The effect of opiates on the terminal nerve impulse and quantal secretion from visualized amphibian nerve terminals

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1 Secretion of transmitter from amphibian motor nerve terminal release sites is intermittent, spatially non-uniform and varies considerably throughout the year and during development. The role of opioid receptors in modulating transmitter secretion from amphibian motor nerve terminals is evaluated in this study.

2 Dynorphin-A (24 μM) and morphine (500 μM) did not significantly change the shape of the nerve impulse or the consistency with which it was observed, but decreased evoked quantal secretion by more than 50%. These effects of dynorphin-A and morphine were largely reversed by naloxone (50 μM).

3 Dynorphin-A and morphine did not significantly change either the amplitude or the frequency of spontaneous quantal secretions.

4 There was a uniform decrease in evoked quantal secretion from release sites along terminal branches, irrespective of the quantal content value before drug treatment, indicating no difference in the susceptibility of proximal vs distal release sites to opiates.

5 Increasing the extracellular calcium concentration (0.3 to 0.4 mM) or trains of conditioning-test impulses (25 to 100 Hz) resulted in smaller dynorphin-A or morphine-induced decreases in evoked quantal secretion.

6 The decrease in evoked quantal secretion occurs as a result of a uniform decrease in the probability of quantal secretion from release sites without any affect on the propagation of the nerve terminal impulse. Low probability release sites become effectively silent.

Keywords: Neurotransmission; action potential; opiates; amphibian nerve terminals

Introduction

At the amphibian motor nerve terminal the nerve impulse, evoked and spontaneous quantal secretion can be recorded simultaneously with an extracellular electrode if placed within 10 μm of the release sites (del Castillo & Katz, 1956). Using this method, secretion of quanta from small groups of release sites has been shown to be intermittent and spatially non-uniform (Bennett & Lavidis, 1979; D'Alonzo & Grinnell, 1985). The release sites found closest to the point of first axon contact with the muscle fibre membrane have a higher average probability of quantal secretion than more distal release sites (Bennett & Lavidis, 1982; Bennett *et al.*, 1986). Furthermore within the field of recording of the extracellular electrode it was shown by Bennett & Lavidis (1989) that very low probability release sites exist amongst the relatively high probability sites. The probability of quantal secretion of release sites in amphibian motor nerve terminals is modulated during development (Bennett & Lavidis, 1988) and by seasonal factors (Bennett & Lavidis, 1992). For example, during development, approximately 25% of muscle fibres receive dual innervation from adjacent segmental motor nerves, usually one of these terminals has release sites with relatively much lower probabilities of quantal secretion than the other terminal innervating the same muscle fibre (Bennett & Lavidis, 1988). Furthermore, the average probability of quantal secretion of release sites varies by as much as eight fold between late summer and late autumn in the southern hemisphere (Bennett & Lavidis, 1992). Some endogenous chemicals have been shown to produce an affect on quantal secretion from release sites such as, α -melanocyte stimulating hormone (Oren *et al.*, 1987) and adenosine (Ginsborg & Hirst, 1972; Ribeiro & Walker, 1975; Bennett *et al.*, 1991). Opiates such as dynorphin, which is found in relatively high concentrations in brain and spinal cord of *Bufo marinus* (Cone & Goldstein, 1982), or dermorphine found on the skin of frogs (Montecucchi *et al.*, 1981), may act as modulators of quantal secretion by de-

creasing the probability of quantal secretion from release sites. The effects of dynorphin-A, morphine and dermorphine on the secretion of quanta from release sites innervating toad (*Bufo marinus*) iliofibularis muscle fibres have been evaluated in the present study.

At the amphibian neuromuscular junction, high concentrations of morphine (500 μM) produced a decrease in the amount of acetylcholine secreted from cholinergic terminals during nerve stimulation (Frederickson & Pinsky, 1971). Methionine enkephalin has also been shown to reduce the number of quanta secreted during sympathetic ganglion transmission (Hirai & Katayama, 1988) and modulate motor neurone activity in the spinal cord of the bullfrog *Rana catesbeiana* (Suzuki *et al.*, 1987). Also high concentrations of methionine-enkephalin (20 μM) decrease quantal secretion from the release sites of *Rana pipiens* (Bixby & Spitzer, 1983). How these opiates decrease quantal secretion from release sites is not fully understood. Two main mechanisms of how opiates decrease quantal secretion have been proposed. First, in most neuronal cell bodies studied, opiates have been shown to increase the activity of potassium channels resulting in either hyperpolarization of the cell or shortening the duration of the action potential (Pepper & Henderson, 1980; Morita & North, 1982; North & Williams, 1983; Werz & MacDonald, 1985). Second, an alternative mechanism has been proposed from studies conducted on nerve terminals such as the sympathetic varicosities of the mouse vas deferens (Bennett & Lavidis, 1980; Illes *et al.*, 1980) and the amphibian motor nerve terminal (Bixby & Spitzer, 1983). In these preparations, indirect evidence such as overcoming the inhibitory affect of morphine by increasing $[\text{Ca}^{2+}]_o$ and local depolarization of release sites suggest that opiates inhibit a calcium-dependent process rather than conduction failure of the nerve impulse during nerve stimulation. Opiates have been shown to decrease the opening times of calcium channels in dorsal root ganglion cells (Werz &

MacDonald, 1985; MacDonald & Werz, 1986; Gross & MacDonald, 1987). Since we are able in this preparation to record both the nerve impulse and evoked quantal secretion it is possible to test whether the nerve impulse is affected during the decrease in quantal content which occurs when opiates are applied.

Methods

Housing of animals

Toads (*Bufo marinus*) ranging in length from 55 to 70 mm from tail to nose were maintained in a room which was fitted with 15% ultra violet lights. Lights were turned on for 16 h per day. The temperature of the room was maintained between 25° and 30°C and the animals were fed 2 times per week with a mixture of minced meat and fish brittle. Even under these environmental conditions animals still displayed seasonal changes consisting of a uniform decrease in the probability of quantal secretion from release sites during the period May to November (Southern hemisphere; Bennett & Lavidis, 1992).

Tissue preparation

Animals were anaesthetized with tricaine methanesulphonate (MS222, Rural Chemical Industries Australia) and then killed by a cervical fracture. The right iliofibularis muscle with its nerve supply was dissected from its surrounding connective tissue and cut at its tendinous insertions at the pelvis and knee. The muscle was then pinned to the Sylgard covered base of a 3 ml organ bath with the surface where the nerve enters the muscle facing up. The muscle was stretched to approximately 110% of its resting length in the limb to form a flat parallelogram. The preparation was continuously perfused at a rate of 5 ml min⁻¹ with a modified Ringer solution containing (mM): NaCl 99.4, KCl 3.0, MgCl₂ 1.2, NaH₂PO₄ 1.3, NaHCO₃ 16.3, CaCl₂ 0.3 to 0.4, glucose, 7.8. The temperature of the bath was maintained between 12° and 16°C. The reservoir supplying the bath was continuously gassed with 95% O₂ and 5% CO₂, and the pH was maintained between 7.2 and 7.5. The extracellular calcium concentration ([Ca²⁺]_o) was changed by altering the amount of CaCl₂ dissolved in the Ringer solution supplying the bath. Possible changes in the conduction of the nerve impulse were avoided by maintaining the external magnesium concentration above 0.7 mM (Frankenhauser & Hodgkin, 1957).

Visualization of the nerve terminal

The preparation was left bathing in the low [Ca²⁺]_o Ringer for about 40 min while contractions due to nerve stimulation diminished. The muscle was then bathed for 30 s in 3,3-diethylloxardicarbocyanine iodide (DiOC₂(5), 0.1 µM, Yoshikami & Okun, 1984) and then washed with Ringer for 10 min to label fluorescently accumulations of mitochondria corresponding to the nerve terminal branches. Excitation at 560 nm of the DiOC₂(5) resulted in a fluorescence which was visible using a Rhodamine filter set (Olympus BH2 microscope). Terminals were chosen by viewing the fluorescent image via an image intensifier camera (National) on a video monitor (National). The effects of exposing the neuromuscular junction to DiOC₂(5) and fluorescence have been previously described (Bennett *et al.*, 1986). Use of DiOC₂(5), 0.1 µM and 1 to 3 min excitation did not affect the nerve terminal impulse or evoked or spontaneous quantal secretion. To avoid long periods of fluorescing the preparation and the need for repeated applications of DiOC₂(5), the image of the fluorescing terminals was traced onto the video monitor screen at the outset and the fluorescence was turned off. The preparation was then illuminated by a tungsten filament lamp and other visible structures such as pigment cells, blood vessels and bundles of axons were drawn on the video monitor screen 'map'. The position of the

terminals with respect to such structures was rechecked before hunting with an extracellular electrode for the extracellular signs of the nerve terminal impulse or the endplate potential.

Stimulation

The iliofibularis nerve was gently sucked into a pipette filled with the Ringer solution up to a silver/silver chloride wire and stimulated with square wave stimuli of 0.08 ms duration and 8V amplitude. At least 200 samples of evoked release were recorded at 0.25 Hz for each electrode placement.

Recording

Extracellular recordings of the endplate potential give a good approximation of the time course of the synaptic current (Fatt & Katz, 1952; Del Castillo & Katz, 1956; Brock & Cunnane, 1988) and are therefore referred to as the endplate current (e.p.c.) in this study. E.p.cs were obtained with microelectrodes filled with NaCl (2 M) and having resistances of 10 to 30 MΩ. Focal extracellular recordings were obtained by positioning the electrode about 2 to 3 µm on either side of the terminal branch. The external signs of the endplate potential could then be observed on the oscilloscope screen while stimulating the nerve. In most cases small e.p.cs could be recorded within 20 impulses. The electrode was then lifted off the muscle fibre and moved in either direction along the same axis as the terminal branch until the source of these small e.p.cs was located (this procedure involved searching for the most active release site). The external electrode was then lifted off the muscle fibre and moved transversely to improve the recording of the nerve impulse. Two hundred samples of evoked release at a stimulation frequency of 0.25 Hz were recorded before lifting the electrode off the muscle and looking for another active site about 20 µm along the terminal branch.

Extracellular recordings were abandoned if: e.p.cs were mostly less than 1.0 mV in amplitude; presence of e.p.cs without detecting the nerve terminal impulse; any shift greater than 25% in quantal release rates over the first 100 trials as compared to the last 100 trials (see Bennett *et al.*, 1986); any change in the amplitude of the largest recorded e.p.c. at the end of the recording period compared to the beginning.

Avoidance of damage to the terminal by the recording electrode

To avoid the effects of electrode pressure on nerve terminals (Fatt & Katz, 1952) the microelectrode tip, clearly visible on the video monitor, was carefully positioned about 2 to 3 µm from the terminal branch as drawn on the video monitor following the hunting procedure previously described. The frequency of spontaneous miniature endplate current's (m.e.p.cs) was determined at different times, prior to the start of recording evoked release and after having recorded the 200 evoked trials. The frequency of m.e.p.cs normally varied between 0.02 to 0.3 Hz. If this frequency increased by more than 25% of the initial estimate the recording was abandoned (see Bennett *et al.*, 1986). The order of positioning the extracellular electrode at different groups of release sites was randomized along the nerve terminal branch to ensure that the peripheral parts of the branch did not have relatively lower evoked quantal secretion rates than more proximal sites, perhaps due to terminal damage by the recording electrode.

Data analysis

E.p.cs were recorded on an IBM-AT microcomputer using p-Clamp version 5.0 software (Axon Instruments). The interval between two quanta following an impulse had to be greater than 0.14 ms, otherwise they were measured as single quanta. To reduce the chance of this happening, the preparation was kept between 12° and 16°C and the [Ca²⁺]_o less than or equal to 0.4 mM. Selected records were measured by eye from time to

time to monitor the accuracy of the computer analysis, the difference was $\leq 7\%$. Histograms of the number of quanta released were constructed for the e.p.cs and only those which occurred within 5 ms of the negative peak of the nerve impulse were included. The region of recording was restricted to less than a $7\ \mu\text{m}$ radius by only counting e.p.cs with amplitudes $\geq 1.0\ \text{mV}$ (Del Castillo & Katz, 1956; Bennett *et al.*, 1986). Quantal content ($\bar{m}_e$) was determined from the ratio of the total number of quanta released to the number of trials.

Drugs

Drugs were dissolved in different 200 ml capacity reservoirs. Each reservoir was gassed with 95% O_2 and 5% CO_2 . Solutions supplying the preparation bath were changed by 3 way taps. Morphine hydrochloride and naloxone hydrochloride (gifts from the Department of Pharmacology, University of Sydney) were dissolved in distilled H_2O and kept refrigerated as stock solutions at a concentration of 10 mM. Dynorphin-A and dermorphine (Sigma) were dissolved in acetic acid ($40\ \mu\text{M}$) and aliquots placed in vials. Each vial contained dynorphin-A or dermorphine (0.1 ml at a concentration of 10 mM) and was stored at -70°C . Tissues were bathed in drug-containing solution for more than 40 min before recording. Following drug treatment tissues were washed for more than 40 min before determining whether the effects were reversible.

Results

Effect of morphine on $\bar{m}_e$ along motor nerve terminal branches

Extracellular recordings of the e.p.cs and m.e.p.cs (Figure 1) were taken from different positions along terminal branches. The visualized profile of terminals, following $\text{DiOC}_2(5)$ -fluorescence was used to draw the shape of the terminal branches on a video monitor and to determine the distance of the electrode from the point of first axon contact with the muscle. The average number of quanta secreted in the recording field of the electrode per nerve impulse ($\bar{m}_e$) was estimated by taking 200 trials from each position before and after the application of morphine or dynorphin-A to the preparation. Following morphine ($320\ \mu\text{M}$) there was a decrease in the frequency of evoked quantal secretions with an increase in the tails of the e.p.cs (compare in Figure 1, morphine-treated, traces 1 and 3 with control, traces 1 to 5). There was also an increase in the noise level following morphine treatment which lasted only for about 16 ms following the stimulus artefact. This increase in the duration of the e.p.cs and the increase in noise is probably due to the known anti-cholinesterase action of morphine (Haynes & Smith, 1982).

Along each of the four terminal branches studied there was a proximal to distal decline in $\bar{m}_e$ (Figure 2). For example in Figure 2a and b where $\bar{m}_e$ was determined at 8 to 12 μm intervals along the terminal branches, the maximum $\bar{m}_e$ recorded declined proximal-distally. Intercalated amongst the high probability release sites, low probability release sites were recorded (Figure 2a and b). If however, a different procedure (described in the Methods) is used to record from only the highest probability release sites in any region, a profile of the maximum $\bar{m}_e$ is obtained along terminal branches (Figure 2c and d). Of the 21 terminal branches studied, in 6 branches the maximum $\bar{m}_e$ did not significantly decrease along the length of the branches. Only one terminal branch showed an increase in the maximum $\bar{m}_e$ as the electrode was moved in the proximal to distal direction from the point of first nerve contact with the muscle. The rest of the terminal branches (14/21) showed a decline in the maximum $\bar{m}_e$ in the proximal-distal direction. The average decline in $\bar{m}_e$ for all the control terminal branches studied are shown in Figure 3a. There was a significant difference ($P < 0.01$) in the maximum $\bar{m}_e$ only for the distal 60% of the terminal branches (Figure 3a).

Figure 1 The effect of morphine on e.p.cs. Five consecutive nerve stimulations for control and morphine ($500\ \mu\text{M}$)-treated preparations are shown. The extracellular calcium concentration ($[\text{Ca}^{2+}]_o$) was $0.35\ \text{mM}$. Evoked quantal secretions can be seen in traces 1,2,3 and 5 (control) and 1 and 3 (morphine). There is an increase in the duration of the e.p.cs following morphine treatment (compare traces 1 and 3 (morphine) with traces 1,2,3 and 5 (control)). An increase in the noise level is also seen following the administration of morphine (morphine traces 2, 4 and 5), and the duration of this increase in noise is equivalent to the duration of the e.p.cs seen in traces 1 and 3 (morphine). The nerve terminal impulse is also evident, at the left of each recording.

Very high concentrations of morphine ($500\ \mu\text{M}$) but comparable to the concentration used by Frederickson & Pinsky (1971) decreased the frequency of evoked quantal secretions (Figure 1) uniformly along the whole length of the terminal branches (Figures 2a,c and 3a). The 47% decrease in $\bar{m}_e$ induced by morphine ($500\ \mu\text{M}$) was almost reversed by the application of naloxone ($50\ \mu\text{M}$). The ED_{50} for morphine on quantal secretion from amphibian nerve terminals was $510\ \mu\text{M}$ (personal observation). The averaged results of 9 preparations is shown in Figure 3a where the decrease in $\bar{m}_e$ is greater for the proximal site with the remainder of the decrease being uniform for at least 75% of the terminal branches. The relationship between the magnitude of decrease in $\bar{m}_e$ produced by morphine and position from the point of first axon contact with the muscle was determined (Figure 4a). Although there appeared to be a slightly greater percentage decrease in $\bar{m}_e$ for proximal release sites when compared to more distal release sites, the difference was not significant ($P > 0.05$). The relationship between the magnitude of decrease in $\bar{m}_e$ following morphine administration and initial $\bar{m}_e$ (pre morphine administration) was also determined (Figure 4c). Linear regression fit of the data appeared to indicate that the magnitude of the reduction in $\bar{m}_e$ following morphine application appeared to be greatest for high initial $\bar{m}_e$ s however again the difference was not significant ($P > 0.05$). Thus morphine reduces $\bar{m}_e$ along the terminal branches in a uniform manner.

Effect of dynorphin-A on $\bar{m}_e$ along motor nerve terminal branches

Extracellular recordings of the e.p.cs were taken from different positions along terminal branches before and after treatment

with dynorphin-A (Figure 2b and d). The visualized profile of terminals following DiOC₂(5)-fluorescence was used to draw the shape of the terminal branches on a video monitor and to determine the distance of the electrode from the point of first axon contact with the muscle. The $\bar{m}_e$ values at each extracellular electrode position were determined by recording 200 trials from each position before and after dynorphin-A administration. The two terminal branches shown in Figure 2b and 2d demonstrated a significant decrease in $\bar{m}_e$ for the distal half of the release sites. Dynorphin-A produced a relatively uniform decrease in $\bar{m}_e$ along the length of the two terminal branches shown in Figure 2b and 2d. Out of the 8 terminal branches studied, 2 showed no significant decline in $\bar{m}_e$ along the terminal branch while 6 showed a decline at least in the distal half of the terminal branches. Overall there was a significant decline in $\bar{m}_e$ ($P < 0.05$) level among the more distal halves of the terminal branches (Figure 3b).

While dynorphin-A (24 μM) reduced $\bar{m}_e$ along the length of most terminal branches studied, a significant decrease ($P < 0.05$) could be demonstrated only for the proximal region of the terminal branches (Figure 3b). The ED₅₀ of dynorphin-A on quantal secretion at amphibian motor nerve terminal

release sites was 24 μM (personal observation). The relationship between the magnitude of decrease in $\bar{m}_e$ produced by dynorphin-A and distance of release sites from the point of first axon contact with the muscle was evaluated (Figure 4b). Although there was a slightly greater decrease in $\bar{m}_e$ produced by dynorphin-A in the proximal part of the terminal branches studied, the difference was not significant ($P > 0.05$). Regression analysis of the relationship between the magnitude of decrease in $\bar{m}_e$ following dynorphin-A and the initial $\bar{m}_e$ (before dynorphin-A administration) also revealed no significant trend (see Figure 4d and legend). Thus dynorphin-A at a twenty fold lower concentration than morphine reduced $\bar{m}_e$ along the terminal branches in a fairly uniform manner. Naloxone (50 μM) reduced the dynorphin-A induced decrease in $\bar{m}_e$.

The effect of demorphine on $\bar{m}_e$ along nerve terminal branches

The effect of demorphine (83 μM) on $\bar{m}_e$ along four terminal branches was evaluated using the same procedures as described in the two previous sections. There was no significant differ-

Figure 2 The effect of morphine and dynorphin-A on quantal content ($\bar{m}_e$) along the length of nerve terminal branches. Traces of the DiOC₂(5)-fluorescent terminal branches were drawn onto the video monitor and from these measurements of the distance of electrode position from the point of first nerve contact with the muscle estimated. In (a) and (b) the extracellular electrode was placed at about 8 to 12 μm intervals along the length of the terminal branch before and after treatment with morphine (a) and dynorphin-A (b). In (c) and (d) the position of the extracellular electrode was optimized to record the secretion from the most active regions of a terminal branch before and after morphine (c) and dynorphin-A (d) treatment. $\bar{m}_e$ vs distance along the terminal branch measure from the point of first axon contact with the muscle was plotted for each terminal branch. (○) Control data; (●) data after morphine or dynorphin-A administration. The broken lines indicate a release rate less than one quanta per 20 nerve impulses ($\bar{m}_e < 0.05$).

Figure 3 The averaged effect of morphine (a) and dynorphin-A (b) on $\bar{m}_e$ along the lengths of several nerve terminals. The $\bar{m}_e$ determined at various positions along terminal branches, for control, morphine- and dynorphin-A-treated preparations, was normalized with respect to the maximum $\bar{m}_e$ value of the most proximal recorded site in the control preparation. The normalized values were then grouped into 25 μm bins along the length of terminal branches and the mean normalised $\bar{m}_e$ value and its standard deviation calculated. The effects of morphine, 500 μM (a) and dynorphin-A, 24 μM (b) are shown. (○) Means for the control preparations; (●) means for the morphine (a) and dynorphin-A (b)-treated preparations. There was a statistically significant ($P < 0.05$) difference in (a) between control and morphine-treated, and in (b) between control and dynorphin-A-treated for the proximal 75% of the terminals studied.

ence in the $\bar{m}_e$ s of untreated release sites vs treated release sites; the average difference was minus $8 \pm 9\%$ ($n = 4$).

The effect of changing $[\text{Ca}^{2+}]_o$ on $\bar{m}_e$ in control and morphine-treated preparations

The mean quantal content of the e.p.c. was first determined for 0.3 mM, $[\text{Ca}^{2+}]_o$ before raising $[\text{Ca}^{2+}]_o$ to 0.4 mM and re-determining $\bar{m}_e$. Morphine (500 μM) was then added to the bath containing 0.4 mM $[\text{Ca}^{2+}]_o$ and $\bar{m}_e$ estimated. Finally morphine was added to the 0.3 mM, $[\text{Ca}^{2+}]_o$ reservoir and $\bar{m}_e$ redetermined. In the six experiments shown in Figure 5, $\bar{m}_e$ increased by about the same amount (fourth power of $[\text{Ca}^{2+}]_o$) in the presence or absence of morphine. In control preparations the mean power relationship between $\bar{m}_e$ and $[\text{Ca}^{2+}]_o$ was 3.67 ± 0.55 (mean $\pm$ s.d.) while for morphine-treated preparations it was 3.64 ± 0.56 . the decrease in $\bar{m}_e$ of about 47% induced by morphine (500 μM) treatment was reduced by

increasing $[\text{Ca}^{2+}]_o$, there was however no significant difference between the gradient of the $\bar{m}_e$ vs $[\text{Ca}^{2+}]_o$ before and after morphine treatment ($P > 0.9$) indicating that the dependence of $\bar{m}_e$ on $[\text{Ca}^{2+}]_o$ was not changed by morphine.

The effect of dynorphin-A on the ability of the terminal to facilitate during conditioning-test impulses

Following a conditioning nerve impulse there is an increase (facilitation) in the amount of quanta secreted by a test impulse (Figure 6) or subsequent impulses in a train that declines as the interval between conditioning and test impulses increases. The time-constant of decline in facilitation of $\bar{m}_e$ by a test impulse was 30 ms. Dynorphin-A (24 μM) reduced $\bar{m}_e$ of the conditioning impulse by about 49% and this inhibition could be overcome if a test nerve impulse was applied shortly (time interval less than 60 ms) after the conditioning impulse. The time-constant of decay in facilitation was unchanged (34 ms) by dynorphin-A.

The effect of dynorphin-A or morphine on the nerve terminal impulse

Following stimulation of the iliofibularis nerve, nerve impulses are generated which propagate to the last node of Ranvier actively propagate for only about 1/3 the length of the terminal and then passively for the distal 2/3 of the terminal (Brigant & Mallart, 1982). The nerve impulse is clearly visible in the traces shown in Figure 6 even after treatment with dynorphin-A (24 μM) which halved evoked quantal secretion. Morphine and dynorphin-A did not alter the nerve impulse from any position recorded along terminal branches (Figure 7a and b). Each trace in Figure 7a represents the average of 200 trials, the stimulus artefact is shown (sa) followed by the nerve impulse (ni) and the extracellular signs of the endplate current (e.p.c). Neither the shape of the nerve impulse nor the consistency with which it was observed was changed (Figure 7a) following dynorphin-A (24 μM) or morphine (500 μM) treatment or after washing out the drug. There was a decrease in the average size of the e.p.c.s following dynorphin-A treatment with a partial recovery after washing or naloxone (50 μM). This decrease in the averaged e.p.c. amplitude was not caused by a postsynaptic effect since there was no reduction in the size of m.e.p.c.s (Figure 7c). The average size of m.e.p.c.s was: control, 0.91 ± 0.22 mV ($n = 105$, mean $\pm$ s.d.); dynorphin-A treated for 40 min, 0.84 ± 0.19 mV ($n = 57$); after washing out the drug for more than 40 min, 0.88 ± 0.18 mV ($n = 85$). Thus the decrease in e.p.c. amplitude was produced by a decrease in the number of quanta being released and an increase in the number of failures to release any quanta (Figure 7d). Thus, although there was no significant change in the size of the nerve impulse, morphine and dynorphin-A were able to produce a large decrease in $\bar{m}_e$.

Discussion

Is dynorphin contributing to the decrease in probability of quantal secretion along terminal branches?

In most terminal branches studied there was a decline in the number of quanta secreted along the proximal to distal axis of the terminal branch from the point of first axon contact with the muscle fibre (see also Bennett & Lavidis, 1982; D'Alonzo & Grinnell, 1985; Bennett *et al.*, 1986). This however was not always the case: six out of the twenty-one terminal branches studied did not show any decline, with $\bar{m}_e$ remaining uniform throughout the length of the branches. Indeed one out of twenty-one branches studied showed an increase. When the results were normalized with respect to the most proximal section of the terminal branch and results for all the terminal branches averaged there was a significant decline in the maximum $\bar{m}_e$ in the distal half of the terminal branches. The rea-

sons for this decline are not known but a number of possibilities have been considered which include: first, the area of the close apposition (<200 nm) between pre- and postsynaptic membranes decreases in the proximal to distal direction along terminal branches (Bennett *et al.*, 1987; 1989). If the maximum area of the close apposition between nerve terminal and muscle membrane is a measure of the largest active zones it could be argued that the larger active zones have a greater release area available for vesicle release and that this is the determinant of the secretion probability of release sites. Although a reason-

able correlation between close apposition length and $\bar{m}_e$ has been shown (Bennett *et al.*, 1989), the variation in close apposition lengths measured is not as great as the variation in $\bar{m}_e$ of release sites measured. Second, secretion of a quantum of acetylcholine and/or another cotransmitter (ATP) may act back on presynaptic membrane receptors to inhibit subsequent quantal secretions. This may restrict large active zones to secrete only one quantum per stimulus but with a higher probability of release. Although the release of ATP has been demonstrated (Silinsky, 1975) and that in its dephosphorylated

Figure 4 The relationship between percentage change in quantal secretion and either distance of release sites from the point of first axon contact with the muscle fibre or control quantal secretion ($\bar{m}_{e0}$). (a) Percentage change in $\bar{m}_e$ induced by morphine vs distance along terminal branch. Solid line fitted by linear regression to the data (■) has a slope of 0.28 and a correlation coefficient of 0.20. (b) Percentage change in $\bar{m}_e$ induced by dynorphin-A vs distance along terminal branch. Solid line fitted by linear regression has a slope of 0.39 and c.c. 0.22. (c) Percentage change in $\bar{m}_e$ induced by morphine vs control (pre drug) $\bar{m}_{e0}$. Slope of -0.16 and c.c. 0.08. (d) Percentage change in $\bar{m}_e$ induced by dynorphin-A vs control $\bar{m}_{e0}$. Slope of -1.00 and a c.c. 0.39. Dashed lines indicate no change in secretion.

form, adenosine binds to presynaptic purinoceptors to decrease the probability of quantal secretion (Ginsborg & Hirst, 1972; Ribeiro & Walker, 1975; Bennett *et al.*, 1991), addition of purinoceptor antagonists such as theophylline or 8-phenyltheophylline does not lead to any significant increase in quantal content or the variance of quanta secreted under the nerve stimulus frequency used by Bennett *et al.* (1991). Third, opiates such as dynorphin (Cone & Goldstein, 1982) and dermorphine (Montecucchi *et al.*, 1981) have been shown to exist in amphibia. Relatively high concentrations of morphine (Frederickson & Pinsky, 1971; Bixby & Spitzer, 1983) and Met-enkephalin (Bixby & Spitzer, 1983) have been shown to decrease transmitter secretion from amphibian motor nerve terminals. Dynorphin-A and morphine have also been shown to decrease quantal content and the variance in the number of

quanta secreted. This has been interpreted as a decrease in the number of release sites secreting during a nerve impulse since the average probability of quantal secretion calculated from intracellular recordings remained constant (personal observation). As demonstrated here, this decrease in quantal content is due to a uniform decrease in $\bar{m}_e$ along the length of terminal branches irrespective of their initial $\bar{m}_e$. Since the probability of secretion of release sites varies greatly within the field of the extracellular recording electrode (about 7 μm radius), release sites which initially have low probabilities of quantal secretion become effectively silent (they do not register any evoked quantal secretion during the 200 nerve stimulations) when morphine or dynorphin-A is applied. These results indicate the presence of a dynorphin-sensitive receptor on the motor nerve terminal which when activated by exogenously applied dynorphin produces a decrease in evoked quantal secretion without affecting the propagation of the nerve impulse.

It is possible that release sites have different numbers of dynorphin-sensitive opioid receptors and therefore are affected differently by dynorphin-A and morphine. The effect of dynorphin-A and morphine on release sites along the length of terminal branches was evaluated. There was no significant difference in the percentage inhibition produced by dynorphin-A or morphine along the length of terminal branches. The effect of dynorphin-A and morphine on release sites with very different secretion probabilities was next evaluated. No significant difference was found between release sites of very different secretion probabilities. These results indicate that the density of dynorphin-sensitive opioid receptors found on the terminal is uniformly distributed and therefore it is unlikely that opiates contribute to the differences in probability of quantal secretion of release sites along terminal branches.

How do opiates decrease the probability of quantal secretion from release sites?

Opiates can produce a decrease in the release of quanta in two ways: in many neuronal cell bodies, opiates acting on μ -opioid

Figure 5 The effect of morphine on the relationship between $\bar{m}_e$ and $[\text{Ca}^{2+}]_o$. Six experiments are shown. Log $\bar{m}_e$ is plotted against log $[\text{Ca}^{2+}]_o$ and the dashed lines indicate a 4th power relationship. (○) $\bar{m}_e$ values of control branches; (●) results for the morphine-treated preparations.

Figure 6 The effect of dynorphin-A on the secretion of quanta during test-conditioning impulses. Three consecutive test-conditioning trials are shown for control (trials 1, 2 and 3) and dynorphin-A (24 μM , trials 1, 2 and 3)-treated preparation. The interval between conditioning and test impulses is 12 ms. The $[\text{Ca}^{2+}]_o$ was 0.35 mM.

Figure 7 The effect of dynorphin-A on the nerve impulse, quantal size and $\bar{m}$. (a) The average signal of 200 trials is displayed for control (ai), dynorphin-A-treated ($24 \mu\text{M}$, aii) and after washing out the drug (aiii). Amplitude-frequency histograms of the nerve impulse (b) are shown for control (bi), dynorphin-A-treated (bii) and after washing (biii). Amplitude-frequency histograms of the m.e.p.cs (c) are shown for control (ci), dynorphin-A-treated (cii) and after washing (ciii). Number of quanta secreted per nerve impulse during 200 nerve stimulations are shown (d) for controls (di), dynorphin-A-treated (dii) and after washing (diii). The nerve impulse (ni) amplitude was not significantly changed by dynorphin-A (control, 0.39 ± 0.12 (mean $\pm$ s.d.); dynorphin-A-treated, 0.39 ± 0.10 ; after washing, 0.38 ± 0.10). The amplitude of the m.e.p.cs also did not change (control, 0.91 ± 0.22 mV (mean $\pm$ s.d.); dynorphin-treated, 0.84 ± 0.19 mV; after washing, 0.88 ± 0.18 mV). The number of quanta secreted per nerve impulse decreased from 0.52 (control) to 0.26 (dynorphin-A-treated) and then to 0.38 (after washing). The stimulus artefact (sa) and averaged e.p.c. of 200 consecutive stimulations are shown in (a).

receptors affect a G-protein which is directly linked to K^+ channels causing the conductance of K^+ ions to increase and the cells to hyperpolarize making them less excitable or shortening the duration of the nerve action potential (Cherubini & North, 1985; North, 1986; North *et al.*, 1987). Nerve impulse conduction failure has been suggested (Illes *et al.*, 1982; Milner *et al.*, 1982) as a possible mechanism responsible for the opiate-induced decrease in excitatory junctional potential amplitude from the mouse vas deferens (Bennett & Lavidis, 1980). At the release sites of the amphibian motor nerve terminal, morphine and dynorphin-A acting on dynorphin-A-sensitive opioid receptors, decrease the amount of quanta secreted during nerve stimulation without detectably affecting the configuration or the non intermittent nature of the nerve terminal impulse even at these very high concentrations of dynorphin-A or morphine. Thus it is likely that dynorphin-A acting on κ -like opioid receptors produces a decrease in the secretion of quanta possibly by interfering with the influx of calcium ions following nerve stimulation and not by blocking the propagation of the nerve impulse along terminal branches.

Conditions which reduce the effects of opiates on quantal secretion

Effect of changing $[\text{Ca}^{2+}]_o$ on $\bar{m}$. In the absence of morphine, increasing the external calcium concentration from 0.3 to 0.4 mM increases the amount of transmitter secreted by about a 4th power (Dodge & Rahamimoff, 1967). It is likely that more than four calcium ions are required to interact in a co-operative manner on the secretory mechanism before a quantum of transmitter is secreted (Bennett & Lavidis, 1979). The sites of action of these calcium ions involved in the secretory process are not known. Morphine shifted the calcium versus quantal secretion relationship to the right without any change to the 4th power relationship indicating that the dependence was not changed but that the probability of secretion had decreased. This morphine- and dynorphin-A-induced competitive inhibition of the action of calcium ions in the secretory process is similar to that produced by magnesium ions (Jenkinson, 1957; Bennett & Florin, 1974). This conjecture has been challenged by two studies involving the inhibitory effect of normorphine on transmitter secretion from the varicosities

of the mouse vas deferens (Illes *et al.*, 1982; Milner *et al.*, 1982). In both studies relatively high concentrations of normorphine induced a change in the gradient of the relationship between log e.j.p. amplitude and log $[Ca^{2+}]_o$. It is possible that high concentrations of normorphine induce failure in conduction of the nerve impulse along the sympathetic axons of the mouse vas deferens. It has been suggested that morphine directly affects the voltage-dependent calcium channels to inhibit the influx of calcium during nerve stimulation (Werz & MacDonald, 1983; 1985) or that it inhibits the vesicle secretory process. An action on the vesicle secretory process is unlikely since the secretion of spontaneous quanta continues even after evoked quantal secretion has been abolished (personal observation). It is more likely that opiates affect either the entry of calcium ions during nerve stimulation or the action of these calcium ions activating the secretion of vesicles.

Short high frequency nerve stimulation If a test impulse follows a conditioning impulse within 60 ms, there is an increase in the probability of quantal secretion of release sites. This facilitated secretion declines as a single exponential with a time course of about 30 ms (Balnave & Gage, 1977; Bennett & Lavidis, 1989). An inverse relationship exists between $[Ca^{2+}]_o$ and facilitation in $\bar{m}_e$ (Bennett & Lavidis, 1982). The facilitated increase in $\bar{m}_e$ during conditioning-test impulses is probably due to intra-terminal accumulation of calcium ions (residual calcium hypothesis, Katz & Miledi, 1979; Zucker & Fogelson, 1986). In the sympathetic varicosities of the mouse vas deferens, morphine decreases evoked transmitter secretion and increases the level of facilitation during trains of nerve impulses (Einstein & Lavidis, 1984a,b). Similarly in this study dynorphin-A decreased $\bar{m}_e$ and enhanced the ability of release sites to facilitate during conditioning-test impulses without

changing the time course of decay in facilitation, suggesting that the sequestration of calcium ions within the nerve terminal is not affected by dynorphin-A.

In summary this study has demonstrated that dynorphin-A and to a lesser extent morphine, decrease the evoked secretion of quanta from release sites uniformly along the length of visualized terminal branches. Procedures which are thought to increase the intraterminal stores of free calcium ions during nerve stimulation, such as increasing the extracellular calcium ion concentration or use of a conditioning-test set of impulses, decrease the ability of opiates to inhibit quantal secretion. Inhibition of quantal secretion by dynorphin-A and morphine occurs without any noticeable change in the configuration of the nerve terminal impulse. It is likely that dynorphin-A and morphine affect the potential-dependent calcium channels in such a way as to decrease the influx of calcium ions following the successful invasion of the terminal by the nerve impulse. This results in a uniform reduction of the probability of quantal secretion of release sites. The changes in probability of quantal secretion from release sites during different seasons are not produced by regulation of opioid receptors since the release sites were equally responsive to exogenously applied opiates throughout the year. It is possible that the levels of circulating dynorphin or dynorphin-like substances increase during May to November resulting in a general decrease in the probability of quantal secretion.

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