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ELECTROPHYSIOLOGY OF CARDIAC FUNCTION IN TELEOSTS: CHOLINERGICALLY MEDIATED INHIBITION AND REBOUND EXCITATION

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SUMMARY

1. A study has been made on the responses of the plaice (*Pleuronectes platessa*) heart, isolated and *in situ* to differential vagal stimulation using intra- and extracellular recording electrodes. The effects of direct application of acetylcholine and catecholamines and their respective blocking agents are also reported.

2. Vagal stimulation at 7 Hz totally inhibits heart beat whilst stimulation at 2–3 Hz accelerates it. Both these effects are blocked by atropine (10^{-6} g/ml.). Bretylium (10^{-5} g/ml.) and pronethalol (10^{-6} g/ml.) have no effect upon either response to vagal stimulation.

3. On cessation of prolonged inhibitory vagal stimulation there is a marked increase in the heart rate, and in quiescent hearts one or two beats are initiated after stimulation.

4. Vagal stimulation gives rise to a hyperpolarization in atrial cells. It is proposed that all the excitatory effects of vagal stimulation are due to rebound excitation from an inhibitory hyperpolarization. At high frequencies the hyperpolarizations summate to give rise to total inhibition. At lower frequencies of stimulation the heart beat is increased to rates dependent on interaction between the time course of the hyperpolarization and the refractory period of the heart. Both effects are cholinergically mediated.

5. The rebound excitation in response to vagal stimulation (post-vagal tachycardia) persists in response to stimulation of the atrial myocardium in the presence of atropine (10^{-6} g/ml.) and bretylium (10^{-5} g/ml.). It is therefore suggested that this is a response of the muscle cell membrane to vagal stimulation and is not nerve-mediated.

INTRODUCTION

There has been comparatively little electrophysiological work on innervation of the teleost heart and, apart from the work by Gannon & Burnstock (1968) and Gannon (1971), the only general property that has been demonstrated is that there is a cholinergic inhibitory innervation (Von Skramlik, 1935; Laurent, 1962; Campbell, 1970). Kulaev (1957) believed that the positive chronotropic effects of heart rate due to vagal stimulation in the pike resulted from 'active vagal influences'. All vertebrates higher than fish possess both a parasympathetic cardio-inhibitory and a sympathetic cardio-excitatory innervation. Cardio-inhibition is effected by a hyperpolarization of the pace-maker cell membrane caused by vagal stimulation; this effect can be mimicked by acetylcholine. Cardio-excitation on the other hand is effected by an increase in the rate of the diastolic depolarization of the pace-maker cell caused by sympathetic stimulation or the application of noradrenaline (Hutter & Trautwein, 1956).

Preliminary physiological studies on the plaice heart (Cobb & Santer, 1972) have shown that cardio-excitatory and cardio-inhibitory effects can be isolated from one another by differential stimulation which has enabled the following investigations to be carried out concerning the excitation and inhibition of the heart.

METHODS

Approximately ninety plaice (*Pleuronectes platessa* L.) were used in this study.

Plaice of various sizes (8–14 in.) were trawled locally and held in constant circulation tanks. Animals for experiment were decerebrated and pithed before dissecting the cardiac region. Organ-baths and whole animal baths were held to ambient local sea-water temperatures using a water jacket connected to a chiller-thermo circulator. A Ringer solution prepared for the plaice (J. L. S. Cobb, N. Fox & R. M. Santer, in preparation) was used throughout.

Extracellular recording was carried out using conventional suction electrodes of finely drawn polyethylene tubing of 0·1 mm tip diameter, and a differential amplifier.

Intracellular recordings using the floating micro-electrode technique developed by Woodbury & Brady (1956) with an electrode resistance of the order of 25 MΩ were made. A Bak amplifier and a Tektronix 502A oscilloscope were used to record activity.

Stimulation was carried out using Tektronix pulse and wave form generators connected via an optically-coupled gallium arsenide isolation unit providing a constant voltage (C. Roemmélé, in preparation). The stimulating electrode resistance was approximately 20 kΩ and the shocks delivered were just suprathreshold

Perfusion studies on isolated hearts

As described by Falck, von Mecklenburg, Myhrberg & Persson (1966), a cannula was inserted into the sinus venosus as far as the sino-atrial junction and connected to a perfusion apparatus. The apex of the ventricle was connected to a kymograph

for recording contractions of the heart. The heart was perfused at a constant rate with a Ringer solution ionically and osmotically equivalent to the blood of the plaice, at 10° C. Test compounds were applied via a side-tube close to the heart.

Compounds used: acetylcholine chloride, atropine sulphate, adrenaline hydrogen tartrate (B.D.H.), tubocurarine chloride, bretylium tosylate (Burroughs Wellcome), noradrenaline bitartrate monohydrate (Sigma), tyramine, 5-hydroxytryptamine (Koch-light) and pronethalol (Alderlin, I.C.I.). All doses of drugs are given as salts.

RESULTS

Intracellular recording from atrial cell types

The frog and all higher vertebrates have discrete regions of pace-maker cells at the sino-atrial (S-A) region of the heart and an examination of the atrium of the heart of the plaice was made in an attempt to locate a similar region. The initial experiments were carried out recording from the dorsal surface of the heart close to the S-A junction. It is in this

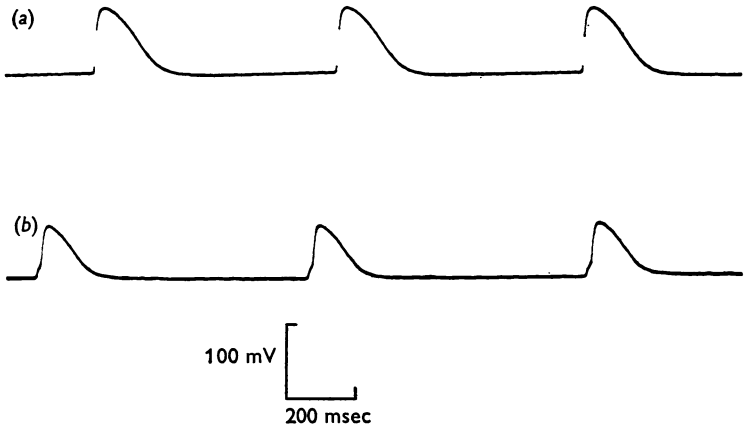

Fig. 1. (*a*) Normal intracellular spike observed in most impaled atrial cells. (*b*) Record showing slow pre-spike depolarization. It is proposed that this slow depolarization is due to electrical events in neighbouring cells failing to propagate between coupled cells but manifesting themselves by electrotonic spread to the impaled cell.

region that one of the two cardiac branches of the vagus breaks up into a plexus. A previous report (Cobb & Santer, 1972) describing a single branch has now been shown to be in error. A second branch has been found innervating the S-A junction on the ventral side. No concentration of cells showing characteristic pace-maker responses was found. In at least 90 % of the cells the action potentials recorded showed a fast depolarization rising from a steady resting potential. The resting potential averaged between 75–78 mV and the spike potential was 85–90 mV

(Fig. 1*a*). Scattered in an apparently random fashion throughout the atrium was a minority of cells showing two different types of potential. The first type, only rarely seen, was a typical pace-maker potential showing a slow diastolic depolarization between characteristic action potentials. The second type arose from a steady resting potential with each spike preceded by a junction potential-like depolarization similar to those seen in frog conducting fibres (Denoit-Mazet & Vassort, 1971) (Fig. 1*b*), but in this case probably representing electrotonic spread from a neighbouring cell. All recorded action potentials showed a falling phase approximately twenty times slower than the rising phase. The duration of the whole potential was about 200 msec. Potentials with these characteristics were obtained from impalements of atrial cells *in situ* in five pithed animals, and from hearts dissected from fifteen animals.

A further survey was undertaken to sample cells of the whole atrium. Hearts were pinned out with either the dorsal or ventral surface uppermost. In other preparations the tube formed by the sinus venosus and atrium was split open so that cells could be impaled from the luminal side of the heart. These studies have not revealed a localized region of cells showing either type of interspike depolarization and support the earlier suggestion that the pacemaker system in fish cardiac tissue is diffuse throughout the atrial myocardium (Laurent, 1962; Jensen, 1965), but it is very difficult to imagine how such an arrangement could function efficiently. A further study may however show that such a localized area exists, as suggested by Wardle (1962) in the flounder (*Platicthys flesus*).

Extracellular recording during and after vagal stimulation

There is a single cardiac branch of the vagus passing over the ocular side of the sinus venosus which either runs singly or branches into a double tract as far as the pericardial membrane. Inside the pericardium this branch of the vagus splits up into a number of large and small tracts running over the sinus venosus before they reach the atrium. This plexus of cardiac nerves also contains the parasympathetic ganglion cell bodies (Santer, 1972; Santer & Cobb, 1972). The stimulating suction electrode was placed over the cardiac nerve where it initially branched over the sinus venosus. The pericardium was opened by a longitudinal slit and a second recording suction electrode was placed on the atrium. Heart beat was always completely blocked by applying stimuli of 0·5 V for 0·03 msec duration at frequencies of 7 Hz, and incompletely blocked at frequencies of 4–7 Hz (Fig. 2*a*). The same results are achieved using a stimulating voltage as high as 40 V. On cessation of stimulation the heart beat returned after a delay of approximately 1–1·5 sec (Fig. 3*c*). The first and subsequent extracellular potentials were smaller in size than those at

resting rate but increased in size until after 4–5 beats they had returned to control values (Fig. 3). An intracellular recording of this phenomenon is shown in Fig. 3*d*. After the cessation of inhibitory stimulation the heart rate increased above the original resting rate for a short period before returning to the resting rate of that particular heart (Fig. 2*a*).

In a minority of experiments (about 1 in 5) low frequency stimulation (2 Hz), using the same stimulus parameters, produced an increase in the heart rate above normal (Fig. 2*b*). Fifty fish were used for the above *in vivo* study.

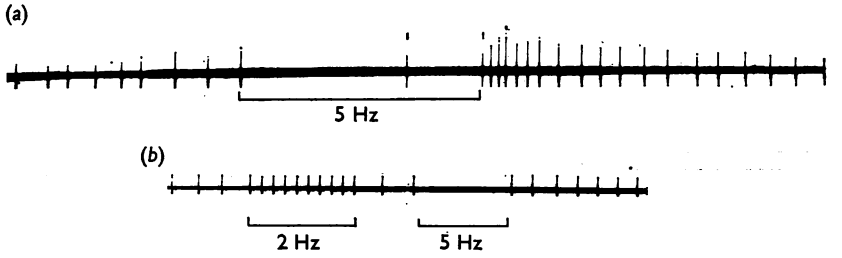

Fig. 2. (*a*) Partial inhibition of heartbeat during vagal stimulation of 5 Hz. (*b*) Excitation and total inhibition to vagal stimulation of 2 and 5 Hz respectively. Stimulus parameters: 0·5 V, 0·03 msec duration.

These experiments were repeated with whole nerve-heart preparations of forty fish dissected out, *in vitro*. These studies have made it clear that the critical factor determining excitation and inhibition due to vagal stimulation is the heart rate of the individual preparation itself. In whole animal experiments the heart rate after pithing remains high (1/sec or more) for long periods, but in dissected preparations it slows to 0·5/sec or slower, sometimes becoming irregular after about 30 min. In these preparations which were beating at a slow rate it was always possible to increase the rate by low frequency vagal stimulation of 3·1 Hz and at even slower frequencies to drive the heart on a 1:1 basis (1·2 Hz) (Fig. 4), whereas this was more difficult to achieve in whole animal preparations. Measurement of the stimulus parameters indicated that a change in frequency from 4 to 4·5 Hz caused a change from excitation to partial inhibition and a change from 3·1 to 6 Hz caused a change from maximum excitation to total inhibition. Maximum excitation was maintained from 3·1 to 2 Hz but below this frequency the heart rate slowed until directly driven at a frequency of 1·2 Hz.

In some isolated preparations where the heart beat had ceased it was possible with low frequency stimulation (2 Hz and below) to restart contractions which continued for the duration of the applied stimulus.

A higher frequency stimulation applied to such quiescent preparations always caused at least one heart beat on cessation of the stimulus, and sometimes several.

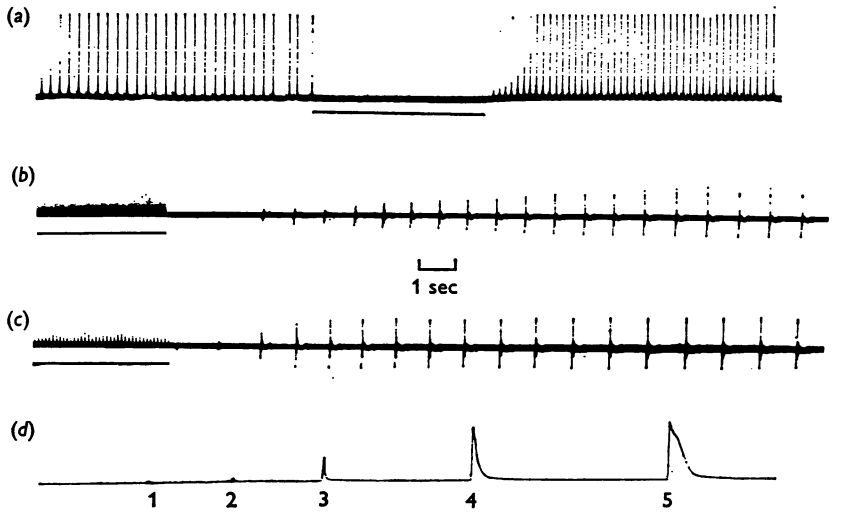

Fig. 3. (*a*) Intracellular recording of electrical activity in an atrial cell following cessation of stimulation at 10 Hz. Note the similar increase in the size of the first few post-stimulus depolarizations as seen with extracellular recording. Bar marks stimulus duration. (*b*) Extracellular recording of the delayed return to normal of the heart beat following cessation of stimulation at 30 Hz. (*c*) Similar record to that in (*b*) but following cessation of 10 Hz stimulation. Note the shorter delay before the return of the heart beat and the more rapid return to normal spike size. (*d*) Intracellular recording from an atrial cell following vagal stimulation of 10 Hz for 5 sec. Note the gradual return to normal spike profile. The difference in the falling phase between events 4 and 5 may be due to long-lasting permeability changes in the atrial cell membrane following prolonged inhibition. The gradual increase in size of events 1–4 is explicable by proposing that events 1–3 are pace-maker potentials in relatively distant cells failing to propagate actively and being conducted decrementally by electrotonic spread through coupled cells. The distance actively propagated increases with each distant pace-maker event until it reaches the impaled cell at event 4. Stimulus parameters for all Fig. 3 records are 0·5 V and 0·03 msec duration.

Intracellular recording during and after vagal stimulation

Impalements of atrial cells with microelectrodes were carried out on several hearts *in situ*, and on forty *in vitro* heart preparations pinned out in a preparation dish. The vagus nerve was again stimulated via a suction electrode. Each pulse applied to the vagus produced a hyperpolarization of 2–3 mV with a duration of approximately 200 msec. Higher frequency

stimulation produced a summation of the hyperpolarizations which eventually produced a steady potential 6 mV below resting potential. The value of this steady potential is approximately half that noted by del Castillo & Katz (1955) in frog heart cells and it is likely that decre-

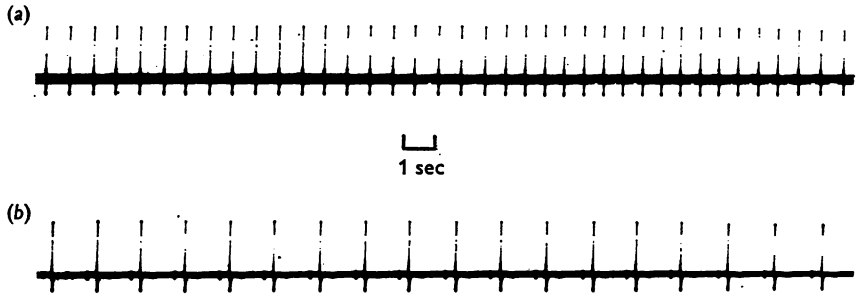

Fig. 4. (*a*) Heart driven by vagal stimulation of 1·2 Hz. (*b*) Control heart rate following cessation of driving stimulus. Stimulus parameters: 0·5 V and 0·03 msec duration.

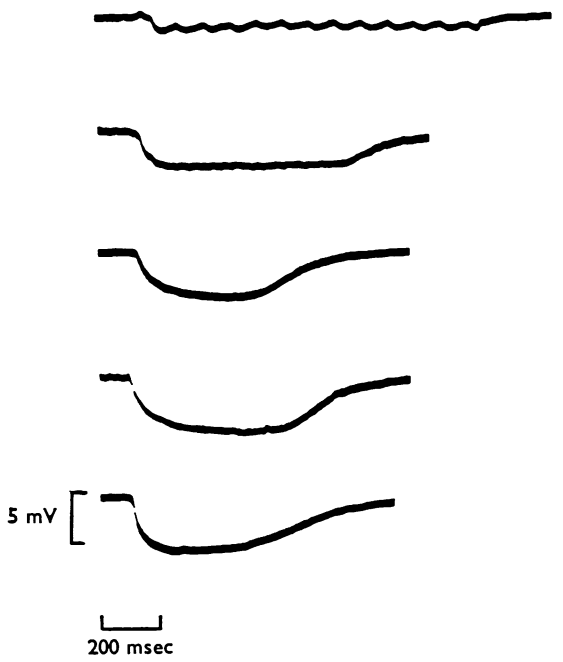

Fig. 5. Hyperpolarization of atrial cell resting potential in response to vagal stimulation at increasing frequencies (10, 20, 30, 35, 40 Hz respectively from top to bottom). Note the increase in the rate of hyperpolarization and the increased amplitude of the hyperpolarization with increased frequency. Stimulus parameters: 0·5 V, 0·03 msec duration.

mental electrotonic spread of conduction would account for this. Responses to different frequencies are shown in Fig. 5.

In preparations where low frequency (2 Hz) stimulation caused a speed-up of heart rate each hyperpolarization was followed by a spike (Fig. 6*a*). It has not proved possible in these recordings to separate the depolarization from the hyperpolarized state into two phases; the spike appears to arise straight from the hyperpolarization. In virtually all

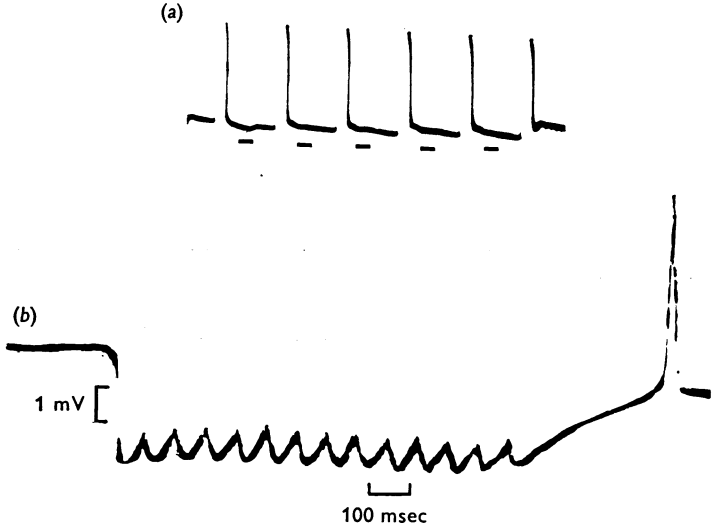

Fig. 6. (*a*) Intracellular recording from an atrial cell showing action potentials following each vagal stimulus (marked by short bar) at low frequency (1 Hz). Hyperpolarization at this gain is not readily visible. (*b*) Intracellular record from an atrial cell showing individual hyperpolarizations to individual vagal stimuli at 10 Hz. Note the depolarization on cessation of the stimulus giving rise to a further rapid depolarization but not to a standard action potential. This presumably represents a propagated event in another region of the atrium manifesting itself in the impaled cell by electrotonic spread through coupled cells. Fig. 6(*b*) retouched.

recordings, even from previously quiescent hearts, after high frequency stimulation had caused a summated hyperpolarization, a single spike, and sometimes two or three, was initiated on cessation of stimulation (Fig. 6*b*). This excitatory effect was observed after vagal stimulation of the heart which raises the possibility that the action potentials observed are a secondary effect caused by a set of intramural excitatory nerves. Alternatively, the 'rebound' effect may be an overcompensatory response of the muscle cell membrane to the preceding hyperpolarization. In order to solve this problem, hearts were stimulated with current from external

electrodes, in the presence of atropine (10^{-6} g/ml.) and bretylium (10^{-5} g/ml.) with the result that nerves to the heart were effectively blocked. The effects of tetrodotoxin on the Teleost heart are as yet unknown, but they are variable on the hearts of higher vertebrates (Narahashi, 1972). For this reason tetrodotoxin was not used to block the cardiac nerves. After an applied hyperpolarizing current to the atrium of ten fish had been stopped, a single action potential was almost always seen but in a few recordings as many as three occurred.

Effects of adrenergic and cholinergic blocking agents

With the whole heart and vagal branch dissected out, a stimulating suction electrode was applied to the vagus nerve and a similar recording electrode to the atrium. When the stimulation electrode had been correctly placed and the heart beat could be inhibited at 7 Hz, the preparation was allowed to achieve a relatively low rate of beating. When the heart was beating, stimulating the vagus at a frequency of 3 Hz caused excitation and at 7 Hz total inhibition, with stimulus parameters of 0·03 msec and 0·5 V.

The effect of drugs was tested on hearts in this experimental condition. Atropine at 10^{-6} g/ml. blocked both excitatory and inhibitory effects of vagal stimulation without altering heart rate. Neither the adrenergic neurone blocker, bretylium tosylate, nor the adrenergic β-blocker, pronethalol, at concentrations up to 10^{-5} g/ml., had any effect on the excitatory or inhibitory responses to vagal stimulation. Also, the transient post-vagal increase in heart rate was not affected by the application of bretylium or pronethalol. Neither drug altered spontaneous heart rate.

Perfusion studies on the effects of drugs on isolated hearts

Acetylcholine. ACh at concentrations from 10^{-6} to 10^{-12} g/ml. was applied to the hearts. Low concentrations (10^{-10}–10^{-12} g/ml.) produced negative chronotropic and inotropic responses whilst higher concentrations (10^{-6}–10^{-9} g/ml.) totally inhibited the heart beat, the duration of inhibition lasting longer at the higher concentrations. A post-inhibitory rebound always occurred consisting in the first instance of a positive inotropic response quickly followed by a positive chronotropic response. Normal heart rate ensued after a period of time varying with the earlier applied concentration of ACh. With high concentrations (10^{-6} g/ml.) of ACh, normal heart rate did not return for as long as 2 min after washing out of the drug. These effects of ACh were blocked by the muscarinic blocker atropine (10^{-7} g/ml.) but not by the nicotinic cholinergic blocker curare (10^{-7} g/ml.). Differential application of ACh to the heart chambers indicated that these results were due entirely to its effect on the atrium,

the ventricle being insensitive to ACh, but excited by electrotonic spread from the atrium.

Catecholamines. Noradrenaline and adrenaline at 10^{-6} g/ml. always produced positive inotropic and chronotropic responses, the heart being about ten times more sensitive to adrenaline.

These responses were blocked by the adrenergic β-receptor blocker pronethalol but not by bretylium at 10^{-6} g/ml. previously added to the perfusate. Both atrium and ventricle were sensitive to catecholamines.

Tyramine. Tyramine at 10^{-4} g/ml. had no effect at all on the heart.

The above results are in general agreement with the report by Falck *et al.* (1966) for the plaice heart.

DISCUSSION

The apparent diffuse occurrence of pace-maker cells throughout the S-A region has made the examination of the effect of the inhibitory innervation upon them a difficult problem. The two types of cells showing interspike depolarization have been previously described: the slow depolarization between action potentials representing the true pace-maker potential (Hutter & Trautwein, 1956), and the more rapid pre-spike depolarization being considered as characteristic of conducting fibre cells in the frog (Denoit-Mazet & Vassort, 1971). It seems more likely that electrotonic spread from propagated action potentials in neighbouring cells would produce identical effects to the latter type of recording. Clearly, the anatomical localization of pace-maker cells in the plaice remains obscure and it is also difficult to conceive how diffusely placed pace-maker cells could operate as efficiently as a single pace-maker region at the S-A junction.

The observation that a very small difference in stimulation frequency caused a change between excitation and inhibition of the heart was also reported by Kulaev (1957) in the heart of the pike. The use of blocking drugs demonstrates that both the excitatory and inhibitory effects of vagal stimulation are mediated cholinergically and not by an adrenergic innervation. All the cells impaled in the S-A region showed a hyperpolarization to a single pulse vagal stimulation and it would therefore seem likely that there is an extensive inhibitory innervation in this region. Anatomical findings confirm this suggestion (Santer, 1972; Santer & Cobb, 1972). The excitatory effect of stimulation at low frequencies and also following the cessation of prolonged inhibitory stimulation results from a rebound phenomenon arising from the hyperpolarization as described in smooth muscle by Bennett (1966) and is probably due to an over-compensation of the muscle cell membrane as a result of the direct

inhibitory action of ACh on the innervated cells. This sympathomimetic effect of ACh has previously been demonstrated in the mammalian heart in which positive inotropic and chronotropic responses have been observed for as long as 2 min after stimulation (Misu & Kirpekar, 1968). These authors indicate that this phenomenon does not arise as a result of an ACh-mediated release of an endogenous sympathomimetic substance as suggested by Hollenberg, Carrière & Barger (1965) but from the direct effect of vagal stimulation on the muscle cells. In the present report, pharmacological studies indicate that, in the teleost, catecholamines do not affect this response and that the observed 'rebound' is a response of the muscle cell membrane to the vagus-induced hyperpolarization. Further support to this theory is provided by the fact that the 'rebound' response persists in preparations electrically hyperpolarized in the presence of both atropine and bretylium. Thus the rebound is not likely to be a secondary nerve-mediated excitation.

The critical threshold between inhibition and excitation can be explained by reference to the time course of the hyperpolarization. High frequency stimulation (7 Hz and above) leads to summation of the inhibitory hyperpolarizations as seen by del Castillo & Katz (1955) in the frog heart, whilst lower frequency stimulation of the atrial cells gives rise to a rebound spike from the hyperpolarization before the next inhibitory stimulus. Clearly the refractory period of the muscle after each spike potential is also involved since it is only at frequencies below 1–2 Hz that the heart can be directly driven. The rebound phenomenon may also be involved in the increase in frequency of heart beat following total inhibition due to vagal stimulation. The exact mechanism of the rebound phenomenon and particularly its relationship to the pace-maker cells is not understood and is at present under investigation. It must be emphasized that it is not yet known whether the rebound excitation is the mechanism whereby the heart rate is speeded up in the intact fish, but a study of the heart rate in such animals and in those under stress may help to solve this problem.

It is interesting to note that the intensity of stimulation but not the frequency or duration used by Gannon & Burnstock (1968) is more than ten times greater than that used by ourselves in order to detect the same effects of varying stimulus frequency on the heart rate. Using stimulation parameters of up to 40 V we achieved identical results to those with lower stimulating voltages. This would preclude the possibility of the lower (0·5 V) stimulating voltage failing to excite non-myelinated sympathetic efferent fibres, should they be present. The effects of atropine and of the adrenergic blockers were identical at higher voltage stimulation. However, Gannon & Burnstock (1968) and Gannon (1971) using blocking

drugs have shown unequivocally that the inhibition is cholinergically mediated and the excitation adrenergically mediated in the trout. The significance of the very precise stimulus frequency-dependence of heart rate using two different neural mechanisms to achieve the same result is not clear. The fact that Gannon & Burnstock (1968) also showed an increase above normal heart rate following inhibition may itself indicate a change in the threshold of pace-makers or a rebound phenomenon to inhibitory stimulation.

There is a change in the height and rate of the falling phase in the first few action potentials following inhibition. Such a phenomenon occurs during and following partial inhibition in the rabbit sino-atrial node tissue (Toda & West, 1965). It may be that the gradual increases in size of action potentials represent active events in cells other than the impaled cell, not being propagated, but spreading electrotonically through the low resistance intercellular junctions. Possibly the spike potential is initiated within a group of pace-maker cells but is only propagated over restricted but increasing distances for the first few spikes. The increased rate of the falling phase of these initial post-inhibitory action potentials is perhaps due to transient permeability changes of the sarcolemma of all cells during inhibition which are longer lasting than the permeability changes causing hyperpolarization and inhibition itself.

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