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Evidence that acetylcholine releases noradrenaline in the sympathetic fibre

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In a previous paper (Burn, 1976) an account was given of the discovery in the period 1930–1933 of the uptake of adrenaline from the blood by sympathetic postganglionic fibres. An account was also given of the discovery of the release of acetylcholine together with the adrenaline-like transmitter following electrical stimulation of sympathetic postganglionic fibres. This discovery was first made by von Euler & Gaddum in 1931, and was confirmed by a series of other workers in 1935 and 1948.

When reserpine was made available in 1953, and was found to diminish greatly the stores of noradrenaline in sympathetic fibres, it became possible to show that electrical stimulation released acetylcholine from sympathetic endings in several more organs. The question arose what was the purpose of the acetylcholine so released? von Brücke (1935), working on the pilomotor muscles of the cat tail, found that the injection of 2 μg acetylcholine into the skin of the tail caused erection of the hairs. A few years later Coon & Rothman (1940) showed that a prior injection of ergotamine prevented this erection. Now acetylcholine is not blocked by ergotamine, and this led Burn in 1958 to consider the possibility that the process of the release of noradrenaline from the sympathetic fibre began with the release of acetylcholine which in its turn released noradrenaline. If this could be shown, the release of noradrenaline from the sympathetic fibre would be similar to the release of adrenaline from the chromaffin cells of the adrenal medulla. This possibility was supported by the observations of Boyd (1960). Boyd's suggestion of a common origin of chromaffin cells and of postganglionic neurons gains support from the work of Douglas & Rubin (1961) who showed that when the adrenal gland of the cat was perfused with a solution containing only sucrose, dextrose and Ca^{2+}, without other inorganic ions, than acetylcholine was effective in causing a discharge of adrenaline. Without Ca^{2+} there was no discharge. Now postganglionic fibres contain acetylcholine, and in them electrical stimulation invokes a response only when Ca^{2+} is present. So the parallel between the mode of release of catecholamines from the adrenal medulla and the release of noradrenaline from the sympathetic fibre is close. It supports the suggestion from the contraction of the pilomotor muscles that acetylcholine release is a step to the release of noradrenaline.

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Release of noradrenaline from the cat hypothalamus in vivo. It is of further interest to observe that the release of noradrenaline has been studied by Philippu, Heyd & Burger (1970). Here also acetylcholine and Ca^{2+} are key substances. The cats were anaesthetized with pentobarbitone and the 3rd ventricle cannulated and [^{14}C]noradrenaline was injected into it. After 4 h the aqueduct was cannulated and the 3rd ventricle was perfused either with artificial cerebrospinal fluid or with Ringer plus tropolone and nialamide. During perfusion the release of total radioactivity from the hypothalamus decreased gradually and after 20 min followed a single exponential decline, which suggested that in the period from 40 to 320 min, catecholamines were released only from intracellular compartments.

The addition of acetylcholine ($2{\cdot}2 \times 10^{-3}$ M) to the perfusion fluid increased the release of catecholamines, and this increase could be demonstrated repeatedly in the same cat. If all Ca^{2+} was removed by perfusing the hypothalamic region of the ventricle with Ringer containing EDTA (10^{-3} M) for 60 min before the collection period, then acetylcholine was ineffective in releasing catecholamines from the hypothalamus. The authors say 'It is therefore likely that in the central nervous system acetylcholine induces the release of noradrenaline from noradrenergic nerve endings by increasing membrane permeability to the calcium ion as in the adrenal gland'. They also found that a rise in Ca^{2+} alone increased the release of noradrenaline from the hypothalamic vesicles, and therefore supposed that *in vivo* the Ca^{2+} ions which enter the cell must act on the subcellular particles and cause the release of noradrenaline.

Action of hemicholinium

In the three situations in which noradrenaline or adrenaline is released, acetylcholine in the presence of Ca^{2+} is certainly an active agent in the hypothalamus and in the adrenal medulla. We must now ask ourselves how we can be certain that it is also the agent in the sympathetic fibre.

Strong evidence has been provided by Rand and his colleagues using one of the hemicholiniums prepared by Schueler (1955). These compounds contain choline incorporated into a six-membered ring through hemi-acetal formation. MacIntosh, Birks & Sastry (1956) thought that the compound HC3 might prevent the synthesis of acetylcholine. They perfused the cat superior cervical ganglion with plasma containing eserine and found that when HC3 was added to the perfusion fluid, the rate at which acetylcholine was released by stimulating the preganglionic fibres was unaffected when the stimulation lasted for 1–2 min only. But with longer stimulation the rate of release of acetylcholine rapidly declined; the ganglion lost its ability to transmit impulses, and also lost its store of preformed acetylcholine. The amount of acetylcholine synthesized in 1 h by the ganglion was reduced from 1·21 μg to 0·1 μg when HC3 was present. They further made the important observation that the effects of HC3 could be antagonized by raising the choline concentration of the perfusing fluid. The authors considered that HC3 competed with choline for transport by a specific carrier mechanism to sites within the neurons where acetylation of choline took place.

The importance of HC3 therefore lay in the fact that just as noradrenaline could be removed from the postganglionic fibre by reserpine, so acetylcholine could be removed from the postganglionic fibre by HC3. Moreover the acetylcholine could be restored at will by adding choline. Here, then, was a method of deciding whether the response to sympathetic postganglionic stimulation depended on the presence of acetylcholine in the fibre.

A detailed study of the action of hemicholinium (HC3) was made by Rand and his colleagues. Brandon & Rand (1961) examined the action of HC3 on the spleen of the cat which they perfused and they recorded the venous outflow by using a drop counter. Stimulation of the splenic nerves in the initial stage of the perfusion caused contraction of the spleen and so increased the outflow from 16 drops per 10 s to 40 drops per 10 s (see Fig. 1). When HC3 (50 μg ml^{-1}) was added to the perfusion fluid, the increase of outflow on stimulation gradually diminished. After 200 min it was much smaller and after 263 min it was zero. At this point the addition of choline chloride (0·5 mg ml^{-1}) to the perfusion fluid restored the increased outflow on stimulation.

Chang & Rand (1960) made observations on the isolated atria of the cat, stimulation being applied to the stellate ganglion, from which only postganglionic fibres run to the heart as Huković (1959) showed when he first made this preparation. Stimulation was at 50 Hz for 50 s, this being applied every 4·5 min. Initially, stimulation caused a rise in rate of 20 beats min^{-1} (see Fig. 2). In the presence of HC3 (0·5 mg ml^{-1}) for 270 min the same stimulation caused a rise of only 4 beats min^{-1} and the increase in amplitude was very much less. When 1 mg ml^{-1} choline chloride was added to the bath, the rise in rate on stimulation

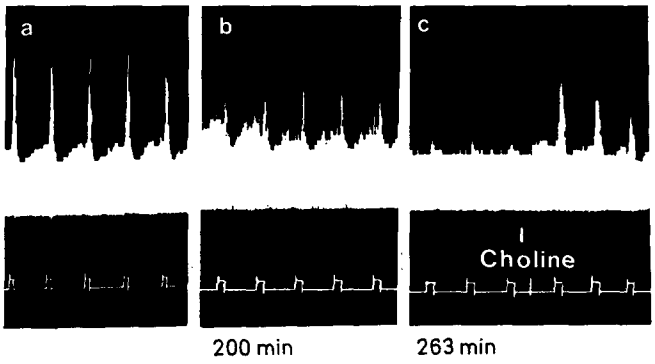

FIG. 1. Cat spleen perfused through the splenic artery (Brandon & Rand, 1961, *J. Physiol.*, with permission). Outflow recorded by Thorp drop counter. Stimulation of splenic nerves caused a contraction of the spleen which expelled an increased outflow from the vein. At the start of the perfusion (a) stimulation caused a rise in outflow from 16 to 40 drops per 10 s. Hemicholinium-(HC3) (50 μg ml^{-1}) was then added to the perfusion fluid, after which there was a gradual fall in the effect of stimulation, (b) shows the effect of stimulation after 200 min, (c) shows that after 263 min, stimulation was practically without effect. However, when choline chloride (0·5 mg ml^{-1}) was added to the perfusion fluid, the stimulation was once more effective.

was much greater being 38 beats min^{-1} and the increase in amplitude was far greater, as Fig. 2 shows.

Chang & Rand also made observations on the vessels of the rabbit ear perfused with Tyrode solution. Electrical stimulation was applied to the postganglionic fibres from the superior cervical ganglion, and this produced vasoconstriction resulting in a fall in outflow. This constriction was matched by injecting noradrenaline into the perfusion fluid as shown in Fig. 3. When HC3 (50 μg ml^{-1}) was added to the perfusion fluid, after 186 min stimulation no longer produced vasoconstriction, but the effect of noradrenaline was slightly increased. After the addition

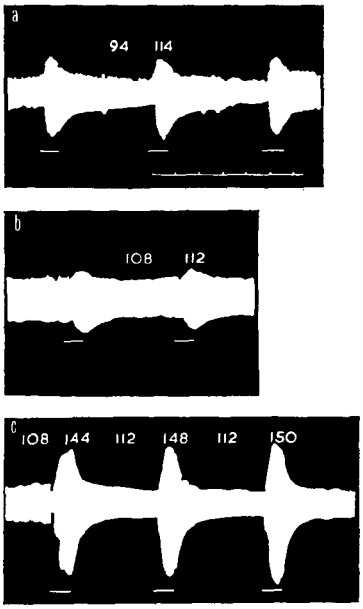

FIG. 2. Experiments with isolated atria of cat (Chang & Rand, 1960, *Br. J. Pharmac. Chemother.*, with permission). The responses of the atria to stimulation of the stellate ganglion at 50 Hz for 50 s every 4·5 min are shown. In (a) stimulation caused a rise in rate of 20 beats min^{-1} together with an increase in amplitude. In (b) the presence of HC3 for 270 min, stimulation caused a rise of only 4 beats min^{-1} and a much smaller increase in amplitude. In (c) after the addition of choline to the bath, stimulation caused the rate to rise by 36 beats min^{-1} and a great increase in amplitude.

of choline chloride to the perfusion fluid, the response to stimulation returned. The same result with the rabbit ear vessels was also obtained by another method, as described in their paper.

Chang & Rand also tested the action of HC3 on cholinergic fibres such as (i) the vagal fibres to the rabbit atria, (ii) the parasympathetic supply to the rabbit isolated vagina, and (iii) the phrenic nerve to

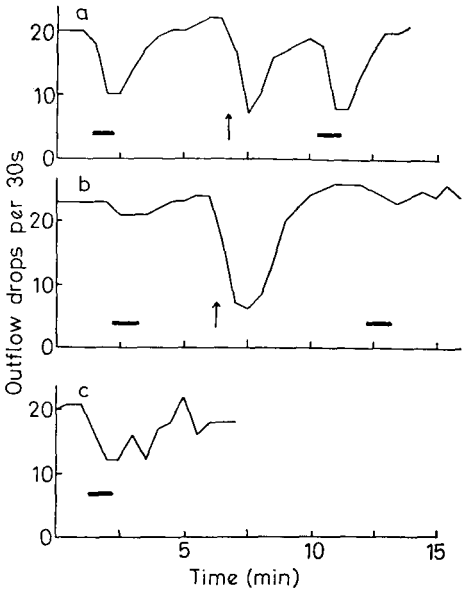

FIG. 3. Experiments on vessels of the rabbit ear (Chang & Rand, 1960, *Br. J. Pharmac. Chemother.*, with permission). Record of outflow per 30 s (a) shows constrictions in response to postganglionic stimulation, followed by response to injection of noradrenaline at arrow, and this followed by a repetition of sympathetic stimulation, (b) shows that the constriction in response to sympathetic stimulation is almost absent after 186 min perfusion with HC3 (50 μg ml^{-1}) but the response to noradrenaline is increased, (c) shows that after the addition of choline chloride to the perfusion fluids, the constrictor response to sympathetic stimulation returned.

the guinea-pig diaphragm. They say 'the similarity in the actions of HC3 in blocking sympathetic nerves and in blocking cholinergic nerves is striking; the effective concentrations of HC3 were approximately equal as far as isolated preparations are concerned; the block depended on the frequency of stimulation, and choline reversed the block'.

Another investigation was undertaken by Rand & Ridehalgh (1965) on the colon of the guinea-pig. When a concentration of HC3 (50 μg ml^{-1}) was used, the time to abolition of the response to stimulation was 335 min. Then the response was restored by choline chloride.

Thus the action of hemicholinium (HC3) in all these trials, on cat spleen, on cat atria, on rabbit ear vessels (in which two methods were used) and on guinea-pig colon was effective in removing the response to sympathetic postganglionic stimulation. This action was due to the cessation of acetylcholine formation since the response returned when an excess of choline chloride was supplied.

Action of tetraethylammonium

While HC3 removes the acetylcholine from the sympathetic fibre, the compound now to be considered has the opposite effect, that of increasing its action. In 1954, Del Castillo & Katz showed that when the action potential at the neuromuscular junction is extended in duration and increased in amplitude, more transmitter is released. Collier & Exley (1963) measured the release of acetylcholine from the rat diaphragm, and showed that it was increased by tetraethylammonium (TEA). Matthews & Quilliam (1964) showed that TEA caused an increased output of acetylcholine from the perfused superior cervical ganglion. Both of these observations concerned nerve endings where acetylcholine was the transmitter. This action of TEA was found to apply also to sympathetic nerves. Thus Thoenen, Haefely & Staehelin (1967) showed that TEA increased the contraction of the spleen caused by postganglionic stimulation and increased the amount of noradrenaline released. Armitage and Burn (unpublished) made observations on the nictitating membrane of cats under chloralose. Stimulation of the postganglionic fibres of the cervical sympathetic evoked a contraction of 5 mm before injection of TEA, while after injection of TEA the stimulation evoked a contraction of 35 mm. To ensure that this increase produced by TEA was not directly connected with the release of noradrenaline, we gave other cats reserpine. Before injection of TEA the effect of postganglionic stimulation on the nicitating membrane of one of these cats was nil, but after injection of TEA, the stimulation caused a contraction of 22 mm. Evidently the effect of TEA on the response to stimulation was due to prolongation of the action potential of acetylcholine. This provided further evidence that acetylcholine was involved in the response to sympathetic stimulation.

Botulinum toxin

Another substance which suggests that acetylcholine is liberated by sympathetic stimulation in order to release noradrenaline is botulinum toxin. Burgen, Dickens & Zatman (1949) showed that this toxin prevented the release of acetylcholine from the terminals of motor nerves such as the phrenic. Rand & Whaler (1965) found that botulinum toxin caused the pilomotor muscles of the cats tail to fail to respond to sympathetic stimulation, this result being obtained in 4 cats. They made further experiments in the Finkleman (1930) preparation of the rabbit intestine in which they found that the inhibitory action of the sympathetic postganglionic fibres was blocked by botulinum toxin after exposure of the intestine to the toxin for 4½ h. This finding was repeated in 17 out of 19 strips of intestine. The toxin did not affect the inhibitory response to adrenaline. There was no change in the inhibitory response of strips to sympathetic stimulation which were not exposed to botulinum toxin in the 4½ h period. These results were to be explained by the action of the toxin in preventing the release of the acetylcholine by sympathetic stimulation.

Acetylcholine in the spleen

Since part of the evidence concerning hemicholinium was obtained by work on the spleen of the cat, it is necessary to refer to the evidence of Fillenz (1970) who has examined the spleen for acetylcholinesterase by electromicroscopic methods and failed to find it. This would suggest that the splenic nerves do not liberate acetylcholine.

Burn & Rand (1960) took cats and under chloralose recorded the spleen volume; in responses to stimulation of the splenic nerves they observed constriction. They then took cats which were given reserpine for two days, and in these, under chloralose, they observed that stimulation of the splenic nerve caused dilatation. This dilatation was increased when eserine was injected and was abolished by the injection of atropine, and the result indicated that nerve stimulation released acetylcholine.

The next observations were made by Brandon & Rand (1961) who took a series of cats from which they removed the spleens and estimated the amounts of noradrenaline and of acetylcholine in them. They then took a series of 11 other cats in which they cut the splenic nerves under anaesthesia. These cats were left 10 to 12 days to allow the splenic nerves to degenerate. The spleens were then removed and noradrenaline and acetylcholine were estimated as before. The results are in Table 1, which gives the mean results for each group of cats.

In the normal spleens the amount of acetylcholine was no less than 41% of the amount of noradrenaline. In the denervated spleens noradrenaline fell to 18·6% of its normal value while acetycholine fell to

Table 1. *Mean results of noradrenaline and acetylcholine from normal and denervated spleens.*

	Normal spleens	Denervated spleens	$\frac{D}{N} \times 100$
Noradrenaline	1·13 $\mu g\ g^{-1}$	0·21 $\mu g\ g^{-1}$	18·6
Acetylcholine	0·47 $\mu g\ g^{-1}$	0·1 $\mu g\ g^{-1}$	21·2

21·2% of its normal value. These figures are fairly close to one another and suggest that each fibre normally contains noradrenaline and acetylcholine in a similar quantitative relation.

Conclusion

The evidence that has been presented all points in the same direction, indicating that the purpose of the release of acetylcholine when postganglionic fibres are stimulated is to liberate noradrenaline. There was the possibility that while some of the noradrenaline was liberated in this way, some might be liberated directly by the nerve impulse. But the work of Rand and his colleagues, showing that when acetylcholine is removed as a result of the action of hemicholinium, sympathetic stimulation can no longer evoke a response, removes this possibility; the action of botulinum toxin supports this, for when the toxin prevents the release of acetylcholine, sympathetic stimulation has no action, though adrenaline itself acts normally.

The reverse situation holds good with tetraethylammonium which extends the duration and increases the amplitude of the action potential where acetylcholine is the transmitter. Here it was found that when cats had been treated with reserpine so that their sympathetic postganglionic fibres contained little or no noradrenaline, injection of TEA greatly increased the response to sympathetic stimulation of the nicitating membrane.

Now that the evidence is so complete, it should not surprise anyone on reflection that the mode of release of noradrenaline is very similar to the mode of release of adrenaline from the adrenal medulla.

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