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Effect of morphine on the nerve terminal impulse and transmitter release from sympathetic varicosities innervating the mouse vas deferens

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1 The effect of morphine on both the propagation of the nerve terminal impulse along the sympathetic varicose axons as well as the evoked and spontaneous transmitter release has been evaluated.

2 Morphine (1 μM) did not significantly change the shape or the regularity by which the nerve terminal impulse was recorded while evoked transmitter release was greatly reduced.

3 Morphine induced a uniform decrease in evoked transmitter release irrespective of the release probability of individual varicosities of their position along terminal branches.

4 Procedures which are thought to increase intracellular calcium concentration such as increasing the extracellular calcium concentration, stimulation of the nerve with trains of impulses and increasing the duration of the action potential with 4-aminopyridine reduced the ability of morphine to decrease evoked transmitter release.

5 Morphine had to act directly on the varicosities to induce a decrease in evoked transmitter release.

6 The decrease in evoked quantal release does not involve an affect on the nerve terminal impulse or the vesicle release process and morphine may affect the dependence of the secretory process on calcium.

Keywords: Opiates; morphine; sympathetic; varicosities; neurotransmission; vas deferens; action potential

Introduction

Opiates have been shown to reduce transmitter release at a variety of synapses (for a review see Duggan & North, 1984). At the neurone soma opiates act on μ-opioid receptors coupled to G-proteins to increase the potassium conductance, hyperpolarizing the neurone soma and preventing the propagation of the action potential (North *et al.*, 1987). At the toad (*Bufo marinus*) neuromuscular junction opiates are thought to act on dynorphin-sensitive opioid receptors to decrease the influx of Ca^{2+} ions into the terminal and so decrease the probability of quantal release (Lavidis, 1995a,b). At the sympathetic varicosities of the mouse vas deferens, morphine decreases the excitatory junction potential amplitude (e.j.p.) and this inhibitory affect can be reduced by increasing the extracellular calcium concentration (Bennett & Lavidis, 1980; Illes *et al.*, 1980; Milner *et al.*, 1982; Einstein & Lavidis, 1984a,b). The interaction between morphine and extracellular calcium ions has been compared by Bennett & Lavidis (1980) to the competitive interaction between extracellular magnesium ions and extracellular calcium ions (Bennett & Florin 1975). This conjecture has however been challenged by Illes *et al.* (1982) and Milner *et al.* (1982) who demonstrated that the relationship between the e.j.p. amplitude and extracellular calcium concentration ($[Ca^{2+}]_o$) was changed when the normorphine concentration exceeded 1 μM. Cunnane & Evans (1988), using large extracellular electrodes to record the nerve terminal impulse (NTI) and excitatory junction currents (e.j.cs) from an unknown number of axons and varicosities, demonstrated that morphine reduced the frequency of recording e.j.cs without increasing the frequency of NTI failure. In this study I have investigated whether morphine reduces transmitter release by impairing propagation of the nerve terminal impulse or by interfering with the secretory process from single axons and known numbers of surface varicosities.

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Methods

Preparation of tissues

Mice (Balb/c) aged between 5 and 7 weeks postnatal were anaesthetized with halothane and killed by cervical fracture. Both vasa deferentia were dissected free from the surrounding tissues. Each vas deferens was pinned to the bottom of a 3 ml capacity bath on a thin layer of Sylgard. The vas deferens was stretched to about 110% of its resting length in the animal. The preparation was continuously perfused at the rate of 3 ml min^{-1} with a modified Tyrode solution of the following composition (mM): NaCl 123.4, KCl 4.7, $MgCl_2$ 1.0, NaH_2PO_4 1.3, $NaHCO_3$ 16.3, $CaCl_2$ 1.0-8.0, glucose 7.8. The temperature of the bath was maintained between 32 and 34°C. The reservoir supplying the bath was continuously gassed with 95% O_2 and 5% CO_2, and the pH was maintained at 7.3. The extracellular calcium concentration ($[Ca^{2+}]_o$) was changed by altering the amount of $CaCl_2$ dissolved in the Tyrode solution supplying the bath.

Stimulation

The prostatic end of the vas deferens was gently sucked into a pipette filled with modified Tyrode solution. A silver/silver chloride wire on the inside of the pipette and one on the outside was used to stimulate the axons running on the surface of the mouse vas deferens using square wave pulses of 0.05 ms duration and 10 to 17 V amplitude. The axons were stimulated continually at 0.2 Hz while searching for the extracellular signs of the nerve terminal impulse (NTI) and the excitatory junction current (e.j.c.) produced by released transmitter activating the postsynaptic receptors (Brock & Cunnane, 1987; 1988).

Visualization of the sympathetic varicosities

The preparation was left bathing in Tyrode solution containing 4 mM $[Ca^{2+}]_o$ for about 20 min. It was then bathed for 30 s in 3.3 diethyloxardicarbocyanine iodide (0.1 μM; $DiOC_2(5)$; Yoshikami & Okun, 1984; Lavidis & Bennett, 1992; 1993a,b) and then washed with modified Tyrode solution for 3 min.

Terminals were chosen by viewing the $DiOC_2(5)$-fluorescent image via an image intensifier camera (Panasonic) attached to an Olympus (BH2) microscope equipped with a rhodamine filter set and the image was then displayed on a video monitor. (National). The effects of exposing the intervaricose axons and varicosities to $DiOC_2(5)$-fluorescence have been previously described (Lavidis & Bennett, 1992). The outline of $DiOC_2(5)$-fluorescing varicosities were traced onto the video monitor screen and the fluorescence was then turned off, to avoid long periods of fluorescence and repeated applications of the $DiOC_2(5)$. The preparation was then illuminated using a tungsten filament lamp and any visible structures such as blood vessels, bundles of axons, connective tissues etc. were also traced on the video monitor. The position of the terminal with respect to such structures was checked by a short period of re-fluorescence before hunting with an extracellular electrode for any signs of the NTI and the e.j.cs generated by released transmitter activating postsynaptic receptors.

Recording

Extracellular recordings of the NTI and e.j.cs were obtained using micropipettes (3 to 15 μm diameter) filled with the modified Tyrode solution. Focal extracellular recordings were obtained by placing the electrode either over the visualised varicosities or about 1 to 2 μm to the side of them. The external signs of the NTI and the e.j.cs could be observed on the oscilloscope after stimulating one end of the mouse vas deferens. The position of the electrode rim with respect to the visualised varicosities was adjusted to increase the amplitude of both the NTI and the e.j.c. In some cases, where varicosities inside and outside the recording electrode had a high probability of transmitter release and the rim of the electrode had formed a slight seal with the surface of the smooth muscle, negative and positive-going e.j.c. complexes were recorded (see Figure 7a & b).

Data analysis

Between 100 and 200 stimuli were collected and recorded on an IBM-AT microcomputer using p-clamp software (version 5.0, Axon instruments). Histograms of the number of quantal releases, amplitude of evoked and spontaneous e.j.cs vs number of observations were constructed. Estimates of the mean number of quanta released per impulse (quantal content, $\bar{m}_e$) were determined by either dividing the total number of quanta released by the number of stimulations or by dividing the average amplitude of the e.j.cs by the average amplitude of the spontaneous e.j.cs.

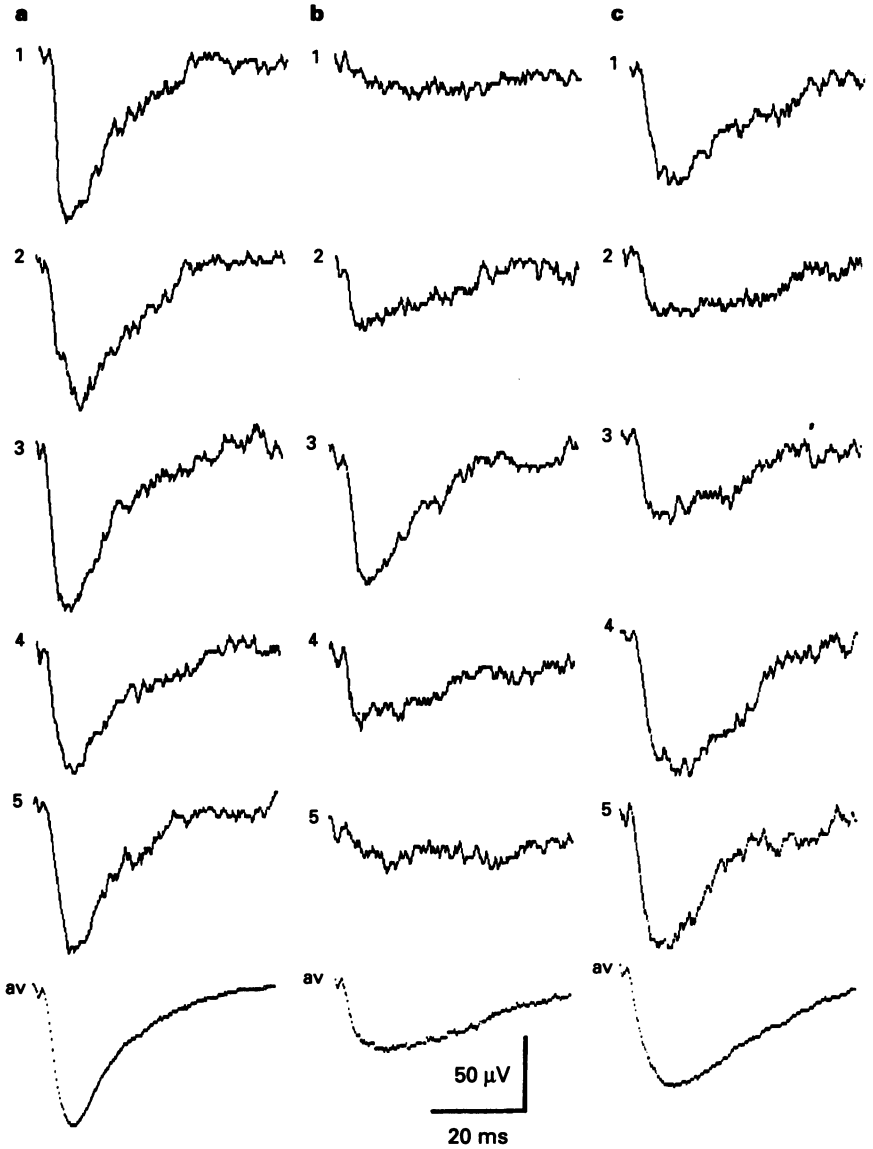

Figure 1 The effect of morphine on excitatory junction currents (e.j.cs). Traces show the electrode-bath potentials generated by the e.j.c. Five consecutive nerve stimulations and the average (av) of 100 stimulations are shown. (a) Controls, (b) morphine (0.5 μM) treated and (c) morphine (0.5 μM) with naloxone (1 μM). $[Ca^{2+}]_o$, 6 mM and $[Mg^{2+}]_o$, 1 mM. Frequency of stimulation was 0.1 Hz.

Drugs

Drugs were dissolved in a second or third reservoir of about 200 ml capacity. 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, The University of Sydney) were dissolved in distilled H_2O and kept refrigerated as stock solutions at a concentration of 1 mM. Stock solutions of $DiOC_2(5)$, (10 mM) dissolved in DMSO were kept refrigerated for up to 6 weeks. From this stock solution 0.1 ml was serially diluted to 10 μM in distilled H_2O. The solution of $DiOC_2(5)$ was finally diluted to 0.1 μM in Tyrode solution.

Results

The effect of morphine on the release of transmitter

The effects of morphine on the extracellular signs of the NTI and e.j.cs were investigated. In high $[Ca^{2+}]_o$ (6 mM) some varicosities showed fairly consistent transmitter release in response to regular (0.1 Hz) nerve stimulation (Figure 1a). If, however, the $[Ca^{2+}]_o$ was lowered to 1 mM transmitter release decreased and was highly intermittent (Figure 2a). A consecutive set of recordings is shown in Figure 1 numbered 1 to 5 with the last trace showing the average of 100 such records. The NTI was present in each of the 100 recordings showing no intermittence. Administration of morphine (0.5 μM) produced a 62% reduction in the release of transmitter (Figure 1b) while the NTI was always present and did not decrease in amplitude. Addition of naloxone to the bath partially reversed the depressive action of morphine on transmitter release (Figure 1c). Again the NTI was unaffected (Figure 1c).

The time course of the e.j.cs was altered by morphine and this effect persisted after the addition of naloxone. The tail of the averaged e.j.cs was increased from having a time constant of about 25 ms in controls to 40 ms following morphine (0.5 μM). The changes in time course of the e.j.cs was not observed for spontaneous e.j.cs (s.e.j.cs, time constant about 40 ms). This difference in time course of the decline in e.j.cs may be a product of the electrode recording from varicosities found either inside or outside the electrode rim. The varicosities located inside the recording electrode yield a negative potential while those outside yield a positive potential (Brock & Cunnane, 1988). Adding the two signals results sometimes in negative e.j.cs having a tail with a faster time course of decline

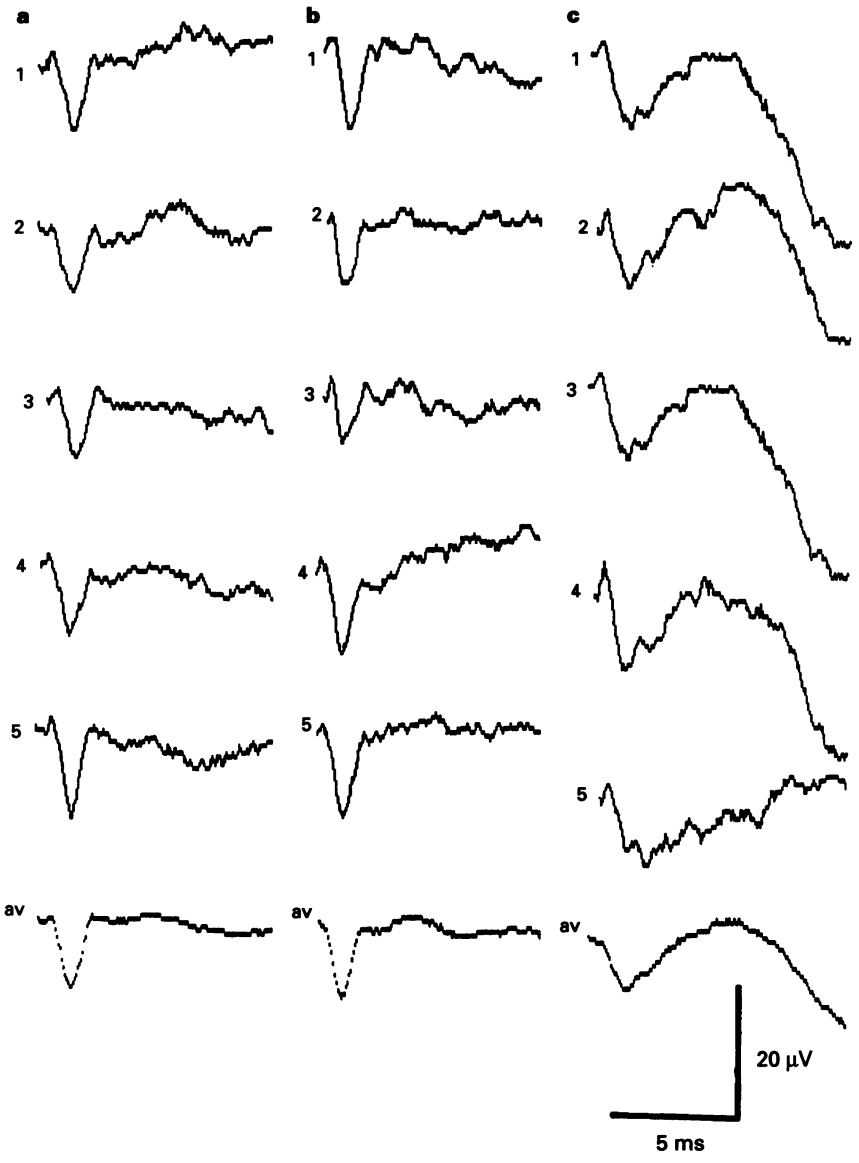

Figure 2 The effect of morphine on the nerve terminal impulse in low $[Ca^{2+}]_o$. Five consecutive nerve stimulations and the average (av) of 20 stimulations are shown. (a) Control, (b) morphine (1 μM) treated and (c) morphine (1 μM) with 4-aminopyridine. $[Ca^{2+}]_o$, 1 mM and $[Mg^{2+}]_o$, 1 mM. Frequency of stimulation was 0.1 Hz.

due to the rising phase of the positive e.j.cs, this is observed in the averaged response shown in Figure 1a. Introducing morphine to the solution bathing the tissue but not the area sealed by the recording electrode decreased transmitter release of the varicosities located outside the recording electrode while the varicosities inside the electrode are less affected by morphine. Loss of the positive signals allows the uncontaminated negative potential to be seen with a time course of decline in the e.j.cs of about 40 ms (Figure 1b and c).

The effect of morphine on the nerve terminal impulse

Extracellular recordings of the NTI were collected for control (Figure 2a), morphine treated (1.0 μM, Figure 2b), and following the addition of 4-aminopyridine (Figure 2c) in $[Ca^{2+}]_o$ of 1 mM. Five consecutive recordings for each treatment are shown along with the average of 20 recordings at the bottom of each column. Morphine did not alter either the amplitude or the duration of the NTI (compare the averaged NTI in Figure 2a and b). Addition of 4-aminopyridine to morphine-treated preparations increased the duration of the NTI (Figure 2c) resulting in an increase in transmitter release. Note that while e.j.cs were not observed in low $[Ca^{2+}]_o$ (Figure 2a and b), 4-aminopyridine was able to evoke transmitter release in preparations that were bathed in morphine (1 μM) and low $[Ca^{2+}]_o$ (1 mM, note in Figure 2c the rising phase of the e.j.cs).

There was no significant difference in the amplitude of the NTI over a morphine concentration range of 0.1 to 1.0 μM. At a morphine concentration of 1.0 μM the amplitude of the NTI was $93 \pm 8\%$ (mean $\pm$ s.e.mean, $n = 8$) compared to control amplitude. In contrast, transmitter release significantly ($P < 0.05$) decreased by $45 \pm 11\%$ at a morphine concentration of 0.1 μM and by $78 \pm 6\%$ ($n = 8$) at a morphine concentration of 1.0 μM (Figure 3). The concentration of morphine which produced a 50% reduction in transmitter release (ED_{50}) was 0.2 μM when the $[Ca^{2+}]_o$ was 4 mM.

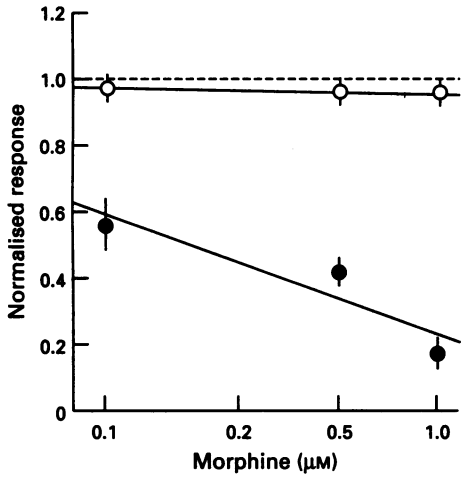

Figure 3 Morphine dose versus excitatory junction current (e.j.c.) amplitude and nerve terminal impulse amplitude. Both e.j.c. and nerve terminal impulse (NTI) amplitudes were normalised with respect to the control e.j.c. or NTI amplitudes. (●) Indicate the mean normalised e.j.c. amplitudes and vertical lines the standard error of the mean. (○) Indicate the mean normalised NTI amplitude and vertical lines the standard error of the mean. Solid lines were fitted by linear regression. The line through the e.j.c. data had a slope of −0.34, with correlation coefficient of 0.82 and the line through the data of the NTI amplitudes had a slope of zero, with correlation coefficient of 0.93. Dashed line indicates no change in response. $[Ca^{2+}]_o$ was 4 mM.

The effect of morphine on transmitter release from varicosities along single terminal branches

The release of transmitter from different groups of varicosities is not uniform and is to a certain extent intermittent depending on $[Ca^{2+}]_o$ (Cunnane & Stjarne, 1982; Brock & Cunnane, 1988; Lavidis & Bennett, 1992; 1993a). Hence it was of interest to examine the effect of morphine on small groups of sympathetic varicosities.

Spatial resolution of recordings depended in part on the electrode tip diameter. The size of the extracellular electrode used to record e.j.cs from visualised sets of varicosities varied from about 3 to 15 μM in diameter. An example of a 6 μM diameter tip electrode is shown in Figure 4. Here the number varicosities recorded from varied from 2 to 3, $\overline{m}_e$ varied from 0.6 to 2.7 and morphine (0.5 μM) decreased transmitter release by 30% to 75%. This decrease was mainly due to a decrease in the probability of quantal release from varicosities (Figure 4). There was no significant difference along terminals in the magnitude of inhibition of release probability induced by morphine. Even in $[Ca^{2+}]_o$ of 4 mM, some visualised varicosities (about 45%) did not show any transmitter release over 100 consecutive stimulations (average secretory probability < 0.01) even though the nerve impulse was clearly visible. In contrast, varicosities with an average release probability of 0.43 to 0.8 could be easily found and at these sites morphine (0.5 μM) produced a decrease in the probability of transmitter release which varied from about 32% to 58%. In 2 of the 23 groups of varicosities studied the highest number of quanta

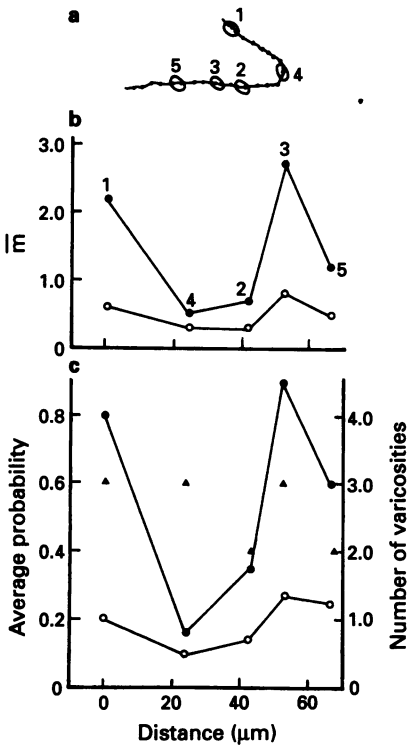

Figure 4 The effect of morphine on evoked quantal release and the average probability of quantal release from sympathetic varicosities, recorded by a 6 μm diameter electrode. (a) Drawing of the visualised terminal branch with varicosities shown as dots along the branch. The extracellular electrode positioned over groups of 2 to 3 varicosities as indicated on the drawing by the elliptical shapes (electrode rim), the numbers indicate the order in which the recordings were taken. (b) The evoked level of transmitter release was determined for each of the groups of varicosities shown in (a) control (●) and after morphine treatment (0.5 μM, ○). (c) The average probability of quantal release of the varicosities recorded from (●,○) and number of varicosities recorded from (▲) is plotted for each electrode placement; (●,▲) controls and (○) morphine treated. $[Ca^{2+}]_o$ was 4 μM.

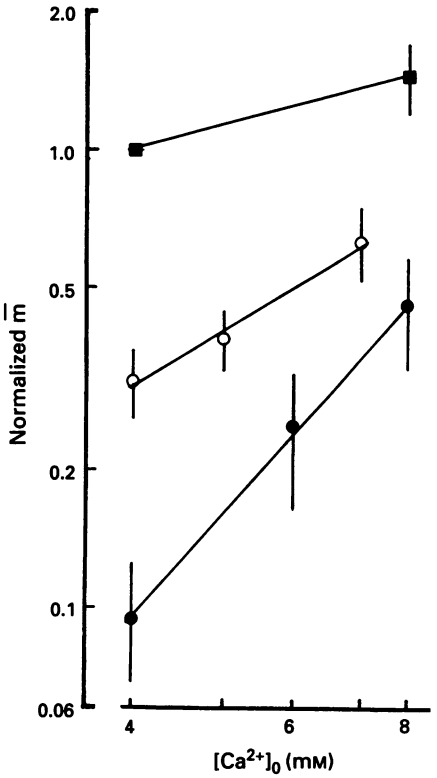

Figure 5 The effect of $[Ca^{2+}]_o$ on the morphine-induced decrease in quantal release. All excitatory junction currents (e.j.cs) were normalised with respect to the average e.j.c. amplitude of controls in $[Ca^{2+}]_o$ of 4 mM. (■) Controls; (○) morphine treated (0.5 μM); (●) morphine treated (1 μM). Lines fitted by linear regression. The slope of the line through the control data was 0.69, for the morphine (0.5 μM), 1.1 and for the morphine (1 μM), 1.8.

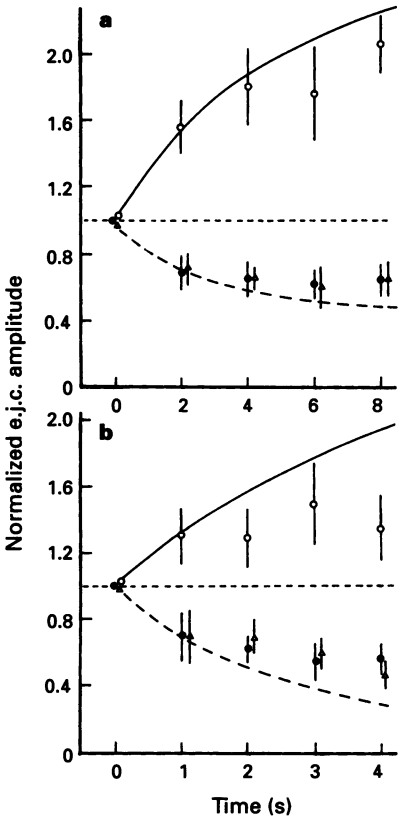

Figure 6 The effect of stimulation frequency on the morphine-induced decrease in quantal release. All excitatory junction currents (e.j.cs) were normalised with respect to the e.j.c. amplitude of the first nerve impulse in the train of 5 impulses. (●) Control; (○), morphine (0.5 μM) treated and (△) morphine (0.5 μM) and naloxone (1 μM) treated. Solid line indicates the theoretical level of facilitation according to the residual calcium hypothesis with a 6 s time course of decline in facilitation (Bennett & Florin, 1974; Einstein & Lavidis, 1984a). Dashed lines indicate the theoretical level of autoinhibition using a time constant of decline in autoinhibition of 4 s.

released per impulse was greater than the number of visualised varicosities observed inside the rim of the recording electrode. It is possible that some varicosities have more than one release site. Alternatively an undetected additional varicosity may have been just one layer of smooth muscle beneath the visualised varicosities and could conceivably have contributed to quantal release during the 100 recordings taken.

The effect of changing $[Ca^{2+}]_o$ on the decrease in $\overline{m}_e$ produced by morphine

There was very little change in $\overline{m}_e$ when $[Ca^{2+}]_o$ was increased from 4 to 8 mM (Figure 5). Addition of morphine (0.5 μM) produced about a 70% decrease in $\overline{m}_e$ while morphine (1 μM) decreased $\overline{m}_e$ by 90%. Increasing $[Ca^{2+}]_o$ mitigated the morphine-induced reduction in $\overline{m}_e$ such that at $[Ca^{2+}]_o$ of 8 mM and morphine (0.5 μM) the percentage inhibition was only 40% while at 1 μM the percentage inhibition was 60%. The slope of the line was dependent on the degree of inhibition in $\overline{m}_e$ produced by morphine (Figure 5).

The effect of high frequency trains of impulses on the morphine induced decrease in $\overline{m}_e$

In high $[Ca^{2+}]_o$ of 4 mM, during the train of nerve impulses there is a progressive decrease in $\overline{m}_e$ by subsequent impulses. This depression in $\overline{m}_e$ may be due to autoinhibition since it could be abolished for at least the 2nd and 3rd impulses if idazoxane or yohimbine was added in the bathing solution to antagonize the presynaptic α_2-adrenoceptors (Lavidis unpublished observation). Morphine decreased transmitter release by the 1st to 3rd nerve impulses in a train and therefore decreased the level of autoinhibition that would normally occur and so increase the relative amounts of transmitter released during the 4th and 5th impulses partially overcoming the morphine-induced decrease in $\overline{m}_e$. The depression in quantal release which occurs during a train of impulses in control (Figure 6a and b, dashed lines) was converted to facilitation when morphine (0.5 μM) was administered (Figure 6a and b, solid lines). Similar results were observed for 1 Hz stimulation. However, the degree of decrease in $\overline{m}_e$ was greater and morphine less effective at overcoming the depression. An inverse relationship between frequency of stimulation and the effect of morphine has been previously shown (Bennett & Lavidis, 1980). The time course of depression was determined to be about 4 to 6 s while that for facilitation was about the same, a more accurate estimate of the time course of depression and facilitation could not be made since the two phenomena have similar time courses.

The effect of morphine on varicosities located inside and outside the recording electrode

When an electrode is placed over a group of varicosities so that some are within the rim of the recording electrode and some are just outside, and a loose seal forms between the rim of the electrode and the surface of the smooth muscle cells, a complex signal is recorded (Figure 7a). There is an initial positive component followed by a slower negative component which finally turns positive again (Figure 7a). This is explained theoretically if an electrode records a negative e.j.c. produced by the release of a quantum from a varicosity inside the electrode and simultaneously records a positive e.j.c. produced by the release of a quantum from a varicosity just outside the elec-

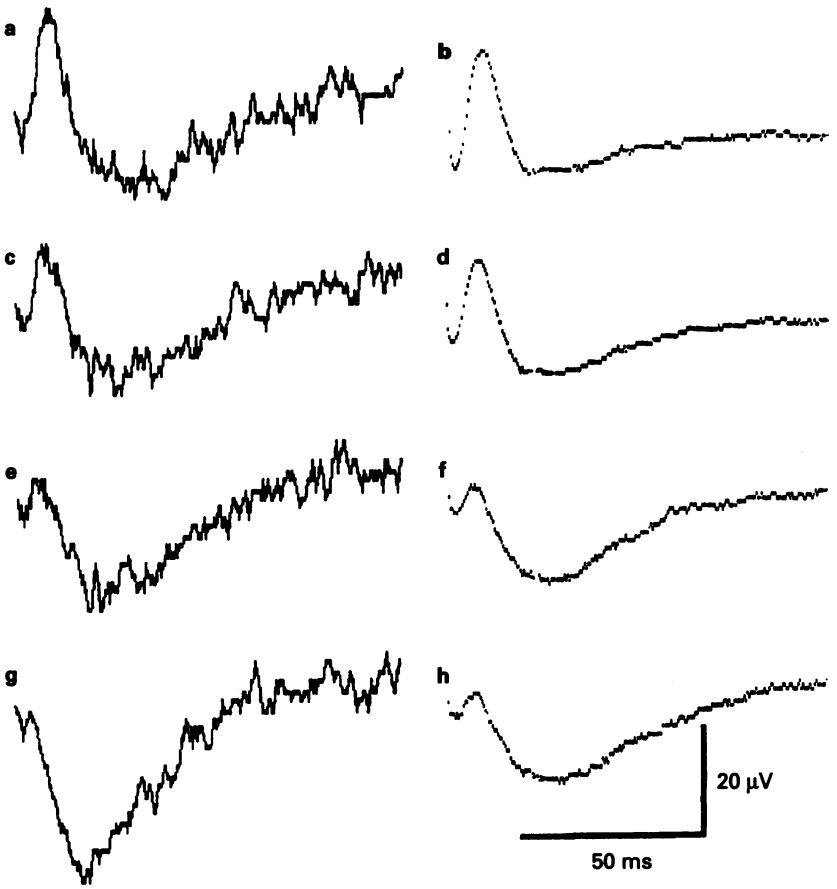

Figure 7 A comparison of the effect of morphine on sympathetic varicosites located inside and outside the recording electrode. When a slight seal forms between the rim of the recording electrode and the surface of the smooth muscle cells a complex signal (a) is recorded by the electrode. Negative excitatory junction currents (e.j.cs) are recorded when varicosities inside the recording electrode release transmitter while positive e.j.cs are recorded when varicosities outside the recording electrode release transmitter, when both inside and outside varicosities simutaneouly release transmitter a complex signal is recorded shown in (a), the averaged signal of 20 stimulations is shown in (b). When morphine (0.5 μM) was added to the bathing solution but not within the solution of the recording electrode, there was a decrease in the positive e.j.c. while the negative e.j.c. become more pronounced, compare the change in the individual traces from (a) to (g) (left column) and the averaged signals from (b) to (h) (right column). (a & b) Control; (c & d) 5 min; (e & f) 10 min; (g & h) 15 min following morphine administration.

trode. When morphine (0.5 μM) was introduced into the bathing solution, the varicosities located outside of the electrode rim are affected by the morphine sooner than those located within the electrode rim. As a result of the delay in diffusion of morphine through the loose electrode seal the complex signal is converted to purely a negative e.j.c. representing just the transmitter release from varicosities inside (Figure 7g and h).

Discussion

The effect of morphine on the terminal nerve impulse and the release of transmitter

In most neurone somas studied, opiates have been shown to increase the conductance of potassium channels causing either a shortening of the duration of the action potential or a hyperpolarization of the neurone soma (Pepper & Henderson, 1980; Morita & North, 1982; North & Williams, 1983). The amplitude and form of the NTI was studied along single terminal branches before and after the introduction of morphine. At all concentrations studied up to 1 μM, morphine altered neither the size nor the shape of the nerve terminal impulse (NTI) while transmitter release was greatly reduced in morphine (1 μM). This result is consistent with the findings of Cunnane & Evans (1988) were they demonstrated using blind placement of large extracellular electrodes over axons and groups of varicosities that morphine does not affect the NTI. Furthermore, recordings taken some 0.5 to 2.0 mm away from the point of nerve stimulation showed consistent nerve action potential propagation in the presence of morphine upstream from the recording electrode. Morphine had to be in direct contact with the varicosities to produce a decrease in quantal release since varicosities inside the recording electrode (morphine free environment) were more slowly inhibited than varicosities outside the recording electrode (exposed to bath morphine). Thus the morphine-induced decrease in transmitter release occurred without any detectable change in the NTI when a slight seal had formed between the rim of the recording electrode and the surface of the smooth muscle.

Are all varicosities equally affected by morphine?

The large variation in transmitter release probability of varicosities in the guinea-pig vas deferens (Brock & Cunnane, 1988) and the mouse vas deferens (Lavidis & Bennett, 1992; 1993a,b) may be due to variation in their susceptibility to endogenous inhibitory agents such as noradrenaline, adenosine, neuropeptide Y or opiates. The possibility that opiates may be involved was checked in this study by taking recordings from different varicosities along single branches before and after treatment with morphine. Although a large variation in the ability of morphine to inhibit quantal release was observed

between release sites there was no consistent difference in the susceptibility of low and high probability varicosities. Nor was there any discernible relationship between morphine-induced inhibition and distance from the point of nerve stimulation. Furthermore when the opiate antagonist, naloxone was applied there was no change in quantal release when using these stimulus paradigms.

Procedures which reverse the inhibitory action of morphine on transmitter release

In the mouse vas deferens if the extracellular calcium concentration is increased from 0.7 to 1.8 mM there is a 2.3 power increase in the size of the intracellularly recorded e.j.p. (Bennett & Florin, 1975). At the neuromuscular junction of amphibia the 4th power relationship between quantal release and $[Ca^{2+}]_o$, however, has been shown not to hold in very low or very high calcium concentrations (see Jenkinson, 1957; Dodge & Rahamimoff, 1967) and may depend on the initial probability of transmitter release. Since the probability of transmitter release from varicosities is highly variant there is considerable variance in the increase of transmitter release when $[Ca^{2+}]_o$ is increased. For example, if a group of varicosities initially had a high probability of transmitter release the increase in release was low (0 to 1 power) when $[Ca^{2+}]_o$ was increased. Alternatively if a group of varicosities had an initial low probability of transmitter release there was a 3 to 4 power increase in release when $[Ca^{2+}]_o$ was increased. An inverse relationship exists between the rate of increase in transmitter release when $[Ca^{2+}]_o$ is increased and the initial probability of transmitter release (see following paper by Lavidis, 1995c).

Morphine decreases the size of the e.j.c. but when $[Ca^{2+}]_o$ was increased from 0.7 to 2.5 mM there was a 2.3 power increase in the size of the e.j.p. recorded with an intracellular microelectrode (Bennett & Lavidis, 1980). The effect of morphine on the relationship between $[Ca^{2+}]_o$ and e.j.c. of varicosities with vastly different (pre-morphine) probabilities of transmitter release was evaluated in the present study. Since morphine decreased the probability of transmitter release, varciosities with initially low probabilities of release did not show any change in the 3rd to 4th power relationship while varcosities with high probabilities of quantal release changed from a 1st to a 2nd or 3rd power when $[Ca^{2+}]_o$ was increased. The effect of morphine in varicosities is to decrease the probability of transmitter release without affecting the inverse relationship between the rate of increase in transmitter release and $[Ca^{2+}]_o$. Overall the effect of morphine was overcome by increasing the extracellular calcium concentration.

Procedures which cause an accumulation of calcium in the varicosities can overcome the decrease in $\overline{m}_e$ produced by morphine. Stimulation at short high frequency trains of impulses produced an increase in e.j.ps that has been attributed to residual calcium being present when subsequent impulses introduce more calcium into the varicosities (Bennett & Florin, 1975). Other mechanisms have been proposed by Smith & Cunnane (1994) to account for facilitation involving intravaricosity Ca^{2+} stores. The degree of facilitation in e.j.p. amplitude can therefore be predicted according to this assumption. This, however, does not take into account the contribution of autoinhibition which occurs when the interval between nerve impulses is less than 10 s (Storey *et al.*, 1981; Marshall, 1983; Brock *et al.*, 1990) and has a time course similar to facilitation (Bennett & Lavidis, 1980). Because of this, experimentally-derived e.j.p. amplitudes are mostly lower than expected values derived from theoretical predictions according to the residual calcium hypothesis. This problem was largely avoided by keeping the probability of transmitter release low by either lowering the extracellular calcium concentration or by treating the preparation with morphine. The decrease in transmitter release which occurs during a train of impulses in high $[Ca^{2+}]_o$ is reversed to facilitation when morphine is added of if the $[Ca^{2+}]_o$ is lowered.

Finally if the terminal nerve impulse is prolonged by blocking potassium channels with 4-aminopyridine there is an increase in the duration of calcium entry into the varicosities resulting in an increase in the probability of transmitter release (Wakade & Wakade, 1981). The morphine induced decrease in transmitter release was reversed in the presence of 4-aminopyridine. In general, procedures which increase the intracellular level of calcium following nerve stimulation reduce the ability of morphine to lower transmitter release.

Can morphine decrease transmitter release from varicosities not directly exposed to drug?

When a recording electrode is placed on top of a group of varicosities and a seal forms between the rim of the electrode and the surface of the smooth muscle, the varicosities located within the rim of the electrode tip produce negative e.j.cs when they release transmitter, while varicosities found just outside of the electrode produce positive e.j.cs when they release transmitter (Brick & Cunnane, 1988; Cunnane & Manchada, 1989; Stjarne & Stjarne, 1989). The complex extracellular recording of transmitter being simultaneously released from inside and outside the electrode make it possible to study the indirect effect of morphine. Morphine when applied to the bath (that is, varicosities outside the recording electrode) initially produced a decrease in the amplitude of the positive potential and finally abolished it; the negative potential persisted over this period (15 to 20 min) but eventually also decreased and was eventually abolished (40 to 50 min). Morphine must act directly on the varicosities to induce a decrease in transmitter release, it is therefore unlikely that morphine affects the propagation of the nerve impulse along terminal branches. Rather, the results suggest that morphine presumably may decrease the opening time or conductance of neuronal Ca^{2+} channels or calcium sequestration after entry. It remains possible that morphine affects potassium channels so as to shorten the opening of the calcium channels without affecting the propagation or the shape of the nerve impulse. To test this hypothesis would require patch clamp recording from the membrane of varicosities.

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