Br. J. Pharmac. (1980), **69**, 185–191

AN ELECTROPHYSIOLOGICAL ANALYSIS OF THE EFFECTS OF MORPHINE ON THE CALCIUM DEPENDENCE OF NEUROMUSCULAR TRANSMISSION IN THE MOUSE VAS DEFERENS

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1 The effects of morphine on the Ca-dependence of the synaptic potential amplitude in the mouse vas deferens have been determined.

2 The synaptic potential increased with a power factor of 2.4 for $[Ca]_o$ between 0.7 mM and 1.8 mM. Morphine (40 nM) decreased the synaptic potential, without altering the second power relationship between the synaptic potential and $[Ca]_o$.

3 Morphine reversed the depression in the synaptic potential which develops during a short high-frequency (10 Hz) train of impulses to facilitation. Consequently the synaptic potential beyond the tenth impulse was unaffected by morphine.

4 Morphine did not alter the facilitation of the synaptic potential which develops during a short low-frequency (≤ 2 Hz) train of impulses in normal $[Ca]_o$. Consequently morphine decreased the synaptic potential for each impulse by about the same percentage amount.

5 Morphine increased the small facilitation in the synaptic potential which occurs during a short low-frequency (≤ 2 Hz) train of impulses in high $[Ca]_o$. This facilitation approximated the predictions based on the assumption that each impulse leaves residual Ca ions bound to receptors involved in transmitter release from the nerve terminal.

Introduction

Evoked transmitter release from sympathetic nerve terminals in the mouse vas deferens is dependent on the binding of Ca ions to a presynaptic receptor (X) (Kirkepar & Misu, 1967; Bennett & Florin, 1975). The facilitation of transmitter release during short low-frequency trains of impulses can be quantitatively described by a transient accumulation of CaX in the terminals following successive impulses in the train (Bennett, 1973a; Bennett & Florin, 1975). The depression of transmitter release which develops during a high-frequency train of impulses is dependent on the amount of transmitter released during the train (Bennett, 1973a) and is therefore also dependent on the formation of CaX.

Morphine blocks the evoked release of transmitter from sympathetic nerve terminals in the mouse vas deferens during short low-frequency trains of impulses (Henderson, Hughes & Kosterlitz, 1972). This blockade is not achieved through interference with impulse conduction in nerve terminals (Kosterlitz & Wallis, 1964), nor by depression due to blockade of uptake of catecholamines into synaptic vesicles (Blosser & Catravas, 1974). The analgesic effects of morphine are suppressed by intracisternal administration of Ca (Kakunga, Kaneto & Nano, 1966), whilst the tissue Ca of rat brain is decreased by morphine (Cardenas & Ross, 1975). This effect is due to a decrease in the Ca levels found in synaptic terminals (Ross, Lynn & Cardenas, 1976; Harris, Yamamoto, Loh & Way, 1976; Yamamoto, Harris, Loh & Leon Way, 1978), and may be associated with alteration by morphine of the binding of Ca ions to phospholipids within the presynaptic membrane (Múle, 1969). The present work is a study of the hypothesis that morphine acts by blocking the binding of Ca ions to the X-receptor in the presynaptic membrane.

Methods

The isolated vas deferens from S.W. and Raub C. (pathogen-free) strain mice were used. The mice were killed by cervical fracture and both vas deferens dissected free and pinned out in a perspex organ bath of 10 ml capacity. The preparations were bathed in a modified Krebs-Ringer-bicarbonate solution of the following ionic composition (mM): Na^+ 151, K^+ 4.7, Ca^{2+} 1.8, Mg^{2+} 1.2, Cl^- 142, $H_2PO_4^-$ 1.3, SO_4^{2-} 1.2, HCO_3^- 16.3 and glucose 7.8. Solutions were bubbled with a gas mixture containing 95% O_2 and 5% CO_2, and maintained at 35 ± 1°C while flowing through the organ bath at about 10 ml/min. Changes in the

0007-1188/80/060185-07 $01.00

extracellular calcium concentration $[Ca]_o$ were made by changing the concentration of $CaCl_2$ present in the reservoir of modified Krebs–Ringer-bicarbonate solution supplying the organ bath. No compensation was made for tonicity changes. In order to avoid possible changes in conduction of nerve impulses the total divalent cation concentration was always above 0.7 mM (Frankenhauser & Hodgkin, 1957).

Two platinum-ring electrodes, 1 mm apart, were placed round the vas deferens and used for intramural nerve stimulation with pulses of 60 V amplitude and 0.03 to 0.1 ms duration (see Bennett, 1973a, b). Intracellular potentials were recorded from the smooth muscle cells with 70 to 100 MΩ glass microelectrodes filled with 2 M KCl. The signals were led through a high impedance unity gain amplifier, displayed on an oscilloscope screen and photographed on moving film as well as being recorded on FM magnetic tape.

The stimulus duration was graded so that the amplitude of the excitatory junction potentials (e.j.ps) did not exceed 15 mV during trains of impulses; corrections for non-linear summation were therefore avoided (Martin, 1955; Bennett, Florin & Pettigrew, 1976). The quality of an intracellular impalement was judged as adequate if on removal of the microelectrode the membrane potential shift was about 60 mV (see Bennett, 1972). The stability of the impalement over long periods was determined by comparing the amplitude-frequency of the miniature excitatory junction potentials (m.e.j.ps) recorded at the beginning of the impalements with those recorded at the end of the impalement. The synaptic potential in each cell is due to transmitter acting on cells throughout the smooth muscle electrical syncytium (Bennett, 1972), so that the amplitude of the e.j.p. at the stimulating electrode does not vary between cells if constant stimulus parameters are used.

The amplitude of the e.j.p. recorded from cells at the stimulating electrode does not vary between preparations if the same stimulus parameters are used (see Bennett & Middleton, 1975a). In determining the effects of morphine on the Ca-dependence of the synaptic potential (Figure 1), the number of nerves stimulated was kept constant by using the same stimulus parameters of 0.1 ms and 60 V, allowing the results from different cells to be pooled for any given $[Ca]_o$.

If $[Ca]_o$ is increased in the range from 0.7 mM to 4.0 mM there is an approximately 10 mV hyperpolarization of the vas deferens smooth muscle cells (Kuriyama, 1964; Bennett, 1967). This contributes a small increase to the e.j.p. amplitude. No correction has been made for the effect of this hyperpolarization, which occurs in both the presence and absence of morphine.

The amplitude of each e.j.p. during short trains of impulses was normalized with respect to the first e.j.p.

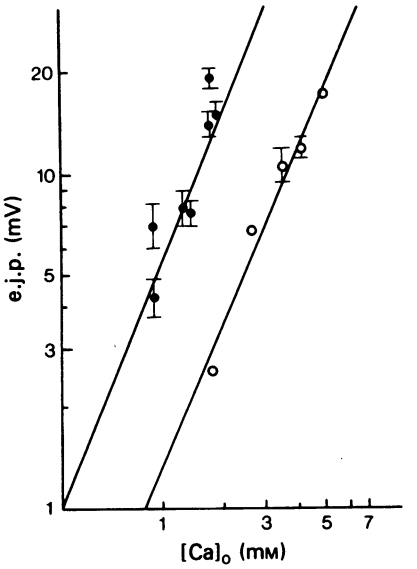

Figure 1 Dependence of the excitatory junction potential (e.j.p.) amplitude on the external calcium concentration $[Ca]_o$, in the absence of morphine (●) and in the presence of morphine (40 nM, ○). Log/log coordinates. Each point was determined from at least 20 e.j.ps in 20 cells from each of 6 preparations. Vertical lines indicate ±s.e. mean for each point, where it is larger than the diameter of the point. Lines are drawn by eye and have a slope of 2.4. Stimulus parameters constant at 0.1 ms and 60 V.

in the train and the normalized data for all preparations pooled. In some cases, changes in e.j.p. amplitude during a short train were compared with predictions based on the residual Ca-receptor hypothesis (see Bennett *et al.*, 1976; Bennett & Fisher, 1977), according to the equation

$$\text{e.j.p. amplitude} = f(j) + 1$$

$$= \left\{ \sum_{i=1}^{j-1} [(1 + f[\Delta t(j-i)])^{1/2} - 1] + 1 \right\}^2$$

where $f(j) + 1$ is the normalized size of the e.j.p. amplitude in response to the jth impulse, Δt is the interval between impulses and $f(\Delta t(j-i))$ is the value of facilitation for the interval $\Delta t(j-i)$; facilitation $f(\Delta t(j-i))$, was equal to $(v - v_o)/v_o$ where v_o is the amplitude of a conditioning e.j.p. and v is the amplitude of a test e.j.p. at an interval $\Delta t(j-i)$ after the conditioning e.j.p. It was found that $f(\Delta t(j-i))$ was unaffected by morphine (40 nM) in low $[Ca]_o$ (≤ 1.9 mM), and was similar to that given in Figure 4 of Bennett (1973a).

The following drugs were added directly to the bathing medium and a delay of at least 0.5 h allowed

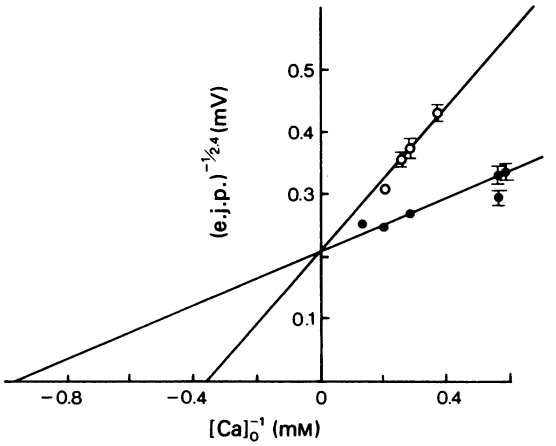

Figure 2 Double reciprocal plot for the relationship between $(e.j.p.)^{-1/2.4}$ and $1/[Ca]_o$. Linear coordinates. (●) = In the absence of morphine; (○) = in the presence of morphine (40 nM). The data shown in Figure 1 have been replotted on these coordinates and lines of best fit drawn by eye.

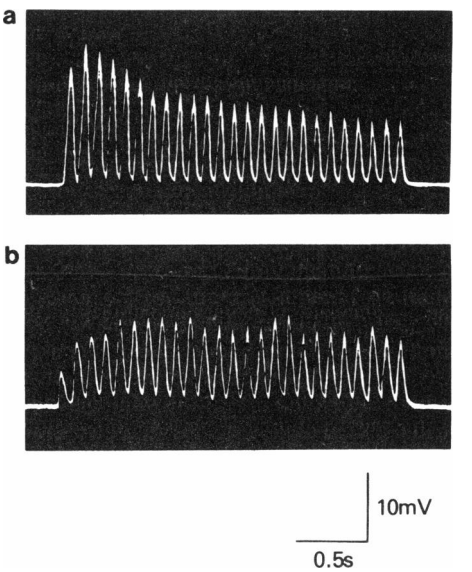

Figure 3 The effects of morphine on the amplitude of the excitatory junction potential (e.j.p.) during short trains of high-frequency impulses (10 Hz). (a) Control, (b) morphine (40 nM), $[Ca]_o$ in both (a) and (b), 3.6 mM.

for equilibration before electrophysiological measurements were made: morphine sulphate (40 nM); naloxone sulphate (300 nM).

Results

The effect of morphine on the $[Ca]_o$ dependence of transmitter release by single impulses

The amplitude of the excitatory junction potential (e.j.p.) due to a single nerve impulse increased with about the second power ($2.4 \pm 0.2(5)$) of the external calcium concentration $[Ca]_o$, over the concentration range from 0.7 mM to 1.8 mM (Figure 1; see also Bennett & Florin, 1975). Increasing $[Ca]_o$ in the presence of a fixed concentration of morphine increased the amplitude of the e.j.p., which again followed a second power (Figure 1), although the curve was shifted to the right of that obtained in the absence of morphine. The e.j.p. returned to its normal amplitude in 30 min following the removal of morphine in any $[Ca]_o$. Furthermore, naloxone blocked the effects of morphine in depressing the amplitude of the e.j.p. at each $[Ca]_o$. Morphine did not alter the amplitude-frequency distribution of the spontaneous miniature excitatory junction potentials (see also Henderson, 1976). A double reciprocal plot of $(e.j.p.)^{-1/2.4}$ against $[Ca]_o^{-1}$ (Figure 2) for data collected in the presence or absence of morphine, shows that morphine acts as a competitive inhibitor of the action of Ca ions in promoting transmitter release. It is therefore possible that morphine acts by competitively blocking the binding of Ca ions to the presynaptic release receptor (X).

The effects of morphine on the release of transmitter during trains of high-frequency impulses

The first few impulses in a short high-frequency train (10 Hz) release successively greater amounts of transmitter, whilst subsequent impulses release less transmitter until a steady-state and depressed release rate is reached (Figure 3a; see also Bennett, 1973a). The extent of this depression during high-frequency stimulation is dependent on the amount of transmitter released by previous impulses in the train. Magnesium which is a competitive inhibitor of Ca ions binding to the X-receptor (Bennett & Florin, 1975), decreases the quantity of transmitter released by the first few impulses and therefore reverses the depression normally observed during high-frequency stimulation to facilitation. Morphine also reverses this depression in the e.j.p., following the first few impulses in a high-frequency train, to facilitation (Figure 3b), in much the same way as does magnesium. This effect of morphine was blocked by naloxone.

Morphine does not reduce the noradrenaline output from the vas deferens (Henderson & Hughes, 1976) or the cat nictitating membrane (Cairnie, Kosterlitz & Taylor, 1961) during trains of high-frequency impulses, This is consistent with the effects of morphine on the e.j.p. By the time a steady-state e.j.p.

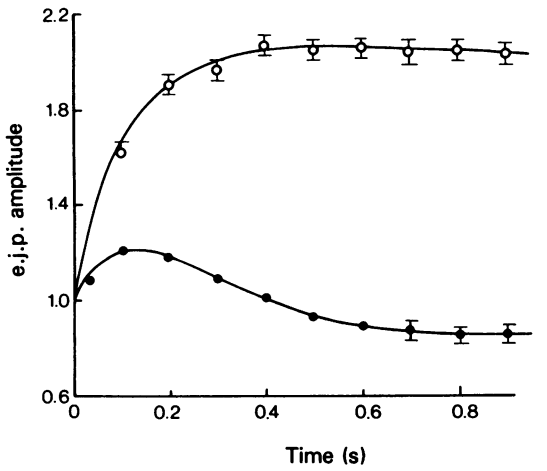

Figure 4 The effect of morphine on the growth of the excitatory junction potential (e.j.p.) during short trains of impulses at high frequencies (10 Hz). (●) = Control; (○) = morphine (40 nM). Each point was determined from at least 10 e.j.ps in three different preparations, and ±s.e. mean for each point is shown where this is greater than the diameter of the point. $[Ca]_o$ in each case was 3.6 mM.

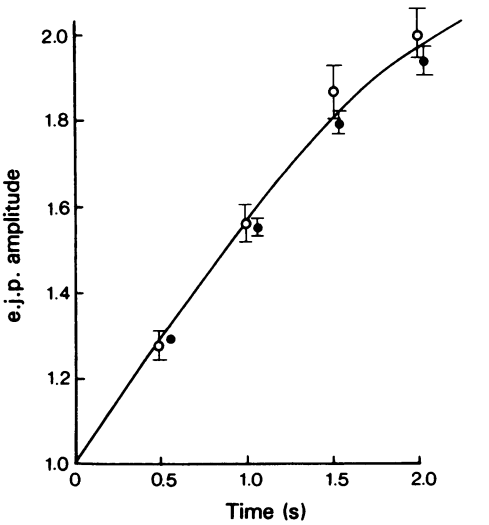

Figure 5 The effect of morphine on the growth of the excitatory junction potential (e.j.p.) during short trains of impulses at low frequency (2 Hz) in normal $[Ca]_o$ (1.8 mM). (●) = Control; (○) = morphine (40 nM). Each point was determined from at least 10 e.j.ps in three different preparations and ±s.e. mean of each point is shown.

amplitude is reached in the presence of morphine during a train of impulses, the output per impulse is over twice that released by the first impulse in the train (Figure 4) and comparable to the steady-state release observed during high-frequency stimulation in the absence of morphine.

The effects of morphine on the release of transmitter during trains of low-frequency impulses

Morphine greatly reduces the noradrenaline (NA) output from both the mouse vas deferens (Henderson & Hughes, 1976) and the cat nictitating membrane (Cairnie *et al.*, 1961) during trains of low frequency impulses. These results are consistent with the failure of morphine to affect the facilitation of the e.j.p. during short trains of impulses at 2 Hz in a normal $[Ca]_o$ of 1.8 mM (Figure 5). As morphine decreases the transmitter released by the first impulse in a train by over two thirds (Figure 1), but does not alter facilitation, it depresses transmitter release during these short trains of impulses.

During short low-frequency trains of impulses in a normal $[Ca]_o$ of 1.8 mM and a high $[Mg]_o$ of 10 mM, successive impulses in a train release successively greater amounts of transmitter, until a steady-state and elevated transmitter release per impulse is reached (see Bennett, 1973a). This facilitation of transmitter release in the absence of any depression can be quantitatively described in terms of each impulse leaving behind residual CaX in the nerve terminal (Figure 6; see also Bennett & Florin, 1975). However, if the $[Ca]_o$ is elevated or the $[Mg]_o$ is reduced, the e.j.p. amplitudes fall below the predictions of the residual CaX hypothesis (Figure 6). If morphine competitively inhibits the action of Ca in promoting transmitter release in much the same way as does Mg, then morphine should prevent the depletion of transmitter which follows impulses in an elevated $[Ca]_o$ or a reduced $[Mg]_o$, allowing the e.j.p. amplitude to facilitate according to the predictions of the residual CaX hypothesis.

The small facilitation in e.j.p. amplitude observed in a high $[Ca]_o$ of 3.6 mM and a $[Mg]_o$ of 1.2 mM during low-frequency trains of impulses was greatly increased by the addition of morphine, and this increase was blocked by naloxone (Figure 6). This facilitation approximated the predictions of the residual CaX hypothesis, in much the same way as does facilitation in the presence of high $[Ca]_o$ with an elevated $[Mg]_o$ (Bennett, 1973a).

Discussion

The effect of morphine on the action of Ca ions in promoting transmitter release by a nerve impulse

It is likely that the transmitter which gives rise to the e.j.p. in the mouse vas deferens is NA. Pretreatment

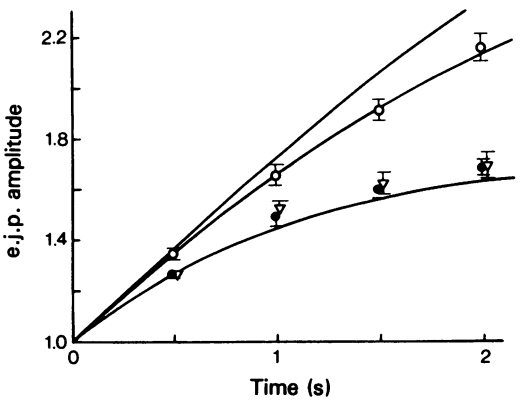

Figure 6 The effect of morphine and naloxone on the growth of the excitatory junction potential (e.j.p.) during short trains of impulses at low frequencies (2 Hz) in high $[Ca]_o$ (3.6 mM). (●) = Control; (○) = morphine (40 nM); △ = morphine (40 nM) plus naloxone (300 nM). Each point was determined from at least 8 e.j.ps in three preparations and ±s.e. mean for each point is shown. Lines drawn by eye. Upper line is the prediction of the residual CaX hypothesis according to equation 1 in the Methods.

of the mouse vas deferens with 6-hydroxydopamine abolishes both the histochemical flourescence due to catecholamines, the e.j.p. and the motor response to nerve stimulation (Furness, Campbell, Gillard, Malmfors, Cobb & Burnstock, 1970; Jones & Spriggs, 1975a). Bretylium and high concentrations of phenoxybenzamine abolish the e.j.p. (Bennett & Middleton, 1975a) whilst guanethidine inhibits the motor response to nerve stimulation (Jones & Spriggs, 1975a; Henderson & Zar, 1976). A cholinergic component of the innervation has been identified histochemically in the mouse vas deferens (Jones & Spriggs, 1975a) and atropine impairs the motor response to field stimulation (Jones & Spriggs, 1975b) without, however, affecting the amplitude of the e.j.p. (Bennett & Middleton, 1975a). It seems unlikely that any significant proportion of the innervation of the mouse vas deferens is cholinergic as 6-hydroxydopamine completely abolishes the e.j.p. as well as the motor response and the effects of atropine on the motor response are themselves blocked by desmethylimipramine (Jones & Spriggs, 1975b). Although the motor response of the vas deferens during short trains of impulses is reduced only 66% following a 97% reduction of the NA levels with reserpine (Marshall, Nasmyth & Shepperson, 1978), this may be due to reserpine reversing the normal depression in e.j.p. amplitude which occurs during a high-frequency train to facilitation (see Figure 2 in Bennett & Middleton, 1975a).

Changes in the amplitude of the e.j.p. are likely to give a good measure of changes in the amount of NA released by nerve impulses under different experimental conditions. There are consistent correlations between relative amounts of NA overflowing from organs under different conditions (Dearnaley & Geffen, 1966; Kirpekar & Misu, 1967; Kopin, Breese, Krauss & Weise, 1968; Stjarne & Wennmalm, 1970; Stjarne, 1973) and relative changes in the amplitude of the e.j.p. (Bennett, 1973a; Bennett & Florin, 1975). The depression of e.j.p. amplitude by morphine and the antagonism of this effect by naloxone (North & Henderson, 1975; Henderson & North, 1976) are therefore to be expected, given that morphine greatly reduces the overflow of NA from the vas deferens during nerve stimulation (Henderson *et al.*, 1972; Hughes, Kosterlitz & Leslie, 1975; Henderson & Hughes, 1976). These observations together with the fact that morphine neither affects the depolarization of smooth muscle cells due to exogenous NA (Henderson & North, 1976), nor the spontaneous miniature junction potentials, indicate that the action of morphine is presynaptic.

The inhibitory effects of morphine on the action of Ca ions in promoting transmitter release are similar to those of the inhibitory effects produced by raising $[Mg]_o$ (Kirkepar & Misu, 1967; Bennett & Florin, 1975). The site where the CaX complexes are formed in the nerve terminal is not known, although recent evidence suggests that the X-receptor is located in the potential-dependent Ca channels of the nerve terminal membrane (Hagiwara, 1975). The entry of Ca ions into axons which accompanies the nerve impulse is blocked by Mg (Baker, Hodgkin & Ridgeway, 1971). Mg may then displace Ca from the X-sites in the potential-dependent calcium channel, decrease the calcium conductance change which accompanies the nerve impulse and therefore block transmitter release. Morphine, as well as the naturally occurring opiates methionine-enkephalin and leucine-enkephalin (Segawa, Murakami, Ogawa & Yajima, 1978), may also block transmitter release by binding to the X-receptors and displacing Ca ions. This would explain the alteration by opiates of Ca binding to phospholipids in the presynaptic membrane (Mulé, 1969) as well as their ability to block transmitter release (Dunlap & Fischbach, 1978).

However, morphine and other opiates may exert this effect on X-receptors in a less direct way. It has recently been shown that 5-hydroxytryptamine promotes transmitter release from nerve terminals in *Aplysia* by first increasing cyclic adenosine 3',5'-monophosphate (cyclic AMP), which in turn increases the calcium influx accompanying a nerve impulse (Shimahara & Tauc, 1977; Klein & Kandell, 1978). Morphine may also modify the binding of Ca to the X-receptor via cyclic AMP.

The effect of morphine on transmitter release during short trains of impulses

What is the basis of the depression in transmitter release that develops during short high-frequency trains of impulses, and which morphine reverses to facilitation? At the cholinergic neuromuscular junction, depression of transmitter release has been attributed to a growing depletion of the numbers of quanta readily available for release from the nerve terminal during a train (Mallart & Martin, 1968; Bennett & Fisher, 1977). However, this depression may be due to a growing nicotinic receptor-mediated autoinhibition of acetylcholine release during a train (Miledi, Molenaar & Polak, 1978). A growing autoinhibition of NA release during a train has also been suggested, in which the NA released by an impulse acts on the nerve terminal to decrease the entry of Ca ions during a subsequent impulse, thus counteracting the effects of residual CaX (Stjarne, 1973; 1975; Bennett & Middleton, 1975b; Starke, 1977; Drew, 1978).

The action of morphine on transmitter release during a high-frequency train may be explained as follows: morphine depresses the amount of NA released by the first impulse in a train by inhibiting the formation of CaX, consequently relieving the extent of the α-adrenoceptor-mediated autoinhibition of the NA released by the second impulse in the train. Facilitation of transmitter release is then enhanced over depression, these antagonistic changes coming into equilibrium during a train when the amount of NA released per impulse is the same in the presence of morphine as it is in its absence (see Figure 3).

We would like to thank Miss J. Stratford for excellent technical assistance. This work was supported by the Australian Research Grants Committee.

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(Received April 10, 1979.
Revised August 7, 1979.)