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THE EFFECTS OF VAGAL STIMULATION AND APPLIED ACETYLCHOLINE ON THE SINUS VENOSUS OF THE TOAD

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SUMMARY

1. The effects of vagal stimulation and applied acetylcholine were compared on the isolated sinus venosus preparation of the toad, *Bufo marinus*.

2. The effects of applied acetylcholine and of low-frequency, or short bursts of high-frequency vagal stimulation were abolished by hyoscine.

3. When intracellular recordings were made from muscle cells of the sinus venosus, it was found that applied acetylcholine caused bradycardia and a cessation of the heart beat which was associated with membrane hyperpolarization and a reduction in the duration of the action potentials. Much of the effect of acetylcholine can be attributed to it causing an increase in potassium conductance, g_K.

4. When slowing was produced by low-frequency vagal stimulation, only a small increase in maximum diastolic potential was detected. During vagal arrest the membrane potential settled to a potential positive of the control maximum diastolic potential.

5. In the presence of barium, much of the bradycardia associated with vagal stimulation persisted. Although the bradycardia produced by added acetylcholine also persisted in the presence of barium, the effects of acetylcholine that could be attributed to an increase in g_K were abolished.

6. Addition of caesium ions produced bradycardia with membrane potential changes similar to those seen during vagal stimulation.

7. The results are discussed in relation to the idea that neuronally released acetylcholine reduces inward current flow during diastole. In contrast applied acetylcholine as well as reducing inward current flow during diastole also increases outward current flow by increasing g_K.

INTRODUCTION

Stimulation of the vagi causes the release of acetylcholine which activates atropine-sensitive receptors to cause slowing and ultimately a cessation of heart beat. In frogs and toads, this effect is thought to be mediated by the rich vagal

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innervation of the sinus venosus (Hartzell, 1980; Campbell, Gibbins, Morris, Furness, Costa, Oliver, Beardsley & Murphy, 1982). In only a few studies have the membrane potential changes which accompany vagal slowing been reported. In frogs, stimulation of the mixed vago-sympathetic nerve slowed the heart rate; this was often but not invariably associated with an increase in maximum diastolic potential of cells in the sinus venosus (Hutter & Trautwein, 1955, 1956). In 'arrested' frog heart cells, vagal stimulation produced membrane hyperpolarization which was mimicked by the local application of acetylcholine (del Castillo & Katz, 1955*a*; Hartzell, 1979). In studies on rabbit heart, vagal stimulation slowed the heart rate but did not increase the maximum diastolic potential in pacemaker cells (Toda & West, 1966), only the rate of diastolic depolarization was reduced (Shibata, Giles & Pollack, 1985).

Acetylcholine acting on cardiac muscarinic receptors has been shown to produce three distinct effects. One of these is an increase in potassium conductance, g_K (Noble, 1975), which leads to membrane hyperpolarization and slowing of the heart rate (Löffelholz & Pappano, 1985). The other two effects result in changes in inward current flow through separate voltage-dependent channels. Acetylcholine suppresses slow inward current (i_{si}, Brown, 1982) through voltage-dependent calcium channels of both amphibian (Giles & Tsien, 1975; Ikemoto & Goto, 1975; Giles & Noble, 1976) and mammalian cardiac muscle (Ten Eick, Nawath, McDonald & Trautwein, 1976; Hino & Ochi, 1980). In the studies on non-pacemaker tissues, lower concentrations of acetylcholine were required to reduce i_{si} than to activate the potassium current. However, it has been shown, in some tissues, that much of this effect depends on the prior facilitation of calcium entry by catecholamines (Fishmeister & Harzell, 1986). The observation that acetylcholine can reduce i_{si} has led to the suggestion that vagal slowing of the heart might, in part, result from a suppression of this current (Egan & Noble, 1987). The third effect of added acetylcholine is to move the activation potential for a hyperpolarization-activated inward current, i_f, to more negative values (DiFransesco & Tromba, 1987). Such an effect would produce bradycardia by suppressing inward current flow during diastole if this current contributed to the diastolic depolarization (see DiFrancesco, 1985; but compare with Hagiwara & Irisawa, 1989)

In the toad, *Bufo marinus*, parasympathetic nerves cause bradycardia by the release of two distinct transmitter substances (Campbell, *et al.* 1982). In this species acetylcholine and somatostatin are co-localized in cardiac parasympathetic post-ganglionic axons. Both acetylcholine and somatostatin cause bradycardia, each acting through pharmacologically distinct receptors. Low-frequency vagal stimulation slows and stops the heart: this effect is readily blocked by hyoscine. In the presence of high concentrations of hyoscine, longer trains of higher frequency vagal stimuli slow the heart rate: this effect is reduced by prior desensitization with somatostatin. The initial aim of this study was to compare the membrane potential changes produced by the two neurally released transmitter substances. This first paper reports on the cholinergic actions of brief periods of vagal stimulation and compares them with the actions of acetylcholine. The observations indicate that neuronally released acetylcholine and exogenously applied acetylcholine activate different subsets of muscarinic receptors. A preliminary account of this work has been presented (Hirst, Edwards, Campbell, O'Shea & Bywater, 1987).

METHODS

Toads, *Bufo marinus*, were anaesthetized by immersion in a solution of 0·5% tricaine methanesulphonate in tap water. Most frequently the preparations consisted of the sinus venosus in continuity with the two atria, but with the ventricle cut away. In addition both the left and right vago-sympathetic trunks were dissected free and left in continuity with the isolated preparation. Each trunk was dissected back to its intracranial roots. Since the sympathetic outflows join the vagi outside the skull, stimulation of the intracranial vagal roots activates only the parasympathetic outflow to the heart (for further details see Campbell *et al.* 1982).

In an initial series of experiments, the preparations were set up to record atrial tension and heart rate as described previously (Campbell *et al.* 1982). The effects of vagal root stimulation on heart rate and tension development were compared with those of bath-applied acetylcholine. In electrophysiological experiments, the preparation was pinned out in a shallow recording chamber, the base of which consisted of a microscope cover-slip coated with Sylgard silicone resin (see Hirst, Holman & Spence, 1973). Fine pins, cut from 100 μm tungsten wire, were placed along the dorsal cut atrio-ventricular border and through the connective tissue around the sinus venosus. Sinus venosus muscle cells were exposed by placing a ring of pins through the sino-atrial aperture (see Hutter & Trautwein, 1956). Care was taken not to damage the atrial septum and not to apply excessive stretch to the partly immobilized, pinned-out region of sinus muscle. Intracellular recordings were made using conventional techniques with fine glass microelectrodes (resistance 80–150 mΩ) filled with 0·5 M-KCl. All membrane potential records were low-pass filtered, cut-off frequency 1 kHz, digitized and stored on disc for later analysis. Vagal fibres were selectively stimulated by drawing the two intracranial roots, some 3 mm central to the vagal ganglia, between a pair of stimulating electrodes (stimulation voltages 3–10 mV, pulse width 1·0 ms). The preparations were continuously perfused with physiological saline (composition, mM: NaCl, 115; KCl, 3·2; $NaHCO_3$, 20; NaH_2PO_4, 3·1; $CaCl_2$, 1·8; $MgSO_4$, 1·4; glucose, 16·7) which had been gassed with 95% oxygen, 5% carbon dioxide and warmed to 25 °C, at a rate of 6 ml/min (bath volume 1 ml). Drugs were added to the preparation by changing the inflow line from the control solution to one containing the appropriate concentration of drug. In some of the experiments involving the application of acetylcholine, a simpler preparation was used: only a small sheet of sinus venosus was pinned out. Drugs used in this study were acetylcholine chloride, barium chloride, caesium chloride, hyoscine sulphate, somatostatin, tetrodotoxin (all obtained from Sigma Chemicals) and nifedipine (Bayer Pharmaceuticals).

RESULTS

General observations

Isolated sinus venosus–atrial preparations beat spontaneously at rates of 40–70 beats/min. Supramaximal bilateral stimulation of the vagi at frequencies of 2–10 Hz, applied for 10 s, caused slowing and arrest. These effects were readily abolished by the addition of hyoscine (1×10^{-6} M) to the perfusion fluid. In the presence of hyoscine, when the frequency of stimulation was increased to the range 10–20 Hz and the stimuli were applied for longer periods (30–60 s), a slowly developing inhibition of rate and force of contraction was observed. Similar responses have been already described for this preparation (Campbell *et al.* 1982).

When acetylcholine was applied to the spontaneously beating sinus venosus–atrial preparation, concentrations of acetylcholine in the range $0·1–1·0 \times ^{-6}$ M caused a fall in the tension developed by each atrial beat, but with little associated change in rate. With higher concentrations of acetylcholine ($1–10 \times 10^{-6}$ M), a graded reduction in rate along with a further fall in tension was observed. Concentrations of acetylcholine in the range $5–10 \times 10^{-6}$ M were required to abolish cardiac activity. These effects of acetylcholine were prevented by the addition of hyoscine (1×10^{-6} M). In these experiments the force of contraction will be largely determined by the force of atrial

contraction whereas the rate will be largely determined by the rate of generation of action potentials by the sinus venosus.

When intracellular recordings were made from sinus venosus cells, rhythmic discharges of action potentials were detected. The frequency of action potential discharge was also in the range 40–70 /min. The action potentials were similar to those recorded from other pacemaker tissues (Hutter & Trautwein, 1956; Noble,

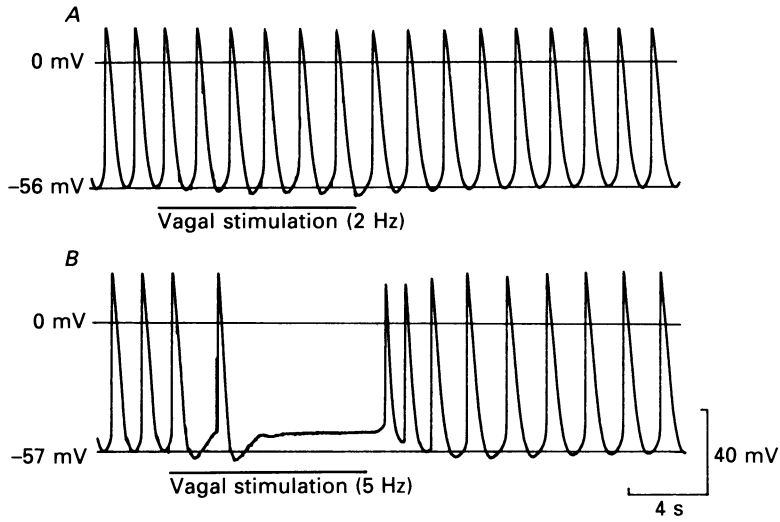

Fig. 1. The effect of vagal nerve stimulation on the membrane potential of a pacemaker cell of toad sinus venosus. In both records the vagi were stimulated with supramaximal stimuli for periods of 10 s. In *A* the stimulus frequency was 2 Hz; in *B* the frequency was 5 Hz. The upper line drawn on each trace represents 0 mV. The lower line on each trace represents the maximum diastolic potential recorded before vagal stimulation; these had values of −56 and −57 mV, respectively. The calibration bars refer to each trace.

1975). The maximum diastolic potentials were often less negative than −60 mV. After a slow diastolic depolarization, action potentials were initiated at a threshold potential of about −50 mV (see for example expansions in Figs 2 and 3). When long-lasting impalements (over 30 min) were obtained from pacemaker cells, that is cells where the diastolic depolarization led smoothly into an action potential, on occasions the action potentials would intermittently be seen to take off more abruptly. We assume that when this occurred a neighbouring region of muscle temporarily had become the dominant pacemaker. In a proportion of recordings, action potentials invariably arose abruptly during the slow diastolic depolarization (see for example expansion in Fig. 4): such recordings were assumed to be from 'driven' or 'follower' cells (Brown, Giles & Noble, 1977). Unless stated otherwise, all data were collected from cells in which, during the majority of the recording period, the diastolic depolarization led smoothly into an action potential.

The rising phases of action potentials recorded from pacemaker cells were brief, lasting some 20–50 ms. The repolarization phase was slower, lasting some 300–500 ms. Action potentials had peak amplitudes, when measured from the maximum di-

astolic potential, in the range 80–100 mV (mean 88·2 mV, S.E.M. ± 1·7 mV, $n = 10$, where each n value, in this and every other case, represents the mean results from a different animal). The action potentials persisted in the presence of tetrodotoxin (TTX, 1×10^{-6} M), albeit at a reduced rate of 20–30 /min. This observation suggests that some TTX-sensitive channels are activated during normal pacemaking activity in the intact sinus venosus preparation. This does not imply that true pacemaker

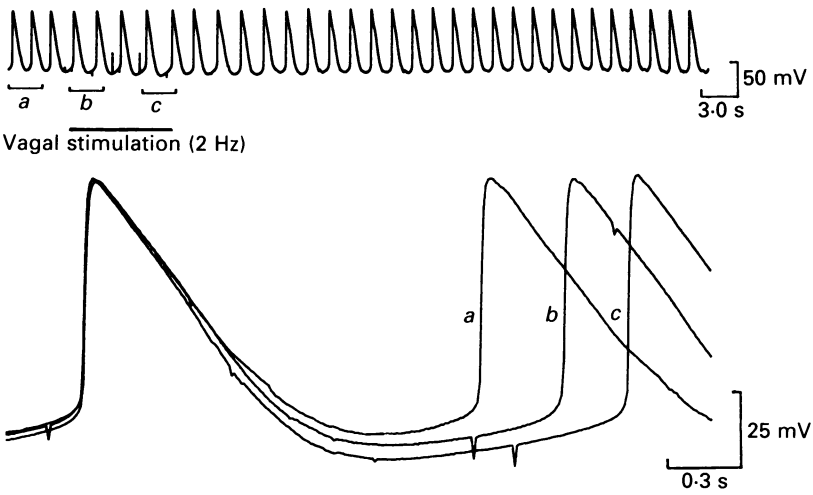

Fig. 2. Effect of low-frequency vagal stimulation (2 Hz for 10 s) on the membrane potential of a pacemaker cell. The upper record shows the membrane potential recorded over a 1 min period at low gain. The lower composite is a series of overlays of selected parts of this record, the areas of expansion being indicated by the three bars (*a*, *b* and *c*) under the upper record. Expansion *a* shows the two action potentials recorded immediately before the start of vagal stimulation. Expansion *b* shows the initial part of the response to vagal stimulation. It can be seen that the time courses of the action potentials are barely affected but that the membrane potential during the diastolic interval is more negative and the rate of diastolic depolarization is reduced. Expansion *c* shows a pair of action potentials later in the stimulus train: again the action potentials are unaffected but the diastolic interval is further increased. The threshold for initiation of action potentials is increased. The upper calibration bars refer to the upper record; the lower bars refer to each of the three expansions.

cells have TTX-sensitive sodium channels (see Giles, van Ginneken & Shibata, 1986), rather it raises the possibility that inward sodium current generated in neighbouring electrically coupled follower cells contributes to net inward current in the pacemaker syncytium during diastole. Action potentials were abolished after prolonged treatment with the calcium antagonist nifedipine (1×10^{-5} M).

Effects of vagal stimulation on pacemaker cells

Bilateral vagal stimulation with low-frequency trains of stimuli (train duration 10 s, stimulation frequency 1–2 Hz) slowed the rate of action potential discharge. This was associated with a variable increase (0–10 mV) in peak diastolic potential and an increase in the threshold potential (1–8 mV) for the initiation of the rapid upstroke

of the action potential: the peak amplitude of the action potential was unchanged (Fig. 1*A*). When the frequency of stimulation was increased (5–10 Hz), vagal stimulation caused the action potential discharge to stop (Fig. 1*B*). During the initial part of the stimulation train, the maximum diastolic potential again increased by a variable amount (0–10 mV, mean 2·5±0·6 mV, $n = 11$). However, when the sinus stopped generating action potentials the membrane potential settled at a potential

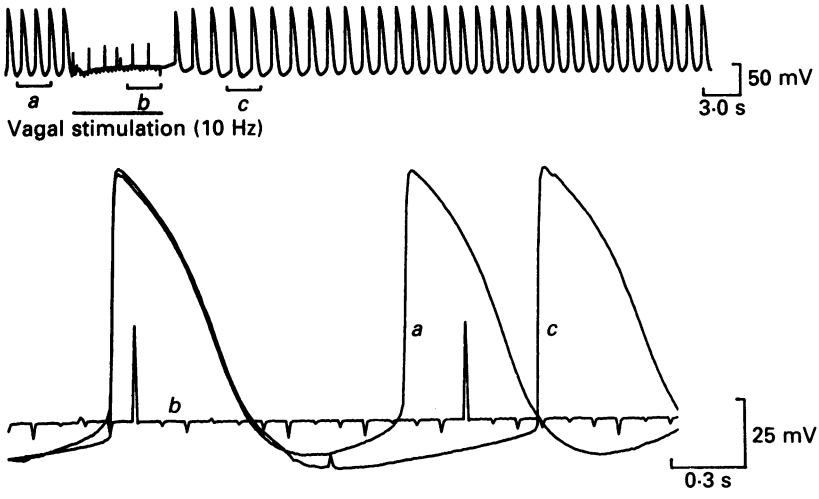

Fig. 3. Effect of higher frequency vagal stimulation (10 Hz for 10 s) on pacemaker action potentials. Expansion *a* shows action potentials before the start of vagal stimulation; *b* overlays this record with the steady potential recorded during the period of electrical quiescence. The expansion *c* shows the action potentials recorded after the reinitiation of beating but while their frequency was still reduced. Note that while the heart is not beating the membrane potential settles at a value just negative of the threshold for initiation of a control action potential. The upper calibration bars refer to the upper record, the lower bars refer to each of the three expansions.

some 2–15 mV (mean 8·3±0·9 mV, $n = 11$) more positive than the control maximum diastolic potential. The absolute potentials which correspond to these values were: maximum diastolic potential of control action potentials, −57·2±1·5 mV ($n = 11$); maximum diastolic potential during vagal slowing, −60·8±1·8 mV ($n = 11$); the steady potential reached during complete vagal inhibition, −49·8±1·7 mV ($n = 11$).

After the period of vagal arrest, action potentials were again generated. The first few action potentials had reduced peak amplitudes (Figs 1*B* and 3). Frequently the first two or three action potentials occurred at brief intervals (see for example Fig. 1*B*) and were not preceded by a slow diastolic depolarization. This suggests that they were being conducted into the point of recording rather than being initiated there. On some occasions this change persisted for some 20–30 s. The effects of vagal stimulation were long-lasting, taking between 30 and 50 s for the control rate to be restored. During the recovery phase the action potentials, like those recorded during vagal slowing, had increased undershoots.

Vagal slowing of the heart rate was not associated with a consistent change in the

time course or amplitude of pacemaker action potentials. Two examples of records examined in detail are shown in Figs 2 and 3. In Fig. 2 the vagi were stimulated at 2 Hz for 10 s. This record was selected to illustrate the largest increase in diastolic potential that we observed during vagal slowing. The peak amplitude of the action potentials was unaffected. This is illustrated by over-laying control action potentials (Fig. 2*a*) with 'slowed' action potentials (Fig. 2*b* and *c*). Moreover, neither the rising

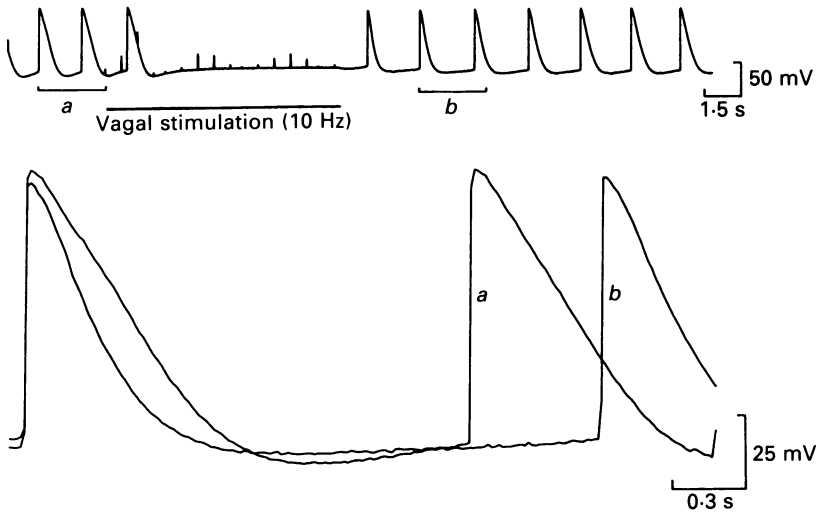

Fig. 4. Effect of vagal stimulation on the membrane potential of a 'driven' sinus venosus cell. During vagal stimulation the membrane potential settled to a value some 15 mV positive of the control maximum diastolic potential. After the period of vagal stimulation the heart rate was slowed but, as can be seen from the two lower expansions (control, *a*; vagal slowing, *b*), there was no increase in maximum diastolic potential. Action potentials, however, had a reduced amplitude and more rapid decay. The upper calibration bars refer to the upper record; the lower bars to each of the two expansions.

phases nor the falling phases were much affected. The only changes were an increase in the diastolic hyperpolarization, a slower rate of diastolic depolarization and an increase in threshold. Similar changes in the configuration of the diastolic membrane potential were recorded in the period preceding cessation of heart beat during more intense vagal stimulation.

Figure 3 illustrates an experiment where the membrane potential was measured during vagal arrest. From the expansion shown in Fig. 3*b* it can be seen that during arrest the membrane potential was slightly negative of the point of inflexion for the rapid rising phase of the control action potential (Fig. 3*a*). After the period of vagal stimulation action potentials were again generated spontaneously, their amplitudes were transiently reduced and their rates of repolarization increased. These effects, unlike the effect on heart rate, were short-lasting: action potentials of normal configuration were again recorded after the second or third beat. During the period of recovery to the resting rate, action potentials had a form similar to that observed during vagal slowing. Again the only changes were an increase in peak diastolic

hyperpolarization and a slower rate of diastolic depolarization. From this record, although the interval between action potentials had increased by about half a second, it can be seen that the peak diastolic potential only increased by some 2–3 mV (Fig. 3*c*).

These observations are consistent with the suggestion that vagal stimulation may cause bradycardia by suppressing inward current flow during diastole (Hutter & Trautwein, 1956).

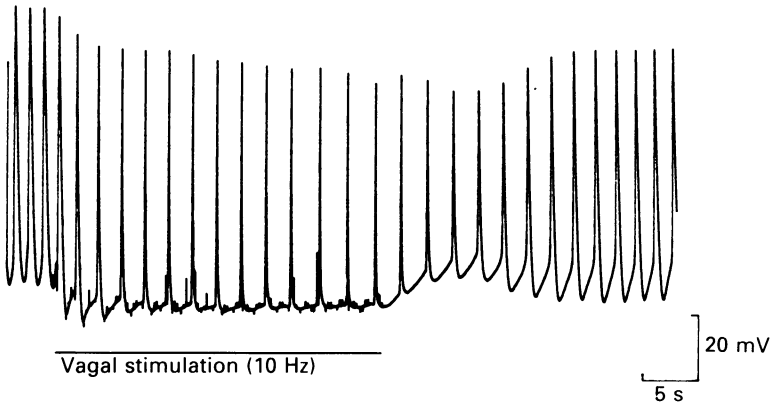

Fig. 5. Membrane potential change recorded from a sinus venosus cell which lay within 1 mm of the sino-atrial aperture. In this cell the maximum diastolic potential during the control period was −69 mV. During vagal stimulation the maximum membrane potential increased to a value of −84 mV. Each action potential initiated during the period of vagal inhibition had a brief time course. After the period of stimulation, action potentials had depressed amplitudes.

Regional variations in responses to vagal stimulation

The preceding section described the membrane potential changes in pacemaker cells during and after vagal stimulation. On a number of occasions, regional variations from this pattern were detected. In most preparations it was possible to find regions in the sinus in which the changing diastolic membrane potential did not lead smoothly into an action potential, rather the cell appeared to be invaded or driven. An example is shown in Fig. 4. It can be seen that during vagal inhibition of these cells, as with pacemaker cells, the membrane potential also settled to a value positive of the maximum diastolic potential; unlike pacemaker cells, at no time did the maximum diastolic potential increase.

A second regional variation was also detected. In three preparations recordings were made from muscle cells lying in close apposition to the sino-atrial aperture. In this region the membrane potential reached during the diastolic interval was more negative than −60 mV, peak potentials in excess of −70 mV being detected. In this region (Fig. 5), vagal stimulation produced profound hyperpolarizations, similar to those detected by Hutter & Trautwein (1956) when they made recordings from conducting fibres. During the slowing produced by vagal stimulation, action

potentials became very brief, having both rapid rising phases and decaying phases. In the same preparations, when the recording site was moved some 2–3 mm into the sinus region, observations like those shown for pacemaker cells (i.e. Figs 1, 2 and 3) were made. When the electrode was placed in the larger atrial muscle bundles outside the sino-atrial ring, action potential with amplitudes and time courses similar to those reported elsewhere for atrial muscle were recorded (Noble, 1975). These

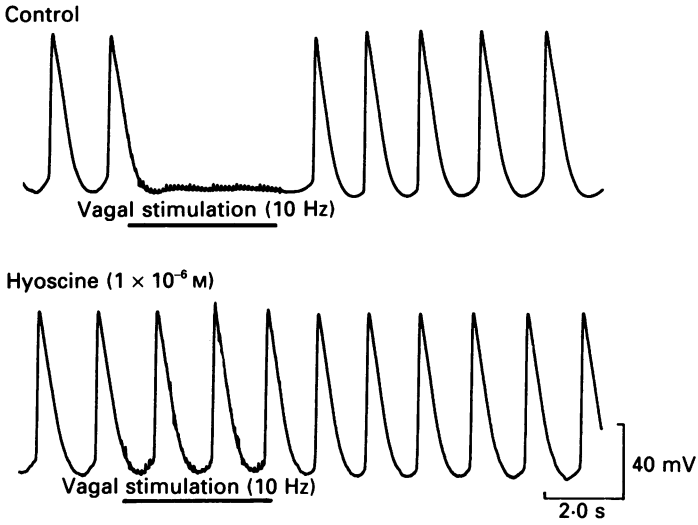

Fig. 6. Effect of hyoscine on the response to a short train of vagal stimuli. The upper trace shows slowing and inhibition of the generation of action potentials in a pacemaker cell. The lower traces shows that the effects of vagal stimulation were prevented by adding hyoscine (1×10^{-6} M) to the perfusion fluid. Calibration bars apply to both traces.

observations suggest that, in regions specialized to conduct action potentials into the atria, vagal inhibition results from the activation of a potassium conductance (see Hutter & Trautwein, 1956; Spear, Kronhaus, Moore & Kline, 1979).

Effect of hyoscine on responses to vagal stimulation

The effects of vagal stimulation were abolished by the addition of hyoscine (1×10^{-6} M) to the perfusion fluid unless long trains of high-frequency stimuli were applied. An example of the effect of hyoscine on the response to a short train of vagal stimuli is shown in Fig. 6. Before treatment, the heart was arrested at a potential positive of the maximum diastolic potential. This response was abolished some 10 min after changing to the hyoscine-containing solution. These observations suggest that the changes in heart rate produced by short trains of vagal stimuli are acting by the release of acetylcholine acting on muscarinic receptors (see also Campbell *et al.* 1982). When longer trains (30–60 s) of high-frequency (10–20 Hz) stimuli were used, vagal stimulation produced a very slowly developing, hyoscine-resistant increase in diastolic potential. Similar potential changes could be produced by superfusing the preparation with physiological solution containing somatostatin (1×10^{-7} M).

The effects of vagal stimulation were examined in three preparations that had been made quiescent by adding nifedipine to the perfusion fluid. In the presence of nifedipine (1×10^{-5} M), the membrane potential was stable with values in the range -35 to -45 mV (mean $39{\cdot}7 \pm 1{\cdot}3$ mV, $n = 3$). Vagal stimulation (frequency 10 Hz) caused both a transient and a sustained hyperpolarization (Fig. 7). During the

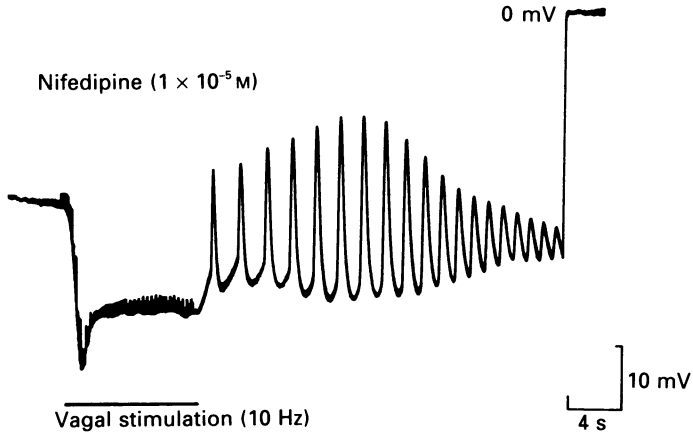

Fig. 7. Effect of vagal stimulation on the membrane potential of sinus venosus cell of a preparation which had been arrested by the calcium antagonist nifedipine (1×10^{-5} M). During vagal stimulation the membrane potential transiently increased from its 'resting value' (-35 mV) to -54 mV. The potential then settled to a steady level of -47 mV, which was maintained for the rest of the stimulation period. After the end of stimulation a series of oscillating potentials occurred. At the end of the record the electrode was withdrawn to check the membrane potential.

sustained hyperpolarization the absolute membrane potentials were similar to those recorded during vagal inhibition of pacemaker cells in the spontaneously beating heart. After the stimulation period the membrane potential slowly returned to its previous value. Often this period of recovery was associated with rhythmical membrane potential changes (Fig. 7).

Comparison between effects of vagal stimulation and superfusion with acetylcholine

Acetylcholine, in varying concentrations, was applied to the sinus venosus–atrial preparation. Visual observation of the preparation indicated that, as with the tension studies, the force of atrial contraction was reduced with concentrations of acetylcholine in the range $0{\cdot}1$–1×10^{-6} M. These concentrations had little effect on the shape or frequency of occurrence of the sinus action potential. When the concentration of acetylcholine was increased in the range 1–100×10^{-6} M the rate of generation of action potentials fell until eventually the heart stopped. Slowing and cessation of beat was associated with an increase in maximum diastolic potential (Figs 8, 9 and 11*A*, *B* and *C*).

The membrane potential changes recorded during the addition of acetylcholine are compared to those recorded during vagal inhibition in Fig. 8. During vagal

stimulation the membrane potential settled to a value some 10 mV positive of the maximum control diastolic potential. In contrast during inhibition by acetylcholine the membrane potential settled to a value some 15 mV negative of the maximum control diastolic potential. The mean potential achieved during maintained perfusion of acetylcholine at a concentration sufficient to abolish activity was $-76{\cdot}0 \pm 1{\cdot}9$ mV

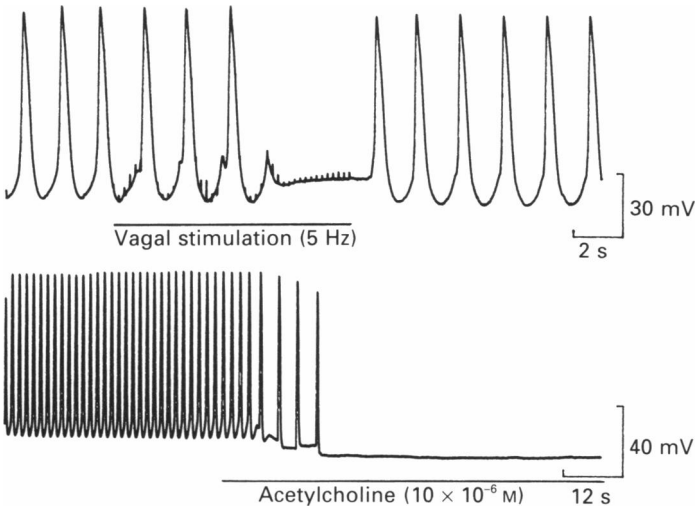

Fig. 8. Comparison between the membrane potential changes recorded during vagal stimulation (above) and superfusion with acetylcholine (below). Note that, in the upper record, during cessation of beat the membrane potential was positive of the maximum diastolic potential. In contrast, the membrane potential became more negative during inhibition by acetylcholine.

($n = 10$); this compares with a mean value of membrane potential of $-49{\cdot}8$ mV (see above) achieved during vagal inhibition. The effects of added acetylcholine, like those of short trains of vagal stimuli, were abolished by hyoscine (1×10^{-6} M).

Several experimental procedures were attempted to try and mimic more closely the effect of vagal stimulation by bath application of acetylcholine. To test whether applied acetylcholine caused hyperpolarization of the entire sinus by activating acetylcholine receptors near the sino-atrial aperture (Fig. 5), preparations of sinus venosus were made in which this region was removed. Hyperpolarization and slowing were still detected. In other experiments only small slips of sinus tissue some 2–3 mm square were pinned out. The bath volume was reduced to 0·1 ml by lowering the fluid level and a solution flow rate of 10 ml/min used. Thus the acetylcholine concentration near the muscle would rise rapidly, perhaps as would happen when acetylcholine is released from nerves. Again bath-applied acetylcholine produced hyperpolarization. When acetylcholine was applied either with slower perfusion rates or for briefer periods of time it also produced hyperpolarization. An example, with expansions of selected regions of the membrane potential record, is shown in Fig. 9. Acetylcholine (10×10^{-6} M) was added rapidly to the chamber. The membrane rapidly hyper-

polarized and the action potential time course changed dramatically before beating stopped. The action potential was slightly increased in amplitude, its overshoot was reduced and its repolarization phase was brief. These observations are consistent with the idea that bath-applied acetylcholine causes hyperpolarization and shortens action potentials by increasing g_K (Noble, 1975). However, the action potentials

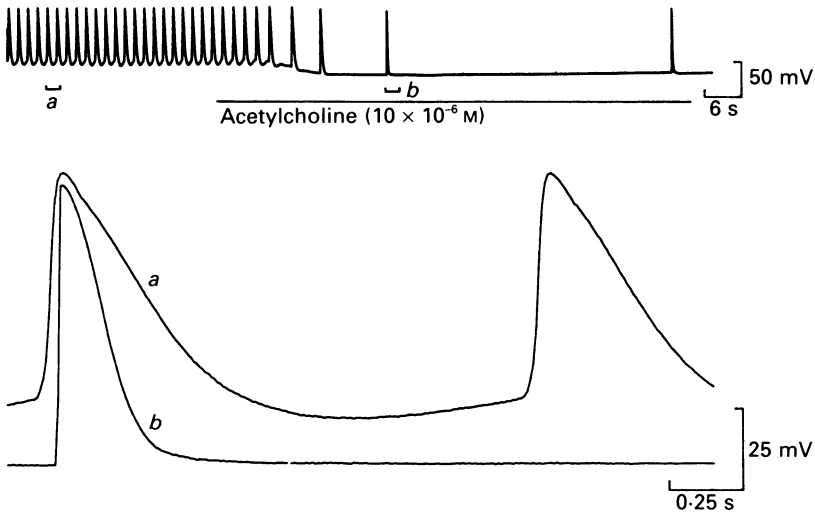

Fig. 9. Effect of rapid application of acetylcholine on the membrane potential of an isolated segment of sinus venosus. The membrane potential increased and stayed at a negative value. The two expansions (*a* and *b*) show control action potentials overlaid with an action potential recorded during the hyperpolarized period. The upper calibration bars refer to the upper record; the lower bars refer to each of the two expansions.

recorded in the presence of added acetylcholine had rapid rising phases and they arose abruptly from quite negative potentials despite the fact that by our criteria the recording was from a pacemaker cell (see Figs 9 and 11*C*). The action potentials that occurred intermittently during a large acetylcholine-induced hyperpolarization were abolished by the addition of TTX. It has been pointed out previously that true pacemaker cells appear to lack TTX-sensitive channels (Giles *et al.* 1986). Our observations on the shapes of action potentials recorded in the presence of added acetylcholine again imply that our recordings from sinus venosus cells reflect the membrane potential changes occurring in both true pacemaker cells and adjacent follower cells that are coupled together as a syncytium. We suggest that the muscarinic hyperpolarization had now reactivated a substantial proportion of sodium channels present in the follower cells. These cells in the presence of added acetylcholine, rather than true pacemaker cells, may now become responsible for the initiation of the rapid action potentials.

Effect of barium ions on the responses to added acetylcholine and vagal stimulation

The preceding sections have indicated that the effects of vagal stimulation on the membrane potential of pacemaker cells are not readily mimicked by the

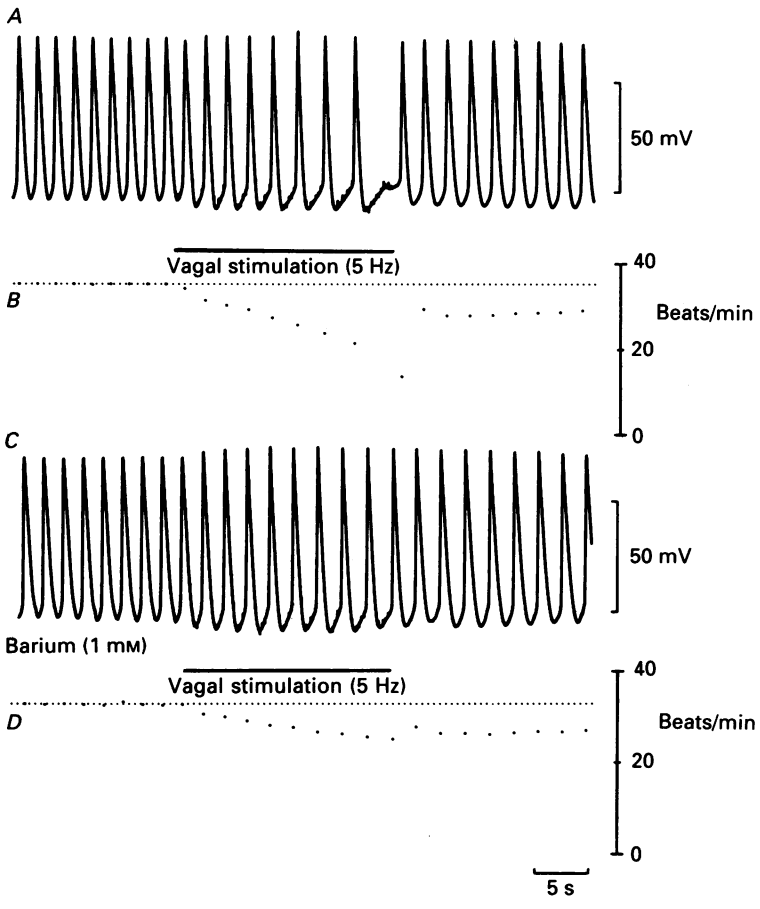

Fig. 10. *A* and *B*, effect of low-frequency vagal stimulation on pacemaker action potentials recorded before and after the addition of barium ions (1 mM) to the physiological saline. The trace *A* shows the response to a 20 s train of vagal stimuli (5 Hz); the trace *B* is a plot of the associated change in the rate of generation of action potentials. Note that during the period of vagal stimulation the peak diastolic potential increased by some 5 mV. Trace *C* shows the response to the same train of vagal stimuli, recorded from the same cell some 5 min after the addition of barium ions; trace *D* is again a plot of the rate of generation of action potentials. Note that the increase in peak diastolic potential still occurred in the presence of barium and that a reduced bradycardia persisted.

addition of acetylcholine. While the effects of added acetylcholine can be readily explained by an increase in g_K, the effects of vagal stimulation seem not to involve much of an increase in g_K. The increase in g_K that accompanies the activation of cardiac muscarinic receptors is prevented by barium ions (Momose, Giles & Szabo, 1984). In an attempt to clarify the mechanism by which vagal stimulation causes bradycardia the effect of barium on vagal stimulation was examined. In each of three experiments a frequency of vagal stimulation was chosen that would reduce the heart

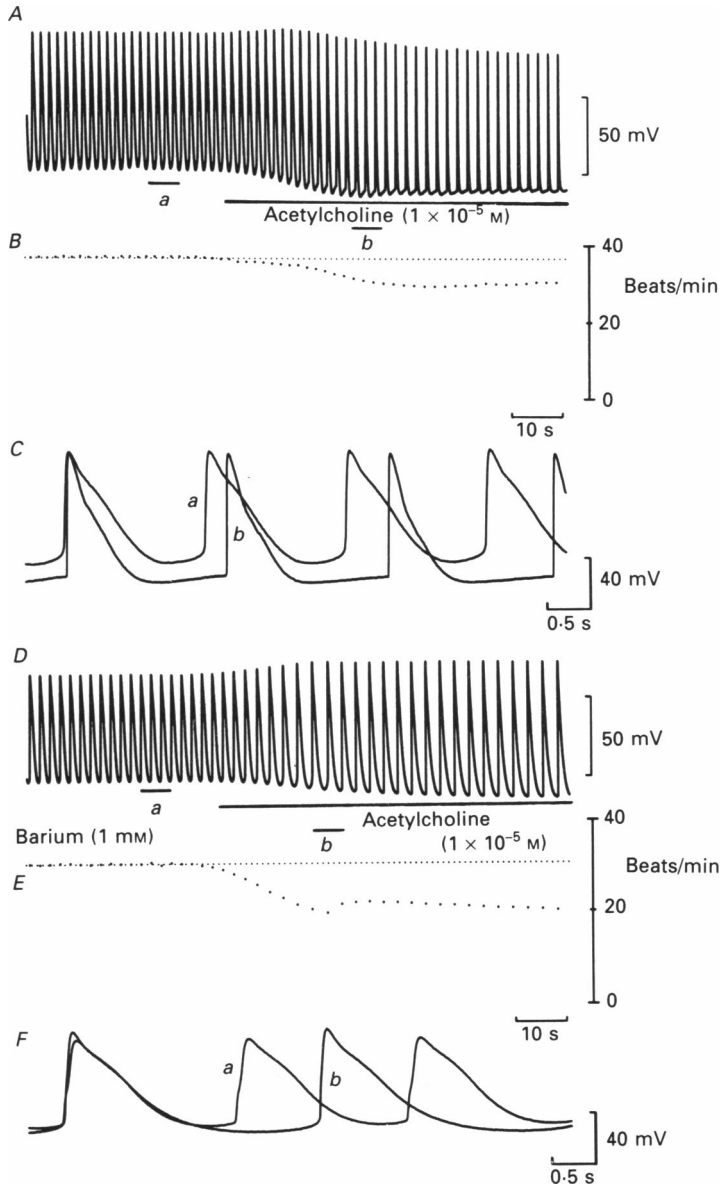

Fig. 11. *A–E*, effect of addition of acetylcholine on pacemaker action potentials recorded before and after the addition of barium ions (1 mM) to the physiological saline. The recording shown in *A* shows the membrane potential change produced by the addition of acetylcholine (1×10^{-5} M) to the tissue fluid. The trace *B* shows the associated change in the rate of generation of action potentials; note that 'slowing' lags behind the increase in peak diastolic potential. The expansions shown in *C* compare the action potentials recorded in control solution (*a*) with those recorded in the presence of acetylcholine (*b*). For further discussion, see text. The records shown in *D*, *E* and *F* are corresponding records made 5 min after the addition of barium ions. Note that the fall in rate of generation of action potentials now is coincident with the increase in peak diastolic potential and that in barium slowing results from a slower rate of diastolic depolarization.

rate from its control value (mean 36·6 ± 1·2 beats/min, $n = 3$) to about half its control rate (mean 21·3 ± 3·7 beats/min, $n = 3$). The increase in peak diastolic hyperpolarization associated with vagal slowing was 5·0 ± 0·8 mV ($n = 3$; Fig. 10*A* and *B*). Barium (1 mM) added to the perfusion fluid, itself caused a slight bradycardia (mean heart rate 34·1 ± 2·0 beats/min, $n = 3$) and reduced both the peak diastolic hyperpolarization and the amplitude of the action potential, each by a variable

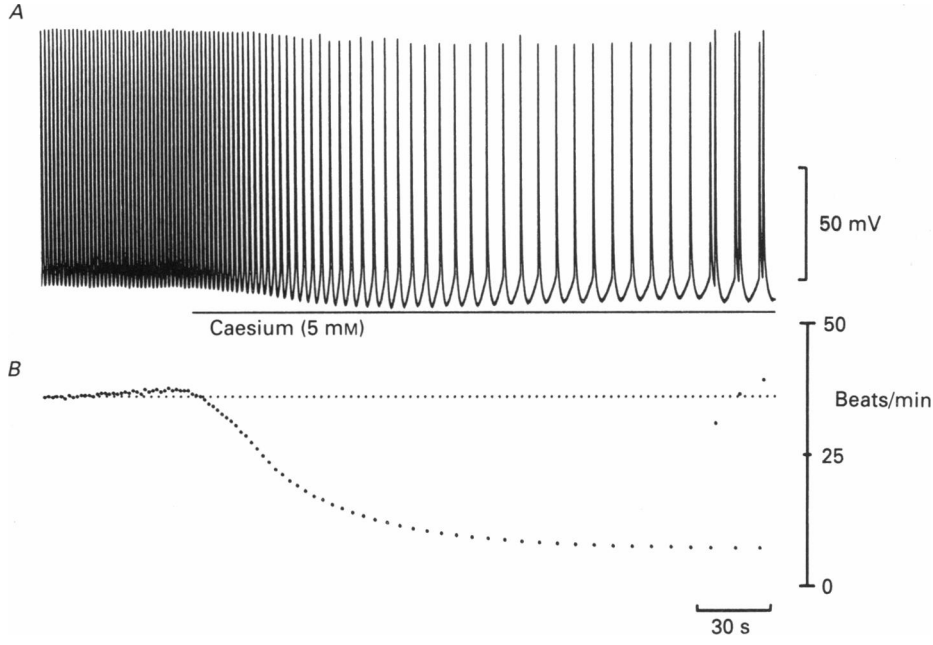

Fig. 12. *A* and *B*, effect of caesium ions (5 mM) on the rate of generation of pacemaker action potentials. The rate of generation of action potentials (*B*) fell to about 25% of the control value after the addition of caesium ions. During this slowing there was an increase in the peak diastolic potential and a slowed rate of diastolic depolarization (*B*). During prolonged exposure to caesium, the pacemaker cells started to generate a rapid repetitive discharge of action potentials (end of record *A*).

amount. The same train of vagal stimuli again produced slowing of the heart rate. The degree of slowing was slightly reduced (mean heart rate 25·9 ± 1·1 beats/min, $n = 3$) but more prolonged. The increase in peak diastolic hyperpolarization was little affected (mean 4·7 ± 0·4 mV; $n = 3$). These observations support the idea that vagal inhibition of pacemaker cells involves an increase in g_K only to a small extent.

In a parallel series of experiments the effects of barium (1 mM) on the responses produced by added acetylcholine (10×10^{-6} M) were examined. The results of an experiment are shown in Fig. 11. It can be seen that added acetylcholine (Fig. 11*A*) caused the peak diastolic potential to increase by 16 mV (mean 18·4 ± 1·4 mV; $n = 5$) and the heart rate to fall from 38 beats/min to 30 beats/min (Fig. 11*B*). However, the fall in heart rate, in this and each experiment, lagged behind the increase in diastolic potential. As has been pointed out before, during the addition of acetylcholine, the action potentials were initiated abruptly and they were brief (see

expansions Fig. 11*C*). In the presence of barium, added acetylcholine continued to slow the heart but the form of the response was different to that seen in the absence of barium. The rapid initial increase in diastolic potential seen in control solution was now absent; it was replaced by a slower increase in peak potential (maximum increase in diastolic potential $11{\cdot}3 \pm 1{\cdot}1$ mV; $n = 5$). The onset of this increase paralleled the onset of the bradycardia (Fig. 11*D* and *E*). When barium was present the addition of acetylcholine no longer changed the rates of repolarization of the action potentials and bradycardia was associated only with a slowed rate of diastolic depolarization (Fig. 11*E*). Thus the effects of added acetylcholine detected in the presence of barium were similar to those observed during vagal stimulation in control solutions (see Figs 2 and 3).

Effect of caesium ions on pacemaker potentials and the responses to vagal stimulation

Our observations have suggested that the effect of vagally released acetylcholine is to slow the rate of diastolic depolarization until a point at which threshold for the initiation of an action potential is no longer reached. Since these effects do not appear to primarily involve an increase in g_K we considered the possibility that neuronally released acetylcholine was reducing inward current flow during diastole. Some of the inward current that flows during diastole may be provided by a hyperpolarization-activated current, i_f, which is sensitive to caesium ions (DiFrancesco, Ferroni, Mazzanti & Tromba, 1986). Furthermore acetylcholine has been shown to move the activation potential for i_f to a more negative value in isolated mammalian pacemaker cells (DiFrancesco & Tromba, 1987). To see if i_f contributed to pacemaker activity in toad sinus venosus cells we examined the effects of caesium ions.

The addition of caesium (5 mM) to the superfusion fluid produced a bradycardia which was in many respects similar to that observed during low-frequency vagal stimulation (Fig. 12). The peak diastolic potential gradually became more negative and the rate of generation of action potentials fell. The amplitude and time course of the action potentials were barely affected but the rate of diastolic depolarization was slowed. In three experiments caesium (5 mM) reduced the heart rate to 24% of its control rate. After prolonged exposure to caesium the heart started to generate extra-systoles (see end of trace shown in Fig. 12). The rates of repolarization of the action potentials recorded just before the occurrence of extra-systolic beats were slowed. This may well reflect the additional ability of caesium ions to block delayed rectification (Hille, 1984).

Lower concentrations of caesium (1–3 mM) produced a less marked bradycardia. When the vagus was stimulated with trains of low-frequency impulses (2 Hz for 10 s) in control solution only slowing was observed (see Fig. 1). In the presence of these low concentrations of caesium, such stimuli caused an abrupt cessation of the heart beat. This might suggest that vagally released acetylcholine and caesium act through a common mechanism.

DISCUSSION

The observed actions of applied acetylcholine on toad pacemaker cells were essentially the same as those previously described for pacemaker cells of both amphibians and mammals. The effects followed a pattern that would be expected if

acetylcholine caused an increase in g_K. Acetylcholine caused membrane hyperpolarization, suppressed the action potential overshoot and increased the rate of repolarization of the action potentials (Hutter, 1957; Trautwein, 1963; Hartzell, 1979). Although muscarinic increases in g_K show time-dependent rectification, with current flow being reduced at depolarized potentials (Noma & Trautwein, 1978; Sakmann, Noam & Trautwein, 1983; Simmons & Hartzell, 1987), this rectification did not appear to be sufficiently intense in toad sinus cells to abolish the acetylcholine-induced outward current flow during the repolarization phase of the action potentials (see also Sakmann *et al.* 1983; Iijima, Irisawa & Kameyama, 1985).

Prolonged high-frequency stimulation of the vagi had some effects on sinus cells that were not blocked by hyoscine. These non-muscarinic actions were presumably associated with the release of somatostatin (Campbell *et al.* 1982) and will be considered in a separate paper. In contrast, the effects of stimulating the vagi at low frequencies or for brief periods at higher frequencies were abolished by hyoscine. These responses were presumably mediated by the release of acetylcholine acting on muscarinic receptors.

Low-frequency vagal stimulation slowed sinus beating with only a small increase in maximum diastolic potential and an increase in the threshold potential for the initiation of action potentials, but did not affect the rate of repolarization of pacemaker action potentials. Much of this slowing persisted in the presence of barium ions which suggests the inhibitory effect of vagal stimulation did not involve predominantly an increase in g_K. When the frequency of vagal stimulation was increased so as to stop the heart, the membrane potential settled at a value positive of the peak diastolic potential. In a simulation showing how an increase in g_K might stop the generation of sino-atrial node action potentials, the membrane potential was predicted to settle at a value close to the maximum diastolic potential. This was preceded by a hyperpolarization of some 25 mV (Egan & Noble, 1987). A hyperpolarization of this magnitude was not detected in the present study nor, for that matter, has it been recorded in any other study of vagal inhibition (Hutter & Trautwein, 1956; Toda & West, 1966; Shibata *et al.* 1985).

An alternative manner in which acetylcholine released by vagal nerve fibres has been suggested to slow the rate of generation of action potentials is to directly inactivate i_{si} (Egan & Noble, 1987). Such a mechanism would increase the thresholds of action potentials, reduce their rates of rise and decrease their amplitudes. The only time that action potentials were depressed in amplitude was when beating restarted immediately after a period of vagal arrest. This transient depression perhaps could have resulted from a short-lasting effect of neuronally released acetylcholine on i_{si}. Alternatively it could have resulted from voltage-dependent inactivation of the channels which carry the i_{si}. These channels have been shown to be activated by periods of hyperpolarization at potentials more negative than -70 mV (Brown, 1982): the absence of a diastolic hyperpolarization during quiescence might well prevent normal reactivation of a proportion of channels. In a simulation that revealed how changing i_{si} might produce slowing, Egan & Noble (1987) have shown that an increase of about 10% in the interval between action potentials is associated with a large decrease in their overshoots. In our experiments, during vagal stimulation a 50% increase in the interval between action potentials was not

associated with a decrease in overshoot. Thus it seems unlikely that vagal inhibition results from a reduction in i_{si}. In addition vagal stimulation was not mimicked by blocking i_{si} with nifedipine, the quiescent potentials differing by about 10 mV. Although this observation is also consistent with the idea that vagally released acetylcholine does not block i_{si}, the interpretation may be complicated since nifedipine in addition to blocking calcium channels also blocks the outward time-dependent K^+ current (i_{K1}, Brown, 1982; Godfraind, Miller & Wibo, 1986).

A final possibility is that neurally released acetylcholine acts to reduce inward current flow during diastole. The source of inward current during diastole is considered to be a background sodium current (Noble, 1984), a transient calcium current ($i_{Ca,T}$, Hagiwara, Irisawa & Kameyama, 1988), a hyperpolarization-activated pacemaker current (DiFrancesco, 1985) or a combination of these. The background sodium current results from a voltage-independent sodium conductance and there is no suggestion that such channels are chemosensitive. The transient calcium current, $i_{Ca,T}$, results from the activation of calcium channels at membrane potentials near -50 mV (Hagiwara *et al.* 1988). These channels rapidly inactivate and thus only contribute to the diastolic depolarization. It is possible that vagally released acetylcholine acts on this current to produce a cessation of heart beat. When this current is blocked with nickel ions, a bradycardia results and the associated membrane potential changes are similar to those which we have recorded during vagal stimulation (Hagiwara *et al.* 1988; see also Shibata *et al.* 1985). However, when recordings were made from arrested hearts (see Fig. 7), the membrane potential settled at a value of about -40 mV. Vagal stimulation produced a hyperpolarization. This effect is unlikely to involve an action on the channels which carry the transient calcium current as these channels are largely inactivated at these potentials (Hagiwara *et al.* 1988). However, the possibility that vagally released acetylcholine reduces a small-steady current that may flow through these channels in the voltage region where the activation curves and the inactivation curves overlap cannot be excluded.

The hyperpolarization-activated pacemaker current allows inward movement of sodium along with outward movement of potassium at potentials more negative than -40 mV. The reversal potential of the resulting current, i_f or i_h, however, is in the range 0 to -20 mV (Yanagihara & Irisawa, 1980; DiFrancesco, 1985). In short, when the membrane potential passes negative to -40 mV, the rectifier is activated and the resultant current, i_f, pushes the potential back towards -40 mV, at which point the current ceases. If vagally released acetylcholine reset the activation voltage of i_f to a more negative potential, say -50 mV, inward current flow during the diastolic interval would be reduced (see DiFrancesco & Tromba, 1987). The rate of diastolic depolarization would decrease. The maximum diastolic potential would increase slightly because of the increased dominance of the outward time-dependent K^+ current, i_{K1}. The threshold depolarization for initiation of action potentials would increase, net inward current over the entire diastolic period being suppressed. In contrast, the membrane potential changes that were positive of the activation potential for i_f, such as those occurring during the action potential, would be unaffected. During vagal arrest, the membrane potential should settle to a value close to the new activation potential (-50 mV) for i_f. Each of these effects has been

seen during vagal slowing and vagal arrest of the sinus. The notion that neural acetylcholine could exert part of its action on i_f receives further support from experiments in which action potentials had been abolished by the calcium antagonist nifedipine. In these tissues, the membrane potential settled to -40 mV, a potential which may reflect the normal activation potential of i_f. In the presence of nifedipine, vagal stimulation causes hyperpolarization (see also Hartzell, 1979) as would be expected if nerve stimulation had either suppressed inward current flow or had moved the activation potential of i_f to a more negative level. In summary, our observations could be explained if acetylcholine released by the vagus reduces inward current flow during diastole by changing the activation potential of i_f. Support for this view could be taken from the finding that caesium ions, which prevent the activation of i_f, produce bradycardia with associated membrane potential changes similar to those detected during vagal stimulation.

However, this interpretation implies that i_f plays a dominant role during pacemaking activity. It should be stressed that there is far from full agreement that this is the case. An alternative view is that i_f is not involved directly in pacemaking. Rather it serves to oppose the hyperpolarizing effect exerted on pacemaker cells by their being coupled to non-pacemaker tissues which have more pronounced peak diastolic potentials (Hagiwara & Irisawa, 1989). Thus the excursions of the pacemaker membrane potentials are restricted to the range of potentials in which true pacemaker currents, for example $i_{Ca,T}$, may exert their effects (Hagiwara *et al.* 1988). In this case an effect of vagally released acetylcholine to move the activation potential of i_f to more negative values would still slow the heart by making the pacemaker cells susceptible to 'electrotonic arrest' (Hagiwara & Irisawa, 1989). Again the ratio between inward current flow and outward current flow would be reduced yet a net increase in g_K would not be involved.

The differences between the muscarinic effects of applied acetylcholine and of acetylcholine released by nerve stimulation suggest that neurally released acetylcholine is not acting on the same population of receptors as that which is activated by applied acetylcholine. At many other synapses, neuronally released transmitter activates specialized subsynaptic receptors (del Castillo & Katz, 1955*b*; Dennis, Harris & Kuffler, 1971) which often have properties different to the extrasynaptic receptors (see Usherwood & Cull-Candy, 1975; Hirst & Neild, 1981; Luff, McLachlan & Hirst, 1987). No such synaptic specialization has been suggested for the vagal innervation of the heart (Löffelholz & Pappano, 1985). However, our observations suggest that vagally released acetylcholine does not have access to the muscarinic receptors that are linked to potassium channels. Yet these receptors are widely distributed in the heart (Hartzell, 1980), so it appears that neuronally released acetylcholine is only effective within a relatively short distance from its sites of release. Clearly within this short distance there are muscarinic receptors that affect inward current flow in pacemaker cells during diastole. Our observations in barium-containing solutions indicate that added acetylcholine has access to these receptors but gives no information as to the extent of their distribution. However, Giles *et al.* (1986) have suggested that only a few pacemaker cells are able to generate an i_f-like current. If all cells in the sinus venosus were able to generate i_f it would be difficult to explain how the vagus could stop the heart unless every cell received a vagal input.

Therefore we speculate that i_f may only be generated in sinus cells that directly received a vagal innervation, and that the muscarinic receptors which influence i_f are limited to such cells, perhaps even confined to a subsynaptic localization.

It is not clear whether vagal inhibition of heart rate might involve a change in the flow of depolarizing current during diastole in other species. Although it is frequently stated that vagal inhibition of pacemaker cells results from an increase in g_K and hyperpolarization, this has not been shown on many occasions. Hutter & Trautwein (1955, 1956) reported that vagal stimulation often caused an increase in membrane potential of the frog sinus venosus but pointed out that the vagus 'often fatigued' and despite a lack of hyperpolarization continued to arrest the heart. Toda & West (1966) reported that, in the pacemaker cells of the isolated rabbit heart, hyperpolarization was not detected during vagal stimulation in normal solutions. More recently Shibata *et al.* (1985) stimulated the mixed parasympathetic and sympathetic nerve supply to rabbit sino-atrial node tissue, in the presence of a β-adrenoceptor antagonist, and obtained records remarkably similar to those shown in this paper. The rate of diastolic depolarization was slowed but much of the action potential time course was unchanged. More recently we have obtained records from guinea-pig sino-atrial node cells during vagal stimulation which are very similar to those reported in this paper (Campbell, Edwards, Hirst & O'Shea, 1989). In contrast, there are many reports that applied acetylcholine causes membrane hyperpolarization in many species (Löffelholz & Pappano, 1985). Clearly further studies are required to answer this point.

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