Analgesic doses of morphine do not reduce noxious stimulus-evoked release of immunoreactive neurokinins in the dorsal horn of the spinal cat

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- 1 Antibody microprobes were used to detect immunoreactive neurokinin A release in the dorsal spinal cord of barbiturate-anaesthetized spinal cats.
- 2 Noxious mechanical stimulation of the ipsilateral hind paw and electrical stimulation (suprathreshold for unmyelinated primary afferent fibres) of the ipsilateral tibial nerve evoked immunoreactive neurokinin A release.
- 3 Systemic morphine, 5 mg kg⁻¹, i.v., did not block immunoreactive neurokinin A release in response to these stimuli.
- 4 Subsequent naloxone administration, 0.5 mg kg⁻¹, i.v., did not alter this stimulus-evoked release.
- 5 Basal levels of immunoreactive neurokinin A were unaltered by morphine or naloxone.
- 6 These results suggest that the analgesic effects of morphine at the spinal cord level are not brought about by activation of presynaptic opiate receptors on neurokinin A containing afferent terminals.

Keywords: Neurokinins; morphine; naloxone; antibody microprobes; cat spinal cord.

Introduction

Substance P and neurokinin A, two members of the tachykinin family of neuropeptides, extensively coexist in the small diameter dorsal root ganglion cells of the rat (Dalsgaard *et al.*, 1985). Both tachykinins have been proposed as important in the transmission of information from nociceptive afferents to spinal or brainstem neurones (Otsuka & Yanagisawa, 1988; Fleetwood-Walker *et al.*, 1990). Studies of the release of these compounds in the spinal cord by use of antibody microprobes have shown both tachykinins to be released in response to a similar range of peripheral noxious stimuli (Duggan *et al.*, 1988b; 1990; Hope *et al.*, 1990a; Schaible *et al.*, 1990) although important differences in noxious stimuli release patterns have also been identified. Noxious stimuli producing detectable tissue damage caused greater release of immunoreactive substance P in the dorsal horn, than noxious, non-damaging stimuli. Noxious cutaneous stimuli also released immunoreactive neurokinin A, but potentiation by tissue damage was not seen. Immunoreactive neurokinin A exhibited a remarkable spread beyond sites of release and persisted following an effective stimulus. In contrast a focal, non-persistent pattern was observed with immunoreactive substance P. These findings suggest that neurokinin A, unlike the relatively short-lived substance P, may not function as a conventional neurotransmitter, but might rather have a long term modulatory role on spinal cord systems.

Ionophoretic administration of morphine (Duggan *et al.*, 1977) and the μ opioid agonist [D-Ala² Me-Phe⁴, Gly-ol⁵] enkephalin (DAMGO) (Fleetwood-Walker *et al.*, 1988) in the region of the substantia gelatinosa selectively suppressed the transmission of impulses from nociceptive afferents to deeper neurones of the dorsal horn. The substantia gelatinosa is the major site of termination of unmyelinated cutaneous afferent fibres (Light & Perl, 1977). Since administration of naloxone in the substantia gelatinosa completely blocked the effects of analgesic doses of systemic morphine on deep neurones of the dorsal horn of the cat (Johnson & Duggan, 1981) it is likely that opiate receptors in the substantia gelatinosa are the major ones mediating the analgesic actions of morphine at the spinal level, whether administered systemically or intrathecally. One mechanism by which morphine could produce

analgesia is by presynaptic inhibition of neurotransmitter release from nociceptive primary afferent terminals. In support of this hypothesis, morphine in high concentrations, when added to the superfusate of either a slice of the upper dorsal horn (Jessel & Iversen, 1977; Mauborgne *et al.*, 1987) or of the spinal cord *in vivo* (Yaksh *et al.*, 1980; Go & Yaksh, 1987) has been shown to reduce stimulus evoked release of immunoreactive substance P. However, systemically administered morphine at analgesic doses has failed to reduce release of immunoreactive substance P evoked by noxious stimulation (Kuraishi *et al.*, 1983; Morton *et al.*, 1990).

With the recognition that a peripheral noxious stimulus results in the central release of a number of neuroactive compounds including amino acids (Kangra & Randic, 1990) and peptides (Duggan *et al.*, 1988b; 1990), a complete study of opiates and release from primary afferents is clearly a complex task. There is evidence that the arrival of impulses in nociceptive afferents produces not only fast transmission to fibres ascending to the brain but also long lasting events such as prolonged facilitation of flexor reflexes (Woolf, 1983; Woolf & Wall, 1986a; Cook *et al.*, 1987; Cervero *et al.*, 1988; Neugebauer & Schaible, 1988; Hoheisel & Mense, 1989; Hylden *et al.*, 1989). It has been proposed that neurokinin A (rather than substance P) is more important in these prolonged spinal events since, following release of immunoreactive neurokinin A, this peptide (or an extended form such as neuropeptide K) diffuses widely and persists in the spinal cord (Hope *et al.*, 1990a). Of note is the finding that in the rat, low doses of morphine suppressed the prolonged facilitation of spinal reflexes produced by nociceptive input, whereas higher doses were required to suppress fast transmission as measured by an unfacilitated nociceptive reflex (Woolf & Wall, 1986b). Such considerations indicate the need for a study of opiates and the central release of neurokinin A. In the present study therefore, the effects of analgesic doses of systemically administered morphine were examined on the noxious stimulus-evoked release of immunoreactive neurokinin A in the dorsal horn of the spinal cord in barbiturate-anaesthetized cats.

Methods

Preparation of antibody microprobes

Antibody microprobes were prepared as previously described (Duggan *et al.*, 1988a). Fine glass micropipettes, heat sealed at

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base and tip, were incubated in a 10% solution of amino-propyltriethoxysilane producing an even layer of a siloxane polymer bearing free amino-groups on the outer surface of the probes. Glutaraldehyde was then used to immobilize protein A (Sigma) onto this polymer; protein A then bound immunoglobulins present in an antiserum raised against the carboxyl terminus of neurokinin A (Peninsula). Although this antiserum is described as anti-neurokinin A, information from the manufacturer indicates it has 100% cross reactivity with neuropeptide K (an N-terminal extended form of neurokinin A), 70–80% cross-reactivity with neurokinin B, but negligible cross-reactivity with substance P. For purposes of the present experiments microprobes are described as detecting immunoreactive neurokinins.

The antiserum lyophilate contained salts from the suspending buffer which limited the possible concentration of resuspended antibody. A dilution of approximately 1:3000 was used. All protein binding outside the spinal cord took place in 5 μ l glass capillaries containing the relevant solutions.

Animal preparation/recording/microprobe analysis

Experiments were performed on 8 cats anaesthetized initially with intraperitoneal sodium pentobarbitone 35 mg kg⁻¹, and maintained by continuous intravenous infusion of sodium pentobarbitone, 3 mg kg⁻¹ h⁻¹. All animals were artificially ventilated with air and when necessary paralysed with gallamine triethiodide, 4 mg kg⁻¹ h⁻¹. Blood pressure was measured continuously via a cannula in the left carotid artery and end tidal CO₂ levels were also monitored and maintained at 4%.

The lumbar spinal cord was exposed by dorsal laminectomy and the spinal cord transected at the level of T13/L1 following injection of 0.1 ml of 2% lignocaine (Astra). The lumbar dura mater was cut longitudinally at the level of L7-S1 and retracted laterally. The exposed cord was then covered with a thin layer of Ringer/agar. At sites of proposed microprobe insertion a small area of agar was removed to form a pool and the underlying pia-arachnoid removed with fine sterile forceps. Such areas were then continuously irrigated with sterile Ringer solution at 37°C. Microprobes were inserted into the spinal cord with stepping motor micromanipulators. The first probe in each new area was used to obtain extracellular recordings to identify the receptive fields, to light brushing of the skin, of neurones encountered along a microprobe track.

Peripheral noxious stimulation was provided either by applying 'crocodile' clips to the pads and interdigital skin of the ipsilateral hind paw in a cycle of 3 min on, 2 min off, for the duration of the stimulus or by electrical stimulation of the ipsilateral tibial nerve (groups of three 0.5 ms square wave pulses at 330 Hz, repeated at 10 Hz, amplitude 20 V). All microprobes were inserted 3 mm into the dorsal spinal cord which, in an adult cat, places the tips in the upper ventral horn. All probes remained in the spinal cord for 30 min following insertion.

Antibody microprobes demonstrate localized binding of an endogenous ligand by their subsequent failure to bind exogenous radiolabelled ligand. Following removal from the spinal cord, microprobes were washed for 15 min in cold phosphate buffered saline containing Tween-80 (0.1%) and then incubated for 24 h at 4°C in a phosphate buffered saline/azide solution of [¹²⁵I]-neurokinin A containing 0.5% bovine serum albumin diluted to give approximately 2000 c.p.m. μ l⁻¹. Following this incubation, microprobes were again washed for 15 min in PBS-Tween with suction applied to the base of the microprobes to draw any labelled neurokinin A out of the tips. The tips were then broken off and fixed to a sheet of paper which was placed in an X-ray film cassette with a sheet of monoemulsion X-ray film (NMB-Kodak). Exposure times were typically in the order of 3–4 weeks reflecting the limited amounts of this antibody which could be bound to the probe surfaces. In each experiment 8 microprobes were used for *in vitro* tests of sensitivity. Four of these were incubated with

10⁻⁷ mol l⁻¹ neurokinin A for 30 min at 37°C prior to incubation in [¹²⁵I]-neurokinin A. The other 4 were simply incubated with the radiolabelled neuropeptide.

The resulting X-ray film images were analysed with an image analysis system employing an Imaging Technology PC Vision Plus frame grabber board operating in a DCS 286e (AT-based) computer. A television (CCD) camera was used as described previously (Hendry *et al.*, 1988) to produce a transverse integration of optical density, with a resolution of 10 μ m, along the length of each microprobe. These integrals were stored on a hard disk together with 32 coded values assigned to various experimental parameters for each microprobe. These codings allowed sorting of microprobes into groups meeting stated criteria. Images from these groups could then be averaged and the differences between control and test groups calculated and assigned levels of statistical significance. It should be emphasized that the spatial resolution of this method is 100 sites (each of 10 μ m) per mm, and that events at each site are considered independently of others in the averaging of images and the calculation of group differences and their significances.

Drug dosage and experimental regimes

The drugs used were administered via an intravenous catheter into the right cephalic vein. Morphine was administered as a 10 mg ml⁻¹ solution in physiological saline, naloxone as 1 mg ml⁻¹ in physiological saline.

As previously demonstrated (Duggan *et al.*, 1990; Hope *et al.*, 1990a) the elevation of immunoreactive neurokinin levels in the dorsal spinal cord, following a discrete stimulus, persists for up to 1 h beyond stimulation. This persistence creates difficulties when testing a compound which may block release, since a series of pre-drug measurements of stimulus-evoked release may result in a build up of immunoreactive neurokinins in the dorsal horn such that any subsequent reduction in release brought about by the drug may not be detectable. Consequently, these experiments examined the basal levels of immunoreactive neurokinins in the absence of any stimulus and then administered morphine prior to measuring stimulus-evoked release of these compounds. Several groups have shown that in the spinal cat, doses of morphine of 0.3 to 4.0 mg kg⁻¹ i.v. reduced the synaptic activation of dorsal horn neurones by peripheral noxious stimuli (Le Bars *et al.*, 1976a,b; Duggan *et al.*, 1980; Johnson & Duggan, 1981). The present experiments used morphine at levels just above the upper limit of this range (5.0 mg kg⁻¹, i.v.) since, with the protocol used, lack of effectiveness of the stimulus would be more readily detected than a partial reduction. A more significant test of the effect of morphine on immunoreactive neurokinin release came from the effects of subsequent naloxone administration. If stimulus evoked release of immunoreactive neurokinins is increased when naloxone is administered after morphine, this indicates that morphine had reduced release of these compounds. The above protocol is not optimal if morphine increases release of immunoreactive neurokinins. A series of experiments was therefore included in this study in which naloxone was administered prior to morphine in an attempt to block such an increase. Comparisons were also made with earlier studies on the effects of stimulus-evoked release of immunoreactive neurokinins in the absence of morphine (Hope *et al.*, 1990a,b).

Results

A total of 97 microprobes coated with antibodies to neurokinin A were inserted into the spinal cord. Additionally, 64 microprobes were used for concurrent *in vitro* tests. These consistently demonstrated suppression of the binding of [¹²⁵I]-neurokinin A by greater than 50% following incubation with neurokinin A 10⁻⁷ mol l⁻¹.

Figure 1 The effects of systemic morphine administration on resting levels of immunoreactive neurokinins in the dorsal horn. Controls: the mean image analysis of microprobes ($n = 11$) inserted into the dorsal spinal cord with no prior or concurrent drug administration and no prior or concurrent peripheral stimulation. Morphine: the mean image analysis of microprobes ($n = 7$) inserted into the dorsal spinal cord immediately following morphine (5 mg kg^{-1} , i.v.) administration and in the absence of prior or concurrent peripheral stimulation. The analysis was performed with a resolution of 33 points per millimetre and with each group the continuous line has joined these points. The (+) s.e.mean at each point is plotted for the morphine group and the (-) s.e.mean at each point is plotted for the control group. The t values ($2t = P < 0.05$) derived from the differences between the means of the two groups are also shown.

Thirty-five probes were inserted 3 mm into the cord and left for 30 min without stimulation of either hind paw or the concurrent administration of any drug. These microprobes formed the basis for assessing possible effects of morphine or naloxone on the basal levels of immunoreactive neurokinins. The remaining 62 probes were inserted into the spinal cord in the presence of peripheral noxious stimuli and/or drug administration.

Effects of morphine and naloxone on basal levels of immunoreactive neurokinins

In four experiments, morphine was the first drug administered and in the remaining experiments naloxone was the first drug administered. Seven probes were inserted into the spinal cord in the absence of prior or concurrent noxious stimuli, and

Figure 2 The effects of systemic naloxone administration on resting levels of immunoreactive neurokinins in the dorsal horn. Controls: the mean image analysis of microprobes ($n = 23$) inserted into the dorsal spinal cord with no prior or concurrent drug administration and no prior or concurrent peripheral stimulation. Naloxone: the mean image analysis of microprobes ($n = 13$) inserted into the dorsal spinal cord immediately following naloxone (0.5 mg kg^{-1} , i.v.) administration and in the absence of prior or concurrent peripheral stimulation. S.e.means for the two groups and t values ($2t = P < 0.05$) derived from the differences of the means are shown as in Figure 1.

Figure 3 The effects of noxious stimulation of the ipsilateral hind paw, following systemic morphine administration on immunoreactive neurokinin levels in the dorsal horn. Controls: the mean image analysis of microprobes ($n = 11$) inserted into the dorsal spinal cord with no prior or concurrent drug administration and no prior or concurrent peripheral stimulation. Morphine (stim): the mean image analysis of microprobes ($n = 17$) inserted into the dorsal spinal cord following morphine (5 mg kg^{-1} , i.v.) administration and during noxious stimulation of the ipsilateral hind paw. S.e.means for the two groups and t values ($2t = P < 0.05$) derived from the differences of the means are shown as in Figure 1.

within 1 min of morphine administration (5 mg kg^{-1} , i.v.). In all cases, this dose of morphine was the first drug administered. The mean image analysis of these probes is illustrated in the upper trace of Figure 1. When this group is compared to the mean image analysis of microprobes inserted in the absence of prior peripheral stimulation in the same experiments (lower trace, 'controls', Figure 1), no significant differences are present as shown in the plot of the t statistics for the differences between these groups of microprobes.

Thirteen probes were inserted into the spinal cord in the absence of prior or concurrent noxious stimulation and within 1 min of naloxone administration (0.5 mg kg^{-1} , i.v.). No prior morphine administration had occurred. The mean image analysis of this group of microprobes and the relevant (no stimulus) controls are shown in Figure 2. The plot of the t statistics derived from the differences between these groups shows that no significant differences are present.

Figure 4 Effect of naloxone following morphine on noxious stimulus evoked immunoreactive neurokinin release. Morphine (stim): the mean image analysis of microprobes ($n = 17$) as described in Figure 3. Naloxone following morphine (stim): the mean image analysis of microprobes ($n = 10$) inserted into the dorsal spinal cord immediately following those illustrated in the morphine (stim) group, and after naloxone (0.5 mg kg^{-1} , i.v.) administration during noxious stimulation of the ipsilateral hind paw. S.e.means for the two groups and t values ($2t = P < 0.05$) derived from the differences of the means are shown as in Figure 1.

An additional 10 probes were inserted into the spinal cord in the absence of prior or concurrent noxious stimulation and within 1 min of morphine administration (5 mg kg^{-1} , i.v.) but in this case morphine immediately followed naloxone (0.5 mg kg^{-1} , i.v.) administration. Again, when compared with the relevant control group no significant differences were found.

In summary, neither morphine nor naloxone, administered in the absence of peripheral noxious stimulation, produced significant changes in detectable levels of immunoreactive neurokinins in the dorsal spinal cord. A combination of these two drugs also failed to alter significantly basal levels of immunoreactive neurokinins.

Effect of morphine on stimulus-evoked immunoreactive neurokinin release

Seventeen microprobes were inserted into the dorsal spinal cord within 90 min of morphine administration (5 mg kg^{-1} , i.v.) and with concurrent noxious stimulation of the ipsilateral hind paw. Noxious pinch and electrical stimulation of unmyelinated primary afferents have previously been shown to be adequate stimuli for evoking immunoreactive neurokinin release in the dorsal spinal cord (Duggan *et al.*, 1990; Hope *et al.*, 1990a). Following morphine administration, noxious stimulus dependent immunoreactive neurokinin release was seen in the present experiments in a zone extending from the spinal cord surface to approximately 1.3 mm into the dorsal grey matter as shown in Figure 3. Although the increases in the levels of immunoreactive neurokinins in the dorsal horn produced by the stimulus appeared comparable to those produced by comparable stimuli in previous studies in which morphine had not been administered (Duggan *et al.*, 1990; Hope *et al.*, 1990a) a small inhibition of release by morphine could only be assessed by the subsequent administration of naloxone.

Eleven probes were inserted into the dorsal spinal cord within 1 min of naloxone (0.5 mg kg^{-1} , i.v.) given after morphine and during peripheral noxious stimulation. These probes were inserted after those represented in Figure 3, i.e. immediately after the period of stimulation which followed morphine administration. Figure 4 shows that naloxone had no effect on the stimulus-evoked levels of immunoreactive neurokinins in the dorsal horn which were observed following morphine. This lack of effect of naloxone suggests that the dosage of morphine used prior to naloxone had not altered the release of immunoreactive neurokinins.

Discussion

When the results of the present experiments are considered with those of Kuraishi *et al.* (1983) and Morton *et al.* (1990), there is little evidence that analgesic doses of opiates administered systemically reduce tachykinin release (either neurokinin A or substance P) from the central terminals of nociceptors. Since such results are at variance with the conclusions of experiments which have examined the effect on substance P release of topically applied opiates, these differences require comment.

Inhibition of substance P release by opiates was first shown by Jessell & Iversen (1977) in a slice preparation of rat trigeminal nucleus. The stimulus used, however, was 47 mM potassium in the perfusate. This will depolarize virtually all neural structures in the slice, and hence the source of substance P is uncertain. Furthermore, the concentration of morphine used was high (10^{-5} M). Using the push-pull cannula technique on rabbit spinal cord *in vivo*, Hirota *et al.* (1985), demonstrated a reduction in the release of substance P following noxious cutaneous stimulation with morphine (10^{-5} M) in the perfusate. Further support for the proposal that opiates impair substance P release from the central terminals of nociceptors came from the experiments of Yaksh *et al.* (1980) and Go & Yaksh (1987), in which morphine (10^{-4} M)

added to a superfusate of intact cat spinal cord *in vivo* reduced the release of substance P into the perfusate following electrical stimulation of unmyelinated primary afferents.

Mauborgne *et al.* (1987), however, found that while the μ -receptor agonist DAMGO (10^{-5} M) increased both high potassium and capsaicin induced release of substance P from dorsal horn slices, the δ -receptor agonist [D-Thr²]-Leu-enkephalin-Thr (DTLET) ($3 \times 10^{-6} \text{ M}$) reduced release in the same preparation. The κ -agonist, U50488H (0.5 – $50 \times 10^{-6} \text{ M}$), did not affect release of substance P (Hamon *et al.*, 1988; Pohl *et al.*, 1989).

It should be noted that the concentrations of drugs used in all of the above studies were high and that no group has found that systemic analgesic doses of morphine reduce substance P release in the dorsal horn of spinal animals. Thus, Kuraishi *et al.* (1983), using push pull canulae in the dorsal horn of the spinal rabbit observed that systemic morphine (10 mg kg^{-1}) failed to reduce immunoreactive substance P release from noxious mechanical stimulation. Using antibody microprobes, Morton *et al.* (1990) failed to observe any effect of morphine given in two dose ranges (1 – 6 mg kg^{-1} and 10 – 20 mg kg^{-1}) on immunoreactive substance P release in the dorsal horn of the spinal cat following noxious mechanical or thermal cutaneous stimulation.

Since studies which have observed effects on tachykinin release have used relatively high concentrations of opiates, is the same true of experiments which have administered opiates microiontophoretically in the substantia gelatinosa and hence may have over-emphasized the importance of effects near the first central synapse of nociceptors? Although concentrations in microiontophoretic experiments are unknown, the finding that naloxone administered in the substantia gelatinosa decreased the effects of systemic morphine, 4 mg kg^{-1} , on deeper neurones (Johnson & Duggan, 1981) does support the importance of opiate receptors in the substantia gelatinosa in mediating the action of systemic morphine on spinal neurones.

When γ -aminobutyric acid acts presynaptically on the central terminals of large diameter muscle afferents, the resultant depolarization is associated with reduced transmitter release and an increased excitability when tested with an adjacent stimulating microelectrode (Curtis *et al.*, 1980). Administering opioids near the central terminals of nociceptors in the substantia gelatinosa however, has resulted in decreased electrical excitability (Sastry, 1980; Carstens *et al.*, 1987), a result not consistent with the well-established mechanism of presynaptic inhibition in the spinal cord. A different mechanism of reducing transmitter release might be if calcium entry with each invading impulse were to be reduced through a shortening of action potential duration. Such a shortening has been observed with micromolar concentrations of opioids acting on cultured dorsal root ganglion neurones (Werz & Macdonald, 1982; Shen & Crain, 1989) but lower (nanomolar) concentrations had the opposite effect (Shen & Crain, 1989). Support for a postsynaptic effect of opioids on intrinsic neurones of the substantia gelatinosa has come from Yoshimura & North (1983) who observed a hyperpolarization of such cells in a slice preparation, and Sastry & Goh (1983) who found many to be excited *in vivo* by iontophoretically administered morphine. The inconsistency in these reports however has not been explained.

Recently Lombard & Besson (1989) have tried to assess the relative importance of pre- and postsynaptic actions of morphine by recording the firing of dorsal horn neurones in decerebrate spinal rats with intact dorsal roots (and an induced peripheral arthritis) and comparable cells in non-arthritic animals with sectioned dorsal roots. Morphine (2 mg kg^{-1} , i.v.) depressed the spontaneous firing of neurones in both preparations but a greater effect was observed in the arthritic animals with intact dorsal roots. Such a result indicates an action by opiates on the pathway from primary afferent terminals to the neurones studied but cannot distinguish between effects on terminals and effects on interneurones interposed between such terminals and the neurones studied.

It has been the conclusion of studies which have observed reductions in tachykinin release with high concentrations of opiates that a presynaptic action on the central terminals of nociceptors is an important component of opiate analgesia (Yaksh & Noueihed, 1985). The present experiments considered with those of Kuraishi *et al.* (1983) and Morton *et al.* (1990) suggest that such an action does not occur significantly

with tachykinin releasing fibres in the spinal cord following clinically relevant doses of morphine.

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