Peptide Systems in the Myenteric Plexus: Extracellular Recording

John T. Williams
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PEPTIDE SYSTEMS IN THE MYENTERIC PLEXUS: EXTRACELLULAR RECORDING

by

John T. Williams

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

March 1979
ACKNOWLEDGMENTS

I am grateful to R. Alan North for his thoughtful guidance throughout my stay in Chicago. I would also like to acknowledge Drs. Graeme Henderson, Alexander Karczmar, Gene Silinsky and Robert Wurster for their aid in preparing the dissertation. Finally, I thank Mrs. Kyoko Kunimoto for her help and kindness.
VITA

John T. Williams was born on June 3, 1950 in Montreal, Canada. He lived and attended high school in Bainbridge, New York. In 1968 he entered St. Lawrence University in Canton, New York and received a Bachelor of Science in chemistry in June of 1972. In June, 1975 he received a Master of Science in biology from the State University of New York at Potsdam.


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1. INTRODUCTION

Peptide hormones such as vasopressin and oxytocin which are found in hypothalamic and pituitary neuroendocrine cells of the central nervous system are known to have physiological roles in homeostasis. Recent advances in peptide sequencing and solid state peptide synthesis techniques have led to important developments in peptide neurobiology.

Techniques have been developed which enable the measurement of minute amounts of specific peptides (radioimmunoassay) and the visualization, histologically and cytologically, of specific peptides within nervous tissue (immunohistochemistry). With the use of radioimmunoassay and immunohistochemistry many peptides once believed to be contained solely in hypothalamic or other neuroendocrine cells in the central nervous system have now been found in neurons throughout the central and peripheral nervous systems. This has led to the suggestion that they may function as neurotransmitter or neuromodulatory agents. In the past ten years the number of peptides for which a neurotransmitter role has been proposed has grown from one (substance P) to at least twelve (see table 1) (Scharrer, 1977; Iversen, Nicoll and Vale, 1978; Hughes, 1978; Walker, 1978).

In order to demonstrate that any of the neuropeptides are neurotransmitter substances, several criteria must be met. First, the peptide under study must be contained within nerve terminals. In addition, it should also be contained within synaptic vesicles which are a char-
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acteristic feature of the presynaptic nerve terminal. A second criterion which must be met is that the substance be released from the terminal in response to a depolarization of the presynaptic neuron. This release must be dependent on the presence of extracellular calcium ions. The most common method by which the release of various putative transmitters has been studied is by depolarizing the cells with a solution having a high concentration of potassium ions.

The use of high potassium solutions to evoke release can be criticised for the following reasons: (a) Osmotic imbalances during the release period must be considered and corrected., (b) Viability of the preparation after such a powerful stimulus should be tested., (c) Reproducible release from the same preparation is usually not observed., (d) In addition to being calcium-dependent, the release of the proposed transmitter should be decreased by tetrodotoxin. Sensitivity of release to tetrodotoxin indicates that the depolarization and subsequent release can be ascribed to action potential generation. Techniques such as application of veratridine or electrical stimulation are less drastic than the use of high potassium solutions, and they affect neurons more specifically, thereby reducing the possibility of extraneous release from other types of cells in the preparation.

The third criterion which must be met is the capability of the presynaptic neuron to synthesize the proposed transmitter; that is to say, the enzymes which are known to be necessary for synthesis of the transmitter must be present. The fourth requirement is that a process by which the transmitter is inactivated either pre- or post-synaptically
is present at the proposed synapse. Finally, the action of the neuropeptide on neuronal excitability and postsynaptic membrane properties must mimic the response to presynaptic nerve stimulation. This criterion requires the use of electrophysiological techniques; this is the approach taken in the present study.

Electrophysiological studies may be carried out in vivo or in vitro. There are advantages and disadvantages to each approach. Studies in vivo leave the anatomy intact and the neurons remain in an environment which approximates normality; functional connections between different areas of the central and peripheral nervous systems are intact. The actions of the peptides on the neuron can be studied in relation to the cell's responses to physiological as well as artificial stimuli. Neurons may even be studied in animals which are conscious and behaving normally. There are also certain limitations to in vivo recordings. The concentration of peptides near the cell being studied is difficult to quantitate when administered systemically or by local application, synaptic influences are sometimes not distinguishable from direct actions on the cell, metabolic and hormonal factors as well as the anesthetic used may interfere with peptide action, the neuronal effects seen after administration of the peptide may be secondary to blood pressure or respiratory changes, diffusional barriers may limit the use of some drugs, and investigation of the ionic mechanisms of any observed effects is rendered difficult.

Studies of neurons in vitro eliminate most of the above problems; however, different problems arise. Studies of single neurons in vitro
are limited to peripheral ganglia, brain slices, or immature neurons grown in tissue culture. In each case, the isolated preparation is maintained in an artificial environment which approximates the conditions in vivo. Even so, explanted adult neurons eventually lose viability in vitro, and the rate at which they do this may limit the experimental approach. The strongest criticism of in vitro techniques is that it is difficult to ascribe functional significance to the effects observed because the neurons are more or less removed from any normal physiological connections. Studies of peripheral ganglia, on the other hand, have demonstrated that those neurons recorded in vivo or in vitro have similar properties including membrane potential, input resistance and synaptic potentials (Skok, 1973). The loss of synaptic transmission in the autonomic ganglia after being maintained in vitro for 15-20 hr correlates well with the degradation of presynaptic terminals after sectioning of the preganglionic fibers (Skok, 1973). It appears that studies of synaptic mechanisms in peripheral ganglia in vitro are not different from recordings made in vivo. Therefore, meaningful results from studies on synaptic mechanisms can be attained with isolated autonomic ganglia.

The present investigation has utilized the in vitro approach to study the action of several endogenous neuropeptides and related substances on the electrical properties of single autonomic neurons. This approach was chosen over in vivo electrophysiological techniques for several reasons. Basic information such as, the direct effects that these peptides have on single neurons, the time course of action and the effective concentration range can be more readily obtained from in
in vitro studies. A study of the physiological role of the neuropeptides is also possible with in vitro techniques using autonomic ganglia since many of the neuropeptides are contained in nerve terminals of these ganglia (see below). The myenteric plexus of the guinea pig ileum is well-suited to study the action of the various neuropeptides for reasons which will become apparent as each individual peptide is discussed.
1.1. OPIATES AND ENDOGENOUS OPIOID PEPTIDES

1.1.1. Opiate Binding Sites

There is a large body of evidence which indicates that the actions of opiates and endogenous opioid peptides are mediated by a common receptor or group of receptors. Areas with high numbers of opiate binding sites were found in the mesolimbic system of the brain; specifically the amygdala, hypothalamus, periaqueductal gray, head of the caudate nucleus and thalamus (Kuhar, Pert and Snyder, 1973; Pert, Kuhar and Snyder, 1975). Certain afferent systems were also shown to be rich in opiate binding sites including the dorsal horn of the spinal cord, the trigeminal nucleus and nuclei of the vagus nerve such as the nucleus tractus solitarius (Atweh and Kuhar, 1977). A presynaptic location of these binding sites was inferred when dorsal root section resulted in a 50% decrease in receptor binding in the dorsal spinal cord (LaMotte, Pert and Snyder, 1976). Similar inferences have been made from the finding that vagotomy leads to a marked reduction in autoradiographic binding of opiates in the nucleus tractus solitarius and nucleus ambiguus (Atweh, Murrin and Kuhar, 1978).

Opiate receptor binding has also been studied in the myenteric plexus of the guinea pig ileum (Creese and Snyder, 1975). Several methods were used to study the opiate receptors in the ileum. The results indicate that the binding properties of receptors in the ileum are much the same as those in the central nervous system. First, the inhibition
of $^3$H-labeled naloxone binding was stereospecific; that is, the active (-)-isomers of three agonists were 10-1,000 times more effective in displacing $^3$H-naloxone binding than the corresponding (+)-isomers. Second, a series of opiates having differing binding affinities in the rat brain were compared to the binding in the ileum. A significant correlation between the relative order of binding affinities was found between the two tissues. Finally, there was good correlation between the ability of agonists to inhibit the electrically induced contraction of the longitudinal muscle-myenteric plexus preparation and their affinities for the receptor in the ileum.

Autoradiographic localization of opiate binding sites in the central nervous system has proved useful in determining the pathways which contain opiate receptors. Unfortunately, no reliable autoradiographic studies on myenteric ganglia have been done.
1.1.2. Endogenous Opioid Peptides

Studies on the distribution of opiate binding sites demonstrated that opiate binding was not associated with any one specific transmitter system (Kuhar, Pert and Snyder, 1973). The demonstration of stereospecific opiate binding sites led to attempts to find endogenous opiate-like substances in the brain. An endogenous ligand for the opiate receptor was isolated from brain tissue, purified, sequenced and synthesized (Hughes, Smith, Kosterlitz, Fothergill, Morgan and Morris, 1975). This ligand was found to be a mixture of two pentapeptides which have been termed methionine-enkephalin (met-enkephalin) and leucine-enkephalin (leu-enkephalin) (for structures see Table 1). The structure of met-enkephalin was shown to be completely represented in the pituitary hormone β-lipotropin and this led to the discovery of a family of endogenous opioid peptides which included α-, β- and γ-endorphin (Table 1).

The opiate-like activity of these peptides has been tested on peripheral, behavioral and opiate binding models of opiate action. Although the peptides had potent opiate-like activity on isolated preparations such as the guinea pig ileum and mouse vas deferens, the opioid peptides had little significant opiate-like activity when administered systemically (Büscher, Hill, Römer, Cardinaux, Classe, Hauser and Pless, 1976). Intracerebral administration of enkephalin resulted in transient antinociception which was reversed or prevented by the narcotic antagonist naloxone. The pentapeptides were found to be metabolically labile, thus accounting for their lack of effect when administered systemically (Miller,
Chang and Cuatrecasas, 1977). The enkephalin molecule is metabolized rapidly by carboxypeptidases and also aminopeptidases (Hughes, Kosterlitz, McKnight, Sosa, Lord and Waterfield, 1978). The rapid metabolism of enkephalin is consistent with a neurotransmitter role, but no specific inactivating enzyme for enkephalin has been isolated yet. The most susceptible region of the enkephalin molecule appears to be the amino terminus where aminopeptidases cleave the tyrosine from the enkephalin. Enkephalin analogs having a (D-Ala) residue in position two are metabolically stable (Pert, Pert, Chang and Fong, 1976). With this substitution, aminopeptidases are unable to cleave the tyrosine-D-alanine bond and the (D-Ala)^2-enkephalin analogs have potent and long-lasting analgesic activity in vivo (Pert et al., 1976).

ß-endorphin, the largest of the endogenous opioid peptides, was first isolated from pituitary extracts (Lee and Chung, 1976). The question arose whether ß-endorphin was the precursor of met-enkephalin. However, the difference in distribution of neurons which contain enkephalin-like immunoreactivity argues against a precursor role for ß-endorphin (Rossier, Vargo, Minick, Ling, Bloom and Guillemin, 1977; Bloom, Battenberg, Rossier, Ling and Guillemin, 1978). A pituitary precursor of ß-endorphin itself, however, has been postulated (Guillemin, Vargo, Rossier, Minick, Ling, Rivier, Vale and Bloom, 1977). Antibodies to ß-endorphin, ß-lipotropin and corticotropin (ACTH) all crossreacted with a large peptide (31,000 daltons) in extracts from a mouse pituitary tumor (Mains, Eipper and Ling, 1977). Tryptic digests of this 31,000 dalton peptide resulted in ACTH, ß-lipotropin, and other endorphin fragments. Furthermore, the concomitant release of ß-endorphin and ACTH from the pituitary
gland during acute stress or long-term adrenalectomy further supports a common precursor (Guillemin et al., 1977). However, the effects of δ-endorphin released during acute stress have not as yet been determined. It seems clear, however, that δ-endorphin secreted by the pituitary into the blood does not result in changes in the levels of δ-endorphin in the central nervous system (Rossier, French, Rivier, Ling, Guillemin and Bloom, 1977). Since δ-endorphin does not cross the blood brain barrier, it may be possible that it may have no action at all or it may have an action on peripheral opiate receptors.

The synthesis of leu- and met-enkephalin has been studied in the rat brain and guinea pig myenteric plexus using (H\(^3\)) tyrosine (Hughes et al., 1978). There is a lag time of 1-2 hr after administration of the labeled tyrosine before labeled leu- or met-enkephalin is found. The synthesis of both peptides was blocked by cycloheximide or puromycin indicating that the pathway is ribosomal. The classical synthetic pathway for proteins is by the ribosomes of the endoplasmic reticulum. Although it is possible that the enkephalins as pentapeptides are synthesized by the classical pathway, it seems more probable, in light of the lag time, that the pentapeptides are products of a pro-enkephalin molecule.

Immunohistochemical localization of leu- and met-enkephalin indicates that the amount of these peptides varies markedly in different areas of the central nervous system (Elde, Hokfelt, Johansson and Terenius, 1976; Simantov, Kuhar, Uhl and Snyder, 1977). In general, the amount of and location in which the opioid peptides were found correlated with the density and localization of opiate receptor binding. There were some exceptions.
The central nucleus of the amygdala contained many immunofluorescent (enkephalin containing) fibers; however, the receptor binding was more evenly distributed in the amygdala (Simantov et al., 1977). The ventral gray portion of the spinal cord has enkephalin containing fibers; however, there is little opiate receptor binding (Simantov et al., 1977). The globus pallidus has the most enkephalin of any area in the brain; however, there are only moderate amounts of receptor binding (Kuhar et al., 1973; Elde et al., 1976; Simantov et al., 1977). These few quantitative differences in the distribution of enkephalin and density of opiate receptors may represent limitations in the techniques used or may also suggest that the opioid peptides act by a mechanism independent of the opiate receptor in some areas.

Subcellular fractionation studies indicate that most enkephalin is contained within the crude synaptosomal fraction of brain homogenates (Smith, Hughes, Kosterlitz and Sosa, 1976; Osborn, Holt and Herz, 1978). A sizable (25%) amount of the recoverable enkephalin was also found in the crude microsomal (p3) fraction (Osborn et al., 1978). This fraction also contains a considerable density of opiate receptor binding sites; however, it was thought that the amount of enkephalin found in this fraction was more than that which would be expected to be bound to the opiate receptors. It was also not possible to account for the enkephalin in the microsomal fraction by contamination, since the supernatant contained only small amounts of enkephalin. The localization of enkephalin in the microsomes may represent an early storage form of enkephalin. Experiments using incorporation of labeled tyrosine are required to test this hypothesis. Ultrastructural immunohistochemical studies may also
aid in demonstrating the relevance of the enkephalin found in the micro-
somal fractions.

A calcium-dependent potassium evoked release of met- and leu-
enkephalin from slices of rat striatum has been demonstrated (Henderson,
Hughes and Kosterlitz, 1978; Iversen, Iversen, Bloom, Vargo and Guillemin,
1978; Osborn, Holt and Herz, 1978). Release was also found to be evoked
by veratridine and this release was blocked by tetrodotoxin, suggesting
that the source of the enkephalin was neuronal (Henderson et al., 1978).
These results satisfy one criterion which must be met before a neuro-
transmitter role can be assigned to the enkephalins.

Leu- and met-enkephalin have been shown to be contained in the
nerve terminals of the myenteric plexus using immunohistochemical (Elde
et al., 1976) and biochemical methods (Hughes, Kosterlitz and Smith, 1977).
There is a limited amount of evidence which indicates that enkephalin is
released by nerve stimulation from the myenteric plexus of the guinea pig
ileum. The contractile response to nerve stimulation (at 0.1 Hz) of the
longitudinal muscle-myenteric plexus preparation exhibits an inhibition
following a 5 min period of stimulation at 10 Hz (Puig, Gascon, Craviso
and Mussachio, 1977). This inhibition in contractile response following
the period of stimulation is also prevented by naloxone, and by the active
(-)-isomer of an opiate antagonist GPA-1943, but is not prevented by the
inactive isomer GPA-1847. This was taken as evidence indicating that
nerve stimulation resulted in release of an endogenous opioid, possibly
enkephalin.
Direct measurement of enkephalin released from the guinea pig myenteric plexus by nerve stimulation has been reported in a single study (Schulz, Wüster, Simantov, Snyder and Herz, 1977). The amount of enkephalin released in these experiments was quite variable in the three tissues studied. Others have also indicated that release of enkephalin from the myenteric plexus for direct measurement is unreliable and variable (Hughes et al., 1978). Using cycloheximide to stop synthesis of enkephalin, the amount of enkephalin present in the myenteric plexus was compared between electrically stimulated and unstimulated tissues. A significant reduction in the amount of enkephalin found in the stimulated preparations was taken as evidence for its release. The depletion of enkephalin from stimulated preparations was also blocked by treatment of the tissue with tetrodotoxin, indicating that the release was from stimulation of neurons.
1.1.3. Actions of Opiates on Single Neurons In Vivo

Single neuron studies on the action of morphine were few prior to the description of stereospecific opiate binding sites (Pert and Snyder, 1973; Simon, Hiller and Edelman, 1973). As the distribution of opiate binding sites was described, electrophysiological studies on single cells were directed toward areas which were high in opiate binding sites.

The localization of receptor binding sites and known physiological pathways involved in pain perception aided the direction of electrophysiological experiments. However, opiates have many effects on neuronal tissue and it became important to demonstrate that the effects observed were directly relevant to the spectrum of effects observed when pharmacological doses of opiates were given to normal animals including man. The relevance of electrophysiological studies on the action of narcotic analgesics therefore required fulfillment of the following criteria. First, the action of the opiates on the cell being studied must be at a concentration comparable to that which reaches that site when a pharmacologically relevant dose of opiates is given to the whole animal. Second, the action of the opiate agonists must be reversed by specific opiate antagonists in appropriate concentrations. Third, the action of the opiates must be stereospecific; that is, the (-)-isomer must be more active than the (+)-isomer of enantiomeric agonists. Likewise, the (-)-isomer of the enantiomeric antagonists should produce a larger degree of antagonism than the (+)-isomer.
With in vivo experiments, it is often difficult to show that the action of an opiate is directly on the cell whose activity is being recorded. Systemic administration may result in any number of indirect effects at distant sites. The alternative method of application of opiates in such experiments is by iontophoresis. This limits any such indirect effects to sites near that of the recording. This advantage of iontophoresis over systemic administration is offset by the problem that with iontophoresis the local concentration of drug around the cell is unknown. A comparison between the effects on a given neuron produced by an analgesic dose of narcotics given systemically, and the effects produced by iontophoretic application often results in a much clearer picture of the site and mechanism of opiate action. Unfortunately, few studies in vivo have used both routes of administration.

When administered either systemically or by iontophoresis, morphine decreases the firing rate of most neurons whether they are spontaneously active or when they are induced to fire with nociceptive stimuli or excitatory agents such as glutamate. One site where the action of morphine has been studied carefully is the locus coeruleus. Noxious stimuli transiently increased the firing rate of neurons of that area (Korf, Bunney and Aghajanian, 1974). This increase in firing rate and also the spontaneous firing rate were inhibited by morphine given systemically (Korf, Bunney and Aghajanian, 1974) or iontophoretically (Bird and Kuhar, 1977). Other regions of the central nervous system where the inhibitory action of morphine has been studied by systemic as well as iontophoretic applications are the mesencephalic reticular formation (Haigler, 1976), sensorimotor cortex (Satoh, Ziegglänsberger and Herz,
1976), spinal motor neurons (Ziegglänsberger and Bayerl, 1976), and lamina IV and V cells from the spinal cord (Duggan, Hall and Headley, 1977). The action of morphine applied by iontophoresis in many other areas of the brain has been studied (Table 2).
Table 2. The action of opiates and opioid peptides studied in various locations in the central nervous system.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>opiates</td>
</tr>
<tr>
<td>E</td>
<td>opioid peptides</td>
</tr>
<tr>
<td>Ion</td>
<td>administered iontophoretically</td>
</tr>
<tr>
<td>Sys</td>
<td>administered systemically</td>
</tr>
<tr>
<td>↑</td>
<td>firing rate increased</td>
</tr>
<tr>
<td>↓</td>
<td>firing rate decreased</td>
</tr>
<tr>
<td>↔</td>
<td>variable effects</td>
</tr>
<tr>
<td>x</td>
<td>indicates reversal by naloxone</td>
</tr>
<tr>
<td>Area</td>
<td>Species</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td></td>
<td>rat, cat</td>
</tr>
<tr>
<td></td>
<td>cat</td>
</tr>
<tr>
<td>Motor neurons</td>
<td>cat</td>
</tr>
<tr>
<td>Renshaw cells</td>
<td>cat, rat</td>
</tr>
<tr>
<td>Brain stem</td>
<td>rat</td>
</tr>
<tr>
<td>Lateral Reticular Formation</td>
<td>rat</td>
</tr>
<tr>
<td>Medulla</td>
<td>rat</td>
</tr>
<tr>
<td>Locus Coeruleus</td>
<td>rat</td>
</tr>
<tr>
<td></td>
<td>rat</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>rat</td>
</tr>
<tr>
<td>Periaqueductal Gray</td>
<td>rat</td>
</tr>
<tr>
<td>Thalamus</td>
<td>rat</td>
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<td></td>
<td>rat</td>
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<tr>
<td></td>
<td>rat</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>rat</td>
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<td></td>
<td>rat</td>
</tr>
<tr>
<td>Striatum (Caudate)</td>
<td>rat</td>
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<tr>
<td></td>
<td>rat</td>
</tr>
<tr>
<td>Cortex</td>
<td>rat</td>
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<td></td>
<td>rat</td>
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<tr>
<td></td>
<td>rat, cat</td>
</tr>
</tbody>
</table>
1.1.4. Actions of Opioid Peptides on Single Neurons In Vivo

Electrophysiological studies of the enkephalins and \( \beta \)-endorphin on single neurons in the central nervous system showed that their effects were similar to those of morphine (Table 2). The route of administration of the peptides has been limited to local applications by iontophoresis or pressure injection. Due to the rapid metabolic degradation of the enkephalins, systemic application was unsuccessful. Development of stable analogs, however, has made systemic administration possible. A study of thalamic neurons demonstrated that iontophoretic as well as systemic administration of D-ala\(^2\), D-leu\(^5\)-enkephalin depressed the increase in firing rate evoked by a noxious stimuli (Hill and Pepper, 1978). This depression was reversed by naloxone given either systemically or iontophoretically. The results obtained with the enkephalin analog were similar to those seen with morphine (Hill and Pepper, 1976) and enkephalin (Hill, Pepper and Mitchell, 1976).

There is a generally good correlation between the density of opiate binding sites and the proportion of cells which are responsive to either morphine or the opioid peptides (Nicoll, Siggins, Ling, Bloom and Guillemin, 1977). It appears that morphine and the opioid peptides have the same action on nerve cells tested throughout the central nervous system. That is to say, both morphine and the opioid peptides decrease firing whether it is spontaneous or evoked by physiological stimuli or excitatory amino acids. At the two sites where excitatory effects of morphine predominate, the opioid peptides also excite (Nicoll
et al., 1977). These sites are the pyramidal cells of the rat hippocampus (Nicoll et al., 1977) and the cat Renshaw cell (Davies and Dray, 1978). There is now evidence that the excitation of hippocampal neurons is due to an inhibition of a tonically active inhibitory interneuron (Zieglgansberger, French, Siggins and Bloom, 1978). An inhibition of an inhibitory interneuron may explain the finding that a small proportion of cells are excited by iontophoretic application of morphine or enkephalin at other sites (brainstem, cortex, cerebellum, caudate nucleus) (Nicoll et al., 1977). Similar considerations may explain the increase in firing of brainstem neurons evoked by morphine, enkephalin, acetylcholine and D,L-homocysteic acid, since the excitations produced by acetylcholine as well as morphine and enkephalin were blocked by naloxone (Davies and Dray, 1978).

In spontaneously active cells, the time course of effects produced by enkephalin administered iontophoretically was generally fast. Effects, whether excitatory or inhibitory, began soon after the onset of iontophoretic current (10-30 s) and the firing rate returned to control within 1 min after termination of the current. Some Lamina IV and V cells in the spinal cord of cat are excited by noxious stimuli. Morphine and enkephalin block the increase in firing rate produced by noxious stimuli, but the time course of this effect is much longer than the inhibition of spontaneously firing cells (Duggan, Hall and Headley, 1977b). The time course of the inhibition produced by morphine and enkephalin on lamina IV and V cells was 30 min and 4-10 min, respectively.
In all previous studies, the release of enkephalin from the iontophoresis electrode has not been measured, and therefore the concentrations at the active site are unknown. The responses to iontophoretically applied enkephalin may be opiate receptor-mediated if they are blocked by the narcotic antagonist naloxone. However, systemic administration of naloxone in a dose sufficient to block the usual pharmacological effects of morphine is more desirable. Because the concentrations of agonist and antagonist near the cell being studied are not known when both are applied by iontophoresis, antagonism by iontophoretic administration of naloxone is of limited value. Indeed, iontophoretic application of naloxone antagonises the inhibitions of neurons in the olfactory bulb of rat produced by \( \gamma \)-aminobutyric acid (GABA) (Dingledine, Iversen and Breuker, 1978). In that study naloxone reversed the effect of morphine in only 2 of 14 cells tested.

Another example of the difficulties inherent in iontophoretic administration of agonists and antagonists comes from the work of Duggan, Hall and Headley (1976; 1977) on cat spinal cord. Iontophoretic application of an enkephalin analog near the cell bodies of lamina IV and V cells decreased both spontaneous firing and the increase in firing rate evoked by noxious or non-noxious stimulation. These effects were determined to be of limited significance for three reasons. First, the effects were not selective for either of the two sensory modalities tested whereas systemic application of morphine selectively blocked nociceptive responses. Second, other agents such as glycine and GABA also blocked increases in firing rate produced by each sensory modality. Third, the inhibitions produced by enkephalin applied by iontophoresis near the
cell body were antagonized by iontophoretic administration of naloxone but were not affected by intravenous administration of naloxone in doses up to 0.6 mg/kg. This dose of naloxone blocked the effect of systemically applied morphine and the effects of enkephalin when iontophoresed into the substantia gelatinosa. These experiments suggest that the concentration of enkephalin near the cell body was greater than that necessary to cause blockade of noxious input and high enough to depress other modalities. On the other hand, iontophoresis of morphine and enkephalin into the substantia gelatinosa selectively depressed nociceptive responses of lamina IV and V cells and this was reversed by low doses of naloxone given systemically (Duggan et al., 1977a).
1.1.5. Actions of Opiates and Opioid Peptides on Single Neurons \textit{In Vitro}

\textit{In vitro} studies have been carried out on peripheral morphine-sensitive neurons of the myenteric plexus of the guinea pig ileum, and on spinal and dorsal root ganglion neurons maintained in culture, all of which have previously been shown to contain opiate receptors.

In cultured dorsal root ganglion neurons two separate studies have indicated a possible presynaptic site of action for the opiates and opioid peptides (Crain, Peterson, Crain and Simmon, 1977; MacDonald and Nelson, 1978). A complex multi-unit potential was recorded in the dorsal horn area of fetal mouse spinal cord explants after electrically stimulating the attached dorsal root ganglion (Crain et al., 1977). This potential was markedly diminished by morphine; the diminution was stereospecific and naloxone-reversible. The concentrations used in the bathing solution were comparable to those which are effective on the contractile response of the guinea pig ileum. Interestingly, the evoked dorsal cord potential was increased by low concentrations of naloxone alone which suggests that an inhibitory endogenous opioid system may have developed in these cultures. In the second study, simultaneous intracellular recordings from dorsal root ganglion cells and spinal cord cells in culture were made. Direct intracellular stimulation of the dorsal root ganglion cell resulted in a mono-synaptic depolarizing potential recorded in the spinal cord cell (MacDonald and Nelson, 1978). The excitatory postsynaptic potential (e.p.s.p.) recorded in the spinal cord cell was depressed by iontophoretic application of etorphine in a
naloxone-reversible manner. The authors concluded that the depression of the e.p.s.p. was due to a decrease in quantal content of the e.p.s.p.: however, calculations of quantal content and quantal size were done by assuming a Poisson distribution and these calculations may be erroneous since there is no evidence that a Poisson distribution of transmitter release occurs in this preparation. No evidence of any postsynaptic effects during application of etorphine were observed.

In another experiment on cultured mouse spinal cord cells, leu-enkephalin was applied iontophoretically near the surface of spinal cord neurons (Barker, Neale, Smith and MacDonald, 1978). Leu-enkephalin decreased the depolarization induced by iontophoretic application of glutamate. This effect was partially reversed by iontophoretic application of naloxone in only some experiments and an opiate receptor-mediated effect may or may not be involved. The action of opiates on single myenteric neurons has also been studied (see Section 1.3.3.).
1.1.6. The Significance of the Endogenous Opioid Peptides

The evidence concerning the location, release and actions of the endogenous opioid peptides strongly supports a neurotransmitter or neuromodulator role for these peptides. No functional opioid synapse, however, has been demonstrated. Also, naloxone has little or no action of its own when administered to normal human subjects. The endogenous opioid system, however, has been proposed to mediate the analgesia produced by acupuncture in man (Mayer, Price and Rafii, 1977) cat and mouse (Pomeranz, 1978) and by electrical stimulation of the periaqueductal gray in man (Akil, Watson, Berger and Barchas, 1978). Administration of naloxone was shown to partially reverse the analgesic effects caused by stimulation produced analgesia in all species tested. The partial reversal, by naloxone, of stimulus produced analgesia (SPA) indicates at least part of the analgesic effect seen could be due to release of opioid peptides. The release of opioid peptides into cerebrospinal fluid following central stimulation also indicates that an endogenous opioid system may play a role in SPA (Terenius, 1978).

Several approaches have been used to study the nature of the antinociceptive effects of acupuncture (Mayer et al., 1977; Pomeranz, 1978). First, the increase in firing rate of nociceptive spinal cord cells in lamina IV and V of cat produced by painful stimulation was blocked by acupuncture in a naloxone-reversible manner (Pomeranz, 1978). Second, the latency of response to a noxious stimulus (heat) in awake mice was increased by acupuncture and this increase in latency was
blocked by prior treatment with naloxone (Pomeranz, 1978). Third, a strain of mice (CBXK) which had a deficit of opiate receptors was used to study acupuncture. This strain of mice showed no significant change in antinociceptive response after acupuncture (Pomeranz, 1978). Finally, naloxone antagonized the increase in pain threshold caused by acupuncture in healthy humans (Mayer, et al., 1977). The most striking effect of the studies on acupuncture was the time course of effects. The onset and offset of analgesia produced by acupuncture were in the order of minutes to hours.

It therefore appears that the antinociceptive responses, behaviorally and at a single cell level, to SPA and acupuncture are, at least in part, mediated by release of endogenous opioids. The site which may be involved in the release of the opioid has not yet been determined. It is of interest to note that hypophysectomy abolished the response to acupuncture in mice (Pomeranz, Cheng and Law, 1977). Since the pituitary is known to contain large amounts of β-endorphin, it may be possible that release of β-endorphin from the pituitary is important in acupuncture analgesia. The metabolic stability of β-endorphin may also account for the prolonged effects produced by acupuncture.
1.2. NEUROPEPTIDES

1.2.1. Substance P

1.2.1.1. General Background. Substance P, a hypotensive and smooth muscle stimulating agent was first extracted from brain and intestine by von Euler and Gaddum (1931). It was not until 1970 that this peptide was isolated in a pure form (Chang and Leeman, 1970). The structure was determined to be an undecapeptide (Chang, Leeman and Niall, 1971) (Table 1) and it was subsequently synthesized (Tregear, Niall, Potts, Leeman and Chang, 1971). The availability of pure, synthetic substance P allowed the development of many avenues of research. A radioimmunoassay was developed which allowed minute quantities to be measured from gross tissue extracts (Powell, Leeman, Tregear, Niall and Potts, 1973). The distribution of substance P in the central nervous system has been studied by measuring the amount of substance P contained in discrete nuclei isolated by microdissection (Brownstein, Mroz, Kiaer, Falkovits and Leeman, 1976; Nakata, Kusuka, Seqawa, Yajima and Kitgawa, 1978). Regions in the central nervous system which contain high concentrations (>1.5 p mole/10 mg wet weight) are the hypothalamus, preoptic area and mesencephalon. The spinal cord contains somewhat less substance P. However, there is about twice as much substance P in the dorsal horn as in the ventral horn of the spinal cord. Using substance P labeled with tritium, a binding site for substance P has been demonstrated in membrane fragments from rabbit brain. This binding site has a dissociation constant (Kd) of 2.7 nM and the order of binding affinities of a
series of analogs correlates well with their biological potencies (Nakata et al., 1978). The distribution of the substance P binding site also correlates well with the distribution of substance P in the central nervous system of rabbit (Nakata et al., 1978).

Immunohistochemical studies also became possible with the availability of pure substance P (Hokfelt, Kellerth, Nilsson and Pernow, 1975). Substance P-like immunoreactivity was found in the neuronal cell bodies and processes. The distribution of immunohistochemically identified substance P correlates well with the distribution determined by the extraction procedures described above. Although immunohistochemistry cannot be used quantitatively, the cellular and subcellular localization is important in determining the physiological role of this peptide. Substance P was generally found in small unmyelinated fibers, often associated with afferent nerve pathways (Hokfelt et al., 1975; Otsuka, Konishii and Takahashi, 1975; Takahashi and Otsuka, 1975; Hokfelt, Elde, Johansson, Luft, Nilsson and Arimura, 1976; Chan-Palay and Palay, 1977; Hokfelt, Ljungdahl, Terenius, Elde and Nilsson, 1977). Substance P-like immunoreactivity has also been found in nerve terminals and varicosities of peripheral ganglia, including sympathetic ganglia, (Hokfelt, Elfvin, Schultzberg, Goldstein and Nilsson, 1977) and in the myenteric plexus (Nilsson, Larsson, Hakanson, Brodin, Pernow and Sundler, 1975; Hokfelt, Johansson, Kellerth, Ljungdahl, Nilsson, Nygards and Pernow, 1977).

Ligation studies of the dorsal roots indicate that substance P is transported towards the spinal cord in nerve axons and may be involved in primary afferent transmission (Otsuka, Konishi, Takahashi and Sato,
Lesion studies using immunohistochemistry and extraction procedures suggest that substance P is also involved in at least three other neuronal pathways: two are the projections to the substantia nigra from the globus pallidus and corpus striatum, and a third is the pathway from the habenula to the interpeduncular nuclei (Brownstein, Mroz, Tappaz and Leeman, 1977; Jessell, 1978; Jessell, Emson, Paxinos and Cuello, 1978). Another short projection within the amygdala has also been described (Emson, Jessell, Paxinos and Cuello, 1978).

The subcellular localization of substance P has been studied in two ways. Cell fractionation of rat brain has shown that substance P is located primarily in the synaptosomal fraction (Cuello, Jessell, Kanazawa and Iversen, 1977). Electron microscopic immunohistochemistry of substance P in the amygdala and substantia gelatinosa demonstrated dense core vesicles which contained substance P-like immunoreactivity (Cuello et al., 1977; Pelletier, LeClerc and Dupont, 1977).

Substance P has been shown to be released from synaptosomal preparations (Schenker, Mroz and Leeman, 1976), slices of hypothalamus (Iversen, Jessell and Kanazawa, 1976), trigeminal nucleus (Jessell and Iversen, 1977) and substantia nigra of the rat (Jessell, 1978) and isolated spinal cord of the rat (Otsuka and Konishi, 1976). These experiments all used high potassium containing solutions to evoke the release; the release was calcium-dependent. In addition, Otsuka and Konishi (1976) and Jessell (1978) observed release of substance P with electrical stimulation and veratridine, respectively. Release of substance P in these preparations therefore
Little is known of the synthesis of substance P. Likewise, no mechanism of inactivation of substance P has been described. Substance P was not found to be actively accumulated in rat brain slices from which it was released (Iversen, Jessell and Kanazawa, 1976). Substance P has been shown to be stable in the postmortem brain (Kanazawa and Jessell, 1976) and in whole blood or plasma (Bury and Mashford, 1977).

If substance P is to satisfy all criteria for a neurotransmitter, diffusion away from the receptor site may play an important role for the inactivation process.

1.2.1.2. Actions on Single Neurons. Since the synthesis of substance P, studies of the action on single neurons have proceeded relatively quickly. Prior to this time, the effects of substance P (obtained from an extract of cattle intestine) on single neurons were inconsistent (Galindo, Krnjević and Schwartz, 1967). This may be attributed to impurities contained in the extract. Recent work has found substance P to be a weak depolarizing or excitatory agent on cells in several locations (Table 3). With the exception of Otsuka and Konishi's work on isolated spinal cord, all studies have been done in vivo using iontophoretic application of substance P. The most striking aspect of the effects produced by substance P was the extended time course of its action compared to other excitatory agents such as glutamate. The onset and offset of the excitatory effect of substance P often occurred over a period of several minutes. The effects were variable and tended to decrease with repeated applications (Krnjević and Morris, 1974; Henry, Krnjević
Table 3. The action of substance P studied in various areas of the central nervous system.

<table>
<thead>
<tr>
<th>Area</th>
<th>Species</th>
<th>Ion</th>
<th>Perfusion</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motor neurons</td>
<td>frog</td>
<td>↑</td>
<td></td>
<td>Konishi and Otsuka, 1974a.</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>↑</td>
<td></td>
<td>Konishi and Otsuka, 1974b.</td>
</tr>
<tr>
<td>Renshaw cell</td>
<td>cat, rat</td>
<td>↑</td>
<td></td>
<td>Henry et al., 1975.</td>
</tr>
<tr>
<td></td>
<td>cat</td>
<td>↑</td>
<td></td>
<td>Davies and Dray, 1977.</td>
</tr>
<tr>
<td></td>
<td>cat</td>
<td>↑</td>
<td></td>
<td>Ryall and Belcher, 1977.</td>
</tr>
<tr>
<td>Cuneate</td>
<td>cat</td>
<td>↑</td>
<td></td>
<td>Krnjević and Morris, 1974.</td>
</tr>
<tr>
<td>Locus coeruleus</td>
<td>rat</td>
<td>↑</td>
<td></td>
<td>Guyenet and Aghajanian, 1977.</td>
</tr>
<tr>
<td>Interpeduncular nucleus</td>
<td>rat</td>
<td>↑</td>
<td></td>
<td>Sastry, 1978a; 1978b.</td>
</tr>
<tr>
<td>Cortex</td>
<td>rat, cat</td>
<td>↑</td>
<td></td>
<td>Henry et al., 1975.</td>
</tr>
<tr>
<td></td>
<td>rat</td>
<td>↑</td>
<td></td>
<td>Sastry, 1978b.</td>
</tr>
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abbreviations: Ion - iontophoretic application  
Perfusion - superfusion of a known concentration on to an isolated preparation  
↑ - indicates that the predominate of substance P was excitatory
and Morris, 1975; Davies and Dray, 1976; Le Gal La Salle and Ben-Ari, 1977; Sastry, 1978). Interpretation of *in vivo* studies on substance P is difficult for five reasons. First, in only two studies was the release of substance P from the iontophoresis electrode measured (Krnjević and Morris, 1974; Belcher and Ryall, 1978). Second, specific antagonists for substance P are not available. Third, the effects of general anesthetics decrease the response of some cells to substance P (Sastry, 1978b). Fourth, none of these studies included the iontophoresis of analogs of substance P. Fifth, substance P in iontophoretic electrodes has been reported to break down over a period of days (Gozlan, Le Gal La Salle, Michelot and Ben-Ari, 1977), even though the amount measured by radioimmunoassay decreased only slightly over extended periods of time (Krnjević and Morris, 1974). This implies that breakdown in the iontophoresis electrode results in fragments which may be biologically less active but which are still immunologically reactive.

Some of the above difficulties are removed in *in vitro* experiments. In the isolated frog spinal cord preparation, bath application of known amounts of substance P caused a marked depolarization associated with an increase in conductance of spinal motor neurons (Konishi and Otsuka, 1974b). This depolarization was determined to be a direct action on the spinal motor neurons since it was still present in solutions which contained low calcium and high magnesium ion concentrations; such solutions blocked the reflex activity induced in the dorsal root and recorded in the ventral root. The specificity of the depolarization produced by substance P was tested using a series of analogs. The potency of the analogs was markedly decreased if any change was made at
the amino terminus of the molecule. Also, if more than five amino acid residues were removed from the carboxy terminal the potency was decreased (Otsuka and Konishi, 1977). The relative order of potency of these analogs on spinal motor neurons corresponds reasonably well with the gut-contracting and hypotensive activity (Otsuka and Konishi, 1977), as well as with their binding affinities (Nakata et al., 1978). The use of a series of structurally related analogs is important to demonstrate possible receptor interactions, since there are as yet no known substance P antagonists. If the relative order of potency of a series of analogs is the same for a number of biological responses and binding studies, then a receptor mediated effect is supported. A major difficulty in the interpretation of Otsuka's work is that the effective concentrations of substance P were 100-1,000 times higher than those effective in contracting smooth muscle or reducing blood pressure. Indeed, a similar point may be raised with all the experiments using iontophoretic application, since the concentration around the cell is not known.

Baclofen (β-(4-chlorophenyl)γ-aminobutyric acid) (Lioresal) was found to antagonize the depolarization of spinal motor neurons produced by substance P (Saito, Konishi and Otsuka, 1975). However, baclofen also decreased the glutamate depolarization. At other synapses, antagonism of the substance P response by baclofen appears to be at least partially non-specific (cat spinal motor neurons - Krnjević, 1977; rat interpeduncular neurons - Sastry, 1978a). Systemic administration of baclofen results in a decrease in synaptic transmission to spinal motor neurons and the cuneate nucleus, but the site of action of systemically-applied baclofen seems to be both pre- and postsynaptic (Krnjević, 1977).
A second discrepancy between the work of Otsuka and that of Krnjević is the proposed mechanisms underlying the depolarization produced by substance P. Krnjević reported a decrease in conductance in cat spinal motor neurons; the reversal potential for this action of substance P was more negative than the resting potential. Otsuka, on the other hand, found an increase in conductance with a reversal potential near zero. Krnjević's results must be interpreted with caution because of the possible activation by substance P of cells which have excitatory or inhibitory synapses on to the spinal motor neuron. Substance P may depolarize an interneuron which releases an excitatory transmitter on to the spinal motor neuron. In this case, substance P and the excitatory transmitter may both depolarize the spinal motor neuron, but do so through different ionic mechanisms.
1.2.2. Neurotensin

1.2.2.1. General Background. Neurotensin was isolated from bovine hypothalamus; it is a tridecapeptide (Table 1) (Carraway and Lee–man, 1973; 1975 a,b). When administered intravenously to rats neurotensin has numerous actions including hypotension, increased vascular permeability, hyperglycemia and hyperglucagonemia. In mice and rats, an intracisternal injection of neurotensin decreases cold tolerance, induces hypothermia, and enhances the depressant effect of phenobarbital (Nemeroff, Bissette, Prange, Loosen, Barlow and Lipton, 1977). These actions are not seen with systemic administration, which probably indicates either an inability of this peptide to cross the blood brain barrier or a relatively fast metabolism in the blood.

The distribution of neurotensin in the central nervous system has been studied using radioimmunoassay and immunohistochemical methods (Uhl and Snyder, 1976; Kobayashi, Brown and Vale, 1977; Uhl, Kuhar and Snyder, 1977). Using immunohistochemistry, neuronal cell bodies and processes containing neurotensin were found in high density in the substantia gelatinosa, trigeminal nucleus, amygdala, anterior pituitary and hypothalamic nuclei. Subcellular fractionation studies indicated that the majority of the neurotensin was associated with a synaptosomal fraction (Uhl and Snyder, 1976). Release of neurotensin from slices of hypothalamus has been demonstrated; the release was evoked by a high potassium solution and required the presence of extracellular calcium ions (Iversen, Iversen, Bloom, Douglas, Brown and Vale, 1978).
Il25-labeled neurotensin binds to membrane fragments from brain homogenates with a high affinity (Kd= 3-8 nM) and the density of binding sites is highest in regions containing large amounts of neurotensin (Lazarus, Brown and Perrin, 1977; Uhl, Bennett and Snyder, 1977). The binding affinity of a series of neurotensin analogs also correlates well with their rank order of potency tested on the hyperglycemic and hyperglucagonemic responses in rats (Uhl et al., 1977). The neurotensin binding sites may be taken to indicate that neurotensin acts on a specific receptor.

The mechanisms by which neurotensin is synthesized, degraded, or removed from the extracellular space have not yet been described. However, enzymic degradation of neurotensin was suggested by studies on extracts of brain and hypothalamus (Dupont and Merand, 1978).

1.2.2.2. Actions on Single Neurons. Some preliminary studies of the action of neurotensin on neurons in the central nervous system have been made. Neurons in several areas which have a relatively high density of binding sites and high levels of neurotensin were studied. These include frontal cortex, hippocampus, striatum and lateral thalamus (Zieglegänsberger, Siggins, Brown, Vale and Bloom, 1978) and laminas I-III of the spinal cord (Miletic and Randic, 1978). The predominant effect produced by iontophoretic application in these areas was excitation which was slow in onset. This excitation long outlasted the period of application. The locus coeruleus also contains neurotensin within cell bodies and nerve terminals but iontophoresis of neurotensin on to these cells inhibits neuronal firing (Scott-Young, Uhl and Kuhar, 1978). The
results from this study differ also in the time course of the effects. The inhibition in firing rate and the return to control occurred rapidly after the onset and offset of iontophoretic current.

Neurotensin causes a contraction of the guinea pig ileum which is blocked by tetrodotoxin, implying an excitatory action on myenteric neurons (Kitabgi and Freychet, 1978). A relaxation of other smooth muscle preparations has been found which was not affected by tetrodotoxin (Andersson, Rosell, Hjelmquist, Chang and Folkers, 1977).
1.2.3. Vasoactive Intestinal Polypeptide

1.2.3.1. General Background. A second polypeptide first isolated from the gut which has properties similar to those of substance P is vasoactive intestinal polypeptide (VIP) (Said and Mutt, 1970a). Originally, the actions of VIP were thought to be confined to the gut since a single pass through the liver almost completely inactivates this peptide (Said and Mutt, 1970b). This 28-amino acid residue peptide (Table 1) causes systemic vasodilation, hypotension, hyperglycemia, stimulates cardiac output and excites respiratory chemoreceptors (Mutt and Said, 1974a). Recently, using radioimmunoassay and immunohistochemistry, VIP has been localized in neurons of peripheral ganglia and central nervous system (Larsson, Fahrenkrug, Schaffalitzky De Muckadell, Sundler, Hansson and Rehfeld, 1976). A dense population of VIP-containing neurons was found in the gut (Larsson et al., 1976; Larsson, 1977a,b; Fux, Hokfelt, Said and Mutt, 1977) and in the mesenteric ganglia complex (Hokfelt, Elfvin, Schulzberg, Fux, Said, Mutt and Goldstein, 1977). In the central nervous system, VIP-containing terminals were found in some hypothalamic nuclei, in parts of the amygdala and in the cortex (Fux et al., 1977). VIP has been shown to occur within neuronal granules of 120 nm diameter by immunohistochemistry (Larsson, 1977a) and in the synaptosomal fraction by subcellular fractionation studies (Gaichetti, Said, Reynolds and Koniges, 1977). Potassium-evoked release of VIP from a synaptosomal preparation by a calcium-dependent mechanism adds further evidence to support a neurotransmitter role for this peptide (Gaichetti et al., 1977).
1.2.3.2. Action on Single Neurons. Little work has been done on the actions of VIP on single neurons. Iontophoretic application of VIP on to cortical neurons results in an increase in firing rate (Phillis, Kirkpatrick and Said, 1978). VIP depolarizes the dorsal and ventral roots of toad spinal cord; however, very high concentrations of VIP (3-6 $\times$ 10$^{-6}$M) are required (Phillis et al., 1978). The high concentration used in this preparation contrasts with the lower doses required for other effects; for example, a dose of only 40 ng/kg increased femoral arterial blood flow by 50% (Said and Mutt, 1970a).
1.2.4. Somatostatin

1.2.4.1. General Background. The hypothalamic peptide somatostatin was first isolated, purified and synthesized in 1973 (Brazeau, Vale, Burgus, Ling, Butsher, Rivier and Guillemin, 1973). This cyclic tetradecapeptide has been shown to inhibit the release of growth hormone in both cultured pituitary cells and pentobarbital-treated rats (Brazeau et al., 1973). An inhibition of the release of other hormones by somatostatin has also been reported (TSH - Siler, Yen, Vale and Guillemin, 1974; insulin - Curry and Bennett, 1974; Curry and Bennett, 1976; glucagon - Fujimoto and Esinck, 1976). More recently, somatostatin has been found by immunohistochemistry and radioimmunoassay at several sites in the central nervous system which are probably unrelated to hormone regulation (Hokfelt, Efendic, Hellerstrom, Johansson, Luft and Arimura, 1975; Hokfelt, Elfvin, Elde, Schultzberg, Goldstein and Luft, 1977; Kobayashi, Brown and Vale, 1977). Extrahypothalamic areas which have high concentrations of somatostatin include the amygdala, nucleus accumbens, medulla, substantia gelatinosa, thalamus and hippocampus. Nerve terminals which contain somatostatin have been observed immunohistochemically in peripheral ganglia including the myenteric plexus (Hokfelt et al., 1975; 1976; Costa, Patel, Furness and Arimura, 1977). Immunocytochemical techniques have localized this peptide to neuronal fibers and varicosities (Petrusz, Sar, Grossman and Kizer, 1977) and subcellular fractionation has indicated that it is concentrated in the synaptosomal fraction (Epelbaum, Brazeau, Tsang, Brawer and Martin, 1977). Release of somatostatin from slices of hypothalamus and amygdala using high potassium
solutions by a calcium-dependent mechanism may imply that, in addition to its neuroendocrine function, somatostatin may act as a neurotransmitter (Iversen, Iversen, Bloom, Douglas, Brown and Vale, 1978).

There is some evidence, from binding studies and behavioral experiments, that the actions of somatostatin may be mediated by opiate receptors (Rezek, Havicek, Lebin, LaBella and Freisen, 1977; Pugsley and Lippmann, 1978). This evidence is weak because 1,000 times more somatostatin than opiates was needed to displace naloxone from its binding site. In fact, somatostatin binding compared with that of dextrorphan (Pugsley and Lippmann, 1978). Large amounts of somatostatin administered intracerebroventricularly resulted in slight antinociception which was only partially reversed by naloxone (Rezek et al., 1977). Somatostatin inhibited the contractile response of the longitudinal muscle-myenteric plexus preparation to nerve stimulation; however, this effect of somatostatin was not reversed by the opiate antagonist, naloxone (Guillemin, 1976).

In the frog isolated spinal cord, the action of somatostatin on the membrane potential of the ventral and dorsal roots has been studied using the sucrose gap technique (Padjen, 1977). Somatostatin hyperpolarized the dorsal roots and this persisted in solutions where synaptic transmission was blocked using a calcium-free solution, and where propagated activity was blocked by a local anesthetic. In this study, the endocrinologically inactive retro-somatostatin did not produce a hyperpolarization. The hyperpolarization produced by somatostatin therefore appears to be a direct action on the dorsal roots; however, the concen-
tration required was 1,000 fold greater than that required to produce endocrine effects.

1.2.4.2. Actions on Single Neurons. Iontophoretic application of somatostatin inhibits neuronal firing in the cortex, hypothalamus and cerebellum (Renaud, Martin and Brazeau, 1975) and in lamina I-III of the spinal cord (Miletic, Kavacs and Randic, 1977). On the other hand, in slices of rat hippocampus, iontophoresis or pressure injection of somatostatin resulted in depolarization and increased excitability of CA1 and CA2 cells (Dodd and Kelly, 1978).

There are no specific antagonists of somatostatin; somatostatin analogs have not been studied and there is no method by which to correlate the concentrations which produce the endocrine effects with the amount of somatostatin which is applied iontophoretically. Moreover, the differences in responses to somatostatin seen among cells of the hippocampus and those elsewhere in the central nervous system were much like those seen with morphine and enkephalin (see section 1.1.4.). The excitation of pyramidal cells of the hippocampus by the opiates and opioid peptides has been shown to be a trans-synaptic action (Zieglgänsberger et al., 1978). The opiates are thought to excite pyramidal cells by inhibiting an inhibitory interneuron. The same type of disinhibition could explain the depolarization seen with iontophoretic application of somatostatin in the hippocampus.
1.3. THE MYENTERIC PLEXUS

1.3.1. Anatomy

The myenteric plexus is a complicated network of neurons which may be involved in the transmission of afferent and efferent impulses to and from the central nervous system as well as being involved in local reflex mechanisms (Schofield, 1968). In the guinea pig ileum, the plexus is a network of neurons arranged in ganglia which are laid out in a relatively structured manner. The ganglia of the plexus are situated in a roughly rectangular arrangement with interconnecting strands containing nerve processes running between the ganglia. The ganglia range from 50-100 \( \mu \text{m} \) in width, are up to 1 mm in length and are 1-3 cells thick. The entire ganglion is surrounded by a basal lamina which isolated the neurons from connective tissue and blood vessels (Gabella, 1972). Each ganglion contains from 20-200 nerve cells and about 2-3 times as many glial cells (Gabella, 1972). The extracellular space within the ganglia is limited by a dense neuropil of nervous and glial origin.

The size of the ganglion cells vary from less than 10 \( \mu \text{m} \) to 35 \( \mu \text{m} \) in diameter (Gabella, 1972; Cook and Burnstock, 1976). Unlike sympathetic and spinal ganglia, there are few nerve cell bodies which are surrounded by satellite cells (Gabella, 1972; Cook and Burnstock, 1976). Using the electron microscope, up to nine types of ganglion cells were distinguished by their size, type of endoplasmic reticulum, appearance and distribution of other cellular organelles and the relationship the neurons had with satellite cells and neuropil (Cook and Burnstock, 1976).
Extraganglionic neurons were present in the interconnecting strands between the ganglia. These cells were seen only occasionally, were unlike other nerve cell types seen in the ganglia and exhibited very few typical synaptic specializations (Cook and Burnstock, 1976).

Several types of axons have also been described using the electron microscope (Gabella, 1972; Cook and Burnstock, 1976). Different types of axons were distinguished by the type of vesicles found near synaptic membrane specializations and included: small granular vesicles (40-60 nm), round agranular vesicles (40-60 nm), flattened agranular vesicles (20-40 nm), elongate structures (up to 350 nm long and 18-30 nm in diameter) and three types of large vesicles with granular cores (Gabella, 1972; Cook and Burnstock, 1976). It was assumed that the small granulated vesicles in nerve terminals contained noradrenaline, and the agranular vesicles which were most frequently seen contained acetylcholine (Gabella, 1972). The presence of cholinergic and noradrenergic terminals in the myenteric plexus is well accepted. Electrophysiological evidence strongly supports the neurotransmitter role of acetylcholine and noradrenaline (see section 1.3.2.2.).

The significance of the remaining types of axon profiles based on the type of vesicles found in the varicosities remains to be determined. Several endogenous agents have been proposed to function as neurotransmitters in the myenteric plexus on the basis of their histochemical and cytochemical locations. The presence of nerve terminals and cell bodies in the myenteric plexus which can take up and synthesize 5-hydroxytryptamine (5-HT) suggests that 5-HT may be another transmitter in the myenteric
plexus (Dreyfus, Bornstein and Gershon, 1977; Dreyfus, Sherman and Gershon, 1977). Electrophysiological evidence on the action and role of 5-HT in the myenteric plexus is inconclusive (see sections 1.3.2.1. and 1.3.2.2.). The myenteric plexus is also thought to have neurons which contain and release purine nucleotides (Burnstock, 1972). More recently, immunohistochemical studies have demonstrated the presence of the following neuropeptides within nerve terminals of the myenteric plexus: enkephalin (leu and met), substance P, somatostatin and VIP (see appropriate sections for references). The location of the cell bodies of origin is not known with certainty. VIP-like and somatostatin-like immunoreactivity have been observed in cell soma within the myenteric plexus such that at least a part of the VIP and somatostatin is intrinsic to the gut. An extrinsic source of enkephalin, substance P and somatostatin has been proposed, but experiments using agents to block axonal transport (colchicine) to increase the content of immunoreactive substances in the cell bodies have not been performed.

The distribution of the neuropeptides within the gastrointestinal tract has not been studied, with the exception of enkephalin. A higher content of met- and leu-enkephalin was found in the duodenum than in other parts of the gastrointestinal tract (Hughes et al., 1977). The significance of this observation remains to be determined. However, it is possible that regional variations in the content of one or more of the neuropeptides may account in part for some functional differences along the gastrointestinal tract.

Histologically, the occurrence of a dense neuropil, the isolation
from blood vessels and connective tissue, and the absence of extracellular space make the cells of the myenteric plexus more like those of the central nervous system than other autonomic ganglia (Gabella, 1972). Functionally, the myenteric plexus also appears to be somewhat different from other autonomic ganglia. The arrangement of neurons within the plexus confers preferential pathways of conduction in the aboral direction (Bayliss and Starling, 1899; Kosterlitz and Lydon, 1971; Hirst and McKirdy, 1975; Yokoyama, Ozaki and Kajitsuka, 1977). The plexus also contains a neuronal type which is functionally distinct from those in autonomic ganglia (see below).
1.3.2. Electrophysiology

The development of techniques to study single myenteric neurons has been made by several groups. The early work utilized extracellular electrodes made of metal or glass (Yokoyama, 1966; Wood, 1970). More recently, intracellular recordings have been performed (Nishi and North, 1973a; Hirst, Holman and Spence, 1974). There are marked differences in the results obtained by these two techniques. The most notable difference is that few spontaneously active cells were found with intracellular techniques, whereas ongoing spike activity was recorded using extracellular electrodes. A description of the methods and results obtained with extracellular and intracellular electrodes is necessary before an understanding of these differences is possible.

1.3.2.1. Extracellular Recording. The first recordings from single myenteric neurons were made by Yokoyama (1966) using extracellular electrodes. Subsequently, Wood (1970) and Ohkawa and Prosser (1972) described a series of different types of neuronal units in terms of their discharge patterns. The experimental methods used by Wood, and Ohkawa and Prosser were similar. Both used the isolated jejunum from cat and metal or metal-filled electrodes having tip diameters of 1-25 μm. The myenteric ganglia were stained with methylene blue for visualization. They described similar types of activity. There were three types of neuronal discharge patterns: (1) mechanosensitive; (2) single spike; (3) burst. Mechanosensitive neurons were stimulated by distortion of the ganglion produced by a glass probe or an electrode having a large
tip diameter. Single spike neurons fired at low frequencies (0.1-2 Hz) with no consistent firing pattern. Burst type neurons periodically discharged a flurry of action potentials followed by periods of silence. Attempts to record activity which was evoked by electrical stimulation were not successful (Ohkawa and Prosser, 1972a,b). The same types of discharge patterns were found in the guinea pig ileum using a similar experimental design (Wood, 1973).

When a glass suction electrode (30-100 µm) was used to record activity, only the single spike and burst units were found (Sato, Takanagan and Takagi, 1973). This type of electrode enabled evoked activity to be recorded (Dingledine and Goldstein, 1976; Yokoyama, Ozaki and Kajitsuka, 1977). There was also a large difference in the ratio of burst units to single spike units encountered when using the different types of electrodes. With metal electrodes, burst units were found five times more frequently than single spike units (Wood, 1970); with glass suction electrodes, burst units were found only occasionally (Sato et al., 1973; Dingledine and Goldstein, 1976).

Dingledine, Goldstein and Kendig (1974) were the first workers to ensure that the unit firing was not secondary to local muscle movement. They isolated 1-3 ganglia using small pins. All previous studies had used methylene blue to visualize the ganglia whereas Dingledine et al. (1974) transilluminated the ganglia and eliminated the need for staining. Methylene blue has been shown to block synaptic transmission in sympathetic ganglia at the concentrations used for staining and, at somewhat higher concentrations, it causes irreversible changes in the
firing patterns in the cat myenteric plexus (Nozdrachev, Gnetov, Kachalo and Fedorova, 1977). It is likely that methylene blue, at the concentrations used for extracellular recording in the myenteric plexus, may have changed the sensitivity of the neurons to some agents.

Neuronal spikes have been reported to be 3-5 ms in duration. The waveform was usually biphasic and sometimes triphasic (Wood, 1975). Biphasic spikes were considered to originate at or near the soma and triphasic spikes to be from a propagated potential coming toward, passing under, and moving away from the electrode. However, extracellular potentials recorded with point microelectrodes may vary considerably in waveform. One important consideration is the time constant of the recording system. As the coupling time constant decreases, the waveform tends to change to a differentiated type of waveform (DeValois and Pease, 1973). It is common to use amplifiers capable of increasing or decreasing the coupling time constant in order to attenuate extraneous slow potentials and line noise which would interfere with spike discrimination. Evaluation of extracellular potential waveform under these conditions is difficult. The waveform of extracellular spikes may also vary with the position of the electrode. As an extracellular electrode is advanced towards a cell in the central nervous system, it is common for the waveform to change (Towe, 1973). Simultaneous recording of intracellular and extracellular spikes has shown marked differences in extracellular waveform when recording from axons, initial segment and soma-dendritic spikes in a given cell (Svaetichin, 1958). Similar considerations must be given to the proximity of the cell being recorded to the electrode in the myenteric plexus. The precise site of recording in the myenteric
plexus has not previously been described. No mention has been made of spontaneous activity recorded in the interconnecting strands between ganglia, although evoked activity from electrical stimulation has been demonstrated (Dingledine and Goldstein, 1976; Yokoyama et al., 1977).

The sensitivity of the three types of units to various transmitters and to drugs is different. Mechanoreceptive and burst type units were relatively insensitive to most drugs, whereas single spike units exhibited marked sensitivity to many agents. All neuronal activity was abolished by tetrodotoxin or local anesthetics, but not by manganese (Sato et al., 1973; Sato, Takayanagi and Takagi, 1974). Single spike units were excited by 5-hydroxytryptamine (5-HT), sodium picrate, caerulein, acetylcholine, nicotine and pentagastrin. These excitations were blocked by morphine as well as by the relevant, more specific antagonist (Sato et al., 1973). The firing rate of single spike units was decreased by α-adrenergic agonists and morphine. A detailed study on the action of morphine on single unit firing in the myenteric plexus was reported by Dingledine and Goldstein (1975, 1976) (see section 1.3.3.).

1.3.2.2. Intracellular Recording Studies. Intracellular recording from myenteric neurons has been accomplished by two groups (Nishi and North, 1973a; Hirst et al., 1974). Similar methods were used by each group. Direct visualization of individual neurons was necessary to allow careful placement of the electrode because the myenteric neurons were highly sensitive to mechanical damage. The use of high resistance (> 60 MΩ) electrodes was also required for stable recordings. The use of
electrodes of lower resistance led to slow depolarization of the cells which was attributed to severe mechanical damage.

The results obtained by Nishi and North (1973a) and Hirst et al. (1974) were essentially the same. Two types of myenteric neurons were distinguished with intracellular recordings. The first type (Type I, Nishi and North, 1973a; S cell, Hirst, et al., 1974) gave multiple spikes with a depolarizing current, received synaptic input which was blocked by nicotinic antagonists and had properties similar to other peripheral ganglion cells. The second type (Type II, Nishi and North, 1973a; AH cell, Hirst et al., 1974) was less excitable, received no synaptic input and had a slow after-hyperpolarization following the action potential evoked by direct or antidromic stimulation. A third type of cell was also impaled which was inexcitable, had a high resting membrane potential and a low input impedance. This type of cell was considered to be a glial cell. Occasional spontaneous neuronal activity was observed; however, spontaneously active cells were probably damaged by the isolation of the ganglia. No evidence of any pacemaker activity was described in any neurons of the myenteric plexus.

The sensitivity of the two types of neurons to pharmacological stimuli (acetylcholine, 5-HT, morphine) appear to be quite different. Iontophoretic application of acetylcholine to type I cells produce a rapid depolarization which was blocked by nicotinic antagonists (North and Nishi, 1973a). Application of acetylcholine to type II cells produced either no effect or a slow depolarization which was blocked by muscarinic antagonists (North, unpublished results).
The results obtained when studying the action of 5-HT and noradrenaline with intracellular electrodes are different than when studied using extracellular electrodes. Noradrenaline inhibited firing of myenteric neurons recorded extracellularly (Sato et al., 1973). The predominant action of 5-HT on the firing rate of myenteric neurons recorded with extracellular electrodes was an increase in firing rate (Dingledine and Goldstein, 1975). 5-hydroxytryptamine produced variable results on the membrane properties of type I cells, but had a prominent action reducing the size of the e.p.s.p. This is due to a presynaptic effect, decreasing acetylcholine release (North and Henderson, 1975). Similar presynaptic effects of noradrenaline have been found (Nishi and North, 1973b). Stimulation of sympathetic nerves to the gut also decreases acetylcholine release without altering membrane properties of the myenteric neurons (Hirst and McKirdy, 1974). The predominant effect that 5-HT has on the type II cell is a slow hyperpolarization with a fall in input resistance (North and Henderson, 1975).

One of the primary differences between results obtained with intracellular and extracellular recording techniques in the guinea pig myenteric plexus was the lack of spontaneous activity recorded using intracellular electrodes, whereas spontaneous neuronal firing was recorded with extracellular electrodes. The high sensitivity of myenteric neurons to mechanical damage seems inconsistent with the use of very large suction electrodes for extracellular recordings. There are similarities, however, for the single spike unit and the burst units in some ways correspond to the intracellular type I and type II cells, respectively. First, the type I cell is more excitable and, if it corresponds to a
single spike unit, this may account for the high frequency of single spike units encountered with a suction electrode. Second, the type I cell is sensitive to acetylcholine through a nicotinic receptor and single spike units are also excited by nicotine. Third, type II cells were insensitive to many pharmacological stimulants as were the burst units encountered with extracellular electrodes (Sato et al., 1974; Dingledine and Goldstein, 1976; Nishi and North, 1973a; North, 1973).
1.3.3. Actions of Opiates, Opioids and Neuropeptides in the Guinea Pig Myenteric Plexus

1.3.3.1. Using the Longitudinal Muscle-Myenteric Plexus Preparation. Electrical stimulation of the nerves in the longitudinal muscle-myenteric plexus preparation induces release of acetylcholine which acts on the longitudinal muscle to cause a contraction. The nerve-mediated contraction of the longitudinal muscle is depressed by narcotic analgesics due to their inhibition of release of acetylcholine from the myenteric plexus neurons (Schaumann, 1957; Paton, 1957). This decrease in acetylcholine output produced by narcotics is blocked by narcotic antagonists in a competitive manner (Kosterlitz and Watt, 1968). The rank order of potency of a series of narcotic agonists in depressing the nerve-mediated contraction of the guinea pig ileum correlates well with the analgesic potency of these agonists in man (Kosterlitz and Waterfield, 1975). There is also a good correlation between the affinity of opiate binding sites in the myenteric plexus and in the rat brain to a series of agonists and antagonists (Creese and Snyder, 1975). The depression of acetylcholine output and the binding of narcotics to opiate receptors in the myenteric plexus are stereospecific as are the analgesic effects of these compounds (Kosterlitz and Waterfield, 1975). Finally, as with the central effects of opiates, cross-tolerance develops between different narcotic agonists (Goldstein and Schultz, 1973) and enkephalin, in the myenteric plexus (Waterfield, Hughes and Kosterlitz, 1976). The neurons of the myenteric plexus therefore appear to have opiate receptors which are much like those in the central nervous system.
which mediate analgesia.

The nerve-mediated contraction of the longitudinal muscle is also depressed by somatostatin and this is not through an interaction with an opiate receptor (see section 1.2.4.). The action of substance P on the nerve-mediated contractile response of the longitudinal muscle is difficult to assess because substance P contracts the longitudinal muscle by a direct action of the smooth muscle. Neurotensin, on the other hand, causes a contraction of the longitudinal muscle which is blocked by tetrodotoxin, suggesting an excitatory effect on myenteric neurons. Since many of the neuropeptides are present in the myenteric plexus and have been found indirectly to change the excitability of myenteric neurons, this preparation may be useful to study the action of the neuropeptides at a single neuron level.
1.3.3.2. Using Extracellular Recording. There have been several electrophysiological studies on the actions of opiates on neurons contained in the myenteric plexus. The first demonstration of an effect of morphine on single myenteric neurons was made by Sato and colleagues (Sato et al., 1973). In this study, only one concentration was used and this was very high (10 μM) compared to the concentration which is effective in inhibiting the contractile response of the longitudinal muscle (ED$_{50} = 100$ nM). Subsequently, studies by Dingledine and Goldstein demonstrated that the inhibition of unit firing produced by morphine was dose-dependent in the same concentration range as that which is effective in inhibiting the release of acetylcholine (Dingledine, Goldstein and Kendig, 1974; Dingledine and Goldstein, 1975; 1976). The inhibition of firing was stereospecific; that is, dextrorphan was without effect (in the concentrations tested), whereas the active isomer, levorphanol, was effective. Naloxone reversed or prevented the action of opiate agonists. Morphine also blocked the effects of a number of excitatory agents such as 5-HT, sodium picrate, caerulein, pentagastrin and acetylcholine (Sato, Takayanagi and Takagi, 1974), which suggests that it is acting directly on the neuron whose activity is being recorded. In fact, strong evidence for such a direct action on the neuron under study was provided by the experiments of Dingledine and Goldstein (1976) in which morphine was shown to be equally effective when synaptic transmission had been completely blocked by omission of calcium ions from the bathing solution. These studies assumed that the neuronal spikes which were being recorded were made from the cell soma; however, there was no clear evidence for this assumption. The effects of pentagastrin and caerulein on myenteric neurons has previously been studied. Both these peptides caused an in-
crease in neuronal firing (Sato et al., 1973). Neither of these peptides was studied in detail and there are no other studies on the action of various peptides on single neurons in the myenteric plexus.

1.3.3.3. Using Intracellular Recording. Using intracellular recordings from myenteric neurons, it has been found that morphine produced a hyperpolarization of the cell soma which was sometimes associated with an increase in conductance (North, 1976; North and Tonini, 1977). This hyperpolarization was stereospecific and blocked by naloxone. However, the ionic mechanism involved has not been determined. Both type I and type II cells appear to be sensitive to morphine; however, the proportion of type I cells which were affected was much greater than that of type II cells. With type II cells, it was common for the effect of morphine to become smaller during the application or with repeated exposures, whereas type I cells more often gave reproducible responses (North and Tonini, 1977). The population of cells in which a hyperpolarization was found was much smaller than the proportion of cells which were found to be sensitive with extracellular recordings.

Studies using intracellular recording to test the action of opioid peptides and other neuropeptides have not yet been done.
1.6. SPECIFIC AIMS

The first aim of the present study was to define the origin and nature of the neuronal activity recorded with extracellular electrodes in order to explain better the differences between results obtained with extracellular and intracellular techniques in the myenteric plexus. This part of the study included: (a) a study of spike waveforms of spontaneous and evoked activity; (b) determination of the locations from which spontaneous activity can be recorded (on the ganglia, on the strands of nerve processes which connect the ganglia and on the smooth muscle); (c) the study of the sensitivity of spontaneous and evoked activity to tetrodotoxin and local anesthetics; (d) a general survey of the types of firing patterns; (e) an investigation of the changes in firing induced by solutions of varying ionic composition.

The second aim of this study was to investigate the action of opiates on single myenteric neurons. This included: (a) testing the sensitivity to opiates of units displaying different types of discharge patterns; (b) confirming that the inhibition of firing is mediated via opiate receptors by antagonizing the effect of opiates with naloxone; (c) examining the action of morphine in solutions with high and low potassium ion concentration and low chloride ion concentration to determine the ionic mechanism of action.

The third aim of the present work was to study the action of several endogenous neuropeptides including leu- and met- enkephalin, β-
endorphin, somatostatin, substance P, neurotensin and VIP on the firing rate of myenteric neurons. These peptides, with the exception of neurotensin, have all been shown to be contained within neurons of the plexus. The effect that each of these peptides has on the firing rate of myenteric neurons, the time course of effects and the concentration range in which they are effective were studied. In addition, where applicable, antagonists of each peptide were studied to determine the specificity of the action of the peptide. For peptides for which there are no available specific antagonists, the effect of several peptides or peptide analogs were studied on a single unit in order to determine the specificity of the effect. Finally, the action of each peptide was studied in a solution which contained no calcium ions in order to eliminate possible trans-synaptic influences. The purpose of these experiments is to help substantiate or refute the hypothesis that the presence of the neuropeptides in the myenteric plexus may indicate that they act as neurotransmitters or neuromodulators at this site.
2. METHODS

2.1. PREPARATION OF TISSUE

Male guinea pigs (300-500 g) were stunned and bled from the neck. A portion of ileum 10-15 cm long excluding the section 5 cm in the oral direction from the ileocecal junction was removed and the luminal contents were gently washed out with Krebs solution. The ileum was then placed in Krebs solution at room temperature. The longitudinal muscle myenteric plexus preparation was prepared as originally described by Ambache (1954) and modified for electrophysiological recording by Nishi and North (1973a). A short piece of ileum (1-2 cm) was slipped on to a glass rod such that the longitudinal muscle myenteric plexus preparation could be separated from the ileum. Small pieces of cotton soaked with Krebs solution were used to gently stroke the ileum in order to tease the longitudinal muscle away from the circular muscle. The longitudinal muscle with the myenteric plexus adherent was first separated from the circular muscle along each side of the mesenteric border and then peeled around to the anti-mesenteric side of the ileum. The longitudinal muscle myenteric plexus preparation was then removed from the anti-mesenteric side of the ileum and placed in a shallow tissue bath. The longitudinal muscle was pinned out as a flat sheet with the myenteric ganglia uppermost. Ganglia were visualized with a dissecting microscope (10-60 x) using transmitted light. Ganglia were considered suitable for electrophysiological experiments if they were free of large amounts of circular muscle and if they contained few or no large cells which stood out clearly.
within the ganglion. Attempts to record from ganglia which did not meet the above requirements were usually futile. The area surrounding 10-15 suitable ganglia was immobilized with several pins (tungsten wire 25 μm in diameter cut into 1 mm lengths) and the remaining portion of the tissue was cut and removed from the bath. When agents which had a direct excitatory effect on the smooth muscle were to be used, single ganglia were surrounded by several pins and thus rendered immobile.

The tissue bath used was similar to that described by Nishi and North (1973a). A rectangular hole the size of a coverslip (24 X 40 mm) was cut from a piece of plexiglass (40 X 80 X 1 mm). A coverslip was then glued to the bottom of the bath and the trough was filled with Sylgard 184 Encapsulating resin (Dow Corning). This clear resin can be transilluminated and is soft enough to allow the use of fine pins to fix the preparation. The Sylgard was then surrounded by a layer of caulk (2 mm high). The caulk enclosed the bath and enabled the indifferent electrodes and perfusion tubes to be fixed to the bath. The bath volume was always less than 1 ml.
2.2. EXTRACELLULAR RECORDING

A glass suction electrode having a tip diameter of 30-90 μm was used for extracellular recording. The suction electrode was prepared by breaking back a longer electrode having a small tip diameter, and viewing the broken tip microscopically. Only electrodes with tips which were flat, smooth and of proper size were selected for recording. Attempts to use smaller glass electrodes or tungsten electrodes (tip diameter 5-10 μm) for recording were not successful. The recording electrodes were filled with Krebs solution and had a resistance of 500-1,000 kΩ. The electrode was tightly connected by a piece of tubing to a syringe (5 or 10 ml). The electrode was placed on the tissue and suction was applied by manually withdrawing the syringe plunger. The amount of suction required for successful recording varied from cell to cell; usually it was sufficient to allow the electrode to be raised 5-10 μm without dislodging it from the tissue. When the electrode had been placed on the tissue and suction applied, the resistance of the electrode typically increased by 200-500 kΩ. This sealing resistance presumably reduced the extracellular shunting of current through the Krebs solution and increased the size of the recorded signal. Attempts to record neuronal activity without suction were not successful. A chlorided silver wire was inserted into the recording electrode, reaching almost to its tip. This wire led directly to the input of a differential AC coupled preamplifier (DC $R_{in}$, 10 MΩ). The indifferent electrode had a tip diameter of 70-150 μm and its tip was placed in the bath close to the site of recording. A chlorided silver wire, at a distant part of the bath, was used to ground the preparation.
Potentials were amplified (1,000 X) and displayed on an oscilloscope (Tetronix 502A). Spike waveform of spontaneous and evoked potentials were studied from photographs of oscilloscope traces. The coupling time constant was 1 s in those experiments in which spike waveform was studied, but in other experiments the coupling time constant was decreased to 2 ms. With a time constant of 2 ms, the spike amplitude was attenuated and somewhat differentiated. However, this was necessary to obtain a sufficiently stable base line to use a window discriminator (Mentor Spike Analyzer). The output of the window discriminator was recorded either on chart paper or counted with a rate analyzer (Frederick-Haer). Histograms were constructed of the number of action potentials in a given time interval (usually 10 s and up to 1 min) either by hand from the chart record or directly from the output of the rate analyzer.

In some experiments, a third glass electrode with a tip diameter of 20-40 μm was used for electrical stimulation. The stimuli used were pulses of 100-500 μs duration. Single pulses or short trains of pulses at frequencies up to 10 Hz were used. The tip of this stimulating electrode was placed so that it just made contact with the preparation, either on the ganglion surface or on the interconnecting strands.
2.3. PERFUSION OF THE TISSUE AND APPLICATION OF DRUGS

Krebs solution continuously perfused the tissue at a rate of 2-4 ml/min, maintained by a peristaltic pump. The composition of the Krebs solution is given in Table 4. The solution was warmed in a heating jacket before it entered the bath such that the bath temperature was maintained at 35-37°C. Drugs were added to the bath by changing the perfusing solution to one which differed only in the content of the drug. There was a delay time of about 1 min between the time when the drug solution was changed and when the drug entered the bath. All figures are drawn such that the periods of drug applications correspond to the times when solutions were changed, without subtracting the delay time.

All glassware that came in contact with peptide solutions was pretreated with a siliconizing compound called sigma-coat (Sigma) to limit the binding of the peptides to the surface of the glass. Solutions of different ion concentrations were always kept isosmotic by changing the concentration of NaCl in the Krebs. The content of ions in these solutions is given in Table 4.

Drug and peptide solutions were prepared fresh daily from stock solutions. Stock solutions were kept frozen for later use. Peptides were checked for stability by comparing the response of a single cell to the same concentration of two peptide solutions. One solution was freshly prepared from solid and the other had been made from a stock solution which had been used for periods up to several weeks. All the peptide solutions appeared to be stable for periods up to several weeks if the
stock solution was kept cool or frozen. The peptides and drugs used in this study are listed in Table 5.
Table 4. The content of salts (mM) in the various Krebs solutions used.

<table>
<thead>
<tr>
<th>Salt</th>
<th>Normal (10 mM)</th>
<th>Low Cl⁻ (12 mM)</th>
<th>High K⁺ (1 mM)</th>
<th>Low K⁺ (1 mM)</th>
<th>Ca²⁺ Free (0 mM)</th>
<th>High Ca²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na isethionate</td>
<td>118</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NaCl</td>
<td>118</td>
<td>110.7</td>
<td>121.7</td>
<td>120.75</td>
<td>106.75</td>
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<tr>
<td>KCl</td>
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<td>4.7</td>
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<td>4.7</td>
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<tr>
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<td>2.5</td>
<td>2.5</td>
<td>0</td>
<td>10</td>
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<tr>
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<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Glucose</td>
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<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>NaHCO₃</td>
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<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
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<tr>
<td>EGTA*</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
</tbody>
</table>

* ethylene-bis-(oxyethylene nitrilo) tetraacetic acid
Table 5. The peptides and drugs used in the present study with the source(s) of each.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>normorphine sufamate or HCl</td>
<td>Dr. E.L. May</td>
</tr>
<tr>
<td>morphine sulphate</td>
<td>Mallinckrodt</td>
</tr>
<tr>
<td>enkephalin, base or HCl</td>
<td>Penninsula, Miles, Dr. B.A. Morgan</td>
</tr>
<tr>
<td>enkephalin, base or HCl</td>
<td>Penninsula, Miles, Dr. B.A. Morgan</td>
</tr>
<tr>
<td>met-leu-</td>
<td></td>
</tr>
<tr>
<td>β-endorphin</td>
<td>Penninsula</td>
</tr>
<tr>
<td>substance P (1-11)</td>
<td>Penninsula, Beckman</td>
</tr>
<tr>
<td>substance P (7-11)</td>
<td>Penninsula</td>
</tr>
<tr>
<td>substance P free acid</td>
<td>Penninsula</td>
</tr>
<tr>
<td>neurotensin</td>
<td>Penninsula, Sigma</td>
</tr>
<tr>
<td>somatostatin</td>
<td>Penninsula, Sigma, Beckman</td>
</tr>
<tr>
<td>VIP</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>naloxone HCl</td>
<td>Endo Laboratories</td>
</tr>
<tr>
<td>hyocine HCl</td>
<td>Sigma</td>
</tr>
<tr>
<td>hexamethonium HBr</td>
<td>Sigma</td>
</tr>
<tr>
<td>baclofen (acid)</td>
<td>Dr. H.K. Proudfit</td>
</tr>
<tr>
<td>lidocaine</td>
<td>ICN·K&amp;K Laboratories</td>
</tr>
<tr>
<td>tetrodotoxin</td>
<td>Sankyo</td>
</tr>
<tr>
<td>propranalol HCl</td>
<td>Sigma</td>
</tr>
<tr>
<td>phenoxybenzamine</td>
<td>Smith, Kline and French</td>
</tr>
<tr>
<td>Mr-2266, Mr-2267</td>
<td>Dr. H. Merz, Boehringer Ingelheim</td>
</tr>
</tbody>
</table>
3. RESULTS

3.1. EXTRACELLULAR RECORDING

3.1.1. Spontaneous Activity

Recordings were made from more than 500 units in tissues removed from 190 guinea pigs. A unit was considered to represent the activity of a single cell on the basis of the uniformity of the spike amplitude, waveform and duration. Neuronal action potentials were clearly distinguishable from smooth muscle action potentials by their different amplitudes and durations (Fig. 1). Smooth muscle action potentials were readily recorded when the recording electrode was placed on the muscle away from a myenteric ganglion. Neuronal activity was only recorded when the electrode was placed on the surface of a ganglion. Neuronal action potentials were blocked by tetrodotoxin (1 μM, n=6) or lidocaine (1 mM, n=2, Fig. 2), whereas smooth muscle action potentials were unaffected by either drug.

The waveform of spontaneous neuronal activity was usually monophasic, spike duration being 2-5 ms. If the coupling time constant of the recording system was decreased, the spike amplitude was reduced and the waveform became biphasic (Fig. 3). During the action potential, the recording electrode usually became negative with respect to the indifferent electrode, although spikes of opposite polarity were occasionally observed. No spontaneous neuronal activity could be recorded from the strands of nerve processes connecting the ganglia within the plexus, although electrically evoked activity was readily recorded from these sites.
Figure 1. Comparison of neuronal and smooth muscle action potentials recorded with extracellular suction electrodes. (a) Different traces from the smooth muscle. Calibration: Horizontal, 20 ms; Vertical, 1 and 2, 100 µV; 3, 200 µV. (b) Neuronal spikes at the same sweep speed. Calibration: Horizontal, 20 ms; Vertical, 50 µV. Coupling time constant in (a) and (b) was 1 s.
Figure 2. Inhibition of firing of a myenteric neuron by lidocaine. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. The solid bar indicates the period during which the bathing solution was changed to one containing lidocaine (1 mM). Lidocaine (1 mM) depressed neuronal firing in a reversible manner.
Figure 3. Spike waveform recorded with extracellular electrodes from myenteric neurons. (a) Coupling time constant 2 ms. 1-3, typical spikes recorded from three myenteric neurons with different electrodes. 4, three different waveforms were recorded with a single electrode in the same position. 5, the same as 4 but spikes were recorded at a slow sweep speed. Calibration: Horizontal (1) 2 ms; (2,3) 10 ms; (4) 20 ms; (5) 2 s. Vertical (1) 100 μV; (2-5) 200 μV. (b) Coupling time constant 1 s. Typical monophasic spike waveforms recorded from three different myenteric neurons. Calibration: Horizontal 5 ms. Vertical (1,2) 100 μV; (3) 50 μV.
(see section 3.1.2.). The strands contain few cell bodies (Gabella, 1972). No neuronal activity was recorded with metal electrodes, glass suction electrodes with a tip diameter less than 30 µm or with electrodes without suction.

In agreement with previous studies, two basic types of firing patterns were found. The first, and most common type (90%), had a discharge pattern that was irregular with a mean rate of 1-3 Hz. The waveform was monophasic (depending on the time constant of the recording system) and had a spike amplitude which ranged from 50-300 µV (Fig. 3). This type of unit was the most likely to continue firing and often lasted for periods of up to 4-5 hr. The firing rate of this type of unit was 5-10 Hz soon after placement of the suction electrode on to the ganglion, but declined to 1-3 Hz within a few minutes. The firing rate usually declined steadily throughout the period of recording, although some units maintained the same firing rate for periods of 4-5 hr. Neurons which stopped firing within the first few minutes of recording exhibited one of two distinct patterns of firing. The first pattern was marked by a declining frequency associated with a steady increase in spike amplitude. When more suction was applied to the electrode, or if the electrode was removed and reapplied to the same point, the unit began to fire again. Units which ceased firing in this manner could usually be reexcited by changing the perfusing solution to one which contained no calcium ions or addition of an excitatory substance (see sections 3.1.3. and 3.4.2.). The second pattern began with a sudden flurry of action potentials associated with decreasing spike amplitude and increasing spike duration. The neuron would continue to fire at high frequency until the spike amplitude
decreased into the background noise. This second discharge pattern was probably due to mechanical cell damage leading to depolarization. Such cells sometimes appeared to recover; that is to say, after a period of 10-15 min a unit would begin to fire again. However, this unit would also begin to fire at a high frequency with the same result as described previously. On rare occasions, such a pattern of firing would continue for periods of up to 1 hr, with a decreasing frequency and number of spikes in each burst. This may correspond to the burst unit described in the experiments of Wood (1973).

A second distinct type of unit made up a small proportion (10%) of the recordings. These units were of large amplitude (150-400 µV) and had a very regular low frequency discharge (0.1-1 Hz). This type of unit was less likely to continue firing for extended periods of time and usually the onset and offset of firing was very abrupt with little change in the firing frequency during the periods which it was active. Units of this type were generally less sensitive to most agents and it seems likely that they correspond to the small population of morphine-insensitive, larger amplitude units described by Dingledine and Goldstein (1976). These units may correspond to the type II cell found with intracellular electrodes and further evidence for this is presented below.

A series of experiments was done to test whether polarizing currents could alter the firing rate of spontaneously active myenteric neurons. While recording from a spontaneously active unit, a stimulating electrode was placed very close to the recording electrode and continuous current (anodal or cathodal) was passed through this electrode.
When an anodal current was passed through the stimulating electrode, the firing rate of the neuron being recorded was decreased markedly. When a cathodal current was being passed through the stimulating electrode, an increase in the firing rate was observed. The changes in firing rate produced by anodal and cathodal currents usually lasted throughout the period during which current was passed, although there was some accommodation observed during the passage of cathodal current. These results indicate that the firing rate of the neurons under study was sensitive to the effects of polarizing currents.
3.1.2. Evoked Activity

A series of experiments was done in which the nerve processes and cell bodies were stimulated, while recordings were made from the ganglia or along the strands of nerve processes which connect the ganglia. When an electrical stimulus was applied to the surface of the plexus at a distance of 100-1,000 μm from the site of recording, two different types of responses were observed. The first type was a potential change which was of short latency, sometimes coming directly (1-3 ms) after the stimulus artifact (Figs. 4, 5, 6). This potential was observed whether recording from the ganglia or from the nerve processes in the connecting strands between the ganglia. The amplitude of this potential was graded with the intensity of the stimulus, and followed the stimulation at frequencies of 10 Hz (Fig. 5). This potential was not blocked by hexamethonium (100 μM) but was blocked reversibly by lidocaine (300 μM and 1 mM, Fig. 6).

The second type of electrically evoked potential closely resembled, in waveform, the spontaneous neuronal spike recorded at the same site. This second type of evoked potential was observed only when recordings were made from the surface of ganglia, not from the interconnecting strands. These potentials (from 1-3 following a single stimulus) were all or nothing spikes which occurred with a latency that was relatively constant for an individual experiment but which varied (from 10-30 ms) among cells (Fig. 4). It is thought that these spikes arise from direct electrical stimulation of the cell or one of its processes or from
Figure 4. Response of myenteric neurons to focal stimulation of the ganglion with single pulses, and the effect of hexamethonium. Each illustration is a single sweep. Stimulation frequency was 0.1 Hz. (a) A single pulse was followed by a small, short latency response and a longer latency response comprising two spikes which were all or nothing and the individual spikes had a waveform similar to the spontaneous potential. (b) 2 min after beginning perfusion with hexamethonium (100 μM). The short latency response was still present. However, the long latency all or nothing potential was blocked. (c) 4 min after the hexamethonium had been washed from the bath. Calibration: Horizontal 10 ms. Vertical 200 μV.
Figure 5. Electrically evoked activity recorded from a myenteric ganglion. Each recording is the last sweep of an oscilloscopy trace following a 10 s train of pulses at the indicated frequency. The evoked activity was of short latency (1-2 ms) and followed a stimulation frequency of at least 10 Hz. At high stimulus frequencies, the activity tended to fractionate, indicating the activity was a compound response. Calibration: Horizontal 1 ms. Vertical 100 μV.
Figure 6a. Electrically evoked activity recorded from a myenteric ganglion. Calibration: Horizontal 1 ms. Vertical 100 µV.

Each recording is a single sweep. Stimulation frequency 0.1 Hz. (1) control. (2) 2 min after changing to a solution which contained lidocaine (1 nM). (3 and 4) 1 and 5 min after washing out the lidocaine.
Ganglion

1 Control

2 1mM Lid.

3 Wash 1 min.

4 5 min

100 µV

1 mS
Figure 6b. Electrically evoked activity recorded from the strands of nerve processes connecting the ganglia. Calibration: Horizontal 1 ms. Vertical 100 μV. The stimulation frequency was 0.1 Hz and each recording is a single sweep. (1) control. A typical waveform of activity recorded from the interconnecting strands consisting of a short latency compound response. (2-4) 1, 2 and 2.5 min after changing to a solution which contained lidocaine (300 mM). (5) 4 min after the solution containing lidocaine had been replaced with a normal Krebs solution.
b Strands

1 Control

2 300 nM Lid. 1 min

3 2 min

4 25 min

5 Wash 4 min

-100 µV
1 mS
synaptic excitation of the cell. It was possible to distinguish synaptically evoked potentials from directly evoked potentials as follows:

(1) Some of the evoked spikes followed at high frequencies of stimulation (up to 10 Hz) with only a small increase in latency. These were not affected by hexamethonium (100 μM) and were considered to be directly evoked spikes. (2) Other potentials were reversibly blocked by perfusing the tissues with hexamethonium (100 μM, Fig. 4). These spikes disappeared when the frequency of stimulation was increased above 1 Hz: this observation supports the interpretation that these are synaptically driven spikes, because the e.p.s.p.'s recorded intracellularly from myenteric neurons decline in amplitude when they are evoked at frequencies above 1 Hz (Nishi and North, 1973a).

The effect of repetitive electrical stimulation on the rate of spontaneously firing cells was studied (n=15). A stimulating electrode was placed close (< 20 μm) to the recording electrode and a train of pulses at 10 Hz for 10-60 s was applied. In a Krebs solution which did not contain acetylcholine receptor antagonists, this stimulation often resulted in movement of the muscle which dislodged the electrode from the ganglion and an increase in firing rate was always observed. When hexamethonium (100 μM) and hyoscine (1 μM) were added to the perfusing solution, muscle movement was eliminated; however, an increase in firing rate usually followed the period of stimulation. The firing rate increased to about 10 Hz and lasted from 30 to 90 s following termination of the stimulation. An electrically evoked inhibition in firing was rarely observed (2 of 15 trials).
3.1.3. Effects of Solutions of Varying Ionic Composition

3.1.3.1. Low Potassium. The effects of a solution containing a low concentration of potassium (1 mM) was tested on 8 units. This solution reversibly decreased the spontaneous firing of only 1 unit and reversibly increased the firing rate in 5 units. The increase in firing frequency was preceded by a transient slowing (30-60 s) in 4 of these units (Fig. 7). The increase in firing rate was usually not maintained throughout the period during which the low potassium solution was perfusing the tissue. This increase in firing rate produced by a low potassium solution, although unexpected, may be explained in terms of the effect that such a solution has on the sodium-potassium pump (Na-K pump). Under these conditions, when the cell is actively firing, the Na-K pump may be important in maintaining the membrane potential. By decreasing extracellular potassium, the pump is inhibited, which may lead to a depolarization and an increase in firing rate. This possibility is supported by experiments with the Na-K, ATPase inhibitor, ouabain. The action of ouabain (1 mM) on the firing rate was tested on 7 units and at least a twofold increase in firing rate was found in 3 units.

3.1.3.2. High Potassium Solution. The effects of a solution containing high potassium ion concentration (12 mM) was tested on 7 units. The firing rate of 3 of these was increased and the firing rate of 3 others was decreased. The increase in firing rate induced by the high
Figure 7. The effect of a low potassium (1 mM) solution on firing rate of a typical myenteric neuron. Ordinate: firing rate (bin width - 30 s). Abscissa: time. During the periods indicated by the filled and open bars, the bathing solution contained normorphine (300 nM, NM) and 1 mM potassium, respectively. The unit showed a typical decline in firing rate with time. Normorphine (300 nM) caused an inhibition in firing rate. The solution containing low potassium increased the firing rate and normorphine prevented the increase in firing rate produced by the low potassium solution. Subsequent application of low potassium solution increased the firing rate even though spontaneous firing had decreased to a very low level.
potassium solution was not maintained throughout the period during which it perfused the tissue and in 2 units was biphasic; that is to say, a transient increase in firing rate was followed by a decrease in firing. The firing rate remained depressed for several minutes after the perfusing solution had been replaced with Krebs solution containing normal potassium (4.7 mM) ion concentration (Fig. 8). A high potassium solution would be expected to depolarize the neurons. With this depolarization, an increase in firing might be expected. The decrease in spike frequency observed in some units and the biphasic response of the firing rate (increase and then decrease) observed in others may be a result of a depolarization beyond the threshold level.

3.1.3.3. Low Chloride. The effect of a solution containing low chloride ion concentration (10 mM) was tested on the firing rate of 12 myenteric neurons. In 11 of these, a small increase in firing rate which was slow in onset and offset was produced (Fig. 9). It appears that myenteric neurons are much less sensitive to changes in chloride ion concentrations than potassium ions. A small (5 mV) depolarization is produced in myenteric neurons in low chloride solutions recorded with intracellular electrodes (North, unpublished results). This small depolarization is consistent with the increase in firing rate observed in the present study.

3.1.3.4. Calcium Free. Calcium-free solutions containing EGTA (1 mM) always markedly increased the firing rate of myenteric neurons (Fig. 10, n=26). Neurons which had ceased to fire action potentials in association with an increasing spike amplitude were often induced to fire
Figure 8. The effect of a high (12 nM) potassium solution on the firing rate of two myenteric neurons. Ordinate: spike frequency (bin width - 30 s). Abscissa: time. During the periods marked by the open and closed bars, the bathing solution was changed to one containing a high potassium (12 nM) and normorphine (NM, 300 nM), respectively. (a) Application of a high potassium solution produced a small depression in firing rate but did not alter the inhibition caused by normorphine. (b) Application of a high potassium solution produced a large increase in firing rate followed by a prolonged depression in firing which returned slowly upon washing the high potassium solution from the bath.
Figure 9. The effect of low chloride (10 nM) solution on the firing rate of a typical myenteric neuron. Ordinate: spike frequency (bin width - 30 s). Abscissa: time. During the periods denoted by the open and closed bars, a low chloride solution (10 nM, isethionate substitution) and normorphine (NM, 300 nM) perfused the tissue, respectively. A solution containing low chloride caused a modest increase in firing rate. The inhibition in firing rate produced by normorphine (300 nM) appeared to be somewhat smaller in a low chloride solution on this neuron.
by changing to a solution which was calcium-free (Fig. 12). Similarly, when recording the firing rate of a single unit, cells which had been silent (not firing) prior to changing to the calcium-free solution, began to fire and interfered with the unit under study.

It was also noted that if the preparation was first perfused with calcium-free solution and then recordings were made, it was much easier to record neuronal activity. This contrasted with the inability to record smooth muscle action potentials in calcium-free solutions.

3.1.3.5. High Calcium. A solution containing high calcium ion concentration (10 mM) was tested on 10 myenteric neurons. In every case, this solution markedly depressed the firing rate (Figs. 10, 22), with a time course which was abrupt upon entry of the solution into the bath and which returned upon changing the perfusing solution back to a Krebs solution containing normal calcium (2.5 mM).
Figure 10. The effect of high calcium (10 mM) and calcium-free solutions on the firing rate of a myenteric neuron. Ordinate: spike frequency (bin width - 15 s). Abscissa: time. The solid bar indicates the period of time during which the bathing solution contained 10 mM calcium solution and the open bar indicates the periods when a calcium-free solution was present. The high calcium solution caused a marked inhibition of cell firing. The calcium-free solution caused a marked excitation which became larger with longer periods of exposure to the solution. This graded increase in firing rate probably indicates incomplete removal of calcium from the bath.
3.2. THE ACTION OF OPIATES

Normorphine was used in the majority of the experiments because its onset and offset of action is more rapid than that of morphine (Kosterlitz, Lord and Watt, 1972). The results are taken from 58 units recorded from tissues taken from 33 animals. Normorphine (100-1,000 nM) reduced the firing rate of 90% of the units tested. The onset of the inhibition produced by normorphine was relatively fast (within 10-20 s after the entry of the normorphine into the bath). The inhibition in firing was rapidly reversed by washing the tissue with drug-free Krebs. Occasionally, a short lasting rebound increase in firing rate was observed upon washing out the normorphine. The inhibition in firing produced by morphine, however, was slower in onset and required a longer period of time for the firing rate to return to control when washing out the drug (Fig. 17). In cells which were sensitive, normorphine (1 μM) completely inhibited firing (n=10) and, in 31 of 37 experiments, normorphine (300 nM) reduced the firing rate to less than 75% of its control (Fig. 11).

The inhibition in firing rate produced by normorphine was antagonized by a 3-10 fold lower concentration of naloxone. The inhibition was reversed by addition of naloxone during the period when normorphine was in the bath and prior treatment of the tissue with naloxone prevented the normorphine-induced inhibition (Fig. 11). These findings are essentially the same as those reported in detail by Dingledine and Goldstein (1975, 1976).
Figure 11. Effect of normorphine on firing of two myenteric neurons and the reversal of this inhibition by naloxone. Ordinate: spike frequency (bin width - 60 s). Abscissa: time. During the periods indicated by the closed and diagonal hatched bars, the bathing solution was changed to one which contained normorphine (NM, 300 nM) and naloxone (Nal, 30 nM), respectively. (a) The firing rate of this myenteric neuron steadily declined throughout the experiment. The depression in firing rate produced by normorphine (300 nM) was completely reversed by a tenfold lower concentration of naloxone (30 nM). This reversal was not due to tachyphylaxis to the effect of normorphine since the inhibition produced by a subsequent application lasted throughout the period which it was in the bath. (b) The firing rate of this unit was maintained throughout the experiment.
Figure 11. Effect of normorphine on firing of two myenteric neurons and the reversal of this inhibition by naloxone. Ordinate: spike frequency (bin width - 60 s). Abscissa: time. During the periods indicated by the closed and diagonal hatched bars, the bathing solution was changed to one which contained normorphine (NM, 300 nM) and naloxone (Nal, 30 nM), respectively. (a) The firing rate of this myenteric neuron steadily declined throughout the experiment. The depression in firing rate produced by normorphine (300 nM) was completely reversed by a tenfold lower concentration of naloxone (30 nM). This reversal was not due to tachyphylaxis to the effect of normorphine since the inhibition produced by a subsequent application lasted throughout the period which it was in the bath. (b) The firing rate of this unit was maintained throughout the experiment.
During the inhibition produced by normorphine (1 µM), the firing rate of these neurons was increased by passing a steady cathodal current through a stimulating electrode close to the recording site. This observation is compatible with a hyperpolarizing action of normorphine on the cell membrane which has subsequently been demonstrated by intracellular recording (North and Tonini, 1977).

The inhibition in firing rate produced by normorphine (300 nM) was not altered when the tissue was perfused with solutions containing 1 mM (n=4) or 12 mM (n=5) potassium ion concentrations (Figs. 7, 8). The action of normorphine (300 nM) was tested on 5 units in the presence of a solution containing 10 mM chloride ion concentration. In 3 of these units, the inhibition of firing produced by normorphine was unchanged from control and, in 2 others, the inhibition in firing appeared to be reduced (Fig. 9).
3.3. THE ACTION OF OPIOID PEPTIDES

These results are based on 192 neurons recorded from tissues taken from 67 animals.

3.3.1. Met-Enkephalin

Application of met-enkephalin (1-300 nM) inhibited the firing of 77 of 96 neurons (Table 6). For a given neuron, the degree of inhibition of firing was dependent on the concentration of enkephalin in the bath (Fig. 12). Met-enkephalin at a concentration of 100 nM was sufficient to reduce the firing rate to 10% or less of the control rate in 90% of sensitive neurons. Lower concentrations of met-enkephalin caused marked inhibitions in many cells (Fig. 12).

The onset of inhibition of firing and the return to control was within 10-15 s of the arrival and washout of met-enkephalin. There was a rebound increase in firing rate following the washout of enkephalin in 40% of cells tested (Figs. 17a, 14). The inhibition in firing was usually maintained throughout the period that met-enkephalin was in the bath. Likewise, the degree of inhibition was reproducible with repeated applications of the same concentration at intervals of 2 min (Fig. 13). With concentrations of met-enkephalin (< 10 nM), the firing rate occasionally (< 15%) returned to control levels during the presence of enkephalin. In such cells, the application of a higher concentration (30 or 100 nM) caused a greater degree of inhibition and this lasted throughout the period of application.
Table 6. The effect of different concentrations of met-enkephalin, leu-enkephalin and β-endorphin in inhibiting the firing rate of myenteric neurons. Inhibition of firing indicates that the spike frequency fell to less than 75% of its control value during the period of drug application and recovered to the original control value following washout. The proportion of neurons which were inhibited by low concentrations of the peptides is artificially high, because low concentrations (met-enkephalin 30 nM or less, leu-enkephalin 100 nM or less) were only tested when the unit was found to be sensitive to a higher concentration.

<table>
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<td>7</td>
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<tr>
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</table>
Figure 12. Inhibition of spike frequency by met-enkephalin in a concentration-dependent manner. Ordinate: spike frequency (10 s epochs). Abscissa: time. The solid bars indicate the periods during which met-enkephalin was included in the bathing solution at the indicated concentration (nM). In this cell, 3 nM met-enkephalin was without effect and a tenfold higher concentration completely suppressed the firing.
Figure 13. Repeated applications of met-enkephalin on a single myenteric neuron. Ordinate: spike frequency (bin width - 15 s). Abscissa: time. Met-enkephalin (100 nM) decreased the firing rate in a reproducible manner during each of five applications with varying durations of exposure (1, 2, 3 and 10 min).
In comparing the inhibition produced by morphine and normorphine with that produced by met-enkephalin, two differences became apparent. First, met-enkephalin produced the same degree of inhibition at a tenfold lower concentration than that of morphine or normorphine (Fig. 17). Second, the time course of the inhibition produced by enkephalin was usually much faster than that produced by morphine and it was also slightly faster than the inhibition produced by normorphine (Fig. 17a). The frequency with which a rebound excitation was observed with the washout of enkephalin was also much higher than that observed for normorphine (Fig. 17a).
3.3.2. Leu-Enkephalin

Leu-enkephalin (10-300 nM) inhibited the firing of 79 of 108 neurons tested (Table 6). The time course of the inhibition produced by leu-enkephalin was similar to that of an equieffective concentration of met-enkephalin. In 90% of sensitive neurons, leu-enkephalin (300 nM) reduced the firing rate by at least 90%. On a given neuron, the degree of inhibition produced by leu-enkephalin was dependent on the concentration in the bath. A 3-5 times higher concentration of leu-enkephalin was usually required to produce the same degree of inhibition as a given concentration of met-enkephalin (Fig. 14, n=12). As with met-enkephalin, the washout of leu-enkephalin was often associated with a marked rebound increase in the firing rate.
Figure 14. Inhibition of spike frequency by met- and leu-enkephalin. Ordinate: spike frequency (bin width - 30 s). Abscissa: time. In this cell, leu-enkephalin (100 nM, leu) had no effect whereas the same concentration of met-enkephalin (100 nM, met) caused almost a complete inhibition in firing. A five fold higher concentration of leu-enkephalin (500 nM) produced an inhibition in firing rate which was similar to that caused by met-enkephalin (100 nM). Note that the inhibition of firing by met-enkephalin remained present throughout a 20 min period of exposure.
3.3.3. β-Endorphin

β-endorphin (1-1,000 nM) inhibited the firing of 35 of 54 neurons (Table 6). The time course of the effects produced by β-endorphin was similar to that of the enkephalins. The rebound increase in firing rate with the washout of β-endorphin was very small and seen only occasionally. The effect of β-endorphin was dependent on the concentration applied (Fig. 15). The most sensitive units were inhibited at a concentration of 3 nM (Fig. 16). It was usually necessary to use a 3-10 fold higher concentration of β-endorphin to equal a response to a given concentration of met-enkephalin (Fig. 17c). In most units, the inhibition in firing produced by β-endorphin was equal or slightly smaller than that caused by an equal concentration of leu-enkephalin (Fig. 25a).
Figure 15. Concentration-related inhibition in firing of a single neuron by β-endorphin. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. Solid bars indicate the periods during which the bathing solution was changed to one which contained β-endorphin in the concentration indicated (nM). β-endorphin caused an inhibition in firing which was related to the concentration in the bath.
Figure 16. Inhibition in firing rate by \( \beta \)-endorphin and reversal of this inhibition by naloxone. Ordinate: spike frequency (bin width = 10 s). Abscissa: time. During the periods denoted by the solid bars and diagonal hatched bar, the bathing solution was changed to one which contained \( \beta \)-endorphin and naloxone, respectively. The numbers above and below the bars indicate the concentration (nM). \( \beta \)-endorphin (30 nM) caused a marked decrease in firing rate which was completely reversed by naloxone (10 nM). Subsequent application of \( \beta \)-endorphin (3 nM) produced increasing amounts of inhibition of firing rate. The loss of effect of the first application of 3 nM \( \beta \)-endorphin may be due to tachyphylaxis following the larger concentration, or due to residual effects of naloxone.
Figure 17. A comparison of the effects produced by met-enkephalin, β-endorphin, morphine and normorphine on three myenteric neurons. (a) Ordinate: spike frequency (bin width = 30 s). Abscissa: time. During the periods indicated, the perfusing solution was changed to one which contained morphine (M, 300 nM, vertically hatched bar) met-enkephalin (met-E, 30 nM, open bar) or normorphine (NM, 300 nM, solid bar). The time course of the inhibition produced by morphine was much longer than that caused by enkephalin or normorphine. About the same degree of inhibition in firing rate was produced by met-enkephalin at a tenfold lower concentration as was produced by morphine and normorphine. A marked rebound increase in firing was also produced by washout of enkephalin.
Figure 17b. Ordinate: spike frequency (bin width - 15 s). Abscissa: time. The inhibition in firing rate produced by met-enkephalin (30 nM) was exactly the same as the inhibition by morphine (300 nM).
Figure 17c. Inhibition of spike frequency by met-enkephalin and β-endorphin. Ordinate: spike frequency (10 s epochs). Abscissa: time. The solid and stippled bars indicate the periods during which the bathing solution contained met-enkephalin and β-endorphin at the concentration indicated (nM). Met-enkephalin (30 nM) produced a marked inhibition in firing rate whereas the initial application of the same concentration of β-endorphin only caused a transient inhibition which was not repeatable. A higher concentration of β-endorphin (100 nM) was required to cause the same amount of inhibition as met-enkephalin (30 nM).
3.3.4. The Effect of Calcium-Free Solutions on the Action of Opioid Peptides

The action of met-enkephalin (n=15) and leu-enkephalin (n=6) were both unchanged in solutions which were calcium-free (Fig. 18). Even perfusion of the tissue with a calcium-free solution for periods of more than 1 hr did not change the action of the enkephalins on sensitive units. If the preparation was perfused with calcium-free solutions before recordings were made, the proportion of neurons which were not sensitive to enkephalin increased to about 50% of those tested. Similar results have been reported for the action of morphine in calcium-free solutions (Dingledine and Goldstein, 1976).
Figure 18. Extracellular recordings from two myenteric neurons. Ordinate: spike frequency (30 s epochs). Abscissa: time. During the periods indicated by the solid bars, the bathing solution was changed to one which contained met-enkephalin; the numbers indicate the concentration in nM. (a) The horizontally hatched bar indicates the period during which the bathing solution was changed to a calcium-free solution which also contained EGTA (1 mM). The initial firing rate of this unit was low, but met-enkephalin (10 nM) caused a small inhibition in firing. The firing rate increased markedly when the bathing solution was changed to a calcium-free solution. Enkephalin, however, still produced a marked inhibition in firing rate. (b) The open bar indicates that the bathing solution was calcium-free and included EGTA (1 mM). The tissue had been perfused with the calcium-free solution for 70 min prior to this experiment. Met-enkephalin caused a marked inhibition in firing rate of this cell. When the bathing solution was changed to one containing a normal calcium concentration, the firing rate of this unit decreased markedly and the effects of enkephalin could not be reliably assessed.
3.3.5. Antagonists

Naloxone (100 nM - 1 μM) usually did not affect neuronal firing, but caused an excitation in approximately 15% of neurons tested (n=30). This increase was usually small (< 2 x control) and short-lived (30-90 s) and only rarely persisted throughout the period of application. An increase in firing rate was also evoked by both the active (levorotatory Mr-2266) and inactive (dextrorotatory Mr-2267) isomers of the benzomorphan antagonist 5,9-diethyl-2-(3-furyl-methyl)-2'-hydroxy-6,7-benzomorphan hydrochloride (Fig. 19, n=6). The active and inactive isomers were equally effective in producing an increase in firing rate; therefore, it seems unlikely that this effect is due to antagonism at the opiate receptor of an endogenously released opioid.

Naloxone (3-30 nM) reversed or prevented the action of the enkephalins and β-endorphin (Figs. 16, 20, 25). Antagonism was considered to be adequate when the inhibitory action was reversed by at least 90%. The mean ratio (dose of enkephalin)/(dose of naloxone) at which antagonism was observed for met-enkephalin was approximately 9:1 (Table 7). Antagonism was also observed with Mr-2266 but not with Mr-2267 (Figs. 19, 21, n=4).

The specificity of the action of naloxone to reverse opioid peptides was tested by examining its ability to reverse the inhibition of the firing of myenteric neurons produced by high calcium solutions (10 nM) (Fig. 22) or somatostatin (10-100 nM) (Fig. 25). Even at high con-
Table 7. The antagonism of the inhibitory action of endorphins by naloxone. Antagonism is indicated when concurrent application of naloxone completely (>90%) prevented or reversed the inhibitory action of the agonist. Concentrations of agonists were: met-enkephalin (30 - 300 nM), leu-enkephalin (100 - 300 nM), β-endorphin (100 - 300 nM). Concentrations of naloxone were 1 - 100 times lower than those of the agonists. In the case of met-enkephalin, the mean ratio agonist concentration: antagonist concentration was 8.7:1 when antagonism was observed (n=15) and 14:1 when antagonism was not observed (n=8).

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</tr>
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</tr>
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<td>5</td>
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Prevention of the effect of met-enkephalin by benzomorphantagonist. Ordinate: (bin width - 10 s). Abscissa: time. The solid bar indicates the period during which the perfusing solution contained met-enkephalin (300 nM). During the periods indicated by the diagonally hatched bars, the solution contained either the inactive (Mr-2267) or the active (Mr-2266) isomer of the benzomorphantagonist. Both the active and inactive isomers caused an increase in the firing rate of this neuron. During the period which the inactive isomer (Mr-2267, 100 nM) was being perfused, met-enkephalin (300 nM) caused a complete inhibition of firing. In the presence of the active isomer (Mr-2266) met-enkephalin (300 nM) produced only a slight decrease in firing rate.
Figure 20. Inhibition in firing produced by met-enkephalin: reversal and prevention of the inhibition by naloxone. Ordinate: spike frequency (bin width - 30 s). Abscissa: time. During the periods denoted by the solid and diagonally hatched bars, the perfusing solution was changed to one which contained met-enkephalin (100 nM) and naloxone (10 nM), respectively. Met-enkephalin produced a nearly complete inhibition in firing rate of this unit. Reversal and prevention of the inhibition in firing rate produced by enkephalin was obtained with a tenfold lower concentration of naloxone. The inhibition in firing rate during subsequent application of enkephalin was not as large as previous applications, probably because of the continued presence of naloxone.
- Met-Enkephalin (100 nM)
- Naloxone (10 nM)
Figure 21. Inhibition of firing rate by β-endorphin and the reversal of this inhibition by a benzomorphan antagonist. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. The solid bars indicate the periods during which the bathing solution contained β-endorphin in the indicated concentration (nM). The inactive (+) isomer of the benzomorphan antagonist (Mr-2267, 30 nM) and the active (-) isomer (Mr-2266, 30 nM) of the benzomorphan antagonists were perfused during the periods indicated. β-endorphin (300 nM) completely stopped the firing of this unit. Pretreatment of the tissue with the inactive isomer (Mr-2267) failed to alter the inhibition produced by β-endorphin whereas pretreatment with the active isomer (Mr-2266) completely blocked the inhibition in firing caused by β-endorphin.
Figure 22. Inhibition in spike frequency of a myenteric neuron with a high calcium solution. Ordinate: spike frequency (bin width - 10 s). Abscissa: time. During the periods indicated by the solid bars, the bathing solution was changed to one which contained a high calcium (10 mM), and the diagonal hatched bar indicates the period in which the perfusing solution contained naloxone (300 nM). The high calcium solution produced an inhibition in firing rate which was not affected by prior treatment with naloxone even at a high concentration.
Naloxone (300 nM)
Ca⁺⁺ (10⁻⁴ M)
concentrations (300 nM), naloxone did not alter the inhibition produced by these agents.

Alpha-receptor antagonists have been shown to reverse the inhibition of the firing of myenteric neurons produced by noradrenaline (Sato et al., 1973). The inhibition in firing rate produced by met-enkephalin was not affected by phenoxybenzamine (n=4), nor was the effect of met-enkephalin blocked by the β-receptor antagonist propranolol (10 μM, n=2).
3.4. NEUROPEPTIDES

3.4.1. Somatostatin

These results are based on recordings made from 70 units from 25 animals. Somatostatin (100 nM - 1 µM) inhibited the firing of up to 80% of myenteric neurons tested. This effect was dependent on the concentration of somatostatin applied (Fig. 23). The firing of the most sensitive units was inhibited by a concentration of 300 pM and 50% of the units were inhibited by somatostatin (30 nM) (Table 8). The inhibition of firing caused by somatostatin occurred within 15 s of the arrival of the somatostatin, reversed equally quickly and usually persisted throughout the period of application (Figs. 23, 25). Higher concentrations of somatostatin sometimes caused a depression of firing which passed off during the continued presence of the peptide. The loss of effect during the application was observed in 42% of units tested with a concentration of 300 nM, 23% of units with 100 nM, 8% of units with 30 nM and in 19% of units with 10 nM. In cells in which the inhibition in firing rate passed off during the presence of somatostatin, the response to somatostatin, even at higher concentrations, disappeared if the interval between applications was less than 9 min. However, repeated applications of the same concentration of somatostatin at intervals of at least 9 min produced similar inhibitions (Fig. 24).

Somatostatin also produced an inhibition in the firing rate in calcium-free solutions. The inhibition in firing produced by somatostatin was not altered by phenoxybenzamine (1 µM, Fig. 24, n=2) or by naloxone (100 nM,
Table 8. The number of neurons in which firing was inhibited by various concentrations of somatostatin. Inhibition indicates that the firing rate fell to 75% of the control rate.

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Fig. 25, n=12). Propranolol (10 μM) did block the inhibition produced by somatostatin in 1 of 5 experiments.

The effects of enkephalin and somatostatin were compared on 51 units. Six were unaffected by either peptide (enkephalin in concentrations up to 300 nM, somatostatin in concentrations up to 100 nM). Ten units were inhibited by enkephalin but not by somatostatin (up to 100 nM). Nine units were inhibited by somatostatin but not enkephalin (up to 300 nM). The other 26 units were inhibited by both somatostatin and enkephalin. In those units where a sufficient number of different concentrations of each peptide were applied, an equal inhibition of firing was produced by somatostatin and enkephalin at about equimolar concentrations. The existence of cells which respond to somatostatin and not to enkephalin (Fig. 23) is strong evidence to support the hypothesis that somatostatin and enkephalin do not act via a common receptor.
Figure 23. Inhibition of spike firing by somatostatin. Ordinate: spike frequency (10 s epochs). Abscissa: time. The entire experiment was carried out in a solution which was calcium-free and contained EGTA (1 mM). During the period indicated by the stippled bars, the bathing solution contained somatostatin in the indicated concentrations (nM). The horizontally hatched bar indicates the period during which leu-enkephalin (300 nM) was included in the bathing solution. Somatostatin produced a concentration-dependent inhibition in spike frequency. The lowest concentration of somatostatin (0.3 nM) appeared to increase the firing rate. However, this was not repeatable (not shown). Leu-enkephalin (300 nM) was without effect on this cell.
Figure 24. Inhibition by somatostatin of the firing of two myenteric neurons. Ordinate: spike frequency (10 s epochs). Abscissa: time. (a) During the periods marked by the solid bars, the bathing solution was changed to one which contained somatostatin (100 nM). The open bar denotes the period during which the perfusing solution contained phenoxybenzamine (POB, 1 µM). Somatostatin (100 nM) caused an inhibition in firing rate which was not maintained throughout the period during which somatostatin was in the bath. The inhibition was reproducible on each of six applications and was still present in solutions which contained phenoxybenzamine (1 µM). (b) The perfusing solution throughout this experiment, and for one hour previous to this experiment, was calcium-free and contained EGTA (1 mM). During the periods marked by the solid bars, leu-enkephalin (LE, 300 nM) was contained in the bathing solution. The diagonal hatched bars represent the periods when the bathing solution contained somatostatin (S, 100 or 300 nM). Somatostatin (100 and 300 nM) and leu-enkephalin (300 nM) inhibited firing of this myenteric neuron. The inhibition caused by somatostatin did not last throughout the period of its application. The inhibition by leu-enkephalin was maintained throughout the period of application.
Spike Frequency (Hz)

Time (min)

Ca\textsuperscript{++} Free Solution

S 100 LE 300 30 100 300 30 30 (nM)

Time (min)
Figure 25. Inhibition of spike firing by opioid peptides and somatostatin, and the sensitivity of these inhibitions to naloxone. Ordinate: spike frequency (30 s epochs). Abscissa: time. During the periods indicated by the solid bars, the bathing solution contained somatostatin (S), leu-enkephalin (LE) and β-endorphin (BE), in the concentrations indicated (nM). The perfusing solution contained naloxone (100 nM) during the period denoted by the diagonally hatched bar. (a) Somatostatin (100 nM), leu-enkephalin (100 nM) and β-endorphin (100 nM) all caused a marked inhibition in firing rate of this unit. Only somatostatin produced the same degree of inhibition in the presence of naloxone. (b) Another unit in which the inhibition produced by somatostatin at lower concentrations (30 nM) was not effected by high concentration of naloxone. (c) A final unit in which the inhibition produced by a low concentration of somatostatin (10 nM) was not effected by a tenfold higher concentration of naloxone (100 nM).
Naloxone 100 (nM)
3.4.2. Substance P

Extracellular recordings were made from 204 units in tissues from 74 animals. Substance P was found to cause a marked increase in the firing rate in up to 80% of neurons. The increase in firing rate was abrupt upon entry of the peptide into the tissue bath (10-15 s) and the firing rate returned to control soon (within 30-60 s) after washing the peptide from the bath. Substance P (1 nM) caused a clear excitation, at least doubling the firing rate, in about 50% of the cells tested (Table 9). Higher concentrations resulted in a greater proportion of cells being excited; up to where 80% of the units tested were found to be sensitive (Table 9). Excitation of the smooth muscle by substance P limited the use of concentrations greater than 30 nM, and it is possible that the increase in firing rate with higher concentrations may have been secondary to muscle movements near the recording site or it may have been due to increased synaptic activity secondary to violent muscle movement away from the recording site. These difficulties were minimized by the use of lower concentrations of substance P and the careful pinning of single ganglia.

The proportion of cells excited by low concentration (300 pM) was less than the proportion of cells excited by higher concentrations (up to 30 nM). This change in proportion of cells excited by increasing concentrations implied a dose-related effect. The response of single cells to substance P was also dose-related, but absolute sensitivities
Table 9. The effect of different concentrations of substance P, substance P pentapeptide and substance P free acid on the firing of myenteric neurons. An increase in firing was determined if the firing frequency at least doubled in the presence of substance P or either analog. About 80% of the units tested were sensitive to substance P (10 nM), no effects were seen with substance P free acid below a concentration of 100 nM and few cells were sensitive to substance P pentapeptide below a concentration of 30 nM.

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varied among units (Figs. 26, 27, 31). The responses observed to repeated applications of the same concentration of substance P were of three types. First, the amplitude of the excitation decreased with second or subsequent applications (Figs. 26a, 27, 31). Second, the excitatory effect remained the same (Figs. 28, 29, 30). Third, the excitatory effect became greater (Fig. 36c). The proportion of units showing these different types of responses to repeated applications was 60%, 30% and 10%, respectively.

Prolonged applications (up to 15 min) of substance P also yielded variable results: most units maintained an increased firing rate throughout the period of application, but in other units the firing rate returned to the control rate before the peptide was washed from the bath. The decrease in response may represent desensitization, but depolarization block or other change in the condition of the cell cannot be detected using extracellular recording.

Substance P produced a dose-dependent increase in firing rate of myenteric neurons in solutions which contained no calcium and EGTA (1 mM) (Fig. 27). In cells which were sensitive to substance P in normal Krebs solution, an equivalent excitation could be produced by a lower concentration of substance P when the neuron was in a calcium-free solution (Table 10). This may be due to the depolarizing effect of the calcium-free solutions (Nishi and North, 1973a). The proportion of cells which were found to be sensitive to substance P was decreased to 40% of those tested in calcium-free solutions (Table 10); similar results were obtained with morphine and enkephalin (see above). Substance P caused an excitation in solutions which contained cobalt chloride (1 mM, n=3).
Figure 26. Excitation by substance P of two myenteric neurons. Ordinate: spike frequency (10 s epochs). Abscissa: time. The solid bars indicate the period during which substance P (SP, concentrations indicated in nM) was contained within the bathing solution. (a) Increasing concentrations of substance P from 1-10 nM produced graded increases in firing rate of this unit. A second application of substance P in the same concentration range resulted in somewhat smaller responses. (b) The vertical hatched bar indicates the period during which the bathing solution contained substance P free acid (SPFA, 100 nM). Substance P caused a concentration-dependent increase in firing of this unit. The substance P free acid analog was about 33 times less potent than substance P on this unit.
Table 10. The effect of substance P in different concentrations on the firing rate of myenteric neurons in a solution which was calcium-free and contained EGTA (1 mM). An increase in firing rate was determined if the firing rate at least doubled in the presence of substance P. About 50% of the cells tested were sensitive to substance P (300 pM) in calcium-free solutions. At the same concentration of substance P (300 pM) in calcium-containing Krebs, less than 30% of the units were sensitive. At higher concentrations (1-10 nM), the number of sensitive units in calcium-free solutions was only 40% of those tested as compared to 60% of units tested in calcium-containing Krebs.

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Figure 27. Increase in firing rate produced by substance P on a myenteric neuron in calcium-free solution. Ordinate: spike frequency (2 s epochs). Abscissa: time. The tissue was continuously bathed in a solution which was calcium-free and contained EGTA (1 mM). The open bars indicate the periods during which the bathing solution contained substance P (0.3-1 nM, SP). Substance P (300 pM) caused a marked excitation of this unit. Repeated applications of the same concentration of substance P resulted in decreasing responses.
Spike Frequency (Hz)

Time (min)

0 15 30 45

0 0.3 0.3 0.3 0.3 0.3 0.3 SP
Substance P was equally effective in the presence of hexamethonium (100 μM, Fig. 28, n=12). This concentration of hexamethonium blocks the fast e.p.s.p. of myenteric neurons (Nishi and North, 1973a). The excitation produced by substance P was not altered by naloxone at a concentration (300 nM, Fig. 30, n=12) which was sufficient to block the action of morphine or enkephalin (Fig. 29, Table 7). Substance P produced an inhibition in 4 units. This inhibition was not prevented by naloxone (300 nM) in the one cell on which it was tested.

The increase in firing rate produced by substance P was not altered by baclofen (5 μM, n=5) (Fig. 30) or baclofen (50 μM, n=9). In two other experiments, the excitation produced by substance P was partially and reversibly blocked by baclofen (50 μM).

The actions of two substance P analogs, substance P pentapeptide (penta-substance P) (1-100 nM) and substance P free acid (3-3,000 nM) (Table 9) were studied on 9 and 17 units, respectively. Both analogs markedly increased the firing rate of myenteric neurons with a time course which was similar to that of substance P. Such excitations were observed only in units which were also excited by substance P. The response to repeated applications of the two analogs was similar to that observed with repeated applications of substance P. Penta-substance P (10 nM) produced an increase in the firing rate in 1 of 6 cells, whereas penta-substance P (30 nM) increased the firing rate in 5 of 6 cells tested (Table 9). No effects of substance P free acid were observed at a concentration below 100 nM, and above this concentration, the proportion of units which were excited was about the same as for substance P. Substance
Figure 28. The effect of acetylcholine antagonists on the response to substance P of a myenteric neuron. Ordinate: spike frequency (10 s epochs). Abscissa: time. Both upper and lower records are taken from the same neuron, but they are separated in time by 30 min. During the periods indicated by the solid bars, the perfusing solution was changed to one which contained substance P (SP, 10 nM). The cholinergic antagonists, hexemethonium (C-6, 100 μM) and hyoscine (Scop. 1 μM) were included in the bathing solution during the period marked by the horizontally hatched bar. The cholinergic antagonists did not alter the excitation of this myenteric neuron by substance P, even after continuous exposure to the antagonists for 30 min (lower record).
Figure 29. Increase in spike frequency by substance P and lack of effect by naloxone. Ordinate: spike frequency (10 s epochs). Abscissa: time. The solid bars indicate the periods during which the perfusing solution was changed to one containing substance P in the given concentrations (nM). During the period denoted by the diagonally hatched bar, naloxone (300 nM) was included in the bathing solution. Substance P caused a marked increase in firing rate which was not affected by naloxone.
Substance P
Naloxone
(nM)

Spike frequency (Hz)

Time (min)
Figure 30. Lack of effect of baclofen on the increase in firing rate produced by substance P on a myenteric neuron. Ordinate: spike frequency (10 s epochs). Abscissa: time. The solid bars represent the periods during which the bathing solution was changed to one which contained substance P (SP, 1 nM) and the diagonally hatched bar indicates the period when baclofen (5 µM) was included in the bathing solution. Substance P (1 nM) produced an increase in firing rate of this unit which was not affected by baclofen.
Baclofen (5 μM) SP (1 nM)
P free acid produced a concentration-dependent increase in spike frequency (Fig. 31).

In order to obtain an equal response on a single unit to a given concentration of substance P, a 10-30 fold higher concentration of penta-substance P and a 100-600 fold higher concentration of substance P free acid was required (Fig. 31). The proportions of cells which were excited by these peptides may be taken as an approximation of relative potency. About 50% of the cells tested with substance P (1 nM) produced an increase in firing rate. In order for 50% of the cells to be affected by penta-substance P and substance P free acid, concentrations of 10-30 nM and 100 nM were required, respectively. Thus, the relative potency of the two substance P analogs to substance P has been tested in two ways: first, by testing different concentrations of each peptide on a single unit and second, by determining the concentration of each peptide which causes an increase in firing rate in 50% of the units tested.
Figure 31. The effects of substance P and two analogs on two different myenteric neurons. Ordinate: spike frequency (10 s epochs). Abscissa: time. The solid, horizontally hatched and vertically hatched bars indicate the periods during which the perfusing solution was changed to substance P, substance P pentapeptide (PSP) and substance P free acid (SPFA), respectively. (a) Substance P produced a concentration-dependent increase in firing rate. On this cell, a concentration of 30 nM substance P pentapeptide produced about the same increase in firing rate as did 1 nM substance P. A 300 fold greater concentration of substance P free acid was required to equal the substance P (1 nM) response on this unit. (b) Substance P (1 nM) produced a reproducible increase in firing rate in this neuron. About a 100 fold larger concentration of substance P free acid and a tenfold larger concentration of substance P pentapeptide were required to equal the substance P response.
3.4.3. Neurotensin

Neurotensin was tested on 54 units in preparations from 37 animals. Neurotensin (1-300 nM) increased the firing rate in about 50% of those cells tested. For a given unit, the increase in firing was concentration-dependent (Fig. 32); however, the sensitivity among units varied considerably. Even at higher concentrations (> 10 nM) only about half of the cells tested were sensitive to neurotensin (Table 11). Little or no smooth muscle movement was observed by microscopic examination during the application of neurotensin and this permitted the use of higher concentrations. The action of neurotensin directly on the smooth muscle has been reported to be inhibitory (Kitabji and Freychelt, 1978). The excitation of myenteric neurons produced by neurotensin had a time course similar to that observed with substance P. The increase in firing rate usually lasted throughout the period of application (up to 15 min) and the effect produced by a given concentration of neurotensin was reproducible on the same unit (Fig. 33): in this respect, the excitations produced by neurotensin differ somewhat from those produced by substance P. The increase in firing rate produced by neurotensin was not altered by solutions containing hexamethonium (100 μM) and hyoscine (1 μM, n=6).

Neurotensin excited neurons in solutions which were calcium-free. The proportion of cells which were sensitive to neurotensin was the same in a calcium-free solution as in solutions with normal concentrations of calcium (Table 11). This suggests that neurotensin is affecting a somewhat different population of cells from those which were sensitive to
substance P or enkephalin. Further evidence for this was found in studies on the action of several peptides on the same cell (see section 3.4.5.).
Table 11. The effect of neurotensin at different concentrations on the firing rate of myenteric neurons in normal Krebs, and in solutions which were calcium-free (+EGTA 1 mM). An increase in firing rate indicates that the firing frequency at least doubled in the presence of neurotensin. The proportion of units sensitive to neurotensin did not change when studied in solutions which were calcium-free.

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Figure 32. Effect of neurotensin on the firing rate of a myenteric neuron. Ordinate: spike frequency (10 s epochs). Abscissa: time. During the periods indicated by the solid bars, the bathing solution contained neurotensin in the indicated concentration (pM). A concentration-dependent increase in firing rate was produced by neurotensin at concentrations ranging from 0.3-10 nM.
Figure 33. Effect of long applications of neurotensin on the firing rate of a myenteric neuron. Ordinate: spike frequency (10 s epochs). Abscissa: time. The solid bars indicate the periods during which the perfusing solution contained neurotensin (1 nM). Neurotensin (1 nM) produced an increase in firing rate which was maintained throughout the period of application (up to 16 minutes).
3.4.4. VIP

The action of VIP (1 pM - 10 nM) was studied on 78 units in tissues from 24 animals. An increase in firing rate was found in 35% of the cells studied (Table 12). The time course of action of VIP was similar to that of substance P and neurotensin. The increase in firing frequency was dependent on the concentration (Fig. 34). Repeated applications of the same concentration of VIP usually resulted in progressively declining responses (Fig. 35). The effective concentrations of VIP were very low: 4 neurons were excited by 1 pM. It is possible that a higher proportion of neurons would have been affected by VIP in concentrations greater than 10 nM. The use of concentrations greater than 10 nM was not attempted for the following reason. One commercially available source of VIP (Calbiochem) contains VIP in 100 ng quantities lyophilized from 1 ml of a solution containing 0.2% bovine serum albumin (BSA). Solutions of VIP exceeding 10 nM therefore contained significant amounts of BSA. In control experiments, the tissue was perfused with a Krebs solution containing BSA (up to 0.2 g/l): this did not alter neuronal firing (n=6).

The action of VIP on myenteric neurons was present when the tissue had been bathed in calcium-free solutions (Table 12, Fig. 36) and in solutions which contained cobalt chloride (1 mM).
Table 12. The effect of different concentrations of VIP on the firing rate of single myenteric neurons in normal and in calcium-free Krebs. An increase in firing rate was determined if the firing frequency increased to at least 50% over control. The proportion of units sensitive to VIP was about 45% at concentrations up to 1 nM. Some units were sensitive to concentrations as low as 1 pM.

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Figure 34. Effect of VIP on the firing rate of a myenteric neuron.

Ordinate: spike frequency (10 s epochs). Abscissa: time.

During the periods indicated by the solid bars, the bathing solution contained VIP in the concentration indicated (pM). VIP (10 pM) caused a marked increase in firing rate of this unit, whereas 3 pM VIP caused only a small increase and 1 pM was without effect.
Figure 35. Effect of repeated applications of VIP on the firing rate of a myenteric neuron. Ordinate: spike frequency (10 s epochs). Abscissa: time. During the periods indicated by the solid bars, the bathing solution contained VIP (300 pM). Application of VIP caused a modest increase in firing rate which became less apparent with repeated exposure and almost disappeared during a longer exposure.
3.4.5. Effects of Excitatory Neuropeptides on the Same Cell

Substance P (1-10 nM), neurotensin (1-100 nM) and VIP (1 pM-1 nM) were studied on each of 53 units. The effects of substance P and neurotensin were initially tested at a concentration of 1 nM and the starting concentration of VIP was usually 10 pM. If an increase in firing rate was observed with at least one of the peptides, at the initial concentration, then a higher concentration (up to 100 times the initial concentration) of the other peptide or peptides which did not previously cause an excitation was applied. When each of the peptides was tested at the initial concentration, 14 (25%) of the units were not affected by any of the peptides and 8 (15%) of the units were excited by all three peptides. The number of cells sensitive to one peptide and not the other two was as follows:

11 cells were sensitive only to substance P,
7 cells were sensitive only to neurotensin
and 1 cell was sensitive only to VIP.

The number of cells which were sensitive to two peptides but not to the third was as follows:

7 cells were sensitive to substance P and neurotensin but not VIP,
3 cells were sensitive to neurotensin and VIP but not substance P
and 2 cells were sensitive to substance P and VIP but not neurotensin.

The existence of cells which respond to one of the three excitatory peptides studied and not the other two strongly suggests that there are populations of cells which are specifically excited by one of these peptides. There is also considerable overlap in the sensitivity to the three pep-
tides between cells of the myenteric plexus.
Figure 36. Effects of two or more excitatory peptides on each of three myenteric neurons. Ordinate: spike frequency (10 s epochs). Abscissa: time. (a) This experiment was done in calcium-free Krebs which contained EGTA (1 mM). During the periods indicated by the open, vertically hatched and solid bars, the perfusing solution was changed to one which contained neurotensin (NT, 1 nM), VIP (1-10 pM) and substance P (SP, 1-10 nM). All three peptides caused an increase in spike frequency. This unit had about a tenfold greater sensitivity to neurotensin than to substance P. VIP had a clear effect at a concentration 1,000 times less than substance P. (b) The solid bars indicate the period during which the bathing solution contained neurotensin (NT, 1 nM), VIP (1 nM) or substance P (SP, 1 nM). Neurotensin and substance P were about equally effective in causing an increase in spike frequency while VIP was without effect. (c) Solutions of substance P (SP, 1 nM) and neurotensin (NT, 1 nM) were perfused during the periods indicated by the solid bars. The spike frequency of this unit was markedly increased by substance P (1 nM) and only slightly affected by neurotensin (1 nM).
4. DISCUSSION

4.1. EXTRACELLULAR RECORDING FROM THE MYENTERIC PLEXUS

Extracellular recording in vitro offers many advantages over recording in vivo. Known concentrations of agonists and antagonists can be added to the bath, the effects of changing ion concentrations in the bathing fluid can be studied and the distinction can be made between direct actions of the drug on the cell and those mediated transsynaptically. However, there are some limitations which are common to both extracellular recording in vitro and in vivo. First, investigation of ionic mechanisms with solutions of altered ionic compositions are difficult to interpret because changes in firing rate are only a rough measure of the action of the agent under study. A change in firing rate can be as a result of many possible factors including membrane potential, membrane resistance, a change in the threshold or sodium inactivation or potassium activation. When studying the change in firing rate produced by a given drug, the experiment is limited to changes in firing rate which vary between complete inhibition caused by an inhibitory substance and depolarization block caused by an excitatory substance. To investigate ionic mechanisms, it must be possible to measure changes in the properties of the neuron which are not bounded by the limits which determine whether the neuron will or will not give an action potential. Properties such as membrane potential, input resistance, threshold and reversal potentials of drugs in normal Krebs solutions and in solutions of altered ion concentrations are much more useful in determination of the ionic mechanism by which a drug acts. Second, using extracellular recording, it is not
possible to study changes in synaptic potentials. Third, in the present study, special attention must be paid to possible changes in firing rate produced by movement of the underlying smooth muscle when recording from myenteric neurons. All movement of the smooth muscle was eliminated in the present experiments by careful pinning of a single ganglion, or by using solutions which were calcium-free or contained cobalt (1 mM).

The primary difference between results from intracellular and extracellular recording of neurons from the guinea pig myenteric plexus is that spontaneous activity was recorded with extracellular electrodes and no spontaneous activity was found when intracellular recordings were made. The site of origin of the extracellular activity could account for this difference between the two techniques. That is to say, intracellular electrodes record activity in the cell soma, whereas extracellular electrodes may record activity from other sites, such as the cell processes or terminals. It is difficult to identify the source of the extracellular activity definitively; however, all indications suggest it is the cell soma. (1) The strongest such indication is that spontaneous action potentials or evoked all-or-nothing potentials were recorded only from the surface of the ganglia and were never recorded from the interconnecting strands. (2) The only activity which was recorded from the interconnecting strands was electrically evoked, graded with the intensity of stimulation and had a multiphasic waveform. Because few cell bodies are found in these strands, it is likely that the graded, short latency, evoked potentials which were recorded, were from cell processes. (3) The waveform and time course of the spontaneous extracellular spikes recorded from the ganglion surface also suggest that the source of the
activity is somatic. The time course of the action potential corresponds to the first time differential of that recorded with intracellular electrodes (Nishi and North, 1973a). The monophasic or slightly biphasic waveform of the potential is usually considered to be somatic (De Valois and Pease, 1973). This waveform indicates that there is a good resistive seal around the electrode. If the resistive seal was smaller, two changes in the extracellular spike would result. First, the amplitude of the spike would become much smaller due to an increased amount of electrical shunting between the recording and indifferent electrodes. Second, one might expect to record the field potentials from the cell which are set up by the membrane currents during the action potential. The field potentials would result in a triphasic waveform which is proportional to the second differential of the change in cell potential with respect to time (Hubbard, Llinus and Quastel, 1969).

The resistive seal around the electrode is sufficiently good that the extracellular potential is coupled to the intracellular potential by the capacitance of the cell membrane. Under these conditions the extracellular spike should be in the opposite polarity to the intracellular spike and the waveform would be proportional to the first differential of the change in potential with respect to time (Hubbard et al., 1969). In practice the polarity and waveform of the extracellular spikes usually conformed to this prediction; however, spikes of opposite polarity and of varied waveform (symmetrically biphasic rather than monophasic) were recorded occasionally. These spikes were more often seen when using large recording electrodes (> 70 μm). There are at least two explanations which may account for the variation in spike polarity and waveform. (1) The
resistive seal around the electrode is not perfect. When recording extracellularly using the sucrose gap method, the resistance between the recording and indifferent electrodes was reported to be usually more than 5 MΩ (Nishi and Koketsu, 1968). Using this method the extracellular potentials are opposite in polarity but similar in waveform and amplitude to the intracellular potentials. Because a sucrose gap involves the resistance of a population of cells over a fairly large (1 mm) gap, it is difficult to compare the resistance seen using sucrose gap with the resistance observed with a suction electrode. However, there is at least a tenfold difference between the resistance measured by the two methods, and this may account in part for the differences in spike polarity, amplitude and waveform seen using the suction electrodes. (2) An entire cell body is not always covered by the suction electrode. When a large electrode (> 70 μm) is used, one might expect that parts of several cell bodies would be covered. If a large part of the cell body were not covered by the electrode, the membrane current produced during an action potential by this part of the cell may result in a large local field potential. These field potentials, and not others, are recorded because of the proximity of the electrode to the source and the relatively large size of the source (cell body as opposed to cell process). If a large part of the cell body is outside the electrode, then the polarity of the spike should be opposite (positive) to that which is recorded when the cell is mostly beneath the electrode. If half of the cell was covered by the electrode, then the waveform might be expected to be symmetrically biphasic.

The small size of the electrorcellular potentials recorded might indicate that their source is a smaller structure than the cell soma.
However, the use of a large, low resistance electrode may result in attenuation of extracellular potentials. In fact, on theoretical grounds, the size of the potential can be predicted (appendix 1). Even if the electrode made a perfect resistive seal around a cell, such that all the current generated by the cell resulted in a potential difference recorded at the electrode, the potential would be only 1-5 mV. If the recording was from a cell process, the maximum potential would be only 50-150 μV. These considerations therefore suggest that the extracellular electrodes are in fact recording potentials which are derived from the soma spike.

Another possible consideration which may explain the origin of the spontaneous extracellular activity is that under the conditions of extracellular recording, the neurons were synthetically driven, and that synaptic activity was absent in the intracellular recording experiments. This possibility was tested in two ways: (1) By the use of calcium-free solutions and (2) by the use of cobalt. Calcium-free solution blocked synaptic transmission (Dingledine and Goldstein, 1976), but had a depolarizing effect on its own (Nishi and North, 1973a). The depolarization of cells in calcium-free solutions may then substitute for the decline in any ongoing synaptic influences such that the firing rate was maintained. Solutions which contain cobalt (1 mM) also block the e.p.s.p. and have no effect or a very small depolarizing effect on membrane potential (Katayama and North, unpublished results). In the present study, the firing rate of myenteric neurons was not affected by solutions which contained 1 mM cobalt (n=6).

It appears that the extracellular electrodes are recording from
the cell soma of myenteric neurons and that the spontaneous activity is not due to ongoing synaptic activity. The neuronal activity recorded with extracellular electrodes is thought to be induced by the large suction electrodes based on the following observations. First, no spontaneous activity was recorded with suction electrodes having a tip diameter less than 30 μm, but electrically evoked activity could be recorded with these small suction electrodes. Second, the firing rate of some units could be increased or decreased by using greater or lesser amounts of suction. Third, sometimes when the electrode was placed on the ganglion with suction and no activity was recorded, excitatory agents added to the bath would cause a unit to begin firing. Likewise, units other than the one being recorded would sometimes begin to fire when excitatory agents were added to the bath.

Although the single spike and burst type firing patterns previously described may represent different functional types of neurons within the myenteric plexus, little physiological significance can be ascribed to this activity since it is thought to be artificially induced by the suction electrode. As a model to study the action of various agents on neuronal firing, it is in some respects analogous to artificial induction of firing in many studies by iontophoretically applied glutamate. The use of mechanical irritation of the cell to induce firing may be, in some respects, an improvement over the use of excitatory amino acids because it eliminates possible interactions (or proposed interactions) between the agent being studied and the excitatory agent used to stimulate the cells.
Mechanical irritation of the myenteric neurons by the suction electrode may also be closer to a physiological stimulus than one might expect since the gut wall is subjected to changes in pressure and marked changes in diameter during the passage of luminal contents through the gastrointestinal tract. The ganglia of the myenteric plexus undergo clear changes in size with the amount of stretch which is put on the longitudinal muscle when pinning out the tissue. Similar changes in size and shape of the ganglia are observed in tissues where the longitudinal smooth muscle is spontaneously active. The movements of peristalsis must also result in mechanical deformations of the ganglia in vivo. It is possible that application of a suction electrode simulates some stage of distension which is present during peristalsis and this results in the firing of myenteric neurons.

The sensitivity to various agents, both excitatory and inhibitory, varied among units. One explanation is that the myenteric plexus is a heterogeneous population of cells and that certain cells are much more sensitive to some agents than are other cells (see sections 3.4.1. and 3.4.5.). Another possibility is that the intensity of stimulation produced by the suction electrode could alter the response of a cell to any given agent. The amount of suction does alter the firing rate of some neurons. It is possible that the degree of inhibition or excitation produced by a given agent could be altered by the amount of suction in the electrode. The sensitivity of units to inhibitory agents also seemed to vary with the size of the suction electrode; units recorded with large (70-90 µm) electrodes being less sensitive than those recorded with smaller electrodes. Presumably, a larger electrode or an increased amount of suc-
tion offered a greater source of stimulation such that it took a greater concentration of inhibitory substance to overcome the stimulation. This explanation may account for the variability in the degree of inhibition or excitation produced by inhibitory or excitatory agents among populations of sensitive units.
4.2. THE ACTION OF OPIATES AND ENDOGENOUS OPIOID PEPTIDES

Morphine has been shown to inhibit the firing rate in 90% of myenteric neurons tested (Dingledine, Goldstein and Kendig, 1974; Dingledine and Goldstein, 1975; 1976). This inhibition was found to be mediated via opiate receptors located on the cell being studied and not mediated through a synaptic mechanism (Dingledine and Goldstein, 1975; 1976). The present results confirm these findings and extend the observations to the endogenous opioid peptides. In addition, the action of morphine was tested in solutions of altered ionic composition. Morphine appeared to be equally effective in solutions containing high potassium, low potassium and low chloride. These results cannot be taken as definitive evidence against the involvement of these ions in the action of morphine, since neuronal firing could still be inhibited by morphine even if the ionic mechanism of its action involved one of these ions. The driving force for a potential change caused by morphine may be shifted markedly by changing the extracellular concentration of potassium or chloride. If the firing rate was dependent only on the potential of the cell, the effect produced by morphine on the firing rate might also be changed by changing the driving force of the ion or ions involved. Since the firing frequency is not dependent only on potential, a change in the driving force produced by changing extracellular ion concentrations may not alter the effect of morphine. For example, it is possible that morphine causes a large increase in conductance, which results in blockade of propagation of the action potential by shunting the current through the membrane. The fall in membrane resistance would still occur in solutions which
markedly alter the driving force of the ion or ions involved in the action of morphine.

The endogenous opioid peptides, met- and leu-enkephalin and β-endorphin all inhibited neuronal firing in the myenteric plexus. These inhibitions are considered to be opiate receptor mediated for the following reasons. First, the effective concentration of agonists were low. Second, the inhibition was blocked or prevented by lower concentrations of the opiate antagonist naloxone. Third, the active benzomorphan antagonist Mr-2267 was effective in blocking the inhibition produced by these peptides whereas an equal concentration of the inactive isomer Mr-2266 was ineffective. The inhibition produced by the opioid peptides was not blocked by phenoxybenzamine, propranolol or by solutions in which synaptic transmission was assumed to be blocked.

Met-enkephalin was found to inhibit the firing rate of myenteric neurons at lower concentrations (about 10 times) than morphine and nor-morphine. However, morphine, met-enkephalin and β-endorphin were equally effective in inhibiting the electrically induced contraction of the guinea pig ileum myenteric plexus-longitudinal muscle preparation (Lord, Waterfield, Hughes and Kosterlitz, 1977). The metabolism of met-enkephalin is one explanation for the differences found between the results obtained using the contractile response and those obtained using extracellular recordings. The effects of metabolism were studied on the contractile response using enkephalin analogs which were more resistant to metabolic degradation. The analogs were up to 6 times more effective than met-enkephalin in inhibiting the contraction and yet compared to met-enkephalin
there was little change in the ability of the analogs to displace naloxone from opiate binding sites (Lord et al., 1977). Thus, it appears that in that preparation, metabolism of met-enkephalin does decrease its effectiveness. The effect of metabolism on the inhibition in firing of myenteric neurons under the conditions of the present experiment are limited. The size of the tissue is small, so that the amount of metabolism is limited by the relative lack of peptidases. The tissue is continuously perfused with fresh Krebs solution, and during the period of peptide application, fresh peptide solution continuously perfuses the tissue. Under the conditions used for extracellular recording, the ganglia of the myenteric plexus are laid out flat such that they are directly exposed to the peptide containing solutions.

It is also possible that the inhibition in firing rate is mediated by a different type of opiate receptor from the one which decreases the release of acetylcholine from the nerve terminals. There is mounting evidence that the myenteric plexus contains a heterogeneous population of opiate receptors (Lord et al., 1977). Demonstration of separate populations of receptors using extracellular recording requires quantitative studies such as determination of pA2 values of antagonists and precise determination of relative potencies of a series of agonists. Such quantitative experiments are rendered impossible because there is too much variability inherent in the method of recording with extracellular suction electrodes (see 4.1.).

There is a large amount of variability among units in the response to a given concentration of a single opiate or opioid peptide such that
only general qualitative statements can be made about the relative potency
of a series of agonists.

A membrane stabilization of hyperpolarization was proposed as
being a possible mechanism of action (Dingledine and Goldstein, 1976).
Subsequently, morphine was found to hyperpolarize a population of neurons
(North, 1976; North and Tonini, 1977). However, the proportion of units
in which the firing rate is decreased is much larger than the population
of neurons in which a hyperpolarization was found. One possible explana-
tion is that extracellular recording selects specifically the population
of neurons which have opiate receptors. The cells which were most often
sensitive to the narcotics using intracellular electrodes were the type
I cells (North and Tonini, 1977). These cells are more excitable than
type II cells (Nishi and North, 1973a), and it is likely that these cells
would fire more readily when stimulated by a large suction electrode.
Another explanation may be that extracellular electrodes record poten-
tials which are only rarely recorded at the soma with intracellular elec-
trodes. There is now evidence from experiments using iontophoretic ap-
plication of enkephalin that the hyperpolarization in the soma originates
in the processes (North, Katayama and Williams, 1979). It is possible
that the firing recorded extracellularly originates from irritation of a
cell process close to the soma (the extracellular electrodes were usually
30-70 μm in tip diameter) and that the hyperpolarization produced by opi-
ates at this site acts to inhibit the firing.

Another difference between the results obtained from intracellular
and extracellular techniques is the occurrence of tachyphylaxis to the
hyperpolarizing effect of the opiate and opioid peptides. If the hyperpolarization recorded at the cell soma was solely responsible for the inhibition in firing rate recorded extracellularly, then why is there little sign of tachyphylaxis to the inhibition in firing rate produced by the opiates or opioid peptides? One possible explanation may be that impalement of a neuron with an intracellular electrode renders the cell unable to respond to the opiates over an extended period of time. Such a change in the cell may be related to leakage of chloride ions from the microelectrode (North and Tonini, 1977). It is also possible that impalement of the neuron results in a slow change in the dendritic-soma conductance ratio, such that potential and resistance changes which originate in the processes may be progressively attenuated when recording at the cell soma. A swelling of the cell soma following impalement has been noted and could result in changes in the dendritic-soma conductance ratio. It is also possible that another mechanism other than hyperpolarization causes the inhibition in firing rate. As previously discussed, one possible mechanism is a marked increase in conductance to some ion such as chloride or potassium which results in blockade in propagation of an action potential down a process at some point. A decrease in resistance was seen in about one half of the myenteric neurons which were hyperpolarized and a measurement of input resistance was made (North, Katayama and Williams, 1979). No change in resistance was ever noted in neurons which were not hyperpolarized by opiates or opioid peptides. This was taken as evidence indicating that the opioid peptides act at a site away from the cell soma. The occurrence of a hyperpolarization with a decrease in input resistance in some cells and not others was perhaps due to variations from cell to cell in the dendritic-soma conductance ratios. The fact that a change in
conductance was never seen in the absence of a hyperpolarization may be interpreted in at least two ways. First, these cells are not sensitive to the opiates of opioid peptides. Second, these cells are sensitive but have a very low dendritic-soma conductance ratio such that potentials in the processes cannot be recorded in the soma. The fact that an increase in conductance was never observed in the absence of a hyperpolarization strongly suggests that the hyperpolarization is at least one mechanism which decreases neuronal excitability and an increase in conductance is a second possible mechanism which is less often recorded in the cell soma.

Studies on the ionic mechanism of action of the opiates and opioid peptides using extracellular recording techniques have been limited as previously described. The same studies using intracellular recording techniques have been limited by the number of cells which give reproducible responses; however, a few isolated experiments have been done which verify the results obtained with extracellular recordings. In one cell, the hyperpolarization produced by met-enkephalin (300 nM) was not altered in a solution containing low chloride (10 nM) and in two other neurons the action of met-enkephalin (300 nM) was still present in solutions which were calcium-free (did not contain EGTA) (North and Williams, unpublished results). Further experiments in solutions of altered ionic composition are required to elucidate the ionic mechanism of the action of the opiates and opioid peptides.

The inhibitions caused by the enkephalins and β-endorphin are mediated through an action directly on the cell under study because they persist in calcium-free solutions. These experiments also indicate that
calcium ions are not required for this action of opiates. Similar re-
sults were obtained for the inhibition of firing produced by morphine
(Dingledine and Goldstein, 1975; 1976). The proportion of units sensi-
tive to enkephalin was reduced in calcium-free solutions as was reported
when studying the action of morphine (Dingledine and Goldstein, 1976).
Although it is not possible to distinguish absolutely different types
of units using extracellular recording, the appearance of morphine and
enkephalin insensitive units in calcium-free solutions strongly suggests
that the insensitive cells are the type II cells of the intracellular
classification. First, the excitability of type II cells has been shown
to increase in calcium-free solutions due to the removal of the calcium-
dependent slow after hyperpolarization following the spike (Nishi and
North, 1973; North, 1973). Second, type II cells have been shown to be
less sensitive to the hyperpolarizing action of morphine when studied
using intracellular techniques (North and Tonini, 1977).

Increasing the calcium ion concentration (10 mM) always decreased
the firing rate of myenteric neurons. This inhibition was not mediated
by opiate receptors but through a stabilizing effect of the calcium ions
on the cell membrane. There are at least two mechanisms by which calcium
can stabilize the neuronal membrane to inhibit spike firing: first, by
suppressing sodium activation (Koketsu, 1965) and, second, by increasing
the intracellular calcium concentration which increased potassium conduc-
tance (Krnjević and Lisiewicz, 1972; North, 1973). The role of calcium
ions in the action of opiates at other sites is somewhat conflicting.
Elevating the extracellular calcium ion concentration in vivo has been
found to overcome the action of morphine (Takemori, 1962; Kakunaga,
Kaneto and Hano, 1966). On the other hand, decreasing calcium levels with EGTA or lanthanum resulted in a potentiation of the antinociceptive effect of morphine in rat (Harris, Loh and Way, 1975). The release of transmitter substances from in vitro preparations which are sensitive to the opiates is decreased by morphine (Paton, 1957; Schaumann, 1975; Henderson and Hughes, 1974). Increasing the extracellular calcium ion concentration increases the release of transmitter from the nerve terminals which are sensitive to opiates (Henderson and Hughes, 1974). However, this effect of calcium is common to all neurotransmission sites, not only those which are sensitive to opiates. The action of calcium probably has a much greater effect on the release of transmitter substances in vivo and in vitro than on the stabilization of neuronal excitability and inhibition of firing which was studied in the present experiments.

The physiological significance of the effects produced by enkephalin and β-endorphin remains to be determined. Concentrations of β-endorphin as low as 3 nM were found to inhibit some myenteric neurons. This concentration is about the same as that which was found in the plasma of rats after stressful stimulation (Guillemin et al., 1977). Stress-induced release of β-endorphin has not been measured in the guinea pig. It seems unlikely that the myenteric plexus is a target organ for blood borne β-endorphin since only a small proportion of myenteric neurons were inhibited by concentrations in the 1-3 nM range. Concentrations of met-enkephalin of 3 nM were much more effective in producing an inhibition of firing in the myenteric plexus. Enkephalin has been shown to be contained within the neurons of the myenteric plexus, and in view of its actions upon single neurons, it seems possible that electrophysiological evidence
for its release by electrical stimulation could be obtained. However, repetitive stimulation (0.1-10 Hz) for up to 1 min only rarely caused any inhibition of neuronal firing and this was never affected by naloxone. Repetitive stimulation more often caused a longer lasting excitation. It is possible that naloxone-reversible inhibitions may have been occluded by concomitant release of other transmitters. The longer lasting excitatory response was not studied in detail. It is possible that this excitation is produced by release of one or another of the excitatory neuropeptides.
4.3. THE ACTION OF SOMATOSTATIN

Somatostatin is found in cell bodies and nerve terminals of the myenteric plexus (Costa et al., 1977) and the present study shows that it causes an inhibition in firing rate of single myenteric neurons. Inhibitions of firing rate were produced by somatostatin in the same concentration range as the effective concentrations of met-enkephalin. Some units were also inhibited by concentrations of somatostatin which were found to inhibit growth hormone release (3 nM) (Brazeau et al., 1973). These results may be taken as evidence in support of a physiological role for somatostatin in the myenteric plexus. The action of somatostatin was probably not mediated through an opiate receptor mechanism for two reasons: first, naloxone never altered the inhibition in firing rate produced by somatostatin and, second, some cells which were not sensitive to high concentrations of enkephalin were found to be sensitive to low concentrations of somatostatin. The inhibition in firing produced by somatostatin was not blocked by phenoxybenzamine or propranolol and persisted in solutions which were calcium-free, indicating the action was not due to release of an inhibitory transmitter and was not dependent on the presence of extracellular calcium.

A population of neurons exhibited tachyphylaxis to the effects of somatostatin. This was more apparent at higher concentrations. However, even at higher concentrations, if the interval between applications was extended, reproducible responses could be obtained. The occurrence of tachyphylaxis to somatostatin contrasts with the effects produced by
enkephalin which produced little or no sign of desensitization. Similar results were obtained with the electrically induced contraction of the myenteric plexus longitudinal muscle preparation (Guillemin, 1976). Somatostatin inhibited the contractile response and marked tachyphylaxis to this inhibitory effect was noted. The concentrations of somatostatin (700 nM - 2.1 µM) used to inhibit the contractile response were much higher than those effective in decreasing the firing rate of myenteric neurons. The inhibition of the contractile response to electrical stimulation by somatostatin was not affected by naloxone. Furthermore, during the periods when the response to somatostatin was desensitized, the effect of β-endorphin was unchanged, which also indicates that somatostatin does not act via opiate receptors.

Iontophoretic application of somatostatin (currents up to 30 nA) decreased the firing rate of cerebellar, cortical and hypothalamic neurons of rat (Renaud et al., 1975) and neurons from lamina I-III of the spinal cord from cat (Miletić et al., 1977; Randić and Miletić, 1978). The inhibition in firing rate produced by somatostatin at these sites, as well as myenteric neurons, may be due to a membrane stabilization or hyperpolarization. However, in the in vivo studies, an indirect action cannot as yet be ruled out. Using the sucrose gap method on the isolated frog spinal cord, somatostatin was found to cause a hyperpolarization recorded from the dorsal roots in solutions where synaptic transmission was blocked (Padjen, 1977). However, the hyperpolarization in frog spinal cord required 100-1,000 times the concentration than that which produced an inhibition in firing of myenteric neurons. It may be that the frog spinal cord preparation is much less sensitive than myenteric neu-
rons or, possibly, the inhibition in firing is due to some other mechanism and hyperpolarizations are seen only at higher concentrations.

Iontophoresis of somatostatin at other sites has caused an increase in neuronal excitability (Dodd and Kelly, 1978; Ioffe, Havicek, Friesen and Chernick, 1978). A depolarization and increased excitability in response to somatostatin was found with intracellular recordings from CA1 and CA2 cells from slices of rat hippocampus (Dodd and Kelly, 1978). An increase in firing rate was also seen in response to somatostatin in sensorimotor neurons from rabbits recorded in vivo (Ioffe et al, 1978).

In the study by Dodd and Kelly (1978), the iontophoresis electrode was filled with somatostatin (3 nM) dissolved in sodium acetate (5 mM, pH = 7.0), and a negative current (196 nA) was used to eject the peptide. In the other study, the iontophoresis electrode was filled with somatostatin (5 nM) in distilled water (pH not indicated) and the peptide was ejected as a cation (10-50 nA). In no study was the amount of somatostatin ejected from the electrode measured. It is possible that the pH of the solution in the electrodes may account for the different polarities of current used in these studies.

The excitatory effects of somatostatin may be due to an indirect action of somatostatin. It is difficult to demonstrate a direct action using iontophoretic application of somatostatin or any other peptide using an in vivo system, and indirect effects were not considered in the study using hippocampal slices (Dodd and Kelly, 1978).
4.4. EXCITATORY PEPTIDES

4.4.1. Substance P

Substance P caused an increase in firing rate in the majority of myenteric neurons. The excitation produced by substance P was not altered by calcium-free solutions, solutions which contained nicotinic and muscarinic antagonists or solutions in which all smooth muscle movements were eliminated. These results indicate that the action of substance P is directly on the neurons from which recordings were made. The effective concentration range (300 pM - 30 nM) in this preparation is comparable with other in vitro preparations (e.g. contraction of the guinea pig ileum - Rosell, Bjarkroth, Chang, Yamaguchi, Wang, Rackar, Fisher and Polkers, 1977 and contraction of the guinea pig vas deferens - Zetler, 1977), and with the Kd (3 nM) for binding sites in brain homogenates using tritiated substance P (Nakata et al., 1978). These correlations may be taken as partial proof that the increase in firing rate produced by substance P on myenteric neurons is mediated by a similar receptor.

It is difficult to compare the concentrations used in iontophoretic studies on single neurons in vivo with the concentrations of substance P used in the present study. However, substance P caused a depolarization of motor neurons in the isolated spinal cord of frog (Konishi and Otsuka, 1974a) and newborn rat (Konishi and Otsuka, 1974b) which required 30-100 times the concentration of substance P which was effective on the myenteric neurons.

There was some tachyphylaxis to the effects of substance P on
myenteric neurons. Desensitization and variable responses to iontophor-etic application of substance P on central neurons has also been reported (Krnjević and Morris, 1974; Henry, Krnjević and Morris, 1975; Davies and Dray, 1976; Le Gall La Salle and Ben-Ari, 1977; Sastry, 1978a). It is difficult to determine, using extracellular recording techniques, the mechanism of this apparent desensitization. It is possible, for example, that depolarization block may account for decreasing effects of substance P.

The specificity of the action of substance P was tested with two analogs (penta-substance P (7-11) and substance P free acid). Studies of structure activity relationships of substance P on the depolarization of newborn rat spinal motor neurons have demonstrated that even small changes in the amino terminus of the substance P molecule drastically alters the biological activity (Otsuka and Konishi, 1977). Removal of the first 5 amino acid residues from the carboxy terminus did not alter the biological potency significantly. However, the activity dropped off markedly with further elimination of carboxy residues. The penta-substance P (7-11) analog was at least 50 times less potent than substance P on the rat spinal motor neurons (Otsuka and Konishi, 1977). In the present study, the pentapeptide analog was about 30 times less active than substance P. The substance P free acid analog has not been studied in other preparations. However, removal of the terminal methionine residue decreased the biological potency by 2,000 fold, as measured on the rat spinal motor neurons (Otsuka and Konishi, 1977). These results, and those of the present study, are consistent with the observation that small changes in the amino end of the substance P molecule can markedly change its biological potency.
Baclofen, a proposed substance P antagonist (Saito et al., 1975), was ineffective on the substance P response in myenteric neurons and had no effect on its own. The action of baclofen at other sites has been reported to be GABA-like (Krnjević, 1977). Baclofen decreases the firing rate of spontaneously firing neurons and blocks excitations produced by acetylcholine and glutamate as well as substance P in the substantia nigra (Davies and Dray, 1976), spinal neurons (Henry and Ben-Ari, 1976) and interpeduncular neurons (Sastry, 1978a). It appears that baclofen only antagonizes substance P effects when baclofen itself has a depressant action.

The present results show that substance P is effective in low concentrations and these are similar to those in which it binds to rat brain homogenates (Nakata et al., 1978). These results are not inconsistent with a neurotransmitter or neuromodulator role of substance P in the myenteric plexus. Further study on the action of substance P using intracellular techniques may provide more conclusive evidence on the role of substance P in the myenteric plexus (see 4.5.).
4.4.2. Neurotensin

Neurotensin produced an increase in the firing rate of myenteric neurons similar to that caused by substance P. The effective concentration range and the time course of action of neurotensin were both about the same as for substance P. The increase in the firing rate was not affected by calcium-free solutions or the presence of cholinergic antagonists. There were some notable differences between the effects of neurotensin and substance P. First, since the longitudinal smooth muscle was not stimulated by neurotensin directly, higher concentrations could be studied. Second, the increase in firing rate produced by neurotensin was more reproducible and did not exhibit tachyphylaxis as did the excitations caused by substance P. Finally, the population of cells which were sensitive to neurotensin was not the same as that for substance P: that is to say, there were cells which were affected by substance P but not by neurotensin and vice versa.

An increase in firing rate in response to neurotensin applied by iontophoresis has been reported at several sites in the central nervous system (frontal cortex, hippocampus, striatum and lateral thalamus) (Ziegglänsberger, Siggins, Brown, Vale and Bloom, 1978). It is not possible to correlate the concentrations used in the present study with studies which used iontophoresic application. However, the effective concentration range in the present study is in the same range (1-100 nM) as that which contracts the guinea pig ileum (Segawa, Hosokawa, Kitagawa and Yajima, 1977; Kitabji and Freychet, 1978).
The physiological significance of the increase in firing rate produced by neurotensin in myenteric neurons is not known because the peptide is not found in neurons of the plexus. Neurotensin is found in high concentrations in endocrine-like cells of the ileum (Sundler, Alumets, Hakanson, Carraway and Leeman, 1977; Sundler, Hakanson, Hammer, Alumets, Carraway, Leeman and Zimmerman, 1977). It is possible that the neurotensin released from these cells in response to local distension may act as a local hormone which alters neuronal excitability.
4.4.3. VIP

VIP caused an increase in the firing rate of a population of myenteric neurons in a concentration range of 1 pM to 10 nM. The use of higher concentrations was prohibited because the peptide was obtained in small quantities (100 ng) which were lyophiled from a solution containing 0.02% BSA. This excitation was a direct action on the neuron under study since VIP was equally effective in solutions which were calcium-free and in solutions which contained cobalt (1 mM).

Excitation of cortical neurons has been reported using iontophoretic application of VIP (Phillis, Kirkpatrick and Said, 1978). Also, in this study, VIP and substance P were applied by superfusion to the isolated toad spinal cord preparation: VIP was 2-3 times more potent than substance P in depolarizing both the dorsal and ventral roots. However, the effective concentrations of VIP and substance P were very high (VIP, 3-6 μM; substance P, 6-20 μM). It was implied that the increase in firing rate of cortical neurons was a result of a depolarizing action of VIP similar to that on the toad dorsal and ventral roots.

The results from the iontophoretic experiments suggest that the neurons were very sensitive to VIP since low concentrations of VIP (300 μM in 100 mM NaCl) were used in the iontophoresis electrodes. The transport number of VIP under those conditions must be very low. The increase in firing rate produced by iontophoretically applied VIP often decreased with repeated applications. The results from the present study on myen-
teric neurons indicate that VIP was effective at concentrations 100 fold less than the effective concentration of substance P, although the population of myenteric neurons which were sensitive to VIP was much smaller than the population sensitive to substance P. The increase in firing rate of myenteric neurons produced by VIP added to the perfusing solution tended to decline with repeated applications of VIP as did the increase in firing of cortical neurons produced by repeated iontophoretic applications.

VIP is an important peptide in the gut, in terms of the large quantities which are contained within the nerve processes and cell somata (Larsson et al., 1976; Fux, Hokfelt, Said and Mutt, 1977). Its action on the firing rate of myenteric neurons may support a possible neurotransmitter or neuromodulator role in the plexus. However, further study with pure VIP is required to demonstrate clearly its possible physiological significance.
4.4.4. The Action of All Three Excitatory Peptides on the Same Unit

The action of all of the excitatory peptides were studied on the same unit in order to determine if different populations of neurons could be distinguished by their sensitivity to one or another of the excitatory peptides (substance P, neurotensin, VIP). The results indicate that there are cells which have different sensitivity to these peptides. There is a large population of cells which are sensitive to more than one peptide. However, there is a significant proportion of cells which are sensitive to only one of the three peptides. The number of cells sensitive to only VIP was very small and this may be due to the use of only low concentrations of VIP. Cells which were sensitive to VIP were also more likely to be sensitive to substance P and/or neurotensin. Units which were sensitive to substance P only, were found most often, followed by those sensitive to only neurotensin. Of the units sensitive to either substance P or neurotensin, 33% were found to be sensitive to both peptides.

The results indicate that there are populations of neurons in the myenteric plexus with varying sensitivity to the excitatory peptides. This variation in sensitivity to the excitatory peptides cannot be as a result of differences in the amount of suction used in the recording electrode or the size of the electrode since all three excitatory peptides were tested on the same cell. The demonstration of different populations of cells which are sensitive to each of the excitatory peptides may be taken to indicate that these peptides have different functional roles in the myenteric plexus. Clear demonstration that these peptides in fact
have functional roles in the myenteric plexus awaits the development of specific antagonists.

Demonstration of different populations of myenteric neurons based on their sensitivity to the excitatory peptides also suggests that the effects of these peptides are specific. If the increase in firing rate was produced by a non-specific mechanism, one might expect that a cell which was excited by substance P would also be excited by a peptide of similar molecular weight such as neurotensin. This type of study is not possible when using iontophoretic application of peptides since the transport number for most of these peptides is not known. In the case of substance P where transport number has been studied, there is marked variation in transport number from one electrode to the next (Krnjević, Henry and Morris, 1974). If the transport number of the other peptides is just as variable, then comparisons of the sensitivity of units to a variety of peptides administered by iontophoresis is impossible to interpret.
4.5. PEPTIDES IN THE MYENTERIC PLEXUS

Iontophoretic application of the neuropeptides onto single neurons in the central nervous system has yielded clear effects on firing rates in cells from various locations which contain the specific peptide under study. Most of the effects produced by the peptides used in the present study on single neurons in the myenteric plexus are the same qualitatively as those which have been found in the central nervous system. The action of endorphins has been studied at several sites in the central nervous system and, in most of these studies, the action of the opioid peptide was determined to be mediated by an opiate receptor because the inhibitions in firing are reversed or prevented by a specific antagonist such as naloxone. With this exception, specific antagonists for the neuropeptides have not been described. As a result, it is difficult to resolve which putative neurotransmitter could be involved in a postsynaptic action in response to presynaptic stimulation, especially when the mechanism of action of two substances is the same. Experiments using cross desensitization is one possible method by which to overcome this problem. Another possible approach is to use agents which deplete specific neurotransmitters (such as p-chlorophenyl alanine in the case of 5-HT, and capsaicin for substance P).

Problems with the specificity of neuropeptide action in the absence of specific antagonists can be eliminated in isolated preparations by using structurally related analogs. Iontophoretic studies with analogs, however, cannot be used quantitatively. At the present time, the
action of neuropeptides has been described in several sites. However, the neurophysiological roles of these peptides in the central and peripheral nervous systems remains to be determined.

The presence of many of the neuropeptides in the gut may seem odd at first. However, demonstration of the presence of these peptides in nerves and also in endocrine-like cells of the gut has supported the theory that endocrine-like cells are the most primitive form of nervous tissue (Pearse, 1978). The endocrine-like cells in the gut share a number of cytochemical and ultrastructural properties with cells which are considered to be neuroendocrine (hypothalamus, pituitary and adrenal medulla) (Pearse, 1968). Pearse (1968) referred to these cells as APUD (amine precursor uptake and decarboxylation) cells based on their ability to take up and decarboxylate amine precursors (5-hydroxytryptophan). Some nerve cells also have this ability. The embryological origin of these cells has been shown, in some cases, to be from neuroectoderm (Pearse and Polak, 1971a, b). Several of the neuropeptides have been found in vesicles contained in APUD cells, neuroendocrine cells and in nerves of the peripheral and central nervous systems. In this way, the neuropeptides appear to be another common link between the endocrine and nervous systems.

The endocrine effects of the hypothalamic peptides such as vasopressin, oxytocin and the releasing factors and possibly somatostatin have been described, as have the actions of many of the gastrointestinal peptide hormones such as gastrin, cholecystokinin, insulin and glucagon. The physiological significance of the peptides contained within the ter-
minals and varicosities of nerves remains to be determined. It is possible that the neuropeptides are merely embryological remnants which have no physiological role in later life. On the other hand, studies of neuropeptides may help to resolve questions concerning the nature of neurotransmitters at some sites (for example, the nature of the nonadrenergic inhibitory transmitter responsible for the relaxation of the smooth muscle in various locations of the gastrointestinal tract). In the presence of atropine, the smooth muscle of the taenia coli relaxes (with a hyperpolarization) in response to nerve stimulation (Burnstock, Campbell, Bennett and Holman, 1963). This hyperpolarization is abolished by tetrodotoxin and is unchanged by guanethidine or sympathetic denervation (Furness, 1969a,b). There is some evidence that the transmitter may be a purine nucleotide. Adenosine triphosphate (ATP) hyperpolarizes the smooth muscle and is contained in nerve terminals of the taenia coli (see review by Burnstock, 1972). The identity of the transmitter, however, has not been determined. Since many of the neuropeptides are also contained in the nerve terminals of the gut, and some neuropeptides are known to have actions directly on the smooth muscle (substance P and neurotensin), it is reasonable to think that a neuropeptide could be the inhibitory neurotransmitter.

Another example where a neuropeptide may act as a neurotransmitter or neuromodulator is in the myenteric plexus. Substance P has been suggested to mediate a slow excitatory synaptic potential seen in some myenteric neurons (Katayama and North, 1978). A depolarization of myenteric neurons associated with a decrease in potassium conductance was found with superfusion of substance P (3-100 nM). Iontophoretic application
also caused a slow depolarization (duration 10-100 s) in some neurons. Stimulation of the nerves (10 Hz for 1 s) also caused a slow depolarization with a similar time course. The depolarization produced by nerve stimulation was abolished in calcium-free, high magnesium (5 mM) Krebs solution such that a synaptic mechanism was indicated, but was not altered by atropine (1 µM) or hexamethonium (100 µM). The slow synaptic potential was also associated with an increase in resistance which probably is due to a decrease in potassium conductance. It is not possible to say with complete certainty, but this evidence is suggestive that substance P is the transmitter at this site. Other possible endogenous substances which may also be involved are VIP and 5-HT. Studies such as these may help to determine the physiological significance of other neuropeptides in the central as well as the peripheral nervous systems.
4.6. PROPOSALS FOR FUTURE WORK

The neuropeptides, met- and leu-enkephalin, β-endorphin, somatostatin, neurotensin, substance P and VIP, had clear effects on the firing rate on neurons of the myenteric plexus.

1. Since the neuropeptides are not distributed equally in various areas of the gastrointestinal tract, studies of their potencies and of the proportion of cells sensitive to the neuropeptides in different areas of the gut may answer questions about their physiological roles. This study may also include extrinsic denervation since some of the peptides (somatostatin, enkephalin and substance P) may originate in cells outside the gut.

2. The action of these peptides on membrane properties and synaptic potentials of myenteric neurons studied with intracellular electrodes would enable the ionic mechanisms of action to be studied in detail. With this method, iontophoretic application of the peptides on to various regions of the cell can be studied and the effects produced by this means of application can be compared to bath applications of known concentrations of the peptides.

3. In order to propose a neurotransmitter or neuromodulator role for any of the neuropeptides in the myenteric plexus, several of the required criteria have yet to be studied. These are outlined in Table 13. Somatostatin, substance P, enkephalin and VIP have been found in nerves of the myenteric plexus. Release of enkephalin from the myenteric plexus by electrical stimulation has been studied with limited success. Release of substance P, somatostatin and VIP from the myenteric
plexus has not been reported. The synthesis of enkephalin has been studied in the myenteric plexus but again, the synthesis of any of the other neuropeptides at this site has not. A mechanism by which the neuropeptides are inactivated or removed from the effector site has not been described for any of the endogenous neuropeptides in the myenteric plexus. Finally, the action of each of the neuropeptides must be mimicked by nerve stimulation. This type of experiment is aided by specific antagonists to compare the antagonism of the effect produced by an exogenously applied agent with the antagonism of the effect produced by nerve stimulation. Unfortunately, the only available antagonists to any of the neuropeptides in the myenteric plexus are those which act on the opiate receptor to block the action of the opioid peptides. Identification of possible neurotransmitters is aided by cross-desensitization experiments between exogenously applied agents and the nerve mediated response. Drugs which deplete specific neurotransmitters from the nerve terminals are also useful. This type of experiment can be done using extracellular techniques. However, interpretation of the results can be difficult. Intracellular recording offers an additional method by which to identify neurotransmitter substances. Using intracellular recordings, the ionic mechanism of action of an exogenously applied agent can be compared to the action and mechanism which results from nerve stimulation. Even in the absence of a specific antagonist, the evidence provided by intracellular recordings may strongly support or refute the neurotransmitter role of the neuropeptides in the myenteric plexus.
Table 13. The present state of criteria which must be satisfied to prove a neurotransmitter role for some neuropeptides in the myenteric plexus.

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<td>I hyperpolarization</td>
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<td>?-endorphin</td>
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<td>ns</td>
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<td>ns</td>
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<td>E increase in firing rate</td>
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<tr>
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<tr>
<td>Somatostatin</td>
<td>yes</td>
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<td>E inhibition of firing</td>
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abbreviations: yes - shown to be present
no - not present
? - studied but still not clear
ns - not studied
E - extracellular recording
I - intracellular recording
SUMMARY

Extracellular recordings from single myenteric neurons were made with large suction electrodes. The origin and nature of the spontaneous neuronal activity was thought to be somatic and induced by irritation of the cell by the large electrode. This conclusion is based on the following observations. First, spontaneous activity was only recorded from the ganglia and not from the interconnecting strands where there are few cell bodies. Second, no spontaneous activity was recorded with small suction electrodes or large electrodes without suction. Third, cells which exhibited a decreasing firing rate with increasing spike amplitude could be induced to fire by increasing the suction or adding excitatory agents to the tissue bath. Spontaneously firing cells were routinely recorded for 1-2 hr and sometimes for periods up to 6 hr. Previous workers had shown that morphine inhibited the firing rate in a stereospecific naloxine reversible manner, indicating this preparation as a useful model to study pharmacological agents.

A number of neuropeptides have been found in nerves of the myenteric plexus, including enkephalin, somatostatin, substance P and VIP. The action of these peptides and neurotensin was tested on the firing rate of myenteric neurons. These peptides were tested in known concentrations, in solutions which completely blocked synaptic transmission, and in solutions containing various specific and non-specific pharmacological antagonists. The endorphins (met- and leu-enkephalin and β-endorphin) reversibly decreased the firing rate by an opiate receptor
mechanism, at concentrations similar to those which inhibit the nerve-mediated contraction of the longitudinal muscle (3-300 nM). The time course of the inhibition was immediate with the entry of the peptide into the bath and washed out quickly with removal of the peptide from the bath. In the same concentration range, somatostatin decreased the firing of myenteric neurons. The inhibition was of the same time course as enkephalin, but in some units there were signs of tachyphylaxis. The inhibition produced by somatostatin was not affected by opiate or adrenergic antagonists.

Substance P and neurotensin produced marked increases in firing in myenteric neurons. The concentration range which produced an excitatory effect (100 pM - 100 nM) was in the same concentrations range as that required to contract the guinea pig ileum. There were some signs of desensitization to substance P with repeated applications whereas the excitations produced by neurotensin were less likely to exhibit desensitization. The excitations produced by substance P and neurotensin were thought to be relatively specific based on two studies. First, two substance P analogs (substance P pentapeptide and substance P free acid) were both markedly less active than substance P on myenteric neurons. The biological activity of these analogs was also less than that of substance P when tested on smooth muscle and spinal cord preparations. Second, substance P and neurotensin affected separate populations of myenteric neurons. That is to say, there were cells that were exquisitely sensitive to substance P and relatively insensitive to neurotensin and vice versa. VIP also produced an increase in firing of myenteric neurons but at concentrations 100-1,000 fold less than the effective concentrations of sub-
stance P and neurotensin. Repeated applications of VIP often resulted in diminution of the response, implying some tachyphylaxis. The population of neurons which is sensitive to VIP is also sensitive to substance P and/or neurotensin: few cells were excited by VIP and not by substance P or neurotensin.


APPENDIX A
APPENDIX A

Determination of spike amplitude recorded with large suction electrodes on theoretical grounds.

(1) Recording from the cell soma. The following assumptions are made:

(a) The cell diameter is 50 µm.
(b) A 50 µm electrode is placed on the cell such that it completely covers the cell.
(c) A perfect resistive seal is formed around the outside of the electrode.
(d) The action potential of the cell is 70 mV with a rise time of 1 ms.
(e) Capacitive current only is measured.
(f) Membrane capacitance = 1 µF/cm².

The surface area for capacitive current.
\[ A = \pi r^2 = 19.4 \times 10^{-6} \text{ cm}^2 \]

Total capacitance.
\[ C = \text{membrane capacitance} \times \text{surface area} \]
\[ C = 1 \ \mu\text{F/cm}^2 \times 19.4 \times 10^{-6} \text{ cm}^2 = 19.4 \times 10^{-6} \ \mu\text{F} \]

Total charge.
\[ Q = C \times V = 19.4 \times 10^{-12} (\text{F}) \times 7 \times 10^{-2} (\text{V}) \]
\[ Q = 1.36 \times 10^{-12} \text{ coulombs} \]

Current generated during rising phase of action potential.
\[ I = \frac{Q}{dt} = 1.4 \times 10^{-12} (\text{C})/10^{-3} = 1.4 \times 10^{-9} \text{ A} \]

Potential drop across an electrode (resistance 1 MΩ).
\[ V = I \times R = 1.4 \times 10^{-9} \times 1 \times 10^6 = 1.4 \text{ mV} \]

Under the given conditions, the potential measured during an action potential would be 1.4 mV. The conditions in practice are much different. There is not a perfect resistive seal around the electrode: in fact, it is only 200-500 kΩ. This resistance is produced by the suction and appears to be important because no activity (spontaneous or evoked) can be recorded without suction. Increasing the amount of suction increases the sealing resistance and also increases the size of the spike recorded. Considering that the resistive seal around the electrode is not perfect, it seems reasonable to assume that the spike amplitude measured in practice must be smaller than the predicted value of 1.4 mV.
(2) Recording from a process. The following assumptions are made:

(a) Cell process is 1 μm in diameter and 50 μm long.
(b) The cell process spikes all along its length at the same time.
(c) The spike is 70 mV and has a rise time of 1 ms.
(d) A perfect resistive seal is made around the electrode.
(e) Capacitive current only is measured.
(f) Membrane capacitance = 1 μF/cm².

The surface area for capacitive current.

\[ A = \pi r L = 3.1 \times 0.5 \times 10^{-4} \times 50 \times 10^{-4} = 77.5 \times 10^{-8} \text{ cm}^2 \]

Total capacitance.

\[ C = 1 \text{ μF/cm}^2 \times 77.5 \times 10^{-8} = 7.75 \times 10^{-13} \text{ F} \]

Total charge.

\[ Q = C \times V = 7.75 \times 10^{-13} \times 7 \times 10^{-2} = 5.4 \times 10^{-14} \text{ C} \]

Current generated during rising phase of action potential.

\[ I = \frac{Q}{dt} = \frac{5.4 \times 10^{-14}}{10^{-3}} = 5.4 \times 10^{-11} \text{ A} \]

Potential drop across an electrode (resistance 1 MΩ).

\[ V = I \times R = 5.4 \times 10^{-11} \times 1 \times 10^6 = 5.4 \times 10^{-5} \text{ V} \]

Under the assumptions listed above, the amplitude of the spike measured during an action potential in a process would be 54 μV. Since the resistive seal around the electrode is not perfect (see appendix A-1), a smaller potential is expected. In addition, the entire length of nerve (50 μm) is not depolarized simultaneously, such that the estimated current is actually smaller than that predicted. Since the amplitude of spontaneous potentials ranged from 50 μV (just over the noise level) to 500 μV, it is unlikely that these potentials originated from single cell processes but probably correspond to the action potential in somata.
The dissertation submitted by John T. Williams has been read and approved by the following committee:

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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.

20 April 1979

Date

Director's Signature