Br. J. Pharmac. (1984), **83**, 853–861

The dependence of excitatory junction potential amplitude on the external calcium concentration in narcotic tolerant mouse vas deferens

R. Einstein & N.A. Lavidis

The Department of Pharmacology, University of Sydney, Sydney, N.S.W., Australia, 2006

1 The dependence of neurotransmitter secretion on external calcium ions during development of opiate tolerance in the mouse vas deferens was studied.

2 The writhing response of mice to an i.p. injection of acetylcholine was inhibited by morphine. Reversal of this antinociceptive effect of morphine during chronic treatment signalled the development of tolerance.

3 Tolerance to morphine at the neuromuscular junction was shown as a reversal of the initial shift of the size of the excitatory junction potential (e.j.p.) vs extracellular calcium concentration relationship back towards the control without any change in the power of 2.4.

4 Facilitation in the amplitude of the e.j.p. occurs with low frequency (2 Hz) stimulation. The initial increase in facilitation induced by morphine was reversed by chronic morphine treatment without any change in the plateau e.j.p. amplitude achieved after a long low frequency train of impulses.

5 At high frequencies (10 Hz) the initial increase in e.j.p. amplitude was followed by a depression. Acute morphine administration decreased the size of the e.j.p., this was followed by an increase in facilitation and a decrease in depression. These effects were reversed after chronic morphine treatment.

6 Tolerance to morphine involves a counteradaptive process which restores the normal entry of calcium ions or its actions within the release sites in promoting transmitter release.

Introduction

Acute administration of morphine to isolated tissues results in a reduction of transmitter release from nerve terminals during electrical stimulation (Paton, 1957; Schaumann, 1957; Henderson *et al.*, 1975; Bornstein & Fields, 1979; Bennett & Lavidis, 1980). In the inferior mesenteric ganglion, the morphine-induced depression of the amplitude of the synaptic potential has been shown to be due entirely to a reduction in the number of quanta released rather than a decrease in quantal size and, although the spontaneous quantal release frequency is depressed by morphine (Bornstein & Fields, 1979), the size of spontaneously released quanta is unaltered (Bornstein & Fields, 1979; Illes & North, 1982). Morphine is therefore thought to act directly on the stimulus secretory coupling mechanism (Henderson & North, 1976; Bornstein & Fields, 1979; North & Viteck, 1980; Marshall *et al.*, 1981; Collier *et al.*, 1981). Mudge *et al.*, (1979) showed that the voltage dependent calcium current produced during stimulation of cultured dorsal root ganglion cells is shortened by enkephalin and that this effect is reversed by naloxone. It is likely therefore, that the morphine-induced inhibition of the evoked release of noradrenaline from the mouse vas deferens is mediated via an inhibition of the voltage-dependent calcium channels directly or indirectly by a K^+mediated hyperpolarization of the terminal (Morita & North, 1982) or by preventing the association of calcium to an intracellular X-receptor involved in transmitter release (Bennett & Lavidis, 1980; Illes *et al.*, 1980).

The development of tolerance to opiates is well documented and can be measured either *in vivo* or *in vitro*. Schulz & Herz (1976) demonstrated a marked tolerance in the ileum from guinea-pigs which had been treated with large doses of morphine before being killed. Similar tolerance was observed in the mouse vas deferens (Cox, 1978). The mechanism of the development of tolerance and dependence is still unclear. No change in opiate receptor binding has ever been observed in tolerant tissues and there is no evidence of any conformational changes in these

receptors (Klee & Streaty, 1974; Hölt *et al.*, 1975). However, it has been shown that following acute administration of morphine there is a marked reduction in the calcium content of brain (Ross, 1977) and synaptosomes (Cardenos & Ross, 1976; Harris *et al.*, 1977; Yamamoto *et al.*, 1978). This initial reduction is reversed by chronic morphine treatment (Lee *et al.*, 1975; Harris *et al.*, 1977; Ross 1977; Yamamoto *et al.*, 1978; Guerrero-Munoz *et al.*, 1978). Schmidt & Way (1980) suggested that tolerance is a counteradaptive process which restores the normal entry of calcium or its action within the release sites in the promotion of transmitter release. We have examined this hypothesis by evaluating the development of opiate tolerance in the mouse vas deferens.

Methods

Tissue preparation

Male mice (Balb C) were divided into four groups of 12 animals each and received either physiological saline (0.1 ml) or morphine, 10, 30 or 100 mg kg^{-1} in a volume of 0.1 ml (s.c.) twice daily for 10 days. Before commencement of chronic morphine treatment (CMT) and 11 h after the evening dose on day five, each group was tested for nociception using the writhing test of Collier *et al.* (1968). One hour after this test the mice were injected with morphine (2 mg kg^{-1}) (controls received saline) and were then retested after 30 min. Electrophysiological studies were conducted on isolated vasa deferentia removed from the mice less than 5 h after the last dose of morphine after 10 days of CMT. The animals were killed by cervical fracture and the tissues immediately mounted in a perspex organ bath (capacity 4 ml), perfused with modified Krebs-Henseleit solution (composition in mM: Na^+ 141, K^+ 4.7, Mg^{2+} 1.2, Cl^- 157, $H_2PO_4^-$ 1.3, SO_2^{2-} 1.2, HCO_3^- 16.3, Ca^{2+} 0.9, glucose 7.8) at a rate of 3 ml min^{-1} and gassed continuously with 95% O_2 and 5% CO_2. The extracellular calcium concentration was varied between 0.7 and 5.0 mM by altering the amount of $CaCl_2$ in the bathing fluid. The temperature in the bath was maintained between 33° and 35°C.

Stimulation

The intramural sympathetic nerves were stimulated with two platinum ring electrodes placed around the vas deferens and about 1 mm apart. The number of nerves stimulated was kept approximately constant by stimulating with single impulses of 60 mV and 0.05 ms duration. With these stimulation parameters the amplitude of the excitatory junction potentials (e.j.ps) did not exceed 18 mV. At low frequency stimulation the e.j.p. amplitude was less than 12 mV (error due to nonlinear summation, 11%). In 20% of the studies involving high frequency stimulation the e.j.p. amplitude after 2–4 impulses reached 18 mV (error due to nonlinear summation, 24%). No correction due to nonlinear summation was carried out since 84% of the e.j.p.s had less than 11% distortion and therefore the main conclusions are not affected (Martin, 1955; Bennett *et al.*, 1976). It was also possible to combine the results from different cells and tissues (Bennett, 1972). There was a minimum interval of 30 s between successive impulses or trains of impulses to allow for the slowest component of decline of facilitation (Bennett, 1973).

Recording

Intracellular potentials were recorded from the smooth muscle cells with glass microelectrodes filled with 2 M KCl and having resistances of 50–90 MΩ. The signals were passed through a high impedence unity gain amplifier and photographed from an oscilloscope display. The quality of intracellular impalement was judged to be adequate if there was a negative shift of at least 50 mV in the recording and if miniature e.j.ps (m.e.j.ps) were present. Facilitation of transmitter release of a conditioning impulse when compared to a test impulse is defined as:

$$\text{Facilitation} = \frac{\text{e.j.p. (test)}}{\text{e.j.p. (conditioning)}}$$

The degree of facilitation during a short train of impulses was compared with the predictions based on the residual Ca-receptor hypothesis (Bennett & Fisher, 1977) according to:

$$\text{Facilitation} = \sum_{i=1}^{j-i} \left\{ [(1 + \text{fn}[(j-1)\Delta t])^{1/2.4} - 1] + 1 \right\}^{2.4}$$

where Δt is the interval between the jth and ith test and conditioning impulses respectively, so that $\text{fn}(j-i)\Delta t$ was determined using Figure 4 and represents the amount of facilitation between the ith and jth impulses. The sum of such increments in facilitation represent the overall facilitation.

Drugs

Morphine hydrochloride was dissolved in saline for administration to mice or in the modified Krebs solution for the isolated organ studies. The Krebs solution containing morphine hydrochloride (5×10^{-7} M) continually perfused the isolated CMT tissues throughout the experiment. For the acute morphine studies, in the controls morphine (5×10^{-7} M) was added to the modified Krebs solution following control recordings.

Results

Effect of tolerance on the writhing response of mice to an i.p. injection of acetylcholine

The effects of initial doses of morphine included a marked increase in spontaneous motor activity, Straub tail, ptosis and piloerection. The obvious reduction of all of these responses during CMT gave a qualitative indication of the development of tolerance. Intraperitoneal injections of acetylcholine before CMT elicited characteristic writhing behaviour, with approximately 6 writhes per 3 min (Figure 1). This response was abolished following acute morphine administration (2 mg kg^{-1}). After 5 days of CMT there was a dose-dependent reversal of the antinociceptive effect of morphine, to the extent that the group receiving the highest chronic dose showed a slight degree of hypernociception in the writhing test (Figure 1).

Effect of tolerance on the amplitude of the e.j.p.

Increasing the external calcium concentration from 0.7 to 1.3 mM increased the amplitude of the e.j.p. recorded from single smooth muscle cells, the amplitude of the e.j.p. was proportional to approximately the second power (2.4) of the extracellular calcium concentration ($[Ca]_o$). Acute addition of morphine shifted this relationship to the right (Figure 2), indicating that a greater $[Ca]_o$ was required in the presence of morphine to elicit equivalent e.j.p. amplitudes. CMT induced a partial reversal of the shift to the right (Figure 2). At no stage was there any alteration of the power relationship between transmitter release and $[Ca]_o$ (Figure 2). The amplitude of spontaneous e.j.ps was unaltered by the acute administration of morphine to both control and CMT vasa deferentia.

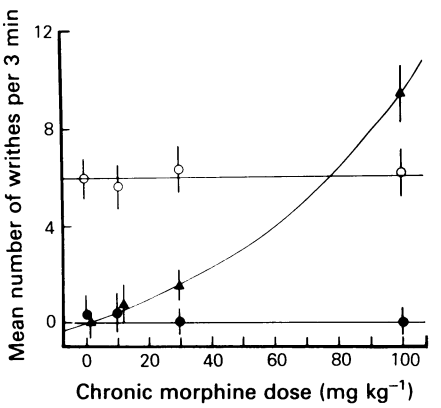

Figure 1 Effect of chronic morphine treatment on the response of mice to an i.p. injection of acetylcholine (Collier *et al.*, 1968). (○) All groups on day 0; (●) all groups on day 0, 30 min after an injection of morphine 2 mg kg^{-1} and (▲) at the end of day 5, 30 min after an injection of morphine 2 mg kg^{-1}. Means ($n = 12$ for each point) are shown with vertical lines representing s.e.mean.

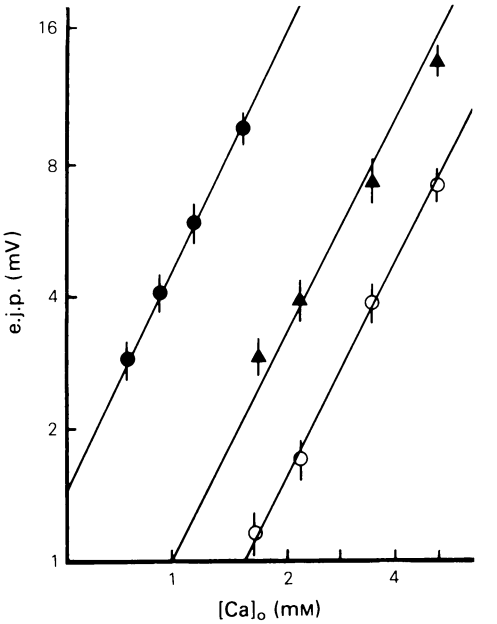

Figure 2 Effect of chronic morphine treatment (CMT) on the relationship between transmitter release (measured as e.j.p. amplitude, Burnstock & Holman, 1961) and extracellular calcium. (●) Controls; (○) controls with morphine; (▲) CMT with morphine. Experimental data ($n > 48$) are shown as mean and vertical lines represent s.e.mean. Lines were drawn using equation 3.

A double reciprocal plot of $(\text{e.j.p.})^{1/2.4}$ vs $[Ca]_o$ for control tissues with added morphine and CMT tissues with added morphine showed that the y-intercept was the same, indicating no change in the e.j.p. (max.) (Figure 3). The reciprocal of the x-intercept represents an equilibrium constant ($K_{[Ca]_o}$). The changes in $K_{[Ca]_o}$ (controls:1.39 mM, controls plus morphine:5.56 mM, CMT plus morphine:3.37 mM) indicated that morphine was acting as a competitive antagonist of calcium and that after CMT the effect of acute morphine was reduced. The lines shown in Figure 2 were drawn by substituting these values for e.j.p. (max) and $K_{[Ca]_o}$ in the equation:

$$\text{e.j.p.} = \text{e.j.p.}_{(\max)} \left\{ \frac{[Ca]_o}{K_{[Ca]_o} + [Ca]_o} \right\}^{2.4}$$

(Dodge & Rahamimoff, 1967; Bennett & Lavidis, 1979).

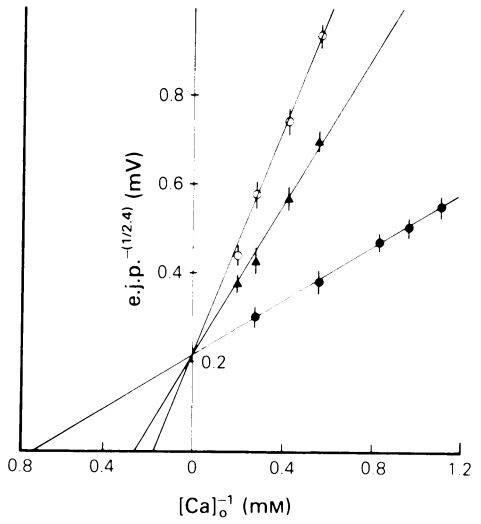

Figure 3 A double reciprocal plot of the amount of transmitter released vs extracellular calcium in control and tolerant mice. (●) Controls; (○) controls with morphine; (▲) chronic morphine-treated (CMT) mice (100 mg kg^{-1}) with morphine (5×10^{-7} M). Means and s.e.mean (vertical lines) of experimental data ($n > 48$) are shown. E.j.p. (max) = 42 mV and $K_{[Ca]_o}$ values were: 1.39 mM, controls; 5.56 mM, controls with morphine; and 3.37 mM, CMT mice with morphine.

Changes in facilitation of transmitter release during low frequency stimulation in the mouse vas deferens throughout the development of tolerance

Facilitation of transmitter release during low frequency stimulation has been described in terms of residual calcium left inside the release sites after each impulse. This calcium is bound to calcium receptors involved in transmitter secretion (Bennett & Florin, 1975). The time constant of facilitation decline (determined using equation 1) was unaltered (approximately 6 s) by acute or chronic morphine treatment (Figure 4). Addition of morphine to the control preparations increased the degree of facilitation. Reducing the extracellular concentration of calcium from 1.8 mM to 1.2 mM in the presence of morphine resulted in a further increase in facilitation. CMT decreased the degree of facilitation of transmitter release so that the CMT preparations maintained in 1.2 mM $[Ca]_o$ and morphine (5×10^{-7} M) were identical to the controls maintained in 1.8 mM $[Ca]_o$ and morphine (5×10^{-7} M) (Figure 4).

Following a short train of impulses the initial reduction of transmitter release produced by morphine was reversed (Figure 5). The rate of reversal was dependent on extracellular calcium, since increasing calcium from 1.8 mM to 5.0 mM progressively increased the amplitude of the first e.j.p. and decreased the degree of facilitation. CMT produced a marked increase in the amplitude of each e.j.p. at any given calcium level and decreased the degree of facilitation (Figure 5). Using the time constant of facilitation decline, at various $[Ca]_o$, in the absence and presence of morphine and equation 2 (residual calcium-receptor hypothesis), it was possible to predict (Figure 6) the response of the release sites to a train of impulses. The initial increase in the degree of facilitation produced by acute morphine was partially reversed by CMT (Figure 6).

Depression in transmitter release from vasa deferentia of tolerant mice

During high frequency stimulation, the first few impulses released greater amounts of transmitter after which there was a decrease in the amount of transmitter released until a steady state was reached. The decrease observed was dependent on the total amount of transmitter released by the first few impulses, which was indirectly dependent on the amount of extracellular calcium. In the vas deferens of non-CMT animals, during a high frequency train

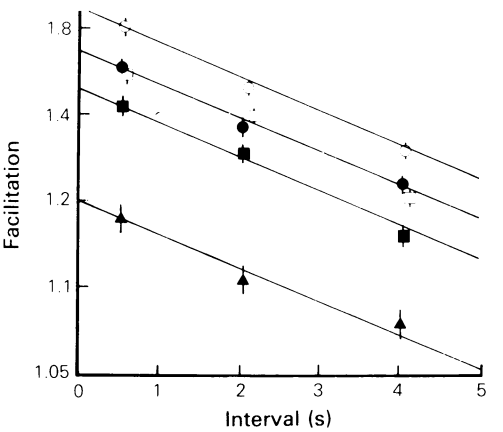

Figure 4 A comparison of the time course of facilitation decay of a conditioning impulse following a test impulse between control and tolerant terminals. (▲) Controls, 1.8 mM $[Ca]_o$; (□) controls, 1.8 mM $[Ca]_o$ and morphine; (○) controls, 1.2 mM $[Ca]_o$ and morphine; (●) chronic morphine treated mice (CMT), 1.2 mM $[Ca]_o$ and morphine; (■) CMT, 1.8 mM $[Ca]_o$ and morphine. Experimental data ($N > 56$) are shown by means and vertical lines show s.e.mean; lines fitted using linear regressions; coefficients > 0.92. The time course of facilitation decay was approximately 6 s.

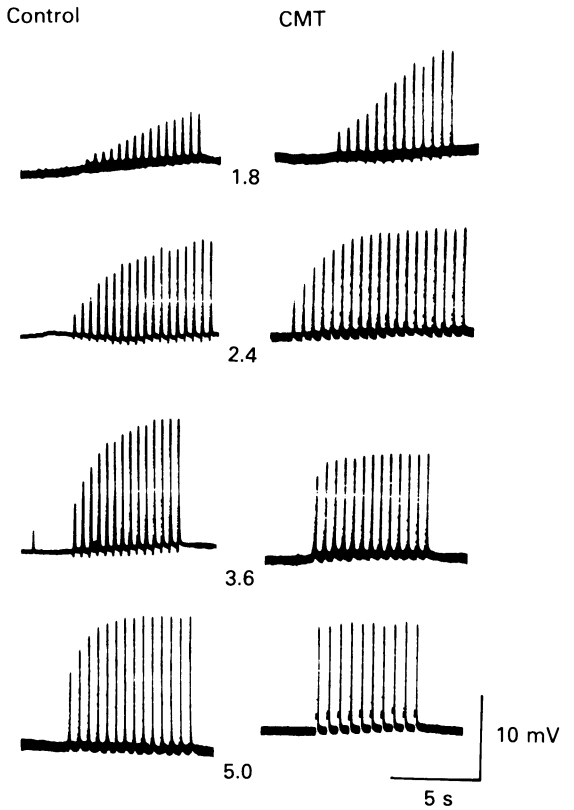

Figure 5 Recordings showing the effect of chronic morphine treatment (CMT) on the facilitation of transmitter released during a short, 2 Hz, train of impulses in various extracellular calcium concentrations (the numbers shown between recordings give the concentration in mM) in the presence of morphine. The CMT dose was 100 mg kg^{-1}.

of impulses in 1.8 mM $[Ca]_o$ following a slight facilitation by the first three impulses there was a gradual decline in the amplitude of the e.j.ps until a steady state was reached, which had the same amplitude as the response to the first impulse. Following addition of morphine to this preparation the first impulse was depressed, but this was quickly reversed by an increase in facilitation and eventually a steady state was reached which was similar in amplitude to the control vasa deferentia (Figure 7). Vasa deferentia from CMT mice in a morphine-free bathing solution containing 1.8 mM $[Ca]_o$ showed a marked increase in the amplitude of the first e.j.p. Subsequent e.j.ps showed some facilitation, after which there was a marked decline in the amount of transmitter released leading to a depressed steady state, which was not observed in the controls at this $[Ca]_o$ (Figure 7). Addition of morphine to these preparations reduced the amplitude of the first e.j.p.; this reduction was greater in the control preparations. Subsequent impulses facilitated transmitter release to a lesser extent in the CMT tissues than in control tissues. The initial increase in transmitter released by the first few impulses was followed by a greater depression of transmitter release from CMT terminals. The notable increase in the facilitation produced by morphine (Figure 8) was effectively reduced by CMT.

Discussion

The dependence of the e.j.p. amplitude on $[Ca]_o$ in vasa deferentia from tolerant mice

The 2.4 power relationship between $[Ca]_o$ and noradrenaline release observed in these studies confirms the findings of others (Bennett & Florin, 1975; Bennett & Lavidis, 1980; Milner *et al.*, 1982) and possibly arises because of the effect of $[Ca]_i$ on the neurosecretory mechanism. For simplicity, a hypothetical receptor binding to Ca^{2+} (Ca-receptor

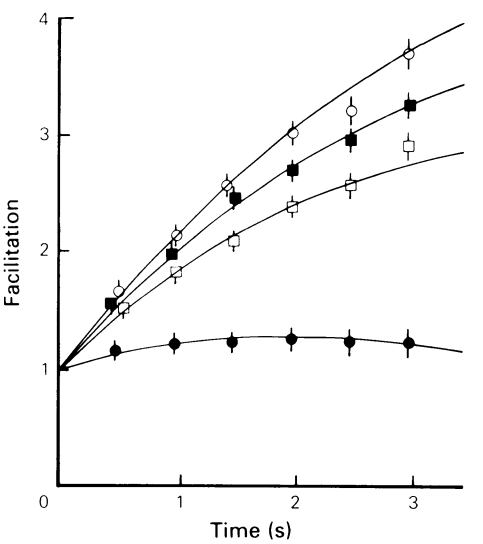

Figure 6 Effect of chronic morphine treatment (CMT) on the facilitation observed during short (2 Hz) trains of impulses in the presence of morphine. (●) Controls, 1.8 mM $[Ca]_o$ without morphine; (■) controls, 0.9 mM $[Ca]_o$ without morphine; (○) controls, 1.8 mM $[Ca]_o$ with morphine; (□) CMT, 1.8 mM $[Ca]_o$ with morphine. Experimental data ($n > 36$) are shown as means and vertical lines represent s.e.mean, lines were fitted using equation 2.

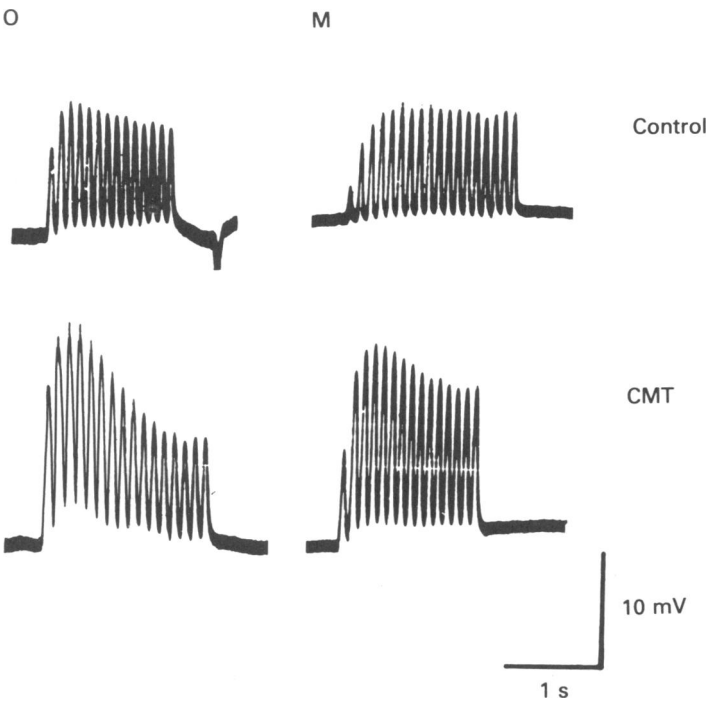

Figure 7 Recordings showing the effect of chronic morphine treatment (CMT) on the responses of the terminal to high frequency (10 Hz) train of impulses. Before (control) and after CMT in the absence (○) and presence (M) of morphine in the bathing Krebs solution.

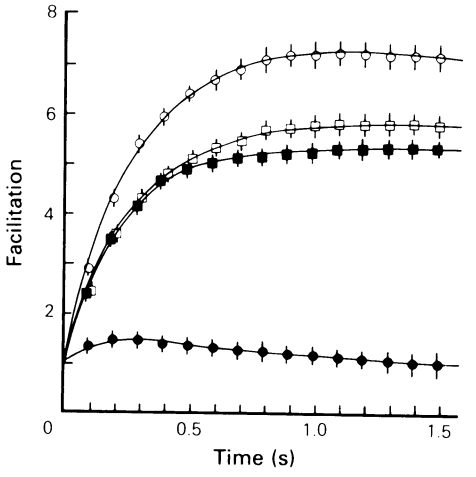

Figure 8 Responses of the terminals to high frequency (10 Hz) train of impulses. (●) Controls, 1.8 mM $[Ca]_o$; (■) controls, 0.9 mM $[Ca]_o$; (○) controls in 1.8 mM $[Ca]_o$ with morphine; (□) chronic morphine-treated (CMT) mice in 1.8 mM $[Ca]_o$ with morphine. Experimental data ($n > 36$) are shown as means and vertical lines represent s.e.mean.

complex) has been used to describe and predict the amount of transmitter released under various conditions (Bennett & Florin, 1975) using the mass action law derived by Jenkinson (1957).

The effect of morphine on e.j.p. amplitude and the antagonism of this effect by naloxone (North & Henderson, 1975; Henderson &. North, 1976) has also been measured in noradrenaline overflow experiments during nerve stimulation (Henderson & Hughes, 1976; Marshall *et al.*, 1981). The depression of transmitter release by morphine is mediated by an effect on Ca^{2+} (Bennett & Lavidis, 1980). This is consistent with the present findings, where acute morphine induced a shift of the e.j.p. vs $[Ca]_o$ relationship to the right without any change in the power relationship. In the myenteric plexus morphine has been shown to cause a hyperpolarization of myenteric neurones and so prevent excitation (North & Tonini, 1977; Morita & North, 1982). This hyperpolarization has not been observed in the inferior mesenteric ganglion (Bornstein & Fields, 1979) nor in the smooth muscle cells of vasa deferentia (North & Vitek, 1980; unpublished observation). Alternatively, morphine may inhibit voltage-dependent calcium channels or prevent the association of intracel-

lular calcium with the receptor which triggers noradrenaline release (Bennett & Lavidis, 1980; Illes *et al.*, 1980). These possibilities are supported by the finding that acute administration of morphine in mice and rats decreases Ca^{2+} levels in synaptosomes and in synaptic vesicles (Harris *et al.*, 1977; Yamamoto *et al.*, 1978). Also Guerrero-Munoz *et al.* (1978) showed that synaptosomes have a viable Ca^{2+} uptake mechanism that can be blocked in a dose-dependent manner by morphine.

Chronic morphine treatment produced a marked elevation of Ca^{2+} in the synaptosomes of both rats and mice (Lee *et al.*, 1975; Harris *et al.*, 1977; Ross, 1977; Yamamoto *et al.*, 1978). An enhancement of the synaptosomal Ca^{2+} uptake was shown in animals rendered tolerant and dependent by morphine pellet implantation (Guerrero-Munoz *et al.*, 1978). Tolerance to morphine at the adrenergic neuromuscular junction was shown as a reversal of the initial shift of the e.j.p. vs $[Ca]_o$ relationship back towards the control, indicating that morphine was less effective at reducing the amount of noradrenaline released for a given $[Ca]_o$. This reversal of the inhibitory action of morphine was achieved without alteration of the amplitude of spontaneous miniature excitatory junction potentials (Illes & North, 1982; personal observation) and without alteration to the resting membrane potential of the smooth muscle cells (North & Vitek, 1980). It is unlikely therefore that changes in the postsynaptic sensitivity to transmitter occur during tolerance. During the development of tolerance the maximum e.j.p. that could be recorded remained constant: it is possible that the increase in e.j.p. amplitude is due to an increase in the probability of transmitter release via a reduction in the $K_{[Ca]_o}$ value.

Facilitation of transmitter release during low-frequency stimulation in vasa deferentia from tolerant mice

During a low frequency train of impulses the amount of transmitter released by successive impulses has been shown to be facilitated, and the degree of facilitation is calcium-dependent (Bennett & Fisher, 1977; Bennett & Lavidis, 1980). Smith & Zucker (1980) have shown that there is no facilitation of the entry of calcium into the terminal during a train of impulses but there is a prolonged rise in the internal calcium concentration. The facilitation of transmitter release has been explained on the basis that each impulse leaves residual calcium bound to the hypothetical receptor (X) involved in linking internal calcium with the secretory mechanism (Bennett, 1973). The results of the present studies show that in a low calcium concentration the amount of transmitter released during a train of impulses can be accurately predicted by the residual calcium receptor hypothesis. As the external calcium concentration increases and the amount of transmitter released by the first impulse increases, the amount of facilitation that can be achieved by subsequent impulses decreases and the steady state in transmitter release is reached sooner.

Morphine reduces the evoked release of transmitter from adrenergic nerve release sites in the mouse vas deferens during short, low frequency trains of impulses (Henderson *et al.*, 1972). This reduction is reversed either by increasing extracellular calcium, by reducing the extracellular magnesium, or by naloxone (Bennett & Lavidis, 1980; Illes *et al.*, 1980). The action of morphine on the release site during trains of impulses can be explained as follows: by reducing the number of Ca-X that are formed, the amount of noradrenaline released by the first impulse in the train is reduced (Bennett & Lavidis, 1980). This results in an increase in the Ca-receptors free to bind to calcium entering the terminal with subsequent impulses and therefore facilitation is enhanced. With the development of tolerance the acute action of morphine was reversed and the responses were similar to those which would be expected in the presence of increased extracellular calcium, decreased extracellular magnesium or naloxone. The increase in facilitation of transmitter release during low frequency stimulation produced by acute morphine was partially reversed by CMT. This reversal could not be explained by a reduction in the sequestering capacity of intraterminal organelles since the time constant of facilitation was unaltered by either acute morphine treatment (Bennett & Lavidis, 1980) or CMT. It is possible, however, that CMT increases the amplitude of the first e.j.p. and reduces facilitation by increasing the affinity of Ca^{2+} for the X-receptor or by increasing the amount of calcium entering the release sites. The observed six fold increase in the rate of $^{45}Ca^{2+}$ uptake by synaptosomes of animals implanted with morphine pellets (Harris *et al.*, 1977; Guerrero-Munoz *et al.*, 1978; Yamamoto *et al.*, 1978) supports the latter possibility.

Depression in transmitter release during high frequency stimulation in vasa deferentia from tolerant mice

During a high frequency train of impulses facilitated release of neurotransmitter is observed for the first few impulses. After this either a steady state is reached or a depression follows, leading to a depressed steady state, depending on the external calcium concentration (Bennett, 1973; Bennett & Florin, 1975; Bennett & Lavidis, 1980). Facilitation can be explained by residual calcium within the release site and the facilitated steady state by saturation of the secretory mechanism. Depression of transmitter re-

lease is likely to be due to either depletion of the amount of transmitter readily available for release (Mallart & Martin, 1968; Bennett & Fisher, 1977) or to autoinhibition via presynaptic α-adrenoceptors (Stjarne, 1975; Bennett & Middleton, 1975; Starke, 1977; Drew, 1978).

The acute action of morphine on transmitter release during high frequency stimulation reduces the amount of internal calcium which binds to the calcium receptors (Bennett & Lavidis, 1980). This increases the free calcium receptors available for binding with newly entering calcium during successive impulses and so leads to a greater degree of facilitation. Because of the reduction in the total amount of transmitter released by the first few impulses, the depression in transmitter release is reduced. Also, a less depressed steady state develops (Bennett & Lavidis, 1980). All these actions of morphine were similar to those which would result from a lowering of extracellular calcium concentration. Development of tolerance to morphine resulted in a gradual reversal of all these effects. The amount of transmitter released by the first impulse was increased, possibly because of a greater influx of calcium and therefore an increased concentration of Ca-X within the release sites. The reduction in free X-receptors would decrease facilitation and the increase in transmitter release during the first few impulses results in increased depression. These results could be predicted and produced experimentally by assuming an increase in the amount of calcium entering the nerve release site of tolerant animals in the presence of morphine.

The acute actions of morphine on the release sites result in a reduction of the amount of transmitter released, via a direct or indirect reduction in the amount of Ca^{2+} influx during stimulation. Tolerance to morphine involves a counteradaptive process which restores the normal entry of calcium, or its actions within the release site, promoting transmitter release.

We are grateful to Professor M.R. Bennett and Dr A.H. Goodman for equipment used in these experiments and to the Coppleson Post Graduate Medical Institute for financial support for this project. N.A.L. is in receipt of a N.H. and M.R.C. post graduate scholarship. We thank Miss J. Wooten and Miss V. Lasaracina for typing this manuscript.

References

BENNETT, M.R. (1972). Autonomic neuromuscular transmission. In *Monographs of the Physiological Society*. No. 30 Cambridge: Cambridge University Press.

BENNETT, M.R. (1973). An electrophysiological analysis of the storage and release of noradrenaline at sympathetic nerve terminals. *J. Physiol.*, **229,** 515–531.

BENNETT, M.R. & FISHER, C. (1977). The effect of calcium ions on the binomial parameters that control acetylcholine release during trains of nerve impulses at amphibian neuromuscular synapses. *J. Physiol.*, **271,** 673–698.

BENNETT, M.R. & FLORIN, T. (1975). An electrophysiological analysis of the effect of Ca ions on neuromuscular transmission in the mouse vas deferens. *Br. J. Pharmac.*, **55,** 97–104.

BENNETT, M.R., FLORIN, T. & PETTIGREW, A.G. (1976). The effect of calcium ions on the binomial statistic parameters that control acetylcholine release at preganglionic nerve terminals. *J. Physiol.*, **257,** 597–620.

BENNETT, M.R. & LAVIDIS, N.A. (1979). The effect of Ca ions on the secretion of quanta evoked by an impulse at nerve terminal release sites. *J. gen. Physiol.*, **74,** 429–456.

BENNETT, M.R. & LAVIDIS, N.A. (1980). An electrophysiological analysis of the effects of morphine on the calcium dependence of neuromuscular transmission in the mouse vas deferens. *Br. J. Pharmac.*, **69,** 185–191.

BENNETT, M.R. & MIDDLETON, J. (1975). An electrophysiological analysis of the effects of reserpine on adrenergic neuromuscular transmission. *Br. J. Pharmac.*, **55,** 79–85.

BORNSTEIN, J.C. & FIELDS, H.L. (1979). Morphine presynaptically inhibits a ganglionic cholinergic synapse. *Neurosci. Letts.*, **15,** 77–82.

BURNSTOCK, G. & HOLMAN, M. (1961). The transmission of excitation from autonomic nerve to smooth muscle. *J. Physiol.*, **155,** 117–133.

CARDENOS, H.L. & ROSS, D.H. (1976). Calcium depletion of synaptosomes after morphine treatment. *Br. J. Pharmac.*, **57,** 521–526.

COLLIER, H.O.J., CUTHBERT, N.J. & FRANCIS, D.L. (1981). Model of opiate dependence in the guinea-pig isolated ileum. *Br. J. Pharmac.*, **73,** 921–932.

COLLIER, H.O.J., DINNEEN, L.C., JOHNSON, C.A. & SCHNIEDER, C. (1968). The abdominal constriction response and its suppression by analgesic drugs in the mouse. *Br. J. Pharmac.*, **32,** 295–310.

COX, B.M. (1978). Stimulus-response relationships in ileum preparations from normal and morphine treated guinea-pigs. *Br. J. Pharmac.*, **26,** 387–388.

DODGE, F.A. & RAHAMIMOFF, R. (1967). Co-operative action of calcium ions in transmitter release at the neuromuscular junction. *J. Physiol.*, **193,** 419–432.

DREW, G.M. (1978). The effect of different calcium concentrations on the inhibitory effect of presynaptic α-adrenoceptors in the rat vas deferens. *Br. J. Pharmac.*, **63,** 417–419.

GUERRERO-MUNOZ, F., CERRETA, K.V., GUERREO, M.L. & WAY, E.L. (1978). Effect of morphine on synaptosomal Ca uptake. *J. Pharmac. exp. Ther.*, **209,** 132–136.

HARRIS, R.A., YAMAMOTO, H., LOH, H.H. & WAY, E.L. (1977). Discrete changes in brain calcium with morphine analgesia, tolerance-dependence and abstinence. *Life Sci.*, **20,** 501–506.

HENDERSON, G. & HUGHES, J. (1976). The effects of

morphine on the release of noradrenaline from the mouse vas deferens. *Br. J. Pharmac.*, **57,** 551–557.

HENDERSON, G., HUGHES, J. & KOSTERLITZ, H.W. (1972). A new example of a morphine-sensitive neuroeffector junction; adrenergic transmission in the mouse vas deferens. *Br. J. Pharmac.*, **46,** 764–766.

HENDERSON, G., HUGHES, J. & KOSTERLITZ, H.W. (1975). The effects of morphine on the release of noradrenaline from the cat isolated nictitating membrane and the guinea-pig ileum myenteric plexus-longitudinal muscle preparation. *Br. J. Pharmac.*, **53,** 505–512.

HENDERSON, G. & NORTH, R.A. (1976). Depression by morphine of excitatory junction potentials in the vas deferens of the mouse. *Br. J. Pharmac.*, **57,** 341–346.

HÖLT, V., DUM, J., BLÄSTIG, J., SCHUBERT, P. & HERZ, A. (1975). Comparison of *in vivo* and *in vitro* parameters of opiate receptor binding in naive and tolerant/dependent rodents. *Life Sci.*, **16,** 1823–1828.

ILLES, P. & NORTH, R.A. (1982). Effects of divalent cations and normorphine on spontaneous excitatory junction potentials in the mouse vas deferens. *Br. J. Pharmac.*, **75,** 599–604.

ILLES, P., ZIEGLAGANSBERGER, W. & HERZ, A. (1980). Calcium reverses the inhibitory action of morphine on neuroeffector transmission in the mouse vas deferens. *Brain Res.*, **191,** 511–522.

JENKINSON, D.H. (1957). The nature of the antagonism between calcium and magnesium ions at the neuromuscular junction. *J. Physiol.*, **138,** 434–444.

KLEE, W.A., & STREATY, R.A. (1974). Narcotic receptor sites in morphine-dependent rats. *Nature*, **248,** 61–63.

LEE, N.M., HO, I.K. & LOH, H.H. (1975). Effect of chronic morphine treatment on brain chromatin template activities in mice. *Biochem. Pharmac.*, **24,** 1983–1987.

MALLART, A. & MARTIN, A.R. (1968). The relation between quantum content and facilitation at the neuromuscular junction of the frog. *J. Physiol.*, **196,** 593–604.

MARSHALL, I., PHILLIPS, D.G.L. & NASMYTH, P.A. (1981). Calcium ions, morphine tolerance and noradrenergic transmission in the mouse vas deferens. *Eur. J. Pharmac.*, **75,** 205–213.

MARTIN, A.R. (1955). A further study of the statistical composition of the endplate potential. *J. Physiol.*, **130,** 114–122.

MILNER, J.D., NORTH, R.A. & VITEK, L.V. (1982). Interactions among the effects of normorphine, calcium and magnesium on transmitter release in the mouse vas deferens. *Br. J. Pharmac.*, **76,** 45–59.

MORITA, K. & NORTH, R.A. (1982). Opiate activation of potassium conductance in myenteric neurons: inhibition by calcium ions. *Brain Res.*, **242,** 145–150.

MUDGE, A.W., LEEMAN, S.E. & FISHBACH, G.D. (1979). Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration. *Proc. natn. Acad. Sci. U.S.A.*, **76,** 525–530.

NORTH, R.A. & HENDERSON, G. (1975). Action of morphine on guinea-pig myenteric plexus and mouse vas deferens studied by intracellular recording. *Life Sci.*, **17,** 63–66.

NORTH, R.A. & TONINI, M. (1977). The mechanism of action of narcotic analgesics in the gunea-pig ileum. *Br. J. Pharmac.*, **61,** 541–549.

NORTH, R.A. & VITECK, L.V. (1980). The effect of chronic morphine treatment on excitatory junction potentials in the mouse vas deferens. *Br. J. Pharmac.*, **68,** 399–406.

PATON, W.D.M. (1957). The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. *Br. J. Pharmac. Chemother.*, **12,** 119–127.

ROSS, D.H. (1977). Calcium content and binding in synaptosomal subfraction during chronic morphine treatment. *Neurochem. Res.*, **2,** 581.

SCHAUMANN, W. (1957). Inhibition by morphine of the release of acetycholine from the intestine of the guinea-pig. *Br. J. Pharmac. Chemother.*, **12,** 115–118.

SCHULZ, R. & HERZ, A. (1976). aspects of opiate dependence in the myenteric plexus of the guinea-pig. *Life Sci.*, **19,** 1117–1128.

SMITH, S.J. & ZUCKER, R.S. (1980). Aequorin response facilitation and intracellular calcium accumulation in molluscan neurones. *J. Physiol.*, **300,** 167–196.

SCHMIDT, W.K. & WAY, E.L. (1980). Effect of a calcium chelator or morphine-chelator on morphine-tolerance development. *Eur. J. Pharmac.*, **63,** 243–250.

STARKE, K. (1977). Regulation of noradrenaline release by presynaptic receptor systems. *Rev. Physiol. Biochem. Pharmac.*, **77,** 1–125.

STJARNE, L. (1975). Basic mechanisms and local feedback control of secretion of adrenergic and cholinergic neurotransmitters. In *Handbook of Psychopharmacology*. ed. Iversen, L.L., Iversen, S.D. & Snyder S.H., Vol 6 pp. 179–233. New York, London:Plenum Press.

YAMAMOTO, H., HARRIS, R.A., HOH, H.H. & WAY, E.L. (1978). Effects of acute and chronic morphine treatments on calcium localization and binding in brain. *J. Pharmac. exp. Ther.*, **205,** 255–264.

(Received August 23, 1983. Revised June 1, 1984.)