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Opiates and Neuro-Effector Transmission in the Mouse Vas Deferens: Intracellular Recordings of Excitatory Junction Potentials

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OPIATES AND NEURO-EFFECTOR TRANSMISSION IN THE MOUSE VAS DEFERENS: INTRACELLULAR RECORDINGS OF EXCITATORY JUNCTION POTENTIALS

by

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VITA

Lauren Vaile Vitek was born April 26, 1953 in Chicago, Illinois. She attended Trinity High School in River Forest, Illinois and graduated in 1971. In September, 1971, she entered Loyola University and in June, 1975, received the degree of Bachelor of Science. She graduated summa cum laude with an Honors degree in biology. Lauren began her graduate studies in pharmacology at the Loyola University Medical Center in July, 1975. She was a National Science Foundation Graduate Fellow from 1975 to 1978, and was also awarded a Basic Science Fellowship (1978-1979) and a Schmitt Dissertation Fellowship (1979-1980) by Loyola University during the course of her graduate work.

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1. INTRODUCTION

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1.11 The pharmacology of opiates: analgesia

For centuries, mankind has known of the medicinal value and psychological effects of opium, which is a powder obtained from the seed of the poppy plant, Papaver somniferum. The major ingredient of opium which is responsible for its pharmacological effects is the alkaloid morphine.

Morphine and other opiates produce analgesia (Jaffe & Martin, 1975); this action is the basis for the extensive clinical use of these drugs. The analgesia produced by morphine is selective in that it occurs without loss of consciousness and without obtunding other sensory input. There are two aspects to pain: pain as a discriminative sensation, and pain as a subjective experience. The subjective experience of pain encompasses the emotional reactions to the painful sensation, which include anxiety, fear and "suffering." Opiates are especially effective in relieving this subjective aspect of pain. Other pharmacological effects of opiates include respiratory depression, meiosis, decreased intestinal motility and psychological actions such as drowsiness and mental clouding (Jaffe & Martin, 1975).

Animal models are used to assess morphine-induced analgesia. The subjective aspect of pain as "suffering," which is the component of pain most amenable to relief by morphine, cannot be measured in animals

therefore the effect of morphine is tested on the responses of animals to stimuli that are considered noxious, such as radiant heat, irritating chemicals or pinching of the skin. One such method in common use is the tail-flick test in rodents (D'Amour & Smith, 1941). In this procedure the tail of a restrained rat or mouse is placed in a groove, and a high intensity lamp is positioned above the groove. The experimenter measures the time interval between switching on the lamp and the response of the animal to the heat generated by the lamp. This response is a characteristic twitch of the tail. Opiates increase the duration of time before the animal flicks its tail. Although this test is useful for evaluating the antinociceptive activity of drugs, D'Amour & Smith (1941) make no claims that the results can be extrapolated to predict the analgesic activity of drugs against deep-seated, continuous pain such as that involved in carcinoma.

Other procedures for evaluating the antinociceptive activity of opiates include placing a rodent on a hot plate and measuring the time before it licks its paws or jumps (hot-plate test), and tests which involve placing a clamp on the tail of a rodent and evaluating either vocal responses or attempts to bite the clamp.

1.12 The opiate receptor

Opiates produce analgesia by binding to receptors on neurons and thus altering the activity of those neurons. A receptor is a macromolecular entity on the membrane of a cell that binds a given class of structurally similar molecules with high affinity and selectivity; the

interaction between the molecule and the receptor produces a characteristic response (Goldstein, Aranow & Kalman, 1974). Saturable, high affinity binding sites for opiates have been identified in isolated neuronal tissue (Pert & Snyder, 1973a; Simon, Hiller & Edelman, 1973; Terenius, 1973) and have been localized to homogenate fractions containing synaptic membranes (Pert, Snowman & Snyder, 1974). Although direct evidence is lacking that these binding sites are equivalent to the opiate receptors, the two share essential features (Hollt & Wuster, 1973). For example, the affinity of a series of opiate compounds for binding sites parallels their pharmacological potency in a given tissue (central nervous system: Pert & Snyder, 1973b; peripheral nervous system: Creese & Snyder, 1975). Also, opiates bind to these sites with much higher affinity than do compounds without opiate activity. Lastly, both binding and receptor activity are stereospecific; levo-isomers of opiates are more effective than dextro-isomers of the enantiomeric pair.

Opiate binding sites are unevenly distributed in the brain (Hiller, Pearson & Simon, 1973; Kuhar, Pert & Snyder, 1973). Some areas, such as structures associated with the limbic system, exhibit a high amount of binding, whereas others (cerebellum, white matter) have little or no binding.

1.13 Endogenous opioid peptides

It is intuitively unlikely that high affinity, stereospecific binding sites should exist in brain in order to interact with alkaloids

extracted from the seed of the poppy. A search for endogenous agonists for the opiate receptor resulted in the discovery of the enkephalins (Hughes, Smith, Kosterlitz, Fotherfill, Morgan & Morris, 1975). The enkephalins are two structurally related pentapeptides that differ only in their carboxy-terminal amino acid: Tyr-Gly-Gly-Phe-Met for Met^5-enkephalin and Tyr-Gly-Gly-Phe-Leu for Leu^5-enkephalin. Opiate-like substances have also been isolated from extracts of pituitary gland. One of these substances, β-endorphin, is a 31-amino acid peptide with the sequence of Met^5-enkephalin at its carboxy terminus (Cox, Goldstein & Li, 1976). β-endorphin, administered by the intracerebral route (0.5 - 1.0 μg) to a mouse, has potent antinociceptive activity as assessed by the hot-plate and tail-flick methods (Loh, Tseng, Wei & Li, 1976). In contrast, high doses of Met^5-enkephalin (EC_{50} = 75 μg) and Leu^5-enkephalin (EC_{50} = 240 μg) must be administered intracerebroventricularly to produce a short-lasting (2-5 min) antinociception in mice (Buscher, Hill, Romer, Cardinaux, Closse, Hauser & Pless, 1976). This result can be explained by the rapid enzymatic degradation of these peptides. Structural analogs of the enkephalins which are resistant to enzymatic degradation have been synthesized. One such analog, $D\text{-}Ala^2\text{-}Met^5$-enkephalinamide (DAEA), has potent, long-lasting (up to 2 h) antinociceptive activity (tail-flick test) if injected (5 or 10 μg) directly into the periaqueductal region of rat brain (Pert, Pert, Chang & Fong, 1976).

1.2 Effects of opiates on single neurons

1.21 In vivo

Morphine and other opiates alter the firing rate of single neurons as measured by extracellular recordings from intact animals. Several criteria are used to ascertain if a given alteration in neuronal activity induced by application of an opiate drug is mediated by interaction with opiate receptors. The effect should occur with low concentrations of opiate agonists. The effect should be stereospecific: mimicked by (-) isomers but less effectively by (+) isomers of opiate agonists, and blocked by (-) isomers but not (+) isomers of opiate antagonists. Also, the effect should be prevented or reversed by low concentrations of naloxone. The most common stereospecific, naloxone-reversible effect of opiates is inhibition of neuronal firing (Zieglgansberger & Fry, 1978). Such neuronal activity may be spontaneous or induced by iontophoretic application of excitatory substances such as L-glutamate. The activity of single neurons in the frontal regions of the cerebral cortex, the striatum, thalamus, brain stem and dorsal horn of the spinal cord is inhibited by systemic or iontophoretic application of opiates. Two exceptions to this generalization are pyramidal cells of the hippocampus and Renshaw cells of the ventral horn of the spinal cord; opiates predominantly excite these neurons. The correlation between the sensitivity to opiates of neurons in a given brain region and the density of opiate binding sites in that same region is good. For example, morphine inhibits the firing of

neurons in the noradrenergic nucleus locus coeruleus (Korf, Bunney & Aghajanian, 1974), an area that has a high density of opiate binding sites (Pert, Kuhar & Snyder, 1976).

North (1979a) discusses the advantages and limitations of extracellular recordings of neuronal activity in vivo. One advantage is that neurons are observed in their natural environment with relationships and connections to other cells intact. Also, the cells can be anatomically and functionally defined. The methods of applying drugs to neurons create difficulties in interpretation of data from extracellular recordings in vivo. Problems of access and metabolism attend systemic administration of drugs. A substance applied systemically could produce an effect on a given neuron by changing the activity of a separate population of neurons which project to the recording site, or could alter neuronal activity by an effect on respiration, blood pressure or blood flow. Application of a drug by iontophoresis largely eliminates these concerns. But results obtained by iontophoretic application of drugs should be interpreted with caution because the transport number for the drug and the location of the pipette relative to the neuron under study vary. The concentration of drug at the neuronal receptor sites is unknown. A substance applied by iontophoresis could be producing an effect either by a direct action on the neuron under study, or by presynaptic modulation of synaptic input to the neuron. An example of the latter circumstance is the excitation of hippocampal pyramidal cells by morphine; this excitation is actually due to disinhibition in that morphine is depressing the activity of

nearby basket cells which tonically inhibit the pyramidal cells (Zieglgansberger, Siggins, French & Bloom, 1978).

Actions of opiates on neuronal activity in vivo can also be influenced by the method of preparation of the animal and its physiological condition at the time of the recording. Thus morphine is more effective in inhibiting firing of dorsal horn cells in the spinal cord of animals that are spinalized rather than decerebrate; in decerebrate animals the cells are already influenced by a strong descending inhibition (see North, 1979a). Also, the effect of morphine on the activity of neurons in the dorsal raphe area of the periaqueductal gray region depends on the presence or absence of general anaesthesia (Urca & Liebeskind, 1979). The initial activity level of the neurons as influenced by anaesthesia may determine the presence or direction of an effect of morphine on firing rates. There is some evidence that opiates are more likely to inhibit a neuron that is firing at a high frequency (North, 1979a).

1.22 In vitro

Tissues containing neurons or nerve processes which possess opiate receptors can be removed from the animal and maintained in a viable condition for long periods of time. The use of such preparations offers several advantages over the use of in vivo preparations. The ionic and chemical environment of the tissue may be controlled and drugs can be applied in known concentrations by superfusion. The site of action of drugs can be inferred by intracellular recordings from single cells or by measurement of transmitter release from nerve

terminals. The use of anaesthetic drugs is avoided.

Neurons which are sensitive to opiates can be found in various parts of the peripheral nervous system. The myenteric plexus of the guinea-pig ileum is a well-studied example. Morphine inhibits the activity of the ganglion cells of the myenteric plexus (Satoh, Takayanagi & Takagi, 1973; Dingledine & Goldstein, 1976). The activity recorded by the extracellular glass suction electrodes arises from mechanical irritation of the cells by these electrodes (North & Williams, 1977). Enkephalins and β-endorphin also inhibit the firing of these cells (North & Williams, 1976; Williams & North, 1979).

Neurons grown in tissue culture have also been used to study the actions of opiates. Several effects of Leu^5-enkephalin applied by iontophoresis to cultured mouse spinal neurons have been observed during intracellular recordings from these cells (Barker, Gruol, Huang, Neale & Smith, 1978; Barker, Smith & Neale, 1978). Rapid depolarizations (in 10% of cells) and slow hyperpolarizations (in 33% of cells) have been observed upon application of enkephalin to cell somata. Both responses are associated with a change in membrane input resistance. In other cells (12/37 tested), enkephalin directly depresses excitability by elevating the threshold for generation of action potentials by intracellular injection of depolarizing current, an action that is sometimes antagonized by naloxone (3/6 cells tested). The depression of excitability is not associated with changes in resting membrane properties in the majority of cells (8/12). A third action of enkephalin in these cells is modulation of responses to iontophoretic

application of amino acids. For example, glutamate-induced depolarizations are either inhibited (28/32 cells) or not affected (4/32 cells) by Leu^5-enkephalin. The depression of the glutamate response is not accompanied by changes in resting membrane properties, and can be partially prevented by co-iontophoresis of naloxone together with enkephalin (6/10 cells).

Caution should be exercised in evaluating the relevance of these studies to physiological actions of opiates *in vivo*. Cultured neurons grow in monolayers, whereas brain structure is three-dimensional. Definite alterations from the usual neuronal organization and physiology thus occur. However, such cells are useful for studying drug actions on neuronal membrane properties and on monosynaptic transmission.

Maintenance of slices of brain tissue *in vitro* is a difficult technique that offers promise for elucidating the mechanisms of action of opiates on central neurons without the problems associated with *in situ* recording (see above). Intracellular recordings have been made from neurons located in the nucleus locus coeruleus of slices of guinea-pig pons (Pepper & Henderson, 1979). When these cells are superfused with solutions containing normorphine (300 nM - 3 μM), hyperpolarizations associated with a decrease in input membrane resistance are observed (Henderson, personal communication). These hyperpolarizations may underlie the decrease in activity of these neurons when morphine is iontophoretically applied to them *in situ* (Korf *et al.*, 1974).

1.23 Effects of prolonged exposure

Repeated administration of narcotics leads to the development of tolerance to their acute effects. Tolerance is a "state in which repetition of the same dose of a drug has progressively less effect, or in which the dose needs to be increased to obtain the same degree of pharmacological effect as was caused by the original dose" (Isbell & Chrusciel, 1970). Repeated exposure to narcotics also produces dependence. Dependence is a state, "resulting from the interaction between a living organism and a drug" (Isbell & Chrusciel, 1970), characterized by the appearance of an abstinence or withdrawal syndrome when morphine administration is discontinued or when the action of morphine is terminated by a narcotic antagonist such as naloxone (Jaffe, 1975).

1.231 In vivo

Neurons can be exposed to morphine in vivo for long periods of time by treatment of an animal with repeated systemic injections of morphine, a single injection of a sustained release formulation of the drug or by implantation of morphine-containing pellets. After animals are chronically treated with morphine, the drug becomes less effective in depressing spontaneous or evoked firing of single neurons in several brain regions (Satoh, Zieglgansberger & Herz, 1976; Aghajanian, 1978; Dafny, Brown, Burks & Rigor, 1979). These results may indicate that tolerance to the inhibitory action of morphine has developed. Another interpretation is that only the activity of morphine-insensitive neurons is recorded because the activity of morphine-sensitive

cells is suppressed by the continuous presence of the drug *in vivo*. Or the observed tolerance may be a secondary consequence of systemic changes in the levels of circulating hormones (Gibson & Pollock, 1975).

A greater percentage of neurons respond to the iontophoretic application of naloxone with an increase in their firing rate in morphine-pretreated animals as compared to naive animals (Frederickson, Norris & Hewes, 1975; Fry, Zieglgansberger & Herz, 1978). Extracellular recording from single neurons *in vivo* cannot distinguish between at least two mechanisms for this excitatory action of naloxone. The neurons could increase their firing rate in response to naloxone as a result of altered synaptic input such as the enhanced release of an excitatory transmitter or the decreased release of an inhibitory transmitter from nerve terminals. Or the morphine-sensitive neurons themselves could become more excitable. In either case, the effect of naloxone could be simply a reversal of the depression of neuronal activity caused by the continuous presence of morphine, and not a manifestation of precipitated withdrawal. One could argue against this possibility by demonstrating that the neurons in morphine-pretreated animals fire spontaneously at the same frequency as they do in naive animals (Aghajanian, 1978).

1.232 *In vitro*

The cellular adaptation which results in tolerance takes place if neurons are continuously exposed to opiates *in vitro* as well as *in vivo*, as has been demonstrated with the opiate-sensitive neurons of the

myenteric plexus of the guinea-pig ileum. If tissues are incubated for 24 h in Krebs solution containing morphine (1 μM), then the firing rate of single neurons usually is not changed by higher concentrations of morphine (10 or 30 μM) (North & Karras, 1978b). The activity of these neurons is usually depressed by much lower concentrations of opiates (10 nM - 1 μM normorphine: North & Williams, 1977). The tolerance to morphine occurs in these cells even if the tissues are incubated *in vitro* for 24 h with hyoscine and hexamethonium together with morphine; these drugs inhibit synaptic activity in the myenteric plexus. Thus transsynaptic activity is not required for the development of tolerance to opiates at the level of the single neuron.

The addition of naloxone to myenteric neurons which have been chronically exposed to morphine *in vitro* results in a marked increase in their firing rate (North & Karras, 1978b). The firing frequencies seen under these circumstances are never observed in naive tissues. Thus naloxone produces a withdrawal syndrome in these neurons; this phenomenon may be characterized by an enhanced excitability of the cells.

1.24 Cellular sites of action

The action of opiate drugs on single neurons has thus been examined in both *in vivo* and *in vitro* preparations. This work has revealed that opiates alter neuronal activity by three mechanisms (see North, 1979b). First, opiates can have a direct postsynaptic effect on a neuron, modifying its membrane properties so as to change its excita-

bility. A second action, also postsynaptic, is neuromodulation, or modification of the response of the neuron to transmitters. This latter effect occurs without changes in resting membrane properties; inhibition of transmitter-induced activation or inactivation of ionic currents could be involved. A third mechanism of action of opiates is presynaptic inhibition, or alteration of evoked transmitter release from axon terminals.

A presynaptic location of opiate binding sites in some brain regions is suggested by results from binding studies and autoradiography. For example, a significant reduction in the binding of [^{3}H]-naloxone in the upper dorsal horn of the spinal cord is the result of cutting the dorsal roots (Lamotte, Pert & Snyder, 1976). This suggests that the binding sites are associated with the primary afferent terminals. The results of deafferentation of the vagus and accessory optic systems in the rat also point to a localization of [^{3}H]-diprenorphine binding sites to the small diameter unmyelinated sensory fibers of these tracts (Atweh, Murrin & Kuhar, 1978). However, the possibility that these binding sites are postsynaptic in location and are reduced in number by deafferentation because of transsynaptic degeneration cannot be dismissed. Definitive localization of opiate binding sites to either nerve terminals or nerve cell bodies in brain awaits refinements in anatomical techniques such that autoradiography can be applied to electron micrographs of nerve tissue.

1.3 Effects of opiates on transmitter release

Opiates inhibit transmitter release from neurons in several parts of the peripheral autonomic nervous system. Two methods are used to estimate transmitter release from autonomic nerve terminals (see Starke, 1977). One method is to measure the mechanical response of the postsynaptic effector cell, the smooth muscle. The general procedure is to suspend the tissue in an organ bath through which heated Krebs solution is superfused. One end of the tissue is fixed to a rod or loop at the bottom of the bath, and the other end is attached to a force or a linear-motion transducer. Field stimulation of the intramural nerves is applied by two wire or ring electrodes situated at the top and bottom of the bath. The transmitter released from the nerve terminals in response to electrical stimuli produces either a change in length or a change in tension of the organ. Any change in this mechanical response that occurs upon addition of a drug to the bathing medium may reflect a modification of transmitter release, but it may also result from postsynaptic effects of the drug. Such effects include changes in sensitivity to transmitter, or changes in the efficiency of the excitation-contraction coupling process of the muscle. Postsynaptic effects of a drug are less likely if the drug in question does not change the response of the organ to direct application of transmitter substance.

Another method of measuring nerve-evoked transmitter release is to measure the amount of transmitter overflowing into the superfusing Krebs solution in response to stimulation of nerves either with elec-

trical stimulus pulses or with depolarizing solutions of high potassium ion concentration. The overflow evoked by such stimuli is distinguished from the "spontaneous" overflow. Overflow represents the difference between the amount of transmitter released from nerves and the amount of transmitter taken back up into cells after its release (such reuptake may be neuronal or extraneuronal). Overflow of transmitter may be estimated chemically or by bioassay. Another technique for estimating overflow is to preload the tissue with radiolabelled transmitter and to count the radioactivity released into the superfusion fluid. It is important to exclude an action of a drug on transmitter reuptake before concluding that the drug alters transmitter release as estimated by the overflow method.

1.31 Guinea-pig ileum

Morphine inhibits the contractile response of the longitudinal muscle and also the release of acetylcholine evoked by coaxial stimulation of the guinea-pig ileum with electrical pulses of supramaximal strength (Paton, 1957). The action of morphine is localized to neurons of the myenteric plexus (see above) because acetylcholine release from tissues which contain only longitudinal muscle and the myenteric plexus is also sensitive to morphine (Paton & Aboo Zar, 1968).

These actions of opiates on myenteric plexus-longitudinal muscle strips of guinea-pig ileum are stereospecific and prevented or reversed by naloxone (see Kosterlitz & Waterfield, 1975). Enkephalins also inhibit cholinergic neurotransmission in the guinea-pig ileum (Hughes

et al., 1975b; Waterfield, Smokcum, Hughes, Kosterlitz & Henderson, 1977).

1.32 Mouse vas deferens

A mouse isolated vas deferens which has been stripped of its connective tissue sheath consists mainly of smooth muscle cells and the axons and nerve terminals which innervate them. The noradrenaline which is released from these nerve terminals in response to electrical field stimulation is most likely the transmitter which produces the contractile response of the muscle (see below). Muscle contractions elicited by supramaximal electrical stimulation at a frequency of 0.1 Hz are inhibited by morphine (70 nM - 2.3 μM) (Henderson, Hughes & Kosterlitz, 1972). The inhibitory effect of morphine and other opiates on neurotransmission in this tissue occurs with low concentrations of drug, is stereospecific (the (-) isomers but not the (+) isomers of opiate agonists are active), and can be prevented or reversed by naloxone (Hughes, Kosterlitz & Leslie, 1975). Fulfillment of these criteria indicate that this action in the mouse vas deferens is mediated by specific opiate receptors similar to those found in brain tissue. The relative agonist potencies of a series of opiates in causing an inhibition of neuro-effector transmission in the mouse vas deferens correlate with the potencies of these same compounds in producing analgesia in man (Hughes et al., 1975a). The electrically-evoked contractions of the muscle are also inhibited by Met^5-enkephalin, Leu^5-enkephalin and β-endorphin (Hughes et al., 1975b; Waterfield

et al., 1977). The inhibitory potencies of normorphine (Hughes et al., 1975a) and Met^5-enkephalin but not Leu^5-enkephalin (Hart, Kitchen & Waddell) are increased by reducing the current strength used to elicit the muscle contraction.

The spontaneous overflow of labelled catecholamines (after incubation of the vas deferens in solutions containing either [^{3}H]-tyrosine or [^{3}H]-noradrenaline) is not affected by morphine (Hughes et al., 1975a). Morphine (Henderson et al., 1972) and enkephalin (Waterfield et al., 1977) do inhibit the overflow of noradrenaline evoked by electrical stimulation of the intramural nerves. The concentrations of opiates which inhibit noradrenaline overflow are similar to the concentrations which inhibit the muscle contractions. The effect of opiates on transmitter release is dependent on the frequency of nerve stimulation used to evoke the release: morphine (1 μM) depresses the fractional noradrenaline output per pulse at a stimulation frequency of 1.5 Hz but not at 15 Hz (Henderson & Hughes, 1976).

Opiates and opioid peptides therefore inhibit nerve-evoked muscle contractions and transmitter release in the mouse vas deferens. The response of the smooth muscle to exogenous noradrenaline is, however, not altered by these substances (Henderson & Hughes, 1976). These results indicate that the opiate receptors in this tissue are located presynaptically on the adrenergic nerves and not postsynaptically on the smooth muscle cells.

The potency of morphine and opioid peptides in inhibiting neurotransmission in the vas deferens is dependent on the strain of mouse

used for the experiment. For example, morphine (1 μM) inhibits contractions and noradrenaline release from vasa removed from T.O. mice, but not from vasa of C57/BL mice (Henderson & Hughes, 1976). On the other hand, Met^5-enkephalin and Leu^5-enkephalin are approximately equipotent in vasa from both of these strains (Waterfield, Lord, Hughes & Kosterlitz, 1978). In a study of seven different strains of mice, Szerb & Vohra (1979) found no correlation between sensitivity to normorphine and sensitivity to Met^5-enkephalin. The contractions of the isolated vas deferens of the rat are not inhibited by either morphine or the enkephalins except in very high concentrations. This tissue is, however, sensitive to β-endorphin, with an EC_{50} of 130 nM for inhibition of the muscle contractions (Lemaire, Magnan & Regoli, 1978). This effect of β-endorphin on the rat vas deferens is reversed by naloxone.

The differential sensitivities to opiates and opioid peptides of vasa from different species and from different strains of the same species suggests a heterogeneity of receptor populations. The concept of multiple types of opiate receptor originated with Martin and his colleagues (Martin, Eades, Thompson, Hupplier & Gilbert, 1976). Based on the behavioral and electrophysiological effects of various morphine derivatives which they observed in the chronic spinal dog preparation, these investigators postulated the existence of three classes of opiate receptor. In their scheme, typical morphine-like compounds interact with the μ receptor. Another system of classification of opiate receptors was proposed by Lord, Waterfield, Hughes & Kosterlitz (1977) based on the following evidence. First is the comparison of the pharmacolog-

ical potencies of opiates and opioid peptides in the guinea-pig ileum and mouse vas deferens bioassay systems. The mouse vas deferens is less sensitive to morphine than is the guinea-pig ileum; the opposite is true for the potencies of the enkephalins. β-endorphin is equally effective in these two systems. Secondly, measurement of the potency of opiates and opioid peptides in inhibiting the binding of labelled ligands to homogenates of guinea-pig brain reveals the presence of two distinct binding sites. Some opiates are more potent inhibitors of the binding of [^{3}H]-naloxone whereas others preferentially inhibit the binding of [^{3}H]-Leu^5-enkephalin. Lord et al. (1977) propose that the sites which preferentially bind naloxone and morphine are μ receptors and the sites which preferentially bind Leu^5-enkephalin are δ receptors. In the guinea-pig ileum, enkephalins interact mainly with the μ receptor, whereas in the mouse vas deferens they probably interact with the putative δ receptor. To account for the selective sensitivity of the rat vas deferens to β-endorphin, a receptor which preferentially binds this substance (the ε receptor) has been postulated (Wuster, Schulz & Herz, 1979).

The vasa deferentia of mice which are sensitive to enkephalins but not morphine (C57/BL strain) presumably possess many δ receptors but few μ receptors. Vasa of mice of the T.O. strain, which are sensitive to normorphine, presumably possess comparatively more functional μ receptors. Apparently the ratio of μ- and δ-receptors in the mouse vas deferens is variable and genetically determined (Waterfield et al., 1978). Recently specific binding sites for [^{3}H]-Met^5-enkephalin, [^{3}H]-

dihydromorphine and [^{3}H]-naltrexone have been demonstrated for the first time in the vas deferens of the T.O. strain of mouse (Leslie & Kosterlitz, 1979). Such binding can only be observed if tissue homogenates are incubated with labelled ligand at 0° C, and not at 37° C. The affinity of naltrexone for opiate binding sites is lower in the mouse vas deferens than in the guinea-pig ileum. Because the opiate antagonists naltrexone and naloxone preferentially bind to the μ receptor and bind less potently to the δ receptor, this result is compatible with the view that the mouse vas deferens has relatively more δ receptors than the guinea-pig ileum. More accurate quantitation of μ and δ binding sites awaits refinements of experimental technique that will improve the ratio of specific to non-specific binding of opiates that can be measured in the mouse vas deferens.

1.33 Other neuro-effector junctions

Opiates and/or opioid peptides inhibit transmitter release at several other autonomic neuro-effector junctions. Examples of such systems include adrenergic neurotransmission in the cat nictitating membrane (Trendelenburg, 1957), cholinergic neurotransmission in the sinoatrial node-right atrium preparation of the rabbit (Kennedy & West, 1967) and non-adrenergic inhibitory transmission in the guinea-pig taenia coli (Shimo & Ishii, 1978). It is not known why opiates inhibit neuro-effector transmission in some, but not all, autonomic end-organs (see Henderson, Hughes & Kosterlitz, 1978), although a selective distribution of opiate receptors is a likely possibility.

1.34 Slices of brain

Opiates can modify the evoked release of putative neurotransmitter substances from brain slices. In slices of rat cerebral cortex preincubated with [^{3}H]-noradrenaline, the spontaneous overflow of tritium is not altered by morphine. Morphine (100 nM - 10 μM) did decrease the augmentation in the overflow of tritium during electrical field stimulation at frequencies of 0.3-3 Hz (Montel, Starke & Weber, 1974). Met^{5}-enkephalin (100 nM - 10 μM) had the same effect (Taube, Borowski, Endo & Starke, 1976). In these experiments, the depressions of stimulus-evoked overflow by morphine and enkephalin were both blocked by naloxone. Opioids reduce the release of dopamine from striatal slices (Loh, Brase, Sampath-Khanna, Mar, Way & Li, 1976) and the release of substance P from the trigeminal nucleus (Jessel & Iversen, 1977), whilst increasing the release of purines from cortical tissue (Fredholm & Vernet, 1978). In these latter three studies, overflow of labelled putative transmitter was induced by superfusion with high potassium solutions.

In order to evoke the release of a measurable amount of radiolabelled substance from brain slices or peripheral neuro-effector junctions, high potassium solutions or a high frequency of electrical stimulation are usually employed. In the former instance, such concentrations of potassium far exceed any that would be encountered physiologically in the extracellular fluid. Concerning the latter circumstance, morphine is a more effective inhibitor of transmitter release at low rather than at high frequencies of nerve stimulation (Henderson

& Hughes, 1976). Measurements of overflow of labelled substances are thus not the best experiments for evaluating the actions of opiates on transmitter release. Transmitter release evoked by single pulse stimulation of nerves is most sensitive to the inhibitory action of opiates.

1.35 Effects of prolonged exposure

The nerve-mediated contractions of the isolated myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum are less sensitive to inhibition by morphine if the tissue is prepared from a morphine-pretreated animal (Goldstein & Schulz, 1973; Ward & Takemori, 1976; Johnson, Westfall, Howard & Fleming, 1978). The degree of tolerance, as assessed by the ratio of the EC_{50} value for morphine in preparations from morphine-pretreated vs. naive animals, ranges from 3 up to 18-fold. This variation results from differences in experimental protocols among investigators, such as the schedule of administration of morphine and the time elapsed between removing tissue from the animal and testing the degree of tolerance (North & Karras, 1978a). Most studies of the induction of tolerance in the guinea-pig ileum involve placing the tissue in drug-free Krebs solution after its removal from the animal. This procedure results in withdrawal of the tissue from the morphine to which it has been continuously exposed in vivo. If strips of myenteric plexus-longitudinal muscle from morphine-pretreated guinea-pigs are maintained in Krebs solution containing normorphine and then challenged with naloxone, the muscle exhibits a contracture (Schulz & Herz, 1976). This response to naloxone rapidly disappears if the tissue is washed in morphine-free Krebs solution.

The contracture results from an action of naloxone on neurons in the myenteric plexus and not from a direct action on the smooth muscle because it is blocked by tetrodotoxin and atropine.

Tolerance in the guinea-pig ileum is not accompanied by a change in the sensitivity of the smooth muscle to acetylcholine (Goldstein & Schulz, 1973; Johnson et al., 1978). Therefore tolerance must develop to the effects of opiates on the cholinergic neurons of the myenteric plexus. Opiates have two actions on these cells: inhibition of their firing rate and inhibition of the amount of acetylcholine they release (see above). The tolerance to opiates observed with the contractions of the muscle may be a consequence of tolerance to either or both of these effects. The release of acetylcholine depends both on the number of neurons excited by a given stimulus and on the amount of acetylcholine secreted by the nerve terminals of the activated neurons.

For neurons with opiate receptors on their nerve terminals, two mechanisms can explain the development of tolerance to the action of morphine. The nerve terminals themselves may adapt to the continuous presence of opiates so that, during withdrawal, transmitter release in response to nerve impulses is greatly enhanced as a result of this compensation (see Montel, Starke & Taube, 1975). On the other hand, the postsynaptic receptors may become supersensitive, allowing synaptic transmission to return to its normal level ("tolerance") in the face of a continued depression of transmission by morphine. Upon withdrawal of morphine, transmitter release would return to normal, but the postsynaptic supersensitivity would result in a neuronal abstinence syndrome (see Llorens, Martres, Baudry & Schwartz, 1978).

1.4 Role of cAMP in the actions of opiates on neurons

Adenosine 3',5'-cyclic monophosphoric acid (cAMP) is formed from ATP by the membrane-bound enzyme adenylate cyclase. Cyclic AMP is believed to be an intracellular "second messenger" that plays a crucial role in the recognition and transduction of signals generated by the binding of hormones and transmitters to the external surfaces of excitable membranes (see Nathanson, 1977). The active substance cAMP is hydrolyzed to the inactive 5'-AMP by cAMP-specific phosphodiesterases.

Three pieces of evidence implicate cAMP in the actions of opiates. Firstly, mice that have received injections of cAMP are less sensitive to the antinociceptive effect of morphine (Ho, Loh & Way, 1973). Secondly, opiates inhibit the prostaglandin-stimulated formation of cAMP in homogenates of rat brain (Collier & Roy, 1974a). Finally, opiates inhibit the enzyme adenylate cyclase in neuroblastoma x glioma hybrid tumor cells (Sharma, Nirenberg & Klee, 1975). These results led to the proposal that the effects of opiates, such as analgesia, are mediated by an inhibition of the adenylate cyclase system in opiate-sensitive neurons (Collier & Roy, 1974b). The relationship between cAMP and opiates in both *in vivo* and *in vitro* systems has therefore been extensively studied.

1.41 Effects of adenosine cyclic nucleotides and phosphodiesterase inhibitors on the actions of opiates

Ho *et al.* (1973) assessed the effects of cyclic nucleotides on the antinociceptive activity of morphine by using the tail-flick test

in mice. This group found that the intracerebral administration of cAMP (28 μg/mouse) 1 or 35 h before a test dose of morphine produces a significant decrease in the effectiveness of morphine in inhibiting the tail-flick response. Furthermore, the intravenous administration of cAMP or its dibutyryl derivative, dbcAMP (10 mg/kg), 6 h before the morphine injection also lessens the effectiveness of morphine. The cyclic nucleotides are effective even if these substances are administered 24 h before the tail-flick experiment.

It is difficult to envision how a small quantity of a rapidly degraded substance such as cAMP, administered peripherally, can alter the action of a drug which acts on centrally located neurons. Morphine and cAMP could be acting at two completely different sites, for example two anatomically distinct populations of neurons, in altering the response of an animal to noxious stimuli. It is impossible to detect if intracerebral or intravenous administration of cAMP is elevating intracellular levels of cAMP in the neurons involved in the antinociceptive activity of morphine. The site of action of cAMP could even be peripheral, affecting either the afferent (stimulus perception) or the efferent (ability of animal to effect movement) limbs of the nociceptive reflex. One substance can also alter the action of another by changing the metabolism or distribution of the latter. The effectiveness of the peripheral administration of cAMP addresses these possibilities.

An interaction of cAMP and morphine at the level of the single neuron has been tested in vivo. Neurons in lamina V of the spinal cord (spinalized cats) increase their firing rate in response to noxious

cutaneous stimuli (heat) applied to their peripheral fields. The activity induced in these cells by noxious stimuli and recorded by extracellular electrodes is reduced by iontophoretic application of morphine (30-250 nA of current, for 4-35 min) into the substantia gelatinosa (Duggan, Hall & Headley, 1977). This effect of morphine is not changed by the simultaneous iontophoretic application of both monobutyryl cAMP and a phosphodiesterase inhibitor (at currents of 150 nA) through the same multi-barrelled pipette (Duggan & Griersmith, 1979). By themselves these compounds do not alter the responses of the neurons to noxious input if administered into the substantia gelatinosa using similar injection currents in other experiments. The administration of phosphodiesterase inhibitors in this and in other experiments is intended to increase the intracellular concentrations of cAMP by preventing metabolism of this substance.

Can pretreatment with cAMP or phosphodiesterase inhibitors block the effects of morphine on isolated tissues? The isolated guinea-pig ileum myenteric plexus-longitudinal muscle preparation has been used to address this question. The action of morphine on this tissue can be measured in three ways: inhibition of the nerve-evoked twitch of the muscle, inhibition of evoked acetylcholine release and inhibition of the firing of single myenteric neurons. If the twitch response of the muscle is studied, phosphodiesterase inhibitors interact with morphine in various ways. Morphine is less potent in producing an inhibition of the muscle contraction in the presence of the phosphodiesterase inhibitor theophylline (500 μM) (Sawynok & Jhamandas, 1976), although other investigators report no effect on the action of morphine of a

higher concentration (3 mM) of theophylline (Gintzler & Musacchio, 1975). Morphine is more effective in the presence of the phosphodiesterase inhibitors RO 20-1724 (100 μM) and dipyridamole (10 nM), which themselves also inhibit the twitch. But the inhibitions of the contractile responses by norepinephrine and adenosine are also enhanced in the presence of these compounds. SQ 20,006 (100 μM), on the other hand, has no effect on the action of these inhibitory compounds, or on the action of morphine (Gintzler & Musacchio, 1975). Thus not all phosphodiesterase inhibitors are active. In these studies an action of the phosphodiesterase inhibitor at a postsynaptic site, for example on the response of the muscle to transmitter or the efficiency of the contractile mechanism, cannot be ruled out. This objection is overcome by studying the evoked release of acetylcholine from the myenteric plexus.

Sawynok & Jhamandas (1979) measured the release of acetylcholine (by radioenzymatic assay) from longitudinal muscle-myenteric plexus strips. Release was evoked by electrical stimulus pulses (36 V, 0.5 ms) at a frequency of 0.1 Hz, for periods of 10 min. Morphine (300 nM and 3 μM) inhibited this release. The inhibition of release by morphine was less in tissues perfused with the methylxanthines theophylline (1 mM), caffeine (3 mM) and 1-methyl-3-isobutylxanthine (IBMX) (1 mM) but not in tissues exposed to the non-xanthine phosphodiesterase inhibitors RO 20-1724 (10 μM) or SQ 20,009 (300 μM). The authors attribute the effect of the methylxanthines to an alteration of calcium fluxes in the nerve terminal, and not to an elevation of cAMP levels by these substances, because elevated calcium ion concentrations and a

calcium ionophore also cause an apparent antagonism of the inhibitory action of morphine. Several problems with this release study should be noted. Some of the phosphodiesterase inhibitors themselves modify the release of acetylcholine. Under control conditions, about 20% of the measured release is spontaneous (not blocked by tetrodotoxin). If these compounds change the ratio of spontaneous to evoked release, they could alter the apparent inhibitory action of morphine, as opiates only depress evoked transmitter release. Also, two different concentrations of morphine (300 nM and 3 μM) have equal potency in the presence of the methylxanthines. Lastly, the results are analyzed by the use of multiple t tests instead of by an analysis of variance. This treatment would tend to increase the likelihood of the appearance of statistical significance.

A study has been made of the effect of cyclic nucleotides and a phosphodiesterase inhibitor on the inhibition by morphine of the firing rate of single neurons in the myenteric plexus. In tissues in which cAMP levels would be expected to be elevated (by the administration of cAMP or dbcAMP, by exposure to prostaglandin E_2 and during phosphodiesterase inhibition by IBMX), the action of morphine on these cells was not changed (Karras & North, 1979). This study, which provides the most direct measurement in this preparation of the response of a neuron to occupation of its opiate receptors by morphine, fails to support the hypothesis that the acute action of morphine in altering neuronal firing rates is mediated by a decrease in cAMP levels. On the other hand, the possibility that the chronic actions of opiates in causing

tolerance and dependence could be mediated by a change in the neuronal cAMP apparatus was suggested by these authors.

1.42 The quasi-morphine withdrawal syndrome

The acute systemic administration of phosphodiesterase inhibitors such as theophylline or IBMX results in behaviors that closely resemble those seen in morphine-dependent animals that are undergoing withdrawal, a "quasi-morphine withdrawal syndrome" (QMWS) (Collier, Francis, Henderson & Schneider, 1974; Francis, Roy & Collier, 1975). A subsequent injection of naloxone further intensifies these behavioral effects of the phosphodiesterase inhibitors (Francis et al., 1975; Francis, Cuthbert, Dineen, Schneider & Collier, 1976). Furthermore, the administration of methylxanthine phosphodiesterase inhibitors increases the intensity of the symptoms of true naloxone-precipitated withdrawal from morphine (Collier & Francis, 1975). Methylxanthines have other actions in addition to inhibition of the enzyme that metabolizes cAMP (see Sawynok & Jhamandas, 1976 & 1979). But the rank order of potency of five phosphodiesterase inhibitors, both xanthines and non-xanthines, in producing a QMWS correlates with their potency for the inhibition of cAMP phosphodiesterase (Francis, Cuthbert, Saeed, Butt & Collier, 1978). The correlation is not perfect, however, as papaverine and SQ 20,006 inhibit cAMP phosphodiesterase but do not induce QMW (Collier, Butt, Francis, Roy & Schneider, 1976).

The QMWS is suppressed by opiate drugs (Collier et al., 1974; Collier et al., 1976), and their relative potencies in doing so correspond to their potencies for eliciting antinociception, for

binding to sites in brain homogenates and for inhibiting neurally-evoked contractions of the guinea-pig ileum (Francis et al., 1978). But pentobarbitone and haloperidol, which are central nervous system (CNS) depressants, also suppress quasi-withdrawal behavior (Francis et al., 1978).

Collier and his colleagues have pointed out the close similarities between the behavior of animals during true morphine abstinence and during quasi-morphine "abstinence" induced by administration of phosphodiesterase inhibitors. These investigators postulate that the QMWS can be attributed to inhibition of brain phosphodiesterase and the consequent increase in cAMP levels in neurons. Therefore they conclude that the opiate withdrawal syndrome elicited by the administration of naloxone to morphine-dependent animals is associated with an increase in levels of cAMP in opiate-sensitive neurons.

Are there alternative explanations for the behavioral similarities between true morphine abstinence and quasi-morphine "abstinence"? If opiate withdrawal involves enhanced excitability of opiate-sensitive neurons, as has been suggested by North & Karras (1978a), then any compound that non-specifically increases the excitability of a similar population of neurons would produce a similar pattern of behaviors. The cell populations affected by phosphodiesterase inhibitors and opiates could overlap but need not be exactly the same, as a given behavior can be elicited by more than one neuronal pathway. Naloxone would further intensify the excitability of these neurons by removing any suppression caused by the presence of endogenous opiates. And any CNS depressant, opiate or otherwise, would suppress such a general

excitatory phenomenon. Once again a major consideration is the site of action - do phosphodiesterase inhibitors limit their actions to the pool of neurons that are responsive to opiates? This question can best be answered by observations on single neurons or simple neuronal systems.

1.43 Effects of opiates on cAMP metabolism

The acute or chronic administration of morphine to an animal has variable effects on cAMP levels or adenylate cyclase activity measured in brain tissue that has been subsequently isolated from the animal (Hamprecht, 1978). The interpretation of these in vivo studies is difficult as biochemical measurements in the relevant populations of morphine-sensitive neurons cannot be made. The majority of activity measured is derived from opiate-insensitive neurons or from non-neuronal brain components such as glia or blood vessels. For example, chronic morphine treatment will elevate the activity of protein kinase, an enzyme that is regulated by cAMP, even in oligodendroglia (Oguri, Lee & Loh, 1976).

The same problem may apply in evaluating the observed alterations in cyclic nucleotide levels in brain homogenates or slices that have been incubated with opiates. If Collier's hypothesis of the mechanism of action of opiates (see above) is correct, then opiates should lower cAMP concentrations in these systems. Havemann & Kuschinsky (1978) measured cAMP levels in striatal tissue of rats in vitro, in the absence and presence of opiates. Slices of striata incubated with morphine (500 nM - 50 μM) accumulate less cAMP than slices incubated

without drugs. This effect is not observed in slices incubated with both morphine (50 μM) and naloxone (50 μM). The decrease in the accumulation of cAMP after incubation with morphine (500 nM) (33%) is approximately the same as the decrease observed after incubation with morphine (50 μM) (26%). A change in the cAMP concentration in slices incubated with opiates could result from drug-induced alterations in the activity of either adenylate cyclase or cAMP phosphodiesterase. Because incubation with morphine (20-50 μM) also lowers (by about 20%) cAMP levels in cell-free homogenates of striata in the presence of IBMX (1 mM), Havemann & Kuschinsky believe the drug is altering cAMP synthesis.

These results in striatal tissues agree with the prediction based on Collier's hypothesis, as do the results of other experiments with homogenates of rat cortex and hypothalamus (Tsang, Tan, Henry & Lal, 1978). Other experiments with brain homogenates, slices or mince systems have not yielded results in agreement with this prediction (for example, Tell, Pasternak & Cuatrecasas, 1975; Katz & Catravas, 1977; Tang & Cotzias, 1978). Such discrepancies in results from brain homogenates and slices may reflect species or regional differences. But more importantly, these biochemical measurements cannot directly relate any opiate-induced changes in neuronal activity with changes in cyclic nucleotide levels in those same neurons. Morphine may alter cAMP levels in brain tissue because of biochemical events subsequent to alterations in resting or active membrane properties, or even by a non-specific action on metabolism. For example, morphine (20 μM - 2 mM) alters cAMP formation in mouse prostate glands in a dose-related manner

(Thomas, Dombrowsky, Mawhinney & Sharifi Hossaini, 1975), presumably by an action unrelated to neuronal opiate receptors.

1.44 Experiments with neuroblastoma x glioma cells

Cells of the neuroblastoma x glioma line NG108-15 exhibit the properties of neurons and possess opiate receptors; opiates bind to these sites with the same relative affinities as they bind to rat brain homogenates (Klee & Nirenberg, 1974). Morphine decreases both basal and prostaglandin-elevated cAMP levels in intact hybrid cells and inhibits adenylate cyclase activity in cell homogenates (Sharma et al., 1975b; Traber, Fischer, Latzin & Hamprecht, 1975). These actions are blocked by concurrent incubation with naloxone. Furthermore, long-term incubation of these cells in morphine (Sharma, Klee & Nirenberg, 1975), enkephalin (Lampert, Nirenberg & Klee, 1976) or endorphin extracted from the pituitary (Goldstein, Cox, Klee & Nirenberg, 1977) causes a compensatory increase in adenylate cyclase activity which tends to overcome the initial inhibition. After this compensation has occurred, removal of the opiate produces a large increase in the activity of the cAMP system to a level greater than before drug incubation. These phenomena have been described as the molecular basis for tolerance and dependence to opiates (Sharma et al., 1975a). Sodium fluoride, which activates basal adenylate cyclase activity and uncouples the enzyme from the hormone-receptor complex, abolishes the difference in enzyme activities between control cells and cells incubated with morphine. Opiates appear to increase the abundance of a high activity form of the

enzyme (Sharma, Klee & Nirenberg, 1977).

The effect of opiates on the biochemistry of the NG108-15 cells is not specific, as stimulation of both muscarinic cholinergic (Traber, Fischer, Buchen & Hamprecht, 1975) and α-adrenergic (Traber, Reiser, Fischer & Hamprecht, 1975) receptors inhibits the activity of adenylate cyclase. Long-term incubation of the cells with carbachol or norepinephrine produces a compensatory increase in adenylate cyclase activity (Klein, Nathanson & Nirenberg, 1976; Sabol & Nirenberg, 1977), just as is seen with incubation in opiates.

Do the results obtained from the neuroblastoma x glioma hybrid cells pertain to the action of opiates in other neuronal systems? The NG108-15 cells are tumor cells that differ in unknown ways from differentiated neurons. They lack the synaptic connections of neurons in their natural environment. The cyclic nucleotide system in these cells responds in general to an inhibition of its activity with a compensatory hypertrophy, whether the initial inhibition is produced by an opiate, adrenergic or cholinergic agonist (see above). Thus the specificity of the response is questionable. Nonetheless, it represents a biochemical correlate, though not necessarily the specific mechanism, of opiate tolerance and dependence in these cells. Some form of physiological compensation must be occurring in neurons in response to chronic treatment with opiates. The involvement of the cyclic nucleotide system in this compensation needs to be studied in different types of neurons at central and peripheral sites.

1.5 Role of the calcium ion in the actions of opiates on neurons

The calcium ion is a key regulator of major functions of neurons, including cellular metabolism, membrane properties and transmitter release. An essential role for the calcium ion as an intermediary of the actions of opiates on neurons has been hypothesized (Kaneto, 1971; Sanghvi & Gershon, 1977; Ross, 1978). The effects of altered calcium ion concentration on actions of opiates and the effects of opiates on calcium levels and metabolism in brain tissue form the basis of this hypothesis.

1.51 The effect of calcium ion on the actions of opiates

The potency of morphine in producing antinociception is lower in mice that have received an injection of calcium ion by the intraventricular route. On the other hand, morphine is more effective in mice that have been injected with the calcium chelator EGTA (Kakunaga, Kaneto & Hano, 1966; Harris, Loh & Way, 1975). These results have been interpreted to suggest that the antinociceptive action of morphine is mediated by a decrease in the availability of calcium at some neuronal site, and that this deficiency can be overcome by administering high doses of calcium.

Are there alternative explanations for the apparent antagonism by calcium of the antinociceptive activity of morphine? Calcium administered into the ventricular system of the brain produces sleep and anaesthesia in some species; these behavioral effects could mask the action of morphine (see Kaneto, 1971). Also, a physiological antagon-

ism of the action of morphine by calcium may occur at the same or different anatomical sites. Increased concentrations of extracellular calcium ion have two important actions on neuronal activity: stabilization of the membrane (Koketsu, 1969) and enhancement of transmitter release (Rubin, 1970). Either or both could antagonize the neuronal actions of morphine without involvement of a calcium link in those actions.

In in vitro preparations containing neurons that bear opiate receptors, opiates can inhibit nerve-evoked muscle contractions, depress transmitter release or alter the electrophysiological properties of neurons. The isolated guinea-pig ileum is an example of a tissue in which all three of these actions of opiates are readily demonstrable. The effect of changing the external concentration of calcium ion on each of these actions of opiates has been tested. In these experiments, a decrease in the effectiveness of morphine in tissues superfused with solutions containing increased concentrations of calcium ion is interpreted to mean that calcium is antagonizing the effect of morphine.

The inhibition by morphine of nerve-mediated muscle contractions of the guinea-pig ileum is reduced in tissues maintained in calcium (5 mM) compared to tissues maintained in calcium (2 mM) (Heimans, 1975). Opmeer & Van Ree (1979) also observed a lowered effectiveness of morphine in guinea-pig ilea maintained in high calcium (5.08 mM) rather than normal calcium (2.54 mM) solutions. In these experiments calcium may be altering the response of the muscle by increasing the efficiency

of excitation-contraction coupling. The measurement of evoked transmitter release in solutions of varying calcium ion concentration eliminates problems associated with direct actions of calcium on muscle. In a preliminary study, Nutt (1968) observed no change in output from strips of guinea-pig intestine maintained in calcium ion concentrations varying over a 10-fold range. But Sawynok & Jhamandas (1979) do see a lesser degree of inhibition by morphine of evoked acetylcholine release from strips of longitudinal muscle-myenteric plexus if the latter are superfused with three times the normal calcium ion concentration. A similar effect occurs in solutions containing the calcium ionophore A23187. A puzzling aspect of this report is that the amount of transmitter released in response to nerve stimulation was the same in high (7.5 mM) calcium as in normal (2.5 mM) calcium. Such a large increase in calcium would be expected to enhance stimulus-secretion coupling.

Opiates still inhibit the firing of single myenteric neurons in solutions that contain no added calcium ion (Dingledine & Goldstein, 1976; Williams & North, 1979), arguing against an essential role for the divalent cation in this action of opiates. Thus even in the same tissue the effect of calcium differs, depending on how the action of opiates is measured. The myenteric plexus is a complex neuronal assembly in which the release of acetylcholine from the autonomic neurons innervating the longitudinal smooth muscle could be influenced in many ways and controlled at multiple sites. A more desirable system for the study of the calcium-opiate interaction is a simple preparation

in which actions of calcium at loci other than on the neurons with opiate receptors can be minimized or discounted.

1.52 The effect of calcium ion on the binding of opiates

Calcium ion could antagonize the actions of opiates in both *in vivo* and *in vitro* experiments by inhibiting the binding of opiates to neuronal receptors. The stereospecific binding of both [^{3}H]-dihydromorphine (DHM) and [^{3}H]-naloxone to brain homogenates is significantly less in solutions containing calcium ion than in solutions containing zero calcium (Pert & Snyder, 1973b; Hitzemann, Hitzemann & Loh, 1974). But the stereospecific binding of [^{3}H]-DHM decreases by no more than 20% with a twenty-fold increase in the calcium ion concentration (1 to 20 mM); in the physiologically relevant range of calcium ion concentrations (1-5 mM), the amount of [^{3}H]-DHM bound varies by no more than 10% (Pert & Snyder, 1974). Thus the binding of opiates to neuronal membranes does not appear to be a major locus of interaction with the calcium ion.

1.53 Effects of opiates on calcium levels and fluxes

The calcium content of whole brain is reduced in mice that have received a large subcutaneous dose of morphine (100 mg/kg but not 20 mg/kg) (Shikimi, Kaneto & Hano, 1967). The same effect occurs in rats that have been injected with morphine (25 mg/kg) (Yamamoto, Harris, Loh & Way, 1978), although antinociception can be produced in these animals by much lower concentrations of morphine. The effect of

morphine administration on calcium levels in brain is dose-related, stereospecific and reversed by naloxone (Cardenas & Ross, 1975). The calcium loss is localized to synaptosomal fractions (Cardenas & Ross, 1976; Harris, Yamamoto, Loh & Way, 1977). Animals that have received prolonged morphine treatment (by pellet implantation) have higher calcium levels in the synaptosomal fractions of their brains (Yamamoto et al., 1978) and an increase in the amount of radioactive label found in isolated nerve ending fractions after the in vivo administration of labelled calcium ion (Harris et al., 1977). These investigators believe that the nerve endings compensate for an initial depletion of calcium caused by the acute administration of opiates. This compensation takes the form of an increase in the content of calcium at these sites.

Several problems are associated with this interpretation of these results. Firstly, morphine is not the only centrally active drug which, when administered to animals, alters brain calcium levels. Ethanol and reserpine also cause an apparent depletion of brain calcium, although pentobarbital does not (Ross, Medina & Cardenas, 1974). Calcium levels in guinea-pig brain stem are also altered during either the excitation or the sleep produced by central stimulants or depressants respectively. General alterations in brain functional activity thus can change the distribution and content of calcium in the brain (see Kaneto, 1971). Secondly, acute administration of morphine (intraperitoneal injection of 6-100 mg/kg) decreases calcium levels to an equal extent in eight different brain regions (Cardenas & Ross, 1975). There is no correlation between the density of opiate binding sites and

the extent of depletion of calcium in a given region. Mice that have received morphine have an equal decrease in the calcium content of the striatum, an area rich in binding sites, as in the cerebellum, an area practically devoid of opiate binding sites (Kuhar et al., 1973). The in vivo administration of morphine thus lowers calcium levels even in neurons that do not bear opiate receptors. One may argue that the calcium effect observed in vivo is likely to be the result of a general change in the functional state of the brain which has been elicited by the administration of morphine. This is the idea proposed by Kaneto (1971). One possible mechanism is related to the respiratory depression caused by morphine. Morphine in vivo increases the pCO_2, causing a decrease in the pH of the brain. An increase in the extracellular concentration of hydrogen ions can decrease the calcium content of nerve (Baker & Honerjager, 1978).

Anionic groups of the proteins and phospholipids of cell membranes attract and bind cations such as calcium. Narcotics inhibit the binding of calcium to phospholipids in vitro, but high concentrations (600 μM) are required and other centrally active drugs share this action. The effectiveness of a given drug correlates with its degree of ionization (Mule, 1969). Isolated synaptic membranes prepared from whole rat brain minus cerebellum bind radiolabelled calcium ions ($^{45}Ca^{++}$) with high affinity (K_D = 840 nM). Low concentrations of levorphanol (0.5-10 nM) added to the incubation mixture non-competitively inhibit this binding, an effect that is stereospecific and blocked by naloxone (10 nM) (Ross & Cardenas, 1977). Both the initial

rate and the maximal level of uptake of $^{45}Ca^{++}$ into homogenates of whole mouse brain are lower if morphine or levorphanol (but not dextrorphan) are added to the incubation mixture or if the homogenates are prepared from mice pretreated with a single injection of morphine (10 mg/kg). In homogenates prepared from animals that have been chronically exposed to morphine (pellet implantation), the time required for maximal uptake of calcium is shortened, whereas the maximum level of uptake is not changed (Guerrero-Munoz, Guerrero & Way, 1978). This study does not distinguish between calcium uptake (energy dependent) and binding (no requirement for metabolic energy) and the relative effect of morphine on each. Therefore similar experiments using lysed synaptosomes measured $^{45}Ca^{++}$ uptake in the presence and absence of ATP (Guerrero-Munoz, Guerrero & Way, 1979). Incubation in morphine decreases uptake only in the presence of ATP; this suggests that morphine inhibits <u>active</u> uptake of calcium by synaptosomes. The postulated inhibition by morphine of calcium uptake into a resting nerve terminal may not be relevant to any actions of morphine in inhibiting stimulus-evoked transmitter release. It is consequently of interest that incubation with levorphanol (100 nM) also reduces calcium uptake in synaptosomes that have been depolarized by a solution containing high potassium (50 mM) (Ross, 1978).

1.6 Neuro-effector transmission in the mouse vas deferens

1.61 Anatomy

The smooth muscle of the mouse vas deferens is innervated by the sympathetic branch of the autonomic nervous system via the hypogastric nerve. Preganglionic sympathetic fibers in the hypogastric nerve synapse in ganglia located close to the vas deferens: this organ is thus innervated by short adrenergic neurons (Sjostrand, 1965). Like most hollow organs, the vas deferens consists of inner circular and outer longitudinal layers of smooth muscle. The following description of the fine structure of the muscle cells and their innervation in the vas deferens of the mouse is a composite of the work of Lane & Rhodin (1964) and Jones & Spriggs (1975b). Individual muscle cells are long (100-300 μm) and thin (diameter less than 10 μm) and are arranged in branching and anastomosing bundles. Adjacent cells often appear to interlock by "peg and socket" structures - an evagination of one cell fits into an invagination of the next. Tight junctions ("nexuses" - apposing membranes fused), which are low resistance pathways for flow of electrical current between cells, are infrequently found in the mouse vas deferens (Yamauchi & Burnstock, 1969). Thus although electrophysiological studies reveal electrotonic coupling among the cells (see below), the structures which allow such coupling have not yet been identified.

The fine unmyelinated sympathetic fibers run for long distances through the smooth muscle before terminating. Large bundles of axons

(about 8 axons/bundle) enclosed in Schwann cell sheaths run between the muscle bundles. Smaller bundles of axons, sometimes with an incomplete sheath, are seen between individual muscle cells. Axons which are devoid of a Schwann cell sheath eventually emerge from these bundles and run singly between cells; these axons often lie in an indentation of a cell or are completely enclosed within smooth muscle cytoplasm (Furness & Iwayama, 1971). The single axons are varicose, with alternate constrictions and dilatations of their diameter at regular intervals. The dilated areas, or nodes, contain numerous synaptic vesicles and some mitochondria and are presumed to be the sites of transmitter release. Nerve varicosities often closely approach the muscle cells; neuromuscular clefts of 7-8 nm are common. All cells appear to be innervated by one or more such close contacts; this is not the case for all smooth muscle organs (see Burnstock, 1970). No postsynaptic specializations of the muscle cells are evident.

Three types of synaptic vesicles are seen within the axonal varicosities. Small dense-core (granular) vesicles are much more numerous than small electron-lucent (agranular) vesicles: approximately 73% of axons contain small granular vesicles only, 22% both agranular and granular vesicles, and 5% agranular vesicles only. A proportion of the axon profiles also contain one or a few large granular vesicles. Small granular vesicles are believed to be associated with the storage of noradrenaline, whereas agranular vesicles are identical to those seen at the skeletal neuromuscular junction and are presumed to be cholinergic. The contents of large granular vesicles are unknown (Burnstock, 1970).

Anatomical studies implicate noradrenaline as the transmitter of the mouse vas deferens. The noradrenaline content of the tissue is high (about 5 µg/g of tissue) (Sjostrand, 1965). The vas exhibits a dense catecholamine fluorescence which is reduced or abolished by pre-treatment of the mouse with 6-hydroxydopamine (Furness, Campbell, Gillard, Malmfors, Cobb & Burnstock, 1970), a toxin which is taken up into noradrenergic nerve terminals, displaces noradrenaline from the vesicles and subsequently causes degeneration of the nerve terminal (Thoenen & Tranzer, 1968). The majority of axons in the mouse vas deferens contain small granular vesicles which are the predominant type in mammalian sympathetic nerves. The dense core of these vesicles is best observed by permanganate fixation of the tissue (Furness et al., 1970) or by pretreatment of the animal with 5-hydroxydopamine (Chiba, 1973). The latter substance is a marker for adrenergic nerve terminals (Tranzer & Thoenen, 1967). A small number (7-8%) of axons found in the mouse vas deferens may be cholinergic (Jones & Spriggs, 1975a). These axons contain small agranular vesicles and acetylcholinesterase reaction product on their surface membrane.

1.62 Mechanical responses

Some investigators have expressed doubt that noradrenaline is the neurotransmitter of the vas deferens (guinea-pig: Ambache & Aboo Zar, 1971; mouse: Jenkins, Marshall & Nasmyth, 1977). The basis of the argument against noradrenaline as the transmitter is three-fold. First, the muscle is insensitive to exogenous noradrenaline. Muscle contractions evoked by adding noradrenaline to the organ bath rarely

compare in amplitude to those evoked by stimulation of the intramural nerves. Secondly, the nerve-mediated twitch response of the muscle is reduced by exogenous noradrenaline. And, finally, neurotransmission is insensitive to blockade by classical α-adrenoceptor antagonists, especially phenoxybenzamine. Each of these results can be explained without abandoning the noradrenaline hypothesis. The insensitivity to exogenous noradrenaline could result from the close packing of the muscle cells. The effect of noradrenaline on the smooth muscle peaks within 30 s, yet in this time the noradrenaline would diffuse into only about 15% of the extracellular space (Jones & Spriggs, 1975b). Transmural stimulation of the nerves effectively activates all of the smooth muscle cells. Also, extra-junctional rather than junctional regions of the postsynaptic cells would be more accessible to exogenous substances. By analogy with skeletal muscle, junctional regions in the vas deferens may be more sensitive to transmitter. Low concentrations of exogenous noradrenaline inhibit the nerve-mediated twitch response of the muscle by an action on nerve terminals. Sympathetic nerves possess presynaptic α-adrenergic receptors: α-adrenergic agonists inhibit and α-adrenergic antagonists potentiate the nerve-evoked release of noradrenaline (see Starke, 1977). In the mouse vas deferens, noradrenaline is less potent in this action than clonidine, but the action of both is blocked by yohimbine (Marshall, Nasmyth, Nicholl & Shepperson, 1978). Yohimbine is an α-adrenergic antagonist that in low concentrations selectively blocks presynaptic receptors. Furness (1974) explains that the ineffectiveness of the α-adrenergic antagonist phenoxybenzamine in blocking the nerve-mediated twitch may be the

result of the closeness of the neuromuscular contacts and the non-specificity of the drug. Thus the highly charged phenoxybenzamine molecule may have difficulty penetrating to the actual site of neurotransmission, which in the mouse vas deferens is well insulated (see above). Also, phenoxybenzamine has other actions besides postsynaptic α-adrenergic receptor blockade, such as antagonism of noradrenaline uptake, inhibition of the enzymatic breakdown of noradrenaline and potentiation of noradrenaline release by blocking the inhibitory presynaptic α-adrenergic receptors. These four latter actions would tend to increase rather than decrease the efficacy of adrenergic transmission. Furthermore, high concentrations of another α-adrenergic antagonist, phentolamine -- 53 μM: Jones & Spriggs (1975b); 360 μM: Henderson (1974) -- do abolish the responses of the vas to both transmural nerve stimulation and exogenous noradrenaline.

Other findings from organ bath physiology experiments further implicate noradrenaline as the transmitter. The mouse vas deferens takes up and stores labelled noradrenaline, and the amount of label released upon nerve stimulation depends on the stimulation frequency and is related to the contractile response of the muscle (Farnebo & Malmfors, 1971). Muscle responses to transmural stimulation are inhibited by guanethidine (Jones & Spriggs, 1975b) and abolished by bretylium but then restored by amphetamine (Henderson, 1974). Also, no nerve-mediated contractions can be elicited in vasa from mice pretreated with 6-hydroxydopamine (Jones & Spriggs, 1975b). These are typical pharmacological responses of an adrenergically innervated organ.

1.63 Electrophysiology

Passive and active membrane responses of smooth muscle can be observed by intracellular recordings from individual cells. Junction potentials, short-lived changes in membrane potential in response to interaction of transmitter with receptors on the smooth muscle membrane, were first described for the vas deferens of the guinea-pig by Burnstock & Holman (1961) and for the vas deferens of the mouse by Holman (1967). The junction potentials of the vas deferens are depolarizing and can be elicited by either stimulation of the hypogastric nerve or by field stimulation of intramural nerves. The excitatory junction potentials (e.j.ps) of the mouse vas deferens can be evoked by both orthodromic and antidromic activation of the intramural nerves, indicating that transmitter is released from the varicosities along the length of the axon, and not just from axon terminals (Furness, 1970). The time to peak (rise time) of the e.j.p. is 10-20 ms, and the duration is 100-250 ms. The amplitude of the evoked e.j.p. is graded with stimulus strength. Increasing the stimulus strength recruits more nerve fibers; up to 15-22 fibers may contribute to the e.j.p. in a given cell (Furness, 1970). Holman (1967 & 1970) observed that spontaneous junction potentials occur with a variable frequency in the mouse vas deferens. Their amplitudes range from just above the noise level of the recording system (about 1 mV) to about 20 mV. These potentials are presumed to result from the spontaneous release of transmitter from the adrenergic nerve varicosities because they persist in the presence of tetrodotoxin (Hashimoto, Holman & McLean, 1967).

The variation in amplitude may be a consequence of transmitter release at different distances from the impaled cell, into synaptic clefts of differing geometry. The actual amount of transmitter released may also vary.

E.j.ps of smooth muscle have been compared to end plate potentials (e.p.ps) of skeletal muscle. E.j.ps are assumed to result from an increase in conductance of the smooth muscle membrane to one or more ions, just as the e.p.p. is the consequence of current flowing due to an increased conductance of the skeletal muscle fiber membrane to sodium and potassium ions (Takeuchi & Takeuchi, 1960). Experiments attempting to define the mode of action of noradrenaline on the post-synaptic membrane are difficult because of the syncytial nature of smooth muscle. Smooth muscle cells are, to a greater or lesser extent, electrotonically coupled to one another. Caution is warranted in interpreting data obtained by intracellular recording from one point in a syncytium (Tomita, 1970; Holman & Nield, 1979). The current applied at one point, for example from a microelectrode placed in a single smooth muscle cell, spreads in three dimensions. The current density decreases rapidly with distance from the current source, as the current is shunted through the low electrical resistance of the surrounding membrane. The area of membrane increases as approximately the cube of the distance from the point of current application. Only a small amount of the current passes across the membrane of the impaled cell. Thus attempts to demonstrate an increased conductance of the smooth muscle membrane during an e.j.p. have been unsuccessful because the e.j.p. recorded in a given cell is largely or entirely the result of

electrotonic spread of depolarization from neighboring cells (Holman, 1970). Similarly, the amplitude of the e.j.p. in a given cell is not altered by changing the membrane potential of that same cell by intracellular injection of current (Holman, 1967). If, however, the entire smooth muscle membrane is depolarized with exogenous noradrenaline (Furness, 1970) or potassium sulphate (Holman, 1970), the amplitude of the e.j.p. is reduced. Interpretation of data obtained in this manner is difficult because these treatments alter the properties of the presynaptic nerve terminals as well as the postsynaptic muscle membrane. If the smooth muscle membrane is depolarized to + 20 mV by a combination of potassium sulphate and intracellular current injection, the polarity of spontaneous potentials is reversed (Holman, 1970). This result may indicate that noradrenaline is increasing the permeability of the membrane to sodium and at least one other ion.

E.j.ps of smooth muscle have also been compared to e.p.ps of skeletal muscle in terms of the quantal nature of transmitter release. The e.p.p. results from the simultaneous release from the motor nerve terminal of many packets (quanta) of transmitter of approximately the same size (del Castillo & Katz, 1954). Transmitter release from autonomic nerves is also assumed to be quantal, but direct proof is lacking. Blakeley & Cunnane (1979) provide some evidence for packeted release of transmitter in the guinea-pig vas deferens. They found that the evoked e.j.p. has intermittent peaks in its rate of rise which are superimposed on a regular, non-intermittent component of the voltage change. In some cells, these peaks or "discrete events" have preferred

amplitudes which are whole number multiples of their smallest amplitude. Furthermore, a few cells demonstrate spontaneous e.j.ps that are of the same amplitude and time course as a discrete event recorded in that same cell. The spontaneous potential and the discrete event are assumed to result from the release of a packet of transmitter from the same varicosity. Thus the rising phase of the e.j.p. has two components: discrete events which represent the packeted release of transmitter directly onto the impaled cell, and a slow non-intermittent component which represents the electrotonic spread of depolarization from neighboring cells.

1.64 Effects of opiates

Intracellular recording of an e.j.p. is a sensitive method of assaying the small amount of transmitter released from the noradrenergic nerve varicosities in response to a single stimulus to the intramural nerves. The inhibition of evoked transmitter release in the mouse vas deferens by opiates is reflected in the depression of e.j.p. amplitude by normorphine (Henderson & North, 1976). Normorphine does not prevent the depolarization of the smooth muscle cells by added noradrenaline, thus confirming the presynaptic location of the opiate receptors.

1.7 Research objectives

The object of this study is to examine the depression by opiates of the amplitude of the evoked e.j.p. in the mouse isolated vas deferens. The study will investigate factors that modify the opiate-induced depression of e.j.p. amplitude and hence will try to clarify the mechanism by which opiates decrease transmitter release by their action on nerve terminals. The specific aims are:

(1) to study the changes that occur in neuro-effector transmission in the mouse vas deferens after chronic exposure to morphine.

(2) to examine the role of cyclic nucleotides and the calcium ion in the acute depressant action of opiates on transmitter release in the mouse vas deferens.

2. METHODS

2.1 Preparation of the tissue

Male mice (25-40 g) of the CF-1 strain (ARS, Madison, WI) were killed by a blow to the head. Both vasa deferentia were dissected free from the pelvic cavity and placed in a Petri dish filled with Krebs solution. Vasa are closely invested with a fine connective tissue sheath containing the nerve and blood supply to the organ. The portion of the sheath containing the main artery was removed. Both ends of the tissue were tied with fine silk thread (6-0) in order to pull them through ring-shaped stimulating electrodes (see below).

The tissue was transferred to a bath constructed of perspex. This bath had a long rectangular channel filled with a silicone rubber resin (Sylgaard, Dow Corning). The bath was secured to the stage of a dissection microscope with modeling clay. The vas deferens was placed at right angles to the length of the Sylgaard-filled trough and the central portion was pinned. A section of the vas was first secured by four large pins arranged in the form of a square or rectangle (2 mm x 2-3 mm); the tissue was stretched by this procedure so that it presented a flat surface as opposed to the rounded contour of unpinned tissue. The close investment of fine connective tissue of the presenting surface was removed with the aid of fine jeweler's forceps. Small pins cut from tungsten wire were used to further stretch and immobilize the portion of the tissue delineated by the four larger pins.

Platinum ring electrodes with an inner diameter approximating the diameter of the vas deferens were placed on either side of the tissue. Each end of the vas was threaded through a ring electrode, and the electrode positioned about 2-3 mm above, and slightly to the side of, the pinned out portion of tissue. A wick of tissue paper kept moist that portion of the muscle which led to the electrode.

The tissue was superfused with Krebs solution of the following composition (mM): NaCl 118, KCl 4.75, KH_2PO_4 0.93, $CaCl_2$ 2.54, $MgCl_2 \cdot 7H_2O$ 1.19, glucose 11 and $NaHCO_3$ 25. This solution was continuously gassed with 95% O_2 and 5% CO_2 in its main reservoir. It then was pumped at a constant flow rate of 1.5 ml/min through a series of three-way taps, through the tubing of a Gilson pump and finally through fine polyethylene tubing. The latter tubing was, before leading to the tissue bath, invested with a jacket containing heated water. The temperature of the Krebs solution at the site of recording was 35-37° C.

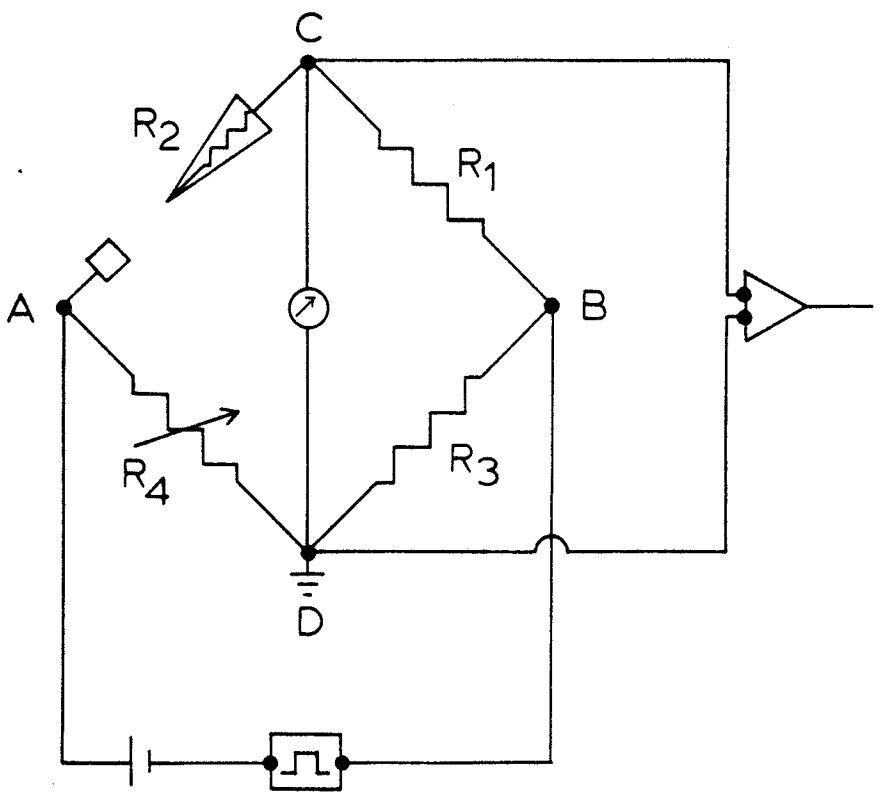
Drugs or solutions of varying ionic composition were applied to the tissue by superfusion. Each drug or solution was contained in a separate reservoir and attached to one of the three-way taps. Turning the tap changed the Krebs solution from the standard solution to another which differed only in its content of drug or in its ionic composition. The "dead time" or the time interval between the turning of a tap and the appearance of the new solution at the tissue varied from 40 s to 1.5 min (depending on tap location, tubing lengths and pump speed).

2.2 Electrical recording

Glass capillary tubing (1.0 or 1.2 mm outside diameter), pulled to a fine tip by a micropipette puller of the horizontal type (Industrial Science, model M1), was used for recording from single smooth muscle cells. The microelectrode was filled with 2 M KCl and attached to a Narishige micromanipulator. A fine chloridized silver wire was inserted into the filled microelectrode. The electrical signal from the silver wire electrode was magnified 100X by the pre-amplifier (Nihon-Kohden model MZ-3B) and displayed on two oscilloscopes (Tektronix 502A and 535A). The amplifiers on the oscilloscopes were DC-coupled. The trace on the 502A oscilloscope was photographed with a Grass kymograph camera (model C4R) on 35 mm Tri-X Pan film (Eastman-Kodak). The second oscilloscope was used for visual observation.

The resistance of the microelectrode was measured by means of a Wheatstone bridge circuit (Figure 1) as described by Araki & Otani (1955). The bridge was balanced, that is, the points C and D were isopotential, if the current flow was equal through each of the parallel arms ACB and ADB. This occurred if $R_1/R_2 = R_3/R_4$. The bridge was calibrated by substituting known resistances for R_2 and adjusting R_4 until no current flowed between points C and D (current trace was monitored on the oscilloscope). The resistance of a given microelectrode could be measured by dialing to the resistance (R_4) which balanced the bridge. Electrodes of resistance 50-80 MΩ were most useful for stable recordings.

Figure 1. Diagram of Wheatstone bridge circuit. The resistances of microelectrodes for intracellular recording were measured using the Wheatstone bridge circuit illustrated. R_1 was a 1,000 MΩ current limiting resistor. The resistance of the microelectrode (R_2) was another arm of the bridge. This arm also contained the lead which grounded the tissue bath. The rest of the bridge consisted of a 10,000 Ω variable resistor which remained fixed at a given value (R_3), and a 1,000 Ω variable resistor which was adjustable (R_4). A test voltage was applied across points A and B of the circuit. A signal related to the potential difference between points C and D was amplified and monitored on an oscilloscope.


C
R2
R1
A
B
R4
R3
D

2.3 Impalement of the smooth muscle cells

The microelectrode was lowered until it just touched the surface of the most superficial layer of smooth muscle. This position was indicated by a sudden unbalancing of the bridge as the electrode tip encountered the resistance of the tissue. The microelectrode was driven through the tissue by a succession of advancements by the micromanipulator, interspersed with gentle tapping of the micromanipulator or table. Cells were impaled by either a gentle tap or by a short duration, sudden increase of the amplifier's capacity compensation. Successful impalement was indicated by a drop in the DC potential of 60 mV or greater.

The resting membrane potentials of cells were measured by sudden withdrawal of the recording electrode into the perfusing solution. Measurements were made only for cells which showed stable resting membrane potentials for longer than 10 min.

2.4 Stimulation of the intramural nerves

The intramural nerves were stimulated by the platinum ring electrodes with single rectangular pulses (500 μs or 1 ms in duration); these pulses were generated by a Grass stimulator (model S88) and were isolated from ground. The preparation was stimulated at 30 s intervals because facilitation of e.j.p. amplitude is not evident at this frequency (Henderson & North, 1976). The stimulus voltage was adjusted so that the unfacilitated e.j.p. was between 10 and 25 mV (usually about 15 mV) in amplitude before measuring the effect of a drug that depressed the e.j.p. These e.j.p. amplitudes were chosen because at lower amplitudes the depressant action of a drug is less accurately measured and at higher amplitudes non-linear summation (Martin, 1955) may become important. The initial e.j.p. amplitude was between 4 and 12 mV before addition of a drug which increased e.j.p. amplitude.

2.5 Prolonged exposure to normorphine *in vitro*

After removing the vas deferens from the animal, the tissues were superfused with Krebs solution containing normorphine (300 nM). After 1-5 h of this treatment, the effect of normorphine (1 and 3 μM) on e.j.p. amplitude was tested. The effect of naloxone (300 nM) on the e.j.p. was tested after 5-8 h of superfusion with normorphine (300 nM).

2.6 Prolonged exposure to morphine *in vivo*

Vasa deferentia from some mice were exposed to morphine *in vivo*; these mice were given 9 intraperitoneal injections of morphine sulphate (doses of 40, 60, 80, 100, 120, 140, 140, 140 and 140 mg/kg) at 8 h intervals. The animals were killed 2 h after the final injection of morphine and their vasa were rapidly removed and placed in Krebs solution containing normorphine (300 nM). Experiments on vasa from morphine-pretreated mice were completed within 5 h of removing the tissue from the animal.

The tissues from morphine-pretreated mice were maintained in Krebs solution containing normorphine to approximate the *in vivo* condition in which morphine is continuously present (North & Karras, 1978a) and to prevent the withdrawal and rapid loss of tolerance which occurs in the mouse vas deferens upon washing the tissue with opiate-free Krebs solution (Cox, 1978). The reasons for using a concentration of normorphine of 300 nM were two-fold. Firstly, this concentration is similar to the concentration used by Schulz & Herz (1976) in their study of morphine tolerance in the guinea-pig ileum. They found that normorphine (200 nM) ideally maintained tissues removed from morphine-pretreated animals in a state of tolerance and dependence. Secondly, by maintaining a tissue in a concentration of 300 nM, a normorphine dose-response curve can be completed without exceeding a 10 μM concentration. Morphine in concentrations above 10 μM inhibits noradrenaline uptake in adrenergic nerves by an action unrelated to the opiate receptor (Montel & Starke, 1973).

2.7 Effects of cyclic nucleotides and phosphodiesterase inhibitors

The acute effects of the cyclic nucleotide cAMP and the dibutyryl derivative of cAMP (dbcAMP), and the acute effects of the phosphodiesterase inhibitors IBMX and SQ 20,006 on the amplitude of the evoked e.j.p. were determined. The effect of normorphine on e.j.p. amplitude was tested on tissues superfused with cAMP (1 mM) and on tissues superfused with the drug combination dbcAMP (500 μM) and IBMX (50 μM). The effects of D-Ala^2-Met^5-enkephalinamide (DAEA) and D-Ala^2-D-Leu^5-enkephalin (DADL) on e.j.p. amplitude were tested on tissues superfused with both IBMX (500 μM) and dbcAMP (250 μM).

2.8 Effects of altered concentrations of calcium ion

The alteration of e.j.p. amplitude caused by halving or doubling the normal calcium ion concentration (2.54 mM) was determined. In these solutions of varying calcium ion concentration, osmolarity was maintained by adjusting the concentration of NaCl. Dose-response curves for the depression by normorphine of e.j.p. amplitude were constructed for vasa maintained in calcium ion concentrations of 1.27 mM, 2.54 mM and 5.08 mM.

2.9 Contractions of the vas deferens

A single vas deferens was suspended in an organ bath (volume 1.5 ml). The tissue was washed with Krebs solution warmed to 37° C and gassed with 95% O_2 and 5% CO_2; this solution was of the same ionic composition as described above except for the omission of magnesium ion (Hughes et al., 1975a). Changes in tension of the vas deferens were recorded isometrically. Single stimulus pulses (voltage maximal, duration 1 ms, frequency 0.1 Hz) were generated by a Grass stimulator (model S4K) and applied to the tissue across two platinum wire electrodes situated at the top and bottom of the organ bath. The effect of normorphine (100 nM - 30 μM) on the responses of the muscle to nerve stimulation was tested.

Dose-response curves for the contractile response to noradrenaline were constructed. Noradrenaline doses were given in ascending order at 12 min intervals. Noradrenaline (1 μM - 1 mM) was applied in the same location in the organ bath relative to the tissue and in the same volume (13 μl). The largest concentrations tested (3 and 10 mM) required larger injection volumes. Stock solutions of noradrenaline in Krebs solution were made fresh daily and contained ascorbic acid (100 μM).

In a few experiments a linear motion transducer was used to record the changes in length of the vas deferens in response either to field stimulation of the intramural nerves (as above) or to an injection of noradrenaline or high potassium solution.

2.10 Collection and analysis of data

The calibration of the oscilloscopes was periodically checked by using a square-wave generator. E.j.p. amplitudes were measured by projecting the oscilloscope traces recorded on film onto calibrated graph paper by means of a film enlarger. The percentage change in e.j.p. amplitude in response to a drug or to a change in ionic composition of the Krebs solution was determined as follows. After a control period of four min or more, the tissue was superfused with drug until the amplitude of at least three consecutive e.j.ps remained relatively constant (no trend toward an increase or a decrease in amplitude was discernible). For each drug application, a plot of e.j.p. amplitude vs. time was drawn. E.j.p. amplitudes during the control period and during the time of maximum drug effect were determined by either drawing a line through the data points (estimated by eye) or by averaging the measured amplitudes. A drug effect was not accepted unless at least a 50% reversal of the effect occurred upon returning to control Krebs solution.

Results were analyzed by calculation of the mean, standard deviation and standard error of the mean using standard formulae (Goldstein, 1964). Student's t test was used to compare the means of two groups which received different experimental treatments. One-way analysis of variance (ANOVA) was used to compare the means of three or more groups. A p value greater than 0.05 was chosen to indicate statistical significance.

2.11 Drugs

Drugs used were: adenosine (Sigma), adenosine 3',5'-cyclic monophosphoric acid (Sigma), D-Ala^2-D-Leu^5-enkephalin (Biosearch), D-Ala^2-Met^5-enkephalinamide (Peninsula), N^6, $O^{2'}$-dibutyryl adenosine 3',5'-cyclic monophosphoric acid, sodium salt (Sigma), 1-ethyl-4-hydrazino-1H-pyrazolo(3,4-b)pyridine-5-carboxylic acid, ethyl ester, hydrochloride (SQ 20,006) (Squibb), 1-methyl-3-isobutylxanthine (Sigma), dl-noradrenaline hydrochloride (dl-arterenol) (Sigma), morphine sulphate (Lilly), naloxone hydrochloride (Endo Laboratories) and normorphine sulphamate (Dr. A. E. Jacobson).

Molar concentrations refer to the final bath concentration of these substances.

3. RESULTS

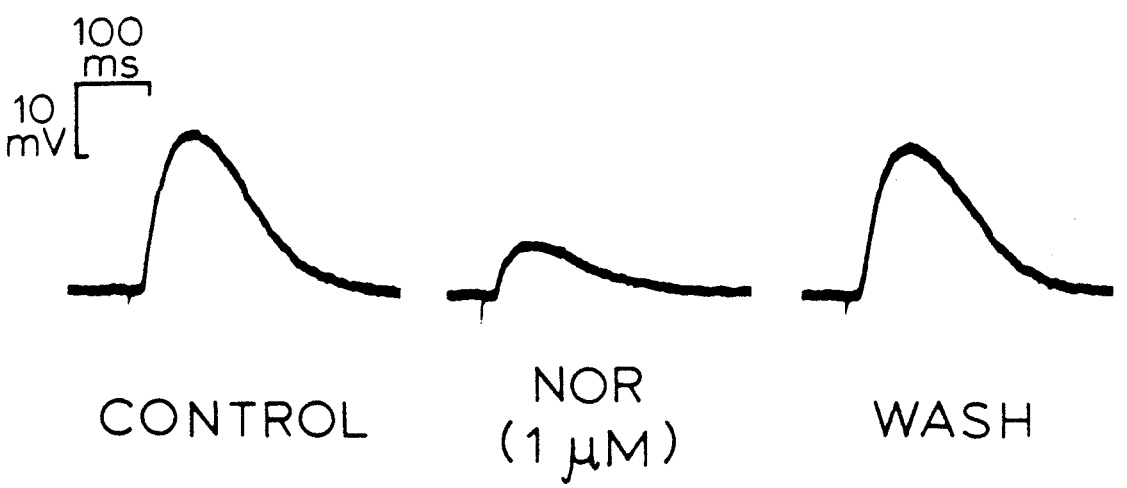
3.1 General observations

The results of the present study are based on recordings from 363 cells (152 mice). Recordings from single cells of up to 3.5 h were possible, although the usual duration of impalement was 20 min to 1 h. The resting membrane potential of individual smooth muscle cells was 62.6 $\pm$ 0.9 mV (mean $\pm$ s.e. mean, n = 16).

E.j.ps evoked by field stimulation of the intramural nerves of the vas deferens could be recorded in every cell successfully impaled; their amplitudes were dependent on the stimulus strength. At a fixed stimulus duration, the stimulus voltage used to evoke an e.j.p. of a given amplitude (about 15 mV) varied from one tissue to the next, and among cells in the same tissue. The voltage for a given cell depended on: the initial positioning of the platinum ring electrodes around the muscle, the location of the cell relative to the electrodes, the position of the recording electrode and the depth of the Krebs solution flowing through the tissue bath.

The time to peak amplitude for an e.j.p. was 25-45 ms, and the time to decay to one-half the peak amplitude was 60-90 ms. The rising phase of the depolarization was steeper in slope and shorter in duration than the falling phase (Figure 2). In some cells graded action potentials were observed; these events could occur on the rising phase, peak or falling phase of an e.j.p. If these potentials obscured

Figure 2. Photographs of excitatory junction potentials (e.j.ps). Photographs of oscilloscope tracings illustrating the waveform and time course of an e.j.p., and the depression of e.j.p. amplitude by normorphine. In this cell, normorphine (1 μM) decreased e.j.p. amplitude by 68%, from 22 mV to 7 mV. This effect washed within 5 min of resuming superfusion with drug-free Krebs solution.


100
ms
10
mV
CONTROL
NOR
(1 μM)
WASH

the peak amplitude of the e.j.p. in a cell, the cell was not used for experimentation.

Spontaneous junction potentials were observed in almost every cell, although their frequency was markedly variable among cells and tissues.

3.2 Effect of opiates and opioid peptides

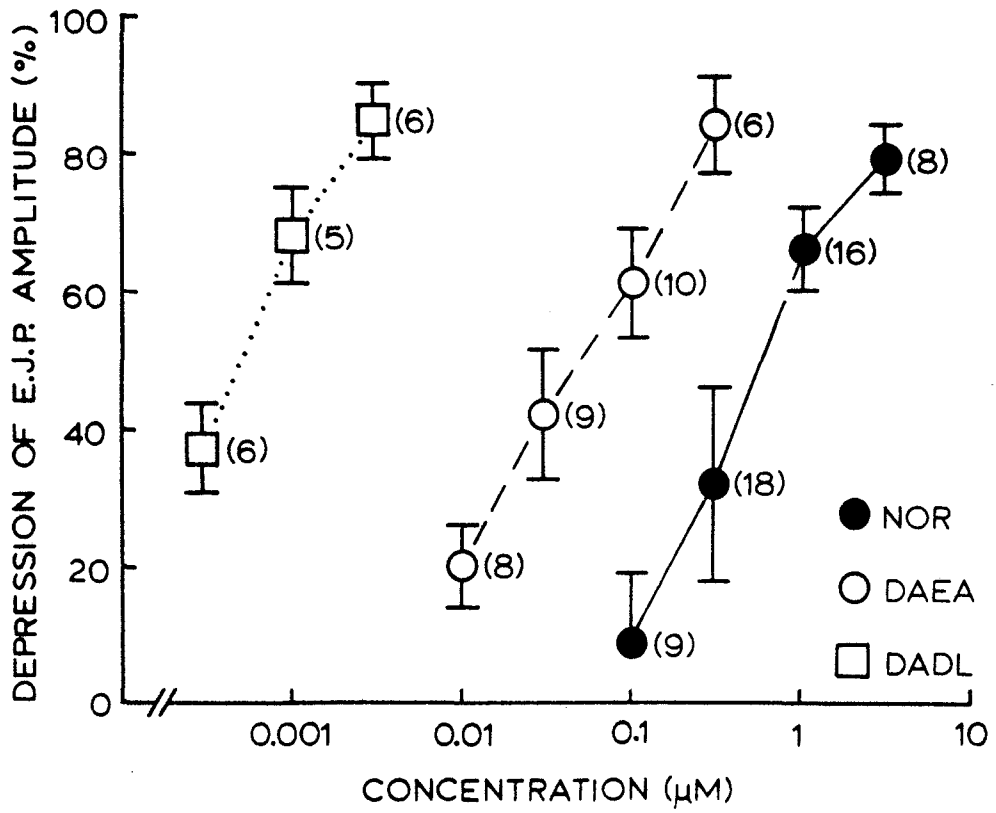
3.21 Normorphine

Normorphine (100 nM - 30 μM) depressed the amplitude of the evoked e.j.p. (Figure 2) but did not alter the resting membrane potential of the smooth muscle cells. Any effect of a drug on membrane potential would be obvious if the change were greater than 3-5 mV. This effect of normorphine was maximal 4-8 min after the onset of drug superfusion, and usually washed within 5-15 min of resuming superfusion with drug-free Krebs solution. The magnitude of the depression of the e.j.p. by normorphine was larger with higher concentrations of normorphine. A composite dose-response curve for normorphine was constructed by pooling results from many cells. Because the duration of impalements was variable, it was not always possible to test all concentrations of normorphine on the same tissue. Occasionally, however, all normorphine concentrations were tested even on a single cell. The EC_{50} for normorphine (concentration that depressed the e.j.p. to 50% of its control amplitude) was 570 nM (Figure 3). An e.j.p. that was depressed in amplitude by normorphine could be restored to its control amplitude by increasing the stimulus voltage.

3.22 Normorphine at different stimulus strengths

Many of the experimental protocols of the present study involved testing the effect of opiates or opioid peptides on vasa which have been exposed to substances or ionic conditions which themselves altered e.j.p. amplitude. Under these conditions the stimulus strength was ad-

Figure 3. Dose-response curves for depression of e.j.p. by normorphine and opioid peptides. A comparison of the dose-response curves for depression of e.j.p. amplitude by normorphine (NOR), D-Ala^2-Met^5-enkephalinamide (DAEA) and D-Ala^2-D-Leu^5-enkephalin (DADL). Bars represent $\pm$ standard deviation. Numbers in parentheses indicate the number of individual cells tested at a given concentration.


DEPRESSION OF E.J.P. AMPLITUDE (%)
100
80
60
40
20
0
(6)
(5)
(6)
(6)
(10)
(8)
(9)
(8)
(16)
(18)
(9)
NOR
DAEA
DADL
0.001
0.01
0.1
1
10
CONCENTRATION (μM)

justed in order to obtain a control e.j.p. of approximately 15 mV in amplitude before testing the effect of an opiate or opioid peptide. It was therefore necessary to investigate if the potency of normorphine was dependent on the initial amplitude of the e.j.p. and on the stimulus strength necessary to evoke it. Normorphine (100 nM, 300 nM and 1 μM) was tested on both high amplitude and low amplitude e.j.ps, often on the same cell or tissue. The degree of depression of the high amplitude e.j.ps (35 mV) was the same as the degree of depression of low amplitude e.j.ps (10 mV) by a given concentration of normorphine when the results from many cells and tissues were pooled. These composite results are summarized in Table 1a. Also, for a given individual cell, normorphine (1 μM) was equally effective in depressing the e.j.p. regardless of the control e.j.p. amplitude in the range 9-38 mV (Table 1b).

3.23 Prolonged exposure to normorphine *in vitro*

Some vasa were incubated in Krebs solution containing normorphine (300 nM) for 1-5 h, and the effect of higher concentrations of normorphine (1 and 3 μM) on e.j.p. amplitude was then tested. An example of such an experiment is illustrated in Figure 4. The potency of normorphine (3 μM) in causing a depression of e.j.p. amplitude in vasa maintained in Krebs solution containing normorphine (300 nM) was not different from its potency in vasa maintained in drug-free Krebs solution. Normorphine (1 μM) was less effective in the vasa exposed to opiate drug *in vitro* (Table 2).

Table 1. Effect of e.j.p. amplitude and stimulus voltage on depression of e.j.p. by normorphine. (a) Composite results. *Mean $\pm$ s.e. mean. Numbers in parentheses indicate the number of cells tested. Depression of high amplitude e.j.p. vs. depression of low amplitude e.j.p. was the same for all three concentrations of normorphine (100 nM, 300 nM and 1 μM) (Students's t test, $p>0.05$). (b) Results from three cells. There was no change in the degree of depression caused by normorphine (1 μM) if tested on e.j.ps of different amplitudes recorded in the same cell. E.j.p. amplitude was changed by increasing or decreasing the stimulus strength.

Table 1.

(a) Composite results

	Amplitude of e.j.p. (mV)*	Percent depression of e.j.p.*
NORMORPHINE (100 nM):		
Large amplitude e.j.p.	35.5 ± 1.8 mV	-17 ± 5.2% (7)
Small amplitude e.j.p.	10.4 ± 0.9 mV	-19 ± 5.8% (6)
NORMORPHINE (300 nM):		
Large amplitude e.j.p.	35.3 ± 1.2 mV	-39 ± 5.0% (6)
Small amplitude e.j.p.	10.7 ± 0.8 mV	-46 ± 3.6% (6)
NORMORPHINE (1 µM):		
Large amplitude e.j.p.	34.0 ± 2.1 mV	-73 ± 3.2% (6)
Small amplitude e.j.p.	11.4 ± 1.3 mV	-66 ± 5.3% (6)

(b) Individual cells

Cell #	Stimulus Voltage	Amplitude of e.j.p. (mV)	Percent depression of e.j.p. by Normorphine (1 µM)
4209-2	1.3	10	75
"	7.6	26	73
4279-2	1.0	9	54
"	10.0	16	63
"	17.0	36	65
4309-1	10.4	10	84
"	23.0	38	87

Figure 4. Effect of normorphine (1 and 3 μM) on e.j.p. after prolonged superfusion with normorphine (300 nM) in vitro. This tissue had been maintained in Krebs solution containing normorphine (300 nM) for 4 h at time zero, and normorphine (300 nM) was also present throughout the recording period. During superfusion with normorphine (1 μM) for 5 min, the e.j.p. was depressed by 50%. During superfusion with normorphine (3 μM), also for 5 min, the e.j.p. was depressed by 78%.

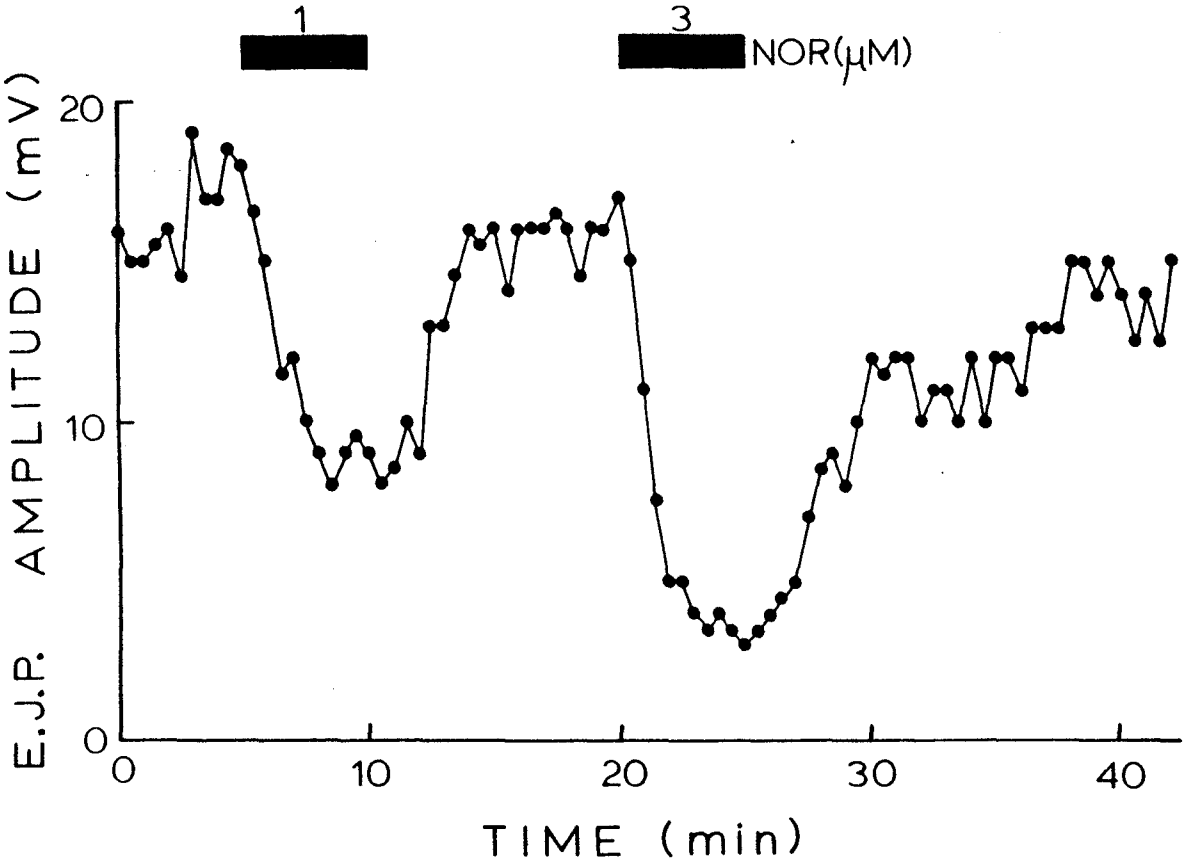

1
3
NOR(μM)
E.J.P. AMPLITUDE (mV)
20
10
0
0
10
20
30
40
TIME (min)

Table 2. Effect of superfusion with drug-free Krebs solution vs. Krebs solution containing normorphine (300 nM) on depression of e.j.p. by normorphine (1 and 3 μM). The depression by normorphine (1 and 3 μM) of amplitudes of evoked e.j.ps was measured in vasa maintained in drug-free Krebs solution or in vasa maintained in Krebs solution containing normorphine (300 nM) for 1-5 h. *Mean ± s.e. mean. Numbers in parentheses indicate the number of cells tested at a given concentration. Values from vasa maintained in drug-free Krebs solution and values from vasa maintained in normorphine-containing Krebs solution were significantly different for normorphine (1 μM) (Student's t test; $p<0.01$) but not for normorphine (3 μM).

	Percent depression of e.j.p.*	
	Nor (1 μM)	Nor (3 μM)
Vasa maintained in drug-free Krebs solution	66 ± 1.5% (16)	78 ± 1.8% (8)
Vasa maintained in Krebs solution containing normorphine (300 nM)	54 ± 2.1% (8)	76 ± 1.5% (8)

3.24 DAEA

DAEA (10-300 nM) decreased the amplitude of the e.j.p. DAEA did not alter the resting membrane potential of the smooth muscle cells. The effect of DAEA on e.j.p. amplitude was maximal within 4 min of the onset of drug superfusion, and quickly reversed (4-6 min) after drug washout. The dose-response curve for the depression of the e.j.p. by DAEA is shown in Figure 3; the EC_{50} for this action of DAEA was 50 nM.

3.25 DADL

DADL (300 pM - 3 nM) decreased the amplitude of the evoked e.j.p. but did not change the resting membrane potential of the smooth muscle cells. Although the maximum effect of this peptide consistently occurred within 4-7 min after onset of superfusion, the time required for washout was variable. In some tissues the e.j.p. amplitude returned to its control amplitude within 5-10 min of resuming superfusion with drug-free Krebs solution. Usually, however, the effect of the higher concentrations of DADL tested (1 and 3 nM) did not completely reverse, even after prolonged washing (20 min or more). The EC_{50} for the depression of e.j.p. amplitude by DADL was 500 pM (Figure 3).

3.26 Naloxone

Naloxone (up to 1 μM) did not change the resting membrane potential of single smooth muscle cells, nor did it change the amplitude of the e.j.p. Naloxone (300 nM) reversed the depression of the e.j.p. caused by superfusion with normorphine (300 nM). Normorphine (300 nM)

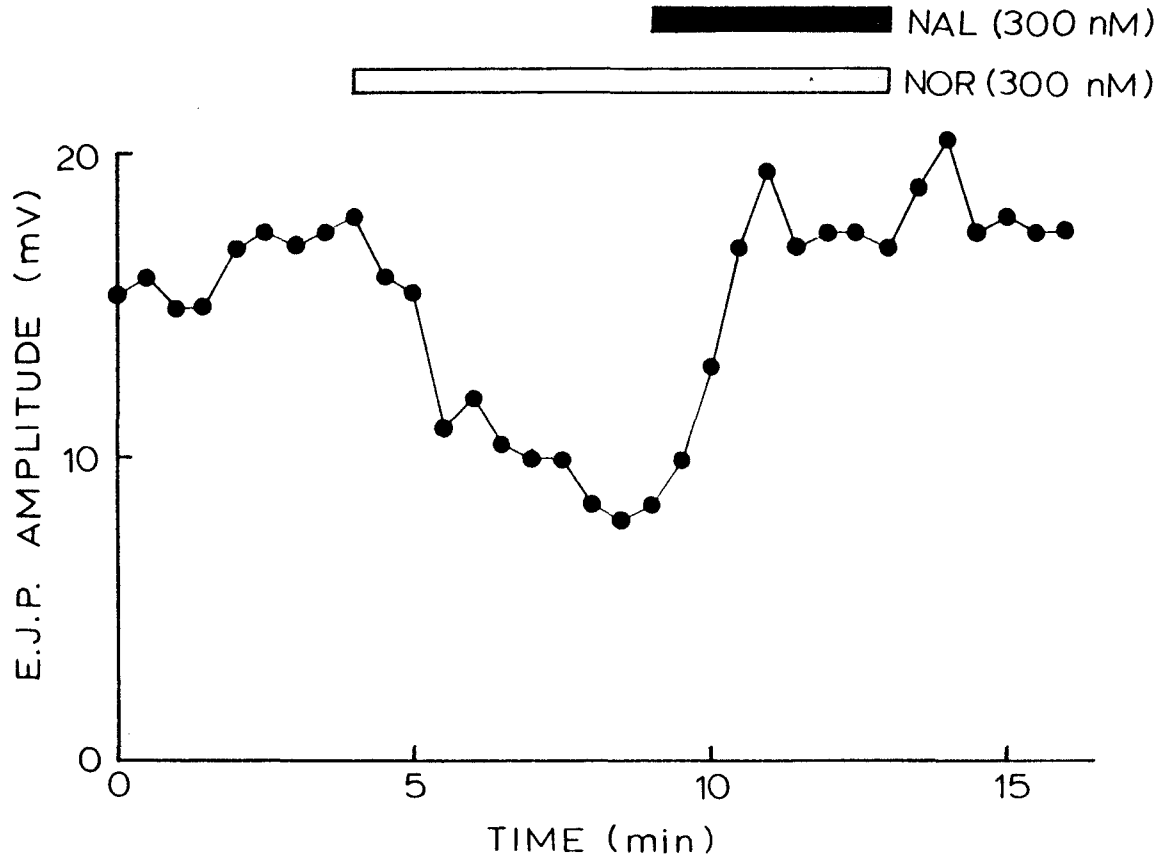
depressed the e.j.p. by an average of 32% (Figure 3). Naloxone (300 nM) reversed this depression, but the e.j.p. amplitude never increased to more than its control value prior to administration of normorphine (Figure 5). In vasa acutely exposed to normorphine (300 nM, for 10 min or less), naloxone increased e.j.p. amplitude by 64 $\pm$ 11% (n = 8). The largest effect observed was a doubling of the e.j.p. in an experiment in which normorphine originally caused a halving of its amplitude.

Naloxone was also tested on vasa that were maintained for 5-8 h in Krebs solution containing normorphine (300 nM). Cells from these preparations responded to naloxone (300 nM) with an increase in e.j.p. amplitude of 184 $\pm$ 21% (n = 4).

3.27 Contractions of the muscle

Short-duration increases in tension of the muscle ("twitches") of the vas deferens occur in response to field stimulation of the intramural nerves. The height of the muscle twitches decreased if normorphine (final bath concentration 100 nM - 1 μM) was injected into the bath. The normorphine EC_{50} was 240 $\pm$ 44 nM (n = 5).

Figure 5. Reversal by naloxone of effect of normorphine on e.j.p. The amplitude of the e.j.p. was depressed by approximately 50% after 5 min of exposure to normorphine (300 nM). This effect was subsequently reversed within 2 min of superfusion with naloxone (300 nM) despite the continued presence of the normorphine.


NAL (300 nM)
NOR (300 nM)
E.J.P. AMPLITUDE (mV)
20
10
0
0
5
10
15
TIME (min)

3.3 Effect of adenosine and cyclic nucleotides

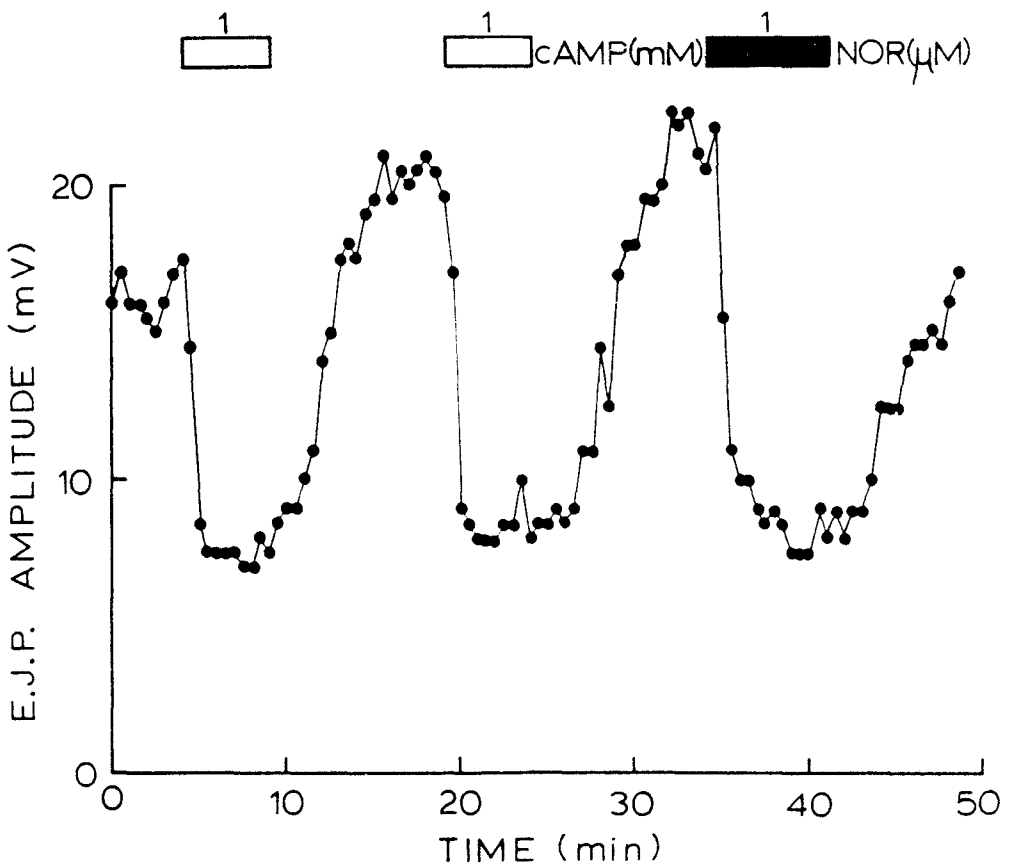
3.31 Adenosine

Adenosine (300 μM and 1 mM) depressed the amplitude of the evoked e.j.p. without changing the resting membrane potential of the smooth muscle cells. This effect was rapid in onset and offset (2-5 min). The magnitude of the effect of adenosine (1 mM) did not diminish if the substance was repeatedly applied to the same cell at intervals of 10-15 min. Adenosine (300 μM) depressed the e.j.p. by 42 $\pm$ 3.0% (n = 11), whereas adenosine (1 mM) depressed it by 64 $\pm$ 3.3% (n = 13).

3.32 Cyclic AMP and dbcAMP

The resting membrane potential of single smooth muscle cells was not changed by the addition of cAMP or dbcAMP (up to 1 mM) to the superfusing solution; both of these substances, however, depressed the amplitude of the e.j.p. The effect of the cyclic nucleotides, like that of adenosine, was rapid in onset and offset (2-5 min). The effect did not diminish with repeated drug applications (at 10-15 min intervals) to the same cell or tissue, as illustrated for cAMP (1 mM) in Figure 6. Cyclic AMP (1 mM) decreased the e.j.p. amplitude by 52 $\pm$ 3.9% (n = 9). The e.j.p. was decreased by 13% and by 30% in the two cells on which cAMP (300 μM) was tested; cAMP (100 μM) had no effect (two cells). DbcAMP (1 mM) decreased e.j.p. amplitude by 22.5 $\pm$ 3.7% (n = 8); lower concentrations of this substance were not tested.

Figure 6. Comparison of effect of cAMP with effect of normorphine on e.j.p. During the first application (for 5 min) of cAMP (1 mM), the e.j.p. was depressed by 57%. The second application (also for 5 min) of the same concentration of cAMP 10 min later resulted in a 61% depression of the e.j.p. The amplitude of the e.j.p. was decreased by 66% after 7 min of superfusion with normorphine (1 μM). In this cell, normorphine was about 1,000 times more potent than cAMP.


1
1
cAMP(mM)
1
NOR(μM)
20
10
0
E.J.P. AMPLITUDE (mV)
0
10
20
30
40
50
TIME (min)

3.4 Effect of phosphodiesterase inhibitors

3.41 IBMX or SQ 20,006 alone

Neither the xanthine IBMX nor the non-xanthine SQ 20,006 (up to 1 mM) altered the resting membrane potential of smooth muscle cells, but both increased the amplitude of the e.j.p. This increase was slow in onset, reaching its maximum after 8-12 min of drug superfusion, and subsequently was slow to reverse upon washing (15-30 min). IBMX (50 μM) increased e.j.p. amplitude 58 ± 9% (n = 6) and IBMX (500 μM) increased it 167 ± 20% (n = 7). A second application of a given concentration of IBMX to the same cell or preparation (after 15 min - 3 h) was less effective than the initial application (Table 3). SQ 20,006 (500 μM) enhanced e.j.p. amplitude by 54 ± 13% (n = 5).

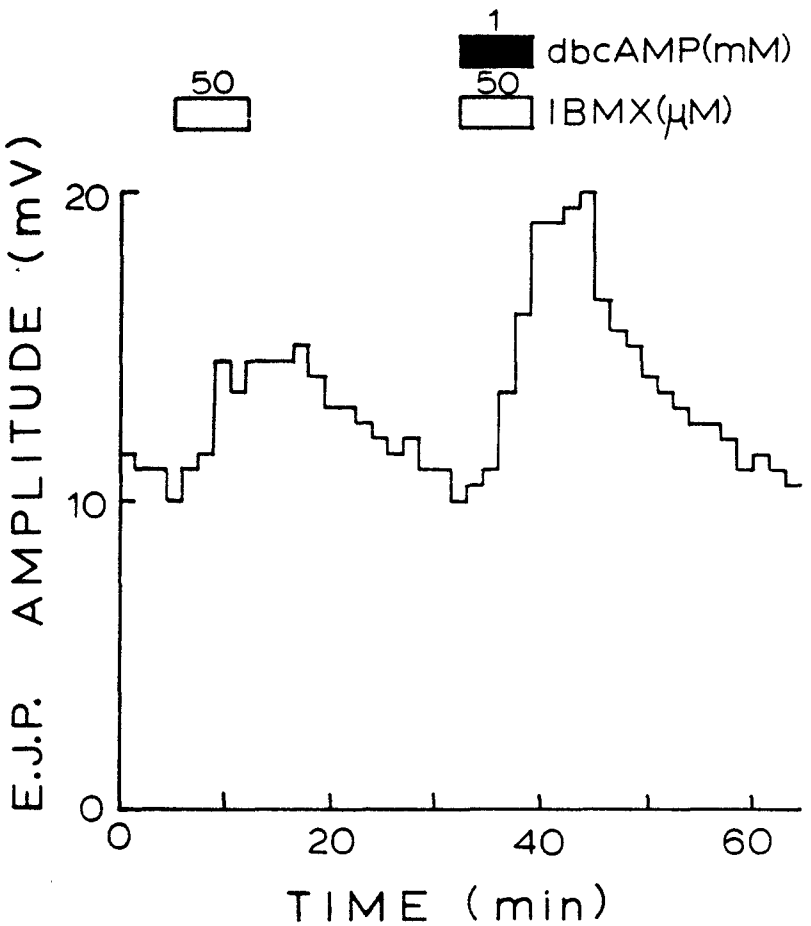
3.42 The combination phosphodiesterase inhibitor and dbcAMP

DbcAMP alone weakly depressed the e.j.p., whereas IBMX increased the e.j.p. (see above). But IBMX and dbcAMP applied together were more effective than IBMX alone in increasing the e.j.p. (Figure 7). Thus IBMX (50 μM) in combination with dbcAMP (500 μM) increased e.j.p. amplitude by 103 ± 16% (n = 5). Also, IBMX (500 μM) + dbcAMP (250 μM) increased it by 182 ± 21% (n = 5). The combination IBMX (500 μM) + dbcAMP (500 μM) increased it by an average of 285% (n = 3). These effects on e.j.p. amplitude were larger in magnitude than those obtained by application of IBMX (50 or 500 μM) alone (Table 3). In one experiment dbcAMP (1 mM), applied during superfusion with IBMX (50 μM)

Table 3. Increase of e.j.p. amplitude by IBMX: effect of concentration and repeated application. *Mean ± s.e. mean. Numbers in parentheses indicate the number of individual cells tested. The effect of IBMX (superfused for 10-15 min) on its second application was less than that on its first application at both concentrations tested (Student's t test; $p<0.01$).

Concentration of IBMX	Increase of e.j.p. amplitude (%)* First application	Increase of e.j.p. amplitude (%)* Second application
50 µM	58 ± 9% (6)	12 ± 6% (5)
500 µM	167 ± 20% (7)	82 ± 10% (6)

Figure 7. Comparison of effect of IBMX alone with effect of drug combination IBMX + dbcAMP on e.j.p. E.j.p. amplitude vs. time is plotted as a histogram. Each bin is the average of the amplitudes of three consecutive e.j.ps. The second application of IBMX (50 μM) to this tissue (for 6 min) resulted in a 32% increase of the e.j.p. (the first application is not shown). Subsequent exposure of the cell to both IBMX (50 μM) and dbcAMP (1 mM) for 6 min produced an increase in e.j.p. amplitude of 95%.

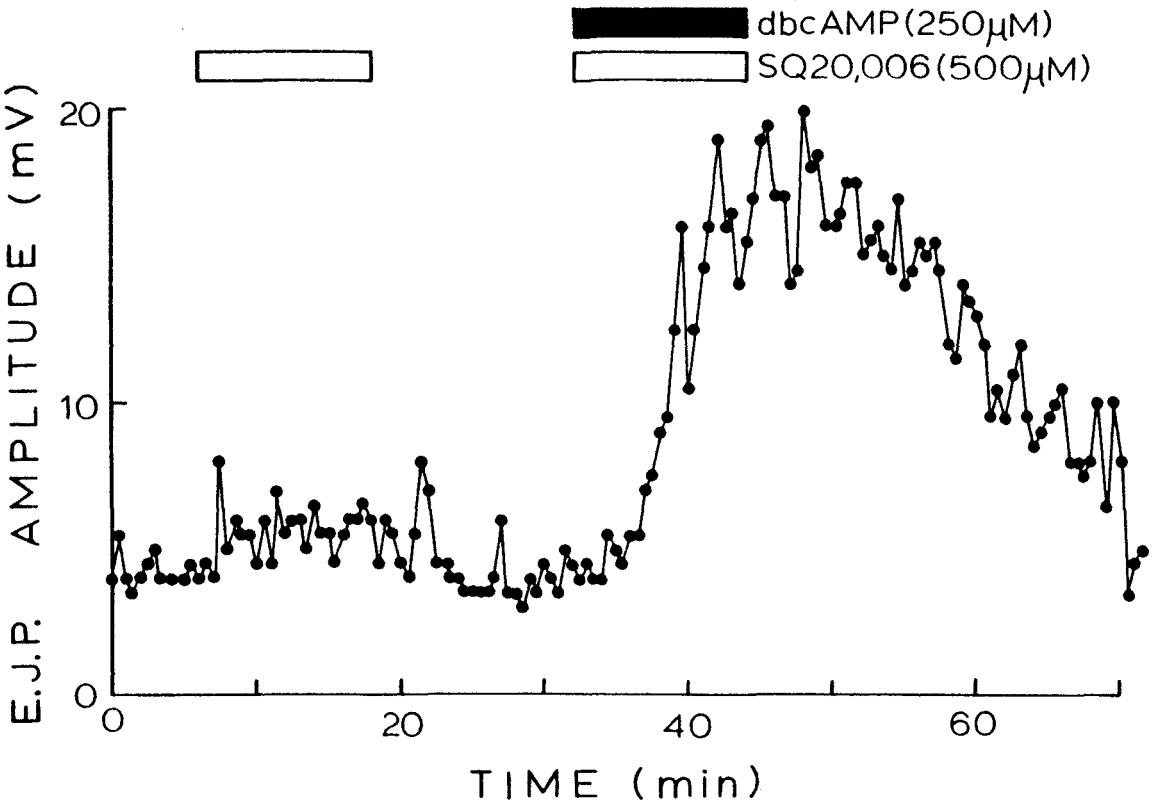

1
dbcAMP(mM)
50
50
IBMX(μM)
E.J.P. AMPLITUDE (mV)
20
10
0
0
20
40
60
TIME (min)

and after the effect of IBMX was maximal, increased e.j.p. amplitude by 59%.

The drug combination SQ 20,006 and dbcAMP was also more effective than SQ 20,006 alone (Figure 8). SQ 20,006 (500 μM) + dbcAMP (250 μM) increased e.j.p. amplitude by 392 $\pm$ 43% (n = 4); by itself, this concentration of SQ 20,006 had only a small effect (see above).

In a few experiments the effect of the drug combination IBMX and dbcAMP was tested on the isotonic contractions of the smooth muscle of the vas deferens. The contraction produced by electrical stimulation of the intramural nerves was reduced by superfusion with Krebs solution containing IBMX (500 μM) and dbcAMP (250 μM). Contractile responses to exogenous noradrenaline (10 μM) and to high potassium solution (50 mM KCl) could not be elicited in the presence of these two compounds.

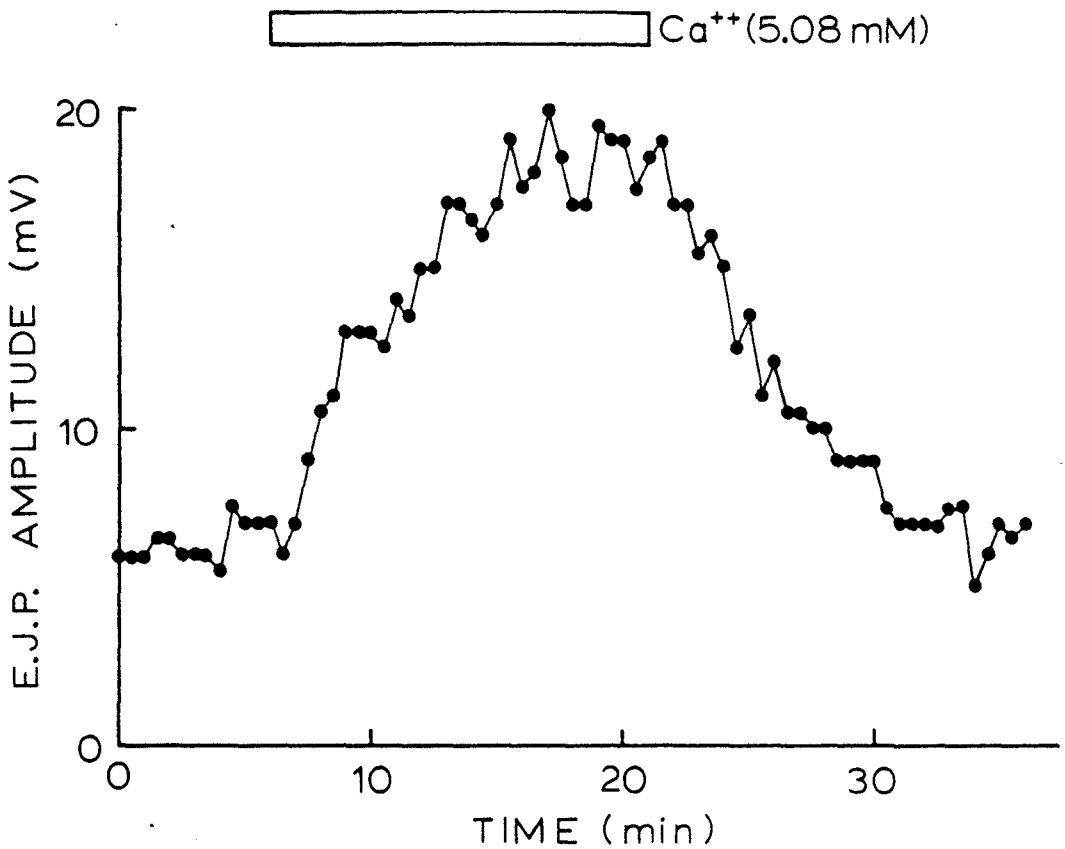
Figure 8. Comparison of effect of SQ 20,006 alone with effect of drug combination SQ 20,006 + dbcAMP on e.j.p. A 12 min exposure to SQ 20,006 (500 μM) produced only a small increment in e.j.p. amplitude (37%) in this cell. Subsequent superfusion (also for 12 min) with the same concentration of SQ 20,006 in combination with dbcAMP (250 μM) resulted in a dramatic 336% enhancement of the amplitude of the e.j.p. The effect of these substances in combination was not apparent until at least 5 min after switching to the drug-containing solution (dead time was about 1 min), and was also slow to reverse. E.j.p. amplitude returned to its control value only after 30 min of washing with drug-free Krebs solution.


dbcAMP(250μM)
SQ20,006(500μM)
E.J.P. AMPLITUDE (mV)
20
10
0
0
20
40
60
TIME (min)

3.5 Effect of altered calcium ion concentration

Doubling the calcium ion concentration of the Krebs solution (to 5.08 mM) resulted in an increase in e.j.p. amplitude of 144 $\pm$ 20% (n = 5), whereas halving it (to 1.27 mM) resulted in a decrease in e.j.p. amplitude of 66 $\pm$ 4.9% (n = 6). These effects of altered calcium ion concentration stabilized within 5-12 min of changing the Krebs solution, and reversed within 10-15 min of resuming superfusion with control Krebs solution (Figure 9). When the calcium ion concentration was doubled or halved, changes in the membrane potential of the smooth muscle cells were less than 5 mV.

Figure 9. Effect of doubling the calcium ion concentration on e.j.p. The amplitude of the e.j.p. in this cell increased almost three-fold (192%) during a 15 min superfusion with Krebs solution containing twice the normal calcium ion concentration. The effect of the changed solution peaked after 10 min and washed within 10-15 min.


Ca++(5.08 mM)
E.J.P. AMPLITUDE (mV)
20
10
0
0
10
20
30
TIME (min)

3.6 Effect of prolonged exposure to morphine *in vivo*

In order to assess the effect of prolonged exposure to morphine on neuro-effector transmission in the mouse vas deferens, vasa from mice that had received morphine injections for three d (see Methods) were studied. Vasa from these mice were compared to vasa from naive mice which received no injections with respect to: resting membrane potential of smooth muscle cells, effects of normorphine, adenosine and naloxone on the e.j.ps, and contractions of the muscle in response to noradrenaline.

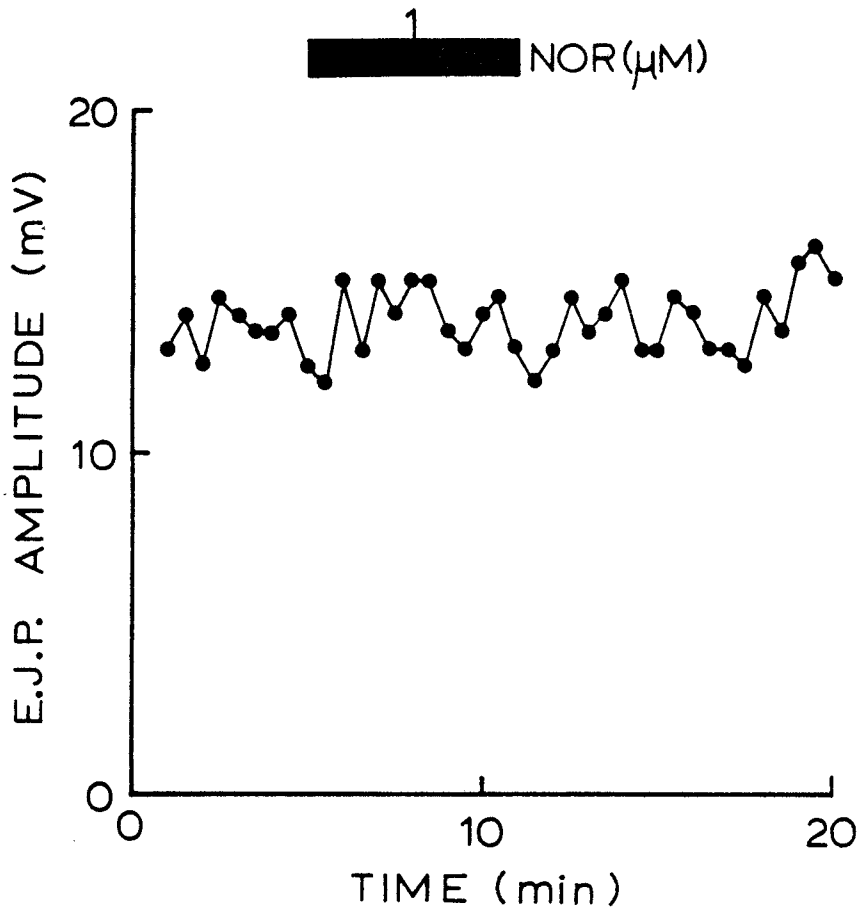
3.61 Membrane potentials

The resting membrane potential of individual smooth muscle cells was 63.0 $\pm$ 2.1 mV (n = 11) in vasa from morphine-pretreated animals. This value was not significantly different from the value of the resting membrane potential in single cells of vasa from naive mice (62.6 $\pm$ 0.9 mV)(Student's t test). Morphine pretreatment produced no obvious change in the frequency and/or amplitudes of spontaneous junction potentials, although careful measurements were not made.

3.62 Normorphine

Vasa from morphine-pretreated mice were maintained in Krebs solution containing normorphine (300 nM) (see Methods). Normorphine (1-30 µM) did not alter the resting membrane potentials of the smooth muscle cells. Normorphine (1 µM) did not depress the e.j.p. in cells from these vasa (Figure 10). Higher concentrations of normorphine (3-30 µM)

Figure 10. Effect of normorphine (1 μM) on e.j.p. (morphine-pretreated mouse). This tissue had been maintained in Krebs solution containing normorphine (300 nM) for 80 min at time zero; this concentration of normorphine was also present throughout the recording period. Application of normorphine (1 μM) for 6 min had no effect on the amplitude of the e.j.p.


1
NOR(μM)
20
10
0
E.J.P. AMPLITUDE (mV)
0
10
20
TIME (min)

decreased the amplitude of the e.j.p. (Figure 11) with a time course similar to the time course of action in vasa from naive mice. The dose-response curve for depression of e.j.p. amplitude by normorphine in vasa from morphine-pretreated mice was shifted to the right of the dose-response curve for this action of normorphine in vasa from naive mice (Figure 12). The EC_{50} for normorphine in vasa from morphine-pretreated mice was 6.6 μM (compared to 560 nM in vasa from naive mice). Individual EC_{50} values for tissues exposed to morphine *in vivo* ranged from 3 to 30 μM, and did not overlap with individual EC_{50} values in vasa from naive mice (270 - 890 nM). Depressions of e.j.p. amplitude by normorphine of 80-90% could be obtained in vasa from both groups of animals.

3.63 Adenosine

Adenosine (300 μM and 1 mM) decreased e.j.p. amplitude without an effect on the resting membrane potential. The effect had the same rapid time course (onset and offset of action within 2-5 min) in tissues from morphine-pretreated mice as in vasa from naive mice (Figure 13). Adenosine (300 μM) depressed the e.j.p. by 40 $\pm$ 6.0% (n = 8). Adenosine (1 mM) depressed the e.j.p. by 70 $\pm$ 4.4% (n = 9). The magnitude of these effects of adenosine in vasa from morphine-pretreated mice did not differ from the magnitude of the effects of adenosine in vasa from naive mice (Table 4).

Figure 11. Effect of normorphine (3 and 10 μM) on e.j.p. (morphine-pretreated mouse). This tissue had been maintained in Krebs solution containing normorphine (300 nM) for 2 h at time zero, and throughout period of recording. The e.j.p. amplitude was decreased by 20% by a 10-fold increase in the normorphine concentration of the Krebs solution (from 300 nM to 3 μM) for 5 min. In this tissue, the e.j.p. was reduced 50% by normorphine (10 μM), applied for 5.5 min. Compare these results with those illustrated in Figure 4.

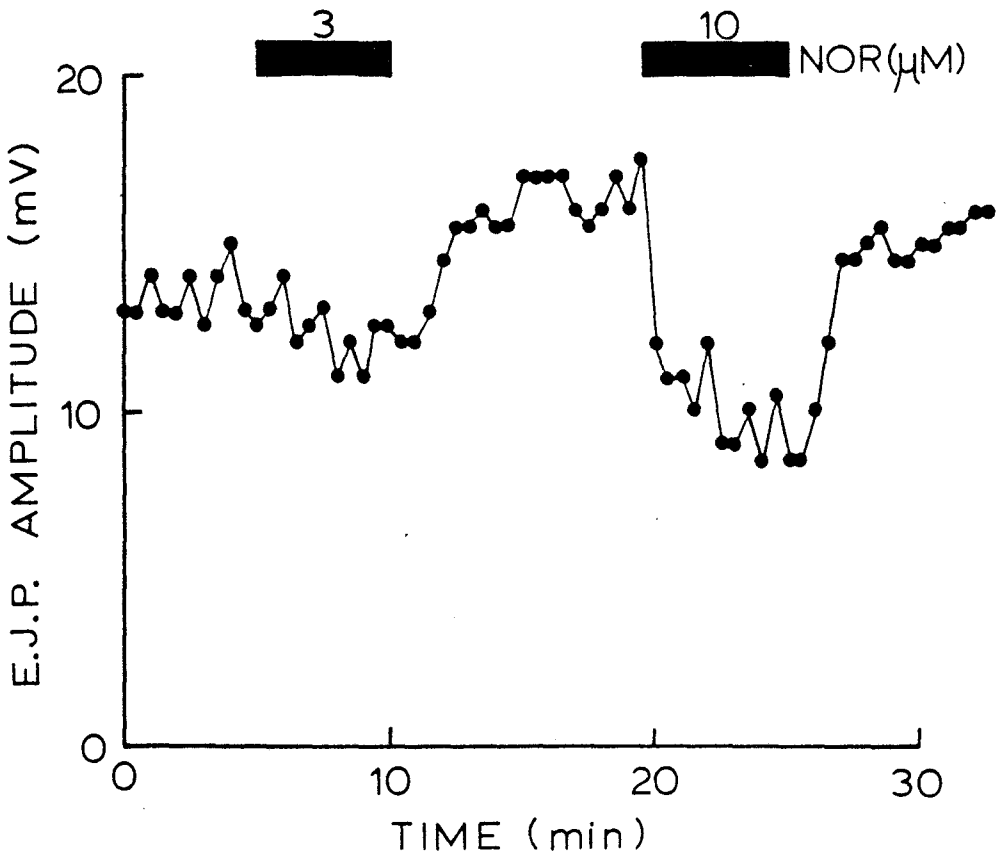

3
10
NOR(μM)
20
10
0
E.J.P. AMPLITUDE (mV)
0
10
20
30
TIME (min)

Figure 12. Normorphine dose-response curves for depression of e.j.p.: naive mice vs. morphine-pretreated mice. Vasa from morphine-pretreated mice were maintained in Krebs solution containing normorphine (300 nM). Bars represent $\pm$ s.e. mean. Numbers in parentheses indicate the number of individual cells tested at a given concentration.

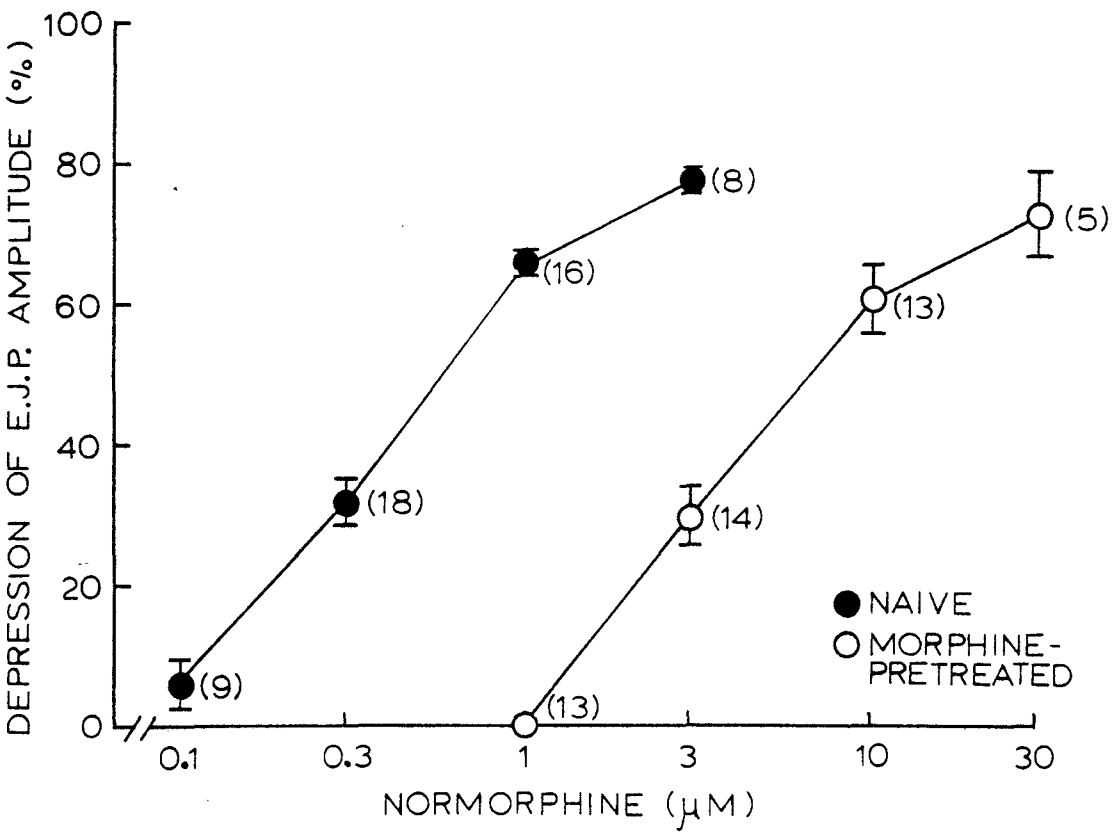

DEPRESSION OF E.J.P. AMPLITUDE (%)
100
80
60
40
20
0
(9)
(18)
(16)
(8)
(13)
(14)
(13)
(5)
NAIVE
MORPHINE-PRETREATED
0.1
0.3
1
3
10
30
NORMORPHINE (μM)

Figure 13. Effect of adenosine on e.j.p. (morphine-pretreated mouse). Adenosine (300 μM) decreased the e.j.p. from 16 mV to 10.5 mV (a 34% depression) whereas adenosine (1 mM) decreased it from 18 mV to 6 mV (a 67% depression). Both concentrations were applied for 5 min. The onset and offset of these effects in this cell were rapid (2 min), and were typical of the time course of the effects of adenosine on cells from both naive and morphine-pretreated tissues.

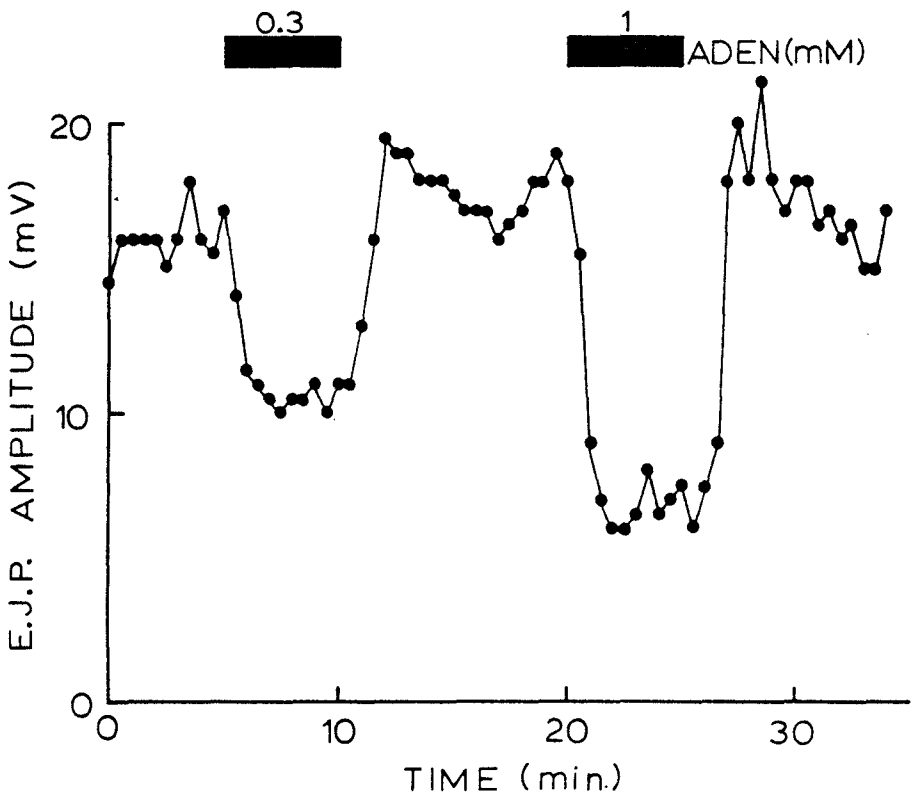

0.3
1
ADEN(mM)
20
10
0
E.J.P. AMPLITUDE (mV)
0
10
20
30
TIME (min.)

Table 4. Depression of e.j.p. by adenosine in vasa from naive and morphine-pretreated mice. *Mean ± s.e. mean. Numbers in parentheses indicate the number of cells tested at a given concentration. There was no difference between values in control (naive) mice and morphine-pretreated mice at either concentration (Student's t test).

	Percent inhibition of e.j.p.*	
	Adenosine (300 µM)	Adenosine (1 mM)
Control (naive) mice	42 ± 3.0% (11)	64 ± 3.3% (13)
Morphine-pretreated mice	40 ± 6.0% (8)	70 ± 4.4% (9)

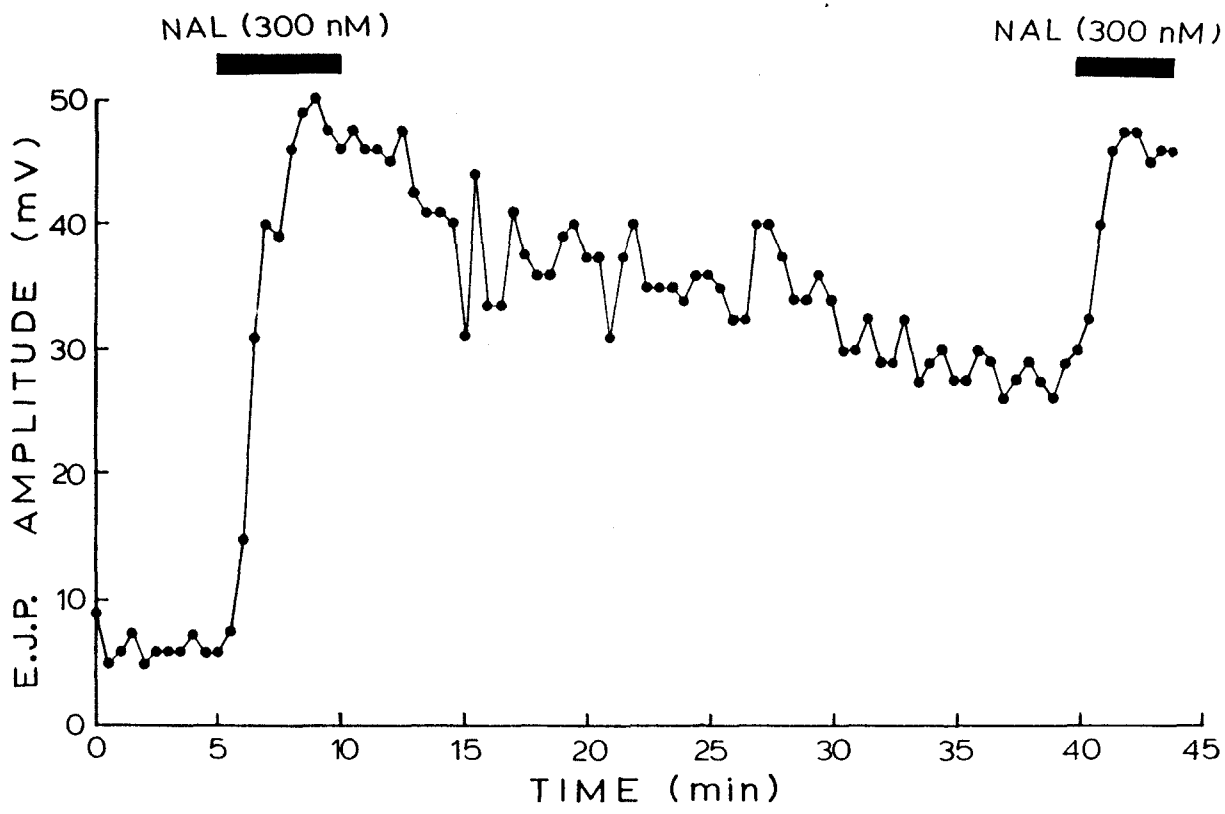
3.64 Naloxone

Naloxone (up to 1 μM) did not alter the resting membrane potential of the smooth muscle cells; nor did it cause any marked change in the frequency of spontaneous e.j.ps when it was applied to the vasa from morphine-pretreated mice. In these vasa (maintained in Krebs solution containing normorphine), changing to a solution that contained both normorphine (300 nM) and naloxone (300 nM) caused a large increase in the amplitude of the e.j.p. (Figure 14). This effect was observed in 9 cells from 9 different tissues; the average increase in e.j.p. amplitude was 360 $\pm$ 37% (range 254% to 600%). The increase in e.j.p. amplitude peaked within 3-5 min of the addition of naloxone to the superfusing Krebs solution, but the effect was slow to reverse (Figure 14). In most instances the impalement was terminated before the e.j.p. amplitude returned to its pre-naloxone value.

3.65 Drug-free Krebs solution

The superfusion of vasa from morphine-pretreated mice (maintained in Krebs solution containing normorphine) with normorphine-free Krebs solution caused an increase in the amplitude of the e.j.p. In the three cells from three different tissues that were tested in this manner, application of drug-free Krebs solution (for 8-11 min) increased e.j.p. amplitude by 365%, 240% and 100% respectively.

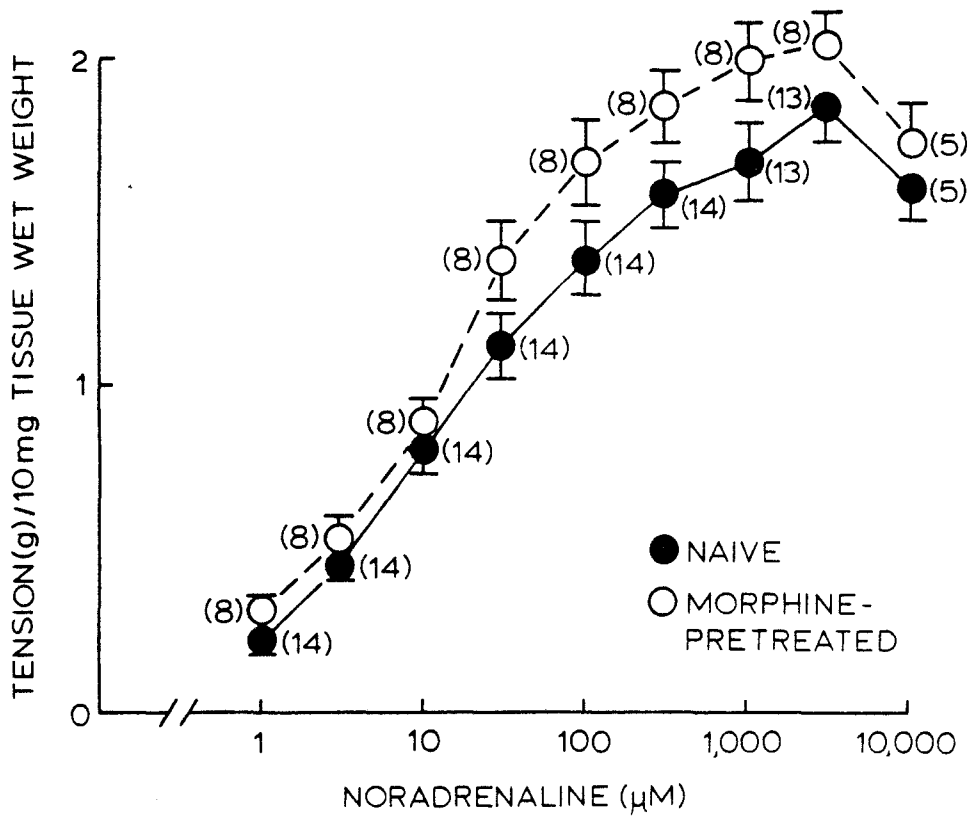
Figure 14. Effect of naloxone on e.j.p. (morphine-pretreated mouse). Normorphine (300 nM) was present throughout the recording period and for 4 h previously at time zero. The addition of naloxone (300 nM) to the superfusing Krebs solution (for 5 min) caused a 7-fold (600%) increase in the e.j.p. This effect was slow to reverse upon washing out the naloxone. A second application of naloxone (300 nM) to the cell was again effective in enhancing the e.j.p. amplitude, although the recording was prematurely terminated before the magnitude and duration of this second effect could be ascertained.


NAL (300 nM)
NAL (300 nM)
E.J.P. AMPLITUDE (mV)
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
TIME (min)

3.66 Contractions of the muscle

The contractions of the smooth muscle of the vas deferens in response to exogenous noradrenaline were measured for tissues prepared from both naive and morphine-pretreated mice. When noradrenaline (1 μM - 10 mM) was added to the organ bath, the vas deferens developed an increase in tension which peaked rapidly and then declined before washout of the drug. The rate of change of tension increased with increasing drug concentration, provided the tip of the injecting needle was in the same position in the organ bath relative to the tissue. With high concentrations of noradrenaline (100 μM - 10 mM), the initial peak tension was followed by a series of rapid fluctuations in tension of lower amplitude. Only the initial peak response was measured. Dose-response curves for these responses of the vasa to noradrenaline were constructed for vasa from both morphine-pretreated and naive mice (Figure 15). The noradrenaline EC_{50} (concentration producing a peak tension which was one-half of the maximum response to this substance) was 10.4 μM in vasa from morphine-pretreated mice and 10.5 μM in vasa from naive mice. The maximum response of the tissue to noradrenaline (expressed as mg tension/10 mg wet weight of tissue) was 2,044 $\pm$ 106 (n = 8) in vasa exposed to morphine *in vivo* and 1,851 $\pm$ 109 (n = 13) in vasa from naive mice. These values were not significantly different (Student's t test). The wet weight of the vasa from morphine-pretreated mice (11.5 $\pm$ 0.6 mg, n = 8) also was not different (Student's t test) from the wet weight of vasa from naive mice (12.7 $\pm$ 0.5 mg, n = 14).

Figure 15. Dose-response curves for contractile responses of the smooth muscle to noradrenaline: vasa from naive mice vs. vasa from morphine-pretreated mice. Bars indicate $\pm$ s.e. mean. Numbers in parentheses indicate the number of tissues tested with a given concentration. At all concentrations, differences between values from control animals and values from morphine-pretreated animals were compared using Student's t test. Only at one concentration of noradrenaline (1 mM) were these values significantly different ($p = 0.04$).


TENSION(g)/10mg TISSUE WET WEIGHT
2
1
0
1
10
100
1,000
10,000
NORADRENALINE (μM)
NAIVE
MORPHINE-PRETREATED
(8)
(14)
(13)
(5)

The nerve-mediated muscle twitches of vasa from morphine-pretreated mice were inhibited by normorphine (1-30 μM). Individual EC_{50} values for normorphine were determined for six vasa from morphine-pretreated mice and ranged from 1.6 μM to 7.8 μM. The normorphine EC_{50} was 4,000 $\pm$ 890 nM (n = 6), which was significantly different from the EC_{50} for the inhibition by normorphine of the muscle twitches in vasa from naive mice (240 $\pm$ 44 nM).

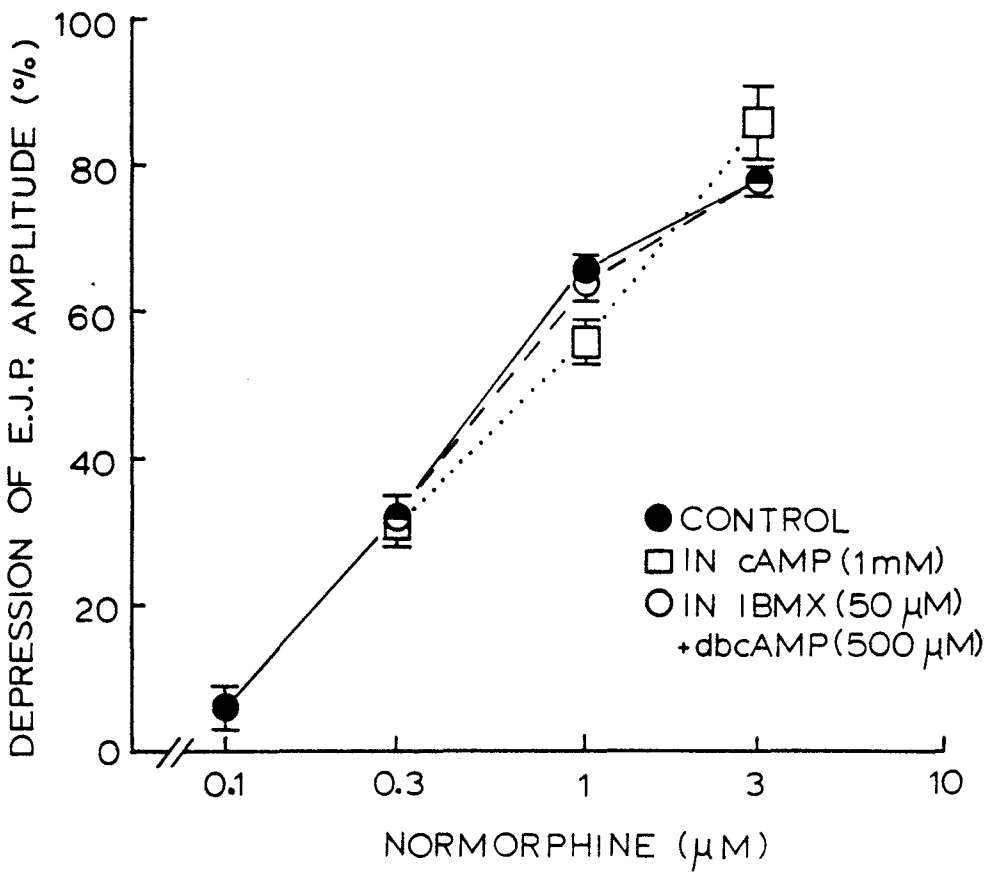
3.7 Effect of cyclic nucleotides and IBMX on the actions of normorphine and opioid peptides

3.71 Normorphine

The effect of normorphine on e.j.p. amplitude was measured in vasa superfused with cAMP (1 mM) and compared to its effect in vasa that were not treated with this substance. This concentration of cAMP depressed e.j.p. amplitude by 52 $\pm$ 3.9% (see above). The tissue was superfused with cAMP (1 mM) for 5-30 min before testing normorphine. Stimulus voltage was adjusted prior to the application of cAMP so that the e.j.p. was between 10 and 25 mV in amplitude before the application of normorphine. Normorphine decreased e.j.p. amplitude in the presence of cAMP (1 mM), with a time course of onset and offset of action similar to its effect in the absence of this nucleotide in other vasa. The EC_{50} for depression of e.j.p. amplitude by normorphine in the presence of cAMP (1 mM) was 700 nM, as determined from the composite dose-response curve (Figure 16). For comparison, the dose-response curve for normorphine in vasa not treated with cyclic nucleotide is also illustrated in this figure.

Normorphine was also tested in preparations that had been superfused with both IBMX (50 µM) and dbcAMP (500 µM) for at least 12 min. This drug combination increased e.j.p. amplitude by 103 $\pm$ 16%, as determined by testing on other tissues (see above). Normorphine still decreased e.j.p. amplitude in the presence of these substances and the time course of onset of its effect was not changed. Often the reversal

Figure 16. Normorphine dose-response curves for depression of e.j.p.: effect of cAMP and the drug combination IBMX + dbcAMP. Dose-response curves for normorphine were constructed using results from tissues that were not pretreated with any drugs, results from tissues exposed to cAMP (1 mM) and results from tissues pretreated with IBMX (50 μM) + dbcAMP (500 μM). Bars represent ± s.e. mean. The number of individual cells tested at each concentration was at least six. Control values and values after incubation in cAMP or incubation in the drug combination IBMX + dbcAMP were compared by one-way analysis of variance at three concentrations (300 nM, 1 μM and 3 μM). No significant effect of these substances on the response to normorphine was found.


DEPRESSION OF E.J.P. AMPLITUDE (%)
100
80
60
40
20
0
0.1
0.3
1
3
10
NORMORPHINE (μM)
CONTROL
IN cAMP (1mM)
IN IBMX (50 μM)
+dbcAMP (500 μM)

of the depression of the e.j.p. caused by normorphine was faster in vasa superfused with IBMX + dbcAMP (Figure 17) than in vasa not treated with these substances. The dose-response curve for normorphine in tissues superfused with both IBMX (50 μM) and dbcAMP (500 μM) was virtually superimposable on the dose-response curve obtained from tissues not exposed to these substances (Figure 16). The normorphine EC_{50} was 590 nM for tissues exposed to IBMX (50 μM) + dbcAMP (500 μM), as compared to 560 nM in drug-naive preparations.

3.72 DAEA

The effect of DAEA on e.j.p. amplitude was tested on vasa exposed to both IBMX (500 μM) and dbcAMP (250 μM) for 12 min or more, and compared to the effect of DAEA on vasa not so treated. In other experiments, IBMX and dbcAMP applied together in these concentrations increased e.j.p. amplitude by 182 $\pm$ 21% (see above). DAEA (10-300 nM) still decreased e.j.p. amplitude in the presence of IBMX + dbcAMP (Figure 18); the time course of this effect was the same as in untreated tissues. The potency of DAEA was slightly enhanced in tissues exposed to IBMX + dbcAMP compared to control tissues, as is evident from the composite dose-response curves (Figure 19). The magnitude of the effect of DAEA in tissues exposed to IBMX + dbcAMP was significantly different from the magnitude of the effect in control tissues at two concentrations (10 nM: $p<0.01$; 100 nM: $p<0.05$; Student's t test). The EC_{50} for DAEA in vasa superfused with IBMX + dbcAMP was 27 nM,

Figure 17. Effect of normorphine (1 μM) on e.j.p. in absence and presence of IBMX (50 μM) + dbcAMP (250 μM): same cell. During the time period indicated by the striped bar and dotted line, stimulus voltage was adjusted (from 23 to 16 volts) to offset the increase of the e.j.p. caused by IBMX + dbcAMP. Normorphine (1 μM) depressed e.j.p. amplitude by 55% in the control solution and by 52% after 12 min of superfusion with the combination of IBMX + dbcAMP. The effect of normorphine washed out faster during exposure to IBMX + dbcAMP.

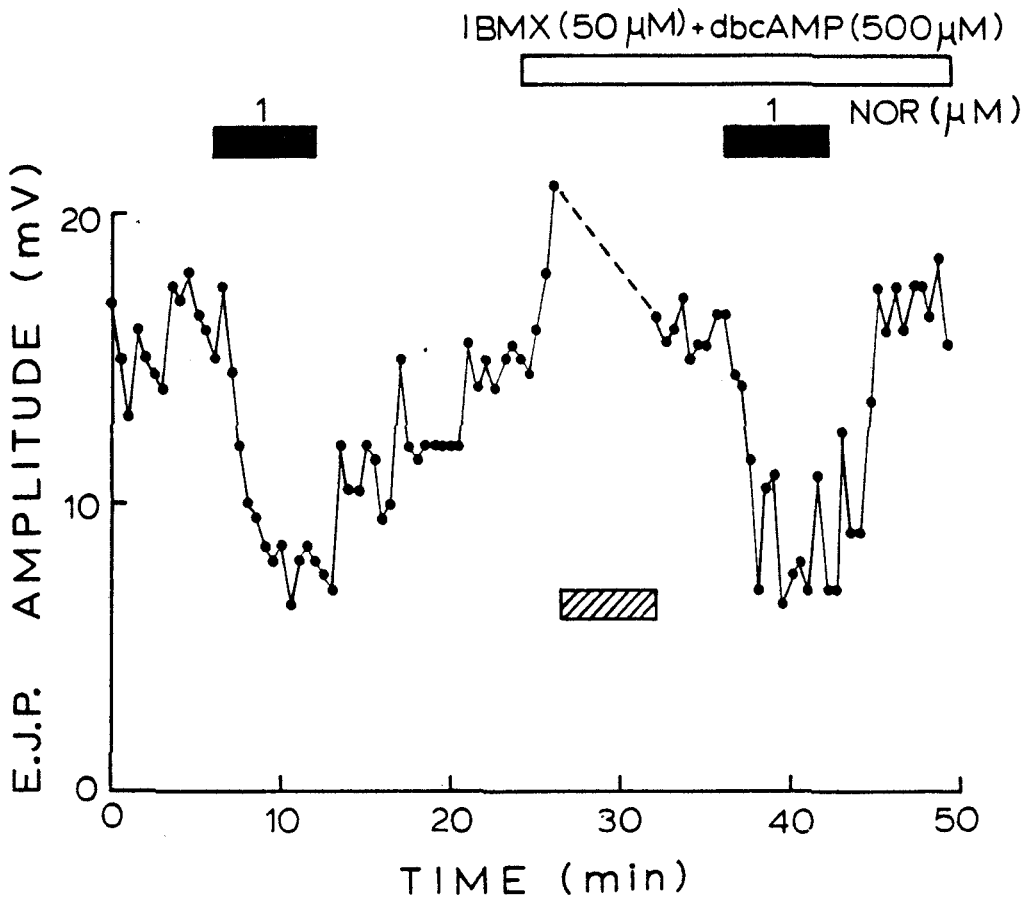

IBMX (50 μM) + dbcAMP (500 μM)
1
1
NOR (μM)
E.J.P. AMPLITUDE (mV)
20
10
0
0
10
20
30
40
50
TIME (min)

Figure 18. Effect of DAEA on e.j.p. during superfusion with IBMX + dbcAMP. This tissue was exposed to IBMX (500 μM) and dbcAMP (250 μM) throughout the period of recording, as well as for 12 min previous to time zero. DAEA (10-300 nM) decreased the e.j.p. amplitude in this cell, and the magnitude of the effect was larger for the higher concentrations.

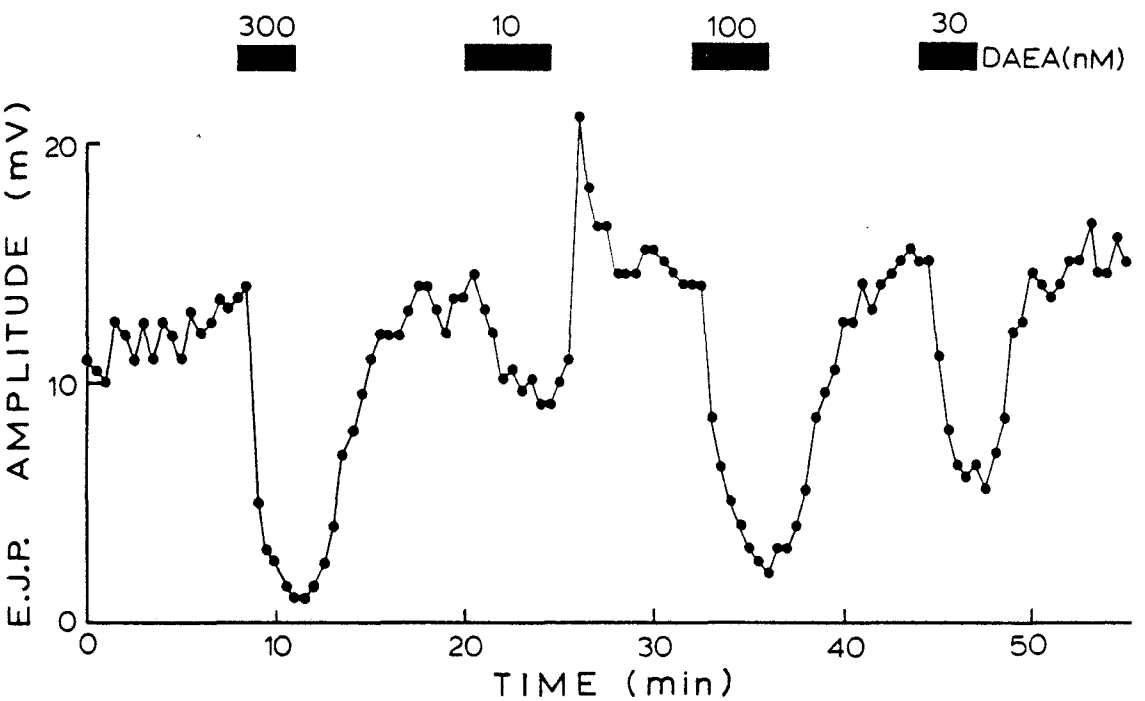
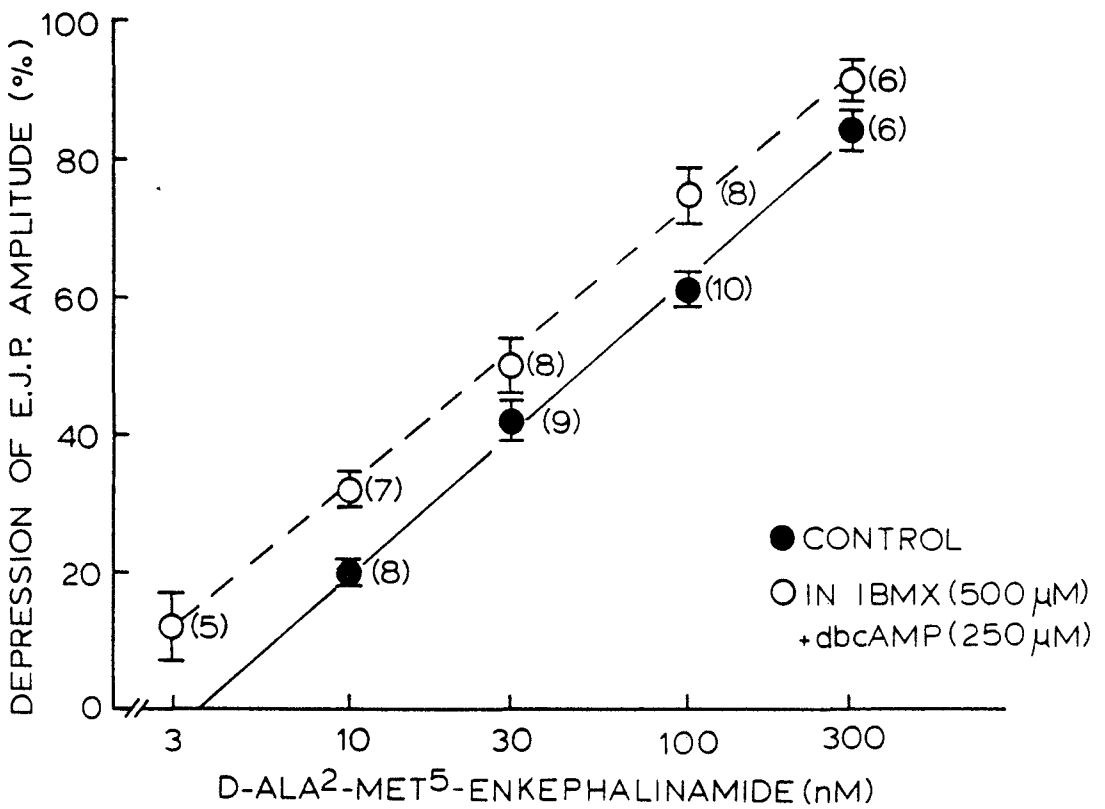

300
10
100
30
DAEA(nM)
E.J.P. AMPLITUDE (mV)
20
10
0
0
10
20
30
40
50
TIME (min)

Figure 19. Dose-response curves for depression of e.j.p. by DAEA: effect of IBMX + dbcAMP. These dose-response curves were constructed using results from tissues not exposed to any drugs and results from tissues superfused with the drug combination IBMX (500 μM) and dbcAMP (250 μM). Bars represent ± s.e. mean. Numbers in parentheses indicate the number of individual cells tested at a given concentration.


DEPRESSION OF E.J.P. AMPLITUDE (%)
100
80
60
40
20
0
3
10
30
100
300
D-ALA2-MET5-ENKEPHALINAMIDE (nM)
(5)
(7)
(8)
(8)
(9)
(6)
(6)
(8)
(10)
CONTROL
IN IBMX (500 μM)
+dbcAMP (250 μM)

whereas the EC_{50} for DAEA in control vasa was 50 nM.

3.73 DADL

The effect of DADL on e.j.p. amplitude was tested on vasa superfused with both IBMX (500 μM) and dbcAMP (250 μM) for 12 min or more, and compared to the effect of DADL on control tissues. This combination of IBMX + dbcAMP increased the e.j.p. amplitude (see above). DADL (300 pM - 3 nM) decreased the amplitude of the e.j.p. in the presence of IBMX + dbcAMP (Figure 20); the time course of this effect was the same as in untreated tissues. Composite dose-response curves for DADL in tissues superfused with IBMX + dbcAMP and in control tissues are illustrated in Figure 21. There were no significant differences between values from these two groups of vasa (Student's t test). The EC_{50} for DADL in the treated vasa was 540 pM and the EC_{50} in control vasa was 500 pM.

Figure 20. Effect of DADL on e.j.p. during superfusion with IBMX + dbcAMP. This tissue was exposed to IBMX (500 μM) + dbcAMP (250 μM) throughout the period of recording, as well as for 12 min previous to time zero. In this cell, DADL (300 pM) depressed the e.j.p. by 30%, DADL (1 nM) depressed it by 61% and DADL (3 nM) depressed it by 83%. It can be seen that the effects of the higher concentrations of the peptide tested (1 and 3 nM) did not completely reverse upon returning to superfusion with normal Krebs solution. This was characteristic of the depressant action of this compound.

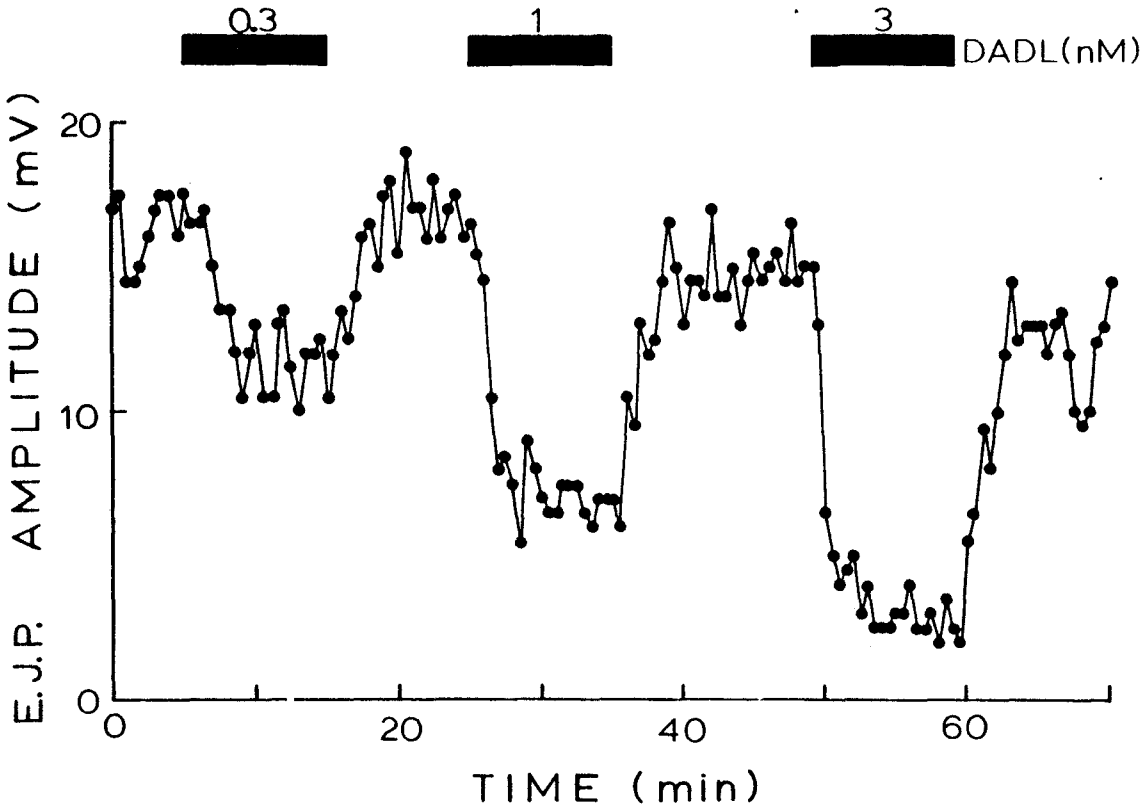
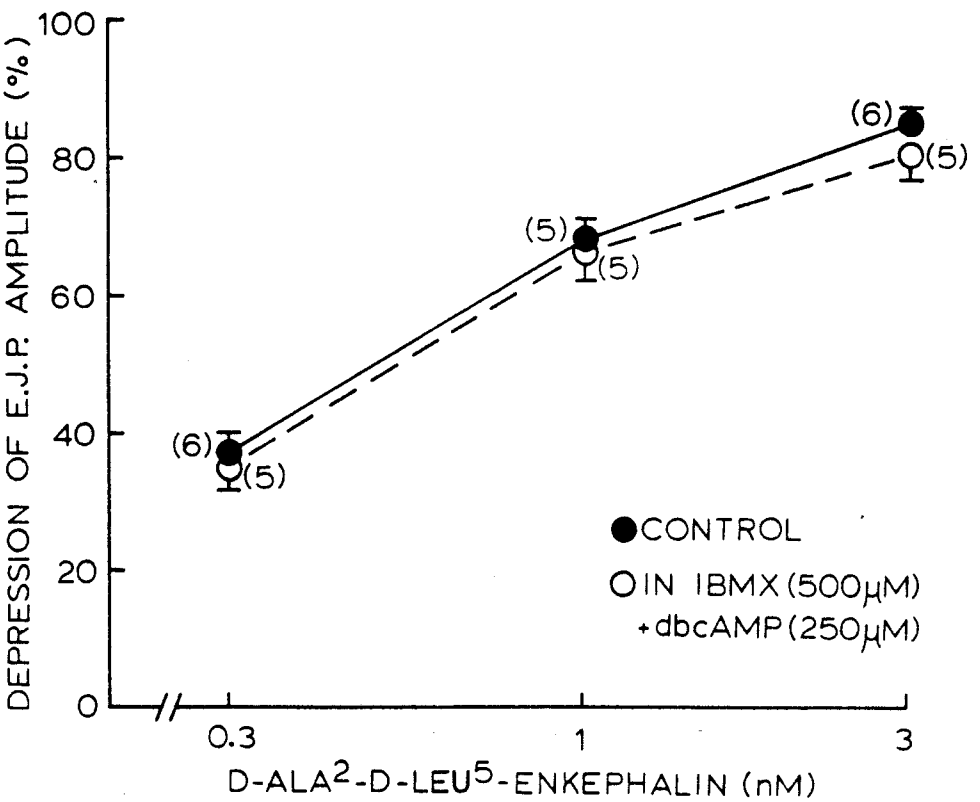

0.3
1
3
DADL(nM)
E.J.P. AMPLITUDE (mV)
20
10
0
0
20
40
60
TIME (min)

Figure 21. Dose-response curves for depression of e.j.p. by DADL: effect of IBMX + dbcAMP. These dose-response curves were constructed using results from tissues not exposed to any drugs, and results from tissues superfused with the drug combination IBMX (500 μM) and dbcAMP (250 μM). Bars represent ± s.e mean. Numbers in parentheses indicate the number of individual cells tested at a given concentration.


DEPRESSION OF E.J.P. AMPLITUDE (%)
100
80
60
40
20
0
(6)
(5)
(5)
(5)
(6)
(5)
CONTROL
IN IBMX (500μM)
+dbcAMP (250μM)
0.3
1
3
D-ALA2-D-LEU5-ENKEPHALIN (nM)

3.8 Effect of altered calcium ion concentration on the action of normorphine

3.81 Stimulus strength adjusted

The effect of normorphine on e.j.p. amplitude was determined in tissues maintained in twice the normal calcium ion concentration (5.08 mM) for 1-6 h. Doubling the calcium ion concentration increased the e.j.p. by 144 $\pm$ 20% in other experiments (see above). The stimulus voltage was adjusted so that e.j.ps were between 10 and 25 mV in amplitude before testing normorphine; it is presumed that this adjustment has compensated for the effect of high calcium on the e.j.p. Normorphine decreased e.j.p. amplitude in tissues maintained in high calcium solution (Figure 22) and the time course of the effect was not different from its time course in normal calcium solutions.

The effect of normorphine on e.j.p. amplitude was also measured in vasa maintained in one-half the normal calcium ion concentration (1.27 mM) for 1-6 h. Halving the calcium ion concentration decreased the e.j.p. amplitude by 66 $\pm$ 4.9% in other experiments (see above). Normorphine was tested on e.j.ps 10-25 mV in amplitude; it is presumed that the stimulus voltage has been adjusted to compensate for the effect of low calcium. Normorphine depressed the e.j.p. in tissues maintained in low calcium solution (Figure 23).

The dose-response curves for the effect of normorphine on e.j.p. amplitude in tissues maintained in high calcium (5.08 mM), normal calcium (2.54 mM) and low calcium (1.27 mM) solutions are illustrated

Figure 22. Effect of normorphine on e.j.p. during superfusion with high calcium solution. The tissue was superfused with Krebs solution containing calcium (5.08 mM) throughout the recording, and for 4.5 h previously. The action of normorphine was related to the concentration applied, and in this cell the following depressions of e.j.p. amplitude occurred: normorphine (100 nM), 26%; normorphine (300 nM), 55%; and normorphine (1 μM), 79%.

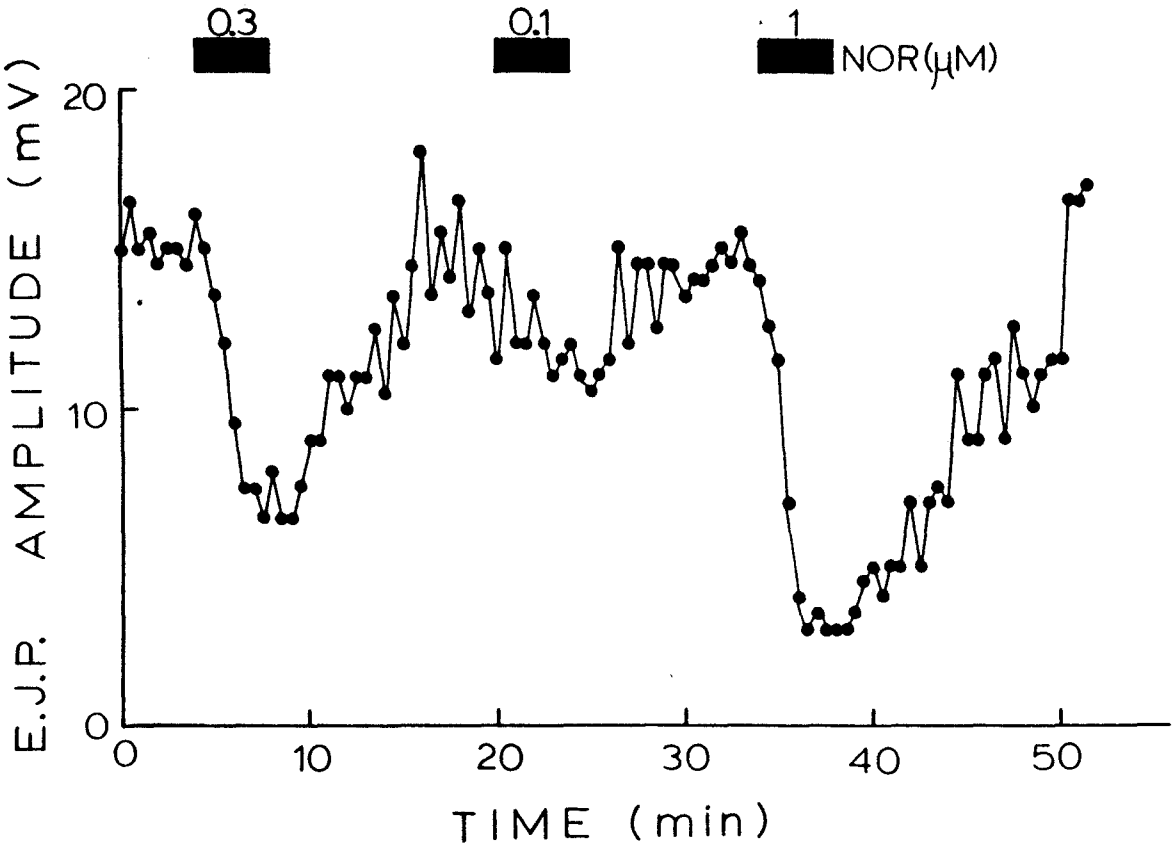
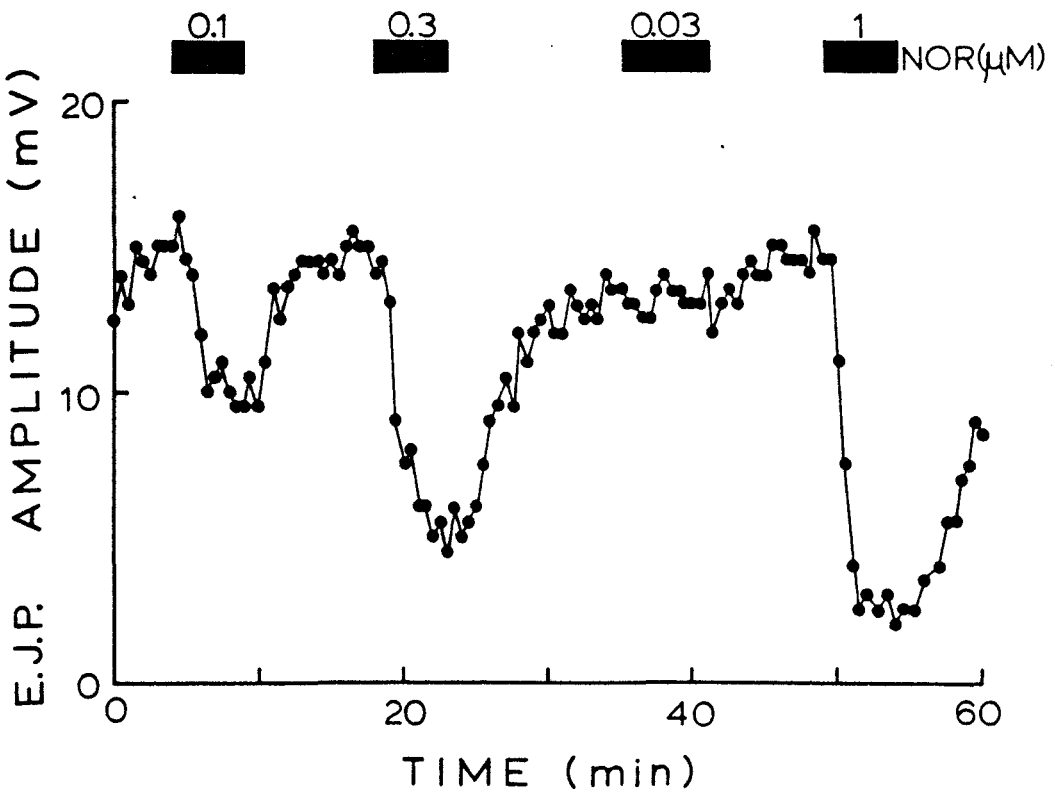

0.3
0.1
1
NOR(μM)
E.J.P. AMPLITUDE (mV)
20
10
0
0
10
20
30
40
50
TIME (min)

Figure 23. Effect of normorphine on e.j.p. during superfusion with low calcium solution. This tissue had been bathed in Krebs solution containing calcium (1.27 mM) for 3 h previous to time zero. Four concentrations of normorphine were tested on the e.j.p. in this cell, which was particularly sensitive to its action. Although normorphine (30 nM) had no effect, normorphine (100 nM) depressed the e.j.p. by 34%, normorphine (300 nM) depressed it by 62% and normorphine (1 μM) depressed it by 83%. The impalement ended before the effect of the final application of normorphine had completely washed out.


0.1
0.3
0.03
1
NOR(μM)
E.J.P. AMPLITUDE (mV)
20
10
0
0
20
40
60
TIME (min)

in Figure 24. Values obtained in high, normal or low calcium solutions were compared at four concentrations of normorphine (100 nM, 300 nM, 1 μM and 3 μM) using one-way analysis of variance. There was a significant relationship between the effectiveness of normorphine and the calcium ion concentration at only one of these concentrations (300 nM: $p = 0.02$). This was due to a difference between the effectiveness of this concentration of normorphine in control vs. low calcium solution (Figure 24). The EC_{50} for normorphine was 270 nM in low calcium solution (1.27 mM), 370 nM in normal calcium solution (2.54 mM) and 410 nM in high calcium solution (5.08 mM) as estimated from the composite dose-response curves (Figure 24).

3.82 Stimulus strength not adjusted

The effect of normorphine (1 μM) in both normal and low calcium solutions was tested on the same cell. The stimulus strength was not changed when the calcium ion concentration was changed (Figure 25). Normorphine (1 μM) depressed the e.j.p. by 74 $\pm$ 3.1% in normal calcium and by 75 $\pm$ 4.8% in low calcium, in the five cells on which it was tested in this manner (Table 5).

Figure 24. Normorphine dose-response curves for depression of e.j.p. in tissues maintained in different calcium ion concentrations. These dose-response curves were constructed using results from vasa maintained in Krebs solutions containing high (5.08 mM), normal (2.54 mM) or low (1.27 mM) calcium ion concentrations. Bars represent $\pm$ s.e. mean. Results from 52 cells (23 tissues).

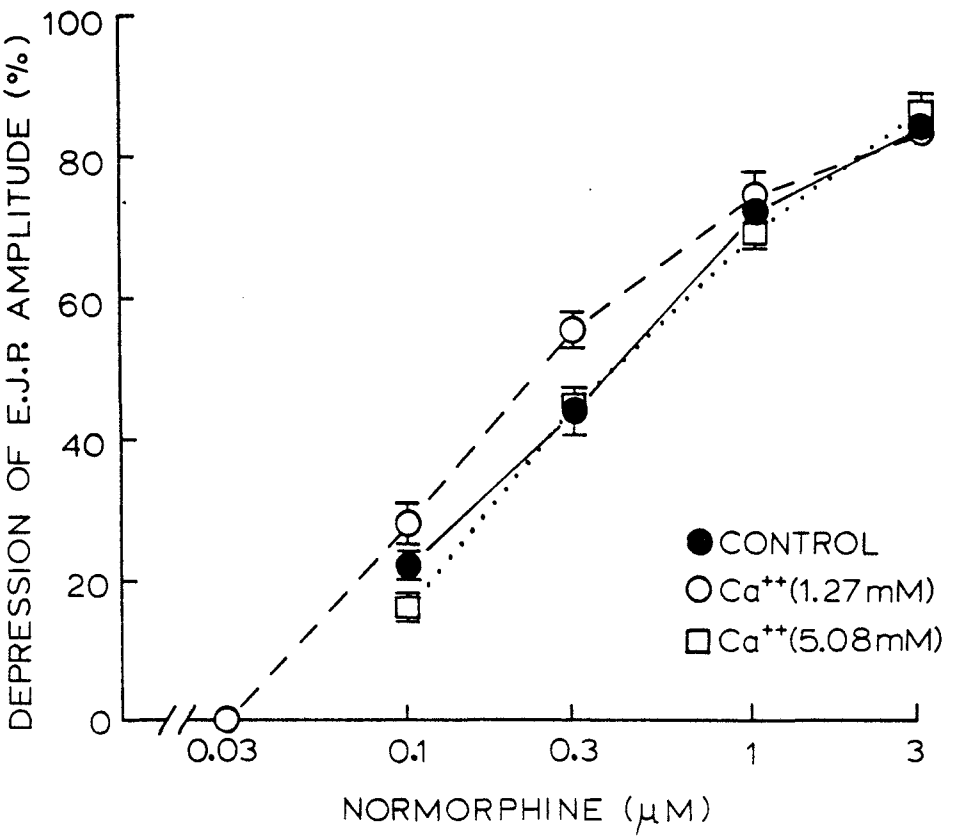

DEPRESSION OF E.J.P. AMPLITUDE (%)
100
80
60
40
20
0
0.03
0.1
0.3
1
3
NORMORPHINE (μM)
CONTROL
Ca++(1.27mM)
Ca++(5.08mM)

Figure 25. Effect of halving the calcium ion concentration on depression of e.j.p. by normorphine: same cell and same stimulation parameters. Continuous record of e.j.p. amplitude during the impalement of a single cell. Stimulation strength was constant throughout the recording. During the time interval indicated by the open bar, the calcium ion concentration of the superfusing solution was reduced from 2.54 mM to 1.27 mM. In this cell, normorphine (1 μM) decreased e.j.p. amplitude by 84% in normal calcium solution and by 64% in the solution with reduced calcium concentration.

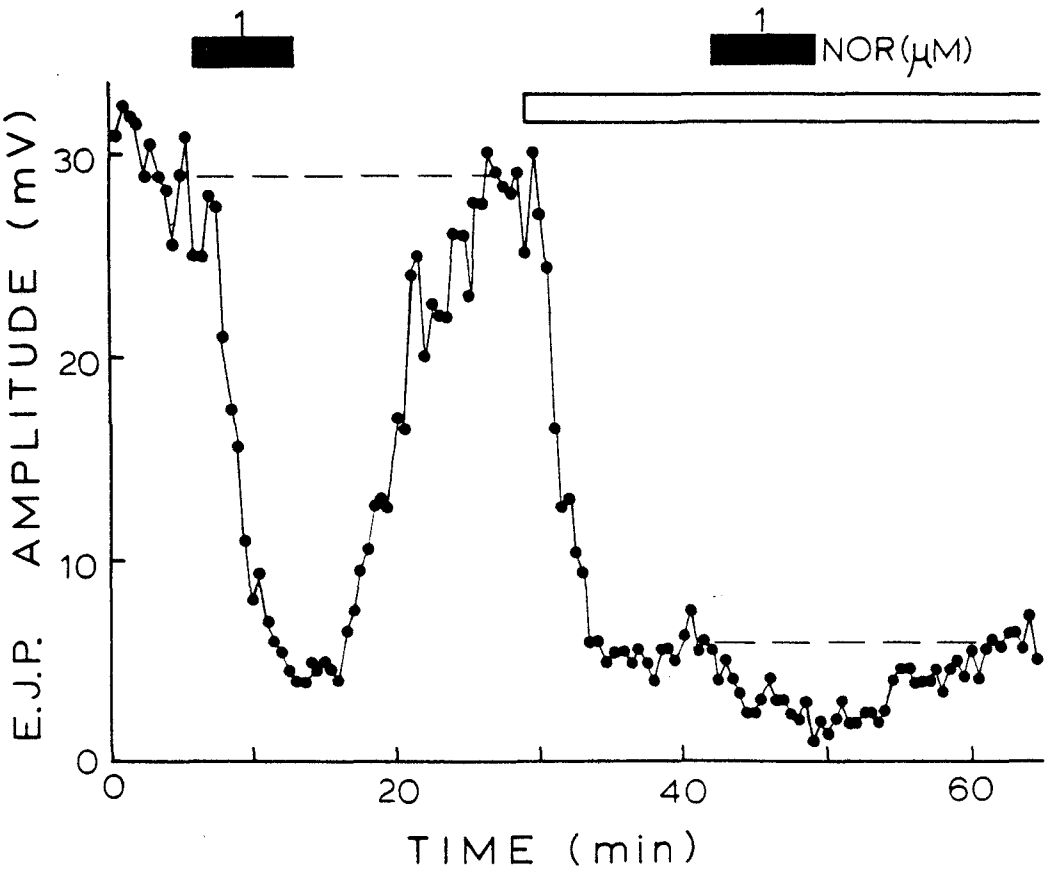

1
1
NOR(μM)
E.J.P. AMPLITUDE (mV)
30
20
10
0
0
20
40
60
TIME (min)

Table 5. Effect of normorphine (1 μM) on e.j.p. in normal and low calcium: same cell. Normorphine (1 μM) was tested on five cells bathed first in solutions with normal calcium (2.54 mM), then in solutions with low calcium (1.27 mM). Stimulation parameters were constant. There was no difference between the mean depression of the e.j.p. by normorphine (1 μM) in normal and low calcium (Student's t test).

	Calcium (2.54 mM)		Calcium (1.27 mM)	
Cell #	E.j.p. amp. (mV)	Depression of e.j.p. (%)	E.j.p. amp. (mV)	Depression of e.j.p. (%)
7259-3	25	74	14	85
8019-5	22	68	8	67
8149-5	29	85	6	64
10169-4	44	68	12	71
10179-1	36	77	10	88
$\bar{x} \pm$ s.e.m.	31 ± 4.0 mV	74 ± 3.1%	10 ± 1.4 mV	75 ± 4.8%

4. DISCUSSION

4.1 E.j.p. amplitude as a measure of transmitter release

Increasing the strength of stimulation of the intramural nerves of the mouse vas deferens will increase the number of nerve fibers that are activated and hence increase the amount of transmitter released into the junctional clefts in the vicinity of a single smooth muscle cell. The amplitude of the e.j.p. increases with strength of stimulation, and hence it can be concluded that e.j.p. amplitude is a quantitative estimate of transmitter release. Other aspects of e.j.p. configuration also vary with stimulus strength, such as the rate of rise of the e.j.p. (in V/s) and the area under the depolarization curve. Why was peak amplitude measured in the present experiments, and not these latter characteristics?

The rate of rise of the e.j.p. increases with stimulus strength, and hence with the quantity of transmitter that interacts with the postsynaptic membrane. But measurements of peak amplitude are easier and more accurate. Small errors in measurements of amplitude as a function of time would produce large changes in the calculated rate of rise, whereas measurements of e.j.p. amplitude in the present study were accurate within $\pm$ 0.5 mV. Furthermore, the rate of rise of the e.j.p. in a particular cell which has been produced by a fixed amount of transmitter is dependent on the distribution of transmitter release sites in the vicinity. Release directly onto the impaled cell would

result in a fast rate of rise whereas release onto neighboring or more distant cells would result in a decreased depolarization rate.

The area under the depolarization is influenced by the rise time, peak amplitude and decay time of the e.j.p. The ionic currents, and hence the conductance changes which produce the e.j.p., have not yet been measured; therefore the duration of transmitter action is not known. In the few smooth muscle cells of the mouse vas deferens in which conductance increases are observed in connection with transmitter action, these increases occur during the early portion of the e.j.p. (Holman, 1970). Thus the duration of the e.j.p. most likely does not reflect continued action of transmitter on the smooth muscle membrane. The falling phase of the e.j.p. probably is determined by the passive electrical properties of the smooth muscle membrane, as postulated by Bywater & Taylor (1980). They found that the time constant of decay of the evoked e.j.p. in the guinea-pig vas deferens was closely similar to the time constant of the smooth muscle membrane in this tissue. Since the final configuration of the e.j.p. is dependent on postsynaptic membrane properties as well as presynaptic events (transmitter release), the area under the depolarization curve was not used as a quantitative measure of transmitter release.

An increase in the amplitude of the e.j.p. results from the action of increasing quantities of transmitter. The transmitter produces the potential change by increasing the ionic conductance of the smooth muscle membrane, but the potential change is not linearly related to the conductance change (Martin, 1955). This non-linear relationship occurs because as the membrane becomes depolarized and

its potential approaches the equilibrium potential of the transmitter, the ionic driving force becomes less. Thus, at the height of the e.j.p., a given quantity of transmitter will produce a smaller potential change than would the same amount of transmitter applied to a resting membrane. To account for the non-linear relationship between transmitter action (conductance change) and e.j.p. amplitude (potential change), the following correction factor may be applied (Martin, 1955):

$$v' = v\ (1 - \frac{v}{V_o})^{-1}$$

where v' = corrected e.j.p., v = measured e.j.p. amplitude, and $V_o = V_E - V_m$ (V_E = equilibrium potential for e.j.p. and V_m = resting membrane potential). The corrected value of the e.j.p. (v') would be an estimate of "transmitter release" and hence would have no units. Any estimate produced by this method would be inaccurate because the noradrenaline equilibrium potential (V_E) is not known (see Introduction, p. 48-49). Also, the actual value of the resting membrane potential (V_m) is difficult to measure accurately because microelectrode impalement of cells probably results in some degree of damage. If, however, an equilibrium potential of 0 mV and a resting membrane potential of -62 mV (see Results, p. 65) are assumed, then e.j.p. amplitudes can be corrected for non-linear summation as shown in Table 6. It is obvious that the greater the amplitude of the e.j.p., the less accurately does the potential change of the membrane reflect the quantity of transmitter released in the vicinity of that membrane. In most of the present experiments, the effectiveness of opiate agonists was tested on e.j.ps of approximately the same amplitude. In a few experiments, such

Table 6. Correction of e.j.p. amplitude to account for non-linear summation. $*v' = v\,(1 - \frac{v}{V_o})^{-1}$ (see text). Assumptions: V_m = -62 mV; V_E = 0 mV.

E.j.p. amplitude (mV) Recorded	Corrected e.j.p. "Transmitter release"
5	5
10	12
15	20
20	30
25	42
30	58
35	80
40	114
45	167
50	250

as effect of stimulus strength on potency of normorphine and in some low calcium experiments, the effect of normorphine is assessed on e.j.ps of different amplitudes. The implications of non-linear summation will be discussed in relation to the results of these studies.

4.2 Depression of e.j.p. by opiates and opioid peptides

Normorphine decreased the amplitude of the evoked e.j.p. in the isolated vas deferens of the mouse, an action that was reversed by naloxone. This confirms the observations of Henderson & North (1976). The EC_{50} for normorphine in the present study (560 nM) was similar to the value obtained by those authors (430 nM), despite the fact that different strains of mice were used in the two studies. The effect of normorphine on the e.j.p. is likely to be the result of a decrease in the amount of transmitter released from the adrenergic varicosities in response to nerve stimulation (see Introduction).

DAEA is an enkephalin analog that is resistant to enzymatic destruction (Pert, Bowie, Fong & Chang, 1976) and it acts primarily on μ receptors (Shaw, Turnbull, Dutta, Gormley, Hayward & Stacey, 1978). It also depressed the e.j.p., and was more potent in this action than normorphine, with an EC_{50} of 50 nM. Such an effect on the e.j.p. of an opioid peptide had previously been demonstrated for Met^5-enkephalin (Waterfield et al., 1977).

A second enkephalin analog, DADL, was also tested on the e.j.p. This opioid peptide is a potent and specific agonist at the δ receptor (Chang & Cuatrecasas, 1979). Neuro-effector transmission in the mouse vas deferens was extremely sensitive to the depressant action of this peptide; the EC_{50} for the depression of e.j.p. amplitude by DADL was 500 pM. DADL was thus about 1,000 times more potent in this action than normorphine, a result which is consistent with the preponderance

of δ-type opiate receptors in the mouse vas deferens (Lord et al., 1977). It is assumed that the decrease in e.j.p. amplitude in the presence of the opioid peptides results from a depression of transmitter release by these substances, as is true for normorphine (Henderson & North, 1976).

4.3 Effect of control e.j.p. amplitude and stimulus strength on the potency of normorphine

The calculated percentage inhibition of e.j.p. amplitude by normorphine was not related to the control amplitude of the e.j.p., because normorphine was as effective on e.j.ps of initially large amplitude (35 mV) as on e.j.ps of initially smaller amplitude (10 mV) (Table 1). Any change in the effectiveness of normorphine (1 μM) as a function of e.j.p. amplitude may not be evident because this concentration falls on the less sensitive (lower slope) portion of the dose-response curve (Figure 3). This caveat does not apply for the lower concentrations of normorphine (especially 300 nM), although the effectiveness of these lower concentrations on e.j.p. amplitude is more variable from cell to cell (note standard deviations in Figure 3). In a few experiments, normorphine (100 nM or 300 nM) was tested on a large amplitude e.j.p. and a small amplitude e.j.p. of the same cell. Depending on the cell, normorphine was more effective, equally effective or less effective on the larger amplitude e.j.p. as compared to the smaller amplitude e.j.p.

More nerve fibers must be excited in order to elicit an e.j.p. of larger amplitude (35 mV vs. 10 mV). The present results indicate that, at least for the nerve fibers with thresholds for excitation in a limited range of stimulus strengths, the sensitivity to normorphine must be similar. In all experiments the stimulus strength used to evoke an e.j.p. was submaximal; in most cells even the large amplitude (30-40 mV) e.j.ps did not give rise to action potentials. If the

potency of normorphine were measured on e.j.ps elicited by supramaximal stimulation, which usually are accompanied by action potentials and muscle contractions, and compared to its potency on submaximal e.j.ps, differences might very well have been found. Hughes et al. (1975a) measured the effect of normorphine on the nerve-evoked muscle contractions of the mouse vas deferens. They observed that normorphine was more effective on vasa stimulated with submaximal current strengths (EC_{50} = 120 nM) compared to its efficacy on vasa stimulated with just supramaximal stimuli (EC_{50} = 440 nM). Submaximal vs. supramaximal e.j.ps were not compared in the present study because only submaximal stimulation strengths were used in all experiments.

If the amplitude of the e.j.p. is corrected using Martin's non-linear summation formula so that the e.j.p. reflects "transmitter release" (Table 6), then the effectiveness of normorphine is apparently greater for large amplitude e.j.ps. For example, normorphine (300 nM) decreases the amplitude of 35 mV e.j.ps by an average of 39%, and decreases the amplitude of 10-11 mV e.j.ps by 46% (Table 1). The percentage depression of the "transmitter release" than these e.j.ps represent is 60% for high release and 48% for low release. The calculations were also made for e.j.ps depressed by normorphine (100 nM and 1 μM), and the results are similar. One possible explanation for the apparent greater effectiveness of normorphine on high levels of transmitter release may be related to the geometry of release in the vas deferens. For large amplitude e.j.ps, more nerve fibers close to the impaled cell are likely to be excited, because the stimulus strength

has increased. An inhibitory effect of normorphine on transmitter release in the vicinity of a given cell would be more likely to result in a measurable decrease in e.j.p. amplitude than would inhibition of transmitter release at more distant sites. Transmitter that is released directly onto or near a cell makes a larger contribution to the amplitude of the e.j.p. than does transmitter released onto distant cells.

4.4 Prolonged exposure to morphine *in vivo*

Intracellular recordings were made from smooth muscle cells of vasa taken either from naive mice or from mice pretreated with morphine. The effects of opiates on neurotransmission in these vasa were compared. The three main questions addressed by this study were as follows. Does chronic morphine pretreatment lead to changes in the resting properties of the smooth muscle cells of the mouse vas deferens? Does tolerance develop to the inhibitory action of morphine at this neuro-effector junction? Can the administration of naloxone *in vitro* to a vas deferens exposed to morphine *in vivo* lead to changes in neuro-effector transmission which are an indication of "dependence"? These aspects will be discussed in turn.

4.41 Resting membrane properties of the smooth muscle cells

Opiates depress transmitter release in the mouse vas deferens *in vitro* (see above). Prolonged exposure to high concentrations of morphine *in vivo* may effectively interrupt neuro-effector transmission in the vas deferens so as to produce a pharmacological denervation. If excitable cells are chronically deprived of their normal physiological stimuli they become more sensitive to excitatory substances. This phenomenon in smooth muscle is postjunctional supersensitivity (Fleming, Urquilla, Taylor & Westfall, 1975). In the guinea-pig vas deferens, postjunctional supersensitivity produced by surgical denervation is accompanied by a depolarization of the smooth muscle cells (Fleming & Westfall, 1975; Fleming *et al.*, 1975). Morphine pretreat-

ment of mice did not alter the resting membrane potentials of single smooth muscle cells of their vasa deferentia. Presumably, during chronic exposure to morphine *in vivo*, neuro-effector transmission is more or less restored to normal due to the development of tolerance to the initial depressant effect of morphine on transmitter release (see below). Under these conditions, postjunctional supersensitivity would not develop. Another possibility is that postjunctional supersensitivity in the mouse vas deferens is not accompanied by a change in the resting membrane potential of the smooth muscle cells. This is true for the smooth muscle cells of the supersensitive rat vas deferens (Goto, Westfall & Fleming, 1978). This is not likely because an increased sensitivity to transmitter did not develop in vasa from morphine-pretreated mice (see below).

Most of the experimental evidence suggests that noradrenaline is the transmitter released from the sympathetic nerve varicosities in the mouse vas deferens, and that the noradrenaline so released mediates the contractile response of the muscle to stimulation of the intramural nerves (see Introduction). Because surgical denervation leads to supersensitivity of smooth muscle to noradrenaline.and other substances (see Fleming *et al.*, 1975), the effect of chronic morphine treatment on the response of the smooth muscle cells of the mouse vas deferens to exogenous noradrenaline was examined. There was no change in the sensitivity of the muscle cells to noradrenaline after chronic morphine treatment. Rae, Neto & de Moraes (1977) reported that long-term treatment of mice with morphine does lead to a supersensitivity of their

vasa deferentia to noradrenaline. The differences between the results of these investigators and the results of the present experiments may be related to differences in the schedule of morphine administration. Rae et al. administered morphine for 10 days, whereas mice were treated for 3 days in the present study. Perhaps any changes in muscle sensitivity related to prolonged morphine treatment have not developed within 3 days (see below). Also, injection of animals twice a day (Rae et al.) as compared to 3 times a day (present study) may result in longer periods of relative withdrawal from morphine. Supersensitivity is a common finding in withdrawal from morphine both in vivo (Pollock, Muir, MacDonald & Henderson, 1972) and in vitro (North & Karras, 1978a).

The noradrenaline EC_{50} for producing a contractile response of the vas deferens in vitro was not different in vasa from mice administered morphine for 3 days as compared to vasa from naive mice. There was, however, a tendency for vasa from morphine-pretreated mice to respond to the higher concentrations of noradrenaline with larger increases in tension (Figure 15). A supersensitivity of smooth muscle which is characterized by an increased maximum response to an agonist, without an associated leftward shift of the dose-response curve to that agonist, has been observed under conditions in which blood corticosteroid levels are elevated. Such conditions include: morphine withdrawal, thyroidectomy, injection of a single dose of reserpine, or chronic administration of corticosterone (Gibson & Pollock, 1975). The stress of daily multiple injections of a drug could also result in elevated levels of plasma glucocorticoids. Perhaps administration of morphine to mice for a longer time in the present experiments would

have resulted in a statistically significant increase in the maximum response of the vas deferens to noradrenaline, an increase which may be only partially developed after three days.

Most importantly, significant tolerance to the effect of opiates on transmitter release (see below) can be demonstrated in vasa which are not supersensitive, or have only a marginally increased sensitivity, to the transmitter noradrenaline. Thus supersensitivity of the post-synaptic effector cell to transmitter does not account for tolerance to morphine in this tissue.

4.42 Tolerance to the effect of normorphine

The EC_{50} for the depression by normorphine of e.j.ps was twelve times higher in vasa from morphine-pretreated mice than the EC_{50} in vasa from naive mice (Figure 12). Transmitter release in vasa from morphine-pretreated mice was thus relatively insensitive to normorphine. The isolated tissue may, therefore, be said to be tolerant to the inhibitory action of normorphine on the nerve terminals. Cox (1978) reported a four-fold degree of tolerance to the inhibitory action of morphine on the nerve-mediated contractions of vasa. These vasa were removed from mice which had been implanted with morphine pellets for five days. A previous study of the effect of opiates on the contractions of vasa from pellet-implanted mice (Waterfield, Hughes & Kosterlitz, 1976) also did not report the high degree of tolerance seen in the present study. The experimental procedures of these studies and the present one differ in two major respects. First, Cox (1978) and Waterfield *et al*. (1976) used pellet implantation, whereas

multiple injections (8 hour intervals) was the method of morphine pretreatment in the present study. Second, in the other studies the contractions of the smooth muscle in response to supramaximal stimulation of the nerves were measured. In the present study the effectiveness of normorphine was assessed on the amplitude of the e.j.p.; the e.j.p. was elicited by submaximal stimulation of the intramural nerves.

The pattern of exposure of the vas deferens to morphine would differ in pellet-implanted mice as opposed to mice receiving multiple injections. Vasa in pellet-implanted mice would be exposed to lower but less variable concentrations of morphine. In contrast, blood levels of morphine would markedly vary in the mice receiving intraperitoneal injections. The plasma concentration of morphine was measured in mice that were treated with the same morphine injection schedule as the animals whose vasa were used for intracellular recordings. Two hours after the last (ninth) morphine injection of 140 mg/kg (the time when the mouse would have been killed and the vasa removed for experimentation), the plasma morphine concentration averaged 6.7 μM (range 3.2 to 13.8 μM; n = 4). Eight hours after the final injection (the time for the next injection, if the treatment schedule would have continued), the morphine concentration in the plasma averaged 900 nM (range 250 nM - 1.5 μM; n = 6) (Wainer, personal communication). Normorphine (1 μM) did not depress the e.j.p. in vasa of morphine-pretreated mice (Figure 10). Thus with this injection schedule, neuroeffector transmission would be periodically restored to normal (when the blood level approached 900 nM) and would be intermittently depressed (when the blood level approached 7 μM). This assumes that the concen-

tration of morphine in the vicinity of the opiate receptors in the vas deferens is similar to the plasma concentration. This schedule of exposure to morphine may be more favorable for the development of tolerance in the mouse vas deferens, as opposed to a schedule of exposure which results in steadily declining blood concentrations (such as pellet implantation).

When the effect of morphine is studied on the contractions of the smooth muscle of the vas deferens, supramaximal stimulation of the intramural nerves is used to elicit these contractions. But the potency of normorphine in inhibiting the muscle contractions is greater if submaximal stimulation currents are used (Hughes et al., 1975a). E.j.ps were elicited by submaximal stimulation strengths, but the effect of normorphine on the e.j.p. was independent of the e.j.p. amplitude, and of the stimulus strength used to evoke it (Table 1). Furthermore, in the present study the potency of normorphine on muscle contractions evoked by supramaximal stimulus strengths was compared to its potency in inhibiting the amplitude of e.j.ps elicited by submaximal stimuli, in different mice of the same strain. In vasa from naive mice of the CF-1 strain, normorphine was as effective or more effective in inhibiting the contractile responses of the muscle (EC_{50} values 165-400 nM) in the vasa tested in this manner, as it was in depressing the e.j.p. (EC_{50} values 270-890 nM) in other vasa. A similar relationship held for vasa from morphine-pretreated mice. In these tissues, the EC_{50} values for normorphine for inhibition of the nerve-evoked muscle contraction (1.6-7.8 μM) were similar to the values

for the inhibition of the e.j.p. (3-30 μM). For these reasons, it is unlikely that the high degree of tolerance (twelve-fold) observed in the present study as opposed to previous studies is related to the method used for assessing the potency of opiates (e.j.p. amplitude) or to the use of submaximal stimulation strengths to elicit transmitter release.

Is it possible that the shift to the right of the dose-response curve for the effect of normorphine on the e.j.p. in vasa from morphine-pretreated mice was a consequence not of *in vivo* exposure to morphine, but of *in vitro* exposure to normorphine (300 nM)? To investigate this possibility, some vasa from naive mice were maintained in Krebs solution containing normorphine (300 nM) for 1-5 hours, and the effect of higher concentrations of normorphine (1 and 3 μM) were tested. Normorphine (1 and 3 μM) still depressed the e.j.p. Although in these vasa normorphine (1 μM) was significantly less effective than in vasa not exposed to opiate drug *in vitro*, this concentration of normorphine never depressed the e.j.p. in vasa from morphine-pretreated mice. The effect of normorphine (3 μM) was not changed by exposure of the tissue to normorphine *in vitro*, whereas it was significantly decreased by exposure of the tissue to morphine *in vivo*. Thus the exposure of the vas deferens to high concentrations of morphine *in vivo*, and not its maintenance in normorphine (300 nM) *in vitro*, produced the tolerance to the effect of normorphine on transmitter release.

At the time the morphine-pretreated mice were killed and their vasa removed for experimentation, blood levels of morphine probably were in the μM range (see above). However, the vasa were maintained

in vitro in Krebs solution containing a lower concentration of normorphine (300 nM). The tissues would therefore be in a state of partial withdrawal during subsequent experimentation. Perhaps the degree of tolerance to morphine attained in vivo by the vas deferens is even greater than was measured in vitro, after the tissue had been partially withdrawn. Normorphine at the concentration used in the present study (300 nM) was able to prevent complete withdrawal of the tissue, because tissues maintained under these conditions were still highly tolerant to normorphine (Figure 12) and responsive to naloxone (Figure 14).

Morphine pretreatment of mice produced a decreased sensitivity to normorphine of neuro-effector transmission in the vas deferens. Did this treatment also produce tolerance to other substances which decrease transmitter release at this site? Adenosine inhibits both neurally-evoked contractions of the muscle and [^{3}H]-noradrenaline release from the isolated vas deferens of the rat. Furthermore, the responses to exogenous noradrenaline are not affected by adenosine; this indicates a prejunctional site of action (Clanachan, Johns & Paton, 1977). The effect of adenosine on the e.j.p. in the mouse vas deferens was investigated so as to determine whether prolonged exposure to morphine brought about changes in the sensitivity of the intramural nerves to adenosine as well as to morphine. Adenosine depressed the e.j.p., but its ability to do so was not changed by morphine pretreatment. That is, morphine pretreatment led to tolerance to the action of morphine without cross-tolerance to adenosine. Similarly, implanting mice with morphine pellets for 3 days does not alter the presynaptic inhibitory effect of the αadrenergic agonist

clonidine (Robson, Gillan, Waterfield & Kosterlitz, 1978).

4.43 Manifestation of "dependence" - effect of naloxone

Naloxone did not change the resting membrane potential of the smooth muscle cells of vasa from morphine-pretreated mice. However, naloxone did cause a large increase in the amplitude of the evoked e.j.p. in those vasa. Was this simply a reversal of the ongoing depression of the e.j.p. by the normorphine present in the bathing solution? Two factors suggest not. First, the vasa should have been tolerant to the normorphine concentration (300 nM) present in the bath fluid because a higher concentration of normorphine (1 μM) did not depress the amplitude of the e.j.p. Second, the increase in e.j.p. amplitude observed upon administration of naloxone to the vasa from morphine-pretreated mice was marked (3.5 to 7-fold). The average increase in amplitude was 360 $\pm$ 37%. When naloxone was used to reverse the depression caused by a short-lasting (10 min) application of normorphine (300 nM) to vasa from naive mice, the e.j.p. amplitude returned only to its control level before application of drugs. This represented an increase in e.j.p. amplitude of 64 $\pm$ 11%.

It is possible that the exposure to normorphine (300 nM) *in vitro* was the cause of the increased response to naloxone in vasa from morphine-pretreated mice. Therefore naloxone was also tested on control vasa which were maintained for 5-8 hours in Krebs solution containing normorphine (300 nM). Cells from these preparations responded to naloxone (300 nM) with an increase in e.j.p. amplitude of 184 $\pm$ 21%. There was a significant change in the effectiveness of naloxone on

e.j.p. amplitude as the duration of exposure to opiate increased, if the responses of naive vasa exposed to opiate for 10 minutes, the responses of naive vasa maintained in opiate for 1-5 hours, and the responses of vasa from morphine-pretreated animals are compared (one-way analysis of variance: $p<0.01$). There was thus a trend toward increased responsiveness to naloxone as the duration of exposure to opiate drug increased.

An increase in the effectiveness of naloxone as a function of duration of exposure to opiates has been observed in whole animal studies, as well as in other in vitro preparations. The dose-response curve for morphine for producing antinociception in the tail-flick test in mice was shifted to the right if naloxone (160 µg/kg) was administered concurrently with the test dose of morphine. The dose-response curve was shifted to a greater extent in mice that have been pretreated with morphine (30 mg/kg) 3 hours before testing. The morphine EC_{50} was not changed by this pretreatment (Tulunay & Takemori, 1974a). Furthermore, in animals rendered tolerant to morphine by multiple injections or by implantation of morphine pellets, naloxone (160 µg/kg) also increased the morphine EC_{50}, and the sensitivity to this effect of naloxone was greater than in animals that have been acutely treated with morphine. The increase in the efficacy of naloxone observed after morphine treatment appeared before tolerance to the antinociceptive activity of morphine developed, and the efficacy of naloxone increased faster than the development of tolerance (Tulunay & Takemori, 1974b). In another series of experiments, slices of corpus striatum were incubated in Krebs solution containing [^{3}H]-morphine, and

the overflow of label during subsequent superfusion with drug-free solutions was measured. The addition of naloxone (1-100 pM) to the superfusate resulted in a dose-related increase in the overflow of label. The sensitivity to this effect of naloxone was increased in slices from mice given morphine pretreatment, either by a single injection of morphine (100 mg/kg) 3 hours before the animal was killed for experimentation, or by pellet implantation 3 days before the experiment. The time course for the development of the enhanced potency of naloxone in striatal slices paralleled the time course for the development of tolerance in the whole animal, as assessed by the tail-flick method (Kitano & Takemori, 1979).

A time-dependent increase in naloxone sensitivity also occurs in the guinea-pig ileum. In this tissue, a naloxone-precipitated muscle contracture is the manifestation of withdrawal from opiate drugs (see North & Karras, 1978a). Naloxone can induce a muscle contracture in a tissue removed from a naive guinea-pig if this tissue is maintained in Krebs solution containing normorphine (200 nM). The degree of tension developed by the muscle in response to naloxone increases as time of exposure to normorphine increases. The effect of naloxone is much enhanced in tissues removed from morphine-dependent animals and maintained in morphine solution (Schulz & Herz, 1976). In the present experiments, the increase in e.j.p. amplitude caused by naloxone in vasa exposed to normorphine (300 nM) was also much greater if the tissue was removed from morphine-pretreated mice as distinct from naive mice. Large increases in e.j.p. amplitude were also observed in

tissues from morphine-pretreated mice if the superfusing solution was changed from one containing opiate (normorphine - 300 nM) to another that was drug-free. These increases were not as large or as consistent as those obtained by superfusion with naloxone. The large increase in e.j.p. amplitude elicited by naloxone in vasa from morphine-pretreated mice may be analogous to the muscle contracture in the guinea-pig ileum. The increase in sensitivity to naloxone of the nerve terminals in the mouse vas deferens may therefore represent "dependence" on morphine in this tissue.

Normorphine inhibits evoked but not spontaneous release of transmitter from the sympathetic nerves in the mouse vas deferens: the amplitudes of evoked e.j.ps are depressed (Henderson & North, 1976) but the frequency and amplitudes of spontaneous e.j.ps are not modified (Henderson, 1976). In the present study naloxone did not cause a large increase in the spontaneous release of transmitter from the nerve terminals of vasa from morphine-pretreated mice. Such a massive release would have been observed either as a depolarization of the smooth muscle cells or as a burst of firing of spontaneous e.j.ps: neither occurred. Thus, it is reasonable that naloxone-precipitated "withdrawal" in the mouse vas deferens is manifest as a dramatic increase in evoked release of transmitter (amplitude of evoked e.j.p.) but not as a change in spontaneous transmitter release.

4.44 Conclusions

The precise nature of the change in neuro-effector transmission in the mouse vas deferens caused by prolonged exposure to morphine *in vivo* is not known. Clearly, though, the change takes place in the nerve terminals which are sensitive to the action of morphine. These nerve terminals become tolerant to the depressant action of morphine on transmitter release. The postsynaptic cells which are not opiate-sensitive did not exhibit changes in resting membrane potential or in sensitivity to transmitter. These findings do not support theories of opiate tolerance and dependence which invoke changes in postsynaptic sensitivity following blockade of transmitter release (Collier, 1968).

It will be of interest to see if tolerance develops in the mouse vas deferens as a consequence of *in vitro* as well as *in vivo* exposure to opiates. The development of tolerance *in vitro* has been demonstrated in the guinea-pig ileum (Hammond, Schneider & Collier, 1976; North & Karras, 1978b) and in explants of fetal mouse spinal cord in tissue culture (Crain, Crain, Finnigan & Simon, 1979). The degree of tolerance to the depressant action of opiates on transmitter release in the mouse vas deferens can be easily quantified by measuring changes in EC_{50} values. This technique would thus be a sensitive way to assess the effectiveness of treatments purported to alter the development of opiate tolerance in other systems, such as protein synthesis inhibitors (see Smith, 1971), peptides (Walter, Ritzmann, Bhargava & Flexner, 1979), cyclic nucleotides (Hammond *et al.*, 1976) and low incubation temperatures (Crain *et al.*, 1979).

4.5 Cyclic AMP and inhibition of transmitter release by opiates

The proposal that cAMP is involved in excitation-secretion coupling in nerve terminals has been examined at both somatic and autonomic neuro-effector junctions. Cyclic AMP has also been considered as a mediator of the presynaptic alterations of transmitter release from sympathetic nerves induced by β-adrenergic agonists, serotonin and, in the present study, opiates.

4.51 Role of cAMP in transmitter release

Calcium is required for the evoked release of transmitter from nerves, but is an increase in the intraterminal concentration of cAMP also a prerequisite? Can cAMP alter the amount of calcium entering nerve terminals in response to a depolarizing stimulus, or the effectiveness of calcium in promoting transmitter release? Cyclic AMP, dbcAMP and IBMX potentiate the electrically-induced but not the spontaneous release of [^{3}H]-dopamine from slices of rat neostriatum (Westfall, Kitay & Wahl, 1976). The role of cAMP in transmitter release has also been examined on peripheral nerves. In the isolated rat diaphragm-phrenic nerve preparation, dbcAMP and theophylline increase the amplitude of the end-plate potential by a prejunctional action on the cholinergic nerve terminal (Goldberg & Singer, 1969). Systemic administration of dbcAMP, theophylline or NaF (an activator of adenylate cyclase) induces spontaneous activity and increases the stimulus-evoked response of single motor axons of cat soleus nerve in vivo (Dretchen, Standaert, Skirboll & Morganroth, 1976). A possible

mechanism for this effect is a facilitation by cAMP of calcium influx into the motor axons (Skirboll, Baizer & Dretchen, 1977; Skirboll, Standaert & Dretchen, 1979).

Cyclic nucleotides and phosphodiesterase inhibitors also can enhance autonomic neurotransmission. In the isolated perfused spleen, the monobutyryl derivative of cAMP (mbcAMP) and the phosphodiesterase inhibitors IBMX, papaverine and RO 20-1724 enhance the nerve-evoked overflow of [^{3}H]-noradrenaline and dopamine-β-hydroxylase (DβH) (Cubeddu, Barnes & Weiner, 1974 & 1975). The effect of the phosphodiesterase inhibitors on evoked noradrenaline release from the spleen does not, however, correlate with their potencies in causing inhibition of splenic phosphodiesterase (Cubeddu et al., 1974; Stjarne, 1979). Also, a derivative of cGMP (8-bromo-cGMP) is as effective in this action as the cAMP analogs (Cubeddu et al., 1975). Wooten, Thoa, Kopin & Axelrod (1973) report an enhancement of the evoked release of [^{3}H]-noradrenaline and DβH from the guinea-pig vas deferens by dbcAMP (100 μM) or theophylline (1 mM). These results could not be repeated by another investigator (Stjarne, 1976). Differences in stimulation parameters and the difficulty of accurately measuring the small amount of DβH released from the vas deferens could account for the discrepancy (Stjarne, 1976 & 1979). Stjarne, Bartfai & Alberts (1979) saw an enhancement of nerve-evoked secretion of [^{3}H]-noradrenaline from the guinea-pig vas deferens in the presence of another analog of cAMP (8-bromo-cAMP) and more specific inhibitors of phosphodiesterase (IBMX and SQ 20,006). None of these studies at either somatic or autonomic

nerve-muscle junctions offers conclusive evidence that cAMP is an essential intermiediate in excitation-secretion coupling. But the presence of cyclic nucleotide derivatives and phosphodiesterase inhibitors, which may produce alterations in the concentration of cAMP in nerve terminals, appears to modify transmitter release at some sites under some experimental circumstances.

In the present study, cAMP and dbcAMP depressed rather than enhanced e.j.p. amplitude in the mouse vas deferens. Because cAMP does not readily penetrate cell membranes, the effect of this substance probably is a result of interaction with adenosine receptors located extracellularly on the presynaptic nerve terminals (Clanachan et al., 1977). Dibutyryl cAMP more readily crosses cell membranes and hence less would remain in the extracellular fluid to activate those receptors. The relative potencies of adenosine, cAMP and dbcAMP for inhibition of e.j.p. amplitude in the mouse vas deferens (adenosine>cAMP> dbcAMP) match their relative potencies for action on adenosine receptors in the guinea-pig ileum (Sawynok & Jhamandas, 1976). In the latter tissue these purine compounds reduce the output of acetylcholine in response to electrical stimulation of the nerves (Takagi & Takayanagi, 1972). Because adenosine inhibits evoked noradrenaline release from the rat vas deferens (Clanachan et al., 1977) and the cat spleen (Mueller, Mosimann & Weiner, 1979), a similar action of compounds acting on the adenosine receptors of the mouse vas deferens is assumed.

The increase of e.j.p. amplitude by IBMX and SQ 20,006 in the

present study is likely to be a presynaptic action on the nerve terminals because these compounds enhance the stimulation-evoked release of noradrenaline and DβH at other sympathetic junctions (see above). A postsynaptic sensitization of the smooth muscle membrane to transmitter is another possible action, which unfortunately could not be disproved. IBMX alone or combined with dbcAMP inhibited the contractile response of the smooth muscle of the mouse vas deferens to both noradrenaline and potassium ion, suggesting a direct interference with excitation-contraction coupling. Postsynaptic actions of phosphodiesterase inhibitors have previously been observed with the pressor response of splenic vasculature (Cubeddu et al., 1974) and the contractile response of the guinea-pig vas deferens (Stjarne et al., 1979).

Is the effect of IBMX on e.j.p. amplitude a result of inhibition of phosphodiesterase or is it caused by another action of this compound? The structure of IBMX is that of a methylxanthine, and methylxanthines such as theophylline increase the entry of calcium across cell membranes (Blinks, Olson, Jewell & Braveny, 1972), mobilize calcium from intracellular storage sites (Johnson & Inesi, 1969) and potentiate calcium-dependent ionic currents, independent of an inhibition of phosphodiesterase (Smith, Weight & Lehne, 1979). Methylxanthines also antagonize the actions of adenosine (Sattin & Rall, 1970) and prostaglandins (Horrobin, Manku, Franks & Hamet, 1977). Although the effect of IBMX in the mouse vas deferens could be caused by any of these actions, it is more likely to be the result of inhibition of phosphodiesterase for several reasons. The non-xanthine phospho-

diesterase inhibitor SQ 20,006 had the same effect. Also, IBMX and dbcAMP applied simultaneously were more effective than IBMX alone. In one cell dbcAMP was applied after the preparation had been superfused with IBMX for several minutes: in this cell dbcAMP increased e.j.p. amplitude. In the absence of phosphodiesterase inhibition the increase in cAMP caused by hydrolysis of intracellular dbcAMP may be prevented by endogenous phosphodiesterase. Phosphodiesterase inhibitors (IBMX or SQ 20,006) would protect the released cAMP from metabolism. Thus the effects of IBMX and dbcAMP would be complementary. Lastly, IBMX enhances transmitter release in another preparation (molluscan neurons), an action which can be mimicked by intracellular injection of cAMP (Klein & Kandel, 1978).

4.52 Cyclic AMP and presynaptic modulation of transmitter release

The enhancement of transmitter release from sympathetic nerve terminals by β-adrenergic agonists may be mediated by an increase in the intraterminal concentration of cAMP. Celuch, Dubocovich & Langer (1978) report that both β-adrenergic agonists and phosphodiesterase inhibitors (papaverine and IBMX) enhance the overflow of [^{3}H]-noradrenaline induced by nerve stimulation in the perfused cat spleen. Propranolol reduces the effect of the phosphodiesterase inhibitors, as well as the effect of the β-adrenergic agonists. This suggests that the phosphodiesterase inhibitors may be enhancing the action of endogenous noradrenaline which, after its release, is interacting with β receptors on the nerve terminals. The β-adrenergic agonists isoprena-

line and terbutaline also enhance the potassium-evoked release of noradrenaline from the pineal gland, an action shared by dbcAMP (Pelayo, Dubocovich & Langer, 1978). Also, a selective inhibitor of cAMP-specific phosphodiesterase enhances noradrenaline release in pineal glands in which the presynaptic α-adrenergic receptors are blocked by yohimbine. The action of another presynaptic modulator, serotonin, may also be mediated by cAMP at a particular ganglionic synapse of Aplysia. Presynaptic facilitation of transmitter release at this synapse by a sensitizing stimulus in a sensory nerve pathway is simulated by extracellular application of serotonin or dbcAMP or by intracellular injection of cAMP (Kandel, Brunelli, Byrne & Castellucci, 1976).

The involvement of cAMP in the inhibition by opiates of transmitter release from axon varicosities of the mouse vas deferens was tested in the present study. Caution is warranted in the interpretation of results from vasa exposed to cyclic nucleotides (cAMP or dbcAMP) because these substances may have postsynaptic effects on the smooth muscle membrane, perhaps altering the sensitivity to the transmitter noradrenaline. Postsynaptic effects of the nucleotides on the response to noradrenaline could not be tested because, like IBMX, these substances inhibit excitation-contraction coupling in smooth muscle. Exposure of the vas deferens to cAMP (1 mM), which depressed the amplitude of the e.j.p., did not alter the potency of normorphine in causing a further depression of transmitter release. These compounds are acting at different receptors, cAMP at the adenosine receptor (see above) and normorphine at the opiate receptor. Substances which bind to these receptors are unlikely to have a common mode of action because

tolerance to the action of opiates is not accompanied by tolerance to the action of adenosine (see above). This result does not support the conclusions of studies which purport to show antagonism of the action of morphine by administration of cAMP (Ho et al., 1973). The effect of normorphine also was unaltered by the drug combination IBMX + dbcAMP. The increase in transmitter release in the presence of this combination may reflect an increase in the intraterminal levels of cAMP (see above). If this is the case, then the potency of the narcotic does not depend on the concentration of cAMP in the nerve terminals which bear the opiate receptors. Enkephalins are more potent than narcotics as inhibitors of transmitter release in the mouse vas deferens (Lord et al., 1977). The enkephalin analog DAEA was more potent than normorphine in depressing e.j.p. amplitude, but its effect was also not blocked by IBMX + dbcAMP in concentrations which produced an even greater enhancement of e.j.p. amplitude and, presumably, intraterminal cAMP concentrations.

Neurotransmission in the mouse vas deferens is much more sensitive to inhibition by δ-type opiate agonists than by μ agonists (Lord et al., 1977). Furthermore, the most direct demonstration that opiates alter cAMP levels comes from studies of hybrid neuroblastoma x glioma cells grown in tissue culture, and the opiate receptors on these cells are almost exclusively of the δ-type (Chang, Cooper, Hazum & Cuatrecasas, 1979). Thus it is possible that cAMP is involved only in the actions of opiates mediated by δ receptors, and not the actions mediated by μ receptors. This is not likely because the depression of the e.j.p. by the specific δ agonist DADL was not changed by IBMX +

dbcAMP.

Is it possible that the acute effects of opiates are mediated by a decrease in neuronal cAMP concentrations, but other factors can account for the inability of cyclic nucleotides and IBMX to block the action of opiates in the mouse vas deferens? Firstly, are these substances (IBMX, dbcAMP) actually increasing the cAMP concentrations at the transmitter release sites in the axon varicosities? The evidence that they are is indirect because the anatomy of the vas deferens precludes the direct measurement of cAMP levels in the nerve terminals. The concentrations of IBMX used in the present study (50 μM, 500 μM) are probably high enough to inhibit cAMP phosphodiesterase because in other systems the IC_{50} for inhibition of cAMP phosphodiesterase by IBMX is approximately 5 μM (Beavo, Rogers, Crofford, Hardman, Sutherland & Newman, 1970; Collier et al., 1976). Furthermore, similar concentrations of IBMX do increase cAMP levels in other tissues (neuroblastoma cells: Hamprecht & Schultz, 1973; slices of anterior pituitary gland: Schofield & McPherson, 1974). Also, the effectiveness of the drug combination IBMX + dbcAMP in enhancing transmitter release in the mouse vas deferens (see above) suggests that these substances are directly or indirectly affecting the transmitter release sites in the varicosities.

The action of opiates on transmitter release may result instead from alterations in cGMP levels in the varicosities. There is evidence that in the rat pineal gland the inhibition of the release of noradrenaline by α-adrenergic agonists is mediated by an increase in the cGMP levels of noradrenergic nerve endings (Pelayo et al., 1978). In this context it is of interest that withdrawal of a tolerant guinea-pig

ileum from morphine renders the preparation insensitive to clonidine, an α-adrenergic agonist that usually is a potent inhibitor of the evoked release of acetylcholine (Robson et al., 1978). Similarly, the ratio of cAMP to cGMP may be important in the actions of opiates, and not the absolute level of either one. IBMX inhibits hydrolysis of cGMP (Kakiuchi, Yamazaki, Teshima, Uenishi & Miyamoto, 1975) as well as hydrolysis of cAMP, and thus may not alter this ratio in the adrenergic nerves so as to block the action of morphine. Further experimentation is needed to confirm or refute this hypothesis that cGMP and not cAMP mediates the inhibition of transmitter release by opiates in the mouse vas deferens.

Opiates inhibit transmitter release in the mouse vas deferens by a presynaptic action on the adrenergic nerves. Another major action of opiates is depression of the firing rate of single neurons. The combined presence of IBMX + dbcAMP was unable to alter the acute inhibitory action of morphine on the firing rate of single myenteric neurons of the guinea-pig ileum (Karras & North, 1979). The opiate-sensitive neurons of both the mouse vas deferens and the guinea-pig ileum are peripheral in location. The effect of cAMP on the actions of opiates on central neurons in vivo has also been tested, and the results are in agreement with the results obtained using peripheral neurons. Thus, the depression by iontophoretically-applied morphine of the activity of lamina V cells in the spinal cord is unaffected by the simultaneous iontophoresis of IBMX or of dbcAMP (Duggan & Griersmith, 1979).

In the neuroblastoma x glioma hybrid cells, opiates acutely decrease cAMP concentrations, but prolonged exposure to opiates produces tolerance to this effect in that the cAMP concentrations return to normal despite the continuous presence of opiate drug (Sharma et al., 1975b). It has been suggested (Karras & North, 1979; Collier, 1980) that cAMP is involved in the chronic but not the acute actions of opiates. There is evidence against the involvement of cAMP in the acute actions of opiates on transmitter release (present study) and on the firing rate of single neurons (Karras & North, 1979; Duggan & Griersmith, 1979). The involvement of cAMP in the long-term effects of morphine, that is, the development of tolerance and dependence, remains to be tested.

4.6 Calcium ion and inhibition of transmitter release by opiates

4.61 Presynaptic modulation of transmitter release: role of calcium

Transmitter release is a function of the concentration of calcium ion at release sites within nerve terminals. Therefore it has been proposed that substances which modulate transmitter release alter calcium availability or action at these sites.

The release of noradrenaline from sympathetic nerve endings can be inhibited by α-adrenergic agonists, muscarinic agonists and prostaglandins (see reviews by Starke, 1977; Westfall, 1977). The effects of these substances on transmitter release have several features in common. First, only noradrenaline release that is dependent on the presence of extracellular calcium is inhibited. Release evoked by stimulation of nerves or by direct depolarization of nerve terminals with high concentrations of potassium is sensitive, whereas spontaneous or tyramine-induced release is insensitive. Second, the degree of inhibition by these substances appears to depend on the concentration of calcium ion in the extracellular fluid; high calcium decreases and low calcium increases their effectiveness. Lastly, these substances are more potent at low as opposed to high frequencies of nerve stimulation. The facilitation of transmitter release that occurs at high frequencies of stimulation has been explained by an increase in the amount of calcium available at the transmitter release sites (Younkin, 1974). These findings are interpreted as evidence that calcium has a pivotal role in presynaptic inhibition.

The inhibition of transmitter release by opiates is also considered to be mediated by a decrease in calcium availability for similar reasons. Thus, in the mouse vas deferens morphine depresses the release of noradrenaline evoked by nerve stimulation (Henderson et al., 1972) or high potassium solutions (Sim & Henderson, personal communication), but not the spontaneous release of noradrenaline measured either biochemically (Hughes et al., 1975a) or electrophysiologically (Henderson, 1976). Spontaneous transmitter release is not dependent on the extracellular calcium ion concentration (Hashimoto & Holman, 1967; Hubbard, Jones & Landau, 1968; Rubin, 1970). Also, the depression by morphine of the neurally-mediated contractions of the smooth muscle and of the release of noradrenaline from the adrenergic varicosities of the mouse vas deferens is frequency-dependent; morphine is more potent at low than at high frequencies of nerve stimulation (Henderson & Hughes, 1976). At other sites of neurotransmission which are sensitive to opiates, the potency of the opiates appears to depend on the extracellular calcium ion concentration. For example, DAEA depresses the amplitude of e.p.s.ps evoked by preganglionic stimulation of prevertebral sympathetic ganglia, an effect that is more marked in low calcium (0.7 mM)-high magnesium (2 mM) solutions than in solutions containing higher concentrations of calcium ion (Konishi, Tsunoo & Otsuka, 1979). Also, opiates depress the negative slow waves recorded in fetal explants of mouse spinal cord during stimulation of the attached dorsal roots; this action is reduced in high calcium solutions (3-5 mM vs. 1 mM) (Crain, Crain, Peterson &

Simon, 1978).

More direct evidence for modulation of calcium currents by substances that presynaptically alter transmitter release has been provided by studies of ganglion cells in vitro. The action potential of the soma of immature chick dorsal root ganglion cells has a calcium-dependent plateau phase which is accentuated in high calcium solutions or in solutions containing barium (Dichter & Fischbach, 1977). The duration of this plateau phase is shortened by noradrenaline and serotonin (Dunlap & Fischbach, 1979), as well as by the opioid peptide DAEA (Mudge, Leeman & Fischbach, 1979). This effect of DAEA on the ganglion cell bodies correlates with its inhibition of the release of substance P from the nerve terminals of these cells, although the postulate that the same mechanism is responsible for both effects is inference. It is unlikely that the membranes of a neuron's cell body and its axon terminals are identical with respect to distribution, density and properties of ionic channels as well as susceptibility of those channels to external regulation. The presence of high calcium concentrations is required to demonstrate clearly the effect of the opioid peptide in these cells, whereas transmitter release is purported to be less sensitive to inhibition by opiates under these conditions (see above). Also, the alteration of the action potential plateau could result either from a decrease in the inward calcium current or an increase in an outward potassium current. A change in the inward calcium current during presynaptic inhibition could be measured by impaling nerve terminals and voltage-clamping their membrane potential. Studies at an inverte-

brate synapse come closest to achieving this ideal situation. In the abdominal ganglion of Aplysia, presynaptic inhibition of transmitter release from the identified cholinergic cell L10 occurs in response to stimulation of specific sensory fiber pathways. Transmitter release can be assayed by measuring the amplitude of synaptic potentials in follower cells of the L10 neuron. If the L10 neuron is voltage-clamped in the presence of tetrodotoxin, depolarizing pulses elicit graded transmitter release, suggesting at least partial control of the membrane potential of the nerve terminals (Shapiro, Castellucci & Kandel, 1980a). These step depolarizations of the soma, if carried out during pharmacological blockade of sodium channels (with tetrodotoxin) and potassium channels (with the combination of tetraethylammonium ions, 4-aminopyridine and barium), elicit an inward current carried presumably by calcium ions. During presynaptic inhibition caused by stimulation of the sensory pathway, the transient inward calcium current elicited by step depolarizations is decreased (Shapiro et al., 1980b).

The mouse vas deferens preparation offers no convenient way of assessing the effect of opiates on the transient calcium currents associated with transmitter release from the axon varicosities. Therefore the amplitude of the e.j.p. was used as a measure of transmitter release and the ability of normorphine to depress this transmitter release was measured in the presence of different calcium ion concentrations. Halving or doubling the calcium ion concentration led to marked changes in the amount of transmitter released. However, the ability of normorphine to depress transmitter release was little changed by these manipulations.

It may be argued that a different population of nerves was excited in the solutions with altered calcium ion concentration. Stimulus voltage was adjusted to compensate for the effect of altered calcium in order to obtain control e.j.ps of approximately the same amplitude. However, if the stimulus voltage was not adjusted, normorphine was equally effective in low and in control calcium solutions, even though the pre-drug e.j.p. amplitudes differed. As already discussed, the potency of normorphine is independent of the initial amplitude of the e.j.p.

If the effects of normorphine on e.j.p. amplitude as summarized in Table 5 are reevaluated so that corrections for non-linear summation are made, then in some cells normorphine (1 μM) is more effective in normal (2.54 mM) calcium than in low (1.27 mM) calcium. However, the depression of transmitter release by normorphine was not different in normal vs. low calcium if the results are averaged and a statistical comparison made (Table 7).

4.62 Effect of calcium on e.j.p. amplitude - loci of action

Could the alteration in e.j.p. amplitude by calcium ion be explained by actions at sites other than at the adrenergic varicosities which are releasing the transmitter? Calcium alters neuronal excitability by shifting the voltage sensitivity of the system controlling membrane permeability to sodium and potassium; in high calcium, nerves are stabilized and in low calcium, nerves are more excitable (Frankenhaueser & Hodgkin, 1957). By reducing the number of nerves excited, elevated calcium might reduce e.j.p. amplitude, whereas in low

Table 7. Effect of normorphine (1 μM) on "transmitter release" (corrected for non-linear summation) in normal and low calcium: same cell. Results of Table 5 are corrected to account for non-linear summation using the conversion formula of Table 6.*

	Depression of "transmitter release" by normorphine (1 μM)	
Cell #	Calcium (2.54 mM)	Calcium (1.27 mM)
7259-3	-83%	-89%
8019-5	-76%	-71%
8149-5	-92%	-69%
10169-4	-88%	-77%
10179-1	-90%	-90%
	-86 ± 2.9%	-79 ± 4.5%

*There was no difference in the amount of depression of "transmitter release" by normorphine (1 μM) in normal calcium (2.54 mM) vs. low calcium (1.27 mM) in the same cell (Student's t test, $p>0.05$).

calcium more nerves would be excited by a given stimulus voltage, and e.j.p. amplitude would increase. But the relationship between e.j.p. amplitude and external calcium ion concentration in the present experiments was in the opposite direction.

Another possible locus of action of calcium ion in the mouse vas deferens is on the bioassay system used, the postsynaptic smooth muscle cells. The reaction of transmitter with the postsynaptic membrane may be changed. The membrane potential of smooth muscle cells is hyperpolarized by excess calcium and depolarized by a decrease in calcium (Bulbring & Kuriyama, 1963). These changes occur without modification of the ionic content of the cell (see Casteels, 1970). Meaningful measurements of the resistance of the resting or active smooth muscle membrane as a function of the external calcium ion concentration cannot be made with a single microelectrode. Thus any effect on e.j.p. amplitude of calcium-mediated alterations of membrane potential and resistance cannot be determined. However, these effects are likely to be small because the manipulations of calcium ion concentration did not change the membrane potential by more than 5 mV in the present experiments. The most likely site of action of calcium is thus the nerve terminal - a direct change in the stimulus-evoked transient inward calcium current that produces release of transmitter. Bennett & Florin (1975) previously reported that the amplitude of the e.j.p. in the mouse vas deferens is dependent on the extracellular calcium ion concentration. In their experiments the synaptic potential increased with about the second power of the extracellular calcium ion concentration.

4.63 Interactions between calcium and opiates

In the mouse vas deferens, both normorphine (Henderson & North, 1976) and the calcium ion (Bennett & Florin, 1975) change the amplitude of the e.j.p. by acting at the same locus, the presynaptic nerve terminal. But the potency of normorphine is independent of the calcium ion concentration over a four-fold range (1.27-5.08 mM). These results allow two conclusions to be drawn. First, the affinity of normorphine for the opiate receptor and the efficacy of the resulting drug-receptor complex are unlikely to be calcium-dependent. Second, considerable caution is warranted in the interpretation of studies of complex neuronal assemblies such as the central nervous system in vivo or a plexus of neurons in vitro, which claim to demonstrate antagonism of the effects of opiates by calcium. The interaction in these systems may be a functional antagonism which does not allow conclusions to be drawn concerning the mechanism of action of opiates.

The action of opiates has also been tested in neuroblastoma x glioma hybrid cells exposed to differing concentrations of extracellular calcium ion. Opiates and opioid peptides decrease cAMP levels in these cells even if calcium ion is omitted from the incubation medium. The inhibition by Leu^5-enkephalin of PGE_1-induced increases in cAMP levels is independent of the extracellular calcium ion concentration over a wide range (1 μM - 20 mM) (Brandt, Buchen & Hamprecht, 1980).

How can the results in neuroblastoma x glioma hybrid cells (above) and the mouse vas deferens (present experiments) be reconciled

with the experimental evidence in favor of a major role for calcium ion in the actions of opiates? Opiates inhibit calcium uptake into brain synaptosomes (Guerrero-Munoz et al., 1978). In lysed synaptosomes, morphine inhibits only active (ATP-dependent) calcium uptake (Guerrero-Munoz et al., 1979). Furthermore, a high affinity binding site for calcium (K_D = 0.85 μM) located on synaptic membranes is selectively affected by opiates. The binding of calcium to this site is inhibited by preincubation of membranes in levorphanol (0.5-10 nM) and, furthermore, levorphanol can release calcium that has already been bound (Ross & Cardenas, 1977). Perhaps the high affinity, saturable calcium binding and the ATP-dependent calcium uptake are the same functional system. This system would be located on the inner surface of the synaptic membrane. It would be in some way coupled to extracellularly located opiate receptors such that binding of opiates to their receptors would inhibit calcium uptake and binding. The function of such a system might be to maintain a low calcium ion concentration in a particular sub-compartment of the intracellular space. Opiates could act by increasing the calcium ion concentration of this compartment. Such an action could reduce the evoked release by transmitter in two ways. Firstly, the driving force for calcium entry during depolarization of the synaptic membrane could be reduced. Secondly, a calcium-dependent process could be activated which would, in turn, reduce transmitter release. One possible candidate is the calcium-dependent potassium conductance which has been demonstrated in several neuronal systems (see Thompson, 1977). An increased conductance to potassium ion would

hyperpolarize the terminal or pre-terminal membrane and possibly prevent invasion of axon varicosities or by some other mechanism inhibit transmitter release (see below).

4.64 Mechanism of presynaptic inhibition by opiates: speculation

Although the present findings provide evidence against an effect of calcium on the action of morphine, they do not directly refute a possible effect of morphine on calcium action in the nerve terminals. Thus it is possible that morphine is blocking the entry of calcium through its voltage-dependent channels, although if this blockade were competitive, it might be expected to be critically dependent on the external calcium ion concentration. Opiates could directly decrease the transient calcium current by completely closing some calcium channels, by decreasing the conductance of all channels, or by increasing the voltage threshold at which they become activated. The calcium current could be indirectly depressed by decreasing the amplitude or shortening the duration of the presynaptic action potential. At the squid giant synapse a decrease in the amplitude of the spike of the presynaptic terminal causes a decrease in the amount of transmitter released from that terminal (Erulkar & Weight, 1977). An increase in an outward potassium current would shorten the action potential duration and decrease the voltage-dependent calcium current. Such a potassium current may or may not be calcium-dependent (Thompson, 1977). Morphine could also leave unchanged the inward calcium current and instead decrease the amount of calcium available for interaction

with the transmitter release sites. This could be accomplished by increasing the affinity for calcium of another calcium-binding site in the nerve terminal.

Another possible mechanism of action for presynaptic inhibitors of transmitter release is blockade of impulse propagation into the fine terminal ramifications of autonomic nerves. Such a mechanism has previously been considered for the inhibition of transmitter release by α-adrenergic agonists (Stjarne, 1978) as well as by opiates (Sastry, 1978; North, 1979b). Blakeley & Cunnane (1979) provide electrophysiological evidence that transmitter release from a given varicosity in the vas deferens is intermittent, which could reflect a lack of activation of each nerve fiber by every impulse. There is a large increase in the membrane surface area through which activating ionic currents must pass between the adrenergic axon and the nerve varicosity. Such a region would have a lower safety factor for impulse propagation, and hyperpolarization of the membrane would further reduce the safety factor (Van Essen, 1973). Hyperpolarization of neurons by opiates has been observed in the myenteric plexus of the guinea-pig ileum (see North, 1979b), in the locus coeruleus (Henderson, personal communication) and in the frog sympathetic ganglion (Wouters & van den Bercken, 1979). Afferent fibers of the sural nerve terminate in the dorsal horn region of the spinal cord. The excitability of the terminal region of single Aδ fibers of this nerve is decreased by morphine; hyperpolarization of the terminals could produce this effect (Sastry, 1978). Although morphine does not alter the conduction of the action potential in non-terminal regions of the hypogastric or vagus nerves (Kosterlitz

& Wallis, 1964), the membrane of the terminal regions may have different properties. Possibly functional opiate receptors are found only in those regions. Hyperpolarization by morphine of the terminal portion of the preganglionic fibers of the frog sympathetic ganglion has been reported (Wouters & van den Bercken, 1980).

Further experimentation with the mouse vas deferens and with other tissues that have opiate-sensitive transmitter release will hopefully produce more evidence to explain how opiates depress the nerve-evoked release of transmitter substances. As the two predominant effects of opiates in the central nervous system are depression of the firing rate of neurons, and depression of transmitter release from nerve terminals, such information will aid in the understanding of how opiates produce their important pharmacological effects in man.

4.7 Proposals for future experiments

4.71 Effects of prolonged exposure to opiates

The present experiments have demonstrated that *in vivo* exposure of the mouse vas deferens to morphine results in the development of tolerance to the effect of normorphine on transmitter release, as assessed *in vitro*. It is of interest to find out if prolonged *in vitro* incubation of the vas deferens in morphine also leads to tolerance. If *in vitro* tolerance can be demonstrated, then factors which might enhance or block tolerance could be studied. Such factors, which are best studied in *in vitro* as opposed to *in vivo* experiments, include length of incubation time, incubation temperature, calcium ion concentration, and the presence of cyclic nucleotides and phosphodiesterase inhibitors or protein synthesis inhibitors. Incubation of vasa in the presence of D-Ala^2-D-Leu^5-enkephalin (DADL) would address the question of whether tolerance develops to actions of opiates mediated by δ receptors, as well as to effects mediated by μ receptors. The extent of cross-tolerance between μ and δ agonists could be quantitatively assessed in tissues incubated *in vitro* with either a μ agonist (morphine) or a δ agonist (DADL).

4.72 Cyclic nucleotides

The results of testing cyclic nucleotides and phosphodiesterase inhibitors on e.j.ps in the mouse vas deferens raise many questions about the role of cAMP in transmitter release and about the pharmacology of these substances. More phosphodiesterase inhibitors should be

tested on the e.j.p. to see if potency in increasing e.j.p. amplitude is correlated with potency in inhibiting phosphodiesterase, and especially cAMP vs. cGMP specific phosphodiesterases. In order to eliminate any problems of interpretation related to possible postsynaptic effects of these compounds, effects on transmitter release as assessed by overflow studies could be measured.

It was observed that e.j.ps that had been increased in amplitude by IBMX did not facilitate when the stimulation frequency was increased (from 0.033 to 1 Hz). Facilitation usually occurs at a stimulation frequency of 1 Hz. The effect of phosphodiesterase inhibitors on facilitation of transmitter release could be studied, and the role of calcium ion in the action of phosphodiesterase inhibitors also examined.

The role of cyclic nucleotides in tolerance to opiates could be tested by co-incubating vasa deferentia in phosphodiesterase inhibitors or cyclic nucleotide analogs together with morphine in in vitro experiments (see above). If such compounds do alter the development of tolerance, then the relative specificities of cAMP vs. cGMP selective phosphodiesterase inhibitors and cAMP vs. cGMP analogs could be investigated.

4.73 Calcium ion

Experiments which might further elucidate the possible role of the calcium ion in the inhibition of transmitter release by opiates in the mouse vas deferens include:

(1) testing the effect of altered calcium ion concentration on e.j.p. amplitude in the presence of opiates;

(2) measuring the post-stimulus increase in spontaneous potentials, or the spontaneous potentials in the presence of elevated extracellular potassium ion concentration, and determining if opiates change the frequency of these spontaneous events. A component of the spontaneous release under these latter two circumstances is reportedly dependent on extracellular calcium; the calcium dependence of these phenomena would first have to be assessed by the use of calcium channel blockers such as cobalt ion;

(3) loading liposomes with calcium ion and applying them to tissues bathed in zero calcium solution. If transmitter can be released by this method, the effectiveness of opiates on such release could be tested. An inhibition of liposome-induced transmitter release by opiates would imply an action of opiates on a release mechanism other than the depolarization-induced inward calcium current, because liposomes deposit the calcium ion directly into the intracellular compartment.

4.8 Summary

1. Excitatory junction potentials were recorded by impalement of single smooth muscle cells of the mouse vas deferens *in vitro*. The amplitude of these potentials was used as a measure of the release of transmitter, which is most likely noradrenaline, from the varicosities in response to single pulse stimulation of the intramural nerves.

2. E.j.ps were depressed by application of opiates and opioid peptides to the tissue. The EC_{50} values were 560 nM for normorphine, 50 nM for D-Ala^2-Met^5-enkephalinamide (DAEA) and 500 pM for D-Ala^2-D-Leu^5-enkephalin (DADL).

3. The potency of normorphine (100 nM, 300 nM and 1 μM) was the same regardless of the control amplitude of the e.j.p. and of the stimulus strength used to evoke the e.j.p.

4. In order to study the effect of prolonged exposure to morphine on neuro-effector transmission in the vas deferens, two groups of mice were studied. One group was naive and the second group received multiple injections of morphine sulphate (every 8 hours, up to 140 mg/kg) for 3 days. The vasa from morphine-pretreated mice were maintained in Krebs solution containing normorphine (300 nM).

5. Prolonged exposure to morphine *in vivo* did not alter the resting membrane potential of single smooth muscle cells nor the contrac-

tile response of the smooth muscle to exogenous noradrenaline.

6. E.j.ps were depressed by normorphine in vasa from both groups of mice: the EC_{50} was 560 nM for the naive group and 6.6 μM for the morphine-pretreated group. This shift to the right of the normorphine dose-response curve could not be accounted for by the presence of normorphine (300 nM) in the Krebs solution of the morphine-pretreated mice.

7. Adenosine (300 μM and 1 mM) depressed the e.j.p. with equal potency in vasa from both groups of mice.

8. Naloxone (300 nM) caused a marked (3.5 to 7-fold) increase in the e.j.p. amplitude of vasa from morphine-pretreated mice. The *in vitro* exposure of these vasa to normorphine could not account for this large effect. It is believed that the increase in response to naloxone of the nerve terminals may represent "dependence" on morphine in this tissue.

9. These results indicate that prolonged exposure of a mouse vas deferens to morphine *in vivo* leads to the development of tolerance to the normal inhibitory effect of normorphine on transmitter release. This "presynaptic" tolerance is not associated with changes in resting membrane potential or sensitivity to transmitter of the postsynaptic smooth muscle cells.

10. The involvement of adenosine 3',5'-cyclic monophosphoric acid (cAMP) in the action of opiates on transmitter release was tested by measuring the effectiveness of opiates in the presence of drugs which might elevate the cAMP concentrations in the nerve terminals: cAMP, dibutyryl cAMP (dbcAMP) and 1-methyl-3-isobutylxanthine (IBMX).

11. Cyclic AMP and dbcAMP (1 mM) depressed the amplitude of the e.j.p., possibly by interacting with extracellular sites similar to those responsive to adenosine.

12. The phosphodiesterase inhibitors IBMX (50 and 500 μM) and SQ 20,006 (500 μM) increased e.j.p. amplitude; this increase was much greater when the phosphodiesterase inhibitor was applied together with dbcAMP. It is believed that these increases in e.j.p. amplitude represent a presynaptic action on transmitter release, analogous to the effects of phosphodiesterase inhibitors at other sites of neuro-effector transmission.

13. There was no change in the EC_{50} for normorphine in the presence of cAMP (1 mM) or in the presence of the drug combination IBMX (50 μM) and dbcAMP (500 μM). Similarly, the depression of the e.j.p. by the opioid peptides DAEA and DADL was not affected by the combination IBMX (500 μM) and dbcAMP (250 μM).

14. These findings provide evidence against the hypothesis that a reduction in cAMP levels in the nerve terminals is an essential

step in the inhibition by opiates and opioid peptides of transmitter release.

15. It has been proposed that the presynaptic action of opiates may result from a decrease in the depolarization-induced influx of calcium ions that precedes the release of transmitter from nerve terminals. The action of normorphine in the mouse vas deferens was therefore tested in tissues that were superfused with different concentrations of calcium ion.

16. Doubling or halving the calcium ion concentration from its usual value (2.54 mM) caused marked changes in the amplitude of the e.j.p. These alterations in the e.j.p. are likely to result from an effect of calcium on transmitter release.

17. In vasa superfused with different concentrations of calcium ion, the stimulus strength was adjusted so that normorphine was tested on e.j.ps of approximately the same amplitude. Under these conditions the ability of normorphine to depress the e.j.p. was little changed in tissues superfused with calcium (1.27-5.08 mM).

18. In cells in which the stimulus strength was not adjusted when the calcium ion concentration was changed, normorphine (1 μM) was as effective in normal (2.54 mM) as in one-half (1.27 mM) calcium.

19. Although these findings provide evidence against an effect of calcium on the action of normorphine, they do not directly refute a possible effect of normorphine on calcium action in the nerve

terminal. However, the affinity of normorphine for the opiate receptor and the efficacy of the resulting drug-receptor complex are unlikely to be calcium dependent.

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APPROVAL SHEET

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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmacology.

13 May 1980
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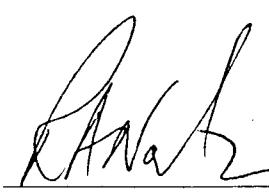
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