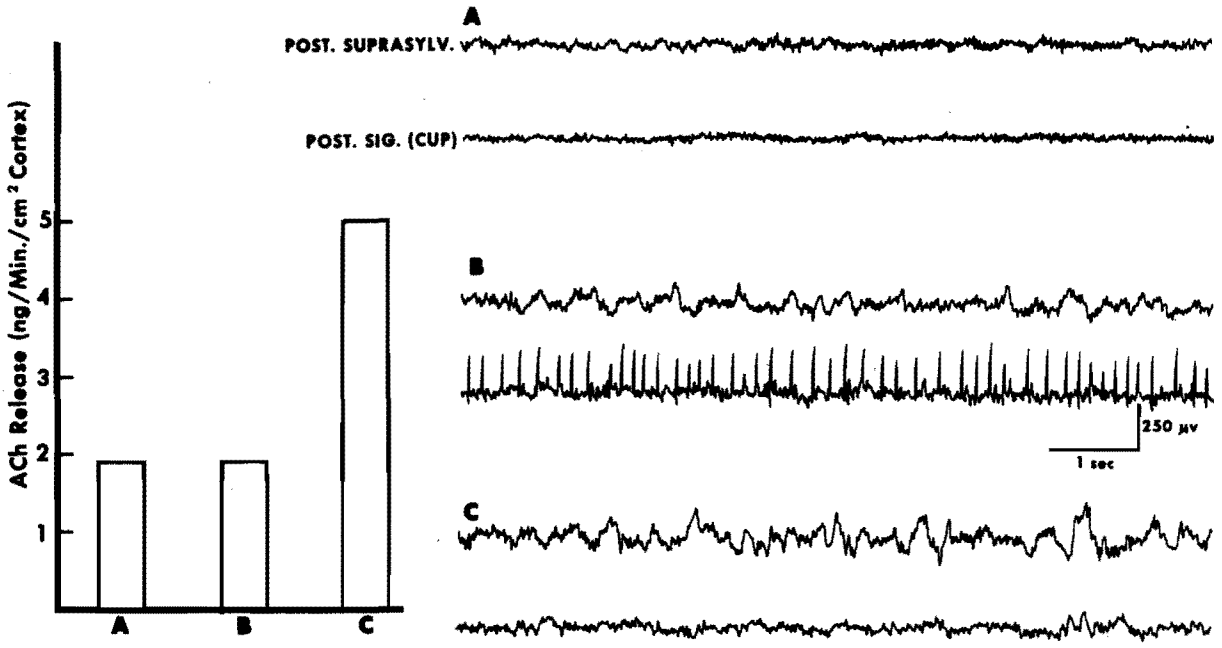
A
POST. SUPRASYLV.
POST. SIG. (CUP)
B
250 μv
1 sec
C
ACh Release (ng/Min./cm² Cortex)
1
2
3
4
5
A
B
C

Figure 7

Figure 8

Release of ACh from the primary somato-sensory cortex of one cat. The first two points represent the ACh output in normal drowsy animal. The other points represent the effect of the stimulation of the mesencephalic reticular formation (SRF) upon the release of ACh. The atropinization of the animal does not prevent the increase in the liberation of ACh by stimulation of the reticular formation. The abscissa represent the time in minutes after the beginning of cortical perfusion with Elliott's solution containing prostigmine.

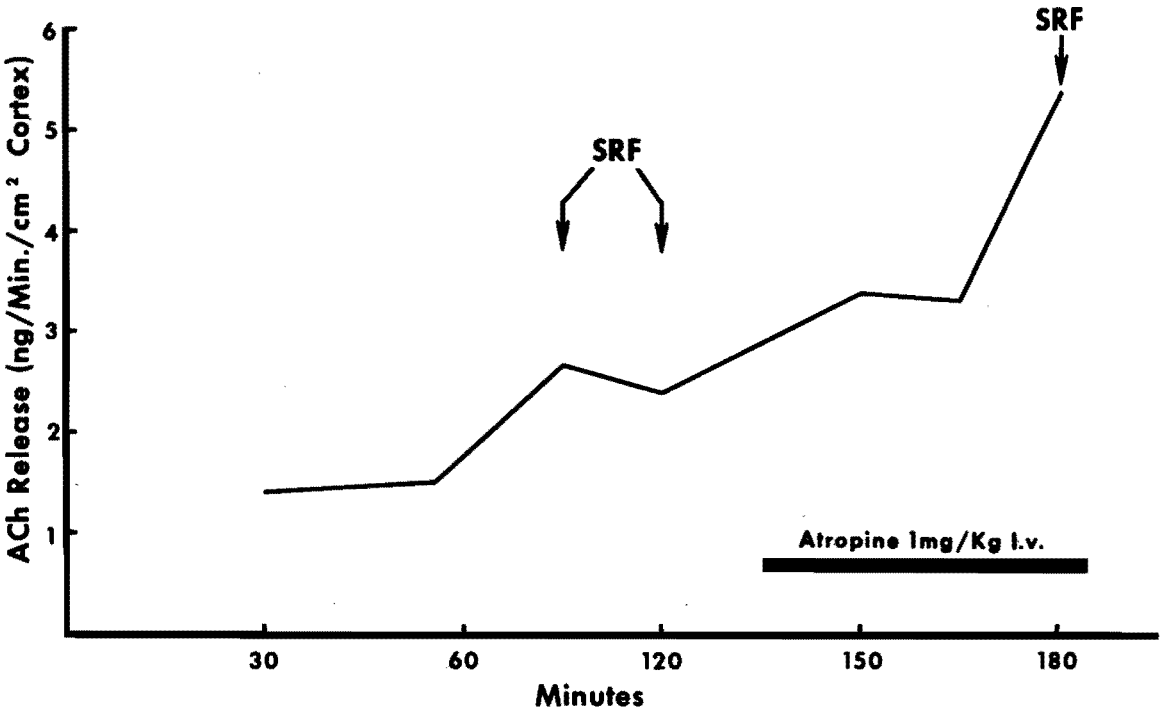
SRF
SRF
ACh Release (ng/Min./cm² Cortex)
6
5
4
3
2
1
Atropine 1mg/Kg I.v.
30
60
120
150
180
Minutes

Figure 8

Figure 9

Effect of direct and transcallosal stimulation on the amount of ACh released from the somatosensory cortex. The first two columns represent the ACh output in the normal awakened animal.

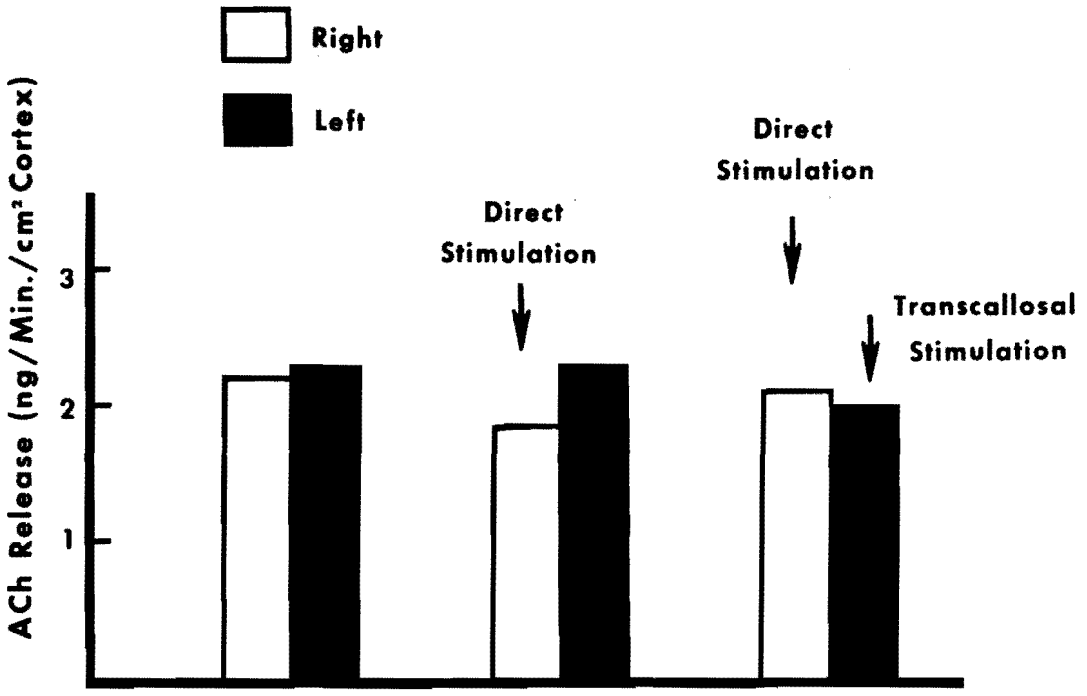
Right
Left
Direct
Stimulation
Direct
Stimulation
Transcallosal
Stimulation
ACh Release (ng/Min./cm² Cortex)
3
2
1

Figure 9

Figure 10

Release of ACh from the primary somato-sensory cortex of one cat. The first two points represent the ACh output in the normal awakened animal. The other points represent the effect of ACh spiking, atropine, nembutal, and direct cortical stimulation on the amount of ACh released. The abscissa represents the time in minutes after the beginning of cortical perfusion with Elliott's solution containing prostigmine.

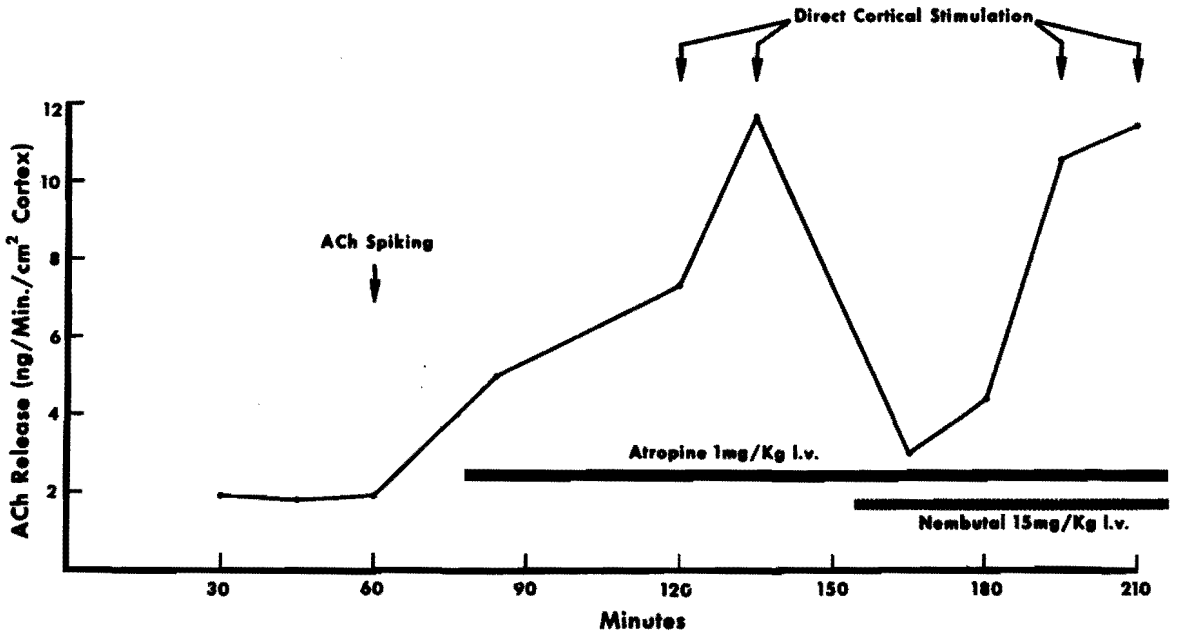
Direct Cortical Stimulation
ACh Spiking
Atropine 1mg/Kg I.v.
Nembutal 15mg/Kg I.v.
ACh Release (ng/Min./cm² Cortex)
12
10
8
6
4
2
30
60
90
120
150
180
210
Minutes

Figure 10

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