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INHIBITION OF NEUROEFFECTOR TRANSMISSION BY MORPHINE IN THE VAS DEFERENS OF NAIVE AND MORPHINE-TREATED MICE

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1 The amplitude of excitatory junction potentials (e.j.ps) recorded intracellularly from smooth muscle cells of the mouse vas deferens varied with the strength of stimulation. Normorphine (0.4, 2 and 10 μM) shifted the stimulus-response curve to the right, without any change in slope. This shift of the curve was proportional to the concentration of the opiate in the bath. Naloxone (0.4 and 2 μM) antagonized this effect of normorphine.

2 The action of normorphine (2 and 10 μM) was studied in vasa deferentia prepared from control mice and mice that had been implanted with morphine pellets. Both groups of tissues were continuously exposed to a low concentration of normorphine (0.4 μM), to simulate the plasma concentration in the morphine-treated mice. Addition of 10 μM normorphine produced a parallel displacement of the curve in vasa deferentia from control animals, and a non-parallel displacement in tissues from morphine pellet-implanted mice. In the preparations from morphine-treated mice a pronounced degree of tolerance to normorphine was observed at a low stimulus strength.

3 Naloxone (0.4 and 2 μM) had a greater effect on vasa deferentia prepared from morphine-treated animals than on tissues from control mice, when both organs were continuously exposed to 0.4 μM normorphine. The difference in the effect of the antagonist in the two groups of preparations was absent when the incubating solution contained 2 μM normorphine.

4 It is concluded that at a low intensity of stimulation the e.j.ps are more readily depressed by normorphine and also the degree of tolerance displayed is larger than at a high intensity of stimulation.

Introduction

In some peripheral opiate-sensitive model systems, namely the guinea-pig ileum (Cox & Weinstock, 1966; Cowie, Kosterlitz & Waterfield, 1978) mouse vas deferens (Hughes, Kosterlitz & Leslie, 1975; Hart, Kitchen & Waddell, 1979) and cat nictitating membrane (Illes & Knoll, 1978), opiate inhibition of transmitter release, and subsequent depression of the contractile responses is dependent on the strength of stimulation. Thus a more marked inhibitory effect can be observed by using submaximal rather than supramaximal stimuli.

Similarly to the acute effects produced by opiates, chronic effects may also be dependent upon the stimulus intensity. Supramaximally stimulated ileal preparations from morphine-treated guinea-pigs show a pronounced reduction in sensitivity to opiate drugs (see Schulz, 1978). In contrast, experiments on the vas deferens of morphine-treated mice have yielded variable results. When studying inhibition of the contractile response using supramaximal stimuli, only a moderate degree of tolerance to morphine was observed (Gillan, Kosterlitz, Robson & Waterfield, 1979; Schulz, Faase, Illes & Wüster, 1980). However, a 10 fold decrease of sensitivity to the opiate was found when depression of the amplitude of excitatory junction potentials (e.j.ps) elicited by submaximal stimuli was examined (Vitek & North, 1979; North & Vitek, 1980).

Excitatory junction potentials recorded from a single smooth muscle cell constitute a sensitive method for detecting changes in noradrenaline release (Burnstock & Holman, 1961). The size of these postsynaptic transients is proportional to the stimulus intensity applied (Furness, 1970). Morphine reduces the release of the transmitter from the mouse vas deferens (Henderson, Hughes & Kosterlitz, 1972; Hughes *et al.*, 1975) and in consequence also depresses the amplitude of e.j.ps (Henderson & North, 1976). The purpose of the present paper was two fold: firstly, to investigate the acute action of normorphine upon e.j.p. amplitudes elicited with varying intensities of stimulation; secondly, to study under similar con-

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ditions the degree of tolerance induced by chronic application of morphine.

Methods

Recording techniques

Isolated vasa deferentia from NMRI mice were used. The mesenteric sheath was carefully removed from the vas deferens, a conical holder inserted into the lumen, and the preparation placed in a 4 ml bath, in a Krebs solution (NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 0.9, $NaHCO_3$ 25 and glucose 11 mM) aerated with 95% O_2 and 5% CO_2 and maintained at 37°C. The flow rate through the bath was 3 ml/min.

The intramural sympathetic nerves were stimulated with two platinum ring electrodes (diameter: 1 mm) placed around the vas deferens and separated by 1 mm. Single pulses of 1 ms duration with voltages as indicated were applied. In order to prevent facilitation, an interval of 20 s was allowed between successive impulses. The strength of stimulation current was determined by monitoring the potential drop over a 10 KΩ resistance placed in series, yielding an essentially constant current stimulus.

Intracellular potentials were recorded from the smooth muscle cells with glass microelectrodes filled with 2.5 M KCl each of 50 to 80 MΩ resistance. The site of recording was 1 mm distant from the nearest stimulating electrode.

Evaluation of results

With constant stimulus parameters, the amplitude of e.j.ps progressively declined as impalements were made increasingly further from the cathode. In order to achieve more uniform data for quantitative evaluation, the stimulus intensity eliciting a response of 20 mV amplitude was determined from the stimulus-response curve. The ratio of these values before and after the application of a substance, gives the stimulus ratio (SR). A stimulus ratio of 1 indicates that the stimulus-response curve was not changed. Throughout the paper SR − 1 values are used, such that a positive value indicates a shift to the right, and a negative value a shift to the left of the curve. The larger the absolute value of SR − 1 the more pronounced is the normorphine or naloxone effect.

Chronic morphine treatment

NMRI mice were chronically treated by implantation of morphine-containing pellets [75 mg free morphine base, prepared according to Gibson & Tingstad (1970)]. The animals received half a pellet on day 1, and a whole pellet on day 2. The vasa were removed on the following day and immediately washed with Krebs solution containing 0.4 or 2 μM normorphine to simulate the plasma concentration of morphine.

Control preparations from naive (i.e. non-implanted) mice, were placed either in drug-free Krebs solution (when the acute normorphine effect was tested) or in a Krebs solution containing 0.4 or 2 μM normorphine. In some of the experiments vasa from naive and placebo-implanted mice were tested for normorphine and naloxone sensitivity, and no difference could be observed between the two groups. The placebo-implanted animals received pellets in which morphine was replaced by cellulose.

Statistical analysis

All observations in a group were made on tissues from different animals. Means ± s.e. mean are given and Student's *t* test was applied for statistical analysis of differences. A probability level of 0.05 or less was accepted as significant.

Results

Effect of normorphine on vasa deferentia from control mice

The amplitude of the e.j.ps varied with the strength of stimulation (Figure 1a). When the e.j.p. exceeded about 35 mV a faster secondary depolarization often appeared on the rising phase or at the peak; this probably represents a graded action potential. When the intensity of stimulation was increased further, e.j.ps gave rise to a well-defined action potential.

A linear relationship between the e.j.p. amplitude and log-stimulus intensity was apparent for e.j.p. amplitudes between 5 and 35 mV (Figure 1b). Normorphine (0.4 μM) shifted the stimulus-response curve of the control vasa deferentia to the right without any apparent change in slope. This also signifies that the inhibitory action of normorphine is inversely related to the strength of stimulation (see also Discussion, Figure 3a). In the example illustrated in Figure 1b the 25 mV e.j.p. was depressed by 19% whereas the 15 mV e.j.p. was depressed by 35% in the presence of normorphine (0.4 μM). Addition of 10 μM normorphine produced a still larger parallel shift of the curve (the slopes of the curves were, 0.4 μM normorphine: 42.09 ± 4.82, +10 μM normorphine: 38.87 ± 3.37, s.e. mean, $n = 6$, not significant). Naloxone (2 μM) antagonized the effect of the opiate.

A gradual increase of the concentration of normorphine (0.4, 2 and 10 μM) produced correspondingly

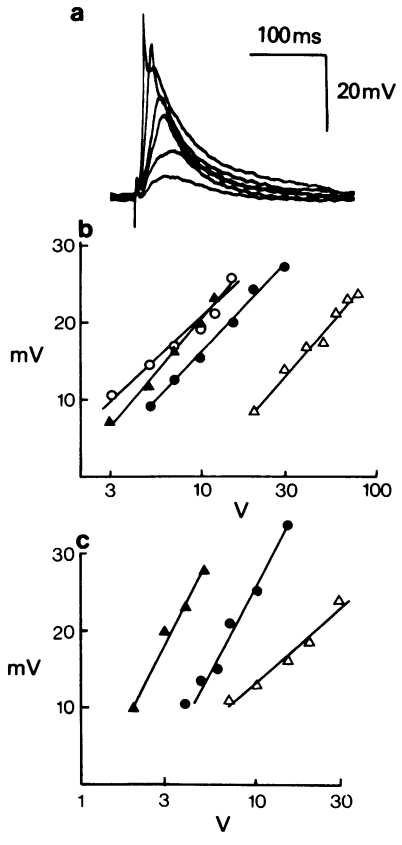

Figure 1 Inhibition of excitatory junction potential (e.j.p.) amplitude by normorphine in the vas deferens of naive and morphine-treated mice. (a) The amplitude of e.j.ps varied with the strength of stimulation. (b) Vas deferens prepared from naive mouse. Plot of log stimulus intensity against e.j.p. amplitude yields a straight line over a certain range. Normorphine 0.4 and 10 μM shifts the stimulus-response curves of the tissue to the right, without any apparent change in slope. (○): Control; (●): 0.4 μM normorphine; (△): +10 μM normorphine; (▲): +2 μM naloxone. (c) Vas deferens prepared from a mouse implanted with morphine pellets. Addition of 10 μM normorphine produces a non-parallel displacement of the control stimulus-response curve obtained in the presence of 0.4 μM normorphine. Symbols as in (a). In this figure each point represents the average of two consecutive measurements. Stimulus parameters: duration, 1 ms; intervals, 20 s. Slopes drawn were estimated.

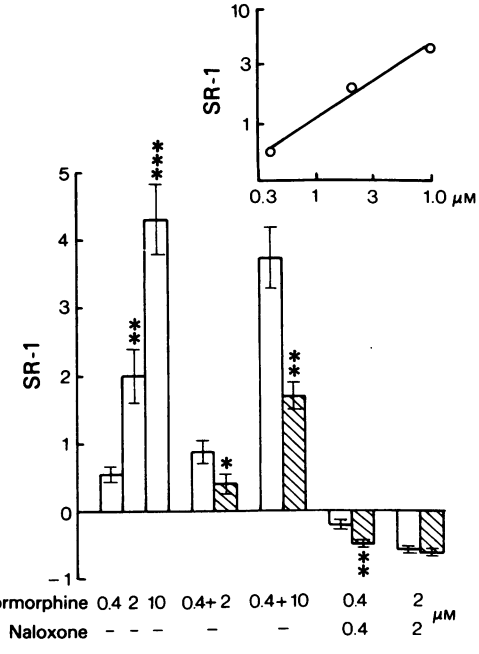

Figure 2 Tolerance to morphine in vasa deferentia prepared from morphine-treated mice. Open columns: naive animals; hatched columns: morphine-pellet-implanted animals. Each column represents the mean of 6 experiments; vertical bars indicate s.e. mean. Normorphine effects obtained on vasa deferentia from morphine-treated mice are compared to results on tissues from naive animals, with the exception of the first set of columns, which compare the effect of 2 and 10 μM normorphine to the SR − 1 value obtained in the presence of 0.4 μM normorphine. Inset: plot of log stimulus ratio − 1 (SR − 1) versus log concentration of normorphine (M) yields a straight line. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ (Student's t test).

larger stimulus ratio − 1 (SR − 1) values (Figure 2). A plot of the log SR − 1 values against the log normorphine concentration (M) yielded a straight line. This fact indicates that the shift of the stimulus-response curve by normorphine was proportional to the concentration of the opiate in the bath.

Effect of normorphine on vasa deferentia from morphine-treated mice

Addition of 10 μM normorphine produced a non-parallel displacement of the stimulus-response curve of vasa deferentia from morphine-treated mice pre-incubated with 0.4 μM normorphine (Figure 1c). In the presence of 10 μM normorphine the curve became less steep, as indicated by a significant decrease in the slope of the curves (0.4 μM normorphine: 55.11 ± 6.41, +10 μM normorphine: 26.78 ± 2.39, s.e. mean, $n = 7$, $P < 0.01$). At low stimuli strength a pronounced tolerance to normorphine was observed. The

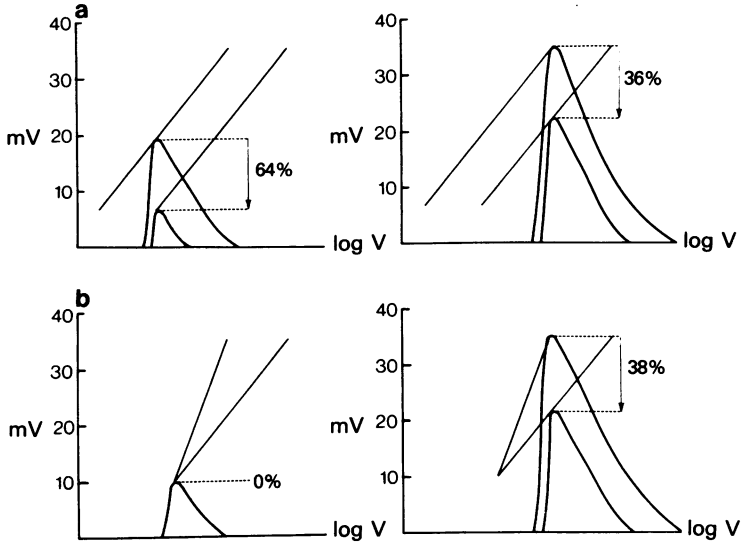

Figure 3 Hypothetical stimulus-response curve in vasa deferentia prepared from naive and morphine-treated mice. (a) Vasa deferentia prepared from naive mice. A parallel shift of the curve by normorphine indicates that e.j.ps of large amplitude are less susceptible to inhibition by the opiate than e.j.ps of low amplitude. (b) Vasa deferentia prepared from morphine-pellet-implanted mice. A non-parallel displacement of the curve indicates that tolerance to the opiate can be shown only by recording low amplitude e.j.ps. Inhibition of high amplitude e.j.ps by the opiate remains similar to that shown in preparation from naive animals.

apparent degree of tolerance decreased gradually as the intensity of stimulation was increased.

The non-parallel displacement of the stimulus-response curve also implies that low amplitude e.j.ps are not more susceptible to inhibition by normorphine than high amplitude e.j.ps (see also Discussion, Figure 3b) as was shown to occur in vasa deferentia from naive mice. The results plotted in Figure 1c show that the amplitudes of the 30 and 20 mV e.j.ps were depressed to a similar extent (by 49 and 48%, respectively) in the presence of normorphine (10 μM).

The SR − 1 values produced by 2 and 10 μM normorphine were significantly lower in vasa deferentia prepared from morphine-treated mice than in tissues from control animals (Figure 2). The differences in the SR − 1 values between the two experimental groups allow for an approximate quantitative evaluation for the effect of 10 μM normorphine. Thus about 5 times more opiate would be needed in vasa deferentia prepared from morphine-treated mice to produce the same stimulus ratio as in control mice.

Effect of naloxone

Naloxone (0.4 μM) antagonized the effect of normorphine (0.4 μM) both in preparations from naive and pellet-implanted mice (Figure 2). However, the shifts of the stimulus-response curve to the left produced by the antagonist were larger in the organs prepared from morphine-treated animals than in those of control mice. In contrast to these results, when the Krebs solution contained a higher concentration of normorphine (2 μM) there was no difference in the shifts of the curves produced by naloxone (2 μM).

Discussion

The present results indicate that normorphine shifts the stimulus-response curve of vasa deferentia prepared from naive mice in a parallel fashion to the right. In contrast stimulus-response curves of tissues prepared from morphine-treated mice were displaced by normorphine in a non-parallel manner.

Morphine has been shown to depress the release of noradrenaline from nerve terminals situated in the mouse vas deferens (Henderson *et al.*, 1972; Hughes *et al.*, 1975), and the mechanism of this action may be a multiple one: firstly, morphine may reduce the supply of calcium to the stimulus-release coupling mechanism (Illes, Zieglgänsberger & Herz, 1979, 1980); secondly, the opiate may in addition cause hyperpolarization of intramural nerve fibres of the vas deferens, as has been suggested to occur in myenteric neurones (North & Tonini, 1977; see also North,

1979). The change in membrane potential would lead to impairment of propagation at the junction between preterminal axons and varicosities (see Haefely, 1972).

In the mouse vas deferens the e.j.p. in a single muscle cell arises both from the local action of transmitter on the cell membrane and from potential changes induced in electrically coupled adjacent cells (Furness, 1970). It has been proposed that each muscle cell is influenced by up to 20 nerve fibres in addition to its own direct innervation. In the present experiments normorphine shifted the stimulus-response curve of the vas deferens to the right in a parallel fashion. This phenomenon may involve both a gradual and all-or-nothing depression of transmitter release from individual nerve terminals. Higher stimulus intensities probably excite new fibres as well as re-exciting previously inhibited fibres.

A parallel shift of the stimulus-response curve by normorphine indicates that e.j.ps of larger amplitude are less susceptible to inhibition by the opiate than e.j.ps of lower amplitude (Figure 3a). Our findings could offer an explanation for the reported ability of the opiates to inhibit the contractile responses of tissue effected by submaximal stimuli more readily than contractions produced by supramaximal stimuli (Hughes *et al.*, 1975; Hart *et al.*, 1979). With increasing strength of stimulation different populations of nerve fibres are excited, and these fibres may exhibit a different sensitivity to opiates (Cowie *et al.*, 1978; Hart *et al.*, 1979). Morphine may depress transmitter release preferentially from those fibres which are excited by relatively low stimuli intensities, whilst less excitable fibres could be more resistant to its action. An alternative explanation may be that non-linear summation of the evoked quanta (see McLachlan, 1978) decreases the inhibitory effect of normorphine on e.j.ps of high amplitude. However, this phenomenon is not likely to explain the pronounced and continuous decrease of opiate sensitivity of the organ by increasing the strength of stimulation.

In tissues from morphine-implanted mice, normorphine (10 μM) caused a non-parallel displacement of the stimulus-response curve. The degree of tolerance calculated for the 20 mV e.j.ps is less than that reported by North & Vitek (1980) using this organ. Normorphine was found to be about 5 times less active in vasa taken from morphine-treated mice than in tissues taken from control animals. The degree of tolerance is not as apparent when higher amplitude e.j.ps are studied (Figure 3b). This may explain the fact that only moderate tolerance was observed when studying the contractile response elicited by supramaximal stimuli (Gillan *et al.*, 1979; Schulz *et al.*, 1979).

In the present experiments, when vasa deferentia continuously exposed to a relatively low concentration of normorphine (0.4 μM) were used, naloxone (0.4 μM) produced a larger shift of the stimulus-response curve to the left in tissues taken from pellet-implanted mice, than in tissues from naive animals. Vitek & North (1979) obtained similar results, and interpreted the observed increase of evoked release of transmitter (amplitude of evoked e.j.p.) as a sign of naloxone-precipitated withdrawal, by analogy to findings obtained in the myenteric-plexus of the guinea-pig (Schulz & Herz, 1976; North & Zieglgänsberger, 1978; see also Schulz, 1978). However, when the vasa were placed in a Krebs solution containing a higher concentration of normorphine (2 μM) which is a better approximation of the actual plasma concentration of opiate in the pellet-implanted mice (Faase, unpublished), no 'withdrawal' could be elicited by naloxone (2 μM). Thus these results cast doubt upon the interpretation of this phenomenon as a withdrawal sign comparable with that obtained in the myenteric-plexus of guinea-pigs.

In conclusion, this paper presents evidence that chronic treatment of mice by morphine renders the nerve terminals in their vasa tolerant to normorphine. The organ is probably innervated by postganglionic nerve fibres of differential excitability. It is tempting to speculate that the more excitable fibres are more sensitive to morphine and also develop a larger degree of tolerance to the opiate and the less excitable fibres a lower degree of tolerance.

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