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The characteristics of acetylcholine release mechanisms in the auditory cortex

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1. The characteristics of the acetylcholine (ACh) release mechanism have been studied in the auditory cortex of rabbits on stimulation of the ipsilateral medial geniculate nucleus.

2. On stimulation of the medial geniculate nucleus the mean release of ACh from the auditory receiving cortex was 6·1 times the spontaneous release; the mean release from other parts of the cortex was 2·2 times the spontaneous release.

3. The frequency of stimulation most effective in evoking ACh release was found to be 10/sec.

4. Both the spontaneous and evoked release of ACh were reduced by 40–65% in the absence of calcium from the solution bathing the auditory cortex, and increased by 15–25% when the calcium concentration in the bathing solution was doubled.

5. The presence of low concentrations of magnesium in the fluid bathing the cortex was essential for the optimal release of ACh, but high magnesium concentrations lowered this release.

6. The presence of triethylcholine (TEC) in the fluid bathing the auditory cortex reduced both the spontaneous and evoked release of ACh. This reduction was reversed in the presence of choline.

7. The effects of calcium, magnesium and TEC on the ACh release mechanism in the cerebral cortex and at the neuromuscular junction are compared.

There is good evidence for the existence of central ascending cholinergic nerve pathways which originate in the reticular formation and in specific thalamic nuclei and terminate in the cerebral cortex. Stimulation of somatosensory nerve pathways, visual pathways and the reticular formation is known to produce an increased release of acetylcholine (ACh) from nerve terminals in the cortex, and when the specific sensory systems are stimulated this release is highest from associated cortical receiving areas (Mitchell, 1963; Kanai & Szerb, 1965; Celesia & Jasper, 1966; Collier & Mitchell, 1966, 1967). It has been suggested that these cholinergic path-

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ways are concerned with arousal and the maintenance of consciousness, and this view is supported by experiments which show an association between central ACh release, behaviour and e.e.g. activity in animals (Celesia & Jasper, 1966 ; Collier & Mitchell, 1967 ; Szerb, 1967).

The auditory pathway and cortex in rabbits has recently been studied and the spontaneous and evoked cortical release of ACh found to be similar to that of other sensory systems (Neal, Hemsworth & Mitchell, 1968). With the auditory system, however, it has been possible to evoke exceptionally well defined increases in ACh release from the cortex by stimulation of the ipsilateral medial geniculate nucleus, and this has allowed a detailed study of the mechanism of cortical ACh release.

The characteristics of this release have been examined by changing the concentration of calcium and magnesium ions in the physiological saline solution bathing the cortex and by the topical application of triethylcholine (TEC). The mechanism of ACh release was found to have several features in common with the characteristics of ACh release from nerve terminals at the neuromuscular junction.

Part of the work described in this paper has been demonstrated to the British Pharmacological Society (Hemsworth & Mitchell, 1967).

Methods

Acetylcholine was collected from the surface of the brain by the Perspex collecting cylinder technique (MacIntosh & Oborin, 1953 ; Mitchell, 1963).

Adult rabbits were anaesthetized with Dial compound (allobarbitone and urethane ; Ciba Ltd. ; 0·7–0·9 ml./kg intraperitoneally). The left auditory cortex and, in some experiments, the somatosensory and visual (non-auditory) areas of the cortex were widely exposed.

Perspex cylinders, which covered 0·5 cm^2 of cortex, were placed on the surface of the brain as described by Collier & Mitchell (1966). The cups were filled with 0·5 ml. Ringer-Locke solution (NaCl 9·0 ; KCl 0·42 ; $CaCl_2$ 0·24 ; $NaHCO_3$ 0·2 ; glucose 2·0 g/l.) at 37° C containing eserine sulphate (10^{-4} g/ml.) and atropine sulphate ($4{\cdot}0 \times 10^{-7}$ g/ml.). The solution collected at the end of the first 30 min period was discarded, but after this samples were collected every 15 min and retained for biological assay. The samples were always assayed within 12 hr of collection. After three to six control collection periods the medial geniculate nucleus was stimulated for a 15 min period ; this sample was then collected and followed by a further three to six control samples.

The medial geniculate nucleus was stimulated by a coaxial electrode inserted stereotactically through a slit in the dura. Stimulation was with rectangular pulses of 1 msec duration at the frequencies specified in the text. The current delivered by the stimulating electrode was monitored by means of an oscilloscope and was maintained at 2–3 mA. The concentrations of calcium and magnesium ions in the bathing fluid were varied by adding or removing calcium chloride or by adding magnesium chloride to the Ringer-Locke solution ; these modifications did not alter the volume and resulted in only very small changes in osmolarity of the solution bathing the cortex.

When the effects of topical application of TEC bromide were studied the same

collection and stimulation procedure was used both in the absence and in the presence of TEC.

Assay of acetylcholine

The solutions were removed from the collecting cups and diluted 1 : 1·8 with distilled water and assayed on the dorsal muscle of the leech sensitized with eserine sulphate (5×10^{-5} g/ml.).

Standard solutions of ACh chloride were prepared in diluted Ringer-Locke solution containing eserine and atropine in the same concentrations as in the diluted test solutions. Calcium ions potentiated and magnesium ions depressed the response of the leech muscle to ACh and therefore, where appropriate, the standard ACh solution was made up in a solution containing the same concentrations of calcium chloride and magnesium chloride as those present in the test samples. TEC also potentiated the response of the leech muscle to ACh and therefore an equivalent amount of TEC to that present in the test samples was added to the standard ACh solution where appropriate.

In most experiments tests were made to check that the activity present was due to ACh or some similar choline ester. The tests used were those described by Mitchell (1963). All results are expressed in terms of ACh chloride (ng) released during a 15 min collection period from 0·5 cm² cortex.

Results

Table 1 shows the mean spontaneous release of ACh from the auditory and non-auditory (visual) cortex on the same side during three to five collection periods in seven experiments. The release varied considerably from animal to animal, but the spontaneous release in any one animal was fairly constant, as shown in Fig. 1.

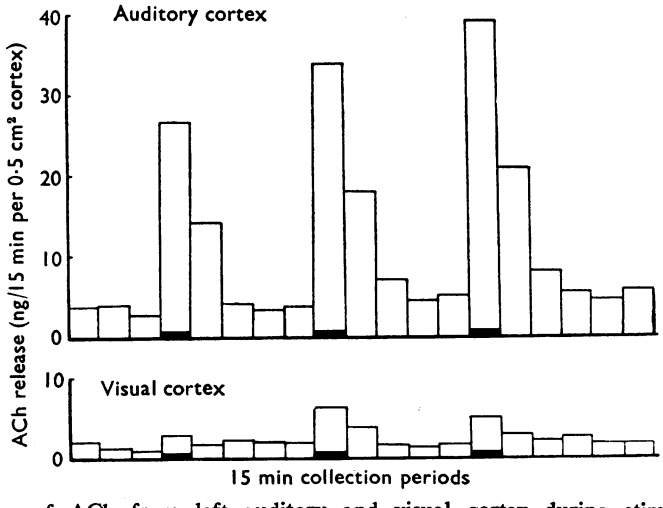

FIG. 1. Release of ACh from left auditory and visual cortex during stimulation of the ipsidateral medial geniculate body. Fifteen-minute periods of electrical stimulation at 10/sec (1 msec duration) are indicated by horizontal bars.

Stimulation of the medial geniculate nucleus caused a large increase in the rate of ACh released from the ipsilateral auditory cortex and a smaller increase from the non-auditory cortex. As shown from the seven experiments in Table 1, the mean evoked release from the auditory cortex was 6·1 times the resting release, whereas that from the non-auditory cortex was only 2·2 times. Figure 1 gives the results of a single experiment with three successive 15 min periods of stimulation of the medial geniculate nucleus. The release was maximal during the stimulation period, it then decreased but was still elevated during the first 15 min after stimulation. The actual amounts of ACh released increased with each successive period of stimulation, but the percentage increase was the same when compared with the resting release determined during the 15 min period preceding each stimulation.

Different frequencies of stimulation

The ACh released from the auditory cortex during stimulation of the medial geniculate nucleus varied with the frequency of stimulation ; a frequency of 10/sec was compared with the release of ACh at one or two different frequencies. As the frequency of stimulation was raised from 0·2 to 10/sec there was a progressive increase in the amount of ACh released, but at frequencies greater than 10/sec there was no further increase in ACh release (Fig. 2).

Calcium ions

When calcium chloride was omitted from the solution bathing the cortex both the spontaneous and evoked ACh output were reduced. The spontaneous ACh release was reduced by 40–65%, and during stimulation the evoked output was reduced by 45–65%. A typical experiment is illustrated in Fig. 3.

In two experiments, increasing the calcium concentration from 0·24 g/l. to 0·48 g/l. increased both the spontaneous and the evoked release of ACh by 15–25%.

Magnesium ions

When magnesium chloride in a concentration equimolar to the amount of calcium chloride present in the Ringer-Locke solution (0·2 g/l.) was included in the bathing

TABLE 1. *Release of ACh from the auditory and the non-auditory (visual) cortex of seven rabbits*

Experiment	1	2	3	4	5	6	7	Mean B/A
Auditory cortex								
A. Mean spontaneous release	4·3	3·6	1·6	6·6	9·4	2·9	4·6	—
B. Release during stimulation	46·8	13·5	6·3	32·3	54·0	27·0	18·9	—
B/A	10·9	3·8	3·9	5·0	5·7	9·3	4·1	6·1
Non-auditory cortex								
A. Mean spontaneous release	1·6	1·8	1·1	1·3	1·2	1·9	2·2	—
B. Release during stimulation	5·1	2·7	2·0	2·9	2·2	6·4	3·7	—
B/A	3·2	1·5	1·8	2·2	1·8	3·4	1·7	2·2

A, Spontaneous release; B, the release evoked by stimulation of the ipsilateral medial geniculate nucleus at 10/sec for a period of 15 min. Each column gives the results from one experiment. The figures refer to ng ACh released into the cup/15 min per 0·5 cm^2 cortex. Spontaneous release is the mean of three to five collection periods taken immediately before stimulation.

fluid there was an increase in both the spontaneous and evoked release of ACh as shown in the upper part of Fig. 4. After the magnesium ions were removed from the bathing fluid the ACh output, both for the spontaneous and evoked release, decreased to approximately the same level as occurred before the addition of magnesium ions. When larger concentrations of magnesium chloride (0·4–1·0 g/l.) were included in the bathing fluid a decrease in both spontaneous and evoked ACh release was obtained. In the experiment shown in the lower part of Fig. 4 a concentration of 0·4 g/l. magnesium chloride in the bathing fluid lowered spontaneous ACh output by 40% and the ACh output during stimulation by 25%. When

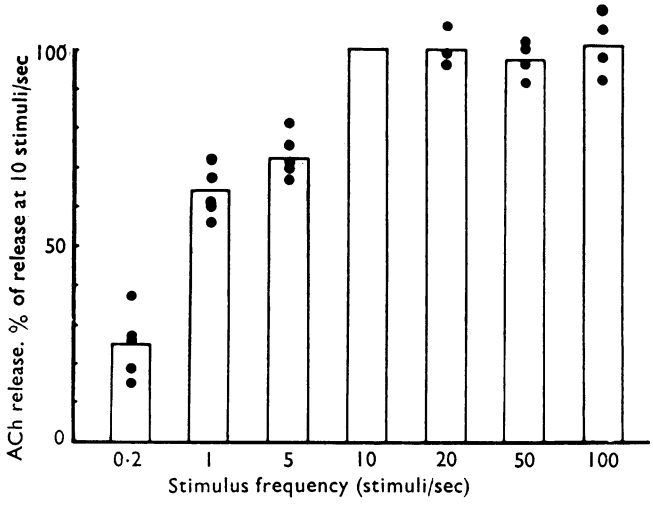

FIG. 2. Release of ACh from the auditory cortex during stimulation of the medial geniculate nucleus at different frequencies. Summary of experiments on fifteen rabbits. In each experiment the release at 10/sec was compared with that at one or more other frequencies. Each column represents the average release at each frequency. Individual results are shown by the filled circles.

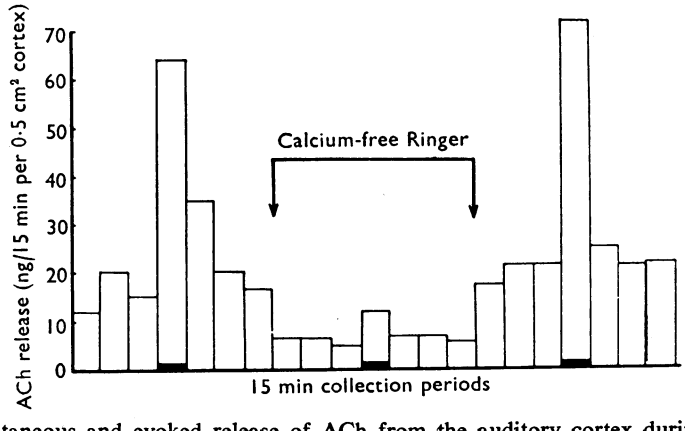

FIG. 3. Spontaneous and evoked release of ACh from the auditory cortex during stimulation of the ipsilateral medial geniculate nucleus at 20/sec (1 msec duration) in the absence and in the presence of calcium. Periods of electrical stimulation indicated by horizontal bars.

magnesium chloride 1·0 g/l. was added to the bathing fluid both the spontaneous and evoked ACh release were lowered by 50–60%.

Triethylcholine

Figure 5 shows the effect of TEC bromide (500 μg/ml.) on both the spontaneous and the evoked release of ACh. The spontaneous release of ACh was reduced by 64% and the evoked release by 67%. After removal of the TEC from the bathing solution there was a return in the amount of spontaneously and evoked released ACh

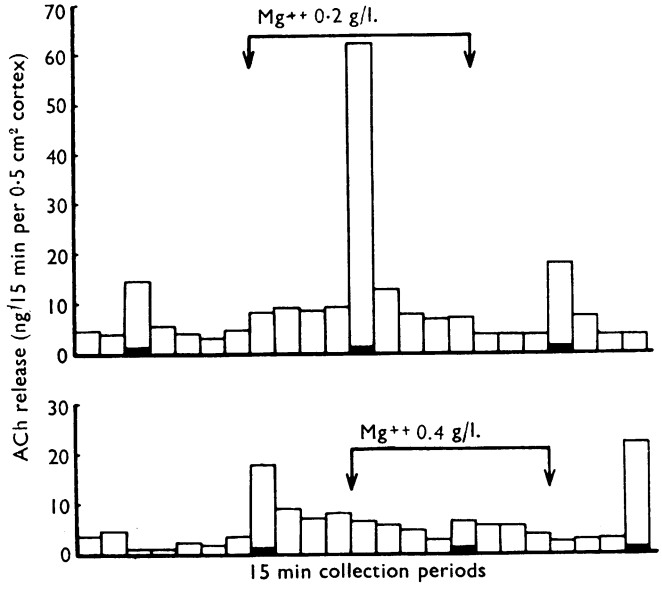

FIG. 4. Magnesium chloride (0·2 and 0·4 g/l.) on spontaneous and evoked ACh release from the auditory cortex during stimulation of the ipsilateral medial geniculate nucleus at 20/sec (1 msec duration). Periods of electrical stimulation indicated by horizontal bars.

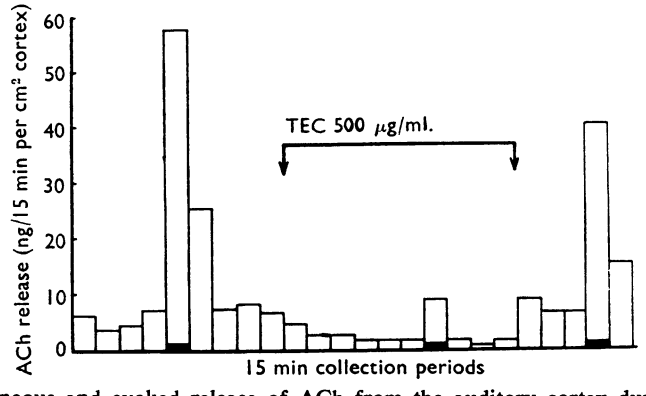

FIG. 5. Spontaneous and evoked release of ACh from the auditory cortex during stimulation of the ipsilateral medial geniculate nucleus at 20/sec (1 msec duration) in the absence and in the presence of topically applied triethylcholine bromide (500 μg/ml.). Periods of electrical stimulation indicated by horizontal bars.

towards normal levels. In four similar experiments TEC reduced both the spontaneous and the evoked ACh release by 60–70%. Choline chloride (150 μg/ml.) added to the bathing fluid, in the continued presence of TEC, partially reversed the effect of TEC on the spontaneous and evoked release of ACh.

Discussion

The spontaneous release of ACh obtained from the auditory cortex in the present experiments is similar to that found previously from the somatosensory and visual cortex of rabbits (Mitchell, 1963 ; Collier & Mitchell, 1966). It is generally agreed that this release is likely to be mediated by a non-specific ascending pathway from the reticular formation.

When the medial geniculate nucleus was stimulated there was a widespread increase in ACh release from the cortex, and this was largest from the auditory receiving area. Table 2 compares these evoked increases from cortical receiving areas with those observed from other areas when the associated afferent pathways were stimulated. In every case the evoked release of ACh from the primary receiving cortex was greater than from other cortical regions.

These results suggest that, although the widespread cholinergic reticulo-cortical pathway contributes to the evoked increase in ACh release from the cortex, there is a second component which produces the higher release in the primary receiving areas. This second component has been suggested to be the augmenting and repetitive after-discharge pathways which run from specific thalamic nuclei to the associated receiving areas of the cortex. The evidence for this view includes the observation that the large ACh release from the primary receiving cortex on stimulation of the thalamic relay nuclei can still be obtained when the influence of the reticular formation has been removed by appropriate central lesions (Collier & Mitchell, 1966, 1967). These conclusions have recently been supported by experiments in which cortical after-discharge synapses have been studied and found to be sensitive to the action of cholinergic drugs and their antagonists (Brownlee & Mitchell, 1968). It is not certain that these ascending pathways themselves release ACh when they are stimulated, or whether they activate intracortical cholinergic neurones with which they may be closely associated. At present, the former possibility would seem more likely because single cell recordings from the cortex during activation of the

TABLE 2. *Evoked increases in ACh release from primary receiving areas and other regions of the cerebral cortex during stimulation of associated afferent pathways*

Type of stimulation	Increased release from associated receiving cortex (× resting release)	Increased release from other regions of cortex (× resting release)
Peripheral nerve *	3·6	2·5
Retina (light) †	4·3	1·9
Lateral geniculate nucleus †	3·4	1·7
Ear (clicks) ‡	3·8	2·0
Medial geniculate nucleus ‡	6·3	2·3

* Kanai & Szerb (1965).
† Collier & Mitchell (1966).
‡ Neal, Hemsworth & Mitchell (1968).

augmenting pathway suggest that only cholinoceptive cells are fired by this procedure (Brownlee & Mitchell, unpublished observations).

Phillis (1968) has claimed that stimulation of a variety of afferent pathways produces an increase in ACh release from the surface of the brain and that this increase is similar in all regions. He therefore argued that the augmenting pathways are unlikely to be cholinergic and that the higher release from receiving areas encountered by other workers could be explained by the activation, presumably by ascending reticulo-cortical pathways, of local intracortical cholinergic fibres. This seems unlikely because non-specific ascending reticular activity is evenly distributed over the cortex (French, 1960).

The explanation for the relatively small increases in evoked ACh output from the receiving areas of the cortex found by Phillis (1968), in contrast to the larger increases found by other authors, is likely to be due, at least in those experiments using auditory and visual stimulation, to the short duration (15 min) of stimulation. These types of stimuli produce a slow increase in ACh release and the maximum is often reached only after 60 min (Collier & Mitchell, 1966 ; Neal *et al.*, 1968). In contrast, direct stimulation of the appropriate specific thalamic nuclei, as in the present experiments, produces rapid increases in ACh release from the cortical receiving areas. The well defined increases in ACh output obtained from the auditory receiving cortex during stimulation of the medial geniculate nucleus made it possible to study the mechanism of this evoked release as well as of the spontaneous release.

The finding that there was a progressive increase in the amount of ACh released as the frequency of stimulation was raised from 0·2 to 10/sec, but that there was no further increase at greater frequency, is similar to the situation when the lateral geniculate nucleus is stimulated (Collier & Mitchell, 1966). It contrasts with the effect of peripheral nerve stimulation, when there is a maximal release at much lower stimulus frequencies (0·5–5/sec) (Mitchell, 1963 ; Phillis, 1968). This lower optimal frequency may reflect the properties of thalamic relay synapses that would be involved during stimulation at a peripheral site but which would not be involved when the thalamic neurones were stimulated directly.

The results obtained with calcium and magnesium suggest a similar mechanism underlying the release of ACh from nerve terminals in the brain and at the neuromuscular junction (del Castillo & Engbaek, 1954 ; del Castillo & Katz, 1954, 1956 ; Hubbard, 1961 ; Elmqvist & Feldman, 1965 ; Hubbard, Jones & Landau, 1968). In both situations optimal release of ACh requires calcium and a minimum amount of magnesium, and in both situations an increase in calcium concentration augments but an increase in magnesium concentration reduces the ACh output.

It is unlikely that the spontaneous efflux of ACh from the cortex can be considered analogous to the resting release of ACh from the unstimulated neuromuscular junction (Mitchell & Silver, 1963), because in the cortex a large proportion of the spontaneous ACh output is probably due to the continuous neuronal activity which occurs in all animals except those which are deeply anaesthetized and in which the ACh efflux falls to undetectable levels (Mitchell, 1963). Because of this, both the spontaneous and the evoked efflux of ACh from the cortex may be more comparable with the release of ACh produced by stimulation of a motor nerve at different frequencies. If this is true, then the effect of calcium ions on ACh release at the two sites is similar. The interesting feature noted first by Randić & Padjen (1967)

and confirmed in the present experiment is that, in the complete absence of calcium, there is still a proportion of ACh efflux left. This part of the efflux, which is apparently independent of calcium for release, may in fact occur because calcium is not completely absent from the tissues, and again this may be compared with the situation at the neuromuscular junction, where it is also possible to release a small amount of ACh when calcium is absent from the surrounding medium. If the tissue calcium is depleted with EDTA then this last fraction of ACh is no longer released (Elmqvist & Feldman, 1965 ; Hubbard *et al.*, 1968).

Unlike Randić & Padjen (1967), we have found that reduced calcium does not abolish evoked release, but only reduced the usual increase. This difference may be attributable to the different afferent pathways stimulated in the two types of experiments.

The results obtained with TEC also show a similarity with results obtained at the neuromuscular junction, where synthesis of ACh is blocked by TEC, probably by competition with choline transport across the presynaptic cell membrane. The block, which is reversed by choline, occurs only when the motor nerve is stimulated rapidly, thus depleting the ACh store (Bowman & Rand, 1961 ; Bowman, Hemsworth & Rand, 1962 ; Bowman & Hemsworth, 1965 ; Bull & Hemsworth, 1965). Applied to the surface of the brain TEC does not abolish but reduces both the spontaneous and the evoked ACh release. This effect is also reversed by choline. The reduction by TEC of the spontaneous release, which is of the same order as that of the evoked release, supports the suggestion that cholinergic nerve endings in the cortex are spontaneously active, otherwise TEC would be ineffective.

The qualitatively similar effects of calcium, magnesium and TEC on the ACh release mechanism in the cerebral cortex and at the neuromuscular junction gives added support to the view that cholinergic nerve terminals are present in the cerebral cortex and that ACh is acting as a central neurotransmitter.

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