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Clonidine and morphine increase [^{3}H]-noradrenaline overflow in mouse vas deferens

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1 Field stimulation of mouse isolated vas deferens produced a biphasic contraction that consisted of an initial brief non-adrenergic, non-cholinergic (NANC) twitch, followed by a more prolonged noradrenergic component.

2 Field stimulation of vasa, previously loaded with [^{3}H]-noradrenaline ([^{3}H]-NA), increased the amount of radioactivity in the Krebs bathing solution; 77% of this radioactivity was derived from [^{3}H]-NA.

3 Tetrodotoxin (3×10^{-6} M) abolished the biphasic motor response to field stimulation and the accompanying increased overflow of [^{3}H]-NA.

4 Morphine (10^{-7}–10^{-5} M) inhibited the initial NANC component but potentiated the secondary noradrenergic component of the motor response to field stimulation. Morphine also increased the field stimulation-induced overflow of radioactivity. Naloxone (10^{-6} M) antagonized the effects of morphine on the motor response and also on the overflow of radioactivity.

5 Clonidine (10^{-9}–10^{-7} M) inhibited the initial NANC component but potentiated the secondary noradrenergic component of the motor response to field stimulation. Clonidine also increased the field stimulation-induced overflow of radioactivity.

6 The ability of morphine (10^{-7} M) and of clonidine (10^{-9} M) to potentiate the field stimulation-induced overflow of radioactivity persisted in the presence of a combination of tranylcypromine (10^{-5} M), desmethylimipramine (10^{-5} M) and 17-β-oestradiol (10^{-5} M).

7 The inhibition of the initial NANC component of the motor response to field stimulation produced by morphine and clonidine may be related to the ability of these drugs to potentiate both the secondary noradrenergic component and the overflow of radioactivity, if the NANC transmitter is involved in regulating NA release.

Introduction

The mouse vas deferens has been used to assay opioid peptide activity (Hughes *et al.*, 1975). For this purpose contractions of isolated vasa to electrical field stimulation are recorded and the inhibitory effects of opioid peptides on these mechanical responses determined. In this technique, the presumed presynaptic inhibitory actions of opioids on transmitter release are investigated by measuring the inhibitory effects of opiates on field stimulation-induced contractions of smooth muscle. Such an indirect method is unsatisfactory, since it assumes that out of the complex sequence of events initiated by field stimulation and culminating in smooth muscle contraction, the only one affected by opioids is transmitter release. Some studies have shown a correlation between the inhibitory effects of opiates on field stimulation-induced contractions and inhibition of noradrenaline (NA) release (Henderson & Hughes, 1976). In contrast, clonidine, which also acts presynaptically to inhibit transmitter release, paradoxically enhanced motor responses to field stimulation and potentiated the overflow of NA in the guinea-pig vas deferens (Stjärne, 1975).

One complication that must now be considered and might explain such a discrepancy is the fact that transmission in the vas involves not only NA but also a non-adrenergic, non-cholinergic (NANC), co-transmitter that might be adenosine triphosphate (ATP) (Fedan *et al.*, 1981). Moreover, there is evidence that the two components of the typically-biphasic motor response to field stimulation differ in their susceptibility to blockade by clonidine and opiates, suggesting that release of the two co-transmitters may be unequally affected by these drugs (Forsyth *et al.*, 1986).

In this study, the effects of morphine and clonidine on motor responses of the mouse vas deferens to field stimulation and on the consequent overflow of [^{3}H]-NA in the vas are compared. In particular, we sought to determine if clonidine simultaneously potentiates motor responses of the mouse vas to field stimulation and increases [^{3}H]-NA overflow as it does in the guinea-pig vas, and whether morphine inhibits both overflow of [^{3}H]-NA and contractions. Some of the results described in this paper have been presented to a meeting of the Physiological Society (Forsyth & Pollock, 1986).

Methods

Male Porton mice (25–30 g) were stunned and killed by exsanguination. Vasa deferentia were dissected, freed from connective tissue and transferred to 0.5 ml of Krebs bicarbonate buffer containing 12 μCi of [^{3}H]-NA (500 nM, 43 Ci mmol^{-1}). The Krebs solution (mM: NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and glucose 11.1) was gassed with a mixture of 95% O_2, 5% CO_2. Vasa were incubated in the radioactive solution (37°C, 30 min), inserted into silver ring electrodes and transferred to organ baths (2 ml capacity), and attached to Statham force displacement transducers (resting tension 0.5 g) to record motor responses isometrically. Vasa were continuously superfused with Krebs solution (37°C, 4 ml min^{-1}, 1.5 h) to remove loosely bound [^{3}H]-NA, not taken up into nerves.

At the end of this preliminary washing period, the superfusion was stopped and, with each organ bath filled with Krebs solution (2 ml), vasa were stimulated with a Grass stimulator (model S88) (20 Hz, 5 s, pulse width 0.5 ms, supramaximal voltage). Motor responses were displayed on a Grass Polygraph (model 7). At the end of each period of stimulation and at 2 min intervals between stimulations, the contents of the organ baths were collected in liquid scintillation vials, each containing 10 ml of Ecoscint (National Diagnostics) and the released radioactivity was measured. Each vas was then digested in KOH (1 ml, 0.5 M, 24 h, 60°C) so that the residual radioactivity in the tissue could be determined. Each stimulation-induced release was corrected to take account of the spontaneous, background release measured between stimulations. None of the drugs studied affected the spontaneous release of radioactivity between stimulations. Knowing the total amount of radioactivity released spontaneously between stimulations, the total amount released during stimulations and the amount remaining in the digested tissue at the end of the experiment, the fractional release during each stimulation was calculated. All counts were corrected for quenching.

The [^{3}H]-NA released into the organ baths during stimulation was separated from its metabolites by column chromatography (Graefe *et al.*, 1973). The initial separation was carried out on columns (0.5 cm diameter), each containing 200 mg of sodium acetate-washed alumina (Crout, 1961). Samples were washed through this column first with sodium acetate (0.2 M) to elute the catechol-*O*-methyl transferase (COMT) metabolites, 3-methoxy-4-hydroxymandelic acid (VMA), 3-methoxy-4-hydroxyphenylglycol (MOPEG) and normetanephrine (NMN) (Fraction 1), then with acetic acid (0.2 M and 0.5 M) to elute 3,4-dihydroxyphenylethylglycol (DOPEG) and NA (Fraction 2) and, finally, with HCl (0.2 M) to elute 3,4-dihydroxymandelic acid (DOMA) (Fraction 3). Further separation was performed on columns (0.5 cm diameter), each containing DOWEX resin (1.5 cm, 0.5 ml, pH 1–2, 50 W × 4, 200–400 mesh), previously washed successively with NaOH (2 M), containing 1% w/v Na EDTA, 50°C; water; HCl (2 M); water and finally, HCl (0.01 M). Fraction 1 was separated into 2 subfractions containing VMA and MOPEG, which were eluted with water, and NMN, which was eluted with a mixture of HCl (6 M) and ethanol (50% v/v of each). Fraction 2 was also separated into 2 subfractions, containing DOPEG which was eluted with water, and NA which was eluted with HCl (3 M).

The two phases of the motor response to field stimulation were examined separately. Comparisons were made between the effects of drugs on each component of the motor response and on the fractional release of [^{3}H]-NA, in the absence and presence of a combination of drugs (tranylcypromine 10^{-5} M, desmethylimipramine 10^{-5} M, 17-β-oestradiol 10^{-5} M) to block monoamine oxidase (MAO) and the neuronal and extraneuronal uptake of NA. Mean values were compared by Student's *t* test.

Drugs

The drugs used were atropine sulphate (Macarthy), clonidine (Boehringer-Ingelheim), desmethylipramine (Sigma), morphine hydrochloride (Macarthy), naloxone hydrochloride (Winthrop), [^{3}H]-noradrenaline (Amersham), 17-β-oestradiol (Sigma), phenoxybenzamine hydrochloride (Smith, Kline & French), phentolamine mesylate (Ciba), tetrodotoxin (Sigma), tranylcypromine (Sigma), yohimbine (Light).

Results

Motor responses to field stimulation and the effects of drugs on these contractions

Electrical field stimulation of the isolated vas produced biphasic contractions that consisted of an initial twitch, followed by a more prolonged contrac-

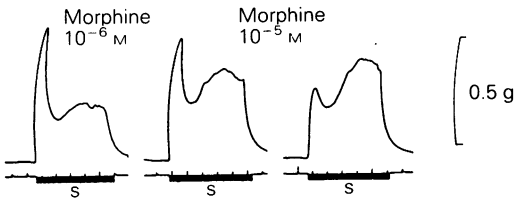

Figure 1 Typical biphasic contractions of the mouse vas deferens to electrical field stimulation at S for the duration of the bar (approximately 5 s). Morphine (10^{-6} M) added between the first and second responses slightly reduced the initial component and potentiated the secondary component. When the concentration of morphine was increased from 10^{-6} M to 10^{-5} M, between the second and third responses, the initial component was reduced and the secondary component was further potentiated.

tion (Figure 1). The initial component was unaffected by atropine (10^{-6} M) or phentolamine (10^{-6} M) but the second component was inhibited by phentolamine (10^{-6} M). Tetrodotoxin (TTX, 3×10^{-6} M) abolished both components of the motor response to field stimulation (Figure 3). Morphine (10^{-7}–10^{-5} M) inhibited the initial component of the motor response to field stimulation and either did not affect or potentiated the second component (Figure 1, 2). Both the inhibitory effect of morphine on the first component and the enhancing effect of morphine on the second component were antagonized by naloxone (10^{-6} M) (Figure 2). Clonidine (10^{-9}–10^{-7} M) also inhibited the initial component of the motor response to field stimulation and either did not affect or more frequently potentiated the second component (Figure 3).

Effects of field stimulation on the overflow of [³H]-NA and the effects of drugs on this overflow

Electrical field stimulation of the vas increased the amount of radioactivity released into the Krebs solu-

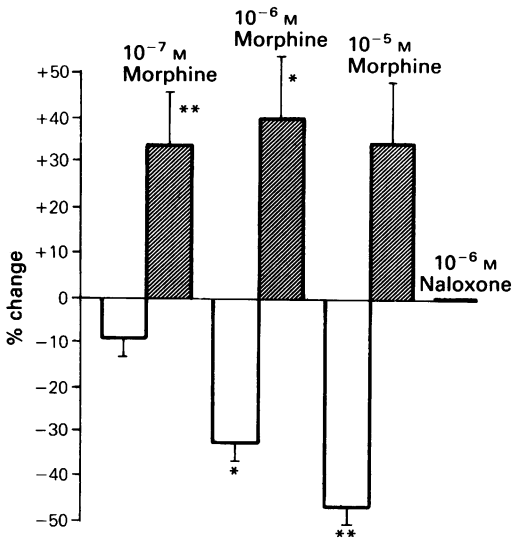

Figure 2 Effects of morphine (10^{-7}–10^{-5} M) on biphasic motor responses of the vas deferens to field stimulation. The results are expressed as percentage changes from controls. The lower, open histograms show the dose-related inhibitory effects of morphine on the initial NANC component of the motor response. The upper shaded histograms show the potentiating effect of morphine on the secondary noradrenergic component of the motor response. Naloxone (10^{-6} M) antagonized the effects of morphine (10^{-5} M) on both phases of the motor response to field stimulation. Each column represents the mean of 6 observations; s.e.mean shown by vertical lines. *$0.05 > P > 0.01$, **$0.01 > P > 0.001$ for comparison with corresponding controls.

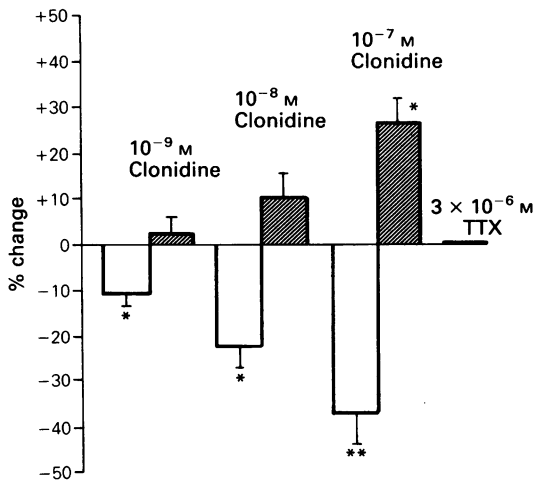

Figure 3 Effects of clonidine (10^{-9}–10^{-7} M) on biphasic motor responses of the vas deferens to field stimulation. The results are expressed as percentage changes from controls. The lower open histograms show the dose-related inhibitory effects of clonidine on the initial NANC component of the motor response. The upper shaded histograms show the potentiating effect of clonidine on the secondary noradrenergic component of the motor response. Tetrodotoxin (TTX, 3×10^{-6} M) blocked both phases of the motor response to field stimulation. Each column represents the mean of 7 observations; s.e.mean shown by vertical lines.
*$0.05 > P > 0.01$, **$0.01 > P > 0.001$ for comparison with corresponding controls.

tion in the organ bath. Such increases coincided with the field stimulation-evoked contractions, after which Krebs solution collected between further stimulations contained only low levels of radioactivity.

TTX (3×10^{-6} M) reduced field stimulation-induced increases in radioactive overflow to basal levels, at a time when motor responses to field stimulation were also inhibited by TTX (Figures 4 and 6). Morphine (10^{-7}–10^{-5} M) increased the overflow of radioactivity in a dose-dependent manner, at a time when the second component of the biphasic response to field stimulation was potentiated (Figure 4). This ability of morphine to enhance overflow of radioactivity was antagonized by naloxone (10^{-6} M) (Figure 5). Clonidine (10^{-9}–10^{-7} M) also increased the overflow of radioactivity in a dose-related manner (Figure 6). This effect of clonidine occurred at a time when the second component of the biphasic response to field stimulation was potentiated.

^{3}H overflow was potentiated by phenoxybenzamine (4×10^{-5} M) but was unaffected by treatment with a combination of tranylcypromine, desmethylimipramine and 17-β-oestradiol. However, in the presence of these drugs, which in combination block MAO and the neuronal and extraneuronal uptake of NA, morphine and clonidine still enhanced ^{3}H overflow (Table 1).

Chromatographic separation of [^{3}H]-NA from its metabolites showed that field stimulation increased the overflow of [^{3}H]-NA. Whereas only 34% of the spontaneous ^{3}H overflow was recovered as [^{3}H]-NA, almost 80% of the radioactivity released into the bathing solution during field stimulation was [^{3}H]-NA (Table 2). In the presence of morphine and clonidine, approximately 70% of the radioactivity in the organ bath following field stimulation was [^{3}H]-NA (Table 2).

Discussion

This study has confirmed that field stimulation-induced motor responses of the mouse vas deferens are biphasic (Forsyth *et al.*, 1986). The first and second

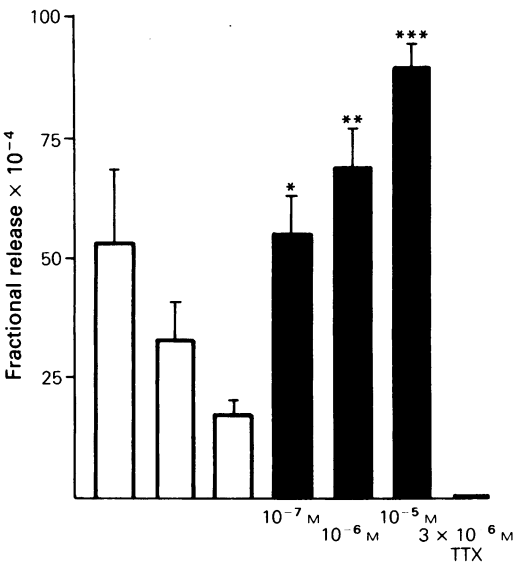

Figure 4 Effect of morphine (10^{-7}–10^{-5} M) on field stimulation-evoked overflow of ^{3}H in the vas deferens. The open histograms show successive control responses prior to drug addition. The succeeding 3 solid histograms show the potentiating effect of morphine added in increasing concentration (10^{-7}–10^{-5} M) in the same experiments. Tetrodotoxin blocked field stimulation-evoked ^{3}H overflow. Each column represents the mean of 6 observations; s.e.mean shown by vertical lines. *$0.05 > P > 0.01$, **$0.01 > P > 0.001$, ***$P < 0.001$ for comparison with control prior to drug addition.

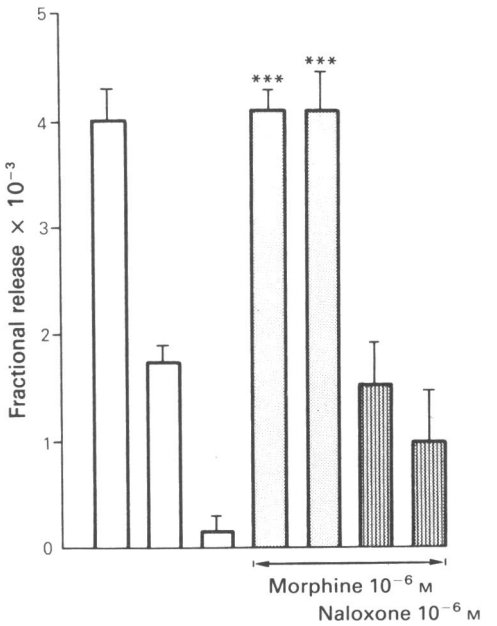

Figure 5 Effect of morphine (10^{-6} M) on field stimulation-evoked overflow of ^{3}H in the vas deferens. The open histograms show successive control responses prior to drug addition. The succeeding 2 stippled histograms show the potentiating effect of morphine (10^{-6} M) in the same experiments. Thereafter, the addition of naloxone (10^{-6} M) (stippled/striped histograms) antagonized the effect of morphine (10^{-6} M). Each column represents the mean of 6 observations; s.e.mean shown by vertical lines. ***$P < 0.001$ for comparison with control prior to drug addition.

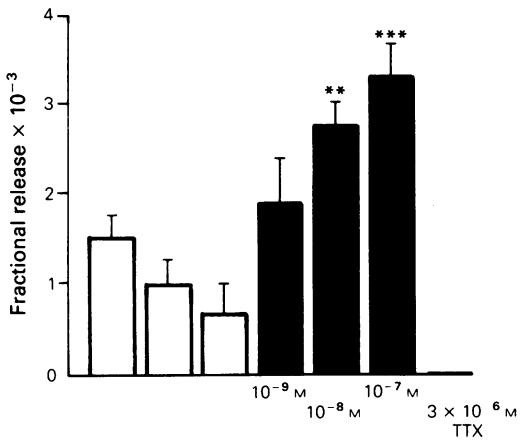

Figure 6 Effect of clonidine (10^{-9} M – 10^{-7} M) on field stimulation-evoked overflow of ^{3}H in the vas deferens. The open histograms show successive control responses before drug addition. The succeeding 3 shaded histograms show the potentiating effect of clonidine added in increasing concentrations (10^{-9}–10^{-7} M) in the same experiments. Tetrodotoxin (TTX, 3×10^{-6} M) blocked field stimulation-evoked ^{3}H overflow. Each column represents the mean of 4 observations; s.e.mean shown by vertical lines.
**$0.01 > P > 0.001$, ***$P < 0.001$ for comparison with control before drug addition.

components of this biphasic response correspond respectively to the NANC and adrenergic components previously characterized in vasa of other species (Meldrum & Burnstock, 1983; Brown *et al.*, 1983). Since both phases of this motor response were inhibited by TTX, which blocks sodium conductance and, therefore, abolishes the action potential in nerves, it appears that the field stimulation-induced contractions were nerve mediated.

In contrast to the inhibitory effect of TTX on both components of the motor response to field stimulation, morphine and clonidine inhibited only the initial (NANC) component. Moreover, unlike TTX, morphine and clonidine either did not affect or potentiated the second (adrenergic) component of the biphasic response. This observation confirms what previous studies have also shown, namely that the two components of the biphasic response can be differentially affected by drugs. Indeed, it is because these two components can be pharmacologically separated, that the biphasic response is believed to reflect the release and action of two transmitters (Booth *et al.*, 1978; Sneddon & Westfall, 1984). Nevertheless, the observation that morphine and clonidine inhibit the first component of the motor response but potentiate the second, is surprising, since several studies have used inhibition of field stimulation-induced motor responses of the vas to assay opiate activity (Hughes *et al.*, 1975; Henderson & Hughes, 1976). However, in such studies, low frequency stimulation is preferred, since the motor responses thus obtained are most sensitive to opiates.

It may be that brief, low frequency stimulation preferentially releases the NANC transmitter, whilst more prolonged, high frequency stimulation liberates also NA, whose release may be unaffected (Jenkins *et al.*, 1975) or, as in this study, potentiated by opiates.

It seems likely that both the inhibitory and excitatory effects of morphine and clonidine on field stimulation-induced motor responses were receptor mediated, since they occurred at low concentrations

Table 1 Effects of drugs on field stimulation-induced ^{3}H overflow into Krebs solution bathing isolated vasa deferentia of the mouse

Drugs	*Concentration* (M)	*% increase in ^{3}H overflow*	*Significance level*
Phenoxybenzamine	4×10^{-5}	241 ± 6(6)	$P < 0.001$
Clonidine	10^{-9}	153 ± 8(7)	$P < 0.001$
Morphine	10^{-7}	220 ± 7(6)	$P < 0.001$
NA disposal blockers:—			
Tranylcypromine	10^{-5}	21 ± 6(6)	$P > 0.5$
Desmethylimipramine	10^{-5}		
17-β-oestradiol	10^{-5}		
Clonidine	10^{-9}		
+ blockers	all 10^{-5}	150 ± 9(6)	$P < 0.001$
Morphine	10^{-7}		
+ blockers	all 10^{-5}	185 ± 10(8)	$P < 0.001$

Mean percentage increases ± s.e.mean (n).

Table 2 Percentage of [^{3}H]-noradrenaline ([^{3}H]-NA) and its ^{3}H-metabolites in Krebs solution bathing isolated, field stimulated vasa deferentia of the mouse

Time	*[^{3}H]-NA (%)*	*[^{3}H]-NMN (%)*	*^{3}H-deaminated metabolites (%)*
Before stimulation	34 ± 8 (5)	33 ± 6 (5)	30 ± 10 (5)
During stimulation	77 ± 8 (5)	16 ± 5 (5)	7 ± 3.5 (5)
During stimulation in the presence of 10^{-5} M morphine	72 ± 4 (4)	18 ± 3 (4)	10 ± 3 (4)
During stimulation in the presence of 10^{-7} M clonidine	70 ± 8.5 (4)	18 ± 5.5 (4)	12 ± 6.5 (4)

Mean percentages ± s.e.mean (*n*).
Deaminated metabolites: 3,4-dihydroxymandelic acid; 3,4-dihydroxyphenylethylglycol; 3-methoxy-4-hydroxyphenylglycol; 3-methoxy-4-hydroxymandelic acid.
NMN = normetanephrine.

and were dose-related. Furthermore, both the inhibitory and the excitatory effects of morphine were antagonized by naloxone.

The overflow experiments demonstrated that morphine and clonidine increased the amount of ^{3}H released by field stimulation and this response too was dose-related, and, in the case of morphine, was reversed by the specific antagonist, naloxone. The effects of clonidine were not reversed by the specific antagonist yohimbine which alone induced an increased overflow of ^{3}H through its blocking action on presynaptic α_2-adrenoceptors.

This study has confirmed that blockade of neuronal and extraneuronal uptake mechanisms for NA does not significantly enhance NA overflow (Langer, 1979). The view that blockade of these disposal mechanisms increases the amount of NA in the synaptic cleft and consequently causes increased activation of the presynaptic α_2-adrenoceptor-mediated negative feedback mechanism and, therefore, reduces release of transmitter (Langer, 1979) is supported by this study. Phenoxybenzamine, which blocks neuronal and extraneuronal disposal mechanisms, but additionally blocks presynaptic α_2-adrenoceptors, potentiated ^{3}H overflow. It appears that the ability of morphine and clonidine to enhance ^{3}H overflow is due to a presynaptic effect on release rather than on any of the disposal mechanisms for NA, since the ability of both drugs to potentiate ^{3}H overflow persisted in the presence of drugs that blocked the disposal mechanisms.

These observations provided futher evidence that morphine and clonidine acted presynaptically to increase NA release, as was suggested by the ability of these drugs in the same experiments, simultaneously to enhance the second (adrenergic) component of the motor response to field stimulation. These results agree in part with those of some workers but contradict others. For example, these observations agree with those of Stjärne (1975), who reported that clonidine paradoxically potentiated motor responses and [^{3}H]-NA overflow in the field-stimulated, isolated vasa of guinea-pig. In contrast, Henderson & Hughes (1976) found that morphine inhibited field stimulation-induced contractions and also inhibited NA release in mouse vasa. It is noteworthy that in the latter study, the stimulation parameters used to investigate the inhibitory effects of morphine on contractions elicited by field stimulation differed from those used to release NA. Contractile responses evoked by trains of 6 pulses at 0.1–1 Hz were readily blocked by opiates and this observation was confirmed in the present experiments. However, quite different stimulation parameters (0.5, 1.5 or 15 Hz with trains of 240 pulses) were used to measure NA overflow (Henderson & Hughes, 1976). No evidence was provided in these experiments to show the effects of morphine on motor responses evoked by field stimulation using these parameters although earlier studies (Henderson *et al.*, 1972) had shown that similar stimulation parameters (1 Hz, 120 pulses) inhibited motor responses and NA release. The present results suggest that the brief low frequency stimulation used by Henderson & Hughes (1976) to produce mechanical responses, susceptible to blockade by morphine, was most likely to have evoked predominantly NANC-mediated contractions. However, the prolonged, higher frequency stimulation used in the separate overflow experiments, would have evoked mechanical responses containing a more substantial adrenergic component that would have been expected, from the present study, to have been resistant to blockade by opiates. It may be that methodological differences explain the discrepancies between the results obtained in this study and those previously reported (Henderson *et al.*, 1972), but this

seems unlikely since other workers, who like Henderson *et al.* (1972), used bioassay to measure NA release found, using the same concentration of morphine and the same stimulation parameters, that morphine did not inhibit NA release (Jenkins *et al.*, 1975).

An important question raised by these results is how can morphine or clonidine selectively inhibit field stimulation-induced release of one transmitter (NANC), whilst increasing the release of the second (NA)? This question is made more difficult by evidence that NA and NANC transmitter are co-transmitters, contained in the same nerves (Fedan *et al.*, 1981; French & Scott, 1983) and indeed, in the same vesicle (Stjärne & Åstrand, 1985).

There is evidence that in nerves which store their co-transmitters in different vesicles, changes in the frequency of stimulation can alter both the quantity and the relative proportions of the co-transmitters released (Bartfai, 1985). Moreover, if either or both transmitters participate in presynaptic auto-inhibition or mutual cross regulation of co-transmitter release, then drugs that affect such mechanisms can be expected to have complex effects.

One explanation of the present results is that morphine and clonidine may selectively inhibit the release of the NANC transmitter, which normally has a presynaptic negative feedback action on NA release. In such circumstances, inhibition of NANC transmitter release, mirrored by inhibition of the predominantly NANC initial component of the biphasic motor response would be accompanied by a reduction in the restraint on NA output, which would, therefore, increase. Such an explanation is, however, difficult to sustain since the two co-transmitters, NA and ATP, are stored in the same vesicles. However, the distribution and possible involvement of a second NANC transmitter in the vas, neuropeptide Y (O'Donohue *et al.*, 1985), remains to be clarified and if it is stored in separate vesicles, then the hypothesis outlined above may be more plausible. In the case of clonidine, a complicating factor that might contribute to its complex effects in the vas is its ability to block presynaptic P_1 purinoceptors, thus preventing the negative feedback of adenosine on transmitter release (Katsuragi & Furukawa, 1985).

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