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OCTOPAMINE RELEASE FROM AN IDENTIFIED NEURONE IN THE LOCUST

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SUMMARY

1. The release of octopamine from the extensor-tibiae muscle preparation of the locust in response to high K^+ saline and DUMETi stimulation has been demonstrated. This release has been measured using a radioenzyme assay for octopamine.

2. Both forms of stimulated release have been shown to be calcium sensitive.

3. Three potential problems of studying the release of transmitters from neuromuscular preparations using radiolabelled compounds have been identified. (a) The cellular location of the labelled compounds is unknown. (b) The site of the high K induced release is unknown. (c) The contraction of the muscle could cause nonspecific release of radioactivity from the muscle.

INTRODUCTION

To demonstrate unequivocally that a particular chemical is released as a functional neurotransmitter from a particular neurone, several conditions have to be fulfilled. The chemical has to be shown to be present and synthesized in the cell. The same physiological response should be obtained from the postsynaptic target cell by application of the putative transmitter and by stimulation of the cell. In addition both these responses should be blocked by the same antagonists. Further, the cell should be shown to be capable of releasing the transmitter upon stimulation, and to possess a means of inactivating the transmitter after release. Most of these conditions have been fulfilled to demonstrate the octopaminergic nature of the modulatory neurone (DUMETi) to the extensor-tibiae muscle of the locust hindleg (Evans & O'Shea, 1977, 1978; O'Shea & Evans, 1979; Evans & Siegler, 1982; Evans, 1981, 1984*a*,*b*; Hoyle, 1975; Hoyle & Barker, 1975). However, a direct demonstration of the release of octopamine in response to DUMETi stimulation has not been reported.

A number of different methods may be used to demonstrate the release of a transmitter from the terminals of a nerve cell. One of the commonest methods is to label the transmitter pool by the administration of radioactive precursors, and then to monitor the radioactivity released from the cell. This method has been used to

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demonstrate the release of octopamine from the endings of cells in the second thoracic nerve roots of the lobster in response to depolarization with a high potassium saline (Evans, Kravitz & Talamo, 1976). The neurotransmitter pool can also be labelled by uptake of the radiolabelled transmitter itself, a technique used frequently in vertebrate tissues in release studies of noradrenaline (e.g. Wakade & Wakade, 1978), 5-hydroxytryptamine (5-HT) (e.g. Göthert, 1980) and gamma-aminobutyric acid (GABA) (e.g. Hammerstad & Lytle, 1976). It has also been used to study the release of octopamine from leech nerve cords in response to potassium depolarization (Webb & Orchard, 1981).

Such techniques, which radiolabel the transmitter pool, are very sensitive, rapid and easy to carry out. However, they have the disadvantage that the chemical identity of the released radioactive compound has to be checked, and distinguished from labelled metabolites and precursors. It is also likely that the labelled compounds will have been taken up by other cells, such as glial and muscle cells, and possibly by nerve terminals that normally do not contain the particular transmitter under study. In addition difficulty is frequently encountered in removing the residual extracellular radioactivity, prior to attempts to release the transmitter from a small population of neurones in the preparation.

Another method commonly employed to demonstrate release is the chemical assay of the released endogenous transmitter, for example, for release of GABA from inhibitory neurones in the lobster (Otsuka, Iversen, Hall & Kravitz, 1966) and for 5-HT release from identified *Aplysia* neurones (Gerschenfeld, Hamon & Paupardin-Tritsch, 1978). This method has also been used to measure the release of octopamine from a half thorax preparation of the locust in response to electrical stimulation (Goosey & Candy, 1982). Although the results are less ambiguous using this method, it is less sensitive and more time-consuming than radiolabelling. Another disadvantage is that it is impossible to use the putative transmitter itself to demonstrate the effects of any presynaptic receptors which might modulate release.

The present paper characterizes the release of octopamine from the terminals of DUMETi in the extensor-tibiae neuromuscular preparation after potassium depolarization and neural stimulation. Release has been followed using both radioenzymatic assay of released octopamine and by radiolabelling of the octopamine pool. In the latter case some further problems associated with the use of such a radiolabelling procedure in a neuromuscular preparation are discussed.

MATERIALS AND METHODS

Maintenance of locusts

Gregarious locusts, *Schistocerca americana gregaria,* were obtained from standard crowded laboratory cultures fed on wheat seedlings and bran. They were kept under a 12 h light, 35 °C: 12 h dark, 25 °C cycle. Adult locusts of both sexes, between 7 and 14 days after the final moult, were used for all experiments.

Incubation of muscles

The metathoracic extensor-tibiae muscle was exposed for incubation as described in O'Shea & Evans (1979) and care was taken to ensure that the tracheae were left

intact. A wall of Vaseline was constructed around the cut edges of the femur and after the radioisotope was added the dissection was sealed with a glass coverslip to prevent desiccation. Under these conditions the tracheae could be seen to inflate and deflate in time with the normal respiratory movements of the locust. The muscle was incubated routinely for 30 min in 25 μl of saline containing DL[3,5-^{3}H] octopamine [22 Ci mmol^{-1} (Amersham), 10 μCi/25 μl, (approx. 2×10^{-5} M)]. The saline had the following composition: 150 mM-NaCl, 6 mM-KH_2PO_4, 4 mM-$CaCl_2$, 4 mM-$KHCO_3$, 2 mM-$MgCl_2$, 90 mM-sucrose and 5 mM-trehalose. For each preparation the radioisotope was dried down under a stream of dry nitrogen at 37 °C, taken up in 25 μl of saline and then added to the muscle. After incubation the muscle was either dissected from the leg and washed in ice-cold saline (four changes, 5 min each) or left *in situ* and washed by superfusion of saline at 1 ml min^{-1} at room temperature (19–22 °C) before being dissected from the leg. The dissected muscle was then added to 100 μl of the extraction buffer consisting of formate/acetate buffer pH 1·9 (0·47 M-formic acid and 1·4 M-acetic acid) and stored at −20 °C. For experiments investigating the potassium-induced release of radioactivity, saline with 50 mM-NaCl replaced by 50 mM-KCl (final K^+ concentration 60 mM) was superfused over the muscle for various periods of time. In all cases DUMETi was stimulated antidromically with hook electrodes on the extensor-tibiae nerve (n5b$_1$) of the leg contralateral to the one being superfused (see Evans & O'Shea, 1978). SETi was stimulated by a pair of hook electrodes on nerve 3b.

Identification of labelled compounds

The extensor-tibiae muscles stored in formate/acetate buffer were frozen and thawed several times and homogenized using a glass rod to release radioactive compounds. The homogenates were then centrifuged at 12 000 ***g*** for 5 min at 20 °C and 40 μl of the supernatant subjected to high-voltage paper electrophoresis (Hildebrand, Barker, Herbert & Kravitz, 1971). These were run on a flat-bed electrophoresis system (Savant Instruments) at 3·5 kV for 3 h with parallel standards of DL-octopamine. Standards were dissolved in supernatant derived from control muscles frozen and thawed in formate/acetate buffer and homogenized. The electropherograms were run through a strip scanner (Packard model 7220/21) and sample areas cut into 1-cm strips for estimation of radioactivity. The strips were placed in 200 μl formate/acetate buffer for 1 h to elute the radioactivity, 5 ml Biofluor (New England Nuclear) was added and the radioactivity was estimated using a Packard Tri-Carb 300C liquid scintillation spectrometer.

The identity of the peaks was confirmed by thin layer chromatography (TLC) of the eluted radioactivity. TLC was carried out on silica gel plates (Camlab) in one of three solvent systems: *n*-butanol saturated with 10 % methylamine ($MeNH_2$); methyl ethyl ketone: propionic acid: water (200: 65: 55): or butan-2-ol: pyridine: acetic acid (glacial): water (300: 5: 20: 50). Tyrosine, tyramine and octopamine standards were run in parallel where applicable and were visualized with diazotized sulphanilic acid.

Radiolabelled compounds released into the superfusate of the muscle were identified as follows. Samples were desalted by passage through an ion exchange column (Dowex 50W, 100–200 mesh, 1 cm×0.8 cm i.d., H^+ form). The column was washed with 5 ml water, the radioactivity eluted with 5 ml 1 M-ammonium hydroxide, the

eluent dried down under vacuum, and the residue taken up in 1 ml absolute ethanol. The ethanol was dried down under a stream of nitrogen, the residue redissolved in 30 μl absolute ethanol and the identity of the products determined by TLC as above. The amount of radioactivity left in the muscle was measured at the end of each superfusion experiment routinely by dissolving the muscle in 0·5 ml Soluene (Packard) at 50 °C and adding 10 ml Biofluor before estimating the radioactivity as above.

Several series of experiments were carried out in which the extensor-tibiae muscle was incubated in [^{3}H]-tyrosine or [^{3}H]-tyramine for various periods of time. These amines are believed to be precursors for octopamine synthesis in insects (see Evans, 1980). Electrophoresis of extracts of these muscles showed very little radioactivity associated with octopamine (<1 % total tissue c.p.m.). Further experiments were carried out, however, exposing these muscles to high K^+ saline and although large quantities of radioactivity were released into the superfusing saline again very little, if any, of the radioactivity released was associated with octopamine. Thus it appeared that the most satisfactory method to label the octopamine pool in the DUMETi terminals was to use [^{3}H]-octopamine.

Release of endogenous octopamine

In some experiments the release of endogenous octopamine from the extensor-tibiae muscle was stimulated by high K^+ saline. Muscles were removed from the femur and rinsed in saline lacking trehalose since only short incubation times were used and since trehalose interferes with the ion exchange procedures used below. Groups of six muscles were placed in 1 ml of saline for 3 min and the saline removed (normal saline sample) and replaced with 1 ml of saline containing 110 mM-K^+ (100 mM-NaCl replaced with 100 mM-KCl). The muscles were left in this saline for a further 3 min and the saline removed (high K^+ saline sample).

In other experiments, release of endogenous octopamine was achieved by stimulation of DUMETi. The muscle was left *in situ* and 30 μl saline applied to the exposed surface. After 3 min this was removed and replaced with a fresh 30 μl of saline. This was carried out five times in each experiment. During the fourth incubation in saline DUMETi was stimulated antidromically by silver hook electrodes placed under the contralateral extensor-tibiae nerve (see Evans & O'Shea, 1978). It was stimulated at a variety of frequencies for the 3 min period using a regime of 30 s stimulation followed by 30 s rest. For each of the 3-min samples, saline from six separate animals was pooled.

Saline samples were passed through a Dowex 50W column as described above to extract any octopamine. After drying down the eluate and redissolving it in 1·5 ml absolute ethanol, the samples were dried down again under vacuum and taken up in 25 μl 0·01 M-formic acid. They were stored frozen until assayed by radioenzyme assay as described in Evans (1978*a*). The twice background sensitivity of the assay is 10–15 pg based on a DL-octopamine standard.

Using a [^{3}H]-octopamine standard the recovery from the Dowex columns was greater than 80 % for all the types of saline used. The authenticity of the radioenzyme assay products was confirmed by TLC as described in Evans (1978*a*) and the presence of phentolamine and yohimbine in the saline (see Results) did not interfere with the assay.

RESULTS

Octopamine uptake and metabolism

Extensor-tibiae muscles incubated in the presence of [^{3}H]-octopamine yield a number of labelled metabolic products. At least four separate electrophoresis peaks can be obtained (see Fig. 1A), after a 30 min incubation. Most of the radioactivity (82 ± 2%; mean ± S.E.M., $N = 9$) is associated with the first and last peaks, which are probably *N*-acetyloctopamine (near the origin) and octopamine respectively, the

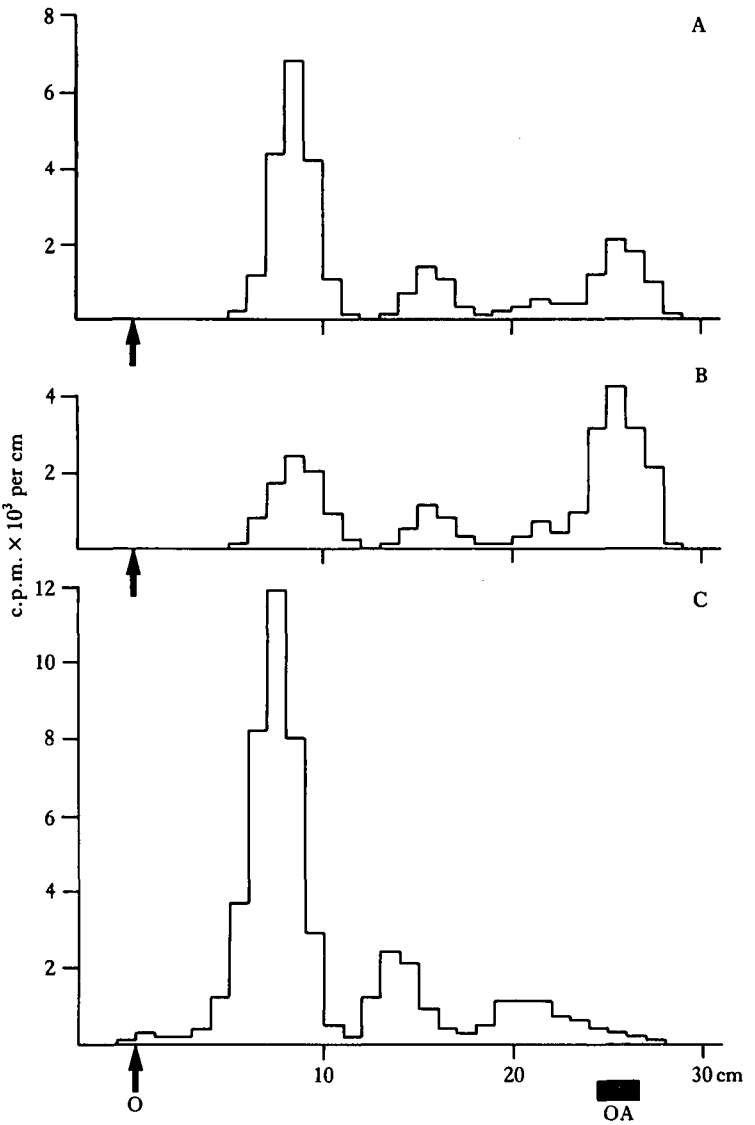

Fig. 1. Radioactivity profiles of electropherograms of extensor-tibiae muscles incubated in [^{3}H]-octopamine and subsequently superfused for 90 min with saline. (A) 30-min incubation period in ordinary saline. (B) 30-min incubation period in the presence of 5×10^{-5} M-harmol. (C) 1-h incubation period in ordinary saline. Abbreviations: O, origin; OA, octopamine.

latter peak accounting for 23 ± 2 % (mean ± S.E.M., $N = 9$) of the radioactivity present in the muscle extract. The two other products are possibly amino acid or sugar conjugates of octopamine (see Maxwell, Moore & Hildebrand, 1980; Evans, 1980). However, after a 1 h incubation period the amount of label associated with the octopamine peak declined to only 3 % (Fig. 1C). In Fig. 1B 5×10^{-5} M-harmol is added to both the incubation and superfusion salines. Harmol is an effective inhibitor of *N*-acetyltransferase activity in mammalian brains (Yang & Neff, 1976) and in locust nervous tissue (P. D. Evans, unpublished). As a result of this treatment the amount of radioactivity associated with octopamine in the muscle extract increases to 49 % and there is a corresponding reduction in that associated with the presumed *N*-acetyloctopamine peak.

The relative amounts of octopamine and *N*-acetyloctopamine in the muscle extracts are the same, for a given incubation time, after the muscle is superfused for 90 min, or washed and then subjected to a release experiment (see below) which usually involves superfusion for a further 30–50 min. However, the cellular location of the labelled octopamine and *N*-acetyloctopamine is not known.

Release of radiolabelled compounds

Effect of high potassium salines

High potassium saline (60 mM-K^+) induces the release of radioactivity from an extensor-tibiae muscle incubated for 30 min in [^{3}H]-octopamine. Fig. 2 shows a

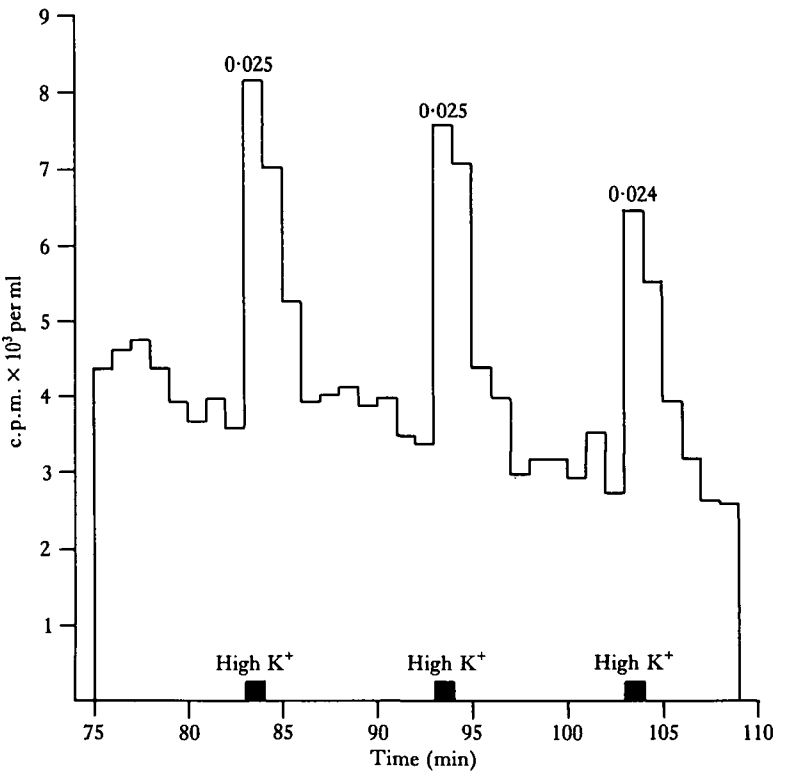

Fig. 2. Effect of repeated pulses of 60 mM-K^+ saline (black squares) on the efflux of radioactivity from an extensor-tibiae muscle incubated in [^{3}H]-octopamine for 30 min. Numbers above the peaks refer to the fractional release of radioactivity (see text).

typical experiment in which three successive pulses of high K^+ produce three discrete peaks of radioactivity in an efflux curve. The reproducibility of the release is shown by the fractional release values for each peak (see numbers above the peaks). The fractional release is the amount of radioactivity in a peak above the background rate of efflux, expressed as a fraction of the total amount of radioactivity in the muscle at that time.

The identity of the radiolabelled products in the background efflux and high K^+ released samples can be determined by ion-exchange chromatography followed by thin layer chromatography (see Fig. 3). In both cases most of the radioactivity in the superfusate samples (70–80 %) is washed out of the column in the water phase and

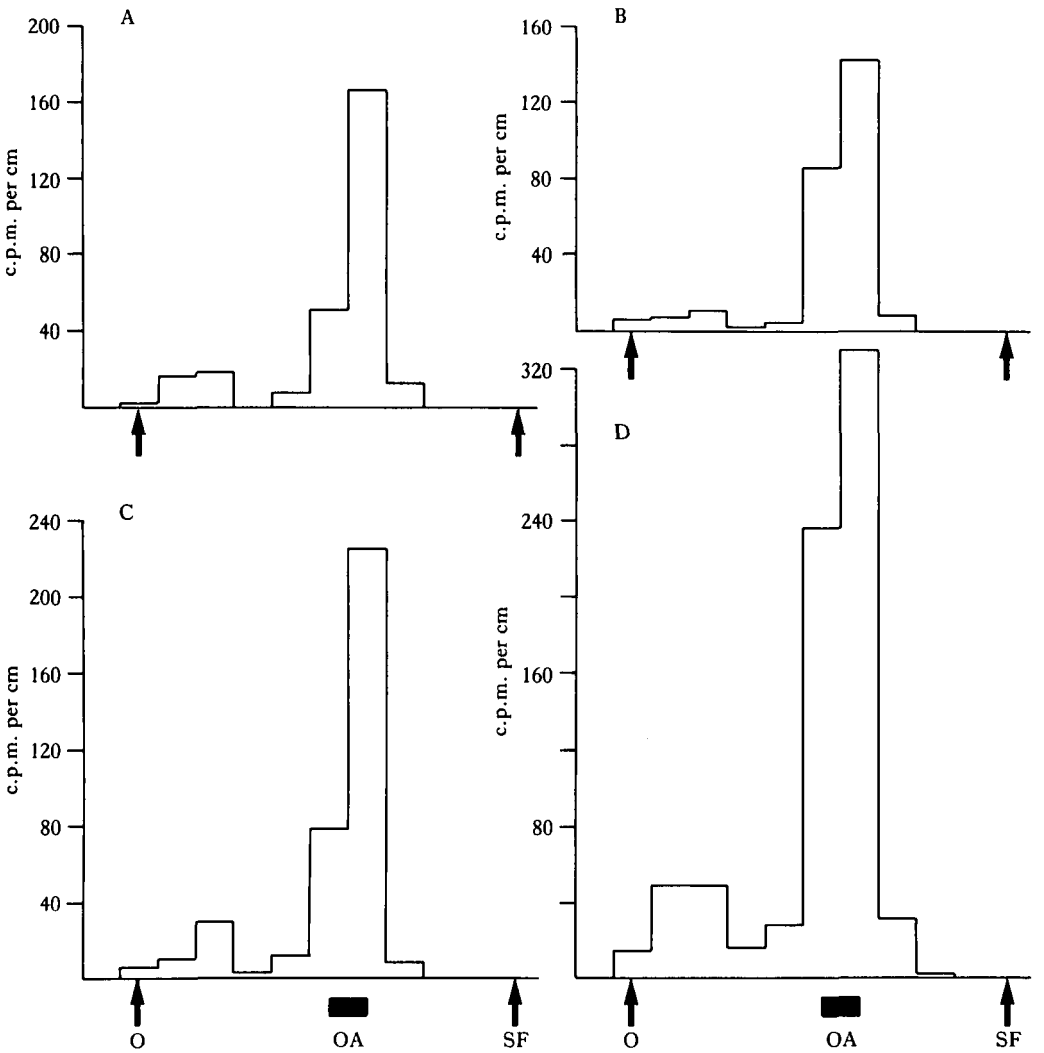

Fig. 3. TLC of the eluent from an ion exchange column loaded with the efflux from the extensor-tibiae muscle incubated in [^{3}H]-octopamine shown in Fig. 2. (A), (B) Background efflux. (C), (D) Efflux in response to 60 mM-K^+ saline. Solvent system *n*-butanol saturated with 10 % $MeNH_2$. SF, solvent front; OA, octopamine; O, origin.

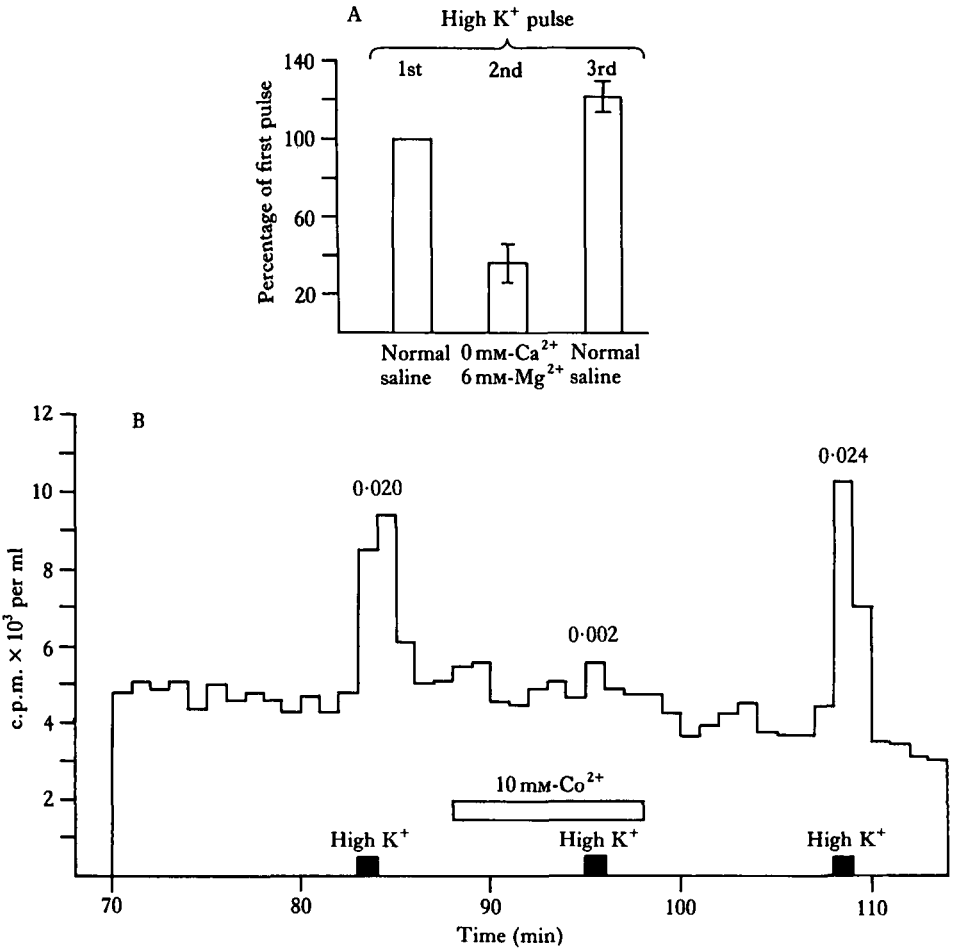

Fig. 4. Calcium dependence of 60 mM-K^+ induced release of radioactivity from extensor-tibiae muscles incubated in [^{3}H]-octopamine. (A) Effect of replacing Ca^{2+} with Mg^{2+} on the second of three high K^+ pulses. Each pulse is expressed as a percentage of the first pulse (taken as 100 %). Values indicate mean ± S.E.M. ($N = 4$). Timing of pulses as in Fig. 2. (B) Effect of 10 mM-Co^{2+} (open bar) on the second of three high K^+ pulses (black squares). Numbers above the peaks refer to the fractional release of radioactivity.

is probably from *N*-acetyloctopamine. Of the radioactivity retained by the column and eluted with ammonium hydroxide at least 80 % co-migrated with octopamine in TLC (Fig. 3). When corrected for the recovery of [^{3}H]-octopamine the background efflux and the high K^+ released radioactivity both contained 27 ± 2 % octopamine (mean ± S.E.M., $N = 5$). These values are very similar to the 23 ± 2 % octopamine remaining in the muscle after superfusion. In addition, when the muscle is incubated and perfused in the presence of 5×10^{-5} M-harmol the percentage of octopamine in both the background efflux and the high K^+ samples is increased in parallel to 34 ± 3 % (mean ± S.E.M., $N = 4$). The results indicate that both the labelled octopamine and *N*-acetyloctopamine are either located in the same cellular compartment or released simultaneously to the same extent from two different sites.

The release of radioactivity from the extensor-tibiae muscle preparation after

incubation in [^{3}H]-octopamine is sensitive to the extracellular calcium ion concentration (Fig. 4). The high K^+ induced release of radioactivity is markedly reduced when all Ca^{2+} ions are replaced by Mg^{2+} ions (Fig. 4A), and reduced to one-tenth of control value in the presence of 10 mM-Co^{2+} ions, which competitively block the entry of Ca^{2+} (Fig. 4B).

Another characteristic of depolarization-induced release of neurotransmitter is that in response to a prolonged depolarization, such as a long, high K^+ pulse, the rate of transmitter release drops from an initial peak to a lower plateau although the membrane remains depolarized (see Evans *et al.* 1976). The potassium-mediated release of radioactivity induced by a 10-min high K^+ pulse declines from an initial peak to a lower level after about 4 min, where it remains despite the continued presence of the elevated potassium saline in the superfusate (Fig. 5).

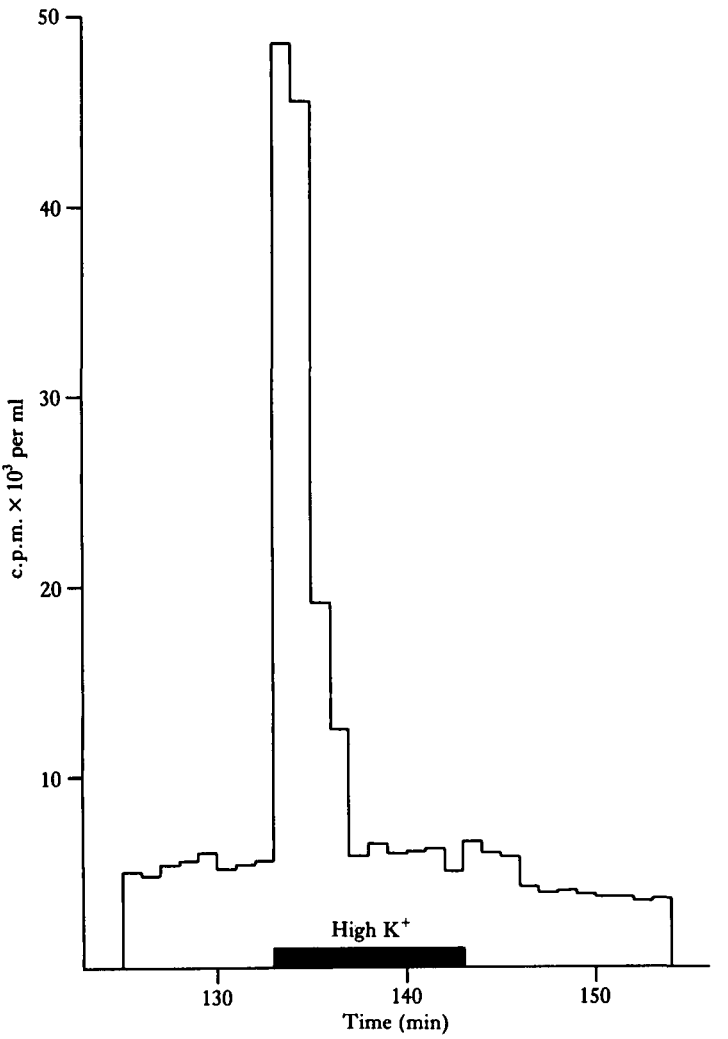

Fig. 5. Effect of prolonged 60 mM-K^+ pulse (black bar) on the efflux of radioactivity from an extensor-tibiae muscle incubated in [^{3}H]-octopamine for 30 min.

Neurotransmitter release can also be induced by increasing the intracellular sodium ion concentration of nerve terminals, for instance by use of the alkaloid veratridine which has been suggested to enhance the sodium permeability of membranes by preventing inactivation of sodium conductance (see Narahashi, 1974). The resulting depolarization activates calcium entry and causes an increased release of transmitter (Paton, 1979). If the extensor-tibiae muscle is superfused with a 1 min superfusion of veratrine ($6\,\mu g\,ml^{-1}$, approx. 10^{-5} M; Sigma, a mixture of alkaloids including veratridine) a large efflux of radioactivity is produced with a fractional release of $0{\cdot}09 \pm 0{\cdot}05$ (mean ± S.E.M., $N = 3$). A second pulse of veratrine produces a much smaller peak, which is only $6 \pm 3\,\%$ (mean ± S.E.M., $N = 3$) of the initial peak. This suggests that, in contrast to the high K^+ pulses used above, a single pulse of veratrine at this concentration can deplete the releasable pool of radioactivity.

The role of muscle contraction in the high potassium mediated release of radioactivity

Although the above properties of the high potassium mediated release are all characteristic of the release of a transmitter from its nerve terminals, the possibility still remains that the effect does not result from a specific release of octopamine from the terminals of the DUMETi neurone. The radioactivity could, for example, be released from another cellular compartment such as glial cells or muscle fibres that have also taken up the labelled octopamine. In addition it could be due to the high K^+ evoked contraction of the muscle allowing access to a further pool of extracellular radioactivity which is then washed from the muscle by superfusion. The latter possibility was tested by stimulating the slow motoneurone (SETi) independently, without stimulating the octopaminergic neurone DUMETi (for details see Evans & O'Shea, 1978). Three discrete peaks of radioactive release are produced by the three periods of SETi stimulation (see Fig. 6). Each peak has a fractional release value, and a time course similar to that caused by the subsequent application of a pulse of high K^+ to the same preparation. Further, continuous stimulation of SETi at 1 Hz throughout the entire experiment failed to prevent a large peak of released radioactivity being induced by subsequent stimulation for 1 min at 20 Hz. A similar result is seen if the above experiments are repeated using stimulation of the fast motoneurone (FETi) to the extensor-tibiae muscle. Direct measurements of the trapping of extracellular radioactivity using [^{3}H]-inulin as an extracellular tracer (not shown) reveal that this is very low under the experimental conditions used and that its release into the superfusate is not increased by SETi stimulation.

The increased efflux of radioactivity in the above preparations induced by both high K^+ depolarization and by SETi stimulation is likely to be the result of release from intracellular sites. Therefore whilst some of the radioactivity released from the preparation in response to high K^+ undoubtedly originates in the terminals of the DUMETi neurone, it is likely that this may be swamped by a large release of radioactivity induced by the depolarization of the glia or muscle fibres. Thus the specific release of radiolabelled octopamine from the terminals of DUMETi can only be demonstrated by the specific stimulation of the DUMETi neurone.

Effect of stimulating DUMETi

A number of different incubation conditions and stimulus regimes were employed in an attempt to demonstrate an increase in the rate of release of radioactivity in

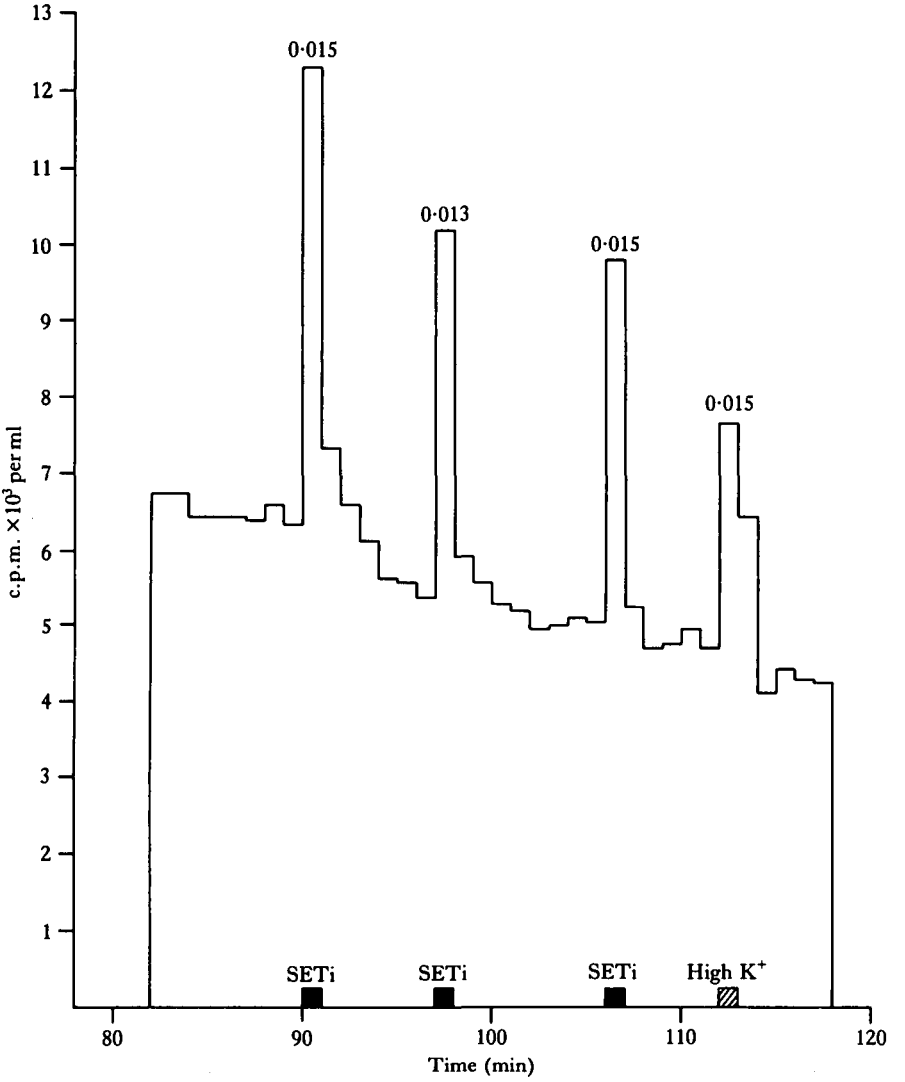

Fig. 6. Effect of repeated stimulation of SETi (black squares) followed by a single pulse of 60 mM-K^+ saline (hatched square) on the efflux of radioactivity from an extensor-tibiae muscle incubated in [^{3}H]-octopamine for 30 min and then superfused for 90 min with saline. SETi was stimulated for three 1-min periods at 20 Hz. Numbers above the peaks refer to the fractional release of radioactivity.

response to DUMETi stimulation. Stimulation of DUMETi either for 1 min at 5, 7 and 10 Hz or for 10 s at 10 Hz with 10 s rest periods for a period of 2 min, produces no significant increase in the efflux rate. Reducing the perfusion rate to 1 ml/5 min and stimulating DUMETi for a 5-min period at 4 and 7 Hz continuously, or with periods of rest, also fails to produce an increase. Further, attempts to block any inhibitory presynaptic octopamine receptors on DUMETi terminals by the addition of 5×10^{-5} M-phentolamine, a potent blocker of octopamine receptors in locusts (see Evans, 1981; Morton, 1984), also fails to reveal such a release as does the addition of 10^{-4} M-yohimbine to the incubation and superfusion salines. In addition the

Table 1. *Release of octopamine from the extensor-tibiae muscle by high K^+ saline measured by radioenzyme assay*

	Normal K^+	High K^+
A. Normal Ca^{2+} saline ($N = 10$)	0·69 ± 0·08	1·20 ± 0·20
B. 0 mM-Ca^{2+}, 6 mM-Mg^{2+} saline ($N = 3$)	0·50 ± 0·16	0·50 ± 0·04
C. Normal Ca^{2+} saline + 10 mM-Co^{2+} ($N = 4$)	0·44 ± 0·06	0·34 ± 0·01
D. Normal Ca^{2+} saline + 10^{-4} M-phentolamine ($N = 4$)	0·92 ± 0·38	2·45 ± 0·74

The results are expressed as pmol of octopamine released per muscle per 3 min and are given as mean ± S.E.M. In A, the increased release of octopamine in the high K^+ saline is significant to $P = 0·005$ using a Wilcoxon matched pairs signed ranks test.

presence in the superfusate of 10^{-4} M-desimipramine, a potent blocker of octopamine uptake in cockroach nerve cord (Evans, 1978*b*), again does not result in a significant increase in the DUMETi-mediated efflux of radioactivity.

It seems very likely that our failure to demonstrate an increase in the release of radiolabel from the extensor-tibiae muscle preparation by stimulating DUMETi is due to a high background of efflux of label from the muscle fibres (although unstimulated). Thus any neurally stimulated release of radiolabel from the much smaller compartment of the DUMETi terminals, would be swamped by the much larger release of radioactivity from the muscle fibres. Thus we examined the release of endogenous octopamine from the DUMETi terminals using a radioenzymatic assay.

Release of octopamine measured by radioenzymatic assay

In initial experiments, the release of endogenous octopamine from the extensor-tibiae muscle was induced using high K^+ saline. Since the terminals of the DUMETi neurone are the only known octopamine containing structures in this muscle, it is likely that they will represent the sole release site for endogenous octopamine upon depolarization by pulses of high K^+. This contrasts with the radiolabelling experiments described above where the high K^+ mediated depolarization releases radioactivity from additional intracellular sites. Radioenzymatic determinations of the amounts of octopamine released into incubation saline containing normal or high levels of K^+ are shown in Table 1. In saline containing normal calcium levels, high K^+ increases the release of endogenous octopamine in nine out of ten pairs of samples.

The high K^+ induced release of endogenous octopamine is calcium sensitive. The replacement of calcium ions in the saline by magnesium ions (Table 1B) or the addition of 10 mM-cobalt ions to saline containing normal calcium levels (Table 1C) blocks the high K^+ mediated release of octopamine. In addition, calcium-free saline, and to a greater extent the presence of cobalt ions, reduce the background efflux of octopamine.

Fig. 7. DUMETi-stimulated release of octopamine from the extensor-tibiae muscle. The release is expressed as pmol of octopamine released per muscle per 3 min (mean ± S.E.M. of at least four determinations). DUMETi was stimulated at 7 Hz over a 3-min period – three 30-s periods of stimulation each separated by a 30-s period of rest (black bars). (A) Release in standard saline. (B) Effect on release of replacing calcium with magnesium. (C) Effect of 10^{-4} M-phentolamine.

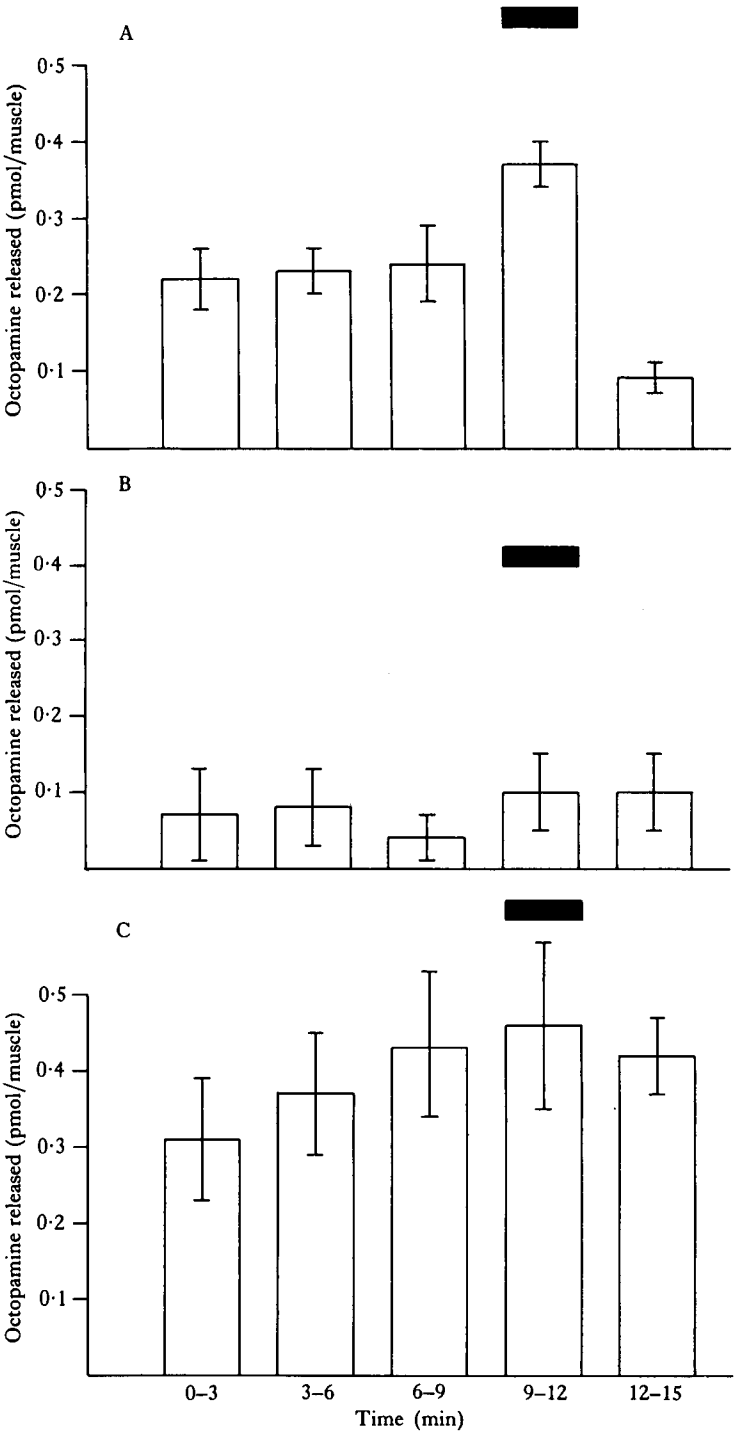
A
B
C
Octopamine released (pmol/muscle)
0·5
0·4
0·3
0·2
0·1
0–3
3–6
6–9
9–12
12–15
Time (min)

Fig. 7

Phentolamine, a blocking agent of octopamine receptors (see Evans, 1981; Morton, 1984), increases the amount of octopamine released by high K^+ saline (Table 1D). Phentolamine (10^{-4} M) increases the difference between the release in normal K^+ saline and that in high K^+ saline by a factor of three. In addition it also increases the basal spontaneous release in one out of four preparations tested. The effect of phentolamine could be explained by the presence of inhibitory octopaminergic autoreceptors on the terminals of the DUMETi neurone, which would normally be activated by released octopamine to feed back and reduce further release.

The release of endogenous octopamine from the extensor-tibiae muscle can also be demonstrated in response to stimulation of the DUMETi neurone. Fig. 7A shows the pooled results obtained for four groups each of six individual muscles incubated in five different changes of saline, the results being expressed as the number of pmol of octopamine released during each 3-min incubation period per muscle. During the fourth incubation period DUMETi is stimulated at 7 Hz for three periods of 30 s, each followed by a rest period of a further 30 s, and the amount of octopamine released is significantly increased. In the period immediately following the DUMETi stimulation the basal release of octopamine is significantly lower than in the three control periods prior to the DUMETi stimulation period.

The effect of DUMETi stimulation is frequency dependent. If DUMETi is stimulated at 4 Hz only one out of four preparations show an increased release of octopamine. At 10 Hz three out of four preparations show an increase but in the pooled data the release during the DUMETi stimulation period is not significantly higher than that observed in the control periods. This reduction of the effectiveness of DUMETi stimulation at higher frequencies is probably due to the failure of the conduction of antidromic impulses across the T-junction of the neurone (see Heitler & Goodman, 1978). Further evidence for this is provided by the observation that the frequency response curve for the modulation of neuromuscular transmission by DUMETi stimulation levels off at frequencies higher than 7 Hz (Morton, 1983).

The calcium dependence of the DUMETi-stimulated release of octopamine can be shown by replacing calcium ions with magnesium ions (Fig. 7B). In this case DUMETi stimulation at 7 Hz fails to increase the amount of octopamine released during the fourth stimulation period. In addition the calcium-free saline also reduces the background spontaneous release of octopamine from the extensor-tibiae muscle during the control periods.

Phentolamine (10^{-4} M), the octopamine receptor blocking agent that causes a large increase in the high K^+ mediated release of endogenous octopamine, however, has no significant effect on the DUMETi-stimulated release (Fig. 7C). This could be due to the smaller amounts of octopamine released by the latter method. This was despite the fact that it significantly increased the spontaneous background release compared to that found in control experiments. In contrast to the findings of Goosey & Candy (1982) on the enhancement of the neural release of octopamine from the locust half thorax, we were unable to demonstrate an effect of 10^{-4} M-yohimbine on either the background or DUMETi-stimulated release of octopamine from the extensor-tibiae muscle preparation.

DISCUSSION

The results of the present study demonstrate directly the release of octopamine from an identified octopamine-containing neurone in the locust. Electrical stimulation of the DUMETi neurone increases the amount of endogenous octopamine released by a mechanism that is calcium sensitive. The amount of octopamine released depends upon the frequency of stimulation, being much less at 4 Hz and 10 Hz than at 7 Hz. This finding agrees well with previous observations that the effects of stimulating the branch of DUMETi in the contralateral leg produce effects in the ipsilateral leg, such as modulation of SETi neuromuscular transmission (Morton, 1983; P. D. Evans, unpublished), number of DUMETi action potentials recorded (Morton, 1983) and changes in cyclic AMP levels (Evans, 1984*a*) which peak between 7 and 10 Hz and decline at higher frequencies. This seems likely to be due to a failure of the conduction of antidromic impulses across the DUMETi 'T-junction' at these frequencies (Heitler & Goodman, 1978). Previous demonstrations of octopamine release in response to electrical stimulation have involved the gross electrical stimulation of a preparation consisting of one half of a locust thorax (Goosey & Candy, 1982) and the stimulation of the motor-ligamental nerve to the lobster pericardial organ (Sullivan, Friend & Barker, 1977). In the latter study the release of [^{3}H]-octopamine, synthesized in the preparation from [^{3}H]-tyramine, was followed and was also found to be frequency dependent and calcium sensitive.

Depolarization of the octopamine-containing terminals of the DUMETi neurone in the extensor-tibiae muscle by use of pulses of high K^+ containing saline also increases the amount of octopamine released. This high K^+ release is again calcium sensitive and in addition it can be shown to be blocked by cobalt ions, which compete for calcium entry sites. The properties of this high K^+ mediated release system for octopamine in the locust are very similar to those demonstrated for the release of [^{3}H]-octopamine synthesized in other preparations from either [^{3}H]-tyrosine or [^{3}H]-tyramine. These included octopamine release from the endings of neurones in the second thoracic roots (Evans, Talamo & Kravitz, 1975; Evans *et al.* 1976) and pericardial organs of lobsters (Evans *et al.* 1976; Sullivan *et al.* 1977), and also from the efferent fibres to the lateral and ventral eyes of *Limulus* (Battelle, Evans & Chamberlain, 1982).

Phentolamine, a potent blocker of octopamine receptors in locusts (Evans, 1981; Morton, 1984), increases both the background and high K^+ stimulated release of endogenous octopamine. In contrast to the results of Goosey & Candy (1982) yohimbine had no effect on the release of octopamine in the present study. The actions of phentolamine could be explained by the presence of inhibitory autoreceptors for octopamine on the DUMETi terminals. Stimulation of these receptors by released octopamine would act as a feedback mechanism to control the subsequent release from the terminals. Similar autoreceptors have been proposed to mediate regulatory actions on the amine-containing neurones in the second thoracic roots of the lobster (Konishi & Kravitz, 1978) and in vertebrate sympathetic nerve terminals (see Langer, 1979). An alternative mode of action for phentolamine would be that it in some way inhibits an inactivation mechanism for octopamine in the muscle, such as an enzymatic process or an uptake mechanism, and thus increases octopamine washout from the

preparation. Gerschenfeld *et al.* (1978) could only demonstrate a neurally-mediated release of 5-HT from giant cerebral neurones of *Aplysia*, if chlorimipramine, a 5-HT uptake blocker, was present in the medium. However, desimipramine, a potent blocker of the high-affinity sodium-dependent uptake of octopamine into cockroach nerve cord (Evans, 1978*b*), had no effect on the release of labelled octopamine in the present study and does not appear to potentiate the modulatory actions of octopamine in this preparation (P. D. Evans, unpublished). Nevertheless, phentolamine does appear to be a potent blocking agent of the sodium-insensitive component of octopamine uptake into cockroach nerve cords (Evans, 1978*b*). This latter uptake system has not yet been examined in the locust extensor-tibiae neuromuscular preparation.

The depression of the basal levels of release of octopamine from the locust extensor-tibiae muscle in the presence of cobalt ions suggests that in normal saline there is a low level of spontaneous activity in the DUMETi neurone. A similar action of cobalt has been noted on the spontaneous release of 5-HT from *Aplysia* ganglia (Gerschenfeld *et al.* 1978) and of acetylcholine at the frog neuromuscular junction (Weakley, 1973). In this connection it is interesting to note that Hoyle & Dagan (1978) have reported that sporadic firing of DUMETi occurs at very low frequencies when locusts are at rest. The total amount of octopamine released during a high K^+ pulse experiment is frequently the same, or in some cases larger than the amount of endogenous octopamine measured in the locust extensor-tibiae muscle (approx. 1–4 pmol/muscle) (Morton & Evans, 1983). This could result from an increased synthesis of octopamine initiated as a result of its release. In similar studies on the release of acetylcholine from cat sympathetic ganglia Birks & MacIntosh (1961) showed that four to five times more transmitter is released than is initially present in the tissue. The release was induced by electrical stimulation in the latter study. However, more recently Collier, Kwok & Welner (1983) have shown that this extra acetylcholine synthesis is only initiated by neuronal stimulation and not by high K^+ saline. At present differences in the synthetic rate of octopamine in insect skeletal muscle after electrical stimulation of DUMETi, and after high K^+ saline pulses, have not been examined.

The present study has also examined the release characteristics of octopamine after incubation of the extensor-tibiae muscle in the presence of [^{3}H]-octopamine. This technique has frequently been used to study transmitter release in the past (see Introduction) and assumes that by analogy with the vertebrate sympathetic nervous system, other aminergic transmitters can be taken up into the nerve terminals that normally release them (see Iversen, 1967). However, it needs to be emphasized that the cellular location of the octopamine taken up and of its metabolites are unknown and could be located in glial, neuronal and muscle compartments as well as in the DUMETi terminals. High K^+ pulses release radioactivity from such preparations in repeated peaks that have the same fractional release values. When analysed the high K^+ released radioactivity contains the same proportion of octopamine (27 %) and *N*-acetyloctopamine as the spontaneously released radioactivity, the activity released by veratrine and the radioactivity left in the muscle after washout of the incubation medium. This contrasts with the release of noradrenaline from dog saphenous vein preparations incubated in [^{3}H]-noradrenaline. Here the spontaneous efflux contain

8·2% noradrenaline and the efflux induced by electrical stimulation contains 39% noradrenaline (Brandao, Paiva & Guimaraes, 1980). This difference could be due to differences in high K^+ and nerve-stimulated release as the *N*-acetyloctopamine could be taken up in glial cells where it could also be released in response to high K^+ saline. An alternative explanation is that both the octopamine and the *N*-acetyloctopamine are taken up by the same mechanism into the same cells and released in unison. It has been suggested that *N*-acetyldopamine and dopamine may be stored in vesicles in certain neurones (may be the same or different) in the cockroach nervous system as they are depleted in parallel by reserpine (Murdock & Omar, 1981). If a similar effect occurred for octopamine it could explain the similarity in the proportions of octopamine and its *N*-acetylated derivative in the spontaneous and high K^+ saline efflux, and in the total tissue radioactivity.

The high K^+ induced release of radioactivity from the extensor-tibiae muscle is sensitive to the extracellular calcium ion concentration, as is the release of other neurotransmitters (Katz & Miledi, 1965). In the presence of a prolonged pulse of high K^+ saline this release is inactivated, in a similar way to the release of octopamine from lobster second thoracic roots (Evans *et al.* 1976) and the release of insect diuretic hormone (Maddrell & Gee, 1974). In all these cases it seems likely that the release declines in the prolonged presence of the potassium depolarizing pulse due to an inactivation of the inward calcium channels (Baker, Meves & Ridgeway, 1973).

In the locust extensor-tibiae muscle a large proportion of the efflux of radioactivity seen under the conditions used in the present study would appear not to be released from the DUMETi terminals. Very similar amounts of radioactivity can be released, with a similar time course to high K^+ saline release, by causing the muscle to contract by selectively stimulating either the slow or the fast motoneurones. The increased efflux of radioactivity could be a result of the mechanical movement of the muscle allowing the perfusing saline better access to the extracellular spaces for washing. Alternatively the depolarization of the muscle and glial cell membranes caused by motoneurone stimulation could cause an increased washout of metabolites. Both high K^+ saline and veratrine will cause the muscle to contract and the high K^+ saline induced contraction will also be sensitive to the extracellular calcium ion concentration. The difficulty in fully removing the source of this radioactivity by washing and continuously stimulating the motoneurone would seem to indicate that it is not extracellular. Instead the [^{3}H]-octopamine could be taken up into glial cells or muscle fibres where it is *N*-acetylated and then gradually released, maybe as the result of the depolarization of the membranes. Some of the [^{3}H]-octopamine could nevertheless be taken up into the DUMETi terminals but its subsequent release swamped by the nonspecific release of radioactivity by high K^+ saline. Attempts to demonstrate a significant release of any [^{3}H]-octopamine from the DUMETi terminals by selective stimulation were not successful under any of the conditions investigated in the present study. This could again be due to a very small amount of stimulated release being lost in the noise level of a high background release of radioactivity from the preparation.

Although it has not been possible to demonstrate the release of octopamine from DUMETi using radiolabelled compounds these experiments do serve to illustrate the problems of studying release of transmitters in this way from neuromuscular preparations. There are at least three major problems: (1) the cellular location of the labelled

compounds is unknown; (2) high K^+ saline will possibly cause release from other cells; (3) the contraction of the muscle could cause nonspecific release of radioactivity from the muscle. A parallel set of difficulties have been pointed out in connection with studies using [^{3}H]-5-HT to study release from nervous tissue (Ascher, Glowinski, Tauc & Taxi, 1968; Gerschenfeld *et al.* 1978). The present study emphasizes that further problems may be experienced in preparations due to muscle contractions.

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