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Effects of hemicholinium and bretylium on the release of autonomic transmitters in the isolated sino-atrial node

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Summary

1. In the isolated, spontaneously beating, sino-atrial node of the rabbit selective electrical excitation of intranodal autonomic nerve fibres results in a biphasic chronotropic response. This chronotropic response (negative followed by positive chronotropism) is due to the release of the autonomic transmitters (acetylcholine and noradrenaline, respectively) from intranodal nerve fibres.

2. In the presence of 2×10^{-4} g/ml hemicholinium, the negative chronotropic (cholinergic) response is abolished while the positive chronotropic (adrenergic) response is unaltered.

3. In the presence of 5×10^{-6} g/ml bretylium, the positive chronotropic response is abolished while the negative chronotropic response is little affected.

4. After blockade of the negative chronotropic response by hemicholinium, bretylium abolishes the remaining positive chronotropic response. The effect of bretylium is not altered in the presence of hemicholinium.

5. Considering currently accepted mechanisms of action for hemicholinium and bretylium, the results of these experiments do not lend support to the cholinergic link hypothesis of adrenergic neuro-effector transmission.

Introduction

According to the hypothesis of Burn & Rand (1959), the release of the adrenergic transmitter, noradrenaline, is mediated through a cholinergic link. That is, stimulation of the adrenergic postganglionic fibre releases acetylcholine which is responsible for the subsequent release of noradrenaline. The drugs hemicholinium No. 3 (α,α-dimethylethanolamino-4,4-bis acetophenone ; HC-3) and bretylium (N-*o*-bromobenzyl-N-ethyl-N,N-dimethyl ammonium *p*-toluenesulphonate), have been particularly useful in the investigation of the cholinergic link hypothesis. HC-3 has been shown to be a specific blocker of cholinergic nerves by inhibiting the production of acetylcholine (MacIntosh, Birks & Sastry, 1956 ; Scheuler, 1960). Bretylium has been shown to be a selective blocking agent of the adrenergic nerves by preventing the release of noradrenaline from nerve endings (Boura & Green, 1959). The use of HC-3 in demonstrating a cholinergic link in adrenergic transmission has met with controversy. While some investigators have shown sympathetic block with HC-3 (Chang & Rand, 1960), others have not (Vincenzi & West, 1965 ; Leaders, 1965).

The mechanism of noradrenaline release caused by stimulation of preparations of isolated heart tissue (such as those used by Vincenzi & West, 1965 ; or by Blinks, 1966) has been questioned. Blinks (1966) was unable to show blockade of sympathetic responses with bretylium using field stimulation. This suggested the possibility that the release of noradrenaline under the conditions of field stimulation is by a mechanism different from the physiological release in which the cholinergic link is proposed. Burn (1968) has suggested that in preparations such as those used by Vincenzi & West (1965) (in which HC-3 does not block sympathetic responses) that a similar unphysiological release of noradrenaline may be involved. Burn further suggested that bretylium could be used to determine whether the sympathetic responses were being produced by " field stimulation ". If so, it was predicted that they would not be amenable to block by HC-3 or by bretylium. This work was designed to investigate this suggestion.

Methods

The methods used in this study were similar to those reported by Vincenzi & West (1965). The technique of " electrorelease " in the isolated sino-atrial node, developed by Amory & West (1962) and Vincenzi & West (1963), has provided a useful model for the study of postganglionic nervous system function. Following the application of carefully controlled electrical pulses to the spontaneously beating sino-atrial node a biphasic chronotropic response occurs. This response is characterized by an initial slowing of the rate followed by an acceleration to above the pre-stimulus rate. These changes in rate, referred to as cholinergic and adrenergic " electrorelease " responses, respectively, are thought to be mediated via the release of acetylcholine and noradrenaline from the intranodal nerve fibres (Vincenzi & West, 1963). With the technique used in this study, as in previous work, it is possible to stimulate the intranodal nerve fibres without exciting the myocardium *per se* (Vincenzi & West, 1963).

Male albino rabbits weighing approximately 2 kg were stunned and killed by bleeding. The sino-atrial node and right atrium were quickly removed and mounted in an acrylic bath maintained at 35° $\pm$0·5° C. The bath was continuously perfused at 4 ml/min with a modified Ringer-Locke solution of the following composition in g/l.: NaCl 8·5, KCl 0·4, $NaHCO_3$ 1·0, dextrose 2·0, $CaCl_2 \cdot 2H_2O$ 0·324. The solution was gassed in an overhead reservoir with a 95% oxygen–5% carbon dioxide mixture. The tissue was allowed to equilibrate for 30–45 min before stimulation was begun. Stimuli were applied directly to the sino-atrial node through 0·25 mm wires insulated to the tip and placed in direct contact with either side of the tissue. Five second bursts of rectangular wave stimuli (6·25 V, 50 Hz) with pulse duration adjusted between 0·1 and 1·0 ms were applied every 180 s throughout each experiment. Recording and evaluation of responses were done by a method previously reported (Vincenzi & West, 1965). Briefly, surface action potentials were obtained with bipolar electrodes placed on the atrial tissue. Each action potential was used to trigger a free-running linear voltage ramp generator, thus giving a record of spontaneous beat interval on a beat-to-beat basis. Responses to stimulation were quantified by defining the longest beat interval following a stimulus as the maximal interval and the shortest beat interval following a stimulus as the minimal interval. The cholinergic responses were then considered proportional to the difference between the maximal and base intervals (base being measured just

before a stimulus burst). Likewise the adrenergic responses were considered proportional to the difference between the base and minimal intervals. Cholinergic and adrenergic responses were calculated as percent increase and decrease in beat interval (respectively) from the base interval (Table 1). Chronotropic responses were further expressed as a percent of the initial chronotropic cholinergic or adrenergic response at the beginning of each experiment (Fig. 1). Standard statistical methods were used to determine means and standard error. Student's t test was used as a test of significance of differences (Goldstein, 1967).

The drugs used were hemicholinium bromide (Aldrich Chemical Co.) and bretylium tosylate (Burroughs). The drugs were dissolved in the perfusing solution and administered by continuous perfusion as described.

Results

Preliminary experiments indicated that 105 min was generally sufficient for HC-3 and bretylium to block the autonomic functions in these preparations. It was decided to evaluate all subsequent experiments at 15 min intervals for 105 min. After equilibration of the tissue the stimulating electrodes were placed in position and the stimulus parameters were selected to give a stable chronotropic response. A 5 s burst was then applied every 3 min for 105 min in every experiment. Drugs were added only after the chronotropic responses were stable. Time 0 (Fig. 1a and b) was then arbitrarily defined, after which thirty-five subsequent bursts of stimuli were supplied.

It was noted in each of the twenty preparations studied that the chronotropic response to stimulation of the node varied with the position at which the electrodes were placed. Responses differed in quality and magnitude, some showing nearly entirely an adrenergic component and others having mainly a cholinergic component. It thus appears that somewhat less than the total population of intranodal autonomic fibres is stimulated under these conditions. For the subsequent measurements it was therefore important to maintain a constant electrode position and stimulus parameters. Since it was desired to examine both cholinergic and sympathetic transmission a position was selected on the node that would give both a negative and a positive chronotropic response to stimulation. Control experiments were run to evaluate the effect of the stimulus programme. Stimulation of the node for nearly 2 h at 3 min intervals caused very little change in the biphasic chronotropic responses (see Table 1). The control cholinergic responses (Fig. 1a) were seen to be less stable than the adrenergic responses.

It is known that HC-3 will block cholinergic function in certain experimental conditions (Scheuler, 1960 ; Long, 1961). It was important in this study to determine if HC-3 would affect adrenergic function as well. In a concentration of 2×10^{-4} g/ml, HC-3 produced a progressive blockade of the cholinergic " electrorelease " response within 60 min (Fig. 1a). On the other hand, the adrenergic " electrorelease " response was not significantly altered by the presence of HC-3 in the conditions of these experiments (Fig. 1b). These findings confirm those of Vincenzi & West (1965) using a slight modification of their technique.

Some experiments were carried out with bretylium alone to serve as partial controls and to observe the effects of bretylium on the adrenergic " electrorelease " response. In these experiments the preparation was stimulated for 105 min as described previously. After 60 min of control stimulation (the time HC-3 was seen

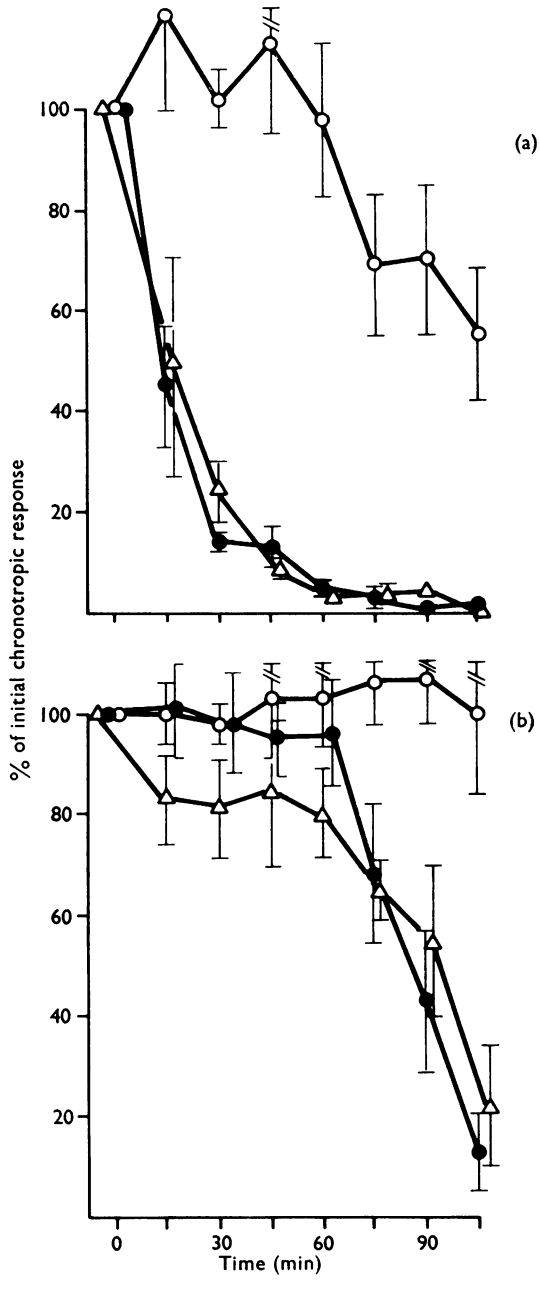

FIG. 1. Chronotropic responses of isolated rabbit sino-atrial node to direct electrical stimulation of intranodal innervation every 3 min for 105 min. (a). Cholinergic responses: ○——○, control experiments; ●——●, influence of 2×10^{-4} g/ml HC-3 added at time zero; △——△, influence of HC-3 (time zero) and 5×10^{-6} g/ml bretylium (added at 60 min). (b), Adrenergic responses: ○——○, control experiments; ●——●, influence of bretylium (5×10^{-6} g/ml) added at 60 min; △——△, influence of HC-3 added at time zero (2×10^{-4} g/ml) and bretylium added at 60 min (5×10^{-6} g/ml). Values represent the percentage of the initial chronotropic responses expressed as a percentage of the initial response at time zero in each set of experiments.

TABLE 1. *Influence of hemicholinium and bretylium on chronotropic responses to stimulation of intranodal autonomic nerves*

	Time (min)				
	0	30	60	75	105
Cholinergic responses					
Control (n=4)	699·3±198·3*	736·9±210·1	715·0±229·7	499·3±222·7	423·8±219·2
HC-3 only (n=5)	479·9±152·6	69·3±23·1	15·2±5·1	9·2±2·7	3·2±0·0
Bretylium only (n=5)	219·9±75·8	193·1±77·2	190·4±80·6	96·9±22·2	96·6±17·9
HC-3+bretylium (n=6)	180·0±24·7	43·7±17·1	1·7±1·1	2·1±1·3	0
Adrenergic responses					
Control (n=4)	28·1±0·9	27·6±1·7	28·9±2·5	29·8±2·1	28·0±4·2
HC-3 only (n=5)	27·7±2·1	22·6±2·6	24·8±3·9	27·4±3·6	25·6±4·4
Bretylium only (n=5)	25·5±2·1	24·9±3·3	24·5±3·1	17·4±3·5	3·2±2·1
HC-3+bretylium (n=6)	22·4±2·5	17·4±1·6	17·0±1·5	14·4±2·1	5·5±2·8

HC-3 was added at time 0 and perfused throughout the experiment; bretylium was added at 60 min.
*Each entry represents the maximum percentage change in the beat interval produced by intranodal nerve stimulation. Values (see **Methods** for calculation) represent means±standard error.

to produce complete block of the cholinergic responses), bretylium (5×10^{-6} g/ml) was given. In the presence of bretylium the adrenergic " electrorelease " response progressively declined and in most preparations was completely blocked within 45 min (Fig. 1b). The cholinergic " electrorelease " response declined somewhat but was not significantly different ($P>0{\cdot}5$) from control responses at 105 min (Table 1). There was no significant change in the basic spontaneous rate during bretylium administration.

It was reported by Burn & Gibbons (1964) that HC-3 inhibited the adrenergic blocking effect of bretylium in guinea-pig ileum. Since the adrenergic response observed in sino-atrial node preparations remained after cholinergic block with HC-3, it seemed necessary to ask whether these responses could be blocked by bretylium in the presence of HC-3. Thus, a final series of experiments was performed to test the effects of bretylium in the presence of a cholinergic block by HC-3. HC-3 (2×10^{-4} g/ml) was given at the beginning of each experiment and was perfused throughout the time course of stimulation. After 60 min with HC-3 present, the cholinergic " electrorelease " response was blocked (Fig. 1a). Bretylium (5×10^{-6} g/ml) was then added. The adrenergic response, which had not been altered by HC-3 (Fig. 1b), diminished and was blocked within 45 min by bretylium in four out of six experiments. (Complete block of the adrenergic response by bretylium was seen in 50 min in the other two preparations.) Compared with experiments in which HC-3 was not present, the adrenergic responses at 105 min were not significantly different ($P>0{\cdot}3$). Thus, bretylium action was not inhibited in the presence of HC-3.

Discussion

The present results should help to demonstrate an important difference between the selective electrical excitation of intramyocardial innervation used in our laboratories from the less selective field stimulation method used by Blinks (1966). Various explanations for the selectivity of the method of " electrorelease " have been offered by Vincenzi & West (1963, 1965). To ensure selectivity the present experiments were done using a point electrode on each side of the tissue (rather than a plate electrode on one side as had been used by Vincenzi & West, 1965). As noted, the responses to stimulation of the node varied with the position on the node at which the electrodes were placed. It is doubtful if such selectivity could be obtained using the field stimulation method (in which, presumably, all excitable cells are depolarized). Certainly small currents applied through two punctate electrodes do not exert " generalized field stimulation " ; in fact, they are even subthreshold for the myocardium.

In these experiments HC-3 was seen to be selective for blocking the cholinergic " electrorelease " response. Bretylium was apparently selective for abolishing the adrenergic " electrorelease " response, and was not affected by the presence of HC-3. The pharmacology of the electrorelease phenomenon (Amory & West, 1962) indicates that the effects of HC-3 and bretylium are on the postganglionic innervation of the sino-atrial node. Blinks (1966) found that bretylium was unable to block the adrenergically mediated positive inotropic effects of field stimulation in isolated atrial preparations. It was suggested by Burn (1968) that such adrenergic transmission was due to an unusual type of noradrenaline release caused by field stimula-

tion. Katz & Kopin (1969) showed that bretylium did not block release of noradrenaline-^{3}H in field-stimulated atria with stimulating currents between 10 and 25 mA. With current strengths of less than 10 mA, however, bretylium was able to block the release of noradrenaline-^{3}H ; the release being calcium-dependent only at these lower strengths. In Blinks' study 50 mA was used while in these experiments the current strength was approximately 5 mA. Hence the adrenergic responses reported here are considered to be of the classical type (based on " physiological " release of noradrenaline). Such responses were clearly amenable to block by bretylium.

As demonstrated previously by Vincenzi & West (1965), HC-3 does not effect the adrenergic " electrorelease " response. It appears that HC-3 acts by depleting the stores of acetylcholine from the intranodal cholinergic nerves under conditions of this kind of experiment. Thus observations with HC-3 (which does block cholinergic transmission but which does not block adrenergic transmission) do not support the notion of an obligate cholinergic link in the release of noradrenaline from adrenergic endings in the sino-atrial node.

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REFERENCES

AMORY, D. W. & WEST, T. C. (1962). Chronotropic response following direct electrical stimulation of the isolated sino-atrial node. A pharmacological evaluation. *J. Pharmac. exp. Ther.*, **137**, 14–23.

BLINKS, J. R. (1966). Field stimulation as a means of effecting the graded release of autonomic transmitters in isolated heart muscle. *J. Pharmac. exp. Ther.*, **151**, 221–235.

BOURA, A. L. A. & GREEN, A. F. (1959). The actions of bretylium. Adrenergic neurone blocking and other effects. *Br. J. Pharmac. Chemother.*, **14**, 536–548.

BURN, J. H. & RAND, M. J. (1959). Sympathetic postganglionic mechanism. *Nature, Lond.*, **184**, 163–165.

BURN, J. H. & GIBBONS, W. R. (1964). The sympathetic postganglionic fibre and the block by bretylium; the block prevented by hexamethonium and imitated by mecamylamine. *Br. J. Pharmac. Chemother.*, **22**, 549–557.

BURN, J. H. (1968). Catecholamines: Pharmacology. In *Recent Advances in Pharmacology*, ed. Robson, J. M. & Stacey, R. S. Boston: Little, Brown & Co.

CHANG, V. & RAND, M. J. (1960). Transmission failure in sympathetic nerves produced by hemicholinium. *Br. J. Pharmac. Chemother.*, **15**, 588–600.

GOLDSTEIN, A. (1967). *Biostatistics: An introductory text*, p. 71. New York: The Macmillan Co.

KATZ, R. I. & KOPIN, I. J. (1969). Electrical field stimulated release of norepinephrine-H^3 from rat atrium; effects of ions and drugs. *J. Pharmac. exp. Ther.*, **169**, 229–236.

LEADERS, F. E. (1965). Separation of adrenergic and cholinergic fibres to the hind limb of the dog by hemicholinium (HC-3). *J. Pharmac. exp. Ther.*, **148**, 238–246.

LONG, J. P. (1961). Hemicholiniums; structure activity relationships and actions on peripheral nervous system. *Fedn Proc.*, **20**, 583–593.

MACINTOSH, F. C., BIRKS, R. J. & SASTRY, P. B. (1956). Pharmacologic inhibition of acetylcholine synthesis. *Nature, Lond.*, **178**, 1181.

SCHEULER, F. W. (1960). The mechanism of action of the hemicholiniums. In *Int. Rev. Neurobiol.*, vol. 2, ed. Pfeiffer, C. C. and Smythies, J. R. New York: Academic Press.

VINCENZI, F. F. & WEST, T. C. (1963). Release of autonomic mediators in cardiac tissue by direct subthreshold electrical stimulation. *J. Pharmac. exp. Ther.*, **141**, 185–194.

VINCENZI, F. F. & WEST, T. C. (1965). Effect of hemicholinium on the release of autonomic mediators in the sino-atrial node. *Br. J. Pharmac. Chemother.*, **24**, 773–780.

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