VIII. LOCAL HORMONES

ACETYLCHOLINE AS A LOCAL HORMONE FOR CILIARY MOVEMENT AND THE HEART

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In the following account I propose to turn from the study of acetylcholine as a humoral transmitter of nerve impulses to a study of its function as a local hormone. A hormone is a substance which excites, and the application of the name has hitherto been to substances which are carried in the blood to their point of action. A local hormone then is an exciting substance which acts locally. Our interest in this type of action began with the heart, and then spread to ciliary movement. Since the evidence of the action of acetylcholine is more complete at the present time for ciliary movement, that will be considered first.

Ciliary movement in the rabbit trachea. We (11) wished to discover whether the movements of the cilia on the tracheal mucous membrane of the rabbit were produced by the action of acetylcholine formed in the membrane. We took out a piece of trachea and pinned it flat on a cork and then studied the transport of graphite particles along the mucous membrane. We began by testing the action of eserine, for we knew that if eserine modified the rate of transport, it would be likely to do so by inhibiting cholinesterase, and that would imply that acetylcholine controlled the movement. We very quickly found that eserine had two actions. Low concentrations accelerated particle transport while high concentrations made it slower. We next found that acetylcholine behaved in a similar way; it accelerated transport when its concentration was low and slowed it when the concentration was high. Atropine and *d*-tubocurarine both slowed the rate of transport. The effect of all these substances was reversed when they were removed.

These results led us to seek the presence of acetylcholine in the mucous membrane; we found that extracts made with acid saline containing eserine answered all the pharmacological tests for acetylcholine which we could apply. We tested the mucous membrane for cholinesterase and found it to be present and to hydrolyze acetyl-β-methylcholine as well as acetylcholine. We tested for the presence of choline acetylase and found that this enzyme system was also present. The mucous membrane contained no nerves, but nerve fibres (though not nerve cells) were present in the submucosa. It remained possible that the acetylcholine which controlled the ciliary movement was liberated from the nerve endings, though this was unlikely because the movement was unaffected by 10 per cent cocaine.

Ciliary movement in Mytilus edulis. We (3) therefore looked for a ciliated tissue free from nerve fibres and found it in the gill plates of the sea mussel, *Mytilus edulis*. Their structure has been described by Orton (12). They are V-shaped gill filaments lying side by side and loosely connected by ciliated

discs. In transverse section each filament consists of a single layer of cubical cells around a central lumen. Tracts of the cells along the long axes of the filament bear large numbers of actively beating cilia.

Our first task was to determine the presence of acetylcholine in the gill plates. Crude acid saline extracts prepared with eserine when examined pharmacologically appeared to contain acetylcholine. The extracts stimulated the frog rectus, inhibited the frog heart, caused a fall of blood pressure in the cat and stimulated the guinea pig ileum. The last three effects were abolished by atropine, and all activity was destroyed either by incubation with cholinesterase or by boiling in alkaline solution.

We then set out to prove the presence of acetylcholine by more rigorous methods. We made an extract by the method of Banister, Whittaker and Wijesundera (1), in which gill plates from 1000 mussels were homogenized in ice-cold acid alcohol, and after treating the cloudy fluid with trichloracetic acid and extracting it with ether, ammonium reineckate was added to precipitate the choline esters. These were collected and dried and converted to chlorides. The solution then obtained was compared with acetylcholine in tests on the frog rectus, the frog heart and the cat blood pressure. Each comparison was made by choosing two doses of the extract and two doses of acetylcholine in the same ratio and carrying out what is often called a "four-point assay". This procedure avoids any unconscious bias, being entirely objective. We found that the extract tested on the frog rectus was equivalent to 64 μg. acetylcholine per ml., tested on the cat blood pressure was equivalent to 61.5 μg. acetylcholine per ml., and tested on the frog heart was equivalent to 60 μg. acetylcholine per ml.; these figures were practically identical, and so showed that the substance in the extract was acetylcholine. We further identified it by paper chromatography. For this purpose the extract was concentrated 6 times more by taking it to dryness, extracting with absolute alcohol and precipitating with chloroplatinic acid. The chloroplatinates were converted to chlorides with powdered silver. Using two different solvents it was found that the activity ran on the paper to the same place as acetylcholine run alongside. The activity was determined by washing off the paper and testing on the frog rectus, and also by colour reactions.

Ciliary movement was studied by two methods. The first was by measuring the rate of particle transport, and the second was by observing the rate of beating of the cilia on an isolated gill filament. This could be done by watching the cilia beating through a microscope in the light of a stroboflash. When the stroboflash was adjusted to a rate corresponding to the rate of the cilia, these appeared stationary. By both methods it was found that eserine accelerated the movement in low concentrations and slowed it in high concentration. The same was true for acetylcholine. The rate was slowed by *d*-tubocurarine. Atropine reduced or abolished the action of acetylcholine. Adrenaline stimulated the ciliary movement. Finally, cholinesterase was found present, which hydrolyzed acetyl-β-methylcholine, and after many attempts a choline acetylase system was demonstrated also. We were thus able to show that acetylcholine was

present, was produced locally in a nerve-free and muscle-free structure, and was responsible for producing the ciliary movement.

The spontaneous contractions of cardiac muscle. If acetylcholine is responsible for the rhythmic contractions of cilia, is it similarly responsible for those of heart muscle? The work of Gray (10) showed that there was a striking resemblance between the effect of changes in pH, in oxygen supply, in monovalent and divalent cations, in osmotic pressure, etc., on ciliary movement in Mytilus and on the sinus region of the frog heart.

I began to consider the heart because of the action of quinidine, which Dawes (7) pointed out was a substance which reduces the action of acetylcholine in all forms of muscle, no matter whether this action is excitor or inhibitor. It seemed possible that quinidine restored a normal rhythm in fibrillation because of such an action. We may suppose, as a first approximation, that the normal contractions are due to the production of acetylcholine which initiates the contraction when it reaches a certain concentration. Two experiments illustrate the effect of quinidine. These have been carried out by Miss Briscoe and me on the isolated auricles of the rabbit heart which maintain rhythmic contractions for long periods when beating in Locke's solution vigorously oxygenated at 29°C.

In the first experiment a low concentration of acetylcholine, 10^{-8} g./ml., has a slightly inhibitory action on the contractions, but in the presence of eserine 10^{-6} g./ml., it arrests the contractions (15). If quinidine (10^{-5} g./ml.) is added to the bath, the contractions begin again. In the presence of quinidine the threshold for acetylcholine is raised, and a concentration which arrested the contractions can arrest them no longer.

A second experiment is as follows. If quinidine 10^{-5} g./ml. is added to a bath containing the freshly set up auricles, the contractions slowly diminish in rate and amplitude and finally stop. The addition of acetylcholine 10^{-6} g./ml. to the bath causes the contractions to begin again. The decline of the rate and amplitude in the presence of quinidine is explained by the hypothesis that quinidine reduces the effect of the endogenous acetylcholine until it fails either to initiate a contraction or fails to make conduction possible. The addition of ACh raises the concentration of ACh above the threshold once more. I mention the failure of conduction as an explanation of the arrest of the beat because quinidine is known to lessen the rate of conduction and ACh is known to accelerate it. The evidence concerning ACh comes from Prinzmetal and his colleagues (13) who found that the intravenous infusion of ACh in dogs shortens conduction time at high rates of stimulation and makes it possible to drive the heart much faster than otherwise. My colleague, Dr. E. M. Vaughan Williams, has also found that ACh increases the rate of conduction in rabbit auricles. Burgen and Terroux (4) state that ACh decreases the rate of conduction in cat auricles, but their evidence appears to depend on the behaviour of carbachol.

I suggest that quinidine arrests the contractions because it renders endogenous ACh ineffective by one means or other: it is a competitive inhibitor for endogenous ACh. If this suggestion is correct, other substances likely to be competitive inhibitors should have a similar action. Now eserine competes with ACh for

cholinesterase; it may therefore compete with ACh for the receptors when it is present in sufficient concentration. In fact, we have found that high concentrations of eserine arrest the auricles, which are then electrically inexcitable and that when ACh is added to the bath the contractions are resumed. The auricles are not brought to rest by high concentrations of neostigmine; they are indeed made to beat faster. Since Rothschuh and Bammer (14) have shown that eserine decreases the rate of conduction in strips of frog ventricle and that neostigmine increases it, and since Vaughan Williams finds that eserine decreases the rate of conduction in rabbit auricles, it is highly probable that the arrest of the beat by high concentrations of eserine is due to a failure of conduction as is the arrest by quinidine. In causing this arrest there is synergism between quinidine and eserine. In the presence of a concentration of eserine which would not cause arrest alone, quinidine causes arrest very quickly. What relation can there be between a high concentration of eserine and quinidine? Why should both cause arrest of the auricles, the beat being resumed on addition of ACh? The answer can only be that the contractions are maintained by ACh which arises endogenously, and that both quinidine and high concentrations of eserine act as competitive inhibitors.

Acetylcholine synthesis. I have spoken of endogenous acetylcholine, and must give the evidence for doing so. If an extract of rabbit auricles is made, acetylcholine is found present in a mean amount of 1.3 μg per g. Moreover, as was shown by Comline (6), the choline acetylase system is present. Miss Bülbring and I (2) were able to demonstrate that the activity of the auricles was related to this system. When the auricles were allowed to beat in Locke's solution, after 24 to 30 hours the contractions became feebler and ceased. When acetylcholine was added to the bath in a concentration from 10^{-7} to 10^{-6} g./ml., the contractions began again. Thus we have a second method demonstrating that acetylcholine not only inhibits but also stimulates contractions, and here again can be seen the gradual change from the stimulant to the inhibitory effect with rising concentration.

We took auricles and having prepared an acetone-dried powder, determined the choline acetylase activity by incubating with choline, citrate and ATP according to the method of Feldberg and Mann (8). We found that powder from fresh auricles was able to synthesize 46 μg. acetylcholine per g. per hr., while the powder from stopped auricles synthesized only 7 μg. Powder from restarted auricles synthesized 37 μg. Thus we found that the choline acetylase activity was directly related to the activity of the auricles when beating in the isolated organ bath. This conclusion was greatly strengthened by two other results which can only be mentioned here though I believe they are important. The first was that the addition of acetylcholine to powder prepared from fresh auricles diminished the synthesis, and the second was that the addition of acetylcholine to powder prepared from stopped auricles increased the synthesis; the effect of acetylcholine on the synthesis was parallel to its effect on the contractions in the bath in both cases. These results gave us a clear foundation for the view that the contractions of the auricles were dependent on acetylcholine formation.

Action of low concentrations of eserine. I have spoken of the action of high concentrations of eserine, and wish now to speak of the action of low concentrations where its predominant action is to inhibit cholinesterase. If ACh plays a part in maintaining cardiac contractions, these contractions must be modified by an anticholinesterase. Such effects were very clearly seen in the heart-lung preparation of the dog on which I worked with Dr. J. M. Walker. The addition of eserine to the reservoir of blood to make a concentration of 3×10^{-6} M caused the heart rate to fall to a mean figure of 62 per cent of the initial value in 5 experiments; in these preparations the vagi were cut and the central nervous system had no circulation. To make sure that the slowing was not due to a slight leakage of acetylcholine from the ends of the cut vagi, Dr. Walker and I carried out 5 experiments in which the vagi were cut 4 days previously. The same concentration of eserine then caused the rate to fall to a mean figure of 75 per cent of the initial value. However, the initial rate was lower, the mean value being 108 instead of 144, and the smaller effect of eserine may be explained by this. A similar effect was produced by neostigmine, Nu 683* and Nu 1250†, and at the concentration of 3×10^{-6} M it is likely that they were acting only by inhibition of cholinesterase. The fall in rate was therefore due to accumulating acetylcholine, and this acetylcholine did not come from the vagal nerve endings. It could have come from ganglion cells in the heart. We tried to throw light on this by injecting hexamethonium. Amounts sufficient to block vagus stimulation (5 mg. per litre) did not modify the action of eserine. Much greater amounts of hexamethonium (30 mg. per litre) reduced, but did not abolish the effect of eserine by what appeared to be a weak atropine-like action.

Double action of eserine on the auricles. While eserine in low concentrations slowed the rate of the auricles as its usual effect, in 10 out of 45 preparations it increased the rate in low concentrations, and decreased it only in higher concentrations. This result resembles the effect of eserine on ciliary movement and appears to be in favour of the view that there is an optimal amount of endogenous acetylcholine for maintenance of the sinus rhythm, and that the rate can be slow either because there is too little acetylcholine or too much. A quickening of the rate by eserine was also observed in isolated frog hearts perfused with Ringer's solution. It is very likely that when auricles are beating in Locke's solution or the frog heart is perfused with Ringer solution, the conditions are slightly suboptimal so that an increase in acetylcholine due to lessened destruction in the presence of an anticholinesterase will sometimes increase the rate. In the heart-lung preparation with a blood circulation giving better conditions, eserine always decreased the rate.

The inhibitory action of acetylcholine. In auricles which ceased to beat after 24 to 30 hours in Locke's solution, or in the presence of quinidine or in a high concentration of eserine, the contractions are resumed when ACh is added to the bath. Further additions may augment the rate and amplitude of the contractions, but a point is reached when ACh produces its normal inhibitory

* Nu 683: dimethyl carbamate of hydroxyphenylbenzyltrimethyl ammonium.

† Nu 1250: p-chlorphenylmethyl carbamate of m-dimethylaminophenol methyl bromide.

effect. Are we to suppose that this inhibitory action is the effect of excess of ACh as is seen in ciliary movement? The answer seems to be no. The effect of ACh on auricles which have almost ceased to beat at the end of 24 hours is inhibitory; its effect on auricles which are almost arrested by quinidine or by a high concentration of eserine is first to cause transient inhibition and then a large increase of rate and amplitude. The inhibition is still there, although it is almost masked by the stimulant action. Burgen and Terroux (4) have shown that ACh causes hyperpolarisation in isolated cat auricles confirming Gaskell's (9) observation of the effect of vagal stimulation on the demarcation potential of the resting turtle auricle. We can conclude from all these observations that the stimulant action of ACh when applied externally is not related to the normal inhibitory action. On the other hand, it may be that so far as endogenous ACh is concerned, excess inhibits; thus the presence of a low concentration of eserine may increase or may decrease the rate of spontaneously beating auricles.

In conclusion, while we are only beginning to understand the cardiac mechanism, the evidence is very strong that the local formation of ACh initiates the contractions. ACh is present, choline acetylase is present, cholinesterase is present. The activity has been shown to be proportional to the choline acetylase activity, and to be governed also by cholinesterase. The long known action of quinidine has been shown to be intimately related to the action of ACh. Whether the origin of the ACh is neurogenic or myogenic we do not yet know, but it is possible that the arguments used by Gaskell and Engelmann to overcome the views of Volkmann will be found to apply also to the origin of this acetylcholine.

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