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SYMPATHETIC NERVE STIMULATION AND APPLIED TRANSMITTERS ON THE SINUS VENOSUS OF THE TOAD

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SUMMARY

1. The effect of sympathetic nerve stimulation on pacemaker cells of the isolated sinus venosus of the toad, *Bufo marinus*, were examined using intracellular recording techniques.

2. Train of stimuli applied to the sympathetic outflow led to a two-component increase in heart rate. Shortly after the onset of stimulation the rate of discharge of pacemaker action potentials increased. After the end of the train of stimuli, the heart rate fell and then again increased to remain high for several minutes.

3. During the early tachycardia, the peak diastolic potential was reduced and the rate of diastolic depolarization increased. During the late tachycardia, the peak diastolic potential and rate of diastolic depolarization were increased; both the amplitude and the rate of repolarization of the action potentials were increased.

4. When membrane potential recordings were made from sinus venosus cells in which beating had been abolished by adding the organic calcium antagonist nicardipine, sympathetic nerve stimulation caused membrane depolarization.

5. The responses to sympathetic nerve stimulation, recorded from beating or arrested hearts, were abolished by bretylium but persisted in the presence of a number of β-adrenoceptor antagonists.

6. Bath-applied adrenaline caused a tachycardia which was associated with a large increase in the amplitudes of pacemaker action potentials. These effects were largely mediated by the activation of β_2-adrenoceptors.

7. In the presence of high concentrations of β-adrenoceptor antagonists, applied adrenaline produced membrane potential changes that although slower in time course were similar to those produced by sympathetic nerve stimulation.

8. Many aspects of the responses to nerve stimulation could be mimicked by applied ATP.

9. The early phase of sympathetic tachycardia was abolished after P_2-purinoceptor desensitization; this phase was also inhibited by dihydroergotamine.

10. The results are discussed in relation to the idea that sympathetic nerve stimulation causes the early tachycardia by increasing inward current flow during diastole, a response involving activation of specialized adrenoceptors and perhaps ATP receptors.

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INTRODUCTION

In most species, stimulation of the sympathetic nerves to heart causes tachycardia. In amphibians, the tachycardia is abolished by ergotamine, with the release of adrenaline being unaffected (Loewi, 1921, 1922), and by agents, such as bretylium, which prevent the release of catecholamines (Morris, Gibbins & Clevers, 1981). Although excitatory β-adrenoceptors are present on amphibian hearts (e.g. O'Donnell & Wanstall, 1982), the responses to sympathetic nerve stimulation are little affected by β-adrenoceptor antagonists (Morris *et al.* 1981). These observations have led to the suggestions either that neuronally released adrenaline activates a specialized junctional adrenoceptor (Morris *et al.* 1981) or that the primary sympathetic transmitter is a purine, perhaps adenosine 5′-triphosphate (ATP), rather than adrenaline (Hoyle & Burnstock, 1986).

Only a few studies have examined the membrane potential changes which accompany the increased rate of generation of cardiac pacemaker action potentials. In frogs, high-frequency stimulation of the mixed vago-sympathetic nerve, in the presence of hyoscine, increases the frequency of generation of action potentials. This response is accompanied by an increase in both the rate of diastolic depolarization and amplitude of action potentials (Hutter & Trautwein, 1955, 1956). In rabbit sino-atrial node cells, sympathetic nerve stimulation increased the heart rate largely by increasing the rate of diastolic depolarization, leaving the amplitudes of the action potentials unchanged (Toda & Shimamoto, 1968; Shibata, Giles & Pollack, 1985): these responses in mammalian tissues were abolished by the non-selective β-adrenoceptor antagonist pindolol (Shibata *et al.* 1985).

When applied to mammalian pacemaker tissues, the sympathetic transmitter noradrenaline changes the properties of at least three distinct voltage-dependent channels which are involved in pacemaking activity (Brown, 1982). Both the inward calcium current (Noma, Kotake & Irisawa, 1980; Brown, 1982) and the delayed rectifier K^+ current are increased (Brown, DiFrancesco & Noble, 1979). The activation potential for the hyperpolarization-activated inward current, i_f, is moved to more positive values (Brown *et al.*, 1979). Each of these effects would be expected to increase the rate of generation of pacemaker action potentials.

The first part of this paper describes the membrane potential changes recorded from pacemaker cells of the sinus venosus of the cane toad, which accompany sympathetic nerve stimulation. The observations raise the possibility that sympathetic nerve stimulation may cause tachycardia by increasing the background sodium current, a current thought to be important during pacemaking activity (Noble, 1984). In the second part of the paper, the responses to sympathetic nerve stimulation were compared with those produced by added adrenaline or ATP. The observations suggest that sympathetic transmission results from the activation of a set of specialized receptors to adrenaline perhaps in co-operation with a set of specialized purinoceptors.

METHODS

Toads (*Bufo marinus*) were anaesthetized by immersion in a solution of 0·5% tricaine methanesulphonate in tap water. The preparations used most frequently consisted of the sinus venosus along 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 the sympathetic chain. Sympathetic nerve fibres were stimulated by drawing the two sympathetic trunks, some 3 mm central to the vagal ganglion, through a pair of platinum ring electrodes (stimulation voltages 7–12 V, pulse width 1·0 ms).

In each experiment, the preparations were pinned out in a shallow recording chamber, the base of which consisted of a microscope cover-slip coated with Sylgard silicone resin (see Bywater, Campbell, Edwards, Hirst & O'Shea, 1989). 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 150–240 MΩ) filled with 0·5 M-KCl. The values of peak diastolic potentials reported in this paper, and those for the amplitudes of the action potentials, are somewhat larger than those usually associated with recordings from pacemaker cells. In this series of experiments we compared the amplitudes of action potentials when recordings were made, in the same region, with microelectrodes with resistances in the range 80–120 MΩ with those using higher resistance electrodes (> 150 MΩ). With the higher resistance electrodes, consistently larger amplitude action potentials were recorded. We assume that the lower resistance electrodes had failed to 'seal' adequately. Therefore all membrane potential records were made using the higher resistance electrodes; such electrodes had the disadvantage that during long-lasting impalements, particularly in beating preparations, drifts in tip potential occasionally occurred. All membrane potential records were low-pass filtered (cut-off frequency 1 kHz), digitized and stored on disc for later analysis. Because of the long duration of the responses to sympathetic nerve stimulation, trains of stimuli were delivered only every 30 min. 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; $MgCl_2$, 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 1·5 ml). In some experiments, the generation of cardiac action potentials was prevented by adding the organic calcium antagonist nicardipine ($1–10 \times 10^{-6}$ M) to the perfusion fluid. Other 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 adrenaline or ATP, a simpler preparation was used: the atria were removed and only the sinus venosus and associated sino-atrial aperture were pinned out. This allowed the bath volume to be reduced to 0·5 ml and so enabled the bathing fluid to be changed rapidly.

Drugs used in this study were; adrenaline bitartrate, ATP disodium salt, bretylium tosylate, caesium chloride, dihydroergotamine tartrate, nicardipine hydrochloride, propranolol hydrochloride, α,β-methylene ATP lithium salt (m-ATP), tetrodotoxin (all obtained from Sigma Chemicals), phentolamine mesylate (Ciba-Geigy Pharmaceuticals), I.C.I. 118551 (erythro-DL-1(7-methylindan-4-yloxy)-3-isopropyl-aminobutan-2-ol), C.G.P. 20712A (2-hydroxy-5-(2-[(2-hydroxy-3-(4[(1-methyl-4-trifluoromethyl)1H-imidazole-2-yl]-phenoxy)propyl)amino]ethoxy)-benzamide monomethane sulphonate) and alkylating pindolol (N^8-(bromoacetyl)-N^1-)3′-(4-indolyloxy)-2-hydroxy-propyl)-(*z*)-1, 8-diamino-*p*-menthane).

RESULTS

General observations

When intracellular recordings were made from sinus venosus cells, rhythmic discharges of action potentials were detected. The frequency of action potential discharge was in the range 31–59 beats/min (mean = 42 beats/min; S.E.M. ± 1·2; $n = 32$, where each n value, in this and every other case, represents the mean result from a different preparation). With most impalements the diastolic depolarization led smoothly into an action potential and such recordings were assumed to have been from pacemaker cells. In about 20% of recordings, action potentials invariably arose abruptly during the slow diastolic depolarization: such recordings were assumed to be from 'driven' or 'follower' cells (Noble, 1975). Pacemaker action potentials were

similar to those described previously for this preparation (see Bywater *et al.* 1989) and like those recorded from other pacemaker tissues (Noble, 1975). The maximum diastolic potentials were in the range −58 to −74 mV (mean −65±0·9 mV, $n = 32$). After a slow diastolic depolarization, action potentials were initiated at a threshold

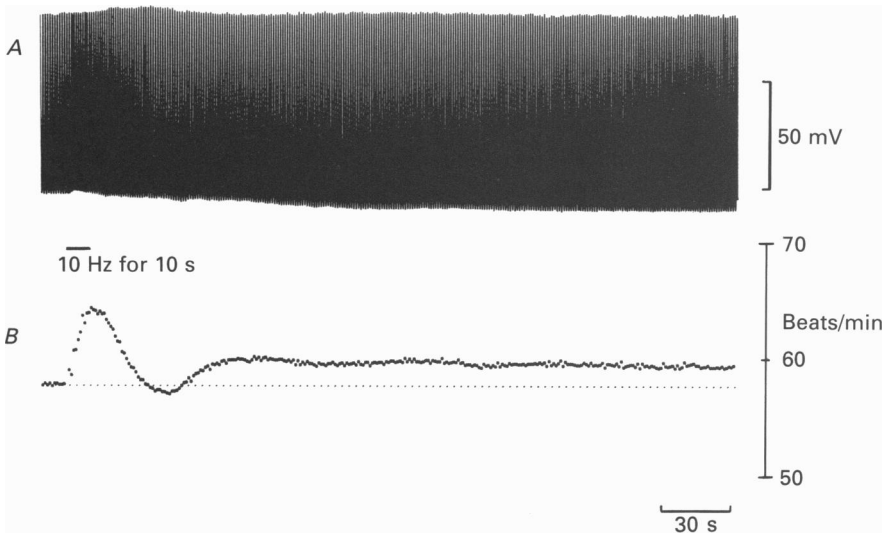

Fig. 1. The effect of bilateral sympathetic nerve stimulation on the generation of action potentials by a pacemaker cell of toad sinus venosus. The sympathetic nerves were stimulated with supramaximal stimuli for 10 s at 10 Hz. The upper record (*A*) shows the action potentials recorded before, during and after this train of stimuli. It can be seen that during the train there is a small decrease in peak diastolic potential. Thereafter, the overshoot potential of the action potentials increased slightly. Subsequently the peak diastolic potential became more negative and the overshoot was a few millivolts positive of control. The lower trace (*B*) shows the instantaneous rate of generation of pacemaker action potentials. During the train of stimuli, the rate of generation of action potentials rapidly increases. After the end of the period of stimulation, the rate of generation of action potentials falls and then rises to remain above control for the rest of the recording. The maximum diastolic potential recorded before sympathetic stimulation was −62 mV. The time calibration bar refers to both traces.

potential of about −50 mV; action potentials had peak amplitudes, when measured from the maximum diastolic potential, in the range 64–114 mV (mean 99±2·1 mV, $n = 32$).

Bilateral sympathetic stimulation with trains of stimuli of variable frequency (train duration 10 s, stimulation frequency 1–10 Hz) increased the rate of pacemaker action potential discharge (Fig. 1) in a biphasic manner. The rate of discharge of action potentials increased during the train of stimuli. After the end of the stimulation train, the frequency fell towards or below control then increased again (Fig. 1*B*). The secondary tachycardia reached a maximum some 30–90 s after the end of the stimulation train and then decayed over the next 10–20 min. Both the magnitude of initial increase in rate, which will be termed the early phase, and the magnitude of the second or late phase, were dependent upon the number of sympathetic stimuli applied. Thus in each of five preparations when 10 impulses were

delivered at 1 Hz, these trains of stimuli produced both small early and small late phases. In the same preparations when 10 impulses were delivered at 10 Hz, the early phase was greater than with 1 Hz but the late responses were identical. With a fixed train length, as the frequency of stimulation was increased so the amplitudes of both phases increased. In a group of ten preparations, using a standard train of 100 impulses delivered at 10 Hz, the mean maximal increase in frequency during the early phase was $10 \pm 1{\cdot}5$ beats/min. The corresponding figure for the late phase was $7 \pm 1{\cdot}1$ beats/min.

During the early phase, the peak diastolic potential was reduced by a few millivolts. The overshoot potential changed during the early phase in a manner that varied from preparation to preparation. In most preparations, the overshoot of the action potentials became more positive (Fig. 1). In others, the overshoot potential decreased. During the late phase the amplitude of the action potentials was slightly increased, the peak diastolic potential was more negative and the overshoot potential was often more positive. The mean amplitude of the control action potentials from this group of ten preparations was $98 \pm 3{\cdot}0$ mV; that of the action potentials during the late phase was $104 \pm 3{\cdot}3$ mV.

Both the early and the late phase of the tachycardia resulted from the release of transmitter from sympathetic adrenergic nerves, both being abolished by bretylium (1×10^{-5} M; see also Morris *et al.* 1981). However, both phases of tachycardia persisted when either propranolol (1×10^{-6} M, six preparations; see also Morris *et al.* 1981) or the selective β_1- and β_2-antagonists C.G.P. 20712A and I.C.I. 118551 (each at a concentration of 5×10^{-7} M, four preparations) were added together to the perfusion fluid. Although they had no effect on either the early or late changes in rate of generation of action potentials, both propranolol and the β_1-selective antagonist altered the amplitudes of the action potentials recorded during the early tachycardia. In the preparations where an increase in amplitude and overshoot of the pacemaker action potentials occurred, this increase was prevented and the overshoot was now depressed. In the preparations where the overshoot was depressed during the early phase, in the presence of propranolol or the β_1-selective antagonist the overshoot was further depressed. Presumably neuronally released adrenaline had activated a population of β_1-adrenoceptors to increase the amplitude of the inward calcium current during the action potentials (see Dukes & Vaughan Williams, 1984). β-Adrenoceptor antagonists had no effect on the increase in action potential amplitude that occurred during the late phase. Both phases of the tachycardia also persisted in the presence of the α-adrenoceptor antagonist phentolamine (1×10^{-6} M, six preparations; see also Morris *et al.* 1981).

In summary, sympathetic nerve stimulation produced a two-component tachycardia; both components were resistant to blockade by conventional α- and β-adrenoceptor antagonists. This report will concentrate on the membrane potential changes underlying the early phase of tachycardia and comment only in passing on the changes occurring during the late phase of tachycardia.

Effects of sympathetic nerve stimulation on pacemaker cells: early phase

During the early tachycardia, changes in the configuration of the action potentials were largely confined to the diastolic interval. As pointed out the peak diastolic potential was reduced (Fig. 2) and the rate of diastolic depolarization was increased

(Fig. 3*A*). Other than this the shapes of the action potentials were little changed. Thus the threshold for initiation of the action potential and rates of repolarization were unchanged (Fig. 3*A*). In a given preparation, three further types of responses were often noted. In some recordings, which were from pacemaker cells, during the

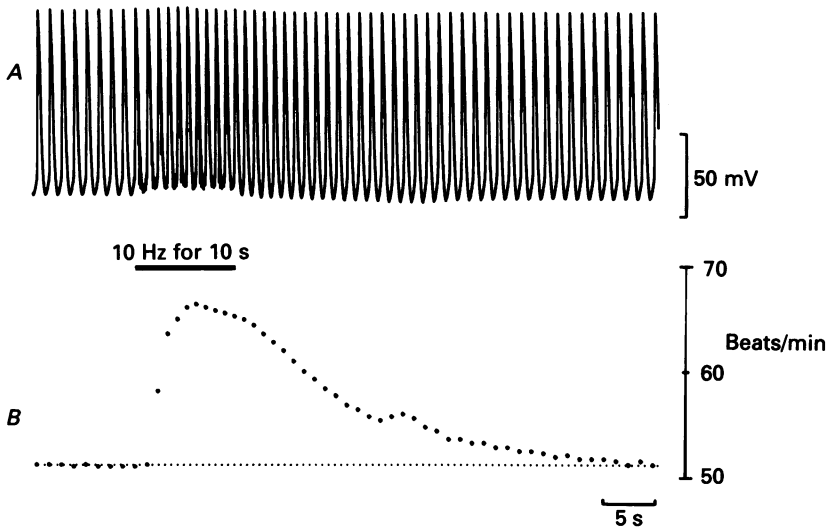

Fig. 2. The effect of sympathetic nerve stimulation on pacemaker action potentials during the early phase of sympathetic tachycardia. The sympathetic nerves were stimulated with supramaximal stimuli for 10 s at 10 Hz. The upper record (*A*) shows the action potentials recorded before, during and shortly after this train of stimuli. During the stimulation train the peak diastolic potential becomes progressively less negative and then returns to control even though the rate of generation of action potentials, shown in *B*, is above control. It can also be seen from trace *B* that the increased rate of generation of action potentials lags behind the start of the stimulation train. After the end of the period of stimulation, the rate of generation of action potentials falls back towards the control rate. The maximum diastolic potential recorded before sympathetic stimulation was −68 mV. The time calibration bar refers to both traces.

tachycardia the smooth diastolic depolarization leading to an action potential was replaced by an abrupt initiation of the action potential. That is the cell appeared to have become a 'driven' cell. This suggests that sympathetic nerve stimulation had made another pacemaker region become dominant and the pacemaker centre had shifted (see Toda & Shimamoto, 1968). In other recordings which were from 'driven' cells, the action potential was initiated at a more hyperpolarized potential. Presumably the nearby pacemaker region was discharging action potentials at a more rapid frequency. In yet other recordings from 'driven' cells, the action potentials became like those recorded from pacemaker cells. Perhaps in these cases, the activity of nearby sympathetic nerve synapses had made these regions become dominant pacemaker areas.

With low-frequency stimulation (1 Hz) the rate of generation of action potentials slowly increased over 5–10 s and then remained constant until stimulation was stopped (Fig. 6*A* and *C*). Thereafter, the rate of generation of action potentials

returned slowly towards control, to rise subsequently in the second phase of tachycardia. With higher frequencies of stimulation (2–5 Hz) the initial tachycardia was more marked, had a more abrupt onset and was again maintained throughout the stimulation period (Fig. 6*E* and *F*). With even higher frequency stimulation

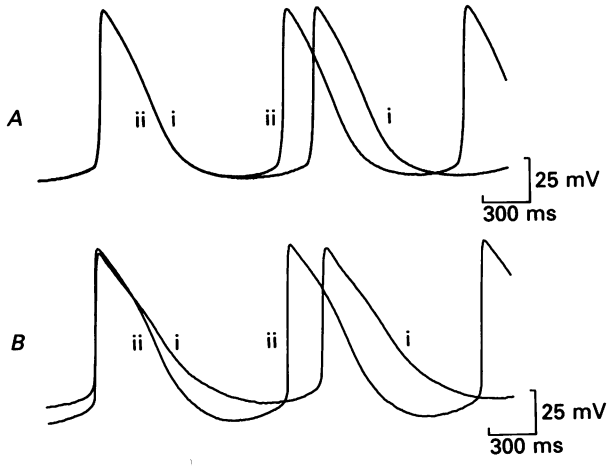

Fig. 3. Effect of sympathetic nerve stimulation on the shapes of pacemaker action potentials during the early and late tachycardias. The traces in *A* show overlays of action potentials recorded before (i) and immediately after (ii) a short train of stimuli (10 mV for 0·5 s). Note that during the early tachycardia, the shape of the action potential is unchanged except during the diastolic interval. During diastole, the peak diastolic potential is less negative and the rate of diastolic depolarization is increased. In the lower traces a pair of control action potentials (i) are overlaid with a pair recorded during the late phase of tachycardia (ii). During the late phase of tachycardia (*B*) the peak diastolic potential and the threshold for initiation of action potentials are more negative, the overshoot potential is slightly more positive, and the rates of action potential repolarization and diastolic depolarization are more rapid. In this illustration the record of the late tachycardia was obtained 4 min after a 10 s train of stimuli delivered at 10 Hz. The peak diastolic potential for the control action potentials in *A* was −64 mV; in *B* it was −67 mV.

(10 Hz) the tachycardia was again rapid in onset. In some preparations the increased rate was maintained until the end of the 10 s train; in others the rate started to fall. Invariably after 30 s of stimulation of 10 Hz, the rate of generation of action potentials had dropped back to only slightly above control but, even so, the early phase was followed by a late phase of tachycardia.

In five preparations the effects of only a few sympathetic stimuli on the rate of discharge of action potentials was examined. The sympathetic supply was stimulated either with a single stimulus or with 2, 3, 5 or 10 impulses each delivered at 10 Hz. The results from one series of these experiments are illustrated in Fig. 4. From the membrane potential record and associated plot of instantaneous rate shown in the upper traces (Fig. 4*A* and *B*), it can be seen that 5 stimuli gave a tachycardia which was apparent about 1 s after the end of the stimulation train. After the peak increase, the rate of generation of action potentials gradually fell back to control over the next 25 s. The time course of the decay of the tachycardia could not usually be described

by a single exponential. The rate of decay often remained constant for several seconds after the peak tachycardia (Fig. 4*C*; 5 and 10 stimuli). Subsequently the onset of the late tachycardia masked the final decay of the early component. Where single exponential functions could be well fitted, their time constant increased with

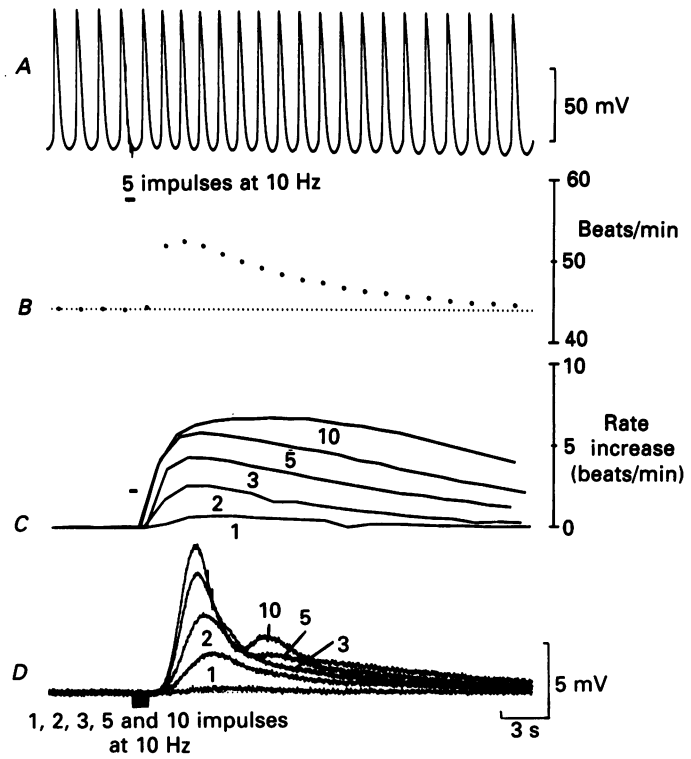

Fig. 4. Effect of single and brief trains of sympathetic stimuli on the membrane potentials of beating and arrested pacemaker cells. The upper traces (*A* and *B*) show the effect of 5 stimuli delivered at 10 Hz on pacemaker action potentials and their rate of generation. Shortly after stimulation, the rate of generation of action potentials increased and then slowly fell back to control level. The traces shown in *C* illustrate the increased rate of generation of pacemaker action potentials produced by 1, 2, 3, 5 and 10 stimuli, each at 10 Hz. A single stimulus produced a small tachycardia. As the number of stimuli was increased the amplitude and duration of the tachycardia were increased. The lower traces (*D*) show the membrane potential changes produced by similar trains of stimuli from a preparation in which pacemaker action potentials had been abolished by nicardipine (1×10^{-5} M). A single stimulus produced a small membrane depolarization. As the number of stimuli was increased, the depolarization became larger and more long lasting. When the depolarization was larger than about 5 mV, regenerative potential changes were superimposed on the depolarization. The time calibration refers to each record. The peak diastolic potential in *A* was −68 mV; the steady potential in *D* was −32 mV.

peak amplitude of tachycardia. From this series of experiments, the 10–90% rise time of the onset of tachycardia was $2{\cdot}3 \pm 0{\cdot}3$ s ($n = 5$). The rate changes produced by the varying number of sympathetic stimuli are plotted in the lower part of the figure (Fig. 4*C*). It can be seen that a single stimulus produced a small increase in

heart rate. As the number of stimuli was increased the tachycardia increased and its time course was prolonged (Fig. 4*C*).

Effects of sympathetic nerve stimulation on pacemaker cells: late phase

As has been pointed out, during the late phase of sympathetic tachycardia the actions potentials were increased in amplitude, and their rates of repolarization and their rates of diastolic depolarization increased (see Fig. 3*B*). As with the early phase, in some cells the action potentials became driven. In some of the recordings from driven cells, during the late phase the action potentials were initiated smoothly after the diastolic depolarization. Apparently, during the late phase of tachycardia, long-lasting shifts in the sites of the dominant pacemaker regions had occurred.

Effects of sympathetic nerve stimulation on arrested pacemaker cells

In a recent study on the effects of vagal stimulation on toad sinus venosus cells, it was suggested that during vagal stimulation neuronally released acetylcholine acted to produce bradycardia by reducing inward current flow during diastole (Bywater *et al.* 1989). From an analysis of vagal inhibitory junction potentials recorded from preparations in which the generation of pacemaker action potentials had been prevented by an organic calcium antagonist, it was suggested that vagal stimulation reduced a background sodium current (Noble, 1984; Bywater, Campbell, Edwards & Hirst, 1990). In the present series of experiments we used similar preparations to see if sympathetic nerve stimulation changed the background conductances.

The generation of pacemaker action potentials was arrested by adding nicardipine (1×10^{-5} M) to the physiological saline. In such preparations the membrane potential was stable and lay in the range -26 to -40 mV (mean $-33 \pm 0{\cdot}5$ mV; $n = 29$); these values are similar to those reported previously using nifedipine (Bywater *et al.* 1989, 1990). Sympathetic nerve stimulation subsequently evoked slow membrane depolarizations. An example is shown in Fig. 5; the sympathetic nerves were stimulated with 10 impulses at 10 Hz and produced a depolarization of about 15 mV. In different preparations the amplitude of the depolarization produced by a similar train of stimuli varied from 3 to 32 mV (mean $9{\cdot}5 \pm 1{\cdot}9$ mV; $n = 10$). These depolarizations, which will be referred to as excitatory junction potentials (EJPs), like the responses to sympathetic nerve stimulation recorded from beating preparations, persisted in the presence of either propranolol (1×10^{-6} M- or phentolamine (1×10^{-6} M) but were prevented by adding bretylium (1×10^{-5} M) to the perfusion fluid. Presumably EJPs result from the release of sympathetic transmitter.

In five preparations the amplitudes and time courses of EJPs produced by only a few sympathetic stimuli were examined. As with the beating preparations described previously (see Fig. 4), the sympathetic supply was stimulated either with a single stimulus or with 2, 3, 5 or 10 impulses, each train delivered at 10 Hz. The EJPs produced by these stimuli are shown in Fig. 4*D*. It can be seen that a single stimulus gave rise to a small membrane depolarization of less than 0·5 mV. As the number of stimuli was increased, EJPs increased in amplitude and they became more prolonged. When the depolarization was larger than about 5 mV, regenerative potential changes

were superimposed on the EJPs: EJPs were then followed by small after-hyperpolarizations. In these five preparations, the mean peak amplitudes of EJPs produced by 1, 2, 3, 5 and 10 impulses were $0{\cdot}4 \pm 0{\cdot}1$, $2{\cdot}7 \pm 1{\cdot}2$, $4{\cdot}4 \pm 2{\cdot}0$, $6{\cdot}4 \pm 2{\cdot}3$ and $8{\cdot}1 \pm 2{\cdot}2$ mV, respectively. It can also be seen that the EJPs occurred after a delay

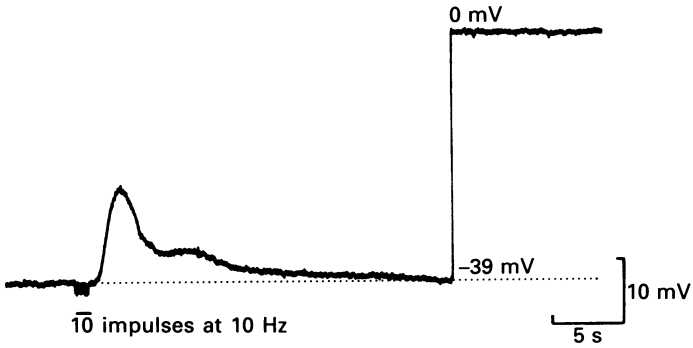

Fig. 5. Response to sympathetic nerve stimulation recorded from a sinus venosus cell in which the generation of pacemaker action potentials had been abolished by nicardipine. The sympathetic nerves were stimulated with 10 impulses delivered at 10 Hz. After a delay of about 1 s, a slow depolarization which lasted for 25 s was recorded. After the end of the depolarization the electrode was withdrawn to check the membrane potential (−39 mV).

of 1–2 s (the delay between the start of the stimulation train (5 stimuli at 10 Hz) and the rise to 10% of the peak response was $2{\cdot}3 \pm 0{\cdot}2$ s). Like the increases in heart rate recorded from beating preparations, EJPs had long time courses (compare Fig. 4*C* and *D*). The time course of decay of those EJPs that were insufficiently large to trigger regenerative potential changes could be roughly described by a single exponential, with a time constant of about 7 s. For EJPs produced by 5 stimuli at 5 Hz the mean value of the time constant at 7·1 s ($\pm 2{\cdot}1$ s; $n = 5$), and the mean value of the rise time was 1·8 s ($\pm 0{\cdot}2$ s; $n = 5$).

When longer trains of stimuli were applied, more persistent depolarizations were recorded. As shown in Fig. 6, the general forms of the membrane depolarizations recorded from arrested preparations were similar to the profiles of the changed rates of generation of action potentials recorded from beating preparations during the early phase of tachycardia. With low-frequency stimulation (1 Hz), the membrane depolarization climbed slowly during the train of stimuli. When stimulation stopped, the depolarization slowly decayed (Fig. 6*B*). With higher frequency stimulation (2–5 Hz), larger depolarizations, more rapid in onset, were recorded (Fig. 6*D* and *F*). Again, regenerative potential changes were superimposed on the larger membrane depolarizations. With longer trains of high-frequency stimuli (30 s at 10 Hz), the depolarization subsided during the period of stimulation. These observations again indicate that sympathetic nerve stimulation produces membrane depolarizations in arrested sinus cells with temporal characteristics similar to those of the early tachycardias recorded from beating preparations. Therefore we suggest that the depolarizations recorded from arrested preparations are a measure of the change in membrane current underlying the early tachycardia.

We considered the possibility that the EJPs resulted from an action of sympathetic

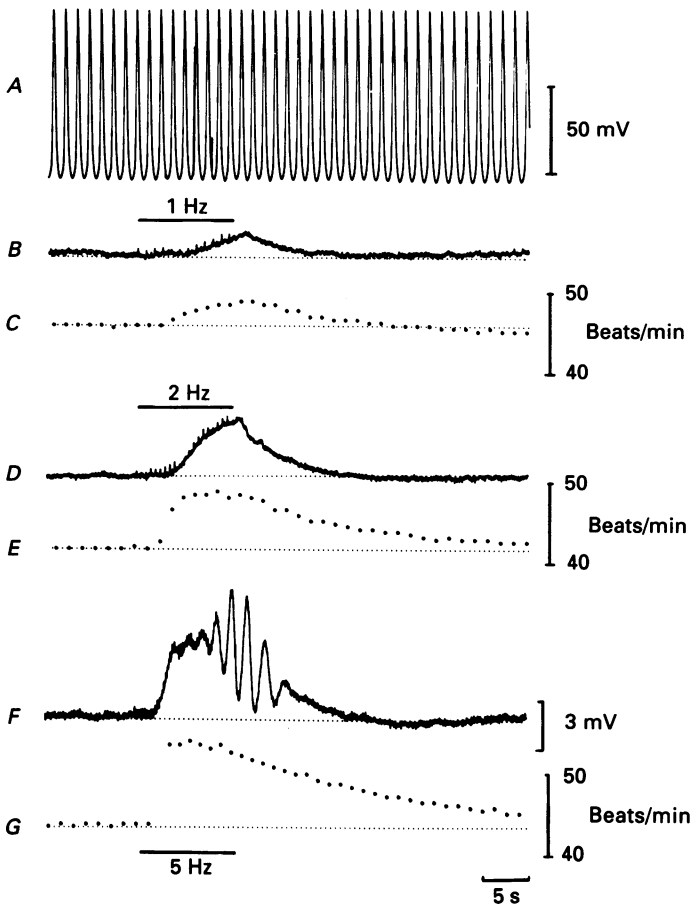

Fig. 6. Comparison between membrane potential responses to maintained sympathetic stimulation in beating and arrested pacemaker cells. In each record, indicated by the continuous bar, the sympathetic nerves were stimulated for 10 s. In *A*, *B* and *C* the stimulation frequency was 1 Hz; in *D* and *E*, the frequency was 2 Hz; in *F* and *G*, the frequency was 5 Hz. The upper trace shows a recording from a pacemaker cell; the associated trace showing the increase in rate produced by low-frequency sympathetic stimulation is shown in *C*. It can be seen that, after a delay, the rate of generation of action potentials slowly increased; after the end of stimulation the tachycardia slowly decayed. In the recording from an arrested pacemaker cell, a few seconds after the start of the stimulation train, a slowly developing depolarization occurred. When the stimulation frequency was progressively increased (*E* and *G*) the tachycardias became more pronounced and occurred at shorter latencies. Similarly in the recordings from the arrested preparation, the depolarizations were larger, their rates of rise were more rapid and their latencies were reduced. In *A*, the maximum diastolic potential during the control period was −67 mV. The 3 mV voltage calibration bar applies to *B*, *D* and *F*; the 'arrested' membrane potential for these records was −35 mV. The time calibration bar applied to all records.

transmitter on the hyperpolarization-activated current, i_f. The activation voltage for this current has been shown to be moved to more positive potentials by catecholamines (Brown *et al.* 1979). If, for example, the activation potential of i_f was changed to −10 mV, a membrane depolarization should occur. However, the EJPs

persisted in the presence of caesium ions (5 mM). The peak depolarization produced by 10 impulses at 10 Hz in control solution was $11{\cdot}0 \pm 2{\cdot}9$ mV ($n = 3$); in the same preparations the same trains of stimuli produced a peak depolarization in caesium-containing solution of $13{\cdot}7 \pm 4{\cdot}3$ mV ($n = 3$). Since caesium ions have been shown to

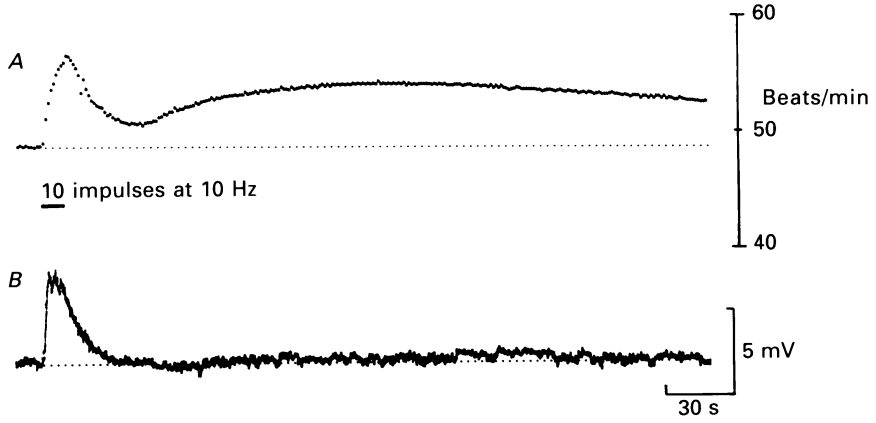

Fig. 7. Comparison between the time course of sympathetic tachycardia recorded from a beating pacemaker cell and the depolarization recorded from an arrested pacemaker cell. The upper trace (*A*) shows the change in rate of generation of pacemaker action potentials produced by a 10 s train of stimuli delivered at 10 Hz. The lower trace (*B*) shows the change in membrane potential recorded from an arrested preparation (control membrane potential −35 mV) in response to an identical train of stimuli. Note that a depolarization is only detected at the time period corresponding to the early phase of tachycardia. The time calibration bar applies to both records.

block i_f in cardiac tissue (DiFrancesco, 1985), it seems unlikely that a change in the activation potential of i_f leads to the initiation of EJPs. In three preparations both the EJPs and the steady membrane potential were unaffected when TEA (10 mM) was added to the perfusion fluid. Although the interpretation of this observation is complicated by the possibility that TEA has increased the release of sympathetic transmitter, it seems unlikely that the depolarization involves a change in the properties of delayed rectifier K^+ channels.

During the time period corresponding to the late phase of sympathetic tachycardia a depolarization was not detected. This is illustrated in Fig. 7. The upper trace (*A*) shows the effect of a 10 s train of stimuli, delivered at 10 Hz, on the rate of generation of action potentials in a beating preparation. The lower trace (*B*) shows the membrane potential change recorded from an arrested sinus venosus preparation in response to an identical train of stimuli. This observation is consistent with the idea that during the late phase of tachycardia, the rate of generation of action potentials is increased by modifying the voltage-dependent channels normally involved in the generation of pacemaker action potentials (for related discussion see Brown, 1982).

Effects of applied adrenaline and ATP on sinus venosus cells

Although the traditional transmitter released by amphibian sympathetic nerves is adrenaline (Loewi, 1921, 1922) both the early and late phases of sympathetic tachycardia persist in the presence of propranolol or phentolamine (Morris *et al.*

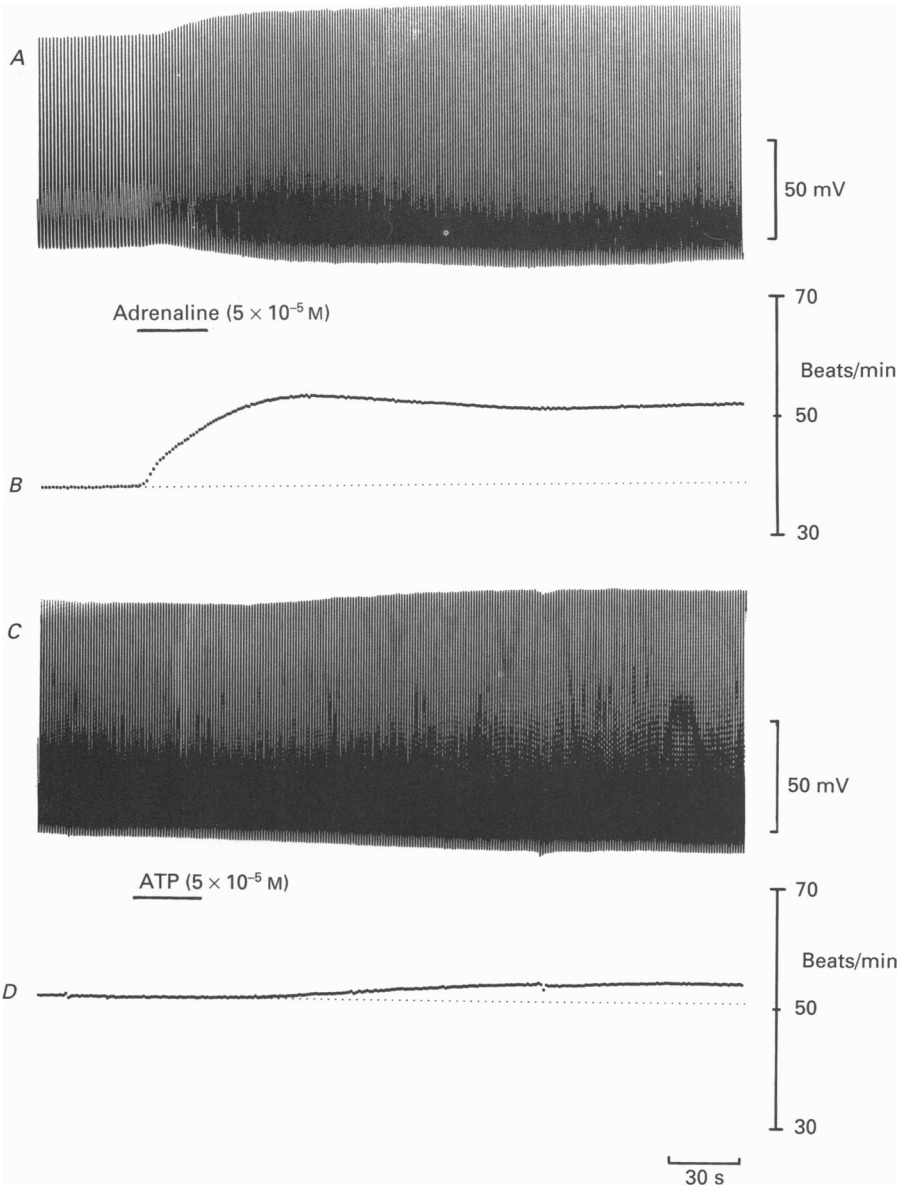

Fig. 8. Effect of adding either adrenaline or ATP to the physiological saline on the amplitudes and the rates of generation of pacemaker action potentials. In each record the fluid inflow line was changed to a solution containing transmitter for 30 s. The upper trace (*A*) and the associated rate plot (*B*) show the effect of adding adrenaline (5×10^{-5} M) to the perfusion fluid. Note that the initial increase in rate of generation of action potentials was associated with a small depression in the amplitudes of the action potentials and a reduction in the peak diastolic potentials. Subsequently both the amplitudes and the rate of occurrence of action potentials were increased. The lower pair of trace (*C* and *D*) shows the effect of adding ATP (5×10^{-5} M) to the perfusion fluid. Note that after a long delay both the rate of generation and the amplitudes of the action potentials are increased. Time calibration bar applies to all records.

1981). In frogs, the positive inotropic effects which accompany sympathetic nerve stimulation also persist in the presence of propranolol but are blocked after desensitization of P_2-purinoceptors with α, β-methylene ATP (m-ATP; Hoyle & Burnstock, 1986). This report led to the suggestion that the sympathetic transmitter on amphibian hearts was ATP. Against this view is the report that applied ATP inhibits the generation of action potentials by frog sinus venosus cells (Hartzell, 1979). Therefore the effects of the two transmitter candidates, adrenaline and ATP, on pacemaker cells of the toad were examined. Unless stated otherwise, the experiments were carried out on preparations of sinus venosus with the atria removed. This allowed the bath volume to be reduced to 0·5 ml and meant that the added transmitter rapidly reached the tissue.

Adrenaline, applied in the concentration range 10^{-7} to 10^{-5} M, caused a persistent increase of the rate of generation and the amplitudes of action potentials. The peak diastolic potential was more negative and the overshoot potential more positive. Similar observations have been made on a number of other pacemaker cells (see Brown, 1982). When higher concentrations of adrenaline ($5–10 \times 10^{-5}$ M) were applied, soon after the start of the application the peak diastolic potential was reduced transiently (see Fig. 8*A*). Thereafter the action potential amplitude increased in the same way as it did when the lower concentrations were used.

The responses to added adrenaline in concentrations up to 1×10^{-5} M were unchanged when the selective β_1-adrenoceptor antagonist, C.G.P. 20712A ($0·5 \times 10^{-6}$ M, five preparations), was added to the perfusion fluid. However, with the further addition of the relatively selective β_2-adrenoceptor antagonist, I.C.I. 118551 ($0·5 \times 10^{-6}$ M), the responses to these concentrations of added adrenaline were greatly reduced. The effects of adrenaline were similarly attenuated either by adding propranolol (1×10^{-6} M, three preparations) to the perfusion fluid or by incubating the tissues in the irreversible non-selective β-adrenoceptor antagonist, alkylating pindolol (1×10^{-5} M, six preparations), for 90 min (Molenaar, Russell, Pitha & Summers, 1988). With either of these antagonists present, adrenaline (1×10^{-5} M) continued to produce a smaller than normal, long-latency tachycardia. When the concentration of applied adrenaline was increased to $5–10 \times 10^{-5}$ M, a two-component increase in the rate of generation of action potentials was invariably detected (Fig. 9). Although these responses were slower in overall time course than those produced by sympathetic nerve stimulation, the two components were otherwise qualitatively similar. These responses did not result from the activation of α-adrenoceptors as they persisted in the presence of phentolamine (1×10^{-6} M, three of the six preparations incubated with alkylating pindolol). It was noted that if the rate of perfusion of the chamber was reduced to 1 ml/min, rather than the usual 6 ml/min, added adrenaline failed to produce a detectable response in the β-blocked tissues.

Applied ATP also increased the rate of generation of action potentials in seven out of eight preparations examined. The concentration of ATP required to cause a tachycardia varied quite markedly from preparation to preparation. In two preparations a clear increase in the rate of generation of action potentials occurred when a concentration of 1×10^{-6} M-ATP was applied. In one preparation a concentration of 1×10^{-4} M-ATP had to be applied before an increase in the rate of generation of action potentials was detected. When the concentration of ATP was

increased above threshold, by a factor of 2 or 3, a maximal acceleration occurred. In the seven preparations that responded to ATP, the maximum frequency at which action potentials were generated never exceeded 60 beats/min. In the single preparation that failed to respond to ATP, at a concentration up to 1 mM, the control

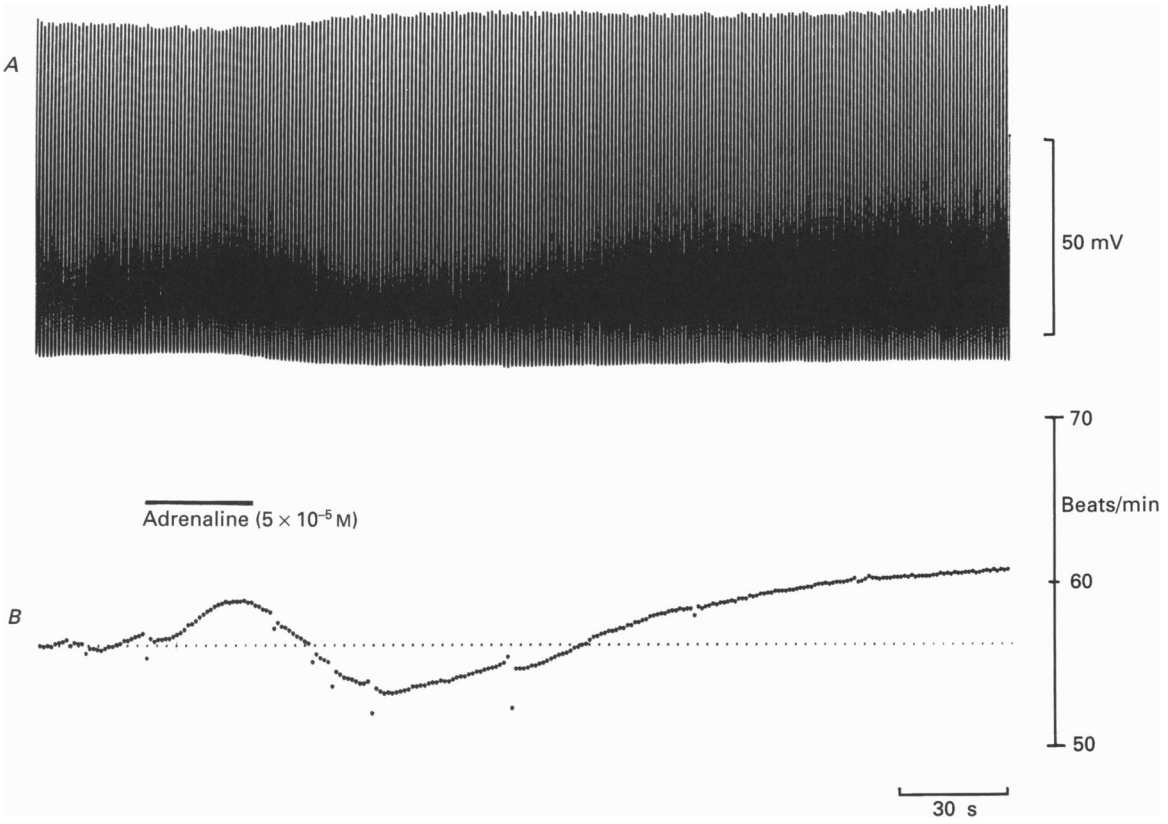

Fig. 9. Effect of rapid application of adrenaline on the membrane potential of an isolated segment of sinus venosus that had been incubated in an irreversible β-adrenoceptor antagonist. In the preparations which had been incubated in alkylating pindolol for 90 min, applied adrenaline caused early and late tachycardias qualitatively similar to those recorded after sympathetic nerve stimulation. Note the small decrease in peak diastolic potential during the early tachycardia and the increased peak diastolic potential during the late tachycardia. Time calibration bar applies to both records.

rate of beating was unusually high, being 58 beats/min. These experiments indicate that in the toad, ATP exerts a positive chronotropic action. The responses to ATP were unaffected by combined β_1- and β_2-adrenoceptor blockade.

ATP ($1–100 \times 10^{-6}$ M) produced changes in the amplitudes and the shapes of the pacemaker action potentials in the same ways as during the late phase of the response to sympathetic nerve stimulation (Fig. 8*D*). In five sinus venosus–atrial preparations the responses to sympathetic nerve stimulation produced by a 10 s train of stimuli delivered at 10 Hz were determined and then compared with the responses to added ATP. The concentration of applied ATP was adjusted so that it

produced a tachycardia similar to that in the late phase of the sympathetic nerve response. In these experiments, sympathetic nerve stimulation increased the rate of generation of action potentials during the late phase from $44{\cdot}6 \pm 1{\cdot}4$ to $51{\cdot}4 \pm 1{\cdot}4$ beats/min and the amplitudes of the action potentials from $105{\cdot}2 \pm 1{\cdot}2$ to $110{\cdot}4 \pm 2{\cdot}0$ mV. The corresponding figures when the tachycardia was produced by added ATP were from $45{\cdot}2 \pm 1{\cdot}6$ to $52{\cdot}8 \pm 1{\cdot}5$ beats/min and from $105{\cdot}2 \pm 1{\cdot}6$ to $114{\cdot}4 \pm 2{\cdot}1$ mV. Using a paired t test these values were found not to be significantly different ($P < 0{\cdot}1$). Taken together with the observation that the late phase of sympathetic tachycardia is abolished by m-ATP (Bramich & Campbell, 1989), the experiments are consistent with the view that ATP acts as an excitatory transmitter on toad sinus venosus cells.

The early phase of the response to sympathetic nerve stimulation could be mimicked by perfusing high concentrations of ATP (0·1–1·0 mM). The rate of generation of pacemaker action potentials increased and the peak diastolic potential was transiently less negative. This phase was rapidly supplanted by a larger increase in the amplitude of the action potentials than that which occurred following the corresponding acceleration produced by sympathetic nerve stimulation. Omitting the preparation which failed to respond to ATP, the mean maximal increase in rate produced by ATP was $8{\cdot}5 \pm 1{\cdot}2$ beats/min and the mean increase in action potential amplitude was $17{\cdot}1 \pm 3{\cdot}7$ mV ($n = 7$). For comparison the largest increase in rate and amplitude of the action potentials that we have detected in any preparation following intense sympathetic nerve stimulation (30 s at 10 Hz) was 14·1 beats/min and 4 mV.

The effects of applied adrenaline and applied ATP were examined in isolated sinus venosus preparations in which the generation of pacemaker action potentials had been abolished by adding nicardipine (1×10^{-5} M) to the perfusion fluid. Adrenaline or ATP each caused a transient membrane depolarization of arrested sinus venosus preparations. In concentrations lower than 1×10^{-5} M adrenaline failed to produce a detectable depolarization. When added to the perfusion fluid, in the range $1–10 \times 10^{-5}$ M, adrenaline caused a depolarization of some 3–10 mV (Fig. 10*A*). These responses to added adrenaline rapidly desensitized. As can be seen from Fig. 10*A*, even during a 30 s application of adrenaline, the membrane depolarization started to decay. In this same preparation, when adrenaline was applied for 2 min, the response was indistinguishable from that produced by the briefer exposure. If adrenaline was re-applied after a short interval, the amplitude of the second response was depressed. If applied at 30 min intervals, consistently sized responses were recorded. In most preparations (ten out of fourteen) the depolarizing response to adrenaline was followed by a small persistent hyperpolarization of 1–3 mV; the nature of the receptors activated during the hyperpolarization has not been investigated further. Like the responses to sympathetic nerve stimulation recorded from arrested preparations, the depolarizations to added adrenaline persisted after β-adrenoceptor blockade (incubation in alkylating pindolol, 1×10^{-5} M, for 90 min or the addition of propranolol, 1×10^{-6} M) and in the presence of phentolamine (1×10^{-6} M).

Added ATP ($1–10 \times 10^{-5}$ M) also produced transient membrane depolarizations (see also Friel & Bean, 1988). As with the responses to added adrenaline, those produced by ATP decayed during sustained perfusion of the ATP-containing solutions. All depolarizations to ATP were followed by hyperpolarizations of some 2–5 mV that

lasted for 20–30 min (Fig. 10*B*). When adrenaline and ATP were added simultaneously, the amplitude of the response was invariably less than the sum of the individual responses. If adrenaline was steadily perfused through the chamber so that the adrenoceptors desensitized, a subsequent addition of ATP no longer

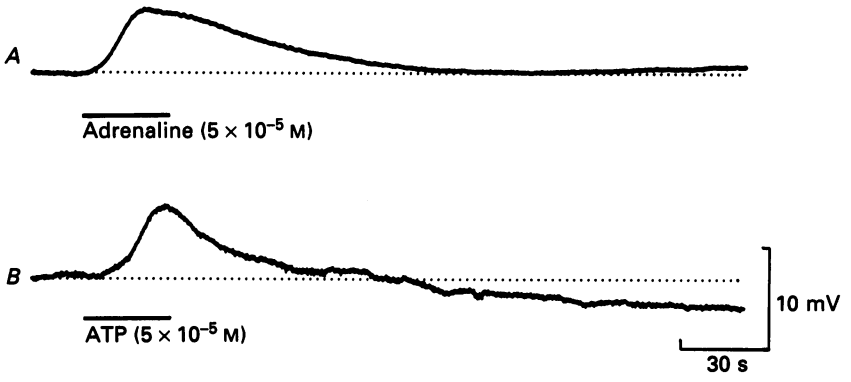

Fig. 10. Responses to applied adrenaline and applied ATP recorded from arrested sinus venosus cells. The upper trace (*A*) shows that adrenaline (5×10^{-5} M) applied for 30 s produced a depolarization. From the lower trace (*B*) it can be seen that ATP (5×10^{-5} M) produced a similar depolarization which was followed by a long-lasting hyperpolarization. The steady membrane potential for both traces was −34 mV; calibration bars apply to both records.

produced a response. Similarly after desensitization to ATP, adrenaline failed to produce a membrane depolarization. These observations suggest that the two putative transmitters are able to activate a common pathway.

In summary, bath-applied adrenaline appears to activate two distinct populations of adrenoceptors on toad pacemaker cells. The adrenoceptors which are most readily activated by adrenaline appear to be of the β_2-type (see also O'Donnell & Wanstall, 1982). These receptors do not readily desensitize. Presumably these receptors modify the properties of the voltage-dependent channels activated during pacemaker activity. A second set of adrenoceptors have a low affinity to adrenaline, are resistant to β-adrenoceptor blockade and rapidly desensitize. The properties of these receptors can be most readily examined when pacemaker voltage-dependent channels have been rendered inactive in arrested preparations. Similarly toad pacemaker cells appear to have two groups of excitatory purinoceptors. One group increases the amplitudes and rate of generation of pacemaker action potentials and these receptors do not readily desensitize. The other group depolarizes arrested pacemaker cells and rapidly desensitizes.

Identity of transmitter responsible for initiation of the early phase of tachycardia

The previous sections have indicated that both adrenaline and ATP can, with the application of an appropriate concentration of transmitter, mimic the responses to sympathetic nerve stimulation. To distinguish between these transmitter candidates we first examined the effects of m-ATP on the responses to sympathetic nerve stimulation and to added adrenaline or ATP. When m-ATP (5×10^{-5} M) was

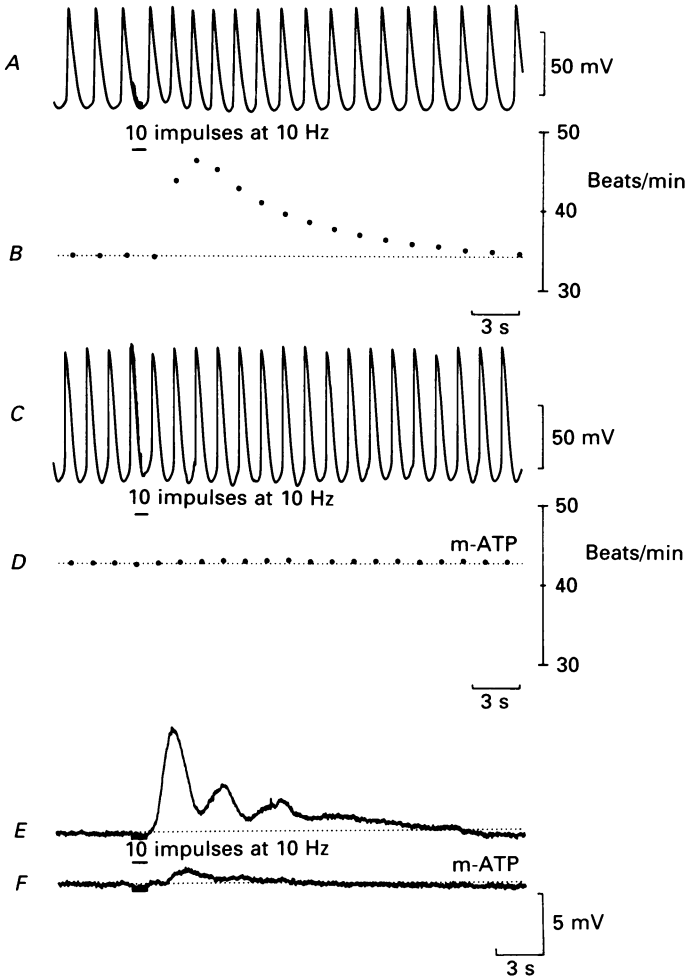

Fig. 11. Effect of desensitization of P_2-purinoceptors with m-ATP on the responses to sympathetic nerve stimulation in beating and arrested preparations of sinus venosus. The upper trace (*A*) and associated plot of instantaneous rate of generation of action potentials (*B*) show the response to 10 stimuli delivered at 10 Hz in control solution. The rate of generation of action potentials increased transiently; peak diastolic potential −62 mV in control. The next pair of traces (*C* and *D*) show the response to the same train of stimuli 1 h after continuous perfusion with m-ATP (5×10^{-5} M). Note that the amplitude and the rate of generation of action potentials are increased; peak diastolic potential −74 mV. Sympathetic nerve stimulation no longer produces a tachycardia. The lower traces (*E* and *F*) show the responses to sympathetic nerve stimulation in an arrested preparation (10 impulses at 10 Hz) in control (*E*) and 1 h after adding m-ATP (5×10^{-5} M) to the perfusion fluid (*F*). The amplitude of the sympathetic EJP is considerably reduced. Steady membrane potential in *E* and *F* was −36 mV.

continuously perfused through the recording chamber there was a persistent increase in the amplitudes and rates of generation of the pacemaker action potentials (Fig. 11*A–D*). The mean amplitudes and rates of generation of control action potentials were $92{\cdot}7 \pm 6{\cdot}8$ mV and $34{\cdot}1 \pm 1{\cdot}0$ beats/min, respectively. After 2 h continuous

exposure to m-ATP (5×10^{-5} M) the amplitudes and rates were $109{\cdot}3 \pm 8{\cdot}2$ mV and $43{\cdot}5 \pm 1{\cdot}8$ beats/min, respectively ($n = 3$). Thus as with the action of ATP on beating preparations, the responses to m-ATP did not appear to desensitize (Fig. 11). However, when m-ATP was present, in each of these three preparations sympathetic

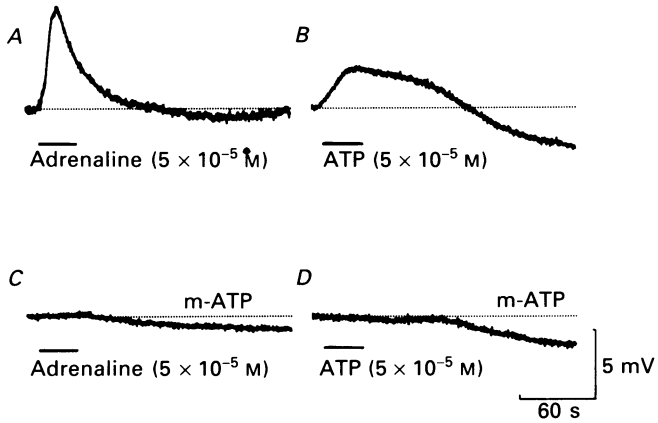

Fig. 12. Effect of desensitization of P_2-purinoceptors with m-ATP on the responses to applied adrenaline and ATP in arrested preparations of sinus venosus. The upper left trace (*A*) shows the response to adding adrenaline (5×10^{-5} M; continuous bar) to the perfusion fluid for 30 s. The upper right trace (*B*) shows the response to adding ATP (5×10^{-5} M); to the perfusion fluid for 30 s. The lower pair of traces show the response to the same concentrations of adrenaline (*C*) and ATP (*D*) 1 h after continuous perfusion with m-ATP (5×10^{-5} M). Note that the transient depolarizations produced by either agonist are abolished but the following hyperpolarizations persist. Steady membrane potential for all records was -33 mV; calibration bars apply to each record.

nerve stimulation failed to produce an additional tachycardia. When m-ATP (5×10^{-5} M) was rapidly applied to arrested sinus venosus preparations, it produced a small (1–3 mV) depolarization which lasted for about 5 min. After this time, the EJPs produced by sympathetic nerve stimulation were rapidly reduced in amplitude (Fig. 11*E* and *F*). In four preparations, a train of ten impulses delivered at 10 Hz produced a mean depolarization of $5{\cdot}9 \pm 1{\cdot}4$ mV; 1 h after adding m-ATP (5×10^{-5} M) to the perfusion fluid, the same stimuli produced a depolarization of $1{\cdot}1 \pm 0{\cdot}4$ mV. This observation might suggest that desensitization of a subset of P_2-purinoceptors blocks the early sympathetic tachycardia.

In arrested preparations when m-ATP (5×10^{-5} M) was added continuously to the perfusion fluid, the responses to both rapidly applied adrenaline or ATP were blocked (Fig. 12). The hyperpolarizing responses which invariably followed the depolarization caused by ATP and which often followed the depolarization to adrenaline were not affected by m-ATP (Fig. 12).

These observations indicate that P_2-purinoceptor desensitization with m-ATP blocks the responses to both ATP and adrenaline. Possible interpretations are that both transmitters activate the same receptor, or m-ATP is not selective in its action, or the adrenaline receptor is rendered inactive by the desensitization of a nearby P_2-purinoceptor.

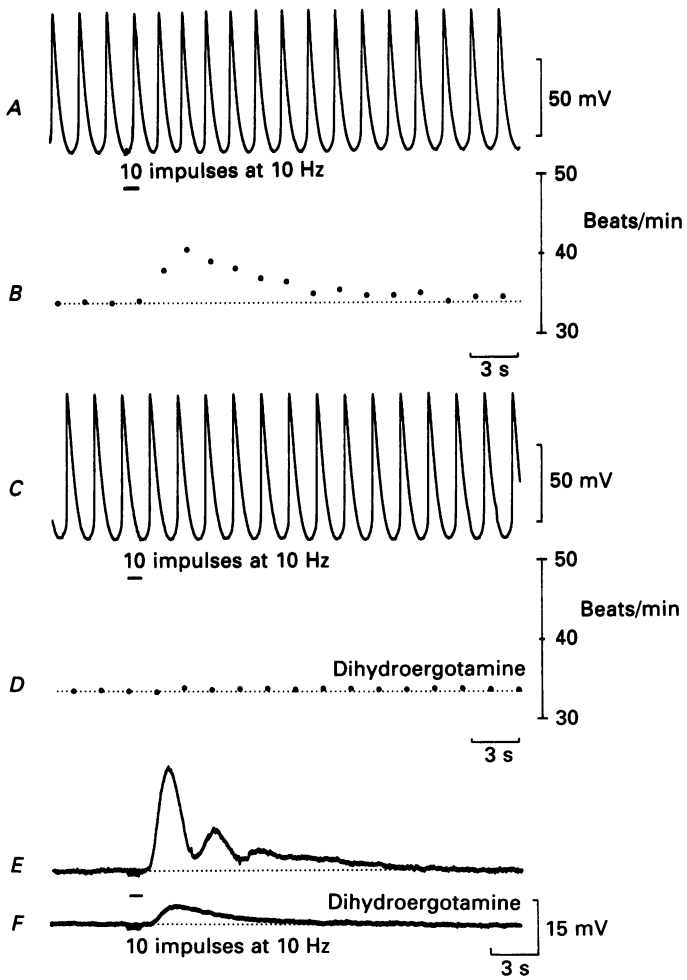

Fig. 13. Effect of dihydroergotamine on the responses to sympathetic nerve stimulation in beating and arrested preparations of sinus venosus. The upper trace (*A*) and associated plot of instantaneous rate of generation of action potentials (*B*) show the response to 10 stimuli delivered at 10 Hz in control solution. The rate of generation of action potentials increased; peak diastolic potential −65 mV in control. The next pair of traces (*C* and *D*) show the response to the same train of stimuli 1 h after adding dihydroergotamine (1×10^{-5} M) to the perfusion fluid. Sympathetic nerve stimulation no longer produces a tachycardia. Note that the amplitude and the rate of generation of action potentials are unchanged; peak diastolic potential −64 mV. The lower traces (*E* and *F*) show the responses to sympathetic nerve stimulation in an arrested preparation (10 impulses at 10 Hz) in control (*E*) and 1 h after adding dihydroergotamine (2×10^{-5} M) to the perfusion fluid (*F*). The amplitude of the sympathetic EJP is reduced. Steady membrane potential in *E* and *F* was −34 mV.

In early studies on sympathetic transmission in amphibian hearts, it was shown that ergotamine blocked sympathetic transmission without preventing the release of sympathetic transmitter (Loewi, 1924; Clark, Chu & Aellig, 1978). Ergotamine was also used to block the effects of sympathetic nerve stimulation on the frog heart

without impairing the effects of vagal stimulation (Hutter & Trautwein, 1956). We therefore examined the effects of dihydroergotamine on the responses to sympathetic nerve stimulation in beating and arrested hearts. In the three preparations examined, dihydroergotamine (1×10^{-5} M) reduced the early tachycardia produced

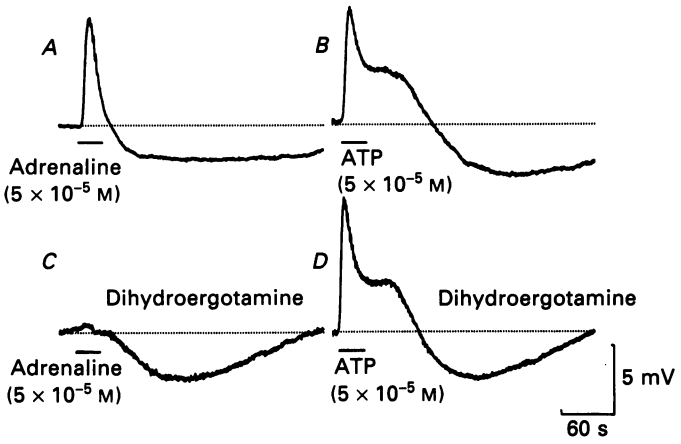

Fig. 14. Effect of dihydroergotamine on the responses to applied adrenaline and ATP in arrested preparations of sinus venosus. The upper left trace (*A*) shows the response to adding adrenaline (5×10^{-5} M; continuous bar) to the perfusion fluid for 30 s. The upper right trace (*B*) shows the response to adding ATP (5×10^{-5} M) to the perfusion fluid again for 30 s. The lower pair of traces show the response to the same concentrations of adrenaline (*C*) and ATP (*D*) 1 h after continuous perfusion with dihydroergotamine (1×10^{-5} M). Note that the transient depolarization produced by adrenaline is abolished but that the response to ATP persists. Steady membrane potential for all records was −34 mV; calibration bars apply to each record.

by ten impulses delivered at 10 Hz without changing the rate of generation or amplitude of pacemaker action potentials (Fig. 13). In preparations arrested with nicardipine, dihydroergotamine ($1–2 \times 10^{-5}$ M) reduced the amplitude of sympathetic EJPs produced by ten impulses delivered at 10 Hz without affecting the resting membrane potential (Fig. 13). The mean amplitude of EJPs in control solution was $14{\cdot}3 \pm 6{\cdot}3$ mV ($n = 5$); the mean amplitude of EJPs in solution containing dihydroergotamine was $3{\cdot}9 \pm 1{\cdot}0$ mV ($n = 5$).

When the responses to added adrenaline or ATP were examined in arrested preparations, it was found that dihydroergotamine selectively abolished the depolarization brought about by added adrenaline, having no effect on the responses to added ATP (Fig. 14). In five preparations the responses to adrenaline (5×10^{-5} M) and ATP (5×10^{-5} M) were determined in control solution and then again in a solution containing dihydroergotamine (1×10^{-5} M). To avoid the problem of desensitization, agonists were added for 30 s at intervals of 30 min: with this regime in control solution, consistently sized responses were obtained for several hours. The mean peak response to adrenaline (5×10^{-5} M) in control solution was $6{\cdot}3 \pm 1{\cdot}1$ mV while in dihydroergotamine-containing solution the mean peak response was $0{\cdot}4 \pm 0{\cdot}2$ mV. In the same five preparations the control response to ATP (5×10^{-5} M)

was 6·3 ± 1·5 mV and the response in dihydroergotamine-containing solution was 6·7 ± 1·7 mV. Taken together these observations suggest that dihydroergotamine distinguishes between the effects of adrenaline on the non-α-, non-β-adrenoceptors and of ATP on P_2-purinoceptors of toad heart. As the response to sympathetic nerve stimulation is also inhibited by dihydroergotamine, presumably these adrenoceptors are activated by neuronally released adrenaline.

DISCUSSION

Effects of sympathetic nerve stimulation on toad pacemaker cells

Sympathetic nerve stimulation caused a long-lasting increase in the rate of generation of action potentials by pacemaker cells of the toad sinus venosus. The response had two components: an early phase and a late phase. During the early phase, the peak diastolic potential was reduced and the rate of diastolic depolarization leading up to the initiation of the subsequent action potential was increased (Toda & Shimamoto, 1968; Shibata *et al.* 1985). The threshold for the initiation of the action potential was unchanged (see also Hutter & Trautwein, 1956). These effects of sympathetic nerve stimulation could be explained if the balance between inward and outward current flow during diastole had been changed. A number of membrane currents are considered to flow during diastole. These include an outward potassium current flowing through delayed rectifier K^+ channels (Brown, 1982), an outward current provided by background potassium channels, an inward current provided by background sodium channels (Noble, 1984), a hyperpolarization-activated inward current, i_f (DiFrancesco, 1985), and a transient inward calcium current, Ca_T (Hagiwara, Irisawa & Kameyama, 1988). Clearly the appropriate change in any one of these currents would explain our observations on beating preparations.

Analysis of the currents involved in the early sympathetic tachycardia is assisted by our observations on preparations which had been arrested by adding an organic calcium antagonist. In these preparations, sympathetic nerve stimulation initiated EJPs. The time course of the summed EJPs was of a similar pattern to that of the early tachycardia recorded from beating pacemaker cells. Hence we suggest that the changes in membrane conductance which give rise to the depolarization recorded from arrested preparations, also give rise to the early accelerations seen in beating preparations.

In the arrested preparations, the membrane potential was stable with values of about −35 mV. Since beating was prevented by nicardipine, the channels (Ca_L channels; Hagiwara *et al.* 1988) responsible for the generation of the long-lasting calcium current would have been blocked. Thus it is unlikely that an effect of sympathetic transmitter on Ca_L channels would be responsible for the initiation of EJPs. At these depolarized potentials, Ca_T channels should be inactivated. If sympathetic nerve stimulation were able to move the reactivation potential of Ca_T channels to much more positive potentials, they could provide an inward current to generate an EJP. We consider this to be unlikely, since their normal reactivation potential is about −70 mV (Hagiwara *et al.* 1988) and a very large change in this potential would seem to be required. It also seems unlikely that EJPs result from the

closure of inward rectifier K^+ channels. These channels do not contribute to the arrested steady potential: the steady potential is unaffected by barium ions (Bywater *et al.* 1990). Furthermore these channels appear to be absent from pacemaker cells (Moore, Clark, Shibata & Giles, 1986). As the EJPs were unaffected by caesium ions it is unlikely that the EJPs reflect a change in the activation potential for i_f (see DiFrancesco, 1985). Hence it appears that voltage-dependent membrane channels are not involved in the generation of EJPs. The remaining pacemaker current is thought to be provided by a background sodium conductance (Noble, 1984), or more correctly by the relationship between the background sodium and potassium conductances. Thus it is possible that the sympathetic transmitter interacts with a receptor to change the balance between the background sodium and potassium conductances. Hence we suggest that neuronally released sympathetic transmitter interacts with a receptor to increase the background sodium conductance (see also Egan, Noble, Noble, Powell, Twist & Yamaoka, 1988). Perhaps it acts on the same channels that have been suggested to close during vagal inhibition (Bywater *et al.* 1990). Alternatively the sympathetic transmitter might activate receptors that are linked to channels which are not involved in on-going pacemaking activity. If such channels increased inward current flow, there would be an EJP in arrested preparations and tachycardia in beating preparations. We are unable to distinguish between the latter two possibilities, nor are we able to comment on whether a similar phenomenon occurs in other species. In the rabbit, sympathetic nerve stimulation also increases the rate of diastolic depolarization and has little effect on the amplitude of the pacemaker action potentials (Toda & Shimamoto, 1968; Shibata *et al.* 1985). Whether or not sympathetic nerve stimulation would initiate a depolarization in arrested mammalian pacemaker cells is not known.

The late phase of sympathetic tachycardia involves different mechanisms than those involved in the first phase. The action potentials had increased overshoots (see also Hutter & Trautwein, 1957). The peak diastolic potential was more negative and the rate of repolarization of the action potentials was more rapid while the rate of diastolic depolarization was again increased. These changes in rate lasted for several minutes even after a brief train of sympathetic stimuli. When recordings were made from arrested preparations, the membrane potential did not change during the time period corresponding to the late phase. We suggest that sympathetic nerve stimulation, as well as causing a transient increase in pacemaker current during the early phase, triggers a long-lasting change in the behaviour of the voltage-dependent channels involved in the generation of action potentials (for related discussion see Brown, 1982). This suggestion requires further study.

Responses of sinus venosus cells to applied adrenaline and ATP

Applied adrenaline increased both the rate of generation and the amplitude of pacemaker action potentials. These responses were largely prevented by a β_2-adrenoceptor antagonist, indicating that in amphibian hearts applied adrenaline most readily activates β-adrenoceptors which resemble mammalian β_2-adrenoceptors (Stene-Larsen & Helle, 1978; O'Donnell & Wanstall, 1982). Applied adrenaline produced depolarizations in arrested preparations which, like those produced by sympathetic nerve stimulation, were resistant to β-adrenoceptor blockade. Unlike

the responses which followed the activation of β-adrenoceptors, those recorded from arrested preparations desensitized during even brief applications of transmitter. Thus toad pacemaker cells have a population of β-adrenoceptors and another population of excitatory adrenoceptors with different pharmacological properties.

Applied ATP also activated two distinct subsets of receptors. One set produced a persistent increase in the amplitudes and rates of generation of action potentials. Evidently toad sinus venosus cells have a population of excitatory receptors for ATP which do not readily desensitize (see also Burnstock & Meghji, 1981; Hoyle & Burnstock, 1986). When applied to arrested pacemaker cells, ATP produced a depolarization similar to that accompanying sympathetic nerve stimulation. These responses readily desensitized during brief exposure to ATP and were blocked after desensitization with m-ATP suggesting that the receptors are of the P_2-type.

Identity of the transmitter released by sympathetic nerves to the toad sinus venosus

The predominant effects of sympathetic stimulation on both beating and arrested preparations were not mediated by the activation of β-adrenoceptors (see also Morris *et al.* 1981; Hoyle & Burnstock, 1986). In contrast, the predominant effects of applied adrenaline on beating hearts involve β_2-like adrenoceptors. At first sight these results suggest that adrenaline is not the sympathetic transmitter substance. However, it is clear that adrenaline does transmit some of the responses to sympathetic stimulation: treatment with a β_1-selective adrenoceptor antagonist prevented the small increase in action potential amplitude that occurred some 10–30 s after the onset of stimulation. At the time the latency between the initiation of an action potential in the sinus venosus and its arrival in the atrium is reduced; this increase in conduction 'velocity' also involves the activation of β-adrenoceptors (Bramich & Campbell, 1989). Since adrenaline is released, one must propose some form of specialization at the sympathetic neuroeffector junction, such that the released adrenaline has little or no effect on what are presumably extrajunctional β_2-adrenoceptors on pacemaker cells. A similar specialization has been proposed at the vagal neuroeffector junction, to explain cholinergic transmission to both amphibian and mammalian pacemaker cells: added acetylcholine increases membrane potassium conductance whereas neuronally released acetylcholine appears to reduce inward current during diastole (Bywater *et al.* 1989, 1990; Campbell, Edwards, Hirst & O'Shea, 1989).

The question remains whether adrenaline is the sole sympathetic transmitter to the toad heart or whether some other substance is involved in a co-transmission. Hoyle & Burnstock (1986) proposed that the non-β effects of sympathetic stimulation to the frog heart were mediated by ATP. Indeed, we found that applied ATP mimicked the responses of toad pacemaker cells to sympathetic stimulation and that both responses were suppressed when m-ATP was present. However, non-β effects of applied adrenaline, mimicking the early responses to sympathetic stimulation, could be seen after β-adrenoceptor blockade in beating preparations or when the consequences of β-adrenoceptor activation had been 'hidden' by preventing beating. These non-α-, non-β-adrenoceptor responses were also suppressed by m-ATP. So the results obtained with m-ATP do not preclude the possibility that adrenaline is the sole transmitter substance. Furthermore, the non-β effects of both sympathetic stimulation and applied adrenaline were antagonized by dihydroergotamine without

a reduction in the effects of ATP. In short, all of our observations would be explained if neuronally released adrenaline activated a population of specialized adrenoceptors which are resistant to α- and β-adrenoceptor blockade but which are blocked by dihydroergotamine. This does not imply that dihydroergotamine is a selective antagonist for these amphibian cardiac receptors, as its affinity for other, primarily α-, adrenoceptors is well documented (see Müller-Schweinitzer & Weidmann, 1978).

The finding that dihydroergotamine blocks the sympathetic tachycardia without affecting the responses to ATP might imply that ATP is not involved in transmission. This might well be the case. However, added ATP did mimic the responses to sympathetic nerve stimulation. Furthermore the sympathetic tachycardia was not apparent when m-ATP was present (Fig. 12). Since m-ATP appears not to effect the release of transmitter from sympathetic nerves (Miyahara & Suzuki, 1987), this suggests that a purine receptor is activated during sympathetic transmission. Perhaps at this and at other sympathetic neuroeffector junctions, a process of purinergic and adrenergic co-transmission involving the same receptor type occurs.

Whether there are any similarities between the processes of transmission at amphibian and mammalian sympathetic junctions on the heart is not known. It is clear that the mammalian sympathetic transmitter interacts with a β-adrenoceptor, for the responses to sympathetic nerve stimulation are abolished by pindolol (Shibata *et al.* 1985). However, the receptors activated by neuronally released noradrenaline may not be of the same type as those most readily activated by applied noradrenaline. In rabbit sino-atrial node cells, the predominant receptor is the β_1-type. When these are activated pacemaker action potentials are increased in amplitude (Dukes & Vaughan Williams, 1984). Changes in membrane potential that resemble those seen during sympathetic nerve stimulation (Toda & Shimamoto, 1968) are, however, recorded when selective β_2-agonists are applied (Dukes & Vaughan Williams, 1984). Such receptors have been found to be concentrated in regions which have a high density of sympathetic innervation (Jones, Molenaar & Summers, 1989).

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