Br. J. Pharmac. (1976), **57**, 341–346

DEPRESSION BY MORPHINE OF EXCITATORY JUNCTION POTENTIALS IN THE VAS DEFERENS OF THE MOUSE

G. HENDERSON & R.A. NORTH[1]
Unit for Research on Addictive Drugs, Marischal College, University of Aberdeen

1 Intracellular recordings were made from smooth muscle cells of the mouse vas deferens. Excitatory junction potentials (e.j.ps) were evoked by stimulation of the intramural nerves.

2 Normorphine (50 nM – 5 μM) depressed the amplitude of the e.j.p. The ED_{50} was 430 nM. The latency of the e.j.p. and the resting membrane potential of the smooth muscle cells were unaffected by normorphine.

3 The depression of the e.j.p. by narcotic analgesic drugs was stereospecific.

4 Naloxone (100 nM) completely reversed the depression of the e.j.p. produced by normorphine (1 μM). Naloxone (100 nM) alone did not alter the amplitude of the e.j.p.

5 Normorphine (1 μM) did not prevent the depolarization of the smooth muscle cells produced by exogenous noradrenaline (10 μM).

6 It is concluded that narcotic analgesic drugs act directly upon the transmitter release sites to reduce the amount of noradrenaline liberated by each nerve impulse.

Introduction

Narcotic agonists inhibit the nerve-mediated contraction of the mouse vas deferens and the cat nictitating membrane. This inhibition is stereospecific and reversed by naloxone (Henderson, Hughes & Kosterlitz, 1972, 1975; Hughes, Kosterlitz & Leslie, 1975). The site of action of the narcotic agonists is thought to be prejunctional because the drugs do not reduce the contraction of the tissues to exogenous noradrenaline (Cairnie, Kosterlitz & Taylor, 1961; Henderson, 1974) but, at both sites, they greatly reduce the output of noradrenaline from the tissue which follows repetitive stimulation of its nerve supply (Henderson *et al.*, 1972; 1975; Hughes *et al.*, 1975).

Excitatory junction potentials (e.j.ps) in response to nerve stimulation have previously been recorded from individual smooth muscle cells of the mouse vas deferens (Holman, 1967; Furness & Burnstock, 1969; Furness, 1970; Bennett, 1973a). Changes in the amplitude of the e.j.p. afford a sensitive method of studying changes in the amount of transmitter released from the presynaptic nerve terminals in response to a single stimulus. The aim of the present experiments was to use this technique to examine the site of action of morphine at this autonomic neuro-effector junction.

[1]Present address: Neurophysiology Laboratory, Department of Pharmacology, Loyola University Medical Center, Maywood, Illinois 60153, U.S.A.

Preliminary results of this work have been published (North & Henderson, 1975).

Methods

Male mice (TO strain), weighing 25–35 g, were killed by cervical dislocation. The vasa deferentia were dissected out and one was placed in a shallow organ bath similar to that described by Nishi & North (1973). The preparation was pinned to the floor of the bath and a portion (1–2 mm) of the outer sheath was gently removed. Glass micro-electrodes filled with 2 M KCl (tip resistance 45–90 MΩ) were used for intracellular recordings. The recorded signals were amplified, displayed and photographed in the conventional manner. The intramural nerves were stimulated by two platinum ring electrodes placed around the vas deferens approximately 2 mm apart and 5 mm from the site of recording. For studies of drug action, the preparation was stimulated with a single rectangular pulse (100 μs to 1 ms duration) at intervals of 30 seconds. The stimulus strength was adjusted so as to evoke control e.j.ps between 15 and 25 mV in amplitude. In measuring e.j.p. amplitudes, no correction was made for non-linear summation (Martin, 1955).

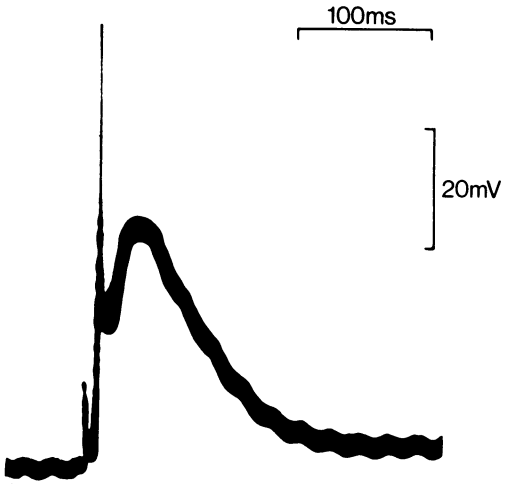

Figure 1 Evoked excitatory junction potential (e.j.p.) giving rise to an action potential. Calibrations: horizontal 100 ms, vertical 20 mV.

The preparation was perfused with a Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, $MgSO_4$ 1.19, KH_2PO_4 0.93, $NaHCO_3$ 25, glucose 11, gassed with 95% O_2 and 5% CO_2. In those experiments in which noradrenaline was added, the Krebs solution contained ascorbic acid (100 μM) and disodium edetate (27 μM). The flow rate of the Krebs solution was 2 ml/min and the bath temperature was 37°C. Drugs used were: bretylium tosylate (Burroughs Wellcome), amphetamine sulphate (Smith, Kline & French), dextrorphan tartrate (Roche Products), hexamethonium bromide (May and Baker), hyoscine hydrobromide (B.P.), levorphanol tartrate (Roche Products), morphine hydrochloride (B.P.), naloxone hydrochloride (Endo Laboratories), (–)-noradrenaline bitartrate (BDH) and normorphine hydrochloride (Dr E.L. May). The concentrations are given in nM or μM.

Results

Characteristics of excitatory junction potentials

The smooth muscle of the mouse vas deferens is not spontaneously active and intracellular recordings were obtained from single cells for periods of up to 4 hours. The resting membrane potential of single cells was between −55 and −65 mV; the value recorded at the moment of impalement remained unchanged throughout the long impalements. Spontaneous e.j.ps were observed in most of the cells impaled. A single stimulus applied to the intramural nerves elicited an e.j.p. in every cell which was satisfactorily impaled. The latency was 6–10 milliseconds. The e.j.p. increased in amplitude as the strength of the stimulus was increased; this increase in amplitude was not smoothly graded but occurred in discrete increments, suggesting that excitation of a limited number of nerve fibres was responsible for the recorded e.j.p. With constant stimulation parameters, the e.j.ps recorded successively from adjacent muscle cells were almost identical. In most cells, the e.j.p. gave rise to a graded action potential. The threshold depolarization for action potential initiation varied between 30 and 40 mV (Figure 1).

When the intramural nerves were stimulated at 0.033 Hz there was neither facilitation nor decline of successive e.j.ps. At higher frequencies of stimulation a progressive increase in amplitude of the e.j.p. occurred during the first 5 to 10 stimuli.

The e.j.ps were unaffected by exposure of the tissue for 15 min to hyoscine (1 μM) or to hexamethonium (100 μM) although they were rapidly abolished by bretylium (50 μM). The action of bretylium was not reversed by washing out the drug, but was reversed by the addition of amphetamine (50 μM).

Effect of normorphine on the e.j.p.

The resting membrane potential of the smooth muscle cells was not changed by the addition of normorphine (50 nM–5 μM) to the perfusing solution. The apparent input resistance of the cells was determined by observing the steady state potential displacement caused by passing pulses of a known current through the recording micro-electrode by means of a bridge circuit; this was not affected by normorphine.

Normorphine reduced the amplitude of the evoked e.j.p. in a dose-dependent manner (Figure 2); it did not affect the latency of the response. Figure 2 represents the pooled data from a number of cells. The threshold concentration at which depression of the e.j.p. was observed was 50 nM and the ED_{50} (calculated from Figure 2) was 430 nM. The e.j.p. was completely abolished by concentrations of normorphine greater than 1 μM. The e.j.p. was smoothly depressed with respect to time, the depression being maximal between 3 and 6 min after changing to a solution containing normorphine (Figure 3). Upon washing out the normorphine, the e.j.p. regained its initial amplitude within a similar period of time. Depression of the e.j.p. was observed in all 37 cells to which normorphine was applied. As has previously been demonstrated on the contractions of the mouse vas deferens to nerve stimulation (Hughes *et al.*, 1975; Kosterlitz, Leslie & Waterfield, 1975) morphine and normorphine were equipotent in depressing the e.j.p.; the time course of the action of morphine was approximately twice as long as that of normorphine and for this reason normorphine was used in the majority of the experiments.

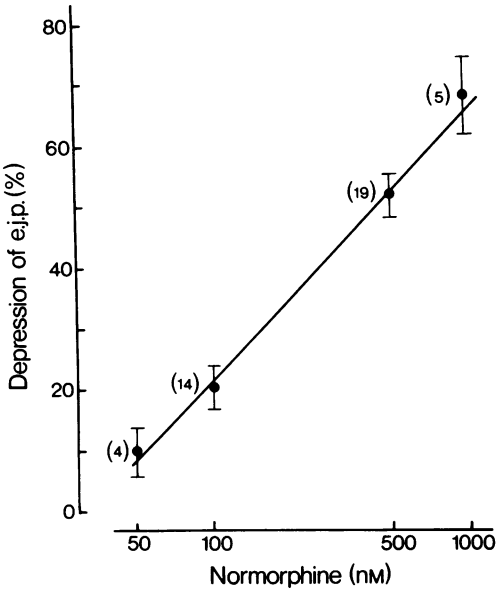

Figure 2 Dose-response curve for the depression of excitatory junction potential (e.j.p.) by normorphine. The tissues were stimulated at 0.033 Hz. Each point is the mean of the number of experiments shown in parentheses. Vertical bars represent s.e. mean. Ordinates: amplitude of the e.j.p. when maximally depressed by normorphine, expressed as a percentage of the control amplitude in the same cell. Abscissae: normorphine concentration (nM).

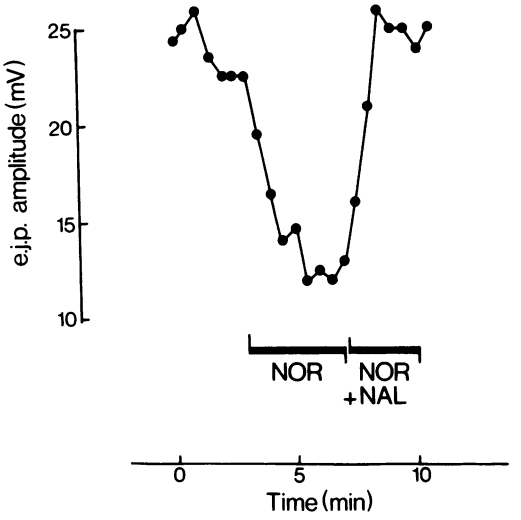

Figure 3 Reversal by naloxone of the reduction in excitatory junction potential (e.j.p.) amplitude caused by normorphine. During the periods indicated, the tissue was exposed to normorphine (NOR, 1 μM) and then to normorphine (1 μM) plus naloxone (100 nM) (NOR + NAL). Ordinates: e.j.p. amplitude (mV). Abscissae: time after impalement (min).

Specificity of narcotic agonist action

Naloxone (100 nM) was added to the bathing solution for periods of up to 20 min without any measurable change in the resting membrane potential or e.j.p. amplitude (Figure 4). However, the reduction of the e.j.p. amplitude produced by normorphine (1 μM) was rapidly reversed by changing to a solution containing naloxone (100 nM) in addition to normorphine (1 μM) (Figure 3). Levorphanol (100 nM) markedly depressed the e.j.p. amplitude whereas a ten-fold higher concentration of its (+)-isomer dextrorphan was without significant effect (Figure 5). Naloxone (50 nM) rapidly and completely reversed the depression of the e.j.p. produced by levorphanol (100 nM) (Figure 5). As on the contractions of the mouse vas deferens (Hughes *et al.*, 1975; Kosterlitz *et al.*, 1975) levorphanol was approximately twice as potent as normorphine in depressing the e.j.p. although the onset and decline of its action were much slower. Maximal depression of the e.j.p. occurred 5–10 min after changing to a solution containing levorphanol. The highest concentration of dextrorphan which was employed (5 μM) depressed the e.j.p. amplitude by not more than 15% of its control value.

Action of exogenous noradrenaline

Concentrations of noradrenaline lower than 10 μM were without effect on the membrane potential of the smooth muscle cells although concentrations of less than 1 μM are capable of causing contraction (Jones & Spriggs, 1975). A similar finding has previously been noted (Holman, 1967) and may be attributed to the inability of low concentrations of noradrenaline to penetrate to the post-junctional receptor sites on the muscle cells lying deep within the tissue from which we recorded. Higher concentrations of noradrenaline often caused a violent contraction of the vas deferens which dislodged the micro-electrode. In experiments in which the micro-electrode was not dislodged, noradrenaline (10 μM) was found to cause a marked depolarization of the muscle cell membrane and a concomitant reduction in amplitude of the e.j.p. (Figure 6). The depolarization produced by noradrenaline varied markedly from cell to cell and at different times within the same cell; this may be due to slight tissue movements. However, the depolarization caused by noradrenaline was not prevented by prior exposure of the tissue to normorphine (1 μM) (Figure 6).

Discussion

The characteristics of the e.j.ps recorded in this investigation were similar to those described by Holman

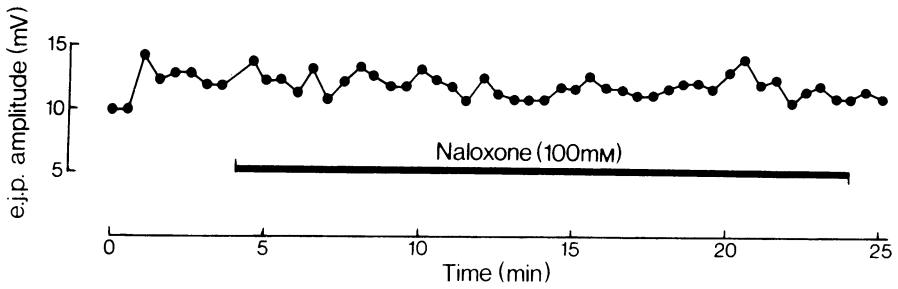

Figure 4 Absence of effect of naloxone on the amplitude of the excitatory junction potential (e.j.p.). The tissue was exposed to naloxone (100 nM) during the period indicated. Ordinates: e.j.p. amplitude (mV). Abscissae: time after impalement (min).

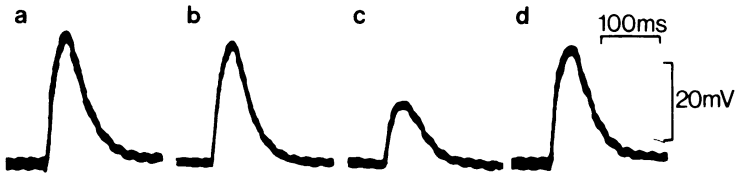

Figure 5 Effects of dextrorphan and levorphanol on the excitatory junction potential (e.j.p.). The tissue was stimulated at 0.033 Hz. (a) Control e.j.p.; (b) e.j.p. when maximally depressed by dextrorphan (1 μM); (c) e.j.p. when maximally depressed by levorphanol (100 nM); (d) e.j.p. 10 min after replacing levorphanol (100 nM) by levorphanol (100 nM) plus naloxone (50 nM). Calibrations: horizontal 100 ms, vertical 20 mV.

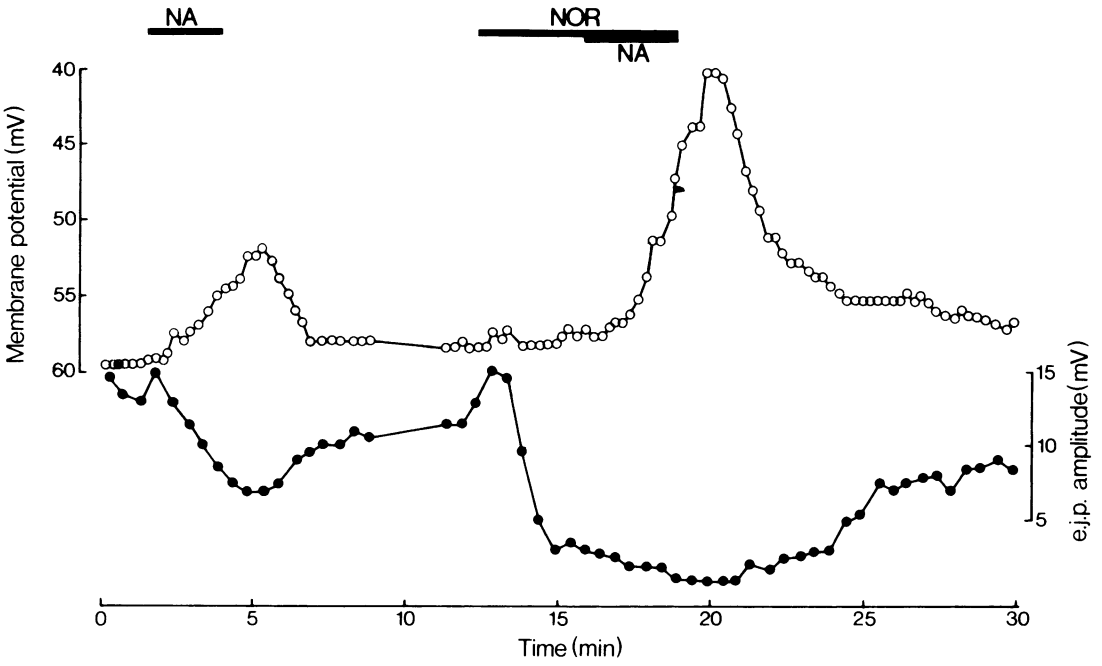

Figure 6 Effect of noradrenaline and normorphine on the membrane potential in a single smooth muscle cell. Ordinates: membrane potential (○); excitatory junction potential (e.j.p.) amplitude (●). Abscissae: time after impalement. The tissue was exposed to noradrenaline (NA, 10 μM) and to normorphine (NOR, 1 μM) during the periods indicated. The depolarization produced by noradrenaline varied markedly from cell to cell and at different times within the same cell; this may be due to slight tissue movements. This depolarization was not prevented by a concentration of normorphine which markedly depressed the e.j.p.

(1967) and Furness (1970). There is good evidence that the e.j.ps recorded from the smooth muscle cells of the mouse vas deferens are due to the release of noradrenaline from adrenergic nerve varicosities. The mouse vas deferens receives a dense adrenergic innervation (Sjöstrand, 1965). As previously described by Holman (1967), exogenous noradrenaline depolarized the smooth muscle cells and reduced the amplitude of the e.j.p. The reduction of e.j.p. amplitude was not a result of the decrease in resting membrane potential (Holman, 1967) but may have been due to an inhibition of neuronal noradrenaline release by exogenous noradrenaline (Starke, 1972). The e.j.p. is abolished by 6-hydroxydopamine (Furness, Campbell, Gillard, Malmfors, Cobb & Burnstock, 1970); it is markedly reduced by reserpine (Bennett & Middleton, 1975) but only by high concentrations of phenoxybenzamine (Bennett, 1973b). Two factors may contribute to the low potency of phenoxybenzamine. First, the mouse vas deferens receives an intimate innervation from close contact varicosities on nerve processes (Lane & Rhodin, 1964; Yamauchi & Burnstock, 1969; Furness & Iwayama, 1971). The concentration of noradrenaline within the synaptic cleft following nerve stimulation will be very large thus requiring a high concentration of phenoxybenzamine to inhibit transmission (Furness, 1974). Second, phenoxybenzamine may increase the concentration of noradrenaline within the synaptic cleft by inhibiting noradrenaline uptake (Avakian & Gillespie, 1968) and by preventing negative feedback on noradrenaline release by an action on presynaptic α-receptors (Starke, 1972). Further evidence of the adrenergic nature of the e.j.p. is obtained from our observations that the e.j.p. is abolished by bretylium; as at other adrenergic synapses (Day & Rand, 1963) the action of bretylium persisted after washing the tissue with drug-free Krebs solution but was reversed by amphetamine. The e.j.p. was unaffected by hyoscine and hexamethonium.

The action of narcotic agonists in depressing the evoked e.j.p. might be at one or more of four sites. First, it may occur at transmission between ganglion cells in the wall of the vas deferens. Such an action can be directly refuted because (a) ganglion cells are extremely sparse within the wall of the mouse vas deferens (Gabella, personal communication), (b) the absence of any effect of hexamethonium suggests that postganglionic elements are being stimulated and (c) the short latency of the e.j.p. described in this paper and by other workers (Furness, 1970) precludes a ganglionic synapse. Second, the narcotic agonists might act on conduction within the intramural nerves. The present observations that the e.j.p. amplitude was reduced in a smoothly graded manner and without change in its latency argue against this interpretation. Local anaesthetic effects of narcotics are non-specific and occur only at 1,000-fold higher concentrations (Ritchie & Armett, 1963; Kosterlitz & Wallis, 1964). Third, the narcotic agonists may directly depress transmitter release by an action at pre-junctional release sites. Fourth, the depression of the e.j.p. amplitude may be due to post-junctional effects of the narcotic agonists. With an intracellular electrode it is difficult to exclude changes in the electrical resistance of the smooth muscle cell membrane as a basis for the reduction in e.j.p. amplitude; in the present experiments, the apparent input resistance of the cells was not changed by normorphine. The observations that the noradrenaline-induced membrane depolarization and the noradrenaline-induced contraction of the isolated vas deferens (Henderson, 1974) are unaffected by normorphine make a post-junctional action of the narcotics even less likely.

The present experiments support the third site of action; namely that narcotic agonists act directly upon the noradrenaline release mechanism. This interpretation is lent weight by the observations that the noradrenaline output following repetitive nerve stimulation is markedly reduced by narcotic agonists irrespective of whether the noradrenaline output is determined by bioassay (Henderson *et al.*, 1972), or by fluorimetric (Henderson, 1974) or radiochemical (Hughes *et al.*, 1975) techniques. The inhibition of noradrenaline output is stereospecific, reversed by naloxone and occurs over the same concentration range as the inhibition of the e.j.p. Jenkins, Marshall & Nasmyth (1975) reported to a recent meeting of the British Pharmacological Society that using a bioassay technique they were unable to observe a depression of the noradrenaline output from the mouse vas deferens by morphine. At present, it is difficult to explain the difference between their findings and the well-documented evidence of the other authors.

The findings of a low ED_{50}, the ineffectiveness of the (+)-isomer and the reversibility of naloxone leave little doubt as to the receptor specificity of the action of the narcotic agonists upon transmitter release in the mouse vas deferens. An endogenous substrate for the morphine receptor (enkephalin) has recently been isolated from pig brain and guinea-pig ileum (Hughes, 1975; Hughes, Smith, Morgan & Fothergill, 1975). In the latter tissue, low doses of naloxone increase the output of acetylcholine evoked by nerve stimulation (Waterfield & Kosterlitz, 1975) and this action might be due to antagonism of endogenous enkephalin. The absence of effect of naloxone alone on the e.j.p. is evidence against the view that enkephalin normally plays a role in controlling transmitter release in the mouse vas deferens.

Supported by a grant from the U.S. National Institute on Drug Abuse (DA 00662) to Dr H.W. Kosterlitz. G.H. is an I.C.I. Post-doctoral Research Fellow. Acknowledgement is made of generous gifts of drugs mentioned in the Methods section.

References

AVAKIAN, O.V. & GILLESPIE, J.S. (1968). Uptake of noradrenaline by adrenergic nerves, smooth muscle and connective tissue in isolated perfused arteries and its correlation with vasoconstrictor response. *Br. J. Pharmac. Chemother.*, **32**, 168–184.

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

BENNETT, M.R. (1973b). An electrophysiological analysis of the uptake of noradrenaline at sympathetic nerve terminals. *J. Physiol., Lond.*, **229**, 533–546.

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

CAIRNIE, A.W., KOSTERLITZ, H.W. & TAYLOR, D.W. (1961). Effect of morphine on some sympathetically innervated effectors. *Br. J. Pharmac. Chemother.*, **17**, 539–551.

DAY, M.D. & RAND, M.J. (1963). Evidence for a competitive antagonism of guanethidine by dexamphetamine. *Br. J. Pharmac. Chemother.*, **20**, 17–28.

FURNESS, J.B. (1970). The excitatory input to a single smooth muscle cell. *Eur. J. Physiol.*, **314**, 1–13.

FURNESS, J.B. (1974). Transmission to the longitudinal muscle of the guinea-pig vas deferens: the effect of pretreatment with guanethidine. *Br. J. Pharmac.*, **50**, 63–68.

FURNESS, J.B. & BURNSTOCK, G. (1969). A comparative study of spike potentials in response to nerve stimulation of the vas deferens of the mouse, rat and guinea-pig. *Comp. Biochem. Physiol.*, **31**, 337–345.

FURNESS, J.B., CAMPBELL, G.R., GILLARD, S.M., MALMFORS, T., COBB, J.L.S. & BURNSTOCK, G. (1970). Cellular studies of sympathetic denervation produced by 6-hydroxydopamine in the vas deferens. *J. Pharmac. exp. Ther.*, **174**, 111–122.

FURNESS, J.B. & IWAYAMA, T. (1971). Terminal axons ensheathed in smooth muscle cells of the vas deferens. *Z. Zellforsch.*, **113**, 259–270.

HENDERSON, G. (1974). The effects of morphine on peripheral adrenergic neuro-effector transmission. Ph.D. Thesis, University of Aberdeen.

HENDERSON, G., HUGHES, J. & KOSTERLITZ, H.W. (1972). A new example of a morphine-sensitive neuro-effector 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.

HOLMAN, M.E. (1967). Some electrophysiological aspects of transmission from noradrenergic nerves to smooth muscle. *Circulation Res.*, **21**, Suppl. 3, 71–82.

HUGHES, J. (1975). Isolation of an endogenous compound from the brain with pharmacological properties similar to morphine. *Brain Res.*, **88**, 295–308.

HUGHES, J., KOSTERLITZ, H.W. & LESLIE, F.M. (1975). Effect of morphine on adrenergic transmission in the mouse vas deferens. Assessment of agonist and antagonist potencies of narcotic analgesics. *Br. J. Pharmac.*, **53**, 371–381.

HUGHES, J., SMITH, T., MORGAN, B. & FOTHERGILL, L. (1975). Purification and properties of enkephalin – the possible endogenous ligand for the morphine receptor. *Life Sci.*, **16**, 1753–1758.

JENKINS, D.A., MARSHALL, I. & NASMYTH, P.A. (1975). Is morphine inhibition of the twitch response of the mouse vas deferens mediated via noradrenaline? *Br. J. Pharmac.*, **55**, 267–268P.

JONES, M.E.L. & SPRIGGS, T.L.B. (1975). Noradrenaline and motor transmission in the vas deferens of the mouse. *Br. J. Pharmac.*, **53**, 323–331.

KOSTERLITZ, H.W., LESLIE, F.M. & WATERFIELD, A.A. (1975). Rates of onset and offset of action of narcotic analgesics in isolated preparations. *Eur. J. Pharmac.*, **32**, 10–16.

KOSTERLITZ, H.W. & WALLIS, D.I. (1964). The action of morphine-like drugs on impulse transmission in mammalian nerve fibres. *Br. J. Pharmac. Chemother.*, **22**, 499–510.

LANE, B.P. & RHODIN, J.A.G. (1964). Cellular interrelationships and electrical activity in two types of smooth muscle. *J. Ultrastruct.*, **10**, 470–488.

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

NISHI, S. & NORTH, R.A. (1973). Intracellular recording from the myenteric plexus of the guinea-pig ileum. *J. Physiol., Lond.*, **231**, 471–491.

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.

RITCHIE, J.M. & ARMETT, C.J. (1963). On the role of acetylcholine in conduction in mammalian non-myelinated nerve fibres. *J. Pharmac. exp. Ther.*, **139**, 201–207.

SJÖSTRAND, N.O. (1965). The adrenergic innervation of the vas deferens and the accessory male genital glands. *Acta physiol. scand.*, **65**, Suppl. 257.

STARKE, K. (1972). Influence of extracellular noradrenaline on the stimulation-evoked secretion of noradrenaline from sympathetic nerves: Evidence for an α-receptor-mediated feed-back inhibition of noradrenaline release. *Naunyn-Schmiedeberg's Arch. Pharmac.*, **275**, 11–23.

WATERFIELD, A.A. & KOSTERLITZ, H.W. (1975). Stereospecific increase by narcotic antagonists of evoked acetylcholine output in guinea-pig ileum. *Life Sci.*, **16**, 1787–1792.

YAMAUCHI, A. & BURNSTOCK, G. (1969). Post-natal development of the innervation of the mouse vas deferens. A fine structural study. *J. Anat.*, **104**, 17–32.

(Received October 1, 1975. Revised November 12, 1975.)