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Effect of opiates on transmitter release from visualized hypogastric boutons innervating the rat pelvic ganglia

Darren A. Warren & [1]Nickolas A. Lavidis

The Narcotics Research Laboratory, The Institute for Biomedical Research, Department of Physiology, The University of Sydney, Sydney, N.S.W., Australia, 2006

1 The effect of opiates on neurotransmission between visualized hypogastric nerve boutons and postganglionic cell bodies has been examined using extracellular recording of nerve bouton impulses (NBIs) and excitatory postsynaptic currents (e.p.s.cs).

2 Morphine (10 to 40 μM) did not affect neurotransmission in the ganglia. Dynorphin-A (4 μM) and U50488H (1 μM) decreased quantal transmitter release and naloxone (10 μM) reversed these effects.

3 Morphine (10 μM), dynorphin-A (4 μM) and U50488H (1 μM) did not affect either the time course or consistency with which the NBI was recorded.

4 Dynorphin-A (1 to 4 μM) and U50488H (1 μM) decreased the average amplitude of e.p.s.cs by increasing the number of failures to release quanta from single or small groups of 2 to 4 boutons during continuous nerve stimulation at 0.1 Hz.

5 The decrease in quantal release induced by dynorphin-A and U50488H in 0.2 to 0.5 mM $[Ca^{2+}]_o$ was readily reversed by increasing the extracellular calcium ion concentration to 1 mM.

6 It was concluded that κ-opioid receptors are located on the boutons of the hypogastric nerve and when activated by κ-opioid receptor agonists reduce quantal release without affecting the NBI.

Keywords: Opiate; dynorphin; pelvic ganglia; neurotransmission; hypogastric nerve; visualized boutons

Introduction

Opiates acting on μ- or δ-opioid receptors increase the conductance of K^+ ions resulting in hyperpolarization of the cell soma (North & Tonini, 1977; Pepper & Henderson, 1980; Werz & MacDonald, 1983; North & Williams, 1983; Mihara & North, 1986; North *et al.*, 1988). Opiates can also act via κ- or infrequently δ-opioid receptors to decrease Ca^{2+} ion conductance (Macdonald & Werz, 1986; Gross & Macdonald, 1987; North *et al.*, 1988; Bean, 1989) during nerve stimulation resulting in a decrease in transmitter release from nerve endings (Bixby & Spitzer, 1983; Lavidis, 1995a, b). The effect of opiates on the mouse hypogastric ganglia (Rogers & Henderson, 1990) and mesenteric ganglia (Bornstein & Fields, 1983) has been studied by use of intracellular recordings of the excitatory postsynaptic potentials (e.p.s.ps) and miniature excitatory postsynaptic potentials (m.e.p.s.ps). In both studies quantal analysis of e.p.s.ps and m.e.p.s.ps suggested that opiates were decreasing quantal release by a presynaptic mechanism. This may occur either by opiates hindering propagation of the nerve bouton impulses (NBI) along the preganglionic axons or by impairing coupling of the NBI to transmitter release possibly by inactivating the voltage sensitive calcium channels.

The rat pelvic ganglia was chosen for this study because the postganglionic cell bodies are large (15–30 μm) and have no dendritic arborisation of their spherical or ovoid shapes (Tabatabai *et al.*, 1986; Snider, 1987; Purves *et al.*, 1988). Each cell is innervated by a single preganglionic axon which forms short strings or clusters of boutons (Rogers *et al.*, 1990; de Groat & Booth, 1993). Locating boutons with an extracellular microelectrode was made easy by $DiOC_2(5)$-fluorescence following careful removal of the capsule and other material covering the ganglia. Large diameter tip electrodes could then be used to record the NBI, e.p.s.c. and the postsynaptic action potential (AP). In this study we have investigated the effects of opiates on neurotransmission between preganglionic nerve fibres of the hypogastric nerve and the postganglionic cell bodies.

[1] Author for correspondence.

Methods

Preparation of tissues

Rats (AAW) aged between 5 and 7 weeks postnatal were anaesthetized with ether and killed by cervical fracture. Both pelvic ganglia were dissected free from the surrounding tissues. Each ganglion was pinned on the bottom of a 3 ml capacity bath on a thin layer of Sylgard (Dow Corning). The preparation was continuously perfused at the rate of 3 ml per min 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, $CaCl_2$ 0.2–1.0, glucose 7.8. The temperature of the bath was maintained between 32°C and 34°C. The reservoir supplying the bath was continuously gassed with 95% O_2 and 5% CO_2 maintaining the pH 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 hypogastric nerve 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 were used to stimulate the hypogastric nerve with square wave pulses of 0.05 ms duration and 10 to 17 V amplitude. The axons were stimulated continually at 0.1 Hz while searching for the extracellular signs of the NBI and the e.p.s.c. produced by released transmitter activating the postsynaptic receptors.

Visualization of the hypogastric boutons

The preparation was initially bathed in Tyrode solution containing 2 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; 1993) and then washed with modified Tyrode solution for 3 min. Boutons were chosen by viewing the $DiOC_2(5)$-fluorescent image via a 50 times long working distance air objective. An image intensifier camera (Panasonic) attached to an Olympus (BH2) microscope equipped with rhodamine filter set was used to display the image on a video monitor (National).

The effects of exposing neurones and terminals to $DiOC_2(5)$-fluorescence have been described previously (Lavidis & Bennett, 1992). The arrangement of $DiOC_2(5)$-fluorescing boutons on the ganglion cells (Figure 1a, b) was traced onto the video monitor screen (Figure 1c, d) 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 with a tungsten filament lamp and any visible structures such as bundles of axons, connective tissues and the geometry of the ganglion cells were also traced on the video monitor (Figure 1c, d). The position of the boutons with respect to such structures was checked by a short period of refluorescence before hunting with an extracellular electrode for any signs of the hypogastric NBI and the e.p.s.cs generated by release transmitter activating postsynaptic receptors.

Recording

Extracellular recordings of the hypogastric NBI and e.p.s.cs were obtained with electrodes filled with modified Tyrode solution and having tip diameters from 5 to 10 μm. Focal extracellular recordings were obtained by placing the electrode either over the visualized boutons or about 1 to 2 μm to the side of them. The external signs of the NBI and e.p.s.cs could be observed on the oscilloscope while stimulating the hypogastric nerve. The position of the electrode rim with respect to the visualised boutons was adjusted to increase the amplitude of both the NBI and the e.p.s.cs.

Data analysis

Between 100 and 200 NBIs and e.p.s.cs were collected and recorded on a Macintosh computer with MacLab and Scope (version 3.3) software. Histograms of the amplitude of e.p.s.cs vs number of observations were constructed including the number of e.p.s.c. measurements which were within the electrical noise level, such recordings were designated as failures to evoke e.p.s.cs. Data are expressed as the mean $\pm$ s.e.mean and n represents the number of experiments. The means were compared by Student's t test; $P<0.05$ was taken to be statistically significant.

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. Dynorphin-A (1-13, Sigma) was dissolved in 40 μM acetic acid (pH about 5) at a concentration of 1 mM, aliquoted into 0.1 ml portions and kept below -20°C. Trans-(±)-3,4-Dichloro-N-(2-(1 Pyrrolidinyl)cyclohexyl) Benzeneacetamide (U50488H) (Sigma) was dissolved in distilled H_2O and kept refrigerated as a stock solution at a concentration of 1 mM.

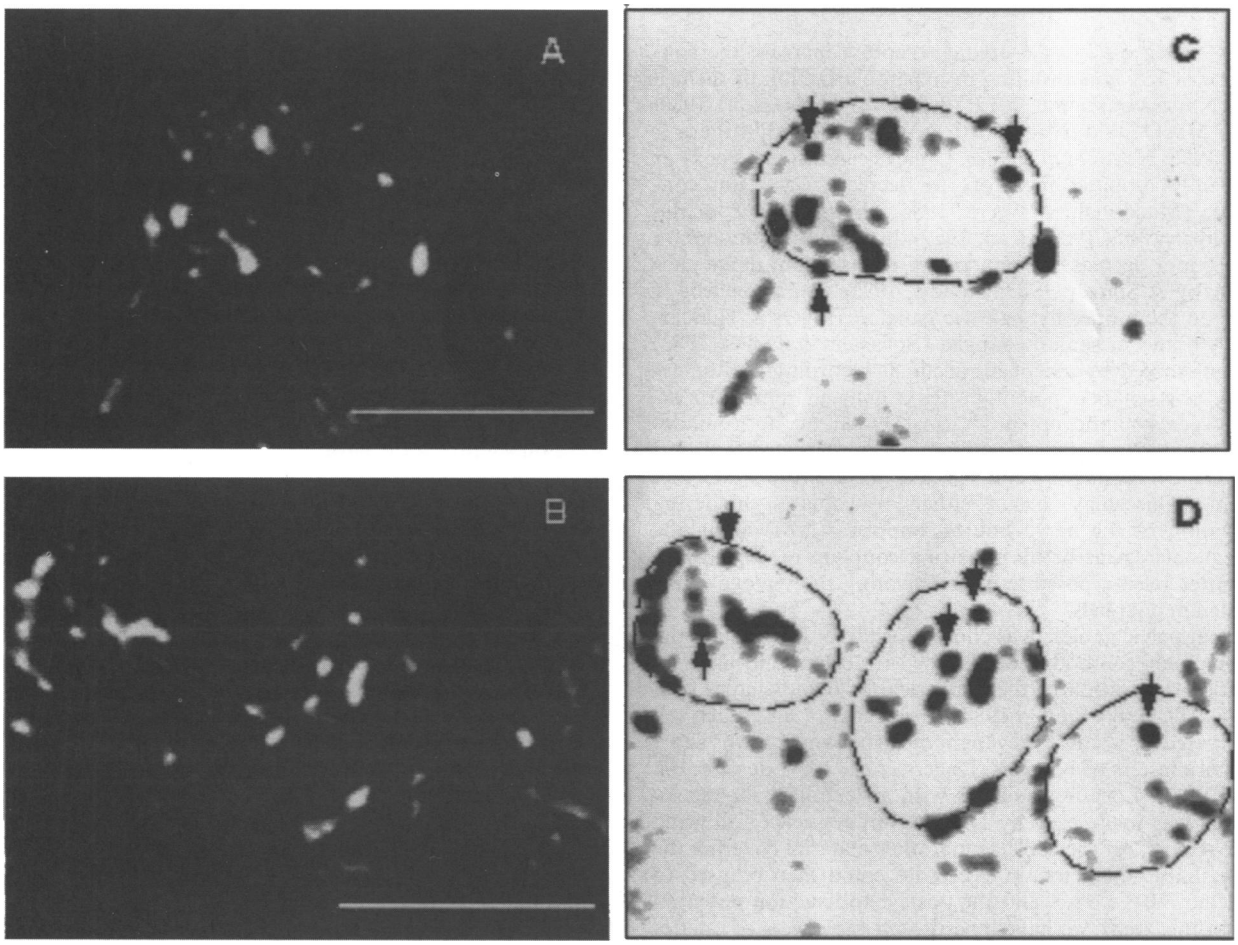

Figure 1 Boutons of the hypogastric nerve innervating ganglion cells viewed by use of $DiOC_2(5)$-fluorescence. The boutons are shown as bright dots about 1 μm in diameter and are located on the surface of the ganglion cell. (a and b), micrographs of $DiOC_2(5)$-fluorescent boutons; (c and d), are drawings of the structures shown in (a and b) respectively. Dashed elliptical structures indicate the shape of the ganglion cells while arrows indicate some of the surface boutons studied electrophysiologically. The calibration bar is 20 μm.

Stock solutions of $DiOC_2(5)$, (10 mM) dissolved in dimethylsulphoxide (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

Recording of NBI and e.p.s.c. from visualized hypogastric boutons

$DiOC_2(5)$-fluorescence was used to visualize the surface hypogastric boutons innervating the ganglion cells (Figure 1). These structures were about 1 μm in diameter and were fluorescing brightly on the surface of the ganglion cells. The extracellular signs of the NBI were recorded with electrodes having tip diameters between 5 and 10 μm (Figure 2). The NBI amplitude varied considerably between preparations but did not vary during the period of recording from single boutons or small group of 2 to 4 boutons. In all recordings the amplitude of the NBI could not be graded when the stimulation voltage was altered; this all or none behaviour of the NBI was taken to mean that we were recording from a single axon.

Effect of opioid receptor agonists on the pre-ganglionic NBI

The effect of opiates on the amplitude, time course and consistency of the NBI was evaluated. Neither the mean amplitude of the NBI nor its variance were affected by U50488H (Figure 3). The variance in NBI amplitude of control and U50488H-treated preparations shown in Figure 3 was similar to the variance in electrical noise during the recording indicating that the amplitude of the NBI is invariant. Morphine (10 μM), dynorphin-A (4 μM) and U50488H (1 μM) did not alter the consistency with which the NBI was recorded over 200 nerve impulses. There was no significant ($P<0.05$) difference in the amplitude of the NBI (Figure 3c and 4) following treatment of the preparations with morphine (10 μM, $n=7$) dynorphin-A (4 μM, $n=4$) or U50488H (1 μM, $n=9$). Naloxone (10 μM) was administered after each treatment and showed no significant change in the amplitude of the NBI (Figure 4).

Effect of opiates on the e.p.s.cs

Extracellularly positioned electrodes over single or small groups of boutons (Figure 1) recorded the NBI and extracellular signs of the e.p.s.c. generated when transmitter released from the bouton activated postsynaptic receptors on the ganglion cell (Figure 2). If these e.p.s.cs were of significant amplitude a postsynaptic action potential was generated on the recovery phase of the e.p.s.cs (Figure 2, control traces 1, 4, 5 and 7). These postganglionic action potentials could be reduced in frequency or abolished by decreasing the extracellular calcium concentration ($[Ca^{2+}]_o$) or by administration of

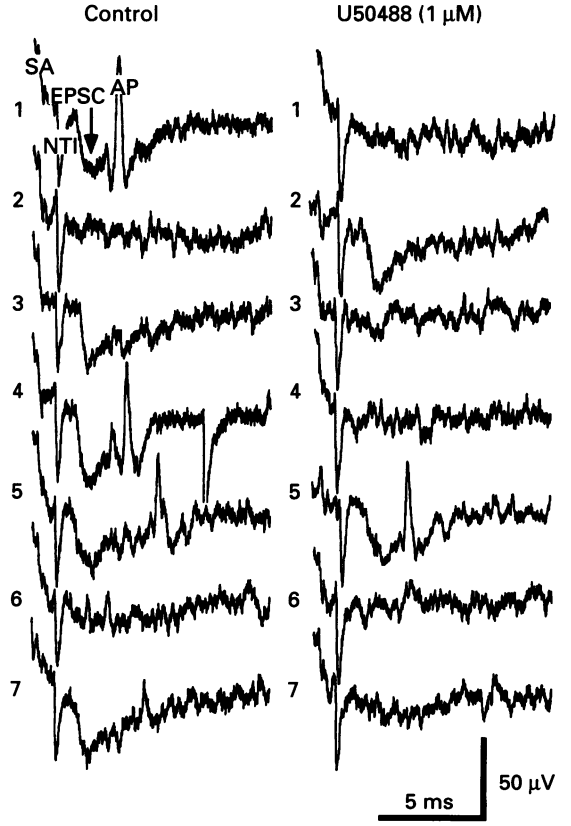

Figure 2 Sample recordings of the stimulus artefact (SA), nerve bouton impulse (NBI), excitatory postsynaptic currents (e.p.s.cs) and the postganglionic action potential (AP). Seven sample traces are shown for control and U50488H (1 μM)-treated preparations. $[Ca^{2+}]_o = 0.3$ mM.

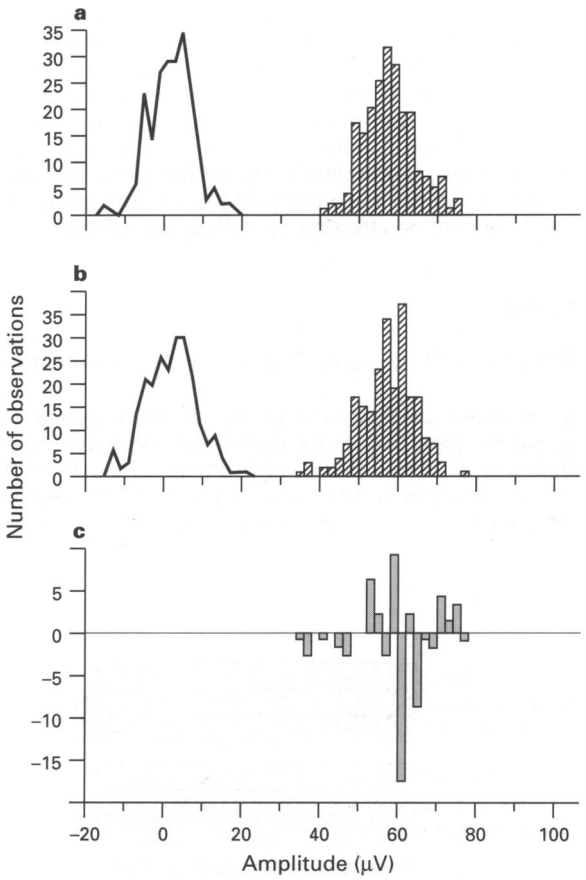

Figure 3 The amplitude distribution of the NBI of a control and U50488H (1 μM)-treated preparation. The amplitude distribution of the electrical noise during the recording of the 200 nerve stimulations is indicated by the clear line histograms while the cross hatched column histograms show the amplitude distribution of the NBIs which were distinguished by their consistent latency. (a) Control recordings; (b) U50488H-treated recordings; (c) the NBI amplitude distribution shown in (a) was subtracted from the NBI amplitude distribution shown in (b). In (c) positive measurements indicate an increase in the frequency of recording NBIs with a certain amplitude in (b) when compared to (a) and negative values indicate a decrease in the frequency of recording NBIs with a certain amplitude in (b) when compared to (a). The frequency of stimulation was 0.1 Hz and $[Ca^{2+}]_o$ was 0.3 mM. Note that the variance in NBI amplitude is similar to the variance of the electrical noise of the recording.

U50488H as shown in Figure 2. Although the average amplitude of the e.p.s.cs decreased in all preparations studied when U50488H was administered, the maximum amplitude of e.p.s.cs did not change significantly. Thus the reduced frequency of postsynaptic action potentials induced by U50488 occurred as a result of a decrease in the frequency of quantal release rather than a decrease in quantal size (i.e. mean quantal size ± s.e.mean for controls was 34 ± 4 μV ($n = 100$) and for U50488H was 35 ± 4 μV ($n = 100$), Figure 5) favouring a presynaptic mode of action for the drug.

The effect of morphine (10 μM), dynorphin-A (4 μM) and U50488H (1 μM) on the average normalised e.p.s.c. amplitude was evaluated (Figure 6). Morphine up to 10 μM did not affect the amplitude of e.p.s.cs ($n = 7$). Application of naloxone (10 μM) to morphine-treated preparations slightly increased the amplitude of e.p.s.cs but this was not significant. Dynorphin-A (4 μM) decreased the amplitude of e.p.s.cs by 32 ± 19% (mean ± s.e.mean, $n = 4$, $P < 0.1$). Naloxone (10 μM) abolished this decrease and again the e.p.s.c. amplitudes were slightly greater than for controls but this increase was not significant. U50488H (1 μM) significantly ($P < 0.05$) decreased the amplitude of e.p.s.cs by 54 ± 12% ($n = 9$) when compared to controls. Naloxone (10 μM) abolished this decrease and again the e.p.s.c. amplitudes were slightly greater than for controls but this increase was not significant (Figure 6).

The decrease in average e.p.s.c. amplitude observed when dynorphin-A (4 μM) and U50488H (1 μM) was administered to the rat pelvic ganglia was predominantly due to an increase in the number of failures to evoke e.p.s.cs during nerve stimulation (Figure 7). U50488H (1 μM) was the most effective inhibitor of evoked transmitter release since it was able to increase the number of failures by 2.8 ± 0.7 times when compared to controls, dynorphin-A (4 μM) increased the failure rate by 2.1 ± 0.6 times when compared to controls while morphine (10 μM) had no effect on the failure rate (Figure 7).

Discussion

Visualisation and recording from the hypogastric boutons

The large ovoid ganglion cells of the rat pelvic ganglia are innervated by single axons arising from the hypogastric nerve (Tabatabai *et al.*, 1986; Snider, 1987; Purves *et al.*, 1988; Rogers *et al.*, 1990). The boutons innervating these large ganglion cells are about 1 μm in diameter and about 30% of the bou-

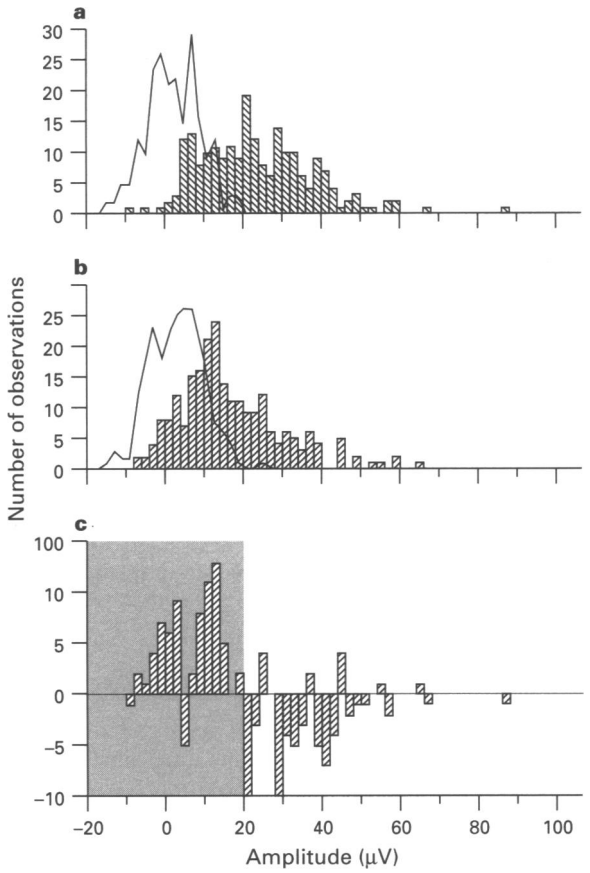

Figure 5 The amplitude distributions of e.p.s.cs for a control and U50488H-treated preparation. (a) Control distribution of e.p.s.c. amplitudes (cross hatched column histogram) and electrical noise (open histogram). (b) U50488H (1 μM) treated distribution of e.p.s.c. amplitudes (cross hatched columns) and electrical noise (open columns). (c) The difference between cross hatched columns in (a) and (b) is plotted, i.e. (a) was subtracted from (b). Positive values on the ordinate scale indicate an increase while negative values indicate a decrease in the occurrence of e.p.s.cs with a particular e.p.s.c. amplitude. The shaded area in (c) indicates the noise level (mean ± 2 s.ds) of the recording. Any measurements of e.p.s.c. amplitudes which fall in the shaded area were counted as failures of the boutons to release quanta while measurements outside the shaded area were counted as quantal releases.

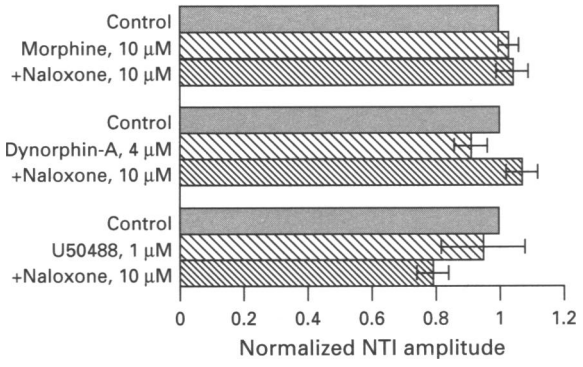

Figure 4 The effect of opiates on the NBI. The average amplitude of the NBI was normalized with respect to the control NBI average amplitude. Drug treatment is indicated on the ordinate scale while the normalized NBI amplitude is shown on the abscissa scale. Bars indicate the mean normalized NBI amplitude and lines indicate the SEM. There was no significant difference between controls and morphine or dynorphin-A or U50488H treated preparations. There was also no significant difference between controls or opiate agonist and naloxone-treated preparations.

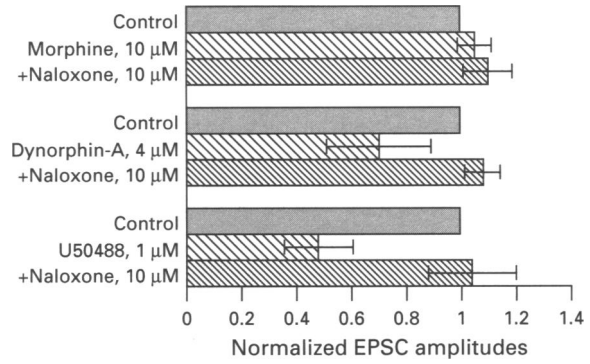

Figure 6 The effect of opiates on e.p.s.c. amplitude. The average amplitude of the e.p.s.cs was normalized with respect to the control e.p.s.c. average amplitude. Drug treatment is indicated on the ordinate scale while the normalized e.p.s.c. amplitude is shown on the abscissa scale. Mean normalized e.p.s.c. amplitude are shown with s.e.mean. There was no significant difference between control, morphine (10 μM)-treated and naloxone (10 μM)-treated preparations. Dynorphin-A produced a significant decrease only at the $P < 0.1$ level. A significant difference between control and U50488H (1 μM, $P < 0.05$)-treated preparations was observed. Naloxone (10 μM) treatment completely reversed the inhibition induced by dynorphin-A and U50488H.

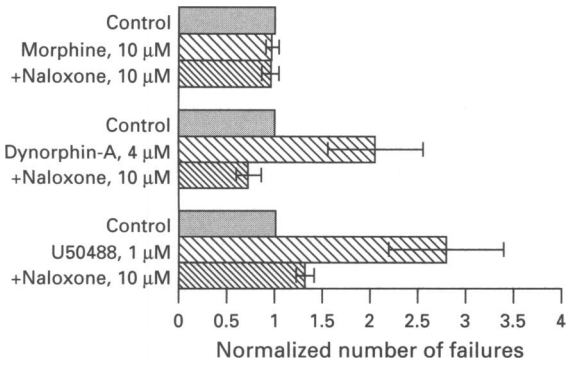

Figure 7 The effect of opiates on the rate of failures in quantal release from hypogastric boutons during nerve stimulation at 0.1 Hz. The average number of failures to release quanta during 200 nerve stimulations was normalized with respect to the control number of failures to secrete quanta. Drug-treatment is indicated on the ordinate scale while the normalized average number of failures is shown on the abscissa scale. Bars indicate the mean normalized number of failures together with s.e.mean. There was no significant difference between control and morphine (10 μM)-treated or naloxone (10 μM)-treated preparations. A significant ($P<0.05$) increase in the number of failures to release quanta was observed following dynorphin-A or U50488H-treated preparations. Naloxone (10 μM) reversed the effects of dynorphin-A and U50488H. The number of failures per 200 stimulations in control preparations varied between 48 and 77%.

tons are arranged in clusters of 2 to 4 (Warren *et al.*, 1995). The size of the individual boutons vaired considerably ranging between 0.3 to 1.6 μm (Warren & Lavidis unpublished observation). When bathed in 0.3 mM $[Ca^{2+}]_o$, the probability of quantal release from these boutons also varied considerably ranging from below 0.005 to nearly 1. A sufficient number of boutons with high probability of quantal release could be found on each ganglion cell even in 0.3 mM $[Ca^{2+}]_o$ to ensure that transmission between the boutons and ganglion cell had a high safety factor.

In this study we considered only the most active boutons since these boutons were still active when the $[Ca^{2+}]_o$ was reduced to very low levels in order to eliminate the postsynaptic action potential. Decreasing both the $[Ca^{2+}]_o$ from 1 to 0.3 mM and the temperature from 34 to 29°C either abolished the postganglionic action potential or delayed it sufficiently to allow reasonable measurements of the e.p.s.c. amplitudes to be made.

Effect of opiates on the NBI and the e.p.s.cs

Opiates have been shown to decrease transmitter release at many synapses including the mouse vas deferens (Henderson *et al.*, 1972; Bennett & Lavidis, 1980; Illes *et al.*, 1980; Lavidis, 1995c) mesenteric neurones (Bornstein & Fields, 1983); amphibian neuromuscular junction (Frederickson & Pinsky, 1970; Bixby & Spitzer, 1984; Lavidis, 1995a, b) and the mouse hypogastric ganglia (Rogers & Henderson, 1990). In all these preparations opiates have been clearly shown to act presynaptically to induce a decrease in quantal release rather than affecting quantal size. Rogers & Henderson (1990) demonstrated a presynaptic locus of action for opiates at the mouse hypogastric ganglia using intracellular recordings of e.p.s.ps and quantal analysis. We have used a more direct approach to investigate the presynaptic locus of action for the opiates using loose patch recording with an extracellular electrode to record the NBI, e.p.s.cs and postsynaptic action potential simultaneously. The present study confirms the presynaptic locus of action of opiates by showing that opiates increase the number of failures to evoke e.p.s.cs without affecting the mean size of e.p.s.cs during low frequency stimulation (0.1 Hz) of the hypogastric nerve. The mean amplitude, time course or consistency with which the NBI was recorded were not affected by any of the opiates tested.

Which opioid receptor is responsible for the decrease in transmitter release?

Morphine is known to be a good μ-opioid receptor agonist and a poor δ- and κ-opioid receptor agonist (Robson *et al.*, 1983). Dynorphin-A and U50488H are known to be fairly specific κ-opioid receptor agonists (Chavkin *et al.*, 1982). In this study morphine was shown not to affect quantal release while dynorphin-A and U50488H decreased quantal release. These results suggest that κ-opioid receptors are located presynaptically on the boutons of the hypogastric nerve. Activation of these κ-opioid receptors results in a decrease in quantal release without any affect on the propagation or the time course of the NBI. This inhibitory effect of κ-opioid receptor agonists was completely abolished by administration of naloxone or by increasing the $[Ca^{2+}]_o$ from 0.3 to 1.0 mM. Similar antagonism of the opiate-induced inhibition of quantal secretion by increasing $[Ca^{2+}]_o$ has been demonstrated in the mouse vas deferens (Bennett & Lavidis, 1980; Illes *et al.*, 1980; Einstein & Lavidis, 1984; Lavidis, 1995c), the amphibian neuromuscular junction (Lavidis, 1995a, b) and the hypogastric ganglia of mouse (Rogers & Henderson, 1990).

In conclusion, the boutons of the hypogastric nerve innervating the rat pelvic ganglion cells seem to possess κ-opioid receptors. When these receptors are activated by dynorphin-A or U50488H there was a decrease in the release of quanta without any effect on the propagation of the NBI. This decrease in quantal release was counteracted by increasing $[Ca^{2+}]_o$, suggesting that the opiate-induced decrease involves an action on the entry of $[Ca^{2+}]_o$ during nerve stimulation.

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