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NEUROHORMONAL MODULATION OF THE *LIMULUS* HEART: AMINE ACTIONS ON NEUROMUSCULAR TRANSMISSION AND CARDIAC MUSCLE

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SUMMARY

The responses of *Limulus* cardiac neuromuscular junctions and cardiac muscle cells to four endogenous amines were determined in order to identify the cellular targets underlying amine modulation of heartbeat amplitude. The amines increased the amplitude of the *Limulus* heartbeat, with dopamine (DA) being more potent than octopamine, epinephrine or norepinephrine. The effect of DA on heartbeat amplitude was not blocked by phentolamine. DA enhanced the contractility of deganglionated heart muscle, with time course and dose-dependence similar to its effect on the intact heart. The amines also enhanced neuromuscular transmission, with time course and dose-dependence similar to their effects upon the intact heart. The amplitude of unitary excitatory junction potentials (EJPs) and frequency of miniature excitatory junction potentials (mEJPs) were increased by DA, while mEJP amplitude was unchanged. Thus DA, and probably the other amines, had a presynaptic effect. Combined actions upon cardiac muscle and cardiac neuromuscular transmission account for the ability of these amines to increase the amplitude of the *Limulus* heartbeat.

INTRODUCTION

Biogenic amines increase the rate and strength of contractions of many neurogenic hearts (Grega & Sherman, 1975; Florey & Rathmayer, 1978; Cooke & Sullivan, 1982; Augustine, Fetterer & Watson, 1982). While the chronotropic effects are thought to be a result of actions on cardiac ganglion neurones (Miller,

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Benson & Berlind, 1984; Augustine & Fetterer, 1985), little is known about how these compounds increase the strength of neurogenic heart contractions.

Amines could increase neurogenic heart contractions in several ways. They might act directly upon cardiac muscle, modulating ionic conductances (Siegelbaum & Tsien, 1983) or affecting subsequent steps in excitation-contraction coupling (Tsien, 1977; Katz, 1979; Kranias & Solaro, 1982; Winegrad *et al.* 1983). Alternatively, amines could increase cardiac neuromuscular transmission. Amines have been shown to enhance transmission at many neuromuscular junctions, *via* actions on transmitter release (Grundfest & Reuben, 1961; Dudel, 1965; Kuba, 1970; Florey & Rathmayer, 1978; O'Shea & Evans, 1979; Weiss, Cohen & Kupfermann, 1978; Bergman *et al.* 1981; Fischer & Florey, 1983) or postjunctional muscle properties (Lingle, 1981). Actions upon these possible targets have not yet been investigated in any neurogenic heart.

The preceding paper demonstrates that octopamine, dopamine, epinephrine and norepinephrine alter heart rate in *Limulus* by acting upon cardiac ganglion neurones (Augustine & Fetterer, 1985). This paper demonstrates further effects upon neuromuscular transmission and cardiac muscle contractility. These agents thus modulate the *Limulus* heart *via* simultaneous actions upon all elements within the cardiac system. This analysis of the cellular actions underlying inotropic modulation of a neurogenic heart also represents one of the few cases where a modulatory role of amines has been described in terms of actions upon multiple cellular elements.

Preliminary reports of this work have appeared previously in an abstract (Watson & Hoshi, 1981) and a review (Watson & Augustine, 1982).

METHODS

Specimens

Male and female *Limulus polyphemus*, with carapace widths of 10–20 cm, were obtained from the supply department of the Marine Biological Laboratory, Woods Hole, MA, Ocean Resources, Elliot, ME, or collected locally near Jackson Estuarine Laboratory, Durham, NH. Animals were held in running or recirculating natural sea water at 15–20 °C.

Hearts were removed according to the procedure described by Augustine *et al.* (1982). When appropriate, the cardiac ganglion and its large side branches were removed to yield deganglionated hearts.

Direct stimulation of heart muscle

In order to stimulate electrically deganglionated heart muscle, the following method was adopted. The long, tubular heart was cut at the third and fifth ostia, yielding a ring of heart muscle. The ring was then cut open and a 1-cm length of insect pin was sutured to one of the lateral edges of the heart muscle and attached to a Grass FT.03 force transducer *via* a piece of thread. Most of the dorsal cardiac muscle was removed, and the other lateral edge was pinned to the bottom of a

Sylgard-filled stimulation chamber. This held the muscle between, parallel to, and within 2 mm of two stainless steel plates which served as stimulation electrodes. This arrangement proved superior to previous methods (Augustine *et al.* 1982) because it measured lateral contractions, which were also measured when recording from the intact heart. In addition, lower voltage stimuli could be used to elicit contractions. The muscle was continuously perfused with saline held at 15 °C in a water-cooled reservoir.

Glutamate-induced contractions

To examine the glutamate response of cardiac muscle, deganglionated hearts were perfused continuously with 15 °C saline, and L-glutamate (10^{-4}–10^{-3} mol l^{-1}) was injected into the chamber in 1–2 ml quantities. Glutamate was added at 5- to 15-min intervals to avoid cumulative desensitization.

Intracellular recordings

Intracellular recordings were obtained from the follower neurones and cardiac muscle of intact hearts using 5–15 MΩ electrodes filled with 3 mol l^{-1} KCl. To permit simultaneous recordings of neurone activity and muscle contraction a small section of cardiac ganglion from the posterior portion of the heart was dissected free from the underlying muscle, without disruption of ganglionic activity. This portion of ganglion was pinned to the recording chamber. A force transducer was attached, *via* thread, to the lateral margin of the remaining heart muscle. Intracellular recordings were made from muscle fibres located near the area from which tension was being monitored. A small piece of nylon mesh was pinned over a section of the heart to stabilize the muscle and permit prolonged recordings (Watson, Augustine, Benson & Sullivan, 1983). Intracellular muscle recordings were obtained from both the interior and exterior of the heart.

To record unitary excitatory junction potentials (EJPs), deganglionated hearts were pinned out and residual motor nerves were stimulated with a suction electrode. EJPs were recorded with or without nylon mesh. In some experiments the EJPs were digitized (1–5 kHz) and averaged with a Nicolet digital oscilloscope. Apparent input resistance of muscle fibres was monitored by passing constant hyperpolarizing current pulses through the recording electrode.

Miniature excitatory junction potentials (mEJPs) were recorded from cardiac muscle fibres with a WPI VF-1 electrometer. Peak-to-peak noise levels were less than 20 μV, which was adequate to detect the small mEJPs found in *Limulus* cardiac muscle (Fig. 8). Preparations were often treated with 2×10^{-7} mol l^{-1} tetrodotoxin (TTX; Sigma) to eliminate spontaneous EJPs and muscle contractions. Miniature EJPs were stored on film using a Grass C4 oscilloscope camera. Data were collected for 1 min out of every 2 min.

Miniature EJPs were analysed on an Apple II plus microcomputer with a programme developed in collaboration with John Langley. This programme can be obtained from W. Watson. Filmed data were entered into the computer *via* a

graphics tablet. Data analysed entirely by hand yielded similar results, but the analysis was much more tedious and limited.

RESULTS

Whole heart amine response

Application of dopamine (DA) to an intact *Limulus* heart caused an increase in the rate and amplitude of heart contractions (Fig. 1A), as previously reported (Augustine *et al.* 1982). The inotropic action of DA was dose-dependent, with a threshold of 10^{-8} mol l^{-1} and an ED_{50} of approximately 10^{-6} mol l^{-1} (Fig. 1B). Octopamine, norepinephrine and epinephrine also increased contraction amplitude, but their effects were less pronounced than those of DA. For this reason, most of the following experiments were per-

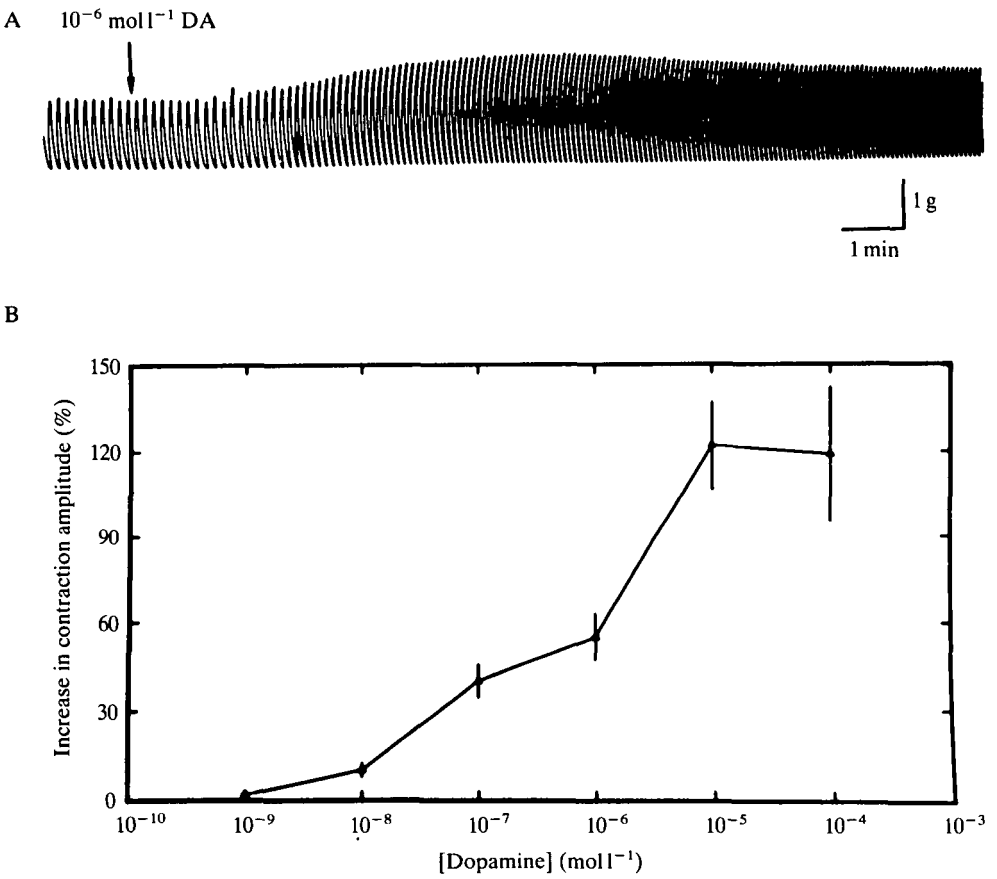

Fig. 1. Inotropic effect of dopamine (DA) on the isolated *Limulus* heart. (A) Tension recording during application of dopamine (10^{-6} mol l^{-1}). DA was continuously perfused, beginning at the arrow. Both heart rate and amplitude increase in response to DA, although the amplitude effect is short-lived. (B) Dose-response curve for the inotropic effect of DA. Each point is the average (±S.E.M.) of at least five responses obtained from different hearts.

formed with DA, although in each type of experiment comparable results were obtained with the other amines.

Phentolamine is a competitive antagonist of amine-induced increases in heart rate in *Limulus* (Augustine *et al.* 1982). However, phentolamine (10^{-5} mol l^{-1}) did not block the inotropic actions of DA (3×10^{-7} mol l^{-1}). In fact, DA responses were 31 % (S.E.M. = 12 %, $N = 8$) greater when phentolamine was present. For comparison, in these same experiments phentolamine reduced the effect of DA on heart rate by 43 % (S.E.M. = 20 %, $N = 8$).

Dopamine actions on cardiac muscle

To examine whether DA caused an increase in heart contraction amplitude by acting directly upon cardiac muscle or by modulating cardiac neuromuscular transmission, several experiments were performed.

DA enhanced electrically-evoked contractions of deganglionated cardiac muscle (Fig. 2A). This effect was dose-dependent, with an ED_{50} of approximately 5×10^{-7} mol l^{-1} (Fig. 2B). There was considerable variability in the response of individual hearts, so that some hearts did not respond to 10^{-7} mol l^{-1} DA. On average, the DA dose-response curve for deganglionated cardiac muscle was similar to that found for DA potentiation of contractions of intact *Limulus* heart (Fig. 1B). When deganglionated hearts were perfused with TTX to block activity of residual motor nerves (Watson *et al.* 1983), there was a slight decrease in contraction strength. However, in the presence of TTX, DA treatment increased contraction strength to the same extent as observed in normal saline. These data suggest that DA is exerting some of its inotropic effects *via* direct actions upon cardiac muscle fibres.

The increase in contraction amplitude of intact hearts observed during DA perfusion had a slow onset, reaching a peak over 5–10 min (Fig. 1A), and often lasted for up to 20 min. However, while the increase in rate persisted as long as the heart was perfused with DA, the inotropic effect of DA declined with maintained exposure to the amine. This secondary decline in heartbeat strength was more prominent at DA concentrations of 10^{-5} mol l^{-1} or higher (Fig. 3A). This effect is apparently a consequence of increased heart rate, rather than desensitization, for it was not observed when deganglionated hearts were directly stimulated at a constant, roughly physiological rate and exposed to DA (Fig. 3B).

If DA acts directly upon cardiac muscle fibres, then it should enhance the contractile response of the muscle to glutamate, the putative neuromuscular transmitter (Abbott, Lang & Parnas, 1969). In 15 experiments performed, DA reversibly enhanced glutamate-induced contractions in deganglionated hearts (Fig. 4A). Occasionally, in the presence of DA, glutamate also produced rhythmic contractions (Fig. 4B). This response was usually obtained with freshly-dissected preparations and, when observed, gradually changed with time to become a single contracture.

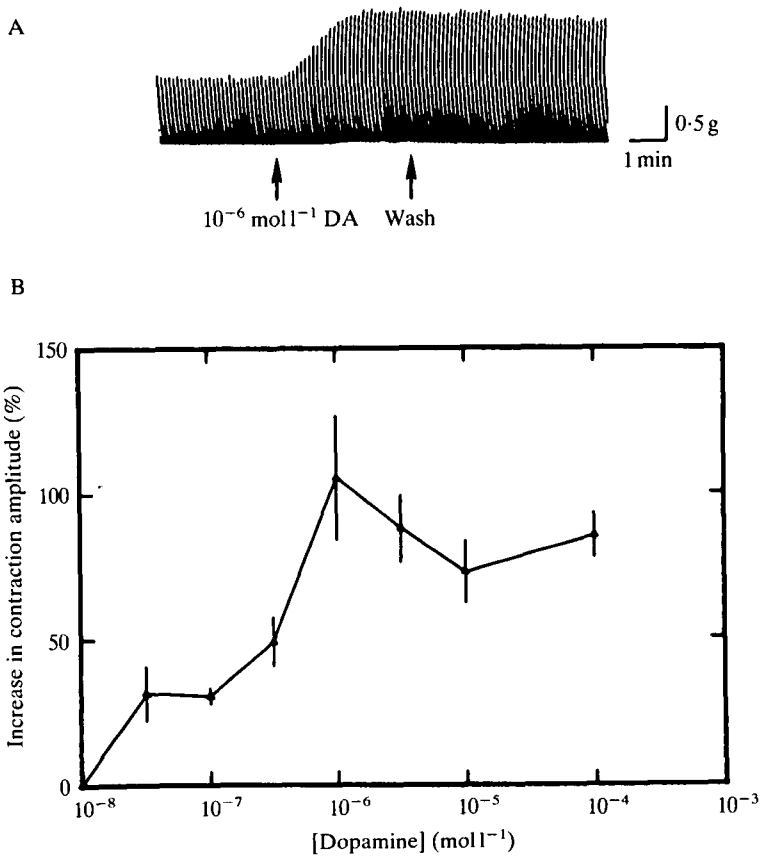

Fig. 2. Effect of dopamine (DA) on electrically-evoked contractions of deganglionated heart muscle. (A) Tension recording from deganglionated heart muscle stimulated at 0·1 Hz. Addition of DA (10^{-6} mol l^{-1}) at the arrow caused an increase in contraction amplitude which slowly returned to control levels during a wash with normal saline. (B) Dose-response curve for the action of DA on evoked contractions of deganglionated heart muscle. Each point represents the mean of at least five responses (±S.E.M.).

Dopamine actions on neuromuscular transmission

To investigate possible actions of DA on cardiac neuromuscular transmission, we recorded from follower neurones and muscle fibres while simultaneously monitoring muscle tension with a force transducer. DA increased the size of individual EJPs within the compound EJP, while decreasing the duration of follower cell bursts and the number of spikes per burst (e.g. Fig. 5). There was no change in the resting potential or apparent input resistance of muscle fibres in 11 experiments of this type. Similar effects were observed with octopamine (10^{-5} mol l^{-1}) and norepinephrine (10^{-5} mol l^{-1}). These data indicate that the amines enhance cardiac neuromuscular transmission in *Limulus*.

To quantify effects of the amines on isolated neuromuscular junctions, we removed the cardiac ganglion and stimulated follower cell axons directly with a suction electrode. Because *Limulus* muscle fibres are multiply innervated (Abbott

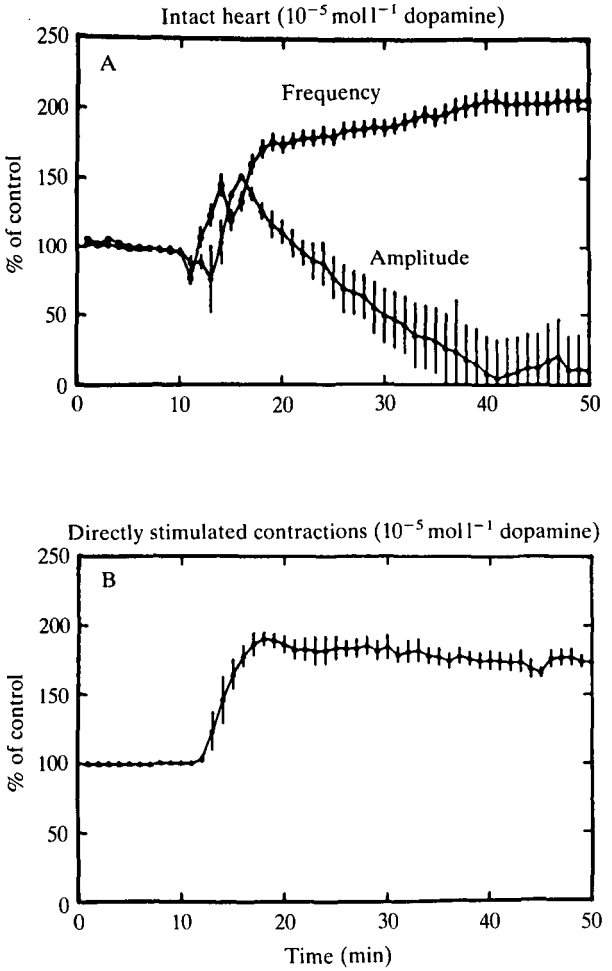

Fig. 3. Time course of dopamine (DA) effects on the rate and strength of *Limulus* heart contractions. All preparations (top, $N=10$; bottom, $N=6$) were treated with DA for 30 min beginning at T = 10 min. (A) 10^{-5} mol l^{-1} DA increased both rate and amplitude of beat of intact hearts. During continual exposure to DA, heartbeat amplitude declined while rate remained elevated. (B) Directly stimulated contractions recorded from deganglionated hearts were also increased by 10^{-5} mol l^{-1} DA, but did not decline during maintained exposure to DA. Stimulation frequency = 0·1 Hz.

et al. 1969; Parnas, Abbott & Lang, 1969), we took special precautions to use only preparations where unitary EJPs could be reliably elicited. DA, as well as epinephrine, octopamine and norepinephrine, increased the amplitude of these unitary EJPs (Fig. 6). DA had no effect at 10^{-7} mol l^{-1} ($N=3$), caused a 64 % (S.E.M. = 17 %, $N=7$) increase in EJP amplitude at 10^{-6} mol l^{-1} and a 109 % increase (S.E.M. = 22 %, $N=5$) at a concentration of 10^{-5} mol l^{-1}. As in the experiments described above, muscle fibre input resistance and resting potential were not altered by DA (Fig. 6).

The effects of DA and the other amines on EJP amplitude had a slow onset and

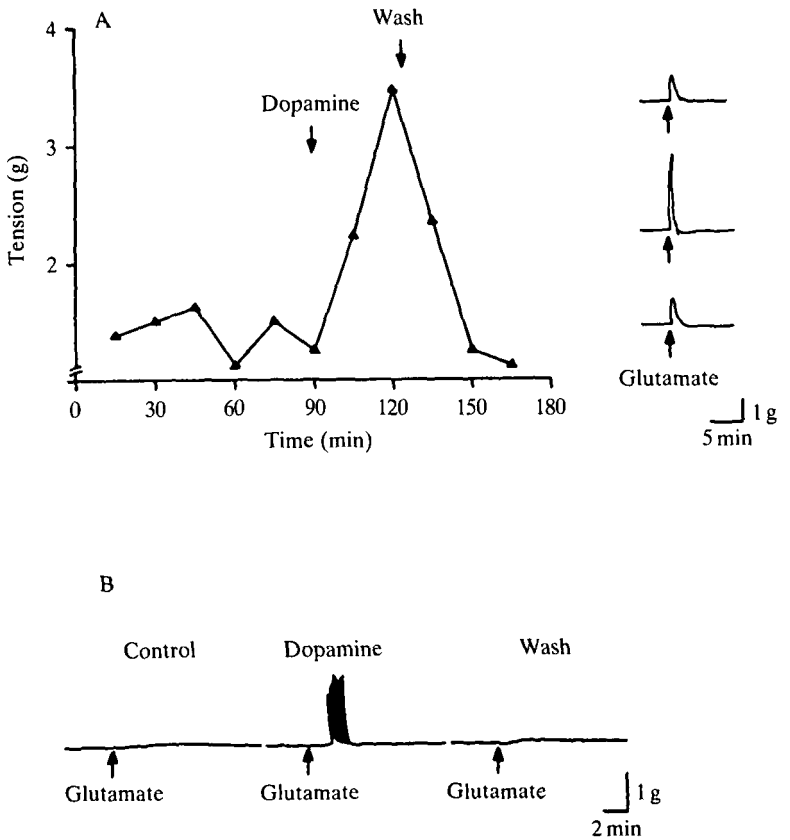

Fig. 4. Dopamine (DA) modulation of glutamate-induced contractions of deganglionated cardiac muscle. (A) Treatment with glutamate (2 ml, 2×10^{-3} mol l^{-1}) resulted in prolonged contractions, which are shown in the insets. Dopamine (10^{-6} mol l^{-1}) caused a reversible increase in the size of glutamate-induced contractions. Insets represent contractions before (top), during (middle) and after (bottom) DA. (B) In some preparations, DA caused the appearance of rhythmic contractions in response to glutamate (2 ml, 10^{-4} mol l^{-1}). This effect was also reversible.

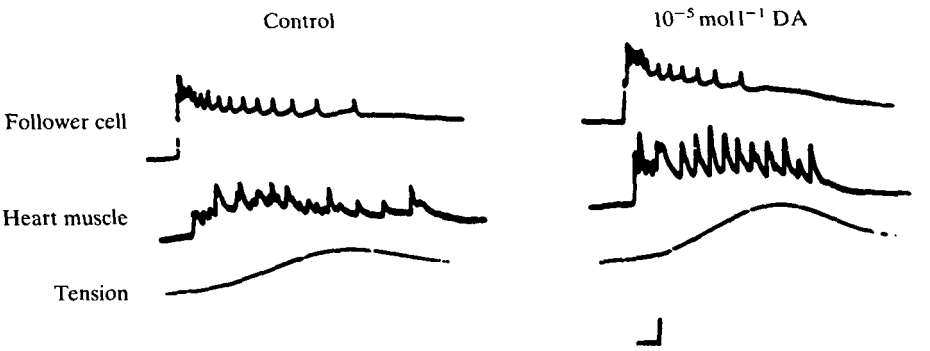

Fig. 5. Dopamine (DA) enhanced compound EJPs recorded from cardiac muscle. Transmembrane potentials were recorded from follower neurones and cardiac muscle cells while simultaneously monitoring contractions with a force transducer. Dopamine (10^{-5} mol l^{-1}) increased follower cell burst rate, decreased follower cell burst duration and the number of spikes per burst, increased the amplitude of heart contractions and increased the amplitude of compound EJPs. Calibration: 10 mV, 5 mV, 1 g; 0·1 s.

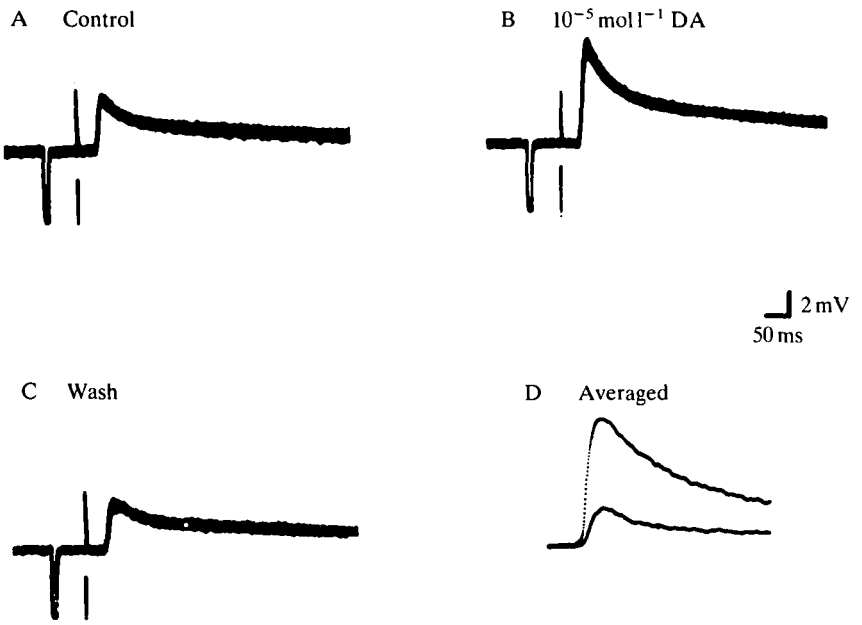

Fig. 6. Dopamine (DA) increased the amplitude of unitary EJPs elicited by 1-Hz stimulation of cardiac ganglion motor nerves. Hyperpolarizing current pulses (0·2 nA) were applied through the microelectrode prior to each stimulus to monitor apparent input resistance. The resting potential of the muscle fibre was −43 mV throughout the experiment. Addition of 10^{-5} mol l^{-1} DA increased the amplitude of EJPs without affecting the apparent input resistance. In D, 32 EJPs in normal saline were averaged and compared to the average of 32 EJPs recorded in the presence of DA.

a long duration (Fig. 7). Although EJPs started to increase in size within a few minutes of DA perfusion, maximum effects were usually not obtained until 10–15 min after its addition. Complete recovery usually required at least 30 min of continuous perfusion with amine-free saline.

To determine whether DA increased EJP amplitude *via* pre- or postsynaptic actions, we analysed spontaneous mEJPs in three different deganglionated heart preparations. As shown in Fig. 8, 10^{-5} mol l^{-1} DA produced a consistent 20·6 % (S.E.M. = 0·4 %) increase in average mEJP frequency, but had no effect on average mEJP amplitude (0 ± 5·6 % S.E.M.). This indicates that DA increases EJP amplitude presynaptically, by enhancing transmitter release.

DISCUSSION

Our experiments describe two ways by which biogenic amines increase the amplitude of *Limulus* heart contractions. First, they interact with presynaptic terminals of cardiac neuromusuclar junctions to increase spontaneous and evoked transmitter release. Second, they act directly upon cardiac muscle cells to enhance contractility.

Presynaptic actions of amines, similar to those described here, have been

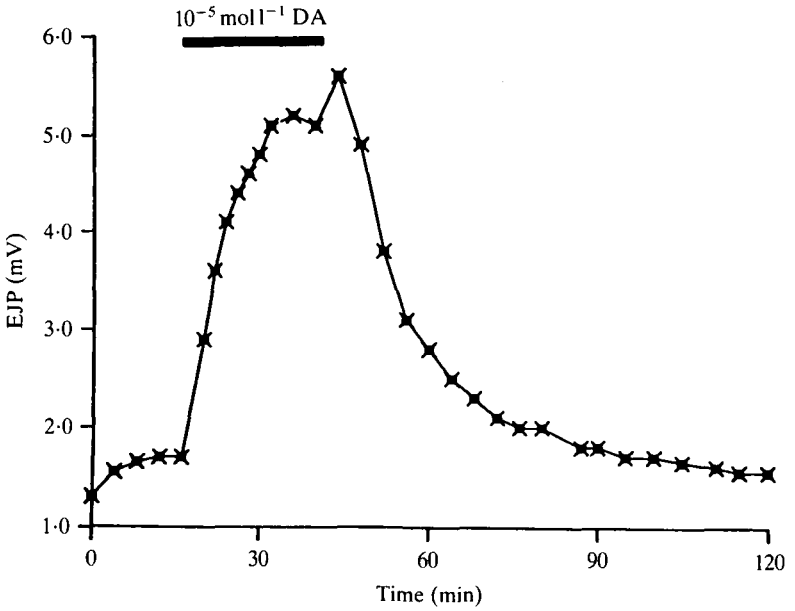

Fig. 7. Time course of dopamine (DA) effects on EJP amplitude. EJPs were recorded from cardiac muscle as in Fig. 6. Addition of 10^{-5} mol l^{-1} DA caused a gradual increase in EJP amplitude which slowly returned to control levels during continuous washing with DA-free sea water.

observed in neuromuscular junctions of other arthropods (Grundfest & Reuben, 1961; Dudel, 1965; Florey & Rathmayer, 1978; O'Shea & Evans, 1979; Glusman & Kravitz, 1982; Fischer & Florey, 1983), molluscs (Weiss *et al.* 1978) and vertebrates (Kuba, 1970; Bergman *et al.* 1981). Presynaptic amine actions are apparently a common strategy for modulating neuromuscular efficacy. In at least one system amines modulate transmission by increasing postsynaptic input resistance (Lingle, 1981), but the lack of effect of DA on mEJP amplitude or muscle fibre electrical properties precludes such an action at the *Limulus* neuromuscular junction.

Although DA increased both spontaneous and evoked release at the cardiac neuromuscular junction, it appeared to have a greater effect on evoked release. DA (10^{-5} mol l^{-1}) increased EJP amplitude by 109 % (see Results), but only increased mEJP frequency by 20 %. This quantitative difference may provide insight into the mechanism of the presynaptic action of DA. One possible explanation is that DA modulates a voltage-gated ion channel; the relatively large effect of DA on evoked release would then reflect the voltage dependence of the channel involved. Alternatively, the presynaptic action of DA could involve an increase in the concentration of Ca^{2+} ions within the nerve terminal. Evoked release might be more sensitive to such a change in Ca^{2+} concentration if transmitter release was a non-linear function of internal Ca (Alnaes & Rahamimoff, 1975; Smith, Augustine & Charlton, 1985).

DA also directly enhanced the contractility of *Limulus* cardiac muscle cells.

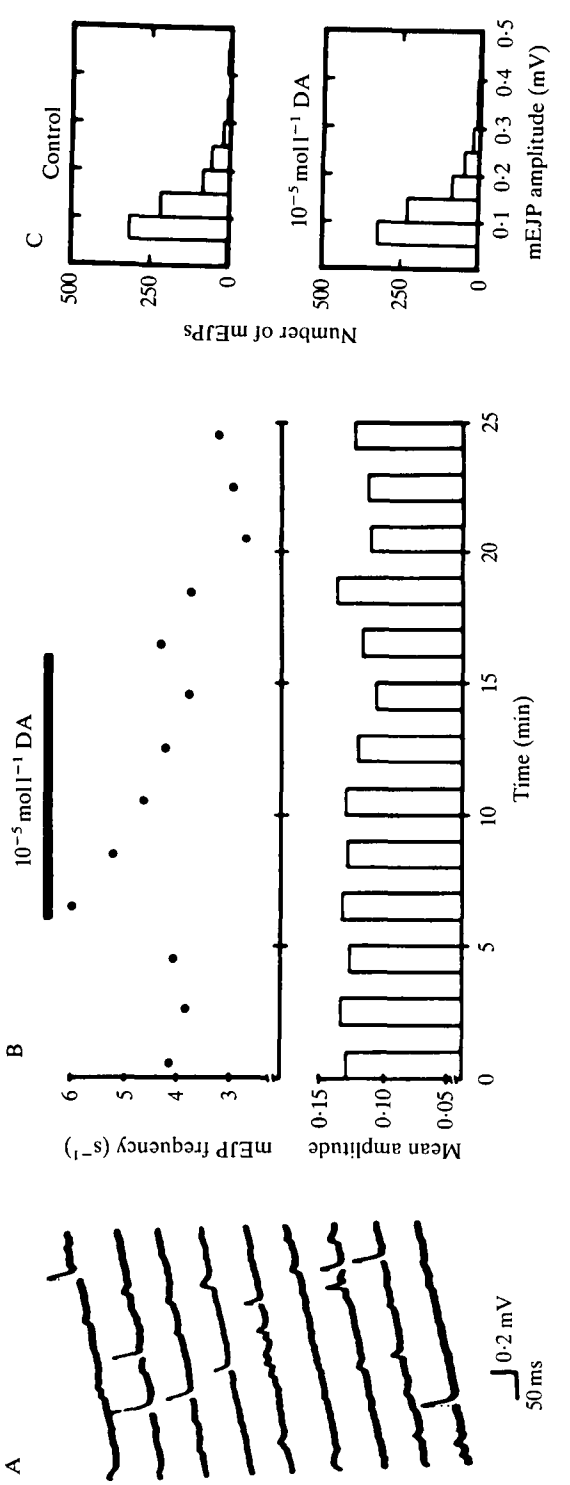

Fig. 8. The effect of dopamine (DA) on cardiac muscle mEJPs. (A)Examples of mEJPs recorded from deganglionated heart muscle in normal saline (10 successive sweeps). (B) Miniature EJP frequency and amplitude during exposure to 10^{-5} mol l^{-1} DA. Miniature EJP frequency increased after addition of DA, while mEJP amplitude was unchanged. Points and bars represent mean values from 1-min bins, which were obtained every 2 min. Standard error bars are not shown because they are very small (less than 10 % of mean for all values). (C) Miniature EJP amplitude distributions in the absence and presence of 10^{-5} mol l^{-1} DA. Although there were more mEJPs after DA addition, only 716 mEJPs were included in each histogram.

This effect occurred in the absence of changes in the membrane potential or input resistance of muscle cells, and thus may be a result of changes in excitation-contraction coupling. This action of DA on *Limulus* cardiac muscle contractility appears similar to the effect of the pentapeptide proctolin (Watson *et al.* 1983). In several experiments DA caused repetitive heart muscle contractions in response to glutamate application (Fig. 4B). These contractions also resemble the myogenic effects of proctolin (Hoshi & Watson, 1981). It is possible that proctolin and DA are exerting some of their effects *via* similar mechanisms. Previous work on the *Limulus* heart failed to demonstrate an action of DA on cardiac muscle contractility (Augustine *et al.* 1982); this may have been due to the less reliable techniques used previously to elicit and record contractions from deganglionated myocardia.

The ability of high DA concentrations to increase contraction strength is short-lived in the intact heart, yet is sustained when the cardiac ganglion is removed (Fig. 3). This difference is presumably a consequence of the increased rate of ganglionic activity produced by DA. One explanation for this is that DA decreases follower cell burst duration (Fig. 5 and Augustine & Fetterer, 1985), which decreases the number of spikes per burst. This would decrease the net depolarization produced in cardiac muscle cells during a heartbeat, which should decrease contraction amplitude. However, such a decrease is not evident at low DA concentrations because of enhanced neuromuscular transmission and muscle contractility. At high concentrations, DA and octopamine produce further increases in heart rate, so that the secondary decrease in contraction amplitude becomes prominent. This secondary decline in heartbeat amplitude probably does not occur *in vivo*, because it is only observed at heart rates which exceed those recorded from an active *Limulus* (Watson, 1979).

Although effects upon both neuromuscular transmission and cardiac muscle contractility are likely to be responsible for the ability of DA to increase *Limulus* heartbeat amplitude, the relative contribution of these two effects is not clear. Both effects are slow, with time courses comparable to the DA response of the intact *Limulus* heart. The dose-response relationships for both these effects are similar to that of the intact heart (Fig. 1), although neuromuscular effects are somewhat less prominent at 10^{-7} mol l^{-1}. At all concentrations, DA increases the amplitude of deganglionated heart contractions by approximately the same amount as in intact hearts. This suggests that neuromuscular effects are quantitatively less important, at least at lower DA concentrations.

In other systems it has been proposed that amines affect both neuromuscular transmission and excitation-contraction (E-C) coupling (Florey & Rathmayer, 1978; Kupfermann *et al.* 1979; Fischer & Florey, 1983), although only one study used stimulation of single muscle fibres to provide direct evidence for effects on E-C coupling (Florey & Fischer, 1983). In the present study, extracellular stimulation of large bundles of muscle fibres was used to examine E-C coupling. This technique is not as direct as intracellular stimulation of single fibres because extracellular current could also release transmitter from motor nerve terminals.

However, since DA also enhanced glutamate-induced contractions (Fig. 4A) and had no effect on postsynaptic receptors activated by the neuromuscular transmitter (Fig. 8C), it is likely that DA does have a direct action upon muscle contractility in *Limulus*.

The action of DA on heartbeat amplitude is not blocked by phentolamine, an antagonist which reduces the effect of DA on heart rate (Augustine *et al.* 1982; Augustine & Fetterer, 1985). This may reflect the involvement of two different amine 'receptors' in the two responses. The amine-induced increase in heart rate behaves more like an octopamine receptor-mediated response (Evans, 1981) because it is activated more effectively by octopamine than DA and it is blocked by phentolamine. The amine-induced increase in contraction amplitude is largest for DA and is not blocked by phentolamine; it might be mediated by a DA receptor (Kebabian & Calne, 1979). Further pharmacological characterization will be necessary to ascribe these various responses to specific receptors. The results of this paper, when combined with those of the previous paper (Augustine & Fetterer, 1985), demonstrate that amines interact with multiple cellular targets in enhancing the *Limulus* heartbeat. Amine modulation of heartbeat rate and duration are primarily due to effects upon cardiac ganglion pacemaker and follower neurones, while enhancement of heartbeat amplitude is due to effects upon neuromuscular transmission and cardiac muscle. Such simultaneous, multiple actions are likely to be produced by neurohormones in other neural and neuromuscular systems.

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