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THE EFFECT OF ANTICHOLINESTERASES ON THE INCREASE IN RATE OF THE ISOLATED HEART IN RESPONSE TO SYMPATHETIC STIMULATION

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According to the hypothesis put forward by Burn & Rand (1959), the release of noradrenaline by an impulse passing down a sympathetic post-ganglionic fibre is accomplished by an initial liberation of acetylcholine. This substance appears to increase the permeability of the post-ganglionic fibre to calcium ions, which then enter the fibre and release noradrenaline (Burn & Gibbons, 1965).

If acetylcholine plays such a part, the amount of noradrenaline released by a train of impulses should be increased by anticholinesterases. Experiments have been made to see if such an effect can be observed when the accelerator fibres to the heart are stimulated. A preparation of the isolated atria of the rabbit with sympathetic innervation has previously been described (Huković, 1959) and the first experiments were made with it. Since no positive results were obtained, it was decided to use the whole heart perfused through the aorta (Huković & Muscholl, 1962) since in this way it was more likely that a substance such as physostigmine would reach the terminations of the sympathetic fibres effectively.

METHODS

As a perfusion fluid, McEwen's solution (1956) was used. This was placed in a Marriotte's bottle from which it passed through a tube containing a stream of very fine bubbles of oxygen + 5% CO_2, then to a coil in a warming bath, and finally to the cannula inserted in the aorta. The rabbits weighed from 2 to 2.5 kg. Each rabbit was stunned by a blow on the head, its throat was cut and it was decapitated. The skin over the thorax was excised, and the muscles joining the forelegs to the chest were divided. The front of the chest was removed and the thymus was gently pulled away with fingers. The pericardium was cut out, and the heart was lifted up to divide the veins and the descending aorta. A ligature was passed around the first part of the aorta. A cannula delivering warm oxygenated McEwen's solution was inserted into the aorta and tied in place. The solution rapidly removed blood from the heart. In the tissue lying on the right side of the trachea the carotid artery, the vagus and the sympathetic nerve could be seen. The sympathetic was separated and a silk thread tied around it. It was then followed down to the stellate ganglion. The vagus was excised. (A small vessel runs in this tissue and care must be taken not to confuse it with the sympathetic.) In approaching the ganglion the dissection was made gently to avoid traction. Usually there were other branches running to the ganglion, and a silk ligature was tied around all of these to include the sympathetic nerve which was separated first. In this way a stronger hold was

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obtained. The tissue lying on the trachea near the ganglion was gently rubbed off towards the ganglion with a finger so as to make it possible to cut away the trachea and oesophagus. The mediastinum behind the heart was then cut as close to the ribs and vertebral column as possible to avoid dividing the terminal fibres of the sympathetic.

The heart and adjacent tissue were removed from the rabbit, and the cannula in the aorta was attached to the Langendorff apparatus, so that the perfusion continued without interruption. The thread attached to the sympathetic fibres was drawn gently into the electrodes (which were of the pattern described by Burn & Rand (1960), until the ganglion was held by the electrodes, in which position it was continuously irrigated. Stimulation was applied by a square-wave stimulator giving supramaximal shocks of a duration of 1 msec at frequencies varying from 0.2/sec to 10/sec. The perfusion fluid contained atropine sulphate 1 μg/ml. and in the later experiments choline chloride 0.5 mg/ml. The temperature varied in different experiments between 32.5° C and 35° C.

The contractions of the heart were recorded on a kymograph by attaching a thread to the apex of the ventricle, the other end of the thread being fastened to a light lever supported by a spring. The heart rate was measured by using a post office counter actuated by a platinum contact on the spring lever, which was arranged to close a circuit through the counter at each contraction. Physostigmine salicylate and prostigmine methylsulphate (neostigmine) were used. Concentrations are expressed in terms of these salts. Stimulation was always applied for 30 sec, and the heart rate was counted for 1 min from the beginning of stimulation.

RESULTS

Plan of experiments

The plan followed was to set up the isolated heart, perfused through the aorta (using for the perfusing fluid a solution containing atropine sulphate, 1 μg/ml.), and then to stimulate the stellate ganglion. Stimulation was carried out at different frequencies. The increase in heart rate was determined, and when sufficient control observations were made, the perfusion fluid was changed to one containing physostigmine or neostigmine, usually in a concentration of 10^{-6} g/ml. This concentration of either substance added to an isolated organ bath greatly increased the action of acetylcholine on rabbit atria (Briscoe & Burn, 1954).

Effect of neostigmine

The details of an experiment in which neostigmine was used are given in Table 1, in which choline was present in the perfusion fluid. Stimulation was applied at 5 frequencies, between 5/sec and 0.2/sec. Stimulation was supramaximal and applied for 30 sec. There was an interval of 5 min between each stimulation and control stimulations at each frequency were repeated three or four times. Thus, the time taken to record the control

TABLE 1

THE EFFECT OF NEOSTIGMINE (1 μG./ML.) ON THE INCREASE IN HEART RATE PRODUCED BY SYMPATHETIC STIMULATION FOR 30 SEC AT VARIOUS FREQUENCIES

Atropine and choline were present throughout

Frequency	5/sec	2/sec	1/sec	0·5/sec	0·2/sec
1st control	45	38	17	4	0
2nd control		39	17	0	
3rd control	44	41	20	1	0
4th control	43	31	18	0	0
1st neostigmine	69	52	25	11	0
2nd neostigmine	67	53	41	24	9
3rd neostigmine	60	48	40		11

stimulations was nearly 2 hr. The increase in rate produced by any one frequency was approximately the same in different trials. Thus the increases for the frequency of 5/sec were 45, 44 and 43 beats/min. At the lowest frequencies of 0.5/sec and 0.2/sec there was no appreciable increase.

The solution perfusing the heart was then changed to one containing neostigmine in a concentration of 1 μg/ml. When this solution reached the heart, the stimulations were begun again, and the increases produced were greater. Thus at 5/sec the increase was 69/min instead of 43/min ; and so on. Whereas at 0.5/sec there was no increase in the control period, there was an increase of 11/min in the presence of neostigmine.

Increase in relation to initial rate

The greater increases observed in the presence of neostigmine were not explained by a lower rate before stimulation was applied. Thus for the fourth set of control observations in Table 1, the initial rates before stimulation at frequencies of 2/sec, 1/sec and 0.5/sec were 158/min, 157/min and 177/min respectively. The increases were 31, 18 and 1. For the first set of observations in the presence of neostigmine the corresponding initial rates were 159/min, 163/min and 177/min, which were about the same as before. Yet the increases due to stimulation in the presence of neostigmine were much greater, being 52, 25 and 11.

Throughout the observations the increase produced by stimulation was not observed to depend on the initial rate. Thus, when the initial rate varied, the increase did not. The three stimulations in the presence of neostigmine at 2/sec started at initial rates of 159/min, 141/min and 131/min, which differed appreciably. Yet the increases were 52/min, 53/min and 48/min, which were almost the same.

Effect on amplitude

Because the contractions of the heart were recorded by a spring lever, the increase in the force of the contraction which accompanied the increase in rate was rarely indicated with any accuracy by the excursion of the lever. Often the increase in the rate diminished the excursion of the lever. However, occasionally a correspondence between the excursion of the lever and the force of contraction seemed to be present. In Fig. 1 (taken from the same experiment as that in Table 1) the control contractions are shown in the upper panels, and the contractions in the presence of neostigmine in the lower panels. The excursion of the lever increased more in response to stimulation at frequencies of 2/sec, 1/sec and 0.5/sec in the presence of neostigmine than it did in response to these stimulations in the control period. In the presence of neostigmine the increase in activity was abrupt in onset, whereas it was very gradual in the controls.

Decline of response

In experiments carried out by Huković & Muscholl (1962), the amount of noradrenaline released by succeeding periods of stimulation declined. In the experiments now described there was sometimes a progressive decline in the effect of stimulation, and the results in Fig. 2 provide an example. The first stimulation at a frequency of 2/sec increased the rate by 40 beats/min. The next stimulation increased it only by 32 ; thus the response

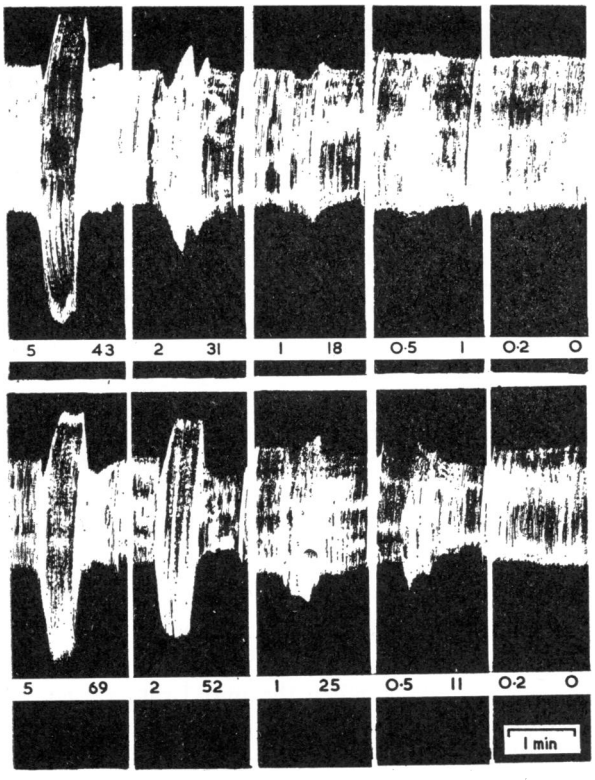

Fig. 1. Contractions of perfused rabbit heart showing the change in amplitude and rate caused by stimulating the right stellate ganglion. Supramaximal shocks were applied for 30 sec at the frequencies /sec shown on the left of each panel. The increase in heart rate is shown on the right of each panel. The upper panels were control observations. The lower panels were observations during perfusion with neostigmine 10^{-6} g/ml. Note the abrupt onset of the responses to frequencies 2/sec, 1/sec and 0.5/sec when neostigmine was present.

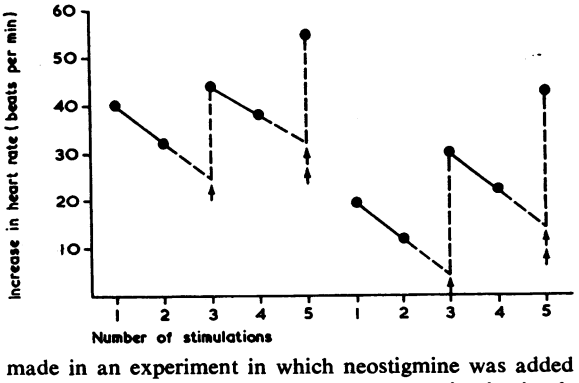

Fig. 2. Observations made in an experiment in which neostigmine was added to the perfusion fluid. Ordinates show the increase in heart rate during supramaximal stimulation for 30 sec (*a*) at 2/sec in the left-hand group of observations, (*b*) at 1/sec in the right-hand group of observations. About 20 min elapsed between each of the five observations at 2/sec, and between each of the five observations at 1/sec. At the single arrow neostigmine 10^{-6} g/ml. was added to the perfusion fluid. At the double arrow the concentration of neostigmine was doubled.

declined along the line shown on the left of the figure. The broken line indicates the probable further fall in the response. Neostigmine (1 μg/ml.) was then added to the perfusion fluid and the next stimulation at 2/sec caused an increase of 44 beats/min. This again declined, and the fourth stimulation increased the rate by 38 beats/min. Before the next stimulation the concentration of neostigmine was doubled, and the response to stimulation then rose to 56 beats/min. In the same experiment similar changes in the response were observed when stimulation was 1/sec, and these changes are shown graphically on the right side of the figure. The initial control increase was one of 20 beats/min, but after the concentration of neostigmine had been raised to 2 μg/ml. the increase rose to 42 beats/min.

Experiments with physostigmine

In Table 2 are given the details of one of two successful experiments with physostigmine. In this experiment the temperature was 33° C and the spontaneous rate of the heart was low. However, because it remained approximately the same throughout the experiment, the influence of physostigmine in increasing the response to stimulation was clearly seen. At the lowest frequency of 1/sec physostigmine increased the response fourfold, at 2/sec it increased it about twice, and at 5/sec it increased it by about 30%.

TABLE 2

THE EFFECT OF PHYSOSTIGMINE (1 μG/ML.) ON THE INCREASE IN HEART RATE PRODUCED BY SYMPATHETIC STIMULATION IN PRESENCE OF ATROPINE

At the frequencies of 1/sec and of 2/sec the total number of shocks was 50. At the frequency of 5/sec the total number of shocks was 125

Frequency	5/sec	2/sec	1/sec
Control	34	16	4
Physostigmine	44	30	16

Some experiments with physostigmine gave negative results. Since the experiments with neostigmine were more successful, it seemed likely that the failures with physostigmine were due to its oxidation in the perfusion fluid. There were occasional experiments with neostigmine which were also unsuccessful, and it was decided to add choline chloride to the perfusion fluid. Birks & MacIntosh (1961) showed that the synthesis of acetylcholine in a perfused ganglion was deficient unless sufficient choline was available. After the addition of choline, all experiments with neostigmine were successful, though whether this was due to the choline or to greater success in setting up the preparation is not known.

TABLE 3

SUMMARY OF RESULTS

Expt.	Drug	Dose (μg/ml.)	Sum of increases: Control	Sum of increases: With drug	Difference (no.)	Difference (%)
1	Physostigmine	1	54	90	36	66
2	Physostigmine	1	39	66	27	69
3	Neostigmine	3	33	71	38	115
4	Neostigmine	2	32	88	56	175
5	Neostigmine	1	62	92	30	48
6	Neostigmine	1	38	64	26	68
	Neostigmine	2	38	98	60	157
7	Neostigmine	1	92	194	102	110

Positive results

A list of successful experiments is given in Table 3. Under the heading of "controls" all the increases caused by stimulation at the different frequencies were added together, and under the heading of "drug" all the increases caused by stimulation at the same frequencies in the presence of the anticholinesterase were added together. The difference was then observed and it was calculated as a percentage of the control figure.

Effect of anticholinesterases on noradrenaline

Experiments were performed to determine whether neostigmine augmented the accelerator action of noradrenaline. These observations were made on the isolated rabbit atria, because of the considerable variation in the effect of noradrenaline when it is injected into the Langendorff preparation. This is probably explained by the variation in the rate of passage of the noradrenaline through the heart when it is given as a single injection. When atria are suspended in a bath, it is possible to ensure that a known concentration is applied. The observations were made in presence of atropine (10^{-6} g/ml.), at a temperature between 28° and 28.5° C. The heart rate was counted at intervals of 1 min for 5 min after each addition of noradrenaline and the maximum increase was recorded. Table 4 shows the results in two experiments; in neither did neostigmne (10^{-6} g/ml.) increase the effect of noradrenaline.

TABLE 4

INCREASE IN A TRIAL RATE CAUSED BY NORADRENALINE ACTING IN THE PRESENCE OF ATROPINE 10^{-6} G/ML. BEFORE AND AFTER ADDITION OF NEOSTIGMINE 10^{-6} G/ML. TO THE BATH

		Control observations		Observations in presence of neostigmine	
Expt.	Noradrenaline concn. in bath (μg/ml.)	Initial rate/ min	Increase/ min with noradrenaline	Initial rate/ min	Increase/ min with noradrenaline
8	0·14	136	10	129	10
		136	5	119	16
		131	17	118	7
		129	14	110	14
		126	11		
		Mean	11·4	Mean	11·75
9	0·086	85	46	76	41
		85	45	79	38
		80	40	71	29
		Mean	43	Mean	36

DISCUSSION

Evidence has come from different sources to show that stimulation of the accelerator fibres to the heart liberates acetylcholine as well as noradrenaline. Folkow, Frost, Haeger & Uvnäs (1948) perfused the heart of the dog and of the cat with a solution containing physostigmine and collected the effluent from the coronary sinus. They tested it on the

leech preparation. They observed that when the stellate ganglion was stimulated, a substance having the properties of acetylcholine appeared in the effluent. Huković (1959) prepared the isolated atria of the rabbit with the sympathetic fibres. When these were stimulated, they caused acceleration. However, if a rabbit were treated with reserpine to remove the noradrenaline, stimulation of the fibres then caused inhibition of the atrial contractions. This inhibition was increased by physostigmine and was abolished by atropine. Day & Rand (1961) made a similar preparation of the atria of the kitten with sympathetic fibres. This responded by acceleration when the sympathetic fibres were stimulated. When guanethidine was added to the bath to prevent the release of noradrenaline, gradually the response to stimulation was reversed and became inhibitory. Boura & Green (1959) found that when an anaesthetized cat had been injected with 1–3 mg/kg bretylium, stimulation of the inferior cardiac nerve, which ordinarily causes acceleration, caused slowing which persisted even when the bretylium injection was increased to 10 mg/kg. The slowing was abolished by atropine, 1 mg/kg.

Not only is there evidence that stimulation of the accelerator fibres can release acetylcholine, but there is also evidence that this acetylcholine is involved in the release of noradrenaline. Chang & Rand (1960) prepared the atria of the kitten, and showed that the acceleration of the rate caused by sympathetic stimulation was reduced and nearly abolished when hemicholinium was added to the bath. Moreover they showed that the full effect of stimulation was restored by the addition of choline to the bath. Birks & Macintosh (1961) demonstrated that, in the perfused superior cervical ganglion, hemicholinium blocked the synthesis of acetylcholine, and that the block was overcome by choline. The observations on the atria of the kitten indicate that hemicholinium prevents the release of noradrenaline, and that its action here also is overcome by choline. This points to a requirement for a normal formation of acetylcholine if noradrenaline is to be released.

The observations described in this paper provide confirmatory evidence of this requirement. For if acetylcholine plays a part in the release of noradrenaline, then anticholinesterases should increase the amount of noradrenaline released.

It would not be expected that the increase would be the same for all frequencies of stimulation. It is likely that at low frequencies such as 0.2/sec and 0.5/sec, much less acetylcholine will accumulate (and therefore much less noradrenaline will be released) because there is time between successive impulses for acetylcholine to be destroyed by cholinesterase. At higher frequencies the time between successive impulses is much shorter, so that cholinesterase has less time to act, and therefore the concentration of acetylcholine will rise much higher and more noradrenaline will be released. A rise in frequency is thus a means of reducing the activity of cholinesterase. The effect of an anticholinesterase should then be greatest when the effect of cholinesterase is greatest —that is, at the lowest frequencies. It is shown in Table 1 that in the presence of neostigmine there was acceleration in response to a frequency of 0.2/sec, to which there was no response whatever in the absence of neostigmine. The only exception to a smaller percentage increase being produced by a higher frequency is in Table 1, where neostigmine increased the acceleration by 47% at 5/sec, but by 37% at 2/sec. However, it increased the acceleration by 94% at 1/sec, and by 600% at 0.5/sec.

These observations cannot be explained on the view that the nerve impulse releases noradrenaline directly, but they provide further evidence in support of the hypothesis of

Burn & Rand (1959) that acetylcholine plays a part in the release of noradrenaline from the sympathetic post-ganglionic fibre, just as it plays a part in the release of adrenaline and noradrenaline from the adrenal medulla.

Similar observations of the action of physostigmine, neostigmine and other anticholinesterases in increasing the effect of sympathetic post-ganglionic stimulation at low frequencies have been made in the nictitating membrane of the cat (Burn, Rand & Wien, 1963) and in the isolated taenia of the guinea-pig (Ng, 1966).

The perfusion experiments were carried out at temperatures below 37° C, because the rabbit heart is then less likely to develop arrhythmias. A final point is that, while neostigmine did not change the response of isolated atria to noradrenaline in the presence of atropine, it might have done so in the absence of atropine.

The experiments were described in a communication to the Physiological Society on June 4, 1966.

SUMMARY

1. The isolated heart of the rabbit with sympathetic fibres attached was prepared and perfused by the Langendorff method with McEwen's solution.

2. The stellate ganglion was held in electrodes in which position it was constantly irrigated.

3. Supramaximal stimuli were applied to the ganglion for 30 sec at frequencies varying from 0.2/sec to 5/sec, and the increase in heart rate was recorded on a counter during 1 min.

4. The perfusion fluid contained atropine 10^{-6} and choline 0.5 mg/ml. Control observations were made, and then physostigmine or neostigmine 10^{-6} was added to the perfusion fluid.

5. The increase in rate caused by stimulation in the presence of the anticholinesterases was greater than in the control period, and stimulation of a low frequency which had no effect in the control period became effective.

6. Neostigmine did not increase the effect of noradrenaline.

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