

## THE ROLE OF CYCLIC AMP IN THE OCTOPAMINERGIC MODULATION OF FLIGHT MUSCLE IN THE LOCUST

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### Summary

The role of cyclic AMP in the octopaminergic modulation of the dorsal longitudinal flight muscles of the locust *Schistocerca gregaria* has been investigated. Several techniques have been used to elevate cyclic AMP levels in this tissue by mechanisms that bypass the receptor activation stage. These include the use of phosphodiesterase inhibitors to block the metabolism of cyclic nucleotides, the use of forskolin, the diterpene activator of adenylate cyclase, and the direct application of permeable and phosphodiesterase-resistant analogues of cyclic AMP. All these approaches can be shown to mimic the modulatory effects of octopamine on the flight muscle. Surprisingly, the phosphodiesterase inhibitors used were not able to potentiate the actions of octopamine on this preparation. Octopamine increases cyclic AMP levels in a similar fashion in all five motor units of this muscle, an effect that is selectively blocked by phentolamine, an  $\alpha$ -adrenergic blocking agent that blocks octopamine receptors in other preparations. In addition, stimulation of the dorsal unpaired median neurone to the dorsal longitudinal flight muscles (DUMDL) results in a frequency-dependent increase in cyclic AMP levels in the muscle that is also blocked by phentolamine. The data presented suggest that the octopamine-mediated modulation of neurally evoked tension in this muscle is brought about by a mechanism that involves an increase in cyclic AMP levels in the tissue.

### Introduction

The biogenic amine octopamine is able to modulate the kinetics of contraction in the dorsal longitudinal flight muscle (DLM) of the locust. It produces an increase in the force generated by each muscle contraction and probably represents an energy-saving adaptation due to the reduced overlap in the duration of twitches in antagonistic muscles (Whim and Evans, 1988). These effects can be mimicked by stimulation of an identified octopaminergic neurone which innervates the DLM. The pharmacological specificity and dependence of these

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responses on the frequency of stimulation of the motor neurone input to the muscle have been characterised (Whim and Evans, 1988), as has the age-dependence of the octopaminergic modulation of this muscle (Whim and Evans, 1989). However, the mode of action of the octopamine receptors mediating these physiological responses has not been determined.

In a range of other insect preparations, including the extensor tibiae muscle of the locust hindleg, many of the prolonged modulatory actions of octopamine have been shown to be mediated *via* changes in the level of the second messenger adenosine 3',5'-cyclic monophosphate (cyclic AMP) (see Evans, 1985a). The flight muscle of the locust, however, is very different from the extensor tibiae muscle in that it is initially a slow muscle but becomes converted to a fast muscle during the first few weeks of adult life. Our initial work on this system (Whim and Evans, 1988, 1989) showed that there were important changes in the response of the flight muscle to octopamine during development, including differences in the octopamine-mediated changes in cyclic AMP levels. Thus, we have attempted to see if the modulatory effects of octopamine on locust flight muscle (Whim and Evans, 1988) are also mediated *via* the octopamine-induced changes in cyclic AMP levels previously described in this muscle (Whim and Evans, 1989). Before it can be concluded that a particular physiological effect of a given neurotransmitter is mediated directly by a cyclic nucleotide, several criteria need to be fulfilled (see Beam and Greengard, 1976). For example, the application of cyclic nucleotides, or drugs that elevate their levels by mechanisms that bypass the receptor activation stage, should mimic all the physiological actions of the agonist. In addition, dose-response curves for the physiological actions of the agonist need to be compared with those obtained for changes in cyclic AMP levels. This is particularly important for studies of octopamine on insect skeletal muscle since in the locust extensor tibiae muscle only the higher-affinity component of the biphasic dose-response curve for the octopamine-mediated increase in cyclic AMP levels correlates with known physiological responses (Evans, 1987). The lower-affinity component of the octopamine-mediated increase in cyclic AMP levels does not correlate with any known physiological processes. Finally, it should be noted that not all of the known actions of octopamine are thought to be mediated *via* increases in cyclic AMP levels. For example, the available evidence suggests that the octopaminergic modulation of a myogenic rhythm in the extensor tibiae muscle of the locust hindleg may involve a mechanism that releases  $\text{Ca}^{2+}$  from intracellular stores (Evans, 1984c), the effects of octopamine on insect blood cells also appear to involve increases in intracellular calcium levels (Jahagirdar *et al.* 1987) and a recently cloned *Drosophila* octopamine receptor expressed in a mammalian Chinese hamster ovary cell line has been shown to inhibit adenylate cyclase activity (Arakawa *et al.* 1990) and also to increase intracellular calcium levels (Robb *et al.* 1991).

The present paper describes experiments in which the intracellular levels of cyclic AMP in the DLM of the locust have been elevated by mechanisms that bypass the octopamine receptor activation stage. These include the inhibition of

the activity of the enzyme phosphodiesterase, which breaks down cyclic AMP, and the direct activation of adenylate cyclase by the diterpene compound forskolin. In addition, octopamine produces an increase in cyclic AMP levels in the DLM by a mechanism with similar pharmacological characteristics to the previously described physiological modulation of this muscle by octopamine. Further, stimulation of the dorsal unpaired median neurone to the dorsal longitudinal flight muscles (DUMDL) results in a frequency-dependent increase in cyclic AMP levels in the muscle.

### Materials and methods

Male locusts (*Schistocerca gregaria* L.) were taken from a crowded laboratory culture fed on wheat seedlings and bran. All experiments were performed at room temperature (20°C). Although locust DLM does not develop its mature adult kinetics until 2 weeks after the terminal moult (Mizisin and Ready, 1986), a study of the development of the octopamine responsiveness of the muscle indicates that both its biochemical and physiological responses to octopamine peak within the first few days after the final moult (see Whim and Evans, 1989). Thus, the present study used adult animals from 1 to 3 days old. However, no qualitative differences in the response of preparations to octopamine were observed with adult animals up to 3 weeks old. Animals were removed from the colony at least 1 h before use to minimize the possible potentiating effects of any released octopamine (Evans, 1981).

Animals were set up for physiological recording of tension from the DLM as described by Whim and Evans (1988). Briefly, animals were immobilized on a block of Plasticine, and the legs and wings removed. A dorsal incision along the length of the thorax exposed the body cavity. The gut and tissues overlying the metathoracic ganglion were then removed, together with any fat adhering to the metathoracic DLMs. Care was taken not to damage the tracheal supply to the muscle. The proximal end of the muscle was dissected clear of its attachment to its mesothoracic homologue and attached to a force transducer, which measured tension in the muscle almost isometrically. The distal end of the muscle was pinned securely to the Plasticine using minuten pins. An operational amplifier signal differentiator was used to measure continuously the rates of contraction and relaxation of neurally evoked tension in the muscle (Buchan and Evans, 1980). All five motor neurones, which innervate the separate motor units of the muscle, were stimulated using a pair of silver hook electrodes placed under the ipsilateral branch of nerve 1 from the metathoracic ganglion after its fusion with nerve 6 from the mesothoracic ganglion. DUMDL was activated selectively by stimulating a pair of hook electrodes on the contralateral branch of nerve 1 (see Whim and Evans, 1988, for further details).

Drugs were superfused at  $1 \text{ ml min}^{-1}$  directly onto the surface of the muscle. They were dissolved in a physiologically isotonic saline (pH 6.8) containing (in  $\text{mmol l}^{-1}$ ); NaCl, 150;  $\text{CaCl}_2$ , 1.3;  $\text{KHCO}_3$ , 4;  $\text{KH}_2\text{PO}_4$ , 6; sucrose, 90. Prep-

arations remained viable in this medium for 4–5 h, although in the present study individual preparations were rarely maintained beyond 3 h. The figures illustrate typical individual experiments all of which were repeated at least three times in the presented format.

For the cyclic AMP assays, male locusts were decapitated and fixed ventral side uppermost on a block of Plasticine. A longitudinal incision along the thorax allowed visual identification of the narrow region separating the dorsal lengths of the two DLMs, enabling accurate bisection. Both the left and right muscles were removed and all adhering fat body and remaining dorsal–ventral musculature cleared. The two muscles, attached to small pieces of cuticle for support, were floated muscle-side down for 10 min in  $10^{-4}$  mol l<sup>-1</sup> 3-isobutyl-1-methylxanthine (IBMX) in locust saline (to inhibit any endogenous phosphodiesterase activity) prior to incubation for a further 10 min in either the control solution ( $10^{-4}$  mol l<sup>-1</sup> IBMX) or the experimental solution (usually a known concentration of octopamine containing  $10^{-4}$  mol l<sup>-1</sup> IBMX). Contralateral muscle blocks from each animal served as unstimulated controls in the majority of experiments. In experiments where DUMDL was stimulated, the preparation was dissected as for the physiological experiments described above and the muscle was superfused *in situ* with locust saline containing IBMX ( $10^{-4}$  mol l<sup>-1</sup>) for 10 min prior to a 1 min period of DUMDL stimulation at different frequencies. At the end of the incubation periods the muscle blocks were pinned to Plasticine using minuten pins, frozen using an aerosol freezer spray and then dissected free from their cuticular supports (see Evans, 1984a). The frozen muscle was either homogenised in 2.5 ml of acidified ethanol (60:1 absolute ethanol: concentrated HCl v/v) or dissected into its five component motor units (see Neville, 1963; Whim and Evans, 1988), and these were separately homogenised in 500  $\mu$ l of acidified ethanol. The muscle homogenates were transferred to 1.5 ml plastic centrifuge tubes together with two 250  $\mu$ l washes of the homogeniser, prior to storage at 4°C for 3–21 days to allow precipitation of protein. The latter was centrifuged down (5 min, 2000 g, room temperature) and dissolved in 200  $\mu$ l of 1 mol l<sup>-1</sup> NaOH. This was assayed for its protein content using the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

The supernatant was evaporated to dryness (80°C for 3 h). Samples were then taken up into 150  $\mu$ l of 0.05 mol l<sup>-1</sup> Tris buffer (pH 7.5) containing 4 mmol l<sup>-1</sup> EDTA and centrifuged at 2000 g for 5 min. Cyclic AMP levels were measured (see Evans, 1984c) using a commercial cyclic AMP assay kit (Amersham International) based on the protein binding method of Brown *et al.* (1972). The kit had a detection limit of 0.04 pmol. Results are expressed as pmol of cyclic AMP per mg of protein or as increases in pmol mg<sup>-1</sup> protein caused by a particular treatment. The increase represents the differences between the levels in experimental and contralateral control muscles.

Cyclic GMP levels were also measured in the extracts of the DLM using the method previously described (see Evans, 1984a).

All drugs were obtained from the Sigma Chemical Co. except

8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (Boeinger Mannheim). Phentolamine mesylate was a gift from Ciba. Stock solutions of forskolin and 1,9-dideoxyforskolin were made by dissolving 1 mg in 100  $\mu$ l of ethanol. Appropriate ethanol controls were run for each experiment.

## Results

### *The effect of phosphodiesterase inhibitors*

The levels of cyclic AMP (and other cyclic nucleotides) within a tissue are dependent on the relative activities of synthetic and degradative enzymes. Elevation of their levels can therefore occur *via* an inhibition of the phosphodiesterase responsible for their breakdown. The methylxanthine IBMX is a potent inhibitor of phosphodiesterase activity in the locust extensor tibiae muscle, where it elevates the levels of both cyclic AMP and cyclic GMP (Evans, 1984a). Fig. 1 shows typical results obtained by applying a 5 min pulse of  $10^{-5}$  mol l $^{-1}$  IBMX on neurally evoked tension in the DLM produced by stimulation of all five of its motor units synchronously at 1 Hz. IBMX increased the amplitude of twitch tension by 36%, increased the rate of contraction by 25% and increased the rate of relaxation by 58%. The responses to IBMX were dose-dependent and exhibited a threshold response between  $10^{-7}$  and  $10^{-6}$  mol l $^{-1}$  IBMX. The most marked effects of IBMX were upon the rate of relaxation of twitch tension, which was also the response that was initiated the most rapidly. All these effects of IBMX were qualitatively similar to the responses of the DLM to octopamine application, although the IBMX responses took longer to develop, presumably because the IBMX has to cross the muscle plasma membrane to reach its site of action.

Other inhibitors of phosphodiesterase produced qualitatively similar results to

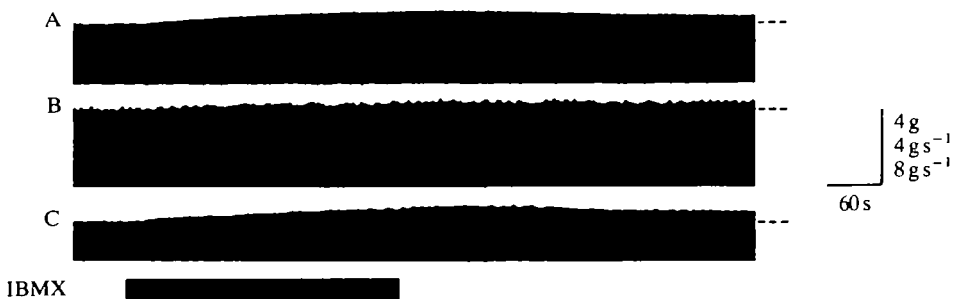


Fig. 1. The effect of a 5 min pulse of  $10^{-5}$  mol l $^{-1}$  IBMX (black bar) on twitch tension generated in the dorsal longitudinal muscle by synchronous stimulation of its five motor units at 1 Hz. (A) The effect on twitch amplitude; (B) and (C) the effects on the contraction rate and relaxation rate of twitch tension, respectively. Dashed lines to the right of the traces indicate basal levels before IBMX addition to the superfusate.

those of IBMX. A 10 min pulse of  $10^{-6}$  mol l $^{-1}$  theophylline also potentiated all the measured parameters of twitch tension and increased the relaxation rate of twitch tension by 18 % (not shown). However, theophylline is a methylxanthine that has been reported to bring about a release of calcium from internal stores in muscle and mammalian neurones (see Neering and McBurney, 1984) in addition to its actions on phosphodiesterase. Thus, we also investigated the effect of papaverine, a non-methylxanthine phosphodiesterase inhibitor, on twitch tension developed in the DLM. A 5 min pulse of  $10^{-4}$  mol l $^{-1}$  papaverine again potentiated all the measured parameters of twitch tension and increased the relaxation rate of twitch tension by 14 % (not shown).

The presence of a phosphodiesterase inhibitor should also potentiate the effects of any neurotransmitter or neuromodulator that mediates its effects by an increase in cyclic nucleotide levels (i.e. the response of the preparation to the joint application of the phosphodiesterase inhibitor and octopamine should be greater than the sum of their effects when applied alone) (see Beam and Greengard, 1976). Surprisingly, however, we were not able to demonstrate any potentiation of the effects of a submaximal dose of DL-octopamine (30 s at  $10^{-7}$  mol l $^{-1}$ ) on the dorsal longitudinal muscle at any of the concentrations used of IBMX (up to  $10^{-6}$  mol l $^{-1}$ ), theophylline (up to  $10^{-4}$  mol l $^{-1}$ ) or papaverine (up to  $10^{-4}$  mol l $^{-1}$ ). Fig. 2 illustrates an example of one of these experiments where the effects of a 30 s pulse of  $10^{-7}$  mol l $^{-1}$  DL-octopamine on the relaxation rate of twitch tension

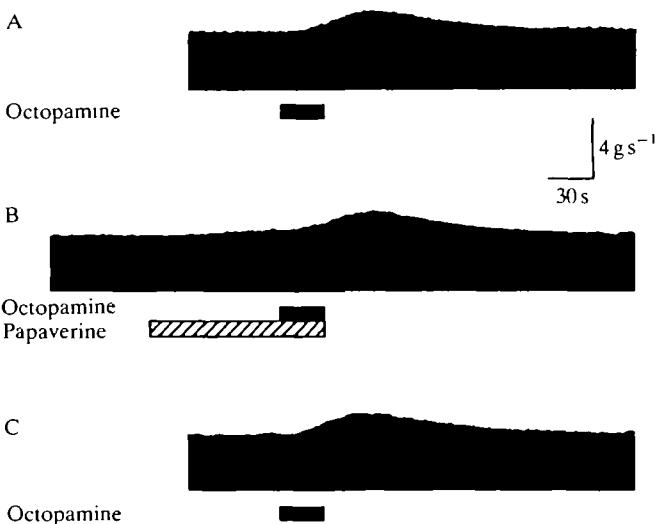


Fig. 2. The additive effect of a 30 s pulse of  $10^{-7}$  mol l $^{-1}$  DL-octopamine on the effect of a 2 min pulse of  $10^{-4}$  mol l $^{-1}$  papaverine on the rate of relaxation of twitch tension in the dorsal longitudinal muscle stimulated at 1 Hz. (A) The effect of an initial pulse of octopamine. (B) The additive effect of a pulse of octopamine given in the presence of papaverine. (C) The effect of a control pulse of octopamine after the papaverine has been washed out. The traces form a continuous record from the same preparation.

merely summated with the effect of  $10^{-4}$  mol l<sup>-1</sup> papaverine when the preparation was exposed to both agents at the same time.

The possibility that this lack of potentiation was due either to a markedly subthreshold dose of each of the phosphodiesterase inhibitors or to the presence of an already 'fully potentiated' muscle were eliminated in experiments where the concentration of the applied phosphodiesterase inhibitor was varied and it was verified that an increase in the concentration of the applied octopamine resulted in an increase in the measured parameters of twitch tension. In all experiments where the concentrations of the phosphodiesterase inhibitors and of octopamine or their lengths of application to the preparations were varied, the responses of the muscle were always purely additive (data not shown).

#### *Effect of the adenylate cyclase activator forskolin*

The direct actions of the phosphodiesterase inhibitors on the DLM muscle of the locust are consistent with the idea that the octopamine-induced physiological responses are mediated *via* a change in cyclic nucleotide levels. Nevertheless, they do not differentiate between the actions of cyclic AMP and those of cyclic GMP, the levels of both of which can be elevated in locust tissues by methylxanthines (Evans, 1984a; Pannabecker and Orchard, 1986). However, the diterpene compound forskolin has been shown to increase cyclic AMP levels selectively in both membrane fragments and intact cells from a wide range of tissues (Seamon and Daly, 1981). Forskolin also selectively increases cyclic AMP levels in the extensor tibiae muscle of the locust hindleg (Evans, 1984a).

Forskolin mimics the effects of octopamine exposure to the DLM. Fig. 3 indicates that a 5 min pulse of  $10^{-5}$  mol l<sup>-1</sup> forskolin can potentiate the amplitude of twitch tension induced by firing the five motor units to the DLM synchronously at 1 Hz by 17% and also the corresponding rates of contraction and relaxation of twitch tension by 20% and 23%, respectively. Again the effects developed and

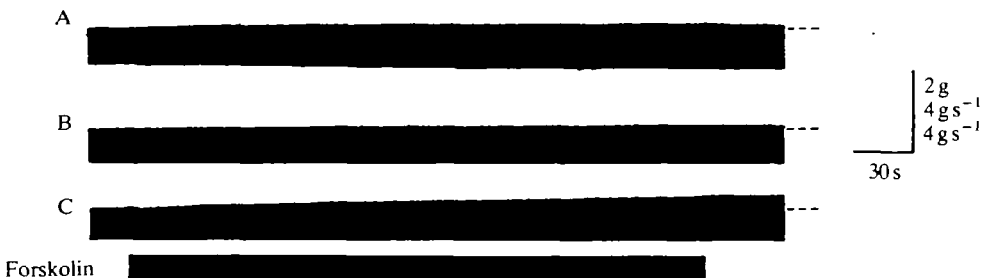


Fig. 3. The effect of a 5 min pulse of  $10^{-5}$  mol l<sup>-1</sup> forskolin (black bar) on twitch tension generated in the dorsal longitudinal flight muscle by synchronous stimulation of its five motor units at 1 Hz. (A) The effect on twitch amplitude; (B) and (C) the effects on the contraction rate and relaxation rate of twitch tension, respectively. Dashed lines to the right of the traces indicate basal levels before forskolin addition to the superfusate.

decayed more slowly than the corresponding octopamine effects. These actions of forskolin were dose-dependent and exhibited a threshold between  $10^{-6}$  and  $10^{-5}$  mol l<sup>-1</sup>. Recently, forskolin has been shown to inhibit a number of membrane transport proteins and channel proteins through a mechanism that does not involve the production of cyclic AMP (see Laurenza *et al.* 1989). However, these cyclic-AMP-independent effects of forskolin can be reproduced by the naturally occurring forskolin analogue 1,9-dideoxyforskolin, a compound that does not activate adenylate cyclase (Baxter and Byrne, 1990). A 5 min pulse of  $10^{-5}$  mol l<sup>-1</sup> dideoxyforskolin does not potentiate twitch tension in the DLM, indicating that the effects of forskolin itself are likely to be mediated by the activation of adenylate cyclase.

In some preparations, but not all, forskolin is also able to increase the sensitivity of the adenylate cyclase complex to receptor activation (Daly, 1984; Seamon and Wetzel, 1984). We attempted to potentiate the actions of a submaximal dose of DL-octopamine (30 s at  $10^{-7}$  mol l<sup>-1</sup>) by applying it to the preparation in the presence of various concentrations of forskolin up to  $10^{-5}$  mol l<sup>-1</sup>. In all cases the effects of octopamine and forskolin on the dorsal longitudinal muscle were purely additive (data not shown). In the presence of doses of forskolin of  $5 \times 10^{-5}$  mol l<sup>-1</sup> and above, 30 s pulses of  $10^{-7}$  mol l<sup>-1</sup> DL-octopamine did not produce any further effects (data not shown).

#### *The effects of cyclic AMP analogues*

Intracellular levels of cyclic nucleotides can also be increased by exposing tissues to exogenous cyclic nucleotides and such applications should also mimic the physiological actions of applying a putative neurotransmitter or neuromodulator that acts through adenylate cyclase activation (Beam and Greengard, 1976). Difficulties have arisen with such experiments since it is often necessary to apply very high levels of cyclic nucleotides before any physiological responses can be observed. This is due to the lack of permeability of cells to many of the analogues and to their rapid metabolism by the phosphodiesterase.

Fig. 4A shows that superfusion of a 2 min pulse of  $10^{-4}$  mol l<sup>-1</sup> dibutyryl cyclic AMP over the surface of a DLM preparation being neurally activated at a frequency of 1 Hz produced a 14% increase in the rate of relaxation of twitch tension. No change was observed in the amplitude of twitch tension and only a very small change in its rate of contraction. Fig. 4B shows that a similar pulse of  $10^{-4}$  mol l<sup>-1</sup> dibutyryl cyclic GMP was without effect on any of the parameters of twitch tension measured in this muscle. However, the 8-(4-chlorophenylthio)adenosine 3',5'-monophosphate cyclic derivative (CPT cyclic AMP) mimicked all the actions of octopamine in this preparation (Fig. 5). This derivative has been reported to be 100 times more effective than dibutyryl cyclic AMP in the activation of cyclic-AMP-dependent protein kinase activity in rat liver and to be more resistant to phosphodiesterase activity (Miller *et al.* 1975). A 1 min pulse of  $10^{-4}$  mol l<sup>-1</sup> CPT cyclic AMP increased neurally evoked twitch tension in the DLM by 19% and the rates of contraction and relaxation of twitch tension by 14%



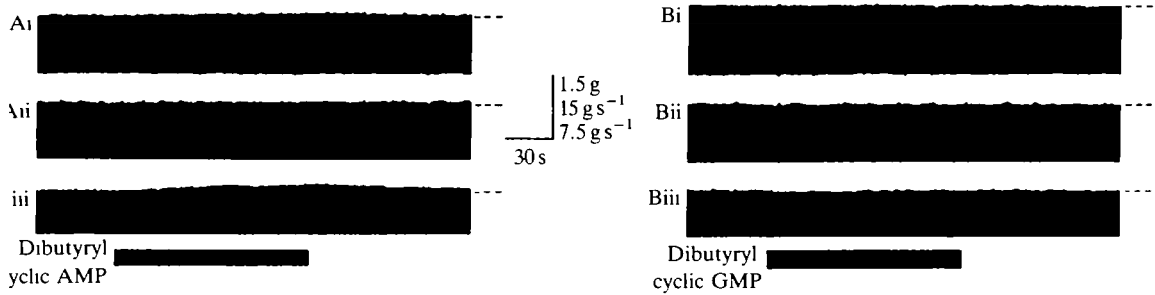


Fig. 4. The effect of (A) a 2 min pulse of  $10^{-4}$  mol  $l^{-1}$  dibutyryl cyclic AMP and (B) a 2 min pulse of  $10^{-4}$  mol  $l^{-1}$  dibutyryl cyclic GMP (black bars) on twitch tension generated in the dorsal longitudinal flight muscle by synchronous stimulation of its five motor units at 1 Hz. (i) The effects on twitch amplitude; (ii) and (iii) the effects on the contraction rate and relaxation rate of twitch tension, respectively. Dashed lines to the right of the traces indicate basal levels before cyclic nucleotide addition to the superfusate.

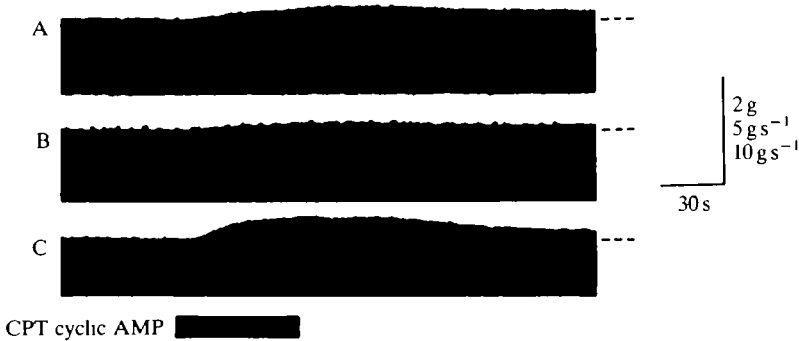


Fig. 5. The effect of a 1 min pulse of  $10^{-4}$  mol  $l^{-1}$  CPT cyclic AMP (black bar) on twitch tension generated in the dorsal longitudinal muscle by synchronous stimulation of its five motor units at 1 Hz. (A) The effect on twitch amplitude; (B) and (C) the effects on the contraction rate and relaxation rate of twitch tension, respectively. Dashed lines to the right of the traces indicate basal levels before CPT cyclic AMP addition to the superfusate.

and 50%, respectively (Fig. 5). The effects of CPT cyclic AMP developed slowly and persisted for several minutes after the end of the CPT cyclic AMP pulse.

#### *The effects of IBMX and forskolin on cyclic AMP levels*

Both IBMX and forskolin mimic the physiological effects of applying octopamine to the DLM. IBMX increases the levels of cyclic AMP in the muscle in a dose-dependent way with a threshold occurring between  $10^{-7}$  and  $10^{-6}$  mol  $l^{-1}$  (Fig. 6A). This is the same threshold observed for the modulatory actions of IBMX on twitch tension in the DLM (see above).

Forskolin also increases cyclic AMP levels in DLM in a dose-dependent manner

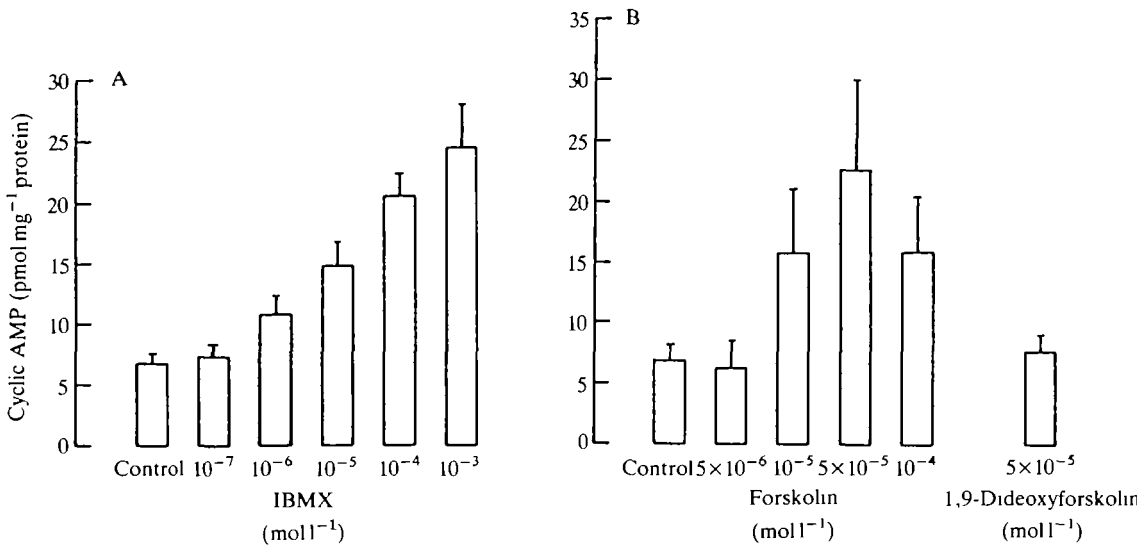


Fig. 6. The effect of IBMX and forskolin on cyclic AMP levels in the dorsal longitudinal muscle. (A) Effect of various concentrations of IBMX on cyclic AMP levels. Muscles were incubated for 10 min before freezing and the results are expressed as picomoles of cyclic AMP per milligram protein. Each value is the mean of four determinations. (B) Effect of various concentrations of forskolin on cyclic AMP levels. Experimental muscles were exposed for 10 min to forskolin plus  $10^{-4}$  mol l<sup>-1</sup> IBMX and control muscles only to IBMX, after each had received a 10 min preincubation in IBMX. The experimental 1,9-dideoxyforskolin muscles were similarly treated. Each value is the mean of eight determinations. Values are expressed as mean+standard error of the mean.

with a threshold occurring between  $5 \times 10^{-6}$  and  $10^{-5}$  mol l<sup>-1</sup> (Fig. 6B). This is again similar to its observed threshold for the modulation of twitch tension (see above). Further, at  $5 \times 10^{-5}$  mol l<sup>-1</sup>, dideoxyforskolin did not produce an increase in cyclic AMP levels above that of the controls (Fig. 6B).

#### *Octopamine-stimulated increase in cyclic AMP levels*

If the potentiation of DLM by octopamine is mediated *via* changes in cyclic AMP levels then it should be possible to demonstrate changes in cyclic AMP levels in the muscle in the physiologically active concentration range for octopamine. We have previously shown (Whim and Evans, 1989) that incubation of the DLM in the presence of octopamine produces a dose-dependent increase in the levels of cyclic AMP in each of the five motor units of the muscle (units 1–5, see Neville, 1963) over a range of DL-octopamine concentrations ( $10^{-8}$  mol l<sup>-1</sup> to  $10^{-3}$  mol l<sup>-1</sup>). The thresholds for a detectable change in cyclic AMP levels occurred between  $10^{-8}$  mol l<sup>-1</sup> and  $10^{-7}$  mol l<sup>-1</sup> DL-octopamine for all five motor units, which is similar to that found for the physiological responses of the muscle to pulses of DL octopamine (Whim and Evans, 1988). Octopamine, at concentrations up to

Table 1. Action of antagonists on basal and octopamine-stimulated increases in levels of cyclic AMP

Drug	Basal level of cyclic AMP (pmol mg <sup>-1</sup> protein)	Octopamine-stimulated increase (pmol mg <sup>-1</sup> protein)
Control	11.3±0.6	1658.8±59.3
Phentolamine	8.5±1.8	10.5±2.8
DL-Propranolol	11.5±1.1	1926.5±241.4

The results are expressed as the octopamine-stimulated increase in cyclic AMP levels ± standard error of the mean ( $N=3$ ) after a 10 min exposure to  $10^{-5}$  mol l<sup>-1</sup> DL-octopamine in the presence of  $10^{-4}$  mol l<sup>-1</sup> IBMX. The measured increase in cyclic AMP levels due to octopamine in the presence of each antagonist ( $10^{-4}$  mol l<sup>-1</sup>) was obtained by comparison with contralateral control muscles exposed only to the antagonist. Both control and experimental muscles received a 10 min preincubation in  $10^{-4}$  mol l<sup>-1</sup> IBMX. The effect of the antagonists on basal cyclic AMP levels was obtained by comparing the results obtained in control muscles with muscles that were incubated only in IBMX.

$10^{-3}$  mol l<sup>-1</sup>, did not change the levels of cyclic GMP in the DLM (data not shown).

The octopamine-mediated increase in cyclic AMP levels in the dorsal longitudinal muscle was selectively blocked by phentolamine, an  $\alpha$ -adrenergic blocking agent that also blocks octopamine receptors in insects (Evans, 1981) (Table 1). Propranolol, a  $\beta$ -blocking agent, did not block the response.

#### *DUMDL-stimulated increase in cyclic AMP levels*

The physiological relevance of the octopamine-mediated increases in cyclic AMP levels in DLM has also been investigated in experiments where DUMDL, the octopaminergic neurone innervating DLM (Whim and Evans, 1988), was stimulated at different frequencies. Fig. 7 shows that stimulating DUMDL for 1 min periods in the presence of  $10^{-4}$  mol l<sup>-1</sup> IBMX produced a frequency-dependent increase in cyclic AMP levels, which peaked at 5 Hz. This is similar to the frequency dependence of DUMDL-induced potentiation of DLM twitch tension (Whim and Evans, 1988, 1989). In addition, the effects of DUMDL on cyclic AMP levels are inhibited by  $10^{-4}$  mol l<sup>-1</sup> phentolamine. This level of phentolamine also reduced the basal level of cyclic AMP in the DLM, which may indicate the spontaneous release of octopamine from DUMDL under control conditions. The extensor tibiae muscle of the locust hindleg shows the same phenomenon (see Evans, 1984a).

### Discussion

The evidence presented in the current paper is consistent with the octopamine-dependent modulation of neurally evoked tension in the DLM of the locust (Whim and Evans, 1988) being mediated *via* an elevation in cyclic AMP levels. The

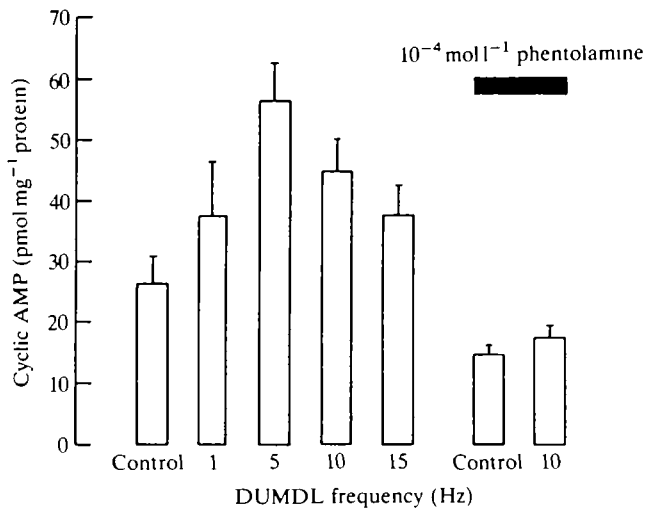


Fig. 7. The effect of DUMDL stimulation at different frequencies on cyclic AMP levels in the dorsal longitudinal muscle in the presence of  $10^{-4}$  mol l<sup>-1</sup> IBMX. The muscles were preincubated for 10 min in IBMX before DUMDL stimulation for 60 s. In the phentolamine blocking experiment  $10^{-4}$  mol l<sup>-1</sup> phentolamine was also included in the preincubation for both the control and experimental muscle sets. The results are expressed as the mean levels of cyclic AMP in pmol mg<sup>-1</sup> protein of the muscle + the standard error of the mean of four determinations.

actions of octopamine are mimicked by elevating the levels of cyclic AMP in the muscle using a variety of mechanisms that bypass octopamine receptor activation.

The application of analogues of cyclic AMP to a DLM contracting at 1 Hz affects all of the three parameters of twitch tension measured but, as with octopamine application, the most marked effects are upon the rate of relaxation of neurally evoked twitch tension. CPT cyclic AMP, a highly permeable analogue that is more resistant to phosphodiesterase activity than is dibutyryl cyclic AMP (Miller *et al.* 1975), was the most effective analogue tested and increased all the parameters of twitch tension measured. Dibutyryl cyclic AMP was less effective and at the concentrations used (up to  $10^{-4}$  mol l<sup>-1</sup>) only produced an increase in the rate of relaxation of twitch tension. However, an equivalent dose of dibutyryl cyclic GMP was totally inactive, suggesting that cyclic GMP is unlikely to be involved in mediating the effects of octopamine on this preparation. This suggestion is further supported by the observation that DL-octopamine (up to  $10^{-3}$  mol l<sup>-1</sup>) does not produce any measurable changes in cyclic GMP levels in the DLM. The responsiveness of the DLM to the application of the above analogues is ten times that found for the extensor tibiae muscle of the locust hindleg (see Evans, 1984*b*) where the actions of octopamine are known to be mediated *via* cyclic AMP (Evans, 1984*a,b*; 1985*b*).

The direct actions of the phosphodiesterase inhibitors, which slow down the catabolism of cyclic AMP, are also consistent with the involvement of cyclic AMP

in the neuromodulatory actions of octopamine on the DLM. IBMX, theophylline and papaverine, a non-methylxanthine phosphodiesterase inhibitor, all increase the amplitude of neurally evoked twitch tension and its contraction and relaxation rates in a manner similar to that found for octopamine. In addition, IBMX has also been demonstrated to increase cyclic AMP levels in a dose-dependent way in DLM. Surprisingly, however, under the conditions used none of these compounds potentiated the actions of simultaneously applied pulses of octopamine. This contrasts with responses obtained in the locust extensor tibiae muscle (Evans, 1984*b*), locust corpora cardiaca (Pannabecker and Orchard, 1986) and locust oviduct (Lange and Orchard, 1986) where the actions of octopamine are all potentiated in the presence of low doses of IBMX. Further, the octopamine-mediated hyperpolarization of Schwann cells from the squid giant axon has also been shown to be potentiated by theophylline and by papaverine (Reale *et al.* 1986).

In spite of these reports, the inability of phosphodiesterase inhibitors to potentiate the actions of biogenic amines is not a novel observation. In a study of the actions of octopamine upon cyclic nucleotide levels in locust flight muscle, Worm (1980) noted that injection of theophylline into locusts prior to the initiation of flight did not alter the flight muscle concentration of cyclic AMP at the time when haemolymph octopamine levels rise dramatically. In a similar fashion, although application of IBMX mimicked the chronotropic and ionotropic actions of octopamine and dopamine on *Limulus polyphemus* heart, the ionotropic action of these two amines was not potentiated in the presence of IBMX (Groome and Watson, 1987). Finally, in an elegant study of the serotonin-induced  $Ca^{2+}$  current increase in snail neurones (Paupardin-Tritsch *et al.* 1986) it was concluded that the serotonin effect was mediated by changes in cyclic GMP level, since both the intracellular injection of cyclic GMP and the application of zaprinast (a cyclic GMP phosphodiesterase inhibitor) mimicked the actions of serotonin. However, zaprinast itself failed to potentiate the actions of serotonin. It seems that phosphodiesterase inhibitors in general, and methylxanthines in particular, can only be shown to potentiate the actions of amines in a limited range of preparations. The reason for this is not known at present.

Additional evidence for the involvement of cyclic AMP in the actions of octopamine on the DLM is derived from the observation that forskolin, the diterpene activator of adenylate cyclase (Seamon and Daly, 1981), increases the amplitude of twitch tension and its rates of contraction and relaxation. As with octopamine, the parameter most affected is that of relaxation rate. Forskolin also increases cyclic AMP levels in the muscle in a dose-dependent manner. The inability of forskolin to potentiate the actions of octopamine has also been found by other workers. Evans and Myers (1986) report that forskolin failed to potentiate the octopamine-stimulated increase in cyclic AMP levels in the locust extensor tibiae muscle. In preparations where forskolin has been shown to potentiate agonist stimulation, its effects are believed to be due to its binding to a site on the guanine nucleotide regulatory protein ( $G_S$ ) in addition to its direct

action on the catalytic subunit of adenylate cyclase (Seamon and Daly, 1986). The lack of potentiation seen with forskolin in the present study may, therefore, be due to the absence of a binding site for forskolin on the G protein present in locust muscle (see Evans and Myers, 1986). Furthermore, other actions of forskolin unrelated to its ability to stimulate adenylate cyclase are known (see Laurenza *et al.* 1989). Thus, in the brain of the fly *Ceratitis capitata*, forskolin has been reported to block the octopamine-dependent activation of adenylate cyclase (Guillen *et al.* 1987). It was suggested that this could occur by a modification of the binding of octopamine to its receptor or by a modification of the interaction between the octopamine receptor and its regulatory G protein (Guillen *et al.* 1987). A partial blocking action of forskolin upon the DLM octopamine receptor might explain why this compound failed to potentiate the actions of exogenously applied octopamine.

In the DLM the observation that 1,9-dideoxyforskolin does not mimic the actions of octopamine again supports the idea that the actions of forskolin in the DLM are mediated *via* the activation of adenylate cyclase. Thus, the direct actions of a number of compounds that elevate cyclic AMP levels by mechanisms that bypass the octopamine receptor activation stage suggest that octopamine receptor activation in the DLM of the locust is likely to mediate its effects *via* a stimulation of adenylate cyclase activity.

The above theory is also supported by the large elevation of cyclic AMP levels observed in the DLM after octopamine application (Whim and Evans, 1989). The  $EC_{50}$  values observed for the different motor units of the DLM (ranging from  $9 \times 10^{-6} \text{ mol l}^{-1}$  to  $3.5 \times 10^{-5} \text{ mol l}^{-1}$ ) were similar to those found in the locust extensor tibiae muscle ( $5.5 \times 10^{-5} \text{ mol l}^{-1}$ ) (Evans, 1984a) and in the firefly light organ ( $3 \times 10^{-6} \text{ mol l}^{-1}$ ) (Nathanson, 1979). They also compare favourably with those found for the stimulation of octopamine-sensitive adenylate cyclase activity in the flight muscle of another locust, *Locusta migratoria* ( $3.3 \times 10^{-6} \text{ mol l}^{-1}$ ) (Lafon-Cazal and Bockaert, 1985). Unfortunately, the octopamine-stimulated cyclic AMP levels in the present study are difficult to compare with those reported in the DLM of *Locusta migratoria* (Worm, 1980) since in the latter study octopamine was injected directly into the insect and its final concentration was unknown.

An interesting difference exists between the octopamine-stimulated increases in cyclic AMP levels found in the DLM and those found in the extensor tibiae muscle preparation. In the extensor tibiae muscle the rising phase of the dose-response curve for octopamine-mediated increases in cyclic AMP levels extends over two log units of concentration before entering the linear phase, indicating the presence of two separate components to the curve (Evans and Myers, 1986; Evans, 1987). It has been suggested that only the higher-affinity component of the two is linked to the known physiological actions of octopamine in this preparation. In the DLM, the corresponding dose-response curve only exhibits a single component, which is closely related to the physiological dose-response curve for the actions of octopamine on this preparation (see Whim and Evans, 1988). The changes in the

cyclic AMP levels described in the present paper are probably linked to the known physiological actions of octopamine on the flight muscle.

This conclusion is further supported by the observation that stimulation of the modulatory octopaminergic neurone to the DLM, DUMDL (Whim and Evans, 1988), also causes a frequency-dependent increase in cyclic AMP levels in the muscle, which parallels its modulatory actions on twitch tension (Whim and Evans, 1988, 1989). A similar frequency-dependent increase in cyclic AMP levels has been shown in the locust extensor tibiae muscle after the stimulation of its modulatory octopaminergic neurone, DUMETi (Evans, 1984a).

The changes in the levels of cyclic AMP observed in the present study can be assumed to reflect almost entirely the results of the activation of postsynaptic octopamine receptors on the muscle fibres themselves, since any changes in the cyclic nucleotide levels within the terminals of the motor neurones will be extremely small compared with those in the muscle fibres. The mechanism whereby the octopamine-mediated changes in cyclic AMP levels effect the observed physiological changes in the DLM is at present unknown.

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