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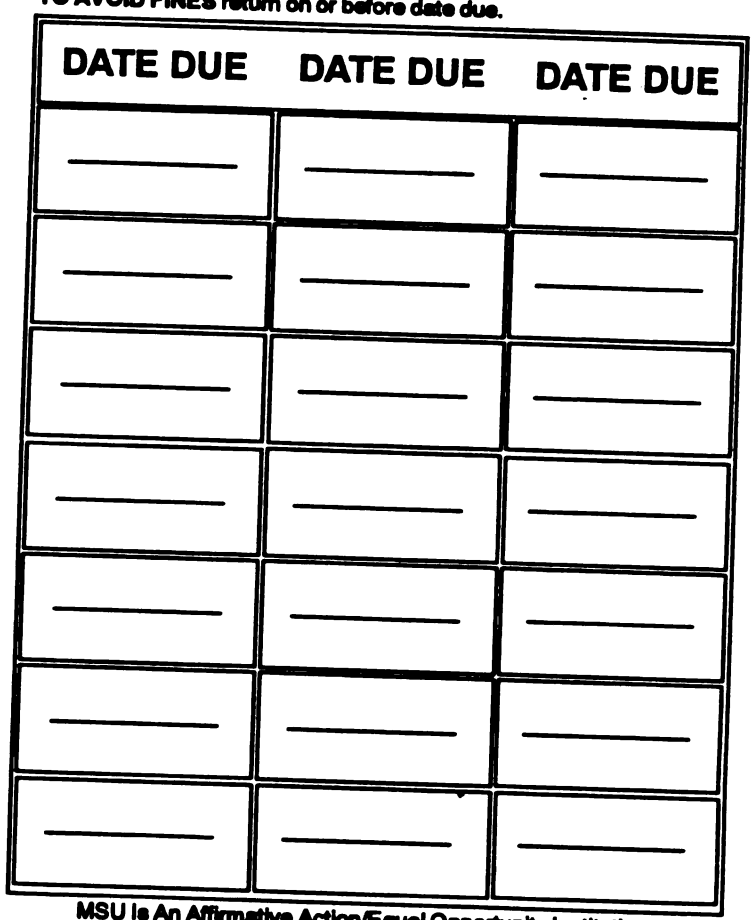

IONIC AND BIOCHEMICAL MECHANISMS OF SLOW SYNAPTIC TRANSMISSION IN GUINEA PIG MYENTERIC NEURONS

By

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ABSTRACT

IONIC AND BIOCHEMICAL MECHANISMS OF SLOW SYNAPTIC TRANSMISSION IN GUINEA PIG MYENTERIC NEURONS

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The goal of this dissertation is to understand the cellular mechanisms of slow synaptic excitation in the enteric nervous system. The questions asked were: what ionic mechanisms generate slow synaptic responses and how do they coupled to intracellular transduction pathways? Single guinea pig myenteric neurons in an *in vitro* preparation with synaptic connections intact were recorded from using conventional electrophysiological techniques.

Previous studies using potential measurements established that slow synaptic responses were associated with a decrease in potassium conductance (G_K). In the present study, the current/voltage relationship for slow synaptic responses was measured with current measurements. By using a mathematical model of current production versus conductance change and pharmacological analysis I was established that some slow synaptic responses were due to a decrease in G_K and simultaneous activation of a conductance increase. Ion substitution and channel blocker experiments established that this was a chloride conductance. Further analysis of conductance changes associated with slow synaptic responses revealed that they consist of a fast decrease in G_K and a slower increase in G_{Cl} (90% and 10% of the total conductance change respectively).

The long latency and time course of slow synaptic responses suggest they are dependent on intracellular signalling pathways. I have established that this pathway is dependent on a G-protein which is pertussis toxin insensitive and is irreversible activated by GTP-γ-S. Activation of adenylate cyclase by forskolin mimicked the response and D609, an inhibitor of phospholipase C, reduced the response indicating both these pathways are activated. Phorbol 12,13 dibutyrate (PDBu) alone mimicked the slow synaptic response and together with forskolin caused a more than additive response indicating that protein kinases are activated. In addition, non-specific protein kinase inhibitors blocked, while a non-specific protein phosphatase inhibitor mimicked the slow synaptic response.

In conclusion the slow excitatory synaptic response is due to a concurrent decrease in G_K and increase in G_{Cl} in some myenteric neurons. These conductances are modulated by G-protein coupled second messenger systems. Forskolin and PDBu sensitive pathways cause inactivation of G_K. The transduction of the G-protein coupled G_{Cl} is unknown.

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I thank my family for their support (both financial and emotional) throughout my long college career. I hope that my mother (Beverly Budzynski) finds happiness in her new marriage and that my father and his partner (John Bertrand & Linda Cavis) continue to pursue their dreams in Hawaii.

Finally, I would like to thank Lisa Marie Decheim whom I love dearly.

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INTRODUCTION

THE ENTERIC NERVOUS SYSTEM

The enteric nervous system (ENS) is located within the wall of the gut. It is composed of two interconnected ganglionated plexuses extending from the mid esophagus to the internal anal sphincter. In 1921 Langley published a comprehensive review of the nomenclature of the newly defined autonomic nervous system in which he classified the ENS as a distinct division. Langley cited three reasons for separating the ENS from the sympathetic and parasympathetic divisions, some of which have been expanded upon by modern researchers.

First, enteric neurons did not appear histologically like other autonomic neurons. While an individual enteric neuron appeared morphologically similar to a neuron in another autonomic ganglia, the diversity of neuronal sizes and types and their profuse projections within enteric ganglia were far more complex. For instance, principal ganglion cells in the sympathetic prevertebral ganglia seldom have more than one process, and seldom ramify within the ganglia before forming a bundle with like fibers and exiting (Szurszewski & King, 1989). In contrast, many enteric neurons have more than one process. Those that have only one process are seen to ramify within the ganglia and to wander in and out of adjacent ganglia without forming fiber bundles (Furness & Costa, 1987). More recently, ultrastructural analysis has extended these observations to the ganglia in which these neurons are situated.

It has been demonstrated that enteric ganglia lack the blood vessels, fibroblasts and collagen fibrils that are present in sympathetic and parasympathetic ganglia (Cook and Burnstock, 1976). The close packing of enteric neurons and enteric glia is similar to the arrangement of cells within the CNS. In addition, the morphological complexity of enteric neurons and their processes is much greater than that observed in autonomic ganglia. Finally, the vasculature supplying enteric ganglia is similar to central vasculature in that the capillaries are non-fenestrated and have tight junctions while other autonomic ganglia typically do not (Gershon and Bursztajn, 1978).

Second, nerve fibers from the CNS were observed to connect with enteric ganglia in a different manner than with other autonomic ganglia. There is a small number of efferent fibers leading from the CNS to the ENS as compared to the large number of neurons in the ENS (Irwin, 1931; Hoffman & Schnitzlein, 1969). The ENS contains between 10^6 to 10^8 neurons, this is similar to the number of neurons estimated to reside within the spinal cord (Irwin, 1931; Furness & Costa, 1980). The ratio of the number of efferent nerve fibers in the vagus nerve to the number of enteric cells is less than 1 : 300 in guinea pig, and approximately 1 : 50,000 in humans. Such a large divergence between preganglionic efferents and postganglionic nerves has not been observed in other autonomic ganglia. Typically, sympathetic and parasympathetic ganglia have innervation ratios that range from 1 : 32 (most divergent) in rabbit superior cervical ganglia to 40 : 1 (most convergent) in rat inferior mesenteric ganglia (Furness & Costa, 1987). While a single extrinsic fiber can theoretically innervate many cells, it has been shown that varicose fibers from the dorsal motor nucleus of the vagus do not form close contacts in over 85% of myenteric ganglia of the ileum

(Kirchgessner & Gershon, 1989). Thus, the ENS retains a high degree of anatomical independence from the CNS.

Third, sympathetic and parasympathetic ganglia operate as relays in reflex arcs mediated by the CNS, by simply repeating the input of the CNS to post-ganglionic neurons. However, the ENS contains reflex pathways which did not involve the CNS. This observation was based on the work of Bayliss and Starling (1899) who found that removal of the sympathetic and parasympathetic innervation of the intestine did not disrupt complex motor behavior such as the peristaltic reflex. They concluded that there was a "law of the intestine". They go on to say, "This law is as follows: local stimulation of the gut produces excitation above and inhibition below the excited spot. These effects are dependent on the activity of the local nervous mechanisms."(Bayliss & Starling, 1899). This was good evidence that the ENS contained the components of a neuronal reflex arc, that is: sensory neurons (which initiate the reflex), interneurons (which spread the reflex) and excitatory and inhibitory motorneurons (which acts on the effector organ)(see - Enteric reflexes and the significance of the slow synaptic response). Thus, the control of the gut by the ENS can be functionally independent of the CNS.

General arrangement of the plexuses

Subsequent investigations have revealed that the ENS is composed of two major plexuses: the myenteric plexus and the submucosal plexus. Original descriptions of the myenteric plexus have been attributed to Auerbach (1862, 1864) while the original descriptions of the submucosal plexus have been attributed to Meissner (1857). For many years these plexuses have bore their names, however ambiguity as to there meaning (introduced by later

investigators) has prompted me to follow the lead set forth in Furness & Costa (1987) in referring to these plexuses simply as the myenteric and the submucosal plexuses. These plexuses are innervated by pre-ganglionic parasympathetic fibers *via* the vagus and pelvic nerves and post-ganglionic sympathetic fibers from the celiac, superior mesenteric and inferior mesenteric ganglion and by primary sensory afferents from the dorsal root ganglia. The ENS sends projections to sympathetic, parasympathetic and pancreatic ganglia in return (Szurszewski & King, 1989; Grundy & Scratcherd, 1989). Thus the ENS does not simply receive information from the CNS but returns neuronal input to it as well.

The submucosal plexus lies between the mucosa and the circular muscle and is responsible for the absorptive and secretive processes of the gut. The myenteric plexus lies between the circular muscle and the longitudinal muscle and is responsible for the control of gastrointestinal motility (Furness & Costa, 1987). Each plexus contains roughly the same number of neurons, however there are a greater number of randomly orientated submucosal ganglia, each containing approximately 8 neurons and a lesser number of linearly orientated myenteric ganglia each containing approximately 100 neurons (Furness, Bornstein, Pompolo, Young & Kunze, 1994).

Morphology of enteric neurons

Dogiel (1899) was the first to give a complete and accurate description of the morphology of enteric neurons, and as a result, this classification now bears his name. He described three types of neuron based on somal size, dendritic structure and axonal projections. Dogiel type I cells are unipolar and have short, tufted dendrites while Dogiel type III cells are unipolar and

have filamentous dendrites. These cells send single axonal projections to the circular or longitudinal muscle or to adjoining ganglia. Based upon this anatomy, Dogiel suggested that type I and type III cells are motor neurons (see - Classification of myenteric neurons). In contrast, Dogiel type II cells are multipolar and have smooth cell bodies with a prominent axon hillock region. These cells send multiple axonal projections to the mucosa and adjoining ganglia. Based upon this anatomy Dogiel suggested that type II cells are sensory neurons (see - Classification of myenteric neurons). All three type are present in the myenteric plexus, but only the Dogiel type II neuron is present in the submucosal plexus.

Ultrastructure

Ultrastructural studies utilizing electron microscopy have identified eight neuronal subtypes based upon the apparent size of the cell and on its internal structure (Cook and Burnstock, 1976). The important structural features were the presence or absence of rough endoplasmic reticulum (which gave the cells an electron dense appearance) and of mitochondria (which stained either pale or electron dense). All cells with in the myenteric and submucosal plexuses appear to receive synaptic input, as vesiculated nerve profiles were identified in close (within 25 μm) contact with cell bodies (either smooth or spiny), dendrites and processes (Furness & Costa, 1987). These synapses have moderate to pronounced pre- and post-synaptic membrane specializations. The appearance of these vesicles suggest in some cases the neurotransmitter present. For example, many terminals contained small dense core vesicles which are usually associated with norandrenergic nerves (Furness & Costa, 1987). Many terminals also contained large granular vesicles which may contain peptides. These vesicles

were found with many other types of vesicle (like small clear vesicles and flattened vesicles) suggesting many terminals utilize more than one transmitter (Komura, *et al.*, 1982).

Electrophysiology of myenteric neurons

Myenteric neurons may be placed into two general electrophysiology categories based upon the work and nomenclature of Hirst, Holman & Spence (1974) and work of Nishi & North (1973). The nomenclature of Nishi & North (1973) has been abandon because of its similarity to the Dogiel classification of cell morphology. The first type is the S neuron which is characterized by its response to presynaptic nerve stimulation. Single electrical stimuli evoke a fast excitatory post-synaptic potential (fEPSP) which can be recorded with an intracellular microelectrode and can be mimicked by application of ACh and blocked by the nicotinic antagonist hexamethonium (see below). Repetitive, high frequency stimulation evokes a train of fEPSPs of decreasing amplitude. This may be due to auto- or pre-synaptic inhibition as post-synaptic responses to ACh are not affected. This stimulus train also evokes a slow synaptic response. Intracellular recordings from other autonomic ganglia reveal similar synaptic potentials (North, 1993). S neurons express conductances such as the M-current or the hyperpolarization activated cation current (I_H) (Tokimasa & Akasu, 1993). Thus, the current/voltage (I/V) relationships for these neurons is linear in the range of physiological potentials and the membrane potential is determined by a background or leak potassium conductance (G_K). S neurons respond to increasing amplitudes of depolarizing current by firing action potential at increasing frequencies (up to approximately 200 Hz)(Furness, 1994).

The action potential in S neurons is followed an afterhyperpolarizations of less than 1 s duration. Another characteristic of S neurons is their insensitivity to γ-aminobutyric acid (Cherubini & North, 1979).

The second type is the AH neuron which is characterized by a long lasting spike afterhyperpolarization (AH) of greater than 1 s duration which serves to limit the firing frequency of the AH neuron. AH neurons respond only weakly, or not at all to single stimuli of presynaptic nerves. This observation led to the conclusion that AH neurons do not receive fast synaptic transmission (Nishi & North, 1973; Hirst, *et al.*, 1974). High frequency stimulation of presynaptic nerves evokes a slow excitatory synaptic response (see - synaptic transmission). AH neurons respond to depolarizing and hyperpolarizing current pulses with a characteristic sag in membrane voltage toward the resting voltage (Wood, 1989). This sag is due to several membrane clamping conductances which are present in AH neurons (Tokimasa & Akasu, 1993). The first membrane clamping conductance is a calcium-activated G_K ($G_{K,Ca}$) which contributes to the resting membrane potential and also gives rise to the long lasting spike AH (Morita, North & Tokimasa, 1982; North & Tokimasa, 1987; Galligan, Tokimasa & North, 1987). The AH is due to activation of $G_{K,Ca}$ by calcium entering the neuron during a calcium spike (Hirst, Johnson & Helden, 1985a, Hirst, Johnson & Helden, 1985b). $G_{K,Ca}$ becomes more or less active when the membrane is either depolarized or hyperpolarized respectively (Hirst, Johnson & Helden, 1985b). The basis of this response is not the voltage dependence of the potassium channel, but the voltage dependence of a leak calcium conductance (Hirst, Johnson & Helden, 1985a). This calcium conductance becomes active at depolarized potentials and inactivated at hyperpolarizing potentials. The second membrane clamping conductance is I_H, this cation conductance activates as the membrane is

hyperpolarized (Galligan, Tatsumi, Shen, Surprenant & North, 1990). As a consequence, the I/V relationship for AH neurons has a characteristic region of low conductance at rest with large increases in conductance more negative and more positive of rest (Tokimasa & Akasu, 1993). The final property of AH neurons is the large depolarization caused by GABA when applied by various methods (Cherubini & North, 1979).

Enteric neurotransmitters

Twenty or more putative neurotransmitters have been described within the gut and include most known neurotransmitters. However, only more classical transmitter molecules meet established criteria: 1) the transmitter must be present in neurons; 2) the transmitter must be released by nerve stimulation; 3) the exogenous application of transmitter must mimic nerve stimulation. A variety of methods have been used to establish that acetylcholine (ACh), norepinephrine, serotonin (5-HT) and the neuropeptide substance P (SP) meet most of these criteria (Furness & Costa, 1987; Wood & Mayer, 1978; Katayama, North & Williams, 1979a).

SP and 5-HT are both putative mediators of the slow synaptic response in the myenteric plexus (see - mediators of the slow synaptic response). A major part of the argument for either substance relies on the evidence that they act as neurotransmitters in the gut. The evidence for SP is as follows. First, SP is present in myenteric neurons as antibodies raised against SP bind with high affinity and reveal a sub-population of neurons and nerve fibers in the myenteric plexus (Bornstein, North, Costa & Furness, 1984). Also, authentic SP has been demonstrated in extracts of myenteric plexus using high performance liquid chromatography (Murphy, Furness, Beardsley & Costa, 1982). Second, SP can be released

from the enteric nerves by distension of the lumen of the gut or by electrical or pharmacological stimulation (Holzer, 1984). Third, SP (or neurokinin-3) receptors are present on myenteric neurons and exogenous SP binds to these receptors with high affinity (Drapeau, Rouissi, Nantel, Rhaleb, Tousignant & Regoli, 1990). The evidence for 5-HT is similar. First, 5-HT, as well as it synthesizing enzymes and uptake molecules have been identified by aldehyde-induced fluorescence (in the presence of monoamine oxidase inhibitors), immunohistochemistry and biochemistry (Gershon, 1981; Furness & Costa, 1987). Second, radiolabelled 5-HT can be released from enteric neurons in a tetrodotoxin-sensitive manner. Third, electrophysiological and pharmacological evidence supports the existence of many classes of 5-HT specific receptors that, when activated, have effects on the electrical properties of enteric neurons.

Neurochemical coding of enteric neurons

Past histochemical techniques have proven inadequate to localize the many neurochemicals within enteric neurons which frequently contain two or more transmitters (Furness & Costa, 1987). Immunohistochemical techniques allow neurons to be simultaneously labeled for several specific chemical markers at the same time. These markers may be the transmitter, like SP, or it may be an enzyme unique to the biosynthesis of the transmitter, like the ACh synthesizing enzyme choline acetyltransferase (ChAT). The occurrence of two or more markers within individual cells has been correlated with functionally and anatomically distinct sub-groups of enteric neurons. This has lead to the idea that enteric neurons can be identified based upon their chemical coding.

In the myenteric plexus there are six or more neurochemically distinct neurons, each containing many neurochemical markers. Several non-transmitter related neuronal markers have been used to simplify this categorization. In the guinea pig ileum, calretinin, a 29 Kd calcium-binding protein has been localized to neurons which conform to Dogiel type I/III morphology and S neuron electrophysiology (Brookes, Steele & Costa, 1991)(see - below). Calbindin, a closely related 28 Kd calcium-binding protein, has been localized to neurons which conform to Dogiel type II morphology and AH neuron electrophysiology (Iyer, Bornstein, Costa, Furness, Takahashi & Iwananga, 1988; Furness *et al.*, 1990) and Dogiel type II neurons which project to the mucosa (Song, Brookes & Costa, 1991)(see - below). These calcium binding proteins do not account for 100% of the neuronal sub-type they are specific for, but are not found in any other sub-types.

Classification of myenteric neurons

Neither an exhaustive nor a complete classification of myenteric neurons can be given by considering only one body of evidence. For example, the electrophysiological classifications listed above place all myenteric neurons in to only two categories when other techniques have clearly demonstrated many different cell types (see - Ultrastructure). The key to understanding enteric circuitry lies in the correlation of different classification schemes. Seemingly a poor example of this methodology is the observation that Dogiel type I/III morphology and S neuron electrophysiology and Dogiel type II morphology and AH neuron electrophysiology are closely related (Furness & Costa, 1987). However, these data can now be applied to other classification schemes that may or may not be compatible with the previous methods. For instance, immunohistochemical techniques can be easily combined

with morphological determinations, but is more difficult to combine with electrophysiology (which has only recently become feasible; Bornstein, Furness, Smith & Trussel, 1991). It is now possible to correlate the neurochemical code of neurons with their morphology, ultrastructure, electrophysiology, pharmacology and in some case their position in enteric reflexes (Furness, *et al.*, 1994; Pompolo & Furness, 1988; Smith, Bornstein & Furness, 1992). These data have recently been incorporated into a computer simulation of the peristaltic reflex (Furness *et al.*, 1994). The work to construct this simulation will likely highlight the areas of information which are currently lacking.

SYNAPTIC TRANSMISSION

The fEPSP

The fEPSP is the primary form of synaptic transmission between enteric neurons. It has been linked to reflex activation of motor neurons and interneurons (Hirst, Holman & Spence, 1975; Smith, Bornstein & Furness, 1992). The fEPSP is mediated primarily by ACh acting at hexamethonium sensitive nicotinic receptors, but in some cases have been found to be mediated by ATP (Galligan & Bertrand, 1994). Single electrical stimuli applied to presynaptic nerve fibres causes a fEPSP that is due to an increase in non-specific cation conductance (Wood, 1989). A fEPSP typically last 20 to 40 ms and may be due to the activation of many individual nerve fibers.

The slow synaptic response

The slow synaptic response can be recorded from many peripheral ganglia as well as in neurons of the CNS. One of the first clear descriptions of the slow synaptic response was in bullfrog sympathetic ganglion cells where inactivation of potassium conductance was shown to be responsible for the observed depolarization elicited by electrical stimulation (Weight & Votava, 1970). In peripheral ganglia, there is a positive correlation between the number of preganglionic inputs a cell receives and the occurrence of slow synaptic responses. In the ENS, neurons receive only a small number of extrinsic inputs, but a large number of intrinsic inputs (Furness & Costa, 1987). Not surprising, 80% or more of enteric neurons respond with a slow synaptic response when interganglionic fiber tracts are stimulated (Bornstein *et al.*, 1984).

Enteric slow synaptic responses have been studied since 1978 when Wood and Mayer first applied trains of stimuli to interganglionic fiber tracts. Previously, extracellular recordings had described neurons that underwent aperiodic burst of action potentials. These characteristics are similar to the burst of action potentials seen during a slow synaptic response (Wood, 1989). Subsequent studies have established that slow synaptic responses are synaptically mediated as they can be elicited at a distance from the cell body, and they are inhibited by calcium channel blockade (Hodgkiss, 1981; Wood and Mayer, 1980b). The slow synaptic response can be reliably evoked by high frequency stimulation of interganglionic fiber tracts. The long-lasting membrane depolarization evoked is usually associated with an increase in membrane input resistance (Johnson, Katayama & North, 1980a; Grafe, Mayer & Wood, 1980). The increase in input resistance is caused by transmitter-induced inhibition

of resting G_K in S neurons and G_K and $G_{K,Ca}$ in AH neurons (Morita, North & Tokimasa, 1982; North & Tokimasa, 1987). During the slow synaptic response the spike AH in AH neurons is inhibited (Grafe, *et al.*, 1980). While most slow synaptic responses are due to inhibition of resting G_K, some slow synaptic responses in enteric neurons are associated with a decrease in input resistance or no change in input resistance. In neurons in which there is a decrease in input resistance the reversal potential for the slow synaptic response is near -10 mV (Mawe, 1990). In neurons in which there is no apparent change in input resistance an estimate of the reversal potential was not possible (Shen & Surprenant, 1993).

The mediator of the slow synaptic response

Many putative neurotransmitters including SP, 5-HT, vasoactive intestinal polypeptide, gastrin releasing peptide, and calcitonin gene-related peptide mimic the slow synaptic response when applied to enteric neurons (Katayama, *et al.*, 1979b; Wood & Mayer, 1978; Palmer, Wood & Zafirov, 1987; Zafirov, Palmer, Nemeth & Wood, 1985). There is strong evidence for SP as the mediator of many slow synaptic responses in myenteric neurons while there is also evidence to suggest that 5-HT mediates a smaller sub-population of slow synaptic responses. Exogenous SP, when applied for only a short time, mimics the latency, time course and conductance changes seen during the slow synaptic response (Johnson, *et al.*, 1981). SP containing nerve fibers are abundant within the myenteric plexus, forming baskets around neuronal cell bodies (Bornstein, *et al.*, 1984). Neuronal substance P receptor antagonists are not available. These antagonists are needed to demonstrate definitively that SP is a mediator of some slow synaptic responses (Surprenant, North & Katayama, 1987).

Senktide (succinyl-[Asp^6, N-Me-Phe^8]-substance P, 6-11) is a selective neurokinin-3 (NK-3) receptor agonist (Hanani, Chorev, Gilon & Selinger, 1988). The NK-3 receptor is believed to be the neuronal tachykinin receptor (Guard, Watson, Maggio, Phon Too & Watling, 1990). Both SP and senktide mimic the slow synaptic response. NK-3 mediated responses are associated with a membrane depolarization, an increase in resistance, an inhibition of resting G_K and inhibition of spike-activated $G_{K,Ca}$ (Katayama & North, 1979a; Hanani *et al.*, 1988; Morita & Katayama, 1992). However, other studies have shown either no resistance change or resistance decreases during depolarizations induced by tachykinin peptides (Galligan *et al.*, 1987). This phenomenon has been ascribed to membrane rectification during uncontrolled depolarizations (Hanani and Burnstock, 1985; Katayama *et al.*, 1979b) or actions of transmitter at sites electrically distant from the recording electrode (Surprenant, 1984). However, recently it has been shown that in submucosal ganglia, SP, muscarine and 5-HT caused a simultaneous increase in a cation conductance and a decrease G_K (Shen & Surprenant, 1993). It was suggested that two conductance changes could mediate the slow synaptic response in some submucosal neurons (Shen & Surprenant, 1993).

The evidence for (and against) 5-HT as a mediator of some slow synaptic responses is similar. Key experiments have shown that 5-HT, when applied exogenously, does not mimic some slow synaptic responses (Johnson, *et al.*, 1981). In addition, selective destruction of 5-HT containing fibers by surgical means does not significantly effect the number of slow synaptic responses found in enteric neurons (Bornstein, *et al.*, 1984). In contrast, non-selective 5-HT antagonists such as methysergide, or the more selective but poorly characterized dipeptide antagonists are effective in blocking the slow synaptic response (Johnson, *et al.*, 1981;Takaki, Branchek, Tamir & Gershon, 1985). Current thinking suggest

that some discrepancy in data gathered by different groups is due to an anatomical difference in SP containing versus 5-HT containing fibers. While SP containing fibers are short, circumferentially orientated (seldom leaving a ganglion) 5-HT containing fibers are long, and only run in the aboral direction. The stimulation parameters (ie. distance and strength) are likely to influence the type of slow synaptic response observed. In this study, only short, circumferentially-orientated stimuli have been used to avoid activation of 5-HT containing fibers.

The signal transduction of the slow synaptic response

The length of the slow synaptic response is on average 1 to 2 thousand times longer than ligand-gated synaptic potentials such as the fEPSP. This alone is suggestive that the transduction of the slow synaptic response is different. ACh acting at muscarinic receptors mimics the slow synaptic response. Experiments by North & Tokimasa (1984) have shown that receptor occupancy is only required for the initial stages of the recorded slow potential. These data were produced by ionophoretically applying ACh followed by immediate pressure application of the higher affinity antagonist hyoscine. Thus, unlike the fEPSP, agonist binding which trigger the slow synaptic response can be separated from the intracellular events which generate the slow synaptic response.

Signal transduction. Receptor:G-protein interactions and diffusible second messengers are a common means of ion channel regulation (Levitan, 1994). In *Aplysia* a firm relationship exists between 5-HT release from sensory nerves leading to activation of 5-HT activation of

second messenger pathways (both protein kinase A (PKA) and protein kinase C (PKC)) in motor nerves which leads to a slow depolarization (Camardo, Shuster, Siegelbaum & Kandell, 1984; Shuster, Camardo, Siegelbaum, & Kandell, 1985). This depolarization has been shown to be due to phosphorylation of a site that is either the channel itself, or a closely associated protein. These channels also display intrinsic phosphatase activity (Camardo, et al., 1985). The slow synaptic response in enteric neurons has similar characteristics suggesting they may also have a similar underlying biochemistry.

G-proteins. The first step in many signal transduction pathways is the activation of a heterotrimeric G-protein (Birnbaumer, 1990). The G-protein is composed of three subunits: the α subunit which binds guanine nucleotides and the closely associated β/γ subunits which seem to serve as a membrane anchor. These G-proteins couple to the superfamily of receptors with 7 membrane spanning regions and to various intracellular enzymes or ion channels (Boyd, MacDonald, Kage, Luber-Narod & Leeman, 1991; Clapham, 1994). G-proteins cycle between an inactive, intact state, and an active, dissociated state. When an activated receptor binds to a G-protein, the G-protein dissociates into α and β/γ subunits. These subunits no longer possess a high affinity binding site for the receptor which now dissociates. The basis of G-protein activity toward down stream elements of the transduction pathway is the exchange of guanine nucleotides on the α subunit. GDP binds preferentially to the receptor dissociated form of the G-protein, while GTP binds to the activated, receptor associated form of the G-protein. GTP binding to the α subunit reduces the α subunits affinity for the β/γ subunits. The active, dissociated α subunit is limited in its effects by the

rate at which it hydrolyses the bound GTP to GDP. Once hydrolysis has occurred, the α subunit moves to rejoin the β/γ subunits and another cycle is started (Birnbaumer, 1990).

Production of diffusible second messengers. While active, the α subunit associates with a number of membrane bound enzymes or channels. Different kinds of G-proteins bind preferentially to different G-proteins. The best characterized G-protein is G_S (stimulatory) which activates adenylate cyclase in many systems. Other G-proteins include G_I (inhibitory) reduces adenylate cyclase activity and may couples directly to ion channels and G_O (other) and G_Q both which couple to phospholipase C (Exton, 1994). Several other G-proteins are specific for the visual system (ie. rhodopsin) or the olfactory system (G_{OLF}) or are poorly characterized at this time (G_Z, G_{12}, G_{13})(Offermannes & Schultz, 1994). These G-proteins can be placed into two categories based upon their sensitivity to the inhibitory bacterial toxin pertussis toxin (PTX). G_S and G_Q are not inhibited by PTX while G_I and G_O are. Activation of adenylate cyclase by G_S leads to the production of cAMP from ATP. cAMP has been shown to gate ion channels and alter gene expression, however, its primary action is the activation of cAMP dependent protein kinase (PKA)(Taylor, *et al.*, 1988). Activation of the ß iso form of PLC by G_O or G_Q leads to production of DAG and a number of other potential messenger substances (Exton, 1994). The identity of these substances depends on the substrate specificity of the PLC. The two most common substrates are phosphatidylinositol (PI) and phosphatidylcholine (PC). Cleavage of either of these lipids by PLC yield DAG, however cleavage of PI also releases the messenger substance IP_3. Cleavage of PC releases phosphocholine, which does not appear to serve as a messenger (Exton, 1994).

Evidence of signal transduction. Several specific lines of evidence suggest that the slow synaptic response is a second messenger mediated event. First, the neurokinin receptors are members of the superfamily of 7 membrane spanning domain receptors of which rhodopsin is the prototype (Gerard, Bao, Xiao-Ping & Gerard, 1993). This type of receptor is know to couple to G-proteins and SP responses in other tissues have been shown to depend of G-proteins (Boyd, *et al.*, 1991). Second, SP when applied to isolated and homogenized myenteric neuron/longitudinal muscle preparations causes accumulation of cAMP and promotes phosophatidylinositol (PI) turnover (Xia, Baidan, Fertel & Wood, 1991; Baiden, Fertel & Wood, 1992; Guard, Watling & Watson, 1988). Third, when activators of second messenger pathways are applied to enteric neurons they mimic many of the changes seen during the slow synaptic response (ie. inhibition of G_K, inhibition of the AH). Forskolin, dibutyrl cAMP and IBMX are effective, suggesting a role of a cAMP/PKA dependent pathway (Nemeth, Palmer, Wood & Zafirov, 1986; Surprenant, 1984). Activators of PKC such as phorbol 12,13 dibutyrate (PDBu), (-)-7-octylindolactam V and 1-oleoyl-2-acetyl-rac-glycerol are effective, suggesting a role of a diacylglycerol/PKC dependent pathway (North, Williams, Surprenant & Christie, 1987; Bertrand & Galligan, 1993b; Pan & Gershon, 1994).

Enteric reflexes and the functional significance of the slow synaptic response

The enteric plexuses control local reflexes within the ENS and participate in several longer reflexes mediated by neurons in the pancreas, prevertebral ganglia and the spinal cord (Furness & Costa, 1987). The most commonly studied local reflex in the isolated ileum is the peristaltic reflex. This reflex is elicited by physiological stimuli such as lumenal distension,

mucosal distortion and application of chemicals to the lumen (Furness *et al.*, 1994). The peristaltic reflex is composed of two distinct components, the ascending reflex is a contraction of the circular muscle oral of the stimulus site, and the descending reflex is a relaxation anal to the stimulus site. This is the essence of Bayliss & Starling's description of the "law of the intestine" (1899).

Analysis of the peristaltic reflex utilizing single and multiple chamber tissue baths has revealed that ascending and descending reflexes are inhibited, but not abolished by either nicotinic ACh antagonist (hexamethonium) or muscarinic ACh antagonist (atropine)(Bartho *et al.*, 1987). Both components involve nicotinic and non-cholinergic neurotransmission from sensory neurons. The ascending component involves nicotinic neurotransmission from interneurons and muscarinic and peptidergic (SP or a related peptide) neurotransmission from excitatory circular muscle motor neurons. The descending component involves nicotinic neurotransmission from interneurons and non-cholinergic inhibitory neurotransmission from circular muscle motor neurons (Holzer, 1989; Tonini & Costa, 1990) which release a combination of VIP, nitric oxide and/or ATP (Furness *et al.*, 1994). It is likely that neurotransmission from the non-cholinergic sensory neurons is in fact mediated by SP and that this part of the reflex is attributable to slow synaptic transmission.

SUMMARY OF GOALS

The goal of this study is to characterize the ionic mechanisms and signal transduction involved in the generation of the myenteric slow synaptic response. These data may be of importance

as the slow synaptic response represents a major mechanism of synaptic transmission within the myenteric plexus. The slow synaptic response leads to an increase in neuronal excitability that enhances all synaptic inputs to a neuron. Over eighty percent of myenteric neurons receive slow synaptic input and in preparations which are left partially intact, slow synaptic responses can elicited by stroking or distension of the mucosa. Thus, understanding the slow synaptic response is fundamental to the understanding of functional enteric circuitry.

Gastrointestinal disorders in highly industrialized countries are often not life-threatening. More often these disorders are related to the patient's discomfort and alteration of lifestyle. These disorders are very common yet poorly understood. Many disorders may have a neurological origin. Examples are irritable bowel syndrome, pseudo-obstructive disorder, and Crohn's disease. Disorders with a known neurological origin include diabetic neuropathies, Hirschsprung's disease and idiopathic megacolon (Bodian, Stephens, & Ward, 1949). The lethal strain of aganglionic piebald and spotted mice are an animal model of these latter two diseases (Bolande, 1975). The mutation leading to the piebald mouse strain is a clear example of the neurological origin of a gastrointestinal disorder (Gershon, 1981). In this disorder, a small section of terminal colon is aganglionic and, through a lack of peristalsis, becomes blocked. It is likely that similar, but less obvious disorders might also arise in this way. For example, loss of only one neuronal sub-type within the ENS could lead to a compromised, but functional gut.

In unindustrialized countries, GI dysfunction is still a leading cause of death, especially among infants. While these diseases probably do not stem from an enteric neurological disorder, treatments targeted at this level are often the only symptomatic cure. Thus,

understanding the neurological functioning of the gut is crucial step toward rational treatment of gastrointestinal disorders.

SPECIFIC GOALS

1. Identify the specific conductance changes associated with the slow synaptic response in myenteric neurons *in vitro*. The preparation used in this study is an ideal model of neuronal function as it contains many ganglia with many intact synaptic connections. Electrophysiological recordings will be obtained using single electrode voltage clamp. Voltage clamp is more favorable for determining the underlying conductances of the slow synaptic response as voltage-activated conductance changes are controlled. These studies will focus on the involvement of a calcium dependent potassium conductance and a novel conductance increase.

2. Identify the intracellular transduction mechanisms responsible for generation of the slow synaptic response. These studies will focus on characterizing the involvement of G-proteins and their coupling to adenylate cyclase and phospholipase C. cAMP/protein kinase A and protein kinase C dependent pathways and their involvement in protein phosphorylization will be characterized by using non-specific kinase inhibitors and a non-specific phosphatase inhibitor.

METHODS

PREPARATION

Tissue dissection

Guinea pigs (male 250-350g, obtained from the Michigan Department of Public Health (Lansing, MI)) were anesthetized *via* an atmosphere generated by aeration of 100% halothane, stunned and bled from the neck. A 5 to 6 cm segment of ileum, taken 10 to 20 cm from the ileocecal junction was removed and placed in oxygenated (95% O_2/5% CO_2) Krebs solution of the following composition (in mM): NaCl, 117; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 2.5; KCl, 4.7; $NaHCO_3$, 25; and glucose, 11. The Krebs solution also contained nifedipine (1 μM, a dihydropyridine calcium channel blocker) and scopolamine (1 μM, a muscarinic antagonist) to reduce movements of the longitudinal muscle during intracellular recordings. These compound have been shown not to affect the normal electrophysiology functioning of the myenteric plexus (Galligan, unpublished results). The piece of ileum was cut open along the mesenteric attachment and pinned mucosal side up in a silastic elastomer-lined Petri dish. The mucosa, submucosa and circular muscle were removed using forceps, leaving the myenteric plexus with attached longitudinal muscle. This preparation was then transferred to the base of a small silastic elastomer-lined recording chamber (volume < 2 mL), stretched and pinned flat. When tissues were incubated with drug, the recording chamber was agitated

briefly (5 min) then placed in a warmed (37 °C), aerated (95% O_2/ 5% CO_2) incubation chamber for approximately 30 min The recording chamber was then transferred to the stage of the inverted microscope and superfused with warmed Krebs solution (34-36 °C) at a flow rate of 3 mL/min. Tetrodotoxin (TTX, a voltage-gated sodium channel blocker, 300 nM) was added at various points through out experiments to block sodium-dependent action potentials and to prevent neighboring cells from contaminating the single cell recordings.

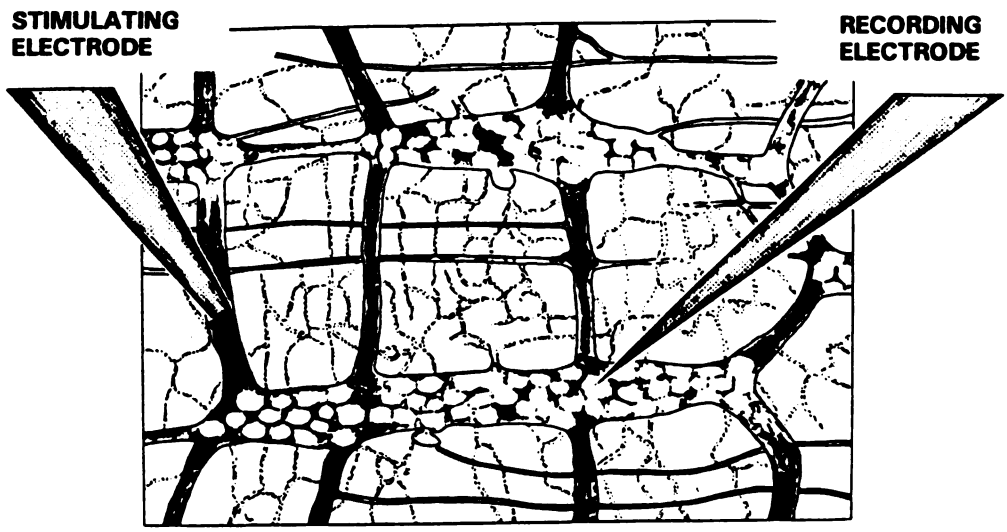
ELECTROPHYSIOLOGY

Myenteric ganglia were visualized at 200x magnification using a Olympus CK-2 inverted microscope (Olympus, Tokyo, Japan) with Hoffman differential interference contrast optics. Glass microelectrodes (Fredrick Hare Company, Brunswick, ME) were pulled to a 80 - 120 MΩ tip resistance (as measured with 2M KCl) with a Narishige PN-3 single stage micropipette puller (Narishige, Tokyo, Japan). Synaptic currents were elicited by focal stimulation (20 Hz, 500 ms train duration, 0.5 ms pulse duration, 40 - 60 V) of circumferentially-orientated interganglionic fiber tracts with a broken-back glass micropipette filled with Krebs solution (Figure 1). Current and voltage measurements were made using a dual purpose single-electrode voltage clamp/current clamp amplifier (Axoclamp 2A, Axon Instruments, Foster City, CA). The voltage clamp switching frequency was 3 kHz and the duty cycle was 70% voltage measuring, 30% current passing. The voltage at the headstage was monitored on a separate oscilloscope to ensure that it had settled to its control level at the time it was sampled. The term "slow synaptic response" may refer to either of these

Figure 1. An electrophysiological preparation utilizing the myenteric plexus

An adaptation from Furness & Costa (1987) illustrating the electrophysiological preparation used in this study. The small intestine has been opened along the mesenteric attachment, pinned flat and stripped of the overlying mucosa and submucosa. The myenteric plexus is composed of ganglia (the oblong structures) each containing 50 to 100 neurons (light circles) and the interganglionic fiber tracts (dark vertical lines). The underlying plexus and longitudinal muscle layer are represented as the light matrix between and under the ganglia. The saline filled stimulating electrode (left) is positioned over interganlionic fiber tract in order to directly evoke action potentials. The saline filled recording electrode (right) is lowered directly into a myenteric neuron. This impalement creates electrical continuity between the electrode and the neuron.

Figure 1



conditions. When under voltage clamp conditions, and current is being measured, the slow synaptic response is referred to as the slow excitatory post-synaptic current (sEPSC), while under current clamp conditions, and voltage is being measured, it is referred to as the sEPSP. In most experiments the holding potential (V_H) was -70 mV. Voltage steps (V_{STEP}) of 10 to 30 mV in amplitude negative to V_H and 300 to 500 ms in duration were evoked at approximately 2.2 s intervals at rest and during agonist- or nerve-mediated current responses. Chord conductances were calculated from the current amplitudes measured at the end of the voltage steps or from I/V relationships. I/V relationships were calculated by measuring the peak amplitude of individual responses at different holding potentials or by measuring the steady state I/V relationship. Steady state I/V relationships were measured by generating a series of voltage steps (300 to 500 ms duration) to different test potentials between -40 and -110 mV at rest and during evoked responses or by manually changing V_H at a rate of 2 mV/sec (Williams *et al.*, 1988). In the case of transient responses (such as sEPSCs and senktide responses) only a few voltage steps could be evoked at peak currents, so several consecutive responses were used.

Analysis of conductance

Chord conductance measurements (see above) were used to determine the contribution of multiple conductance changes to sEPSCs or senktide responses. The observed reversal potential of the conductance increase (-17 mV) suggested that it may be either a chloride conductance or a non-specific cation conductance. In myenteric neurons, the chloride equilibrium potential has been estimated at -18 mV by measuring the reversal potential of $GABA_A$-activated chloride responses using 2 M KCl recording electrodes (Cherubini &

North, 1979; Bertrand & Galligan, 1992a). Similarly, the reversal potentials for a non-specific cation current in myenteric neurons has been estimated to be between -25 and -10 mV by measuring the reversal potential of the hyperpolarization-activated cation current (I_H) or the current activated by acetylcholine acting at nicotinic receptors (Galligan, Tatsumi, Shen, Surprenant & North, 1990; Galligan, Campbell, Kavanaugh, Weber & North, 1989). The results of the analysis described below will hold true for an increase in chloride conductance or an increase in a non-specific cation conductance.

The predicted current resulting from a decrease in potassium conductance combined with a conductance increase (G_{IN}) is given by:

$$\Delta I = \Delta G_K (V_H - E_K) + \Delta G_{IN} (V_H - E_{IN}) \quad \ldots\ldots (1)$$

Where ΔI is equal to the agonist-induced current, ΔG_K is the change in potassium conductance, V_H is the holding potential, E_K is the potassium equilibrium potential (-90 mV), ΔG_{IN} is the increase in conductance (for example, chloride or cation), and E_{IN} is the equilibrium potential for the current passing through G_{IN}. The measured conductance change ΔG is given by $\Delta G = \Delta G_K + \Delta G_{IN}$, and substitution for G_K in equation 1. leads to:

$$\Delta I = \Delta G (V_H - E_K) + \Delta G_{IN} (E_K - E_{IN}) \quad \ldots\ldots (2)$$

This analysis was used to fit I/V relationships with a one or two parameter model in order to determine the significance of ΔG_{IN}. A least squares fit of equation 2 (ΔI as a function of ΔG) was used to determine whether the experimental data were better fit when ΔG_{IN} was zero (one

parameter) or when ΔG_{IN} was allowed to take a value other than zero (two parameter). ΔI and ΔG were measured, V_H was known and E_K and E_{IN} were assigned values of -90 mV and -18 mV respectively. The results of these regressions were then compared using an F-test for one or two parameter equations (Figure 2).

For some cells, I/V relationships were not available and the relative contribution of ΔG_{IN} and ΔG_K were determined from chord conductance determinations made at a single holding potential. This analysis was used to characterize the time course of ΔG_K and ΔG_{IN} where ΔG_K was determined from the calculated value of ΔG_{IN} and the observed ΔG and ΔI.

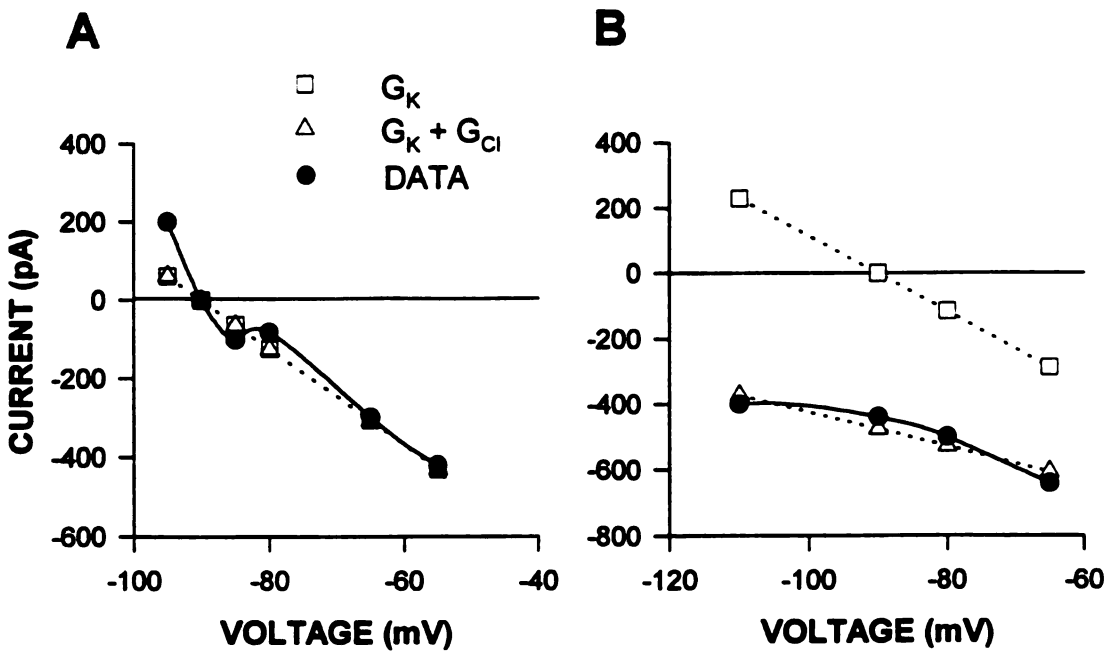
STATISTICS

Data are expressed as the mean ± standard error of the mean. Data were analyzed for significance ($\alpha = 0.05$) using Student's t-test for paired and un-paired data, Tukey-Kramer multiple comparisons test for parametric data or Kruskal-Wallis ANOVA followed by Dunn's multiple ranges test for non-parametric data. Reversal potentials and chord conductances were estimated from I/V plots using a least squares linear regression analysis. Time constants for rise and decay of currents were fit using a double exponential equation. Linear regressions were analyzed for significance using an F-test for an analysis of variance of the goodness of fit for one and two parameter equations. In figure 2, data from two cells (A and B) are plotted versus the best fit G_K and $G_K + G_{Cl}$ equations. We can determine whether the two parameter equations fit the data better by taking the difference between the data and the predicted current (the residuals) at each point, squaring this value and then summing it with

Figure 2. Comparison of one (G_K) and two (G_K and G_{Cl}) parameter equations

Senktide was applied by pressure application at the membrane potentials indicated in two separate neurons (**A** and **B**). These I/V relationships (•) were plotted versus the relationships predicted by a one (□) or two (△) parameter equations. **C.** The columns labelled Voltage and Current represent I/V data, 1. G_K is the best fit line where G_K is negative and is constrained to pass through E_K , 2. G_K+G_{Cl} is the best fit line where G_K is negative and is constrained to pass through E_K and G_{Cl} is positive and is constrained to pass through E_{Cl}.

Figure 2



C

DATA FOR A.

Voltage	Current	1. G_K	2. G_K+G_{Cl}
-55	-420	-430.5	-430.5
-65	-300	-307.5	-307.5
-80	-80	-123	-123
-85	-100	-61.5	-61.5
-90	0	0	0
-95	200	61.5	61.5

n	6	1. G_K	-12.3
SS_1	22680	2. G_K	-12.3
SS_2	22680	2. G_{Cl}	0
df_1	5		
df_2	4		

DATA FOR B.

Voltage	Current	1. G_K	2. G_K+G_{Cl}
-65	-640	-287.5	-606
-80	-500	-115	-528
-90	-440	0	-476
-110	-400	230	-372

n	4	1. G_K	-11.5
SS_1	862981.25	2. G_K	-12
SS_2	4020	2. G_{Cl}	6.8
df_1	3		
df_2	2		

the other points. This sum of square of the residual (SS) for both equations, can now be used to compare the goodness of fit.

$df_x = n - x$, df = the degrees of freedom
n = the number of observations
x = the number of parameters

$$F = \frac{SS_1 - SS_2 / df_1 - df_2}{SS_2 / df_2},$$

SS = the sum of squares of the residuals
$_1$ = designates the one parameter eq.
$_2$ = designates the two parameter eq.

If value of the F-distribution, F_{TABLED} ($\alpha = 0.05$, df_1, df_2) is greater than or equal to F, then the fit is not significantly different. If F_{TABLED} is less than F, then the two parameter equation fits the data significantly better than the one parameter equation. In figure 2A, the F value was calculated to be zero versus a tabled value of 6.3, indicating that a G_K equation fit the data as well a $G_K + G_{Cl}$ equation. In figure 2B, the F value was calculated to be 213.8 versus a table value of 19.2 indicating the $G_K + G_{Cl}$ equation fit significantly better than the G_K equation.

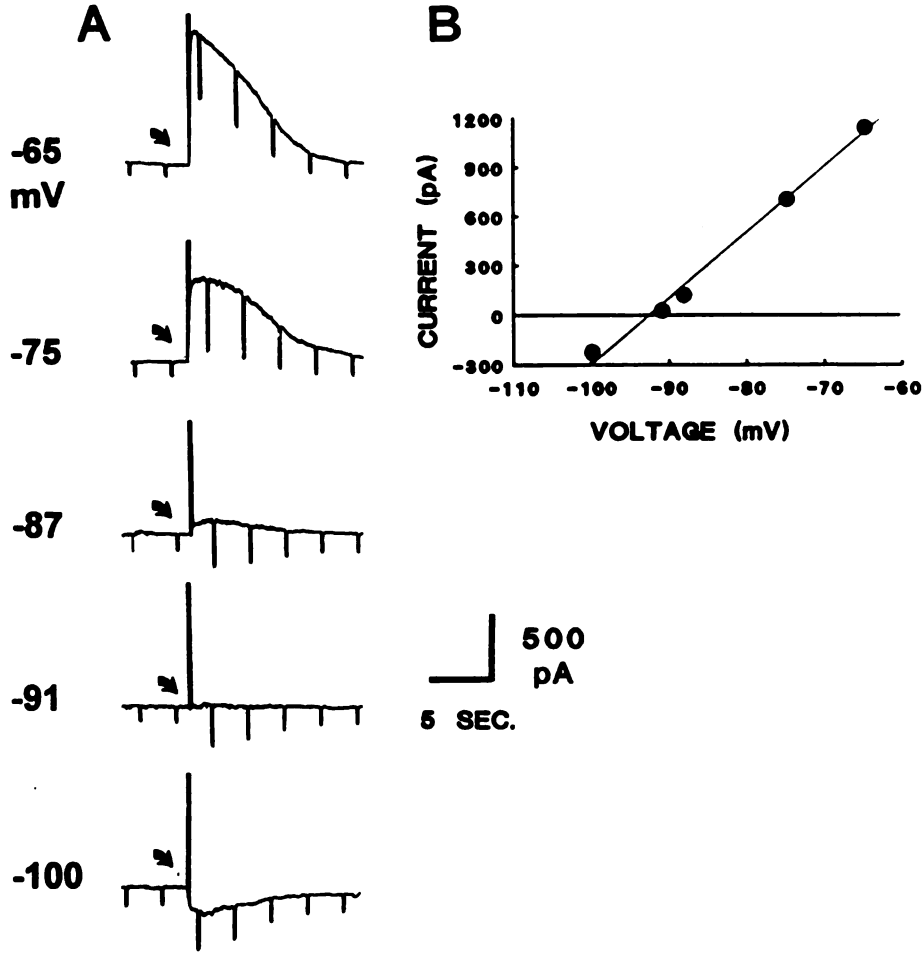
THE AH CURRENT (AHC)

The AHC was elicited by brief depolarizing pulses delivered through the recording electrode (100-500 ms train duration, 20 Hz, +80 mV from V_H, 5 ms pulse duration) which were monitored on an oscilloscope. AHCs were due to an increase in $G_{K,Ca}$ (Morita, North &

Figure 3. The AHC is an increase in $G_{K,Ca}$

A 300 ms train of positive voltage pulses (20 Hz, +80 mV from V_H, 5 ms dur) at the curved arrow (➦) were applied through the recording electrode to a single AH-neuron. Approximately 7 action potentials were initiated in the cell soma. Calcium entry during the spike activates $G_{K,Ca}$. **A.** The AHC was evoked at the indicated holding potentials in 1 to 2 min intervals. V_{STEP} of 20 mV negative of V_H was applied every 2.2 s to reveal the increase in conductance associated with the outward current. **B.** The I/V relationship for (A). The current reversed at -93 mV, near the potassium equilibrium point.

Figure 3


A
B
-65
mV
-75
-87
-91
-100
1200
900
600
300
0
-300
CURRENT (pA)
-110
-100
-90
-80
-70
-60
VOLTAGE (mV)
500
pA
5 SEC.

Tokimasa, 1982)(Figure 3) and were unchanged following application of TTX (300 nM) and completely blocked following application of cobalt (2 mM) in a phosphate-free Krebs solution. Maximal activation of the AHC was determined by increasing the train length (maximum of 500 ms) until this no longer increased AHC amplitude. This value was determined for individual neurons and used for the remaining experiments in that neuron.

DRUGS

Pertussis toxin (PTX) was purchased from List Biological Laboratories (Campbell, CA, U.S.A.), phorbol 12, 13 dibutyrate (PDBu) and calyculin A were purchased from LC Laboratories (Woburn, MA, U.S.A.). All other drugs were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.) PDBu, forskolin, staurosporine and calyculin A were dissolved in 100% ethanol (final concentration $\leq$ 0.3%). Drugs were applied by four methods.

Standard superfusion. Drugs were added directly to the superfusing Krebs solution. Drug concentration is known and is under equilibrium conditions. The average time from the time the tap is turned on the drug containing reservoir to the time drug first arrives in the bath was approximately 40 s. Some compounds such as picrotoxin begin to exert effects as soon as 45 s after the tap is turned. Complete exchange of the recording chamber is estimated to take less than 3 min (bath volume < 2 mL, flow rate > 3 mL/min).

Pressure ejection. Drugs dissolved in Krebs were placed in a glass micropipette with a tip diameter of approximately 20 μM). The pipette was connected to a Picospritzer II (General Valve Corp, Fairfield, New Jersey, USA) and lowered into the recording chamber within 200 μm of the impaled neuron. Drug was ejected from the pipette with brief (10 - 200 ms) pulses of N_2 (5 to 15 p.s.i.). Drugs applied in this way did not reach equilibrium.

Fast/Slow flow superfusion. Drug-containing reservoirs were connected to an array of 300 μm inner diameter capillary tubing which was then lowered into the recording chamber within 300 μm of the impaled neuron. Flow rate was adjusted by changing the height of the reservoirs. At faster flow rates (500 μL/min) drug containing solution were heated by a element contained within the array. Slower flow rates (150 μL/min) did not require heating. The delay from the opening of the tap to the arrival of drug at the impaled neuron was <1 s.

Intracellular application. Drugs were dissolved in 2 M KCl and placed within the recording electrodes. For charged species such as GTP-γ-S or Cs^+ the appropriate holding current was applied to the electrode to hasten the movement of the drug into the neuron.

RESULTS

THE IONIC BASIS OF THE sEPSC

Characterization of neurons

Data were obtained from S neurons and AH neurons (Hirst *et al.* 1974). S neurons received fast and slow synaptic input, had a linear I/V relationship and did not respond to GABA. AH neurons received slow synaptic input, had a non-linear I/V relationship and spike afterhyperpolarizations or aftercurrents of greater than 1 s duration. GABA, applied by pressure produced fast (time to peak < 3 s), inward currents in AH neurons (Cherubini & North, 1979). Recordings lasting from 0.5 to 3 h were made from 50 S neurons and 300 AH neurons in 250 preparations. Extended impalement times, drug application and nerve stimulation were associated with a slowly developing decrease in membrane conductance, a depolarized resting membrane potential and increased neuronal excitability. The resting conductance of neuronal membranes decreased from a value of 16.1 ± 2.3 nS soon after impalement to a stable value of 12.1 ± 1.3 nS 30 min after impalement in S neurons (n = 15), and from 25.7 ± 4.2 nS to 11.9 ± 1.1 nS in AH neurons (n = 17). This decrease in membrane conductance is considered to be an inhibition of resting G_K (Surprenant, North & Katayama, 1987; Wood, 1989). Consequentially, sEPSCs and senktide responses elicited soon after

impalement were composed predominately of a decrease in G_K, while responses elicited later (> 30 min after impalement) were more likely to exhibit an increase in conductance (G_{IN}). In AH neurons, the overall amplitude of the senktide response changed from -429 ± 80 pA within 10 min of impalement to -600 ± 85 pA between 10 and 30 min after impalement to -463 ± 84 pA more than 30 min after impalement ($n = 9$, $p > 0.05$). The amplitude of slow synaptic current changed from -353 ± 114 pA to -299 ± 46 pA to -274 ± 44 pA during a similar time course ($n = 18$, $p > 0.05$).

The sEPSC is due to two conductance changes

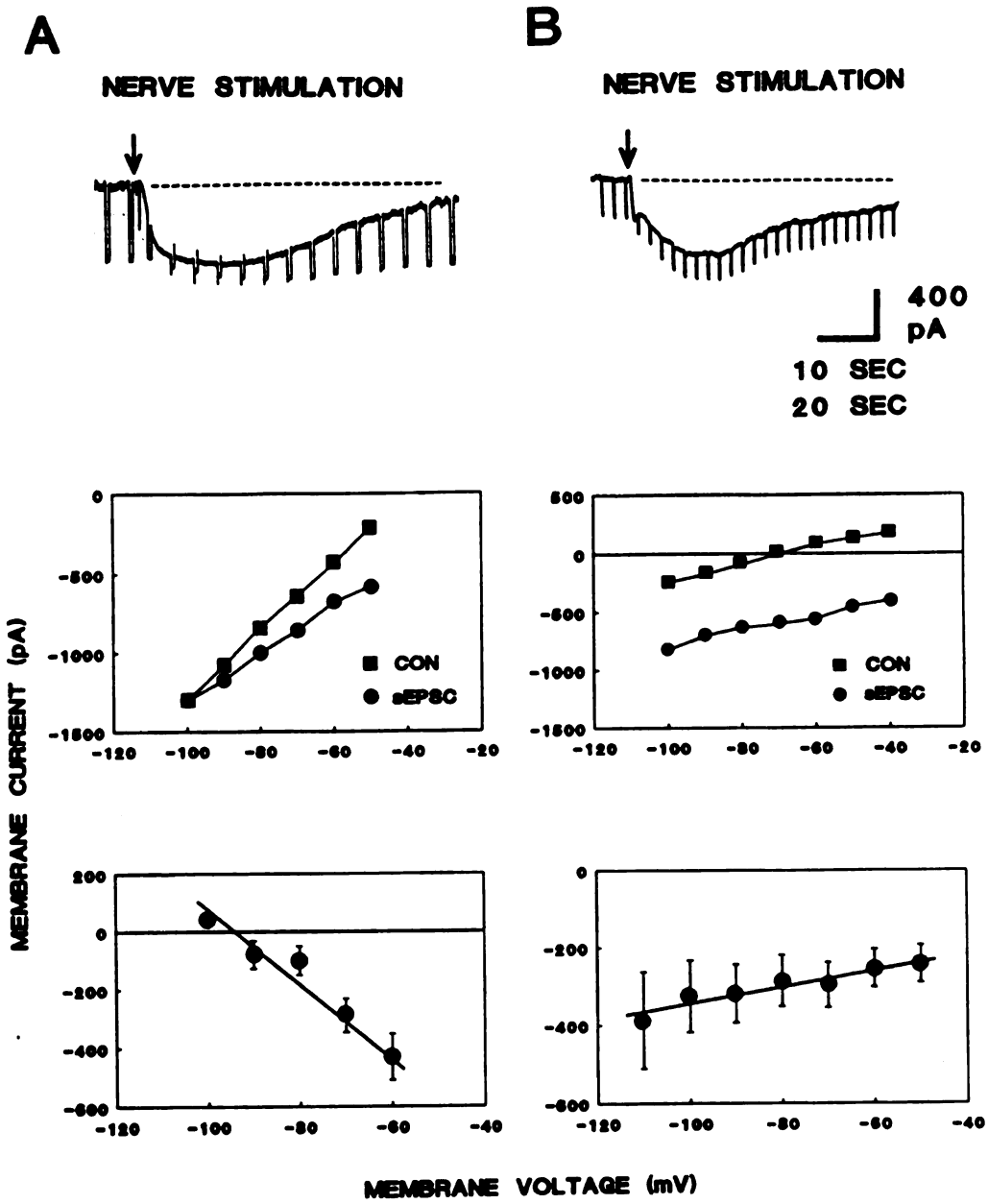
Electrical stimulation of presynaptic nerves 10 - 30 min after impalement elicited sEPSCs in 175/200 neurons. In 160/175 (91%) neurons, the sEPSC was associated with a conductance decrease. In 15/175 (9%) neurons, there was either no measurable conductance change or a biphasic conductance change.

An I/V relationship was obtained for the sEPSC in 24 AH neurons at least 30 min after impalement. In these neurons, peak currents and conductance changes at different holding potentials were known, and were used to fit a one (G_K) or a two (G_K and G_{IN}) parameter model (see *Methods*). Neurons were divided into two categories based on the presence or absence of a significant G_{IN} during the sEPSC. In 17/24 (71%) neurons fiber tract stimulation caused an inward current associated with a 13 ± 3 nS decrease in conductance. The estimated reversal potential of the peak current was -96 ± 3 mV (Figure 4A) and G_{IN} did not make a significant contribution to these responses. The sEPSC in these neurons was considered a *conductance decrease* type response and has been reported by several other groups (Johnson *et al.*, 1980; Grafe *et al.*, 1980). In 7/24 (29%) neurons, the

Figure 4. The sEPSC is due to two conductances

Records were obtained 30 min after impalement. Electrical stimulation of interganglionic fiber tracts (↓) caused a sEPSC. **A.** (top) A sEPSC associated with a conductance decrease (V_H = -70 mV, V_{STEP} = -90 mV); (middle) I/V relationship in the same neuron before (■) and at the peak of two consecutive the sEPSC (●); (bottom) average I/V for sEPSCs recorded from 7 neurons similar to A (middle), the reversal potential for the sEPSC was -96 ± 3 mV. **B.** (top) sEPSC with a biphasic conductance change (V_H = -70 mV, V_{STEP} = -90 mV); (middle) steady state I/V relationship in the same neuron before (■) and at the peak of the sEPSC (●); (bottom) I/V for the sEPSC recorded from 6 neurons similar to B (middle) did not reverse polarity between -40 and -110 mV.

Figure 4


A
NERVE STIMULATION
B
NERVE STIMULATION
400
pA
10 SEC
20 SEC
CON
sEPSC
CON
sEPSC
MEMBRANE CURRENT (pA)
MEMBRANE VOLTAGE (mV)

sEPSC was associated with no observed change in membrane conductance (Figure 4B). The data from these cells were fit best by a model in which there was an approximately equal contribution of G_K decrease and G_{IN}. The peak sEPSC from these 7 neurons did not reverse between -40 and -110 mV and could not be extrapolated to reverse between +50 and -150 mV. These sEPSCs were considered *mixed conductance/non-reversing* type responses. In 4/7 neurons, this relationship did not change during the time course of the sEPSC. In 3/7 neurons, there was a biphasic conductance change, an early conductance decrease was followed by a G_{IN}. The early conductance decrease reversed near -90 mV which is consistent with this being a decrease in G_K (see Figure 6A).

Senktide mimics the sEPSC

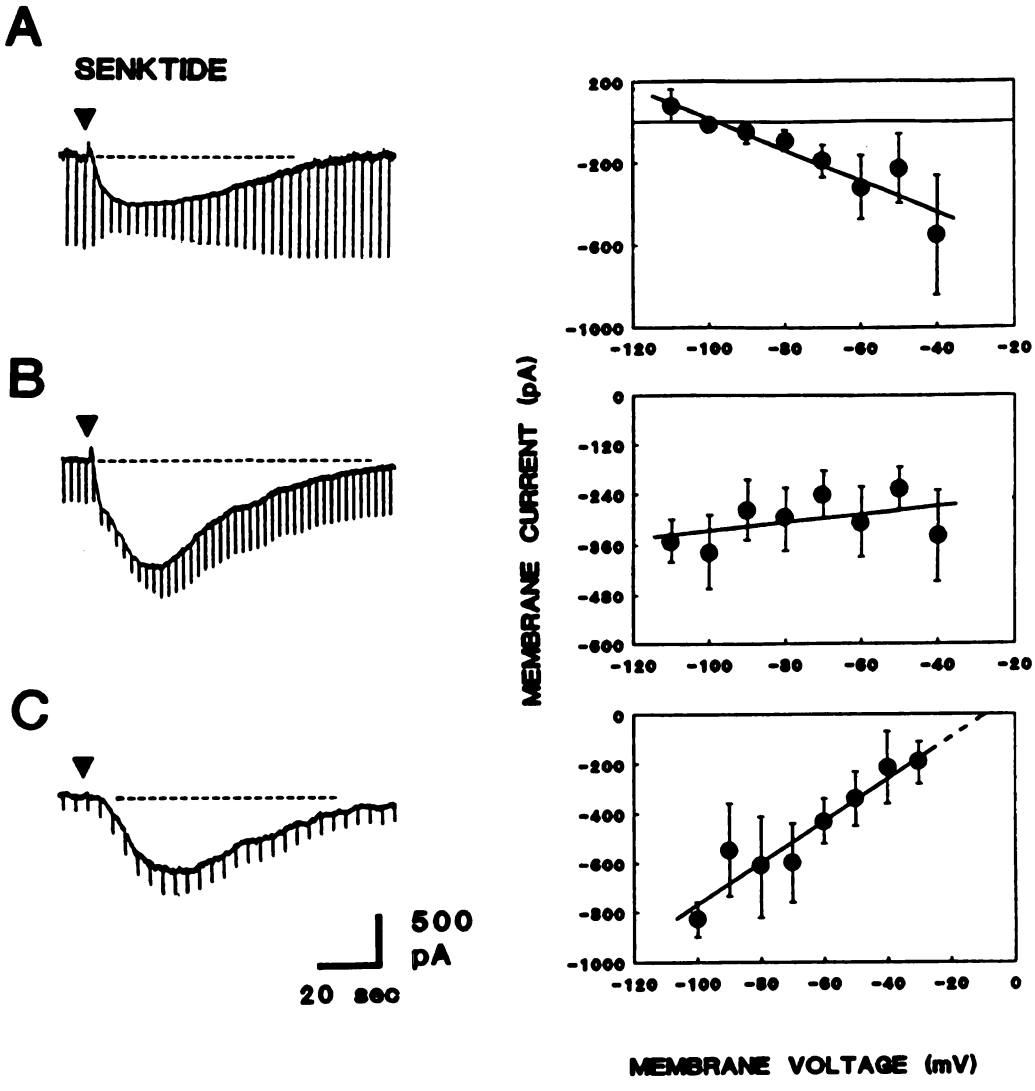
Application of senktide (succinyl-[Asp^6, N-Me-Phe^8]-substance P, 6-11) a selective neurokinin-3 (NK-3) receptor agonist, 10 - 30 min after impalement caused an inward current in 106/139 neurons (Hanani, *et al.*, 1988). In 76/106 (72%) neurons, senktide currents were associated with a conductance decrease. In 23/106 (22%) neurons, the current was associated with either no observed conductance change or a conductance decrease followed by a conductance increase. In 12/106 (11%) neurons the senktide current was associated with a conductance increase.

An I/V relationship, in the presence of TTX (300 nM), was obtained for senktide responses in 41 AH neurons at least 30 min after impalement. In these neurons, peak currents and conductance changes at different holding potential were known, and were used to fit a one (G_K) or a two (G_K and G_{IN}) parameter model. In 20/41 (49%) neurons, senktide caused an inward current associated with a 8.4 ± 5 nS decrease in conductance with a reversal

Figure 5. Senktide mimics the sEPSC

Records were obtained 30 min after impalement. Senktide (3 μM) was pressure applied (▾) and caused an inward current; TTX (0.3μM) present. **A.** (left) A senktide current with a conductance decrease (V_H = -75 mV, V_{STEP} = -85 mV); (right) the I/V from 10 neurons with similar responses; the reversal potential was -94 ± 2 mV. **B.** (left) A senktide current with a biphasic conductance change (V_H = -75 mV, V_{STEP} = -85 mV); (right) the I/V from 7 neurons with biphasic or unclear conductance changes; responses did not reverse. **C.** (left) A senktide current with a G_{IN} (V_H = -65 mV, V_{STEP} = -85 mV); (right) I/V from 5 neurons with similar responses; the estimated reversal potential was -15 mV.

Figure 5


A
SENKTIDE
B
C
500
pA
20 sec
MEMBRANE CURRENT (pA)
MEMBRANE VOLTAGE (mV)

potential of -94 ± 2 mV (Figure 5A). These responses did not contain a significant G_{IN}. These actions of senktide on myenteric neurons were considered *conductance decrease* type responses and are similar to what has been reported by others (Hanani *et al.*, 1988).

In 21/41 (51%) neurons, the I/V relationships could be fit best by a two parameter model in which there was a significant contribution of G_{IN}. In these 21 neurons, there were two subgroups. In the first subgroup, 11/21 neurons, senktide induced a current without an observed conductance change. Simulation showed that this current resulted from similar contribution from G_K decrease and G_{IN}. The peak currents in these 11 neurons failed to reverse polarity between -40 and -110 mV nor could they be extrapolated to reverse between +50 and -150 mV. These actions of senktide were considered *mixed conductance/non-reversing* type responses (Figure 5B). In 6/11 neurons, the relative contribution did not change during the time course of the response. In 5/11 neurons an early G_K decrease was followed by a G_{IN} (see Fig. 6A).

In the second subgroup, 10/21 neurons, senktide caused an inward current that was associated with a large G_{IN} to G_K ratio. This relationship did not change during the course of the response. The estimated reversal potential of the peak current was -17 ± 3 mV. These actions of senktide were considered *conductance increase* type responses (Figure 5C).

Forskolin reduces G_K and reveals a conductance increase during non-reversing sEPSCs

Forskolin mimicked *conductance decrease* type sEPSCs. Forskolin (0.01 - 3 μM) applied by fast flow or superfusion caused a sustained inward current in S and AH neurons. In S neurons, the maximum amplitude of the forskolin (1 μM) current was -132 ± 20 pA (V_H =

-70mV, n = 6) and the reversal potential was -98 ± 5 mV; the forskolin EC_{50} was 0.2 μM. In AH neurons, the maximum amplitude of the forskolin (1 μM) current was -283 ± 49 pA (V_H = -70mV, n = 7) and the reversal potential was -111 ± 4 mV; the forskolin EC_{50} was 0.08 μM. It was concluded that forskolin currents were due to inhibition of G_K. These data are similar to those of others (Nemeth *et al.*, 1987).

Forskolin was applied to neurons with *mixed conductance/non-reversing* type sEPSCs (Figure 6A). Forskolin (1 μM) caused an inward current in these neurons that was due to a decrease in resting G_K. In the presence of forskolin, these sEPSCs were converted to a *conductance increase* type response (Figure 6B). The I/V relationship for the residual sEPSC yielded an estimated reversal potential of -18.8 ± 8 mV with an increase in chord conductance of 5.9 ± 2 nS (n = 5)(Figure 6C).

Before treatment with forskolin, a decrease in G_K accounted for about 70% of the peak total conductance change during the sEPSC and I_K accounted for 30% of the synaptic current (Figure 7A). The small contribution of I_K to the synaptic current can be explained by the small driving force for potassium at a holding potential of -80 mV. During superfusion with forskolin, the contribution of G_K to the sEPSC fell to less than 30% of the absolute conductance change and the peak current was reduced from -200 pA to -50 pA (Fig 7B). Forskolin did not change G_{IN}. Under these conditions, the current generated by G_{IN} accounted for 90% of the synaptic current (see below for a similar analysis of *conductance decrease* type responses).

Figure 6. Forskolin occluded G_K but not the conductance increase

A biphasic sEPSC was evoked (↓) before **A.** and during **B.** superfusion with forskolin (1 μM). In control, the sEPSC was composed of an outward current with a conductance decrease followed by an inward current with no net conductance change. In the presence of forskolin (1 μM) the sEPSC was associated with a G_{IN} and the outward current associated with G_K decrease was occluded (V_{STEP} = -110 mV). **C.** I/V relationship for the sEPSCs in **A.** and **B.**; control (■) and with forskolin (●). The peak control sEPSC did not reverse (range -35 to -100 mV); in the presence of forskolin the peak current reversed at -16 mV and was associated with a 4.5 nS G_{IN}.

Figure 6

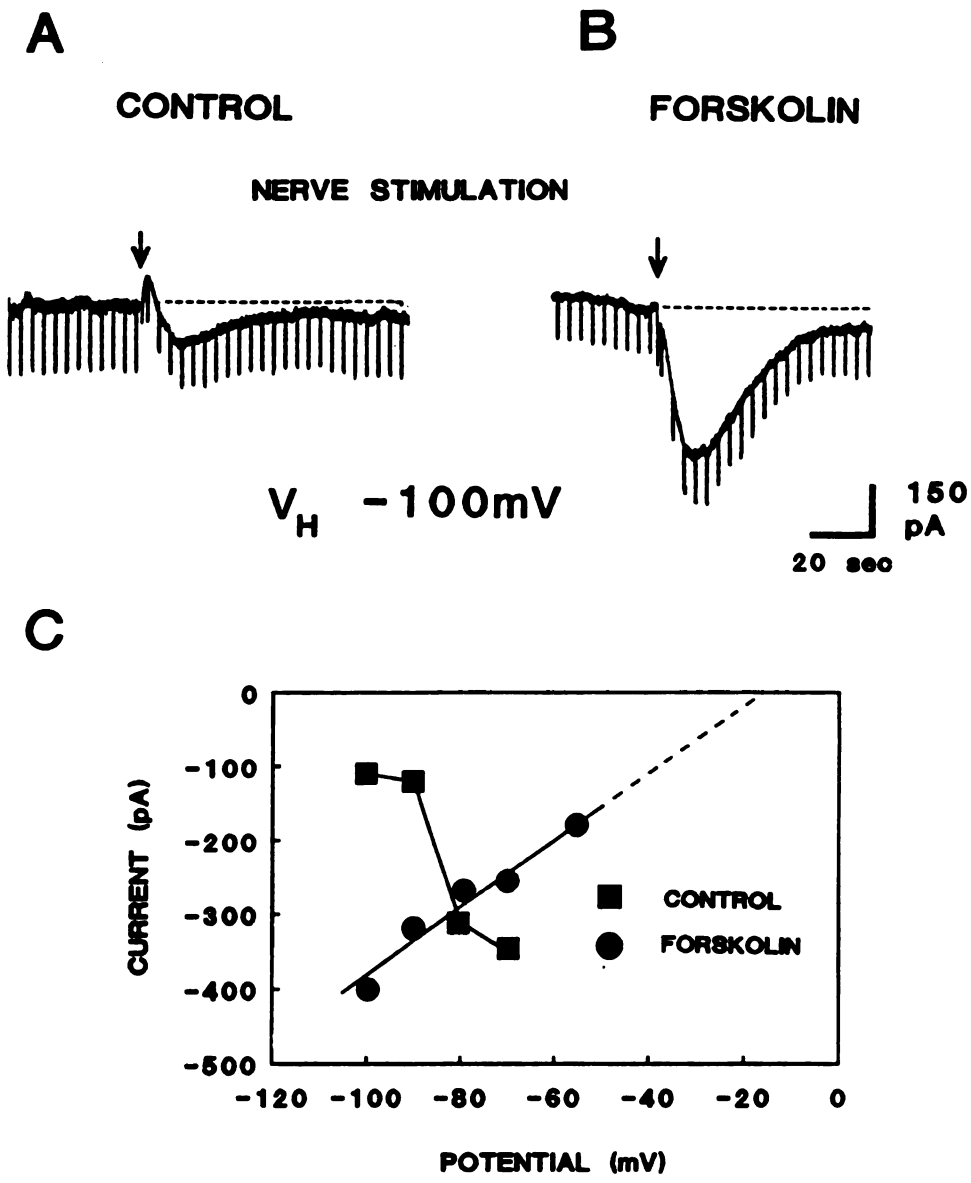
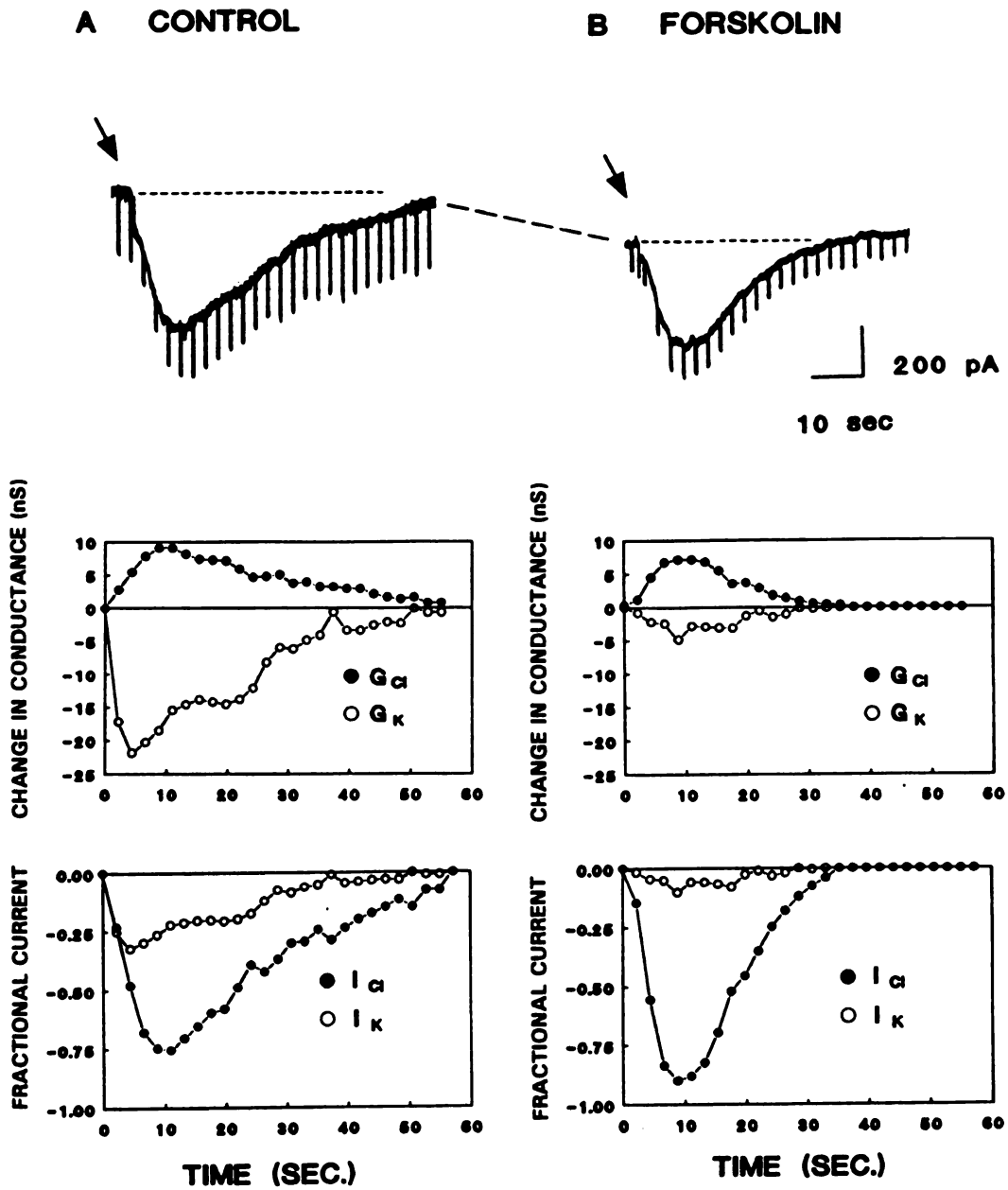

A
B
CONTROL
FORSKOLIN
NERVE STIMULATION
V_H -100mV
150
pA
20 sec
C
CURRENT (pA)
0
-100
-200
-300
-400
-500
-120
-100
-80
-60
-40
-20
0
POTENTIAL (mV)
CONTROL
FORSKOLIN

Figure 7. Analysis of the change in conductance associated with a non-reversing sEPSC

Chord conductance and current measurements were taken every 2.2 seconds during a biphasic sEPSC (V_H = -80mV). The changes in conductance due to G_K and G_{IN} summate and always equal the observed change in conductance. The peak current for each sEPSC was normalized to a value of -1 and the current components were expressed as a fraction of that value. **A.** (Left) Decrease in G_K (○) is 5 to 6 times larger than the G_{IN} (●). (Right) The same sEPSC was divided into two current components based on the ratio of G_K to G_{IN}. I_K (○) is smaller than the current generated by the G_{IN} (●) due to the small driving force for potassium at -80mV (ΔI = -680pA). **B.** (Left) Forskolin (1 μM) specifically occludes the decrease in G_K. (Right) The current generated by G_{IN} is effectively isolated (ΔI = -500pA).

Figure 7


A CONTROL
B FORSKOLIN
200 pA
10 sec
CHANGE IN CONDUCTANCE (nS)
G Cl
G K
FRACTIONAL CURRENT
I Cl
I K
TIME (SEC.)

Forskolin inhibits G_K and reveals a conductance increase during non-reversing senktide responses

Forskolin (1 μM) was applied to neurons exhibiting *mixed conductance/non-reversing* type responses to senktide (Figure 8A) and occluded the G_K decrease caused by senktide (Figure 8B). In the presence of forskolin, these senktide responses were converted to *conductance increase* type responses with no reduction in peak current. In control, the senktide response was -391 ± 78 pA and in the presence of forskolin, the senktide response was -384 ± 81 pA (V_H = -70 mV, $p > 0.05$, n = 6). Currents were estimated to reverse at -14 ± 5 mV (n = 3).

The conductance increase (G_{IN}) is a chloride conductance (G_{Cl})

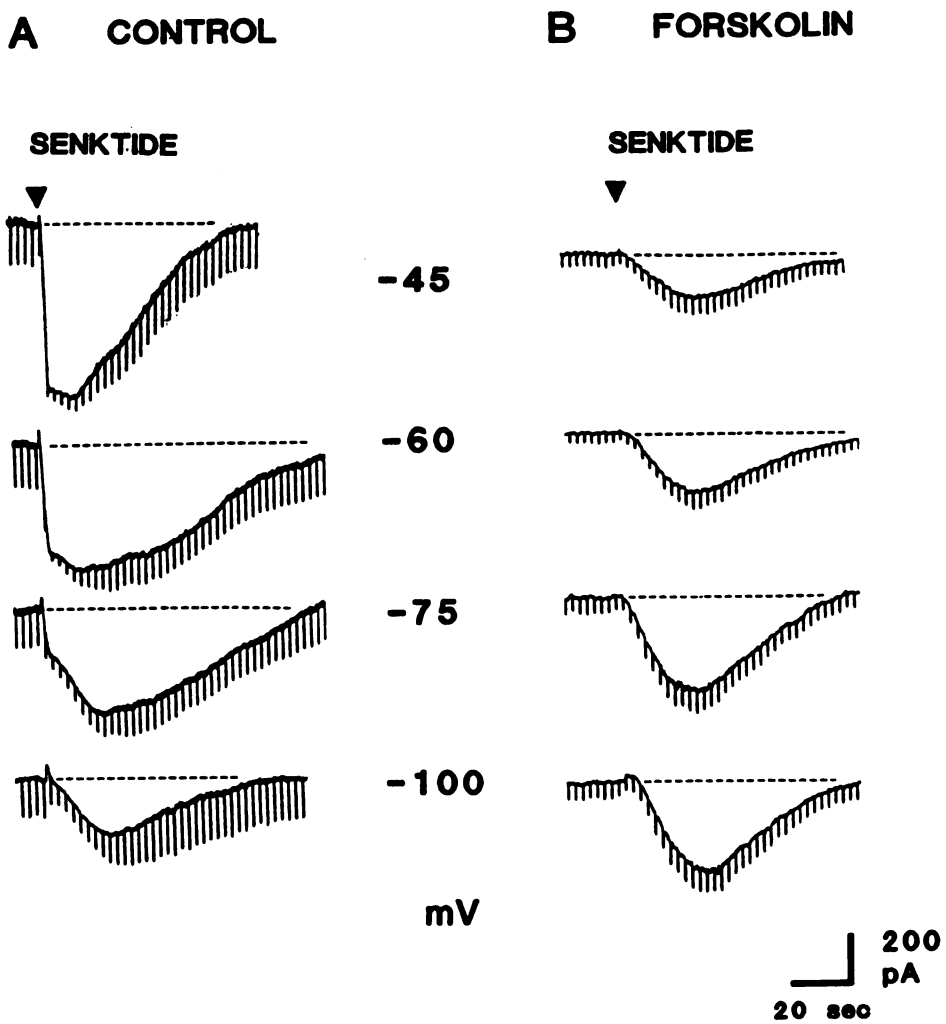
These experiments were carried out under conditions that minimized changes in G_K and blocked $I_{(H)}$(Galligan, *et al.*, 1990). Neurons were recorded from in the presence of forskolin (1 μM) and/or cesium (2 mM) respectively. In addition, responses were recorded 30 min or more after impalement and V_H was near E_K. All responses studied exhibited an observed conductance increase, except where noted.

Ion substitution experiments. Lowering extracellular sodium to 26 mM using choline chloride (117 mM) substitution for sodium chloride did not significantly change the senktide response. The control response was -367 ± 83 pA while in the presence of choline chloride the senktide response was -420 ± 117 pA (V_H = -85 mV, $p > 0.05$, n = 6). Reducing extracellular chloride to 13 mM by substituting sodium isethionate (117 mM) for sodium chloride reduced senktide currents by 79%. Control responses were -619 ± 99 pA while in

Figure 8. Forskolin occludes G_K and isolates the senktide-induced conductance increase

Senktide (3 μM) was pressure applied (▾) to a neuron and evoked a biphasic response at the indicated holding potentials before (A.) and during (B.) superfusion with forskolin (1 μM) (V_{STEP} = 20 mV negative to V_H; TTX 0.3 μM present). **A.** Under control conditions the peak current did not reverse. **B.** In the presence of forskolin, the current was monophasic, was estimated to reverse at -10 mV and was associated with a 5.7 nS increase in conductance.

Figure 8


A CONTROL
B FORSKOLIN
SENKTIDE
SENKTIDE
-45
-60
-75
-100
mV
200
pA
20 sec

the presence of sodium isethionate the current amplitude was -130 ± 51 (V_H = -80 mV, $p < 0.05$, n = 4). Low chloride solutions also reduced sEPSCs that contained a G_{IN} by 78%. Control sEPSCs were -374 ± 35 pA and in the presence of sodium isethionate, sEPSC amplitude was -83 ± 49 pA (V_H = -80 mV, $p < 0.05$, n = 3). The specificity of the actions of reduced chloride solutions were tested under control conditions (ie. no forskolin or cesium), using *conductance decrease* type sEPSCs. The decrease in G_K caused by these sEPSCs was not affected by low chloride solutions; the control response was 243 ± 78 pA, and in the presence of sodium isethionate, the current amplitude was 191 ± 65 pA (V_H = -70 mV, $p > 0.05$, n = 3).

An outward chloride current should be increased by reducing extracellular chloride yet low chloride solutions reduced the G_{IN}. To determine the cause of this discrepancy, similar studies were performed on $GABA_A$-mediated currents. In normal solutions the reversal potential of the GABA response was -18 ± 2 mV (n = 10) while in low chloride solutions the reversal potential was shifted to +5 ± 6 mV (n = 5). On average, when changing from normal to low chloride solutions, the $GABA_A$ slope conductance was reduced from 27 ± 5 nS (n = 10) to 7 ± 6 nS (n = 5). Based on the measured reversal potential of the $GABA_A$ response, and the known extracellular chloride concentration, the intracellular chloride concentration was calculated. When the $GABA_A$ reversal potential was -18 mV and the external chloride concentration was 129 mM, the internal chloride concentration was calculated to be 65 mM. During superfusion with low chloride solutions, the $GABA_A$ reversal potential was +5 mV and the external chloride was 13 mM, the internal chloride concentration was calculated to be 16 mM. This depletion of internal chloride by low chloride solutions

could account for the reduction of sEPSC and senktide-mediated currents that are associated with significant G_{IN}.

The effect of altered intracellular chloride on the GABA and senktide induced chloride currents was also investigated. When recordings were made with a 2M K-acetate or a 2M K-gluconate electrode in normal Krebs solution, the $GABA_A$ reversal potential was shifted to -39 ± 2mV (n = 6) and the internal chloride concentration was calculated to be 28 mM. Similar experiments were attempted to analyze senktide-induced G_{IN}. With K-acetate or K-gluconate electrodes, the chloride equilibrium potential should be shifted from approximately -17 mV to -39 mV. However, no sEPSCs, senktide or forskolin responses could be recorded in 8/11 neurons impaled with 2M K-acetate electrodes and 4/4 neurons impaled with 2M K-gluconate electrodes. In 3/11 neurons impaled with a K-acetate electrode, senktide caused an inward current associated with an observed conductance decrease.

Channel blocker experiments. Tetraethylammonium (TEA) did not reduce the senktide current; the control current was -505 ± 95 pA while in the presence of TEA (10mM) the senktide current was -420 ± 26 pA, (V_H = -70mV, $p > 0.05$, n = 4). Cobalt chloride (2 mM) added to phosphate-free extracellular solution also did not reduce the senktide current. The control senktide response was -529 ± 59 pA while in the presence of cobalt, the senktide current was -485 ± 51 pA, (V_H = -80mV, $p > 0.05$, n = 4). Picrotoxin (30 μM) added to the Krebs solution did not affect the senktide current; in two neurons control responses were -460 and -500 pA and in the presence of picrotoxin, the current amplitudes were -410 and -600 pA (V_H = -80mV).

Niflumic acid (NFA) and mefenamic acid (MFA) inhibit GABA and senktide currents

NFA and MFA are reversible blockers of some chloride conductances (White & Aylwin, 1990). NFA (10 - 300 μM) and MFA (10 - 300 μM) inhibited $GABA_A$-mediated responses. The $GABA_A$ response was inhibited by 77 ± 5 % following superfusion with NFA (300μM) (V_H = -70 mV, n = 3, $p < 0.05$) and by 84 ± 4 % following superfusion with MFA (300μM) (V_H = -70 mV, n = 4, $p < 0.05$). The NFA EC_{50} was 72 μM ($n \geq 3$) and the MFA EC_{50} was 21 μM ($n \geq 3$).

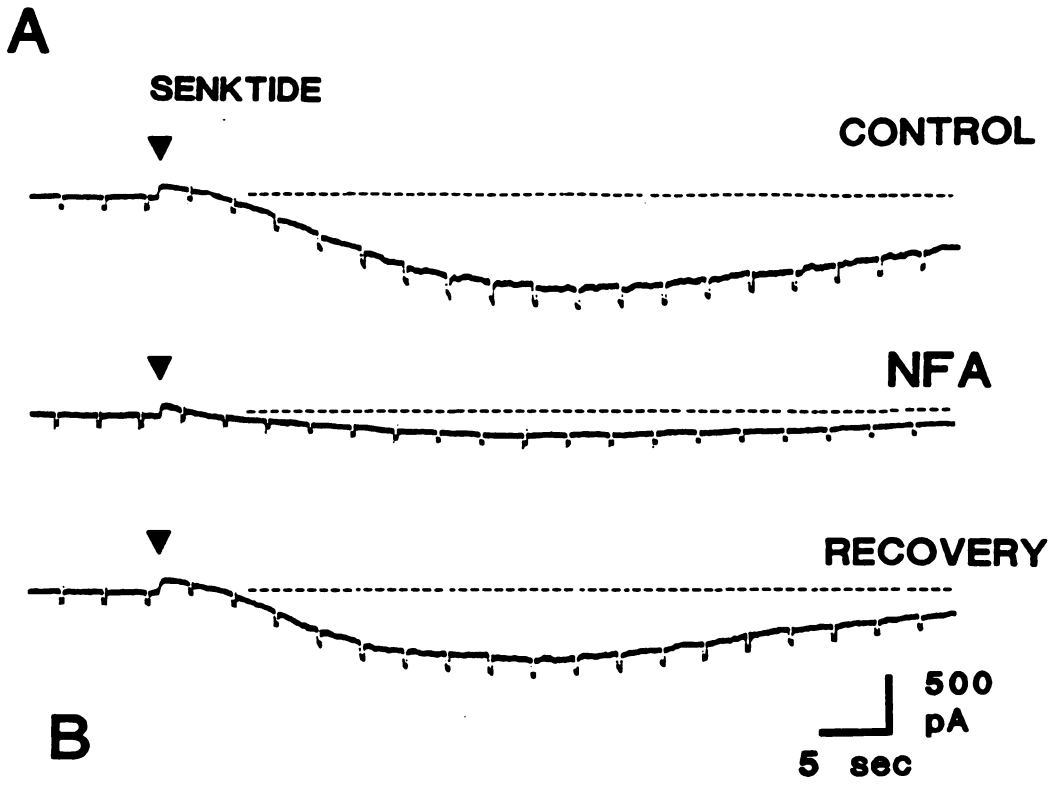
MFA and NFA caused an apparent increase in potassium conductance. NFA (100μM) caused a 3.5 ± 1.3 nS increase in conductance which reversed at 97.5 ± 2.5 (n = 4) and NFA (300μM) caused a 13.4 ± 2.7 nS increase in conductance which reversed at 102.5 ± 5.1 (n = 4). Similar data were obtained with MFA. In order to minimize activation of G_K, a concentration of 100 μM of MFA and NFA was chosen to study the effects of chloride conductance blockade on the senktide induced G_{IN}.

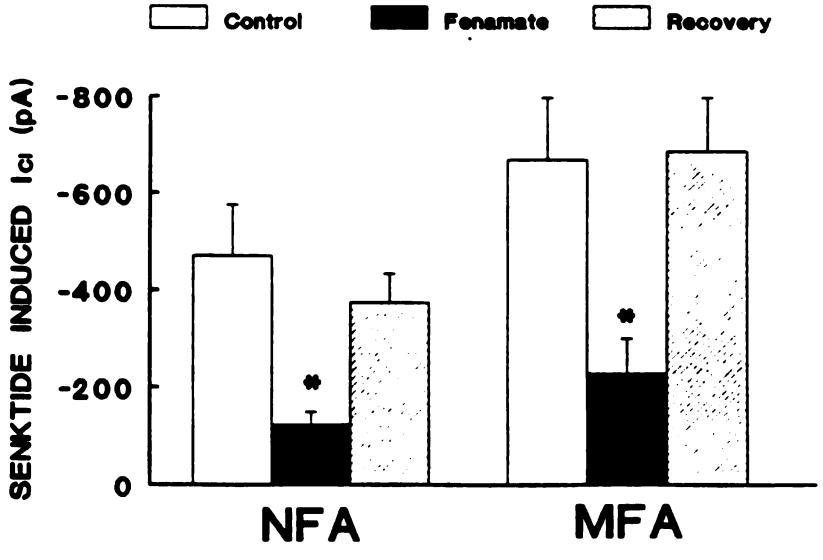
NFA and MFA inhibited the senktide-induced G_{IN} response. Senktide (3 μM) was pressure applied and only responses which exhibited an observed conductance increase were used. NFA (100 μM) and MFA (100 μM), both applied by superfusion, caused a significant block of the senktide-induced current (Figure 9). NFA caused a decrease of peak current from -471 ± 106 pA to -123 ± 27 pA (V_H = -85 mV, $p < 0.05$, n = 4). MFA caused a decrease of peak current from -651 ± 103 pA to -246 ± 57 pA (V_H = -85 mV, $p < 0.05$, n = 4). The specificity of the fenamates was tested under control conditions (ie. no forskolin or cesium). Neither the sEPSC nor the senktide-induced decrease in G_K were affected by the addition of MFA (300 μM). MFA also did not inhibit fEPSPs recorded from S neurons.

Figure 9. Niflumic acid (NFA) and mefenamic acid (MFA) inhibit senktide-induced currents

A. Senktide (3 μM) was pressure applied (▾) and caused an inward current (V_H = -85 mV, V_{STEP} = -105 mV). Forskolin (1μM), present to occlude senktide-induced inhibition of G_K, and TTX (0.3μM) were present. A control senktide response was obtained (top), then NFA (100 μM) was added to the superfusing Krebs 2 min prior to the middle senktide response. The senktide response recovered 5 min after washout of NFA (bottom). **B.** NFA and MFA inhibit senktide currents associated with a G_{IN}. * indicates significant depression of senktide currents in the presence of NFA (n=4) and MFA (n=4) ($p < 0.05$).

Figure 9


A
SENKTIDE
CONTROL
NFA
RECOVERY
B
500
pA
5 sec


Control
Fenamate
Recovery
SENKTIDE INDUCED I_{Cl} (pA)
-800
-600
-400
-200
0
NFA
MFA

Control fEPSPs were 19.8 ± 0.9 mV, and in the presence of MFA (100μM) fEPSPs were 20.5 ± 0.8 mV (V_H = -90mV, $p > 0.05$, n = 3).

NFA and MFA are potent non-steroidal anti-inflammatory drugs (Stutts, Henke & Boucher, 1990). The possibility that inhibition of cyclo-oxygenase mediated the inhibition of chloride currents was tested. Indomethacin (30 - 300 μM) did not inhibit the senktide-induced G_{IN}, the control response was -160 ± 72 pA and in the presence of indomethacin, the senktide current was -193 ± 64 pA (V_H = -80mV, $p > 0.05$, n = 2). Indomethacin (100 - 300 μM) also did not alter the $GABA_A$ response, the control response was -419 ± 95 pA and in the presence of indomethacin, the GABA current was -384 ± 70 pA (V_H = -70, $p > 0.05$, n = 3). Based upon these and the above observations, I conclude that the senktide-induced G_{IN} and most likely the sEPSC-induced G_{IN} are a chloride conductance (G_{Cl}).

A G_{Cl} increase is present in sEPSCs and senktide responses which exhibit an apparent conductance decrease

Distribution of G_K and G_{Cl} in sEPSCs. Forskolin was used to determine the relative distribution of G_K and G_{Cl} in sEPSCs exhibiting an observed conductance decrease. V_H was set to yield the resting G_M (V_H -55 to -70 mV) and changes in chord conductance between this and a step command (V_{STEP} of 20 mV negative to V_H every 2.2 s) were analyzed. Under these conditions, G_{Cl} would become more prominent as was the case with non-reversing sEPSCs (see Figure 6). In 20/35 (57%) neurons, forskolin occluded sEPSCs by 83 ± 7%. This is consistent with these sEPSCs being mediated by a decrease in G_K only. In 15/35 (43%) neurons, forskolin potentiated sEPSCs by 42 ± 25% and the conductance change

during the sEPSC was converted to either an unclear conductance change or an increase in chloride conductance.

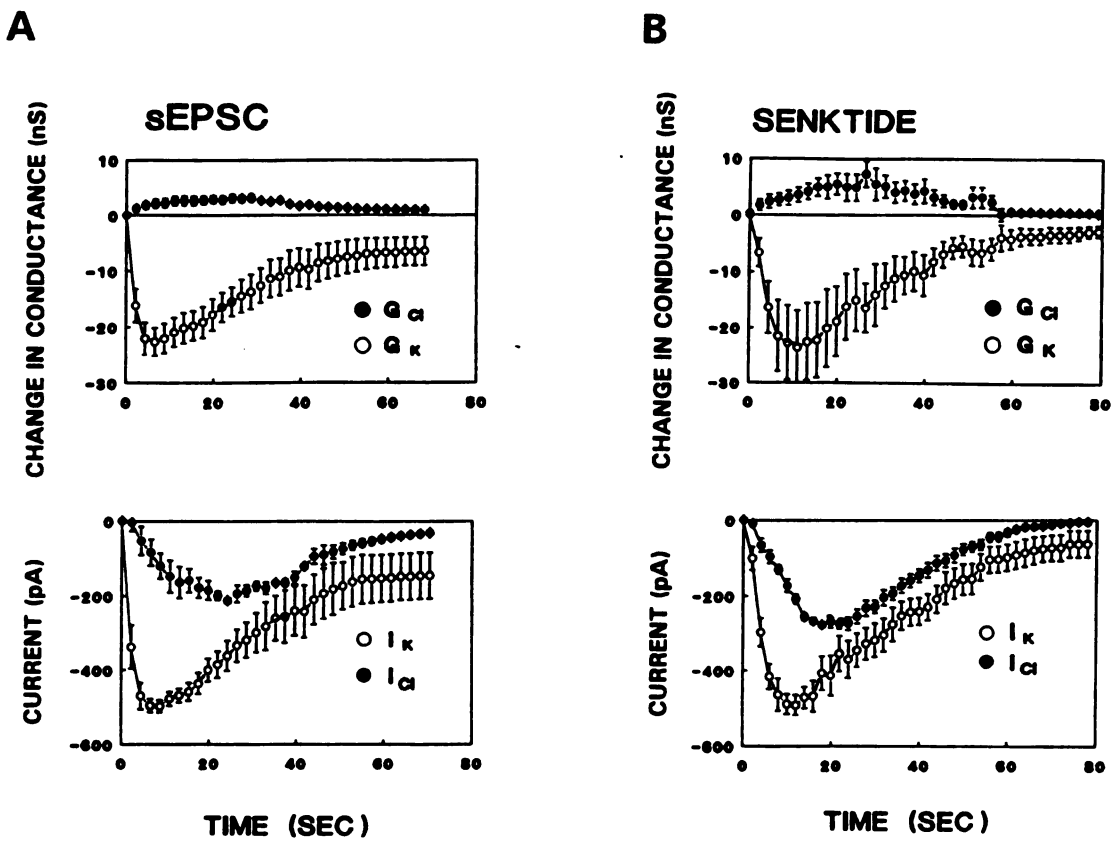
Relative proportions of G_K to G_{Cl} during the sEPSC. In order to determine the relative proportions of G_K and G_{Cl} contributing to sEPSCs exhibiting an observed conductance decrease, changes in chord conductance between the holding potential (V_H -55 to -70 mV) and the step command (V_{STEP} = 20 mV negative to V_H, every 2.2 s) were analyzed. At the peak of the sEPSC, the absolute conductance change was composed of a 91 ± 3% decrease in G_K and a 9 ± 3% increase in G_{Cl}. The times to peak for I_K and I_{Cl} were 8 ± 1 s and 20 ± 2 s respectively. The proportion of G_K to G_{Cl} at the time of peak I_K (G_K = 92 ± 2% , G_{Cl} = 8 ± 2%) was different than at the time of peak I_{Cl} (G_K = 81 ± 3% ,G_{Cl} = 19 ± 3%)(Figure 10A).

Relative proportions of G_K to G_{Cl} during the senktide response. Conductance changes associated with *conductance decrease* type senktide responses were analyzed in the presence of TTX (300 nM). Senktide currents and conductances were measured as described above (V_H -60 to -70 mV, n = 10). At the peak of the senktide-induced current, the absolute conductance change was composed of a 79 ± 12% decrease in G_K and a 21 ± 12% increase in G_{Cl}. The times to peak for I_K and I_{Cl} were 12 ± 2 s and 20 ± 1 s respectively. The proportion of G_K to G_{Cl} at the time of peak I_K (G_K = 81 ± 11% ,G_{Cl} = 19 ± 11%) was different than at the time of peak I_{Cl} (G_K = 66 ± 10% ,G_{Cl} = 34 ± 10%). The rate of rise for I_K (*tau* =

Figure 10. K^+ and Cl^- components isolated from sEPSCs and senktide responses that were associated with an apparent conductance decrease

V_H ranged from -55 to -75 mV (TTX 0.3μM present in B). Current and chord conductance measurements were made every 2.2 s. **A. (Top)** Average conductance changes associated with the sEPSC. G_K was 90% of the ΔG while G_{Cl} was 10% (n = 8). **(Bottom)** Currents were averaged and the values of each component plotted. **B. (Top)** Average conductance changes associated with the senktide response were similar to the sEPSC (n = 10). **(Bottom)** Average senktide-induced currents.

Figure 10


A
sEPSC
CHANGE IN CONDUCTANCE (nS)
10
0
-10
-20
-30
G_{Cl}
G_K
0
20
40
60
80
CURRENT (pA)
0
-200
-400
-600
I_K
I_{Cl}
TIME (SEC)
B
SENKTIDE
CHANGE IN CONDUCTANCE (nS)
CURRENT (pA)
TIME (SEC)

7.2 s) was faster than the rate of rise for I_{Cl} (*tau* = 16.6 s) while the rate of decay for I_K (*tau* = 27.3 s) was slower than that of I_{Cl} (*tau* = 18.6 s) (Figure 10B).

SIGNAL TRANSDUCTION FOR THE sEPSC

The sEPSC and senktide response couple to a G-protein

GTP-γ-S is a non-hydrolyzable analog of GTP which binds to and irreversibly activates an activated G-protein. ATP-γ-S should be inactive and GDP-β-S should inhibit G-proteins under similar conditions. These compounds were dissolved in 2M KCl at their final concentrations and placed in the recording electrode. Impaled neurons were injected with the negatively charged modulator by applying negative holding current (approximately -400 pA) for 5 min before either senktide was applied (3 μM, pressure ejection) or a slow synaptic response was evoked.

GTP-γ-S (10 - 20 mM). Senktide was applied to 8 neurons. In 2 of 8 neurons, the resting membrane conductance appeared to spontaneously decrease and senktide did not produce a current. In 6 of 8 neurons, an average of 2 to 3 senktide responses per neuron could be evoked (Figure 11A,B). These conductances were analyzed using a two parameter model (G_K and G_{Cl}). Senktide was applied twice which caused a -33.5 ± 5.7 nS change in potassium conductance that was sustained at -34.0 ± 5.5 nS (101 % of control) and a peak +2.8 ± 1.2 nS change in chloride conductance that was sustained at +2.8 ± 1.1 nS (100 % of control)(Figure 12A). The sEPSC was evoked in 11 neurons. In 4 of 11 neurons, the resting

Figure 11. GTP-γ-S, but not ATP-γ-S cause irreversible activation of the senktide response

GTP-γ-S was iontophoresised into neurons with negative holding current (-200 to -400 pA) for 5 min. before either senktide application (▼) or nerve stimulation (↓)(see Fig. 3). **A.** Senktide applied to a neuron impaled with standard 2M KCl (upper) or with the addition of GTP-γ-S (10 mM)(lower, V_H = -70mV). In control, senktide responses become smaller over time as the neuron became more excitable. With GTP-γ-S present this process was greatly potentiated. Last application of senktide at ~7 min. **B.** ATP-γ-S (20 mM) does not cause activation of currents (V_H = -70 mV, left), but GTP-γ-S (20 mM) does (V_H = -60 mV, right).

Figure 11

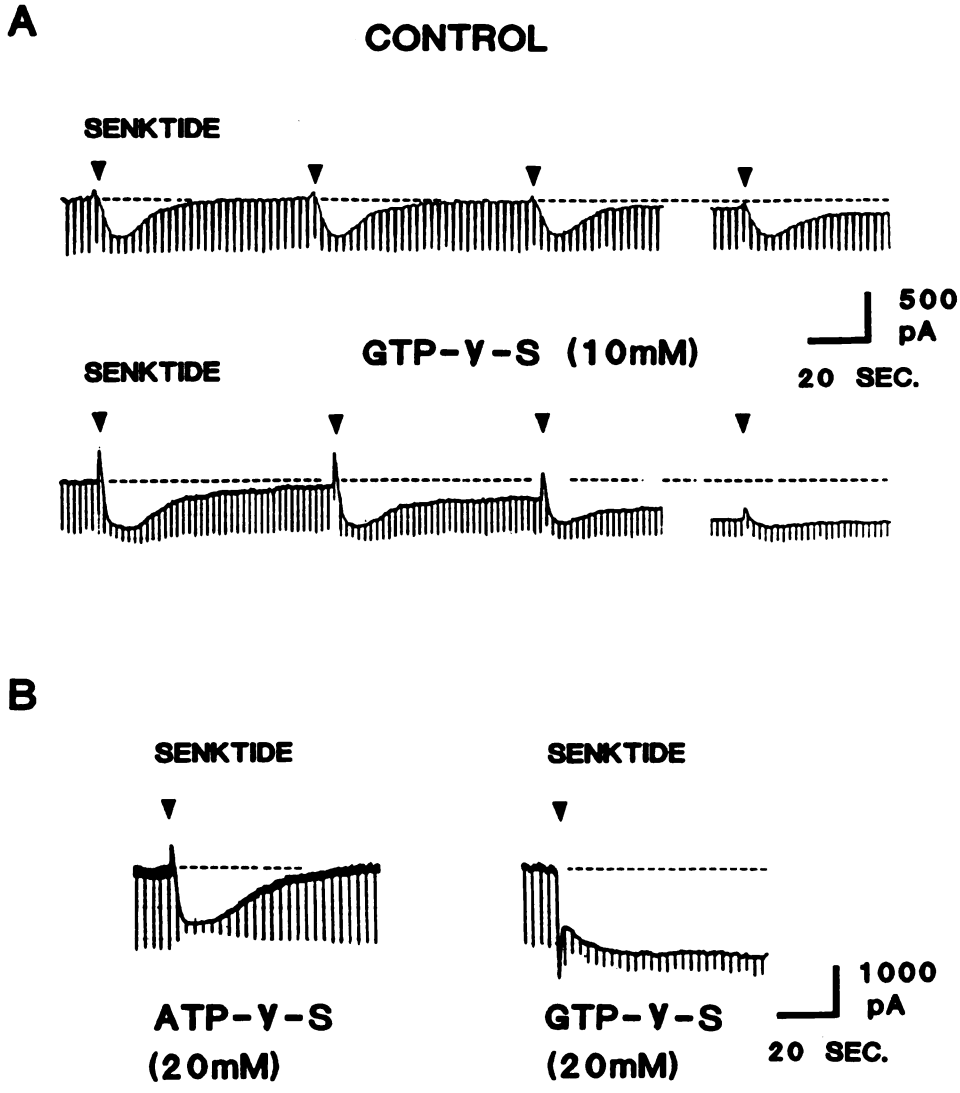
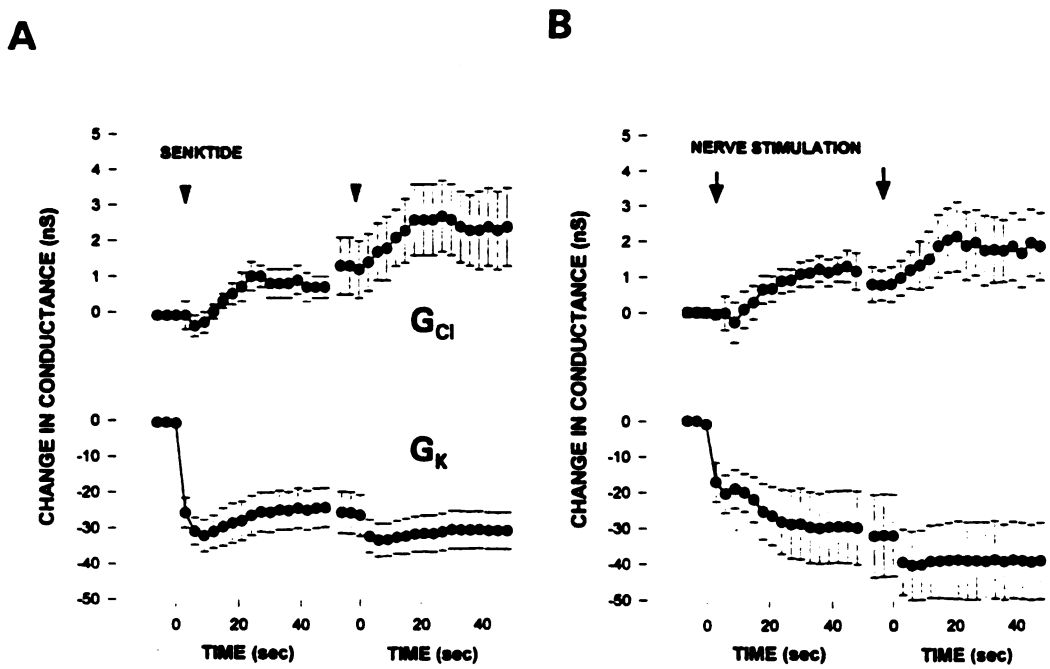

A
CONTROL
SENKTIDE
500
pA
GTP-γ-S (10mM)
SENKTIDE
20 SEC.
B
SENKTIDE
SENKTIDE
1000
pA
ATP-γ-S
(20mM)
GTP-γ-S
(20mM)
20 SEC.

Figure 12. GTP-γ-S causes activation of the sEPSC and senktide response

Recording electrodes were filled with 2M KCl + 20 mM GTP-γ-S. **A.** Senktide (3 μM) was pressure ejected at the (▼) at time zero and at 119 ± 15s (V_H = -79 ± 2 mV; n = 6). Resultant currents were analyzed using a two parameter (G_K and G_{Cl}) model of conductance (see *Methods*). GTP-γ-S was effective in causing irreversible activation of both G_{Cl} (top) and G_K (bottom). Neither current showed significant decay after 5 min (last point). **B.** A sEPSC was evoked at the (↓) at time zero and at 88 ± 11s (V_H = -79 ± 3 mV; n = 7). Analysis of conductances show both G_{Cl} and G_K were sustained at 5 min (last point).

Figure 12


A
B
SENKTIDE
NERVE STIMULATION
CHANGE IN CONDUCTANCE (nS)
G_{Cl}
G_K
TIME (sec)
TIME (sec)
TIME (sec)
TIME (sec)

membrane conductance appeared to spontaneously decrease and senktide did not produce a current. In 7 of 11 neurons, an average of 2 to 3 sEPSCs per neuron could be evoked. In 7 neurons, the sEPSC was evoked twice which caused a peak -40.3 ± 9.6 nS change in potassium conductance that was sustained at -42.9 ± 10.2 nS (106 % of control) and a peak +2.1 ± 1.0 nS change in chloride conductance that was sustained at +2.2 ± 1.2 nS (105 % of control)(Figure 12B).

ATP-γ-S (20 mM). No significant changes in the time course of the senktide response or the sEPSC were observed (V_H = -66 mV). An average of 7 senktide responses per neuron could be evoked in 8 neurons. The initial 2 to 3 responses were -398 ± 50 pA and were sustained -12 ± 50 pA (3 % of control, $p > 0.05$). An average of 4 sEPSCs were evoked in 5 of these 8 neurons. The initial 2 to 3 sEPSCs were -195 ± 32 pA and were sustained at -44 ± 31 pA (33 % of control, $p > 0.05$)(Figure 11B).

GDP-β-S (20 - 60 mM). No changes in senktide or sEPSC generation were observed. Senktide responses were elicited in 4 of 8 neurons with an average of 3 responses per neuron. A sEPSC was evoked in 6 of 9 neurons with an average of 4 responses per neuron.

G-proteins are pertussis toxin (PTX) insensitive

Myenteric plexus. Six preparations were incubated with 25 μg/mL of PTX (holoenzyme) for 35 to 45 min at 25 to 37°C. No attempt was made to determine the contribution of G_{Cl} to these responses. In PTX-treated preparations, a slow synaptic response could be evoked in 21 of 24 neurons (V_H of - 69 ± 2 mV). The average amplitudes were +14 ± 2 mV (n = 12) and -246 ± 58 pA (n = 7) respectively. In control incubated tissue, the values were +16 ± 3

mV (n = 5) and -388 ± 72 pA (n = 5, $p > 0.05$). In PTX-treated preparations, a response to superfused (100 - 300 nM) or pressure applied senktide (3 μM) could be evoked in 21 of 24 neurons. The average amplitudes were +18 ± 2 mV (n = 10) and -426 ± 49 pA (n = 6). In control incubated tissue, the values were +22 ± 2 mV (n = 5) and -354 ± 102 pA (n = 6, $p > 0.05$)(Figure 13A).

Submucosal plexus. In submucosal neurons α_2 receptors are coupled to an increase in G_K *via* a PTX-sensitive G-protein (Surprenant & North, 1988). To verify the effectiveness of our PTX treatment protocol, submucosal preparations were treated and recorded from as described above. In control submucosal preparations, UK 14,304 (1 μM) and noradrenaline (3 μM) which are agonist at the α_2 adrenergic receptor caused a -24 ± 2 mV hyperpolarization (n = 10 in 4 preparations). In PTX-treated tissue these agonist caused a -2 ± 1 mV hyperpolarization (V_H = -57 mV, n = 11 in 2 preparations, $p < 0.05$). In control submucosal preparations, nerve stimulation evoked an α_2-mediated inhibitory post-synaptic potential (IPSP)(Surprenant & North, 1988). The submucosal IPSP was -11 ± 2 mV in control (n = 12) and -1 ± 1 mV in PTX-treated tissue (n = 12, $p < 0.05$)(Figure 13B).

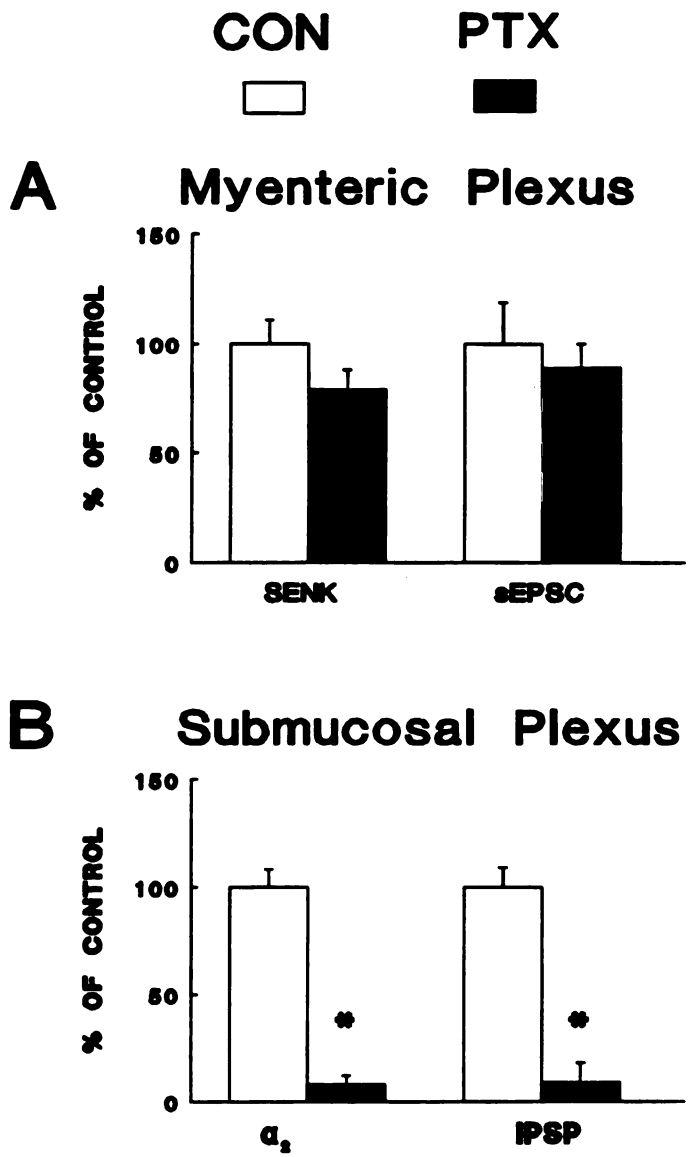
PDBu and forskolin inhibit G_K, but do not activate G_{Cl}

Phorbol ester. PDBu (0.001 to 1 μM) applied by superfusion, caused an inward current with an EC_{50} value of 0.015 μM (n = 5). I/V relationships yielded a reversal potential of -97 ± 1 mV and were tested for the presence of a significant increase in G_{Cl}. In 5 of 5 neurons, a one parameter (G_K) equation fit the data as well as a two parameter equation (G_K and G_{Cl}) indicating that PDBu did not cause a significant increase in conductance. PDBu caused an

Figure 13. The sEPSP is not reduced by PTX

Myenteric plexus and submucosal plexus preparations were incubated with PTX (see *Methods*). Synaptic and agonist responses were evoked in control (CON) and PTX-treated preparations. **A.** In the myenteric plexus, the sEPSP and senktide responses were unchanged in PTX-treated groups ($n = 10$, $p > 0.05$). Data are the average of current and voltage measurements. **B.** In the submucosal plexus, IPSPs and α_2 agonist responses were reduced in PTX-treated tissues ($n = 9$, $p < 0.05$). * indicates significantly different than control ($p < 0.05$).

Figure 13


CON
PTX
A
Myenteric Plexus
% OF CONTROL
150
100
50
0
SENK
sEPSC
B
Submucosal Plexus
% OF CONTROL
150
100
50
0
*
*
α_2
IPSP

inhibition of the AHC with an EC_{50} value of 0.05 μM (n = 6), but did not have a direct effect on the calcium spike. In the presence of PDBu (300 nM), the amplitude of the calcium spike was 95 ± 4 % of control and the duration was 100 ± 2 % of control (n = 3, p > 0.05). The inhibition of the AHC slow synaptic response or senktide is also not due to reduction of the calcium spike. The integrity of the calcium spike was investigated using the methods of Cherubini & North (1984). During the sEPSP, the amplitude of the calcium spike was 100 ± 4 % of control and the duration was 98 ± 2 % of control (n = 3, p > 0.05). In the presence of senktide (300 nM), the amplitude of the calcium spike was 98 ± 2 % of control and the duration was 97 ± 5 % of control (n = 3, p > 0.05).

4-α PDBu did not cause an inward current or reduce the AHC. In the presence of 4-α PDBu (1 μM) the change in current was -32 ± 27 pA and the AHC was 113 ± 31 % of control, in the presence of PDBu (300 nM) the change in current was -400 ± 116 pA and the AHC was 34 ± 13 % of control (V_H = -61 mV, n = 3, p < 0.05)(Figure 14,15).

Forskolin. Forskolin (0.01 to 3 μM) applied by superfusion caused an inward current with an EC_{50} value of 0.08 μM (n = 6) and has been described previously. I/V relationships for 14 neurons yielded a reversed potential of -95 ± 3 mV and were tested for the presence of a significant increase in G_{Cl}. In 11 of 14 neurons, a one parameter (G_K) equation fit the data as well as the two parameter equation (G_K and G_{Cl}) indicating that forskolin did not cause a significant increase in conductance. In 3 of 14 neurons, the two parameter equation fit significantly better than the one parameter equation. These neurons contained a significant conductance increase and reversed more negative than E_K. Forskolin caused an inhibition of the AHC with an EC_{50} value of 0.3 μM (n = 5), but did not have any effect on the calcium

Figure 14. PDBu inhibits resting and spike activated G_K

PDBu was superfused over myenteric AH-neurons. **A.** PDBu (30 nM at the bar) caused a -600 pA sustained inward current which was associated with a decrease in conductance (V_H = -60 mV; V_{STEP} = -10 mV). **B.** The AHC (curved arrow ➦) was inhibited by PDBu (100 nM). Successive AHCs were evoked at the indicated times after start of PDBu superfusion (V_H = -70 mV; V_{STEP} = -10 mV). Control AHC was +440 pA and in the presence of PDBu (at 120 s) was +80 pA. **C.** I/V relationships from 5 AH-neurons. The reversal potential of the PDBu-induced current was -97 ± 1 mV.

Figure 14

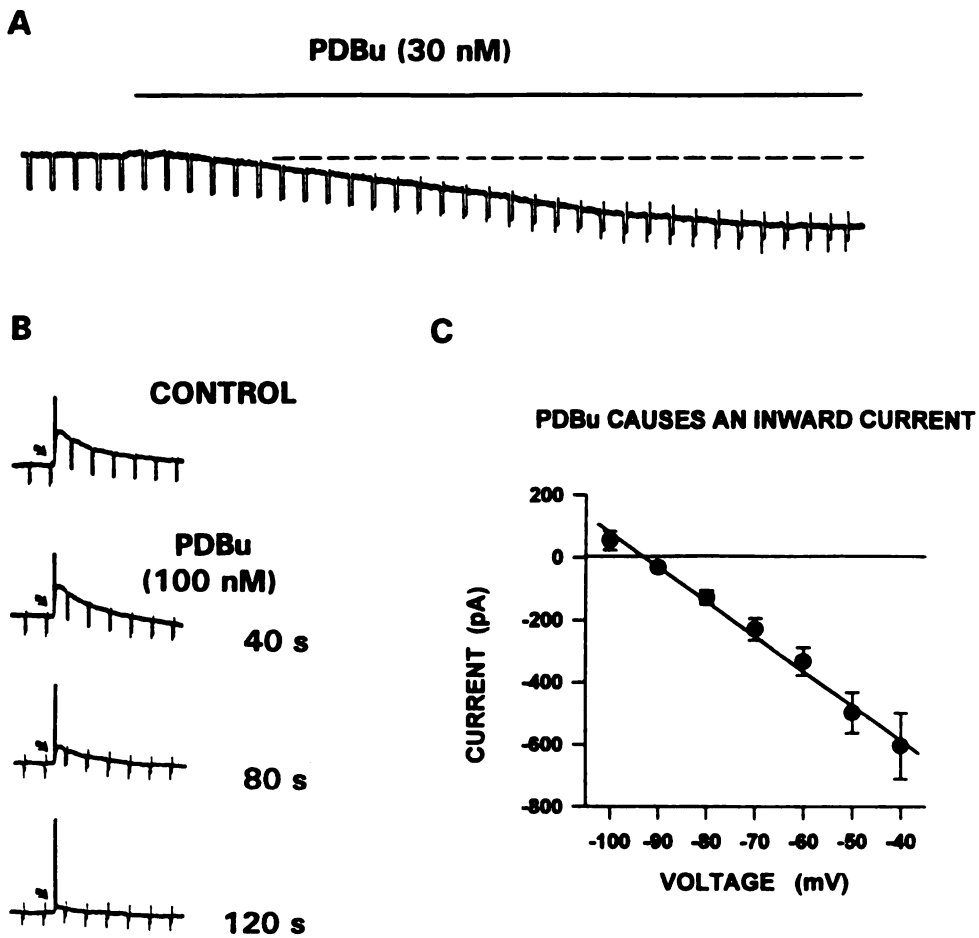
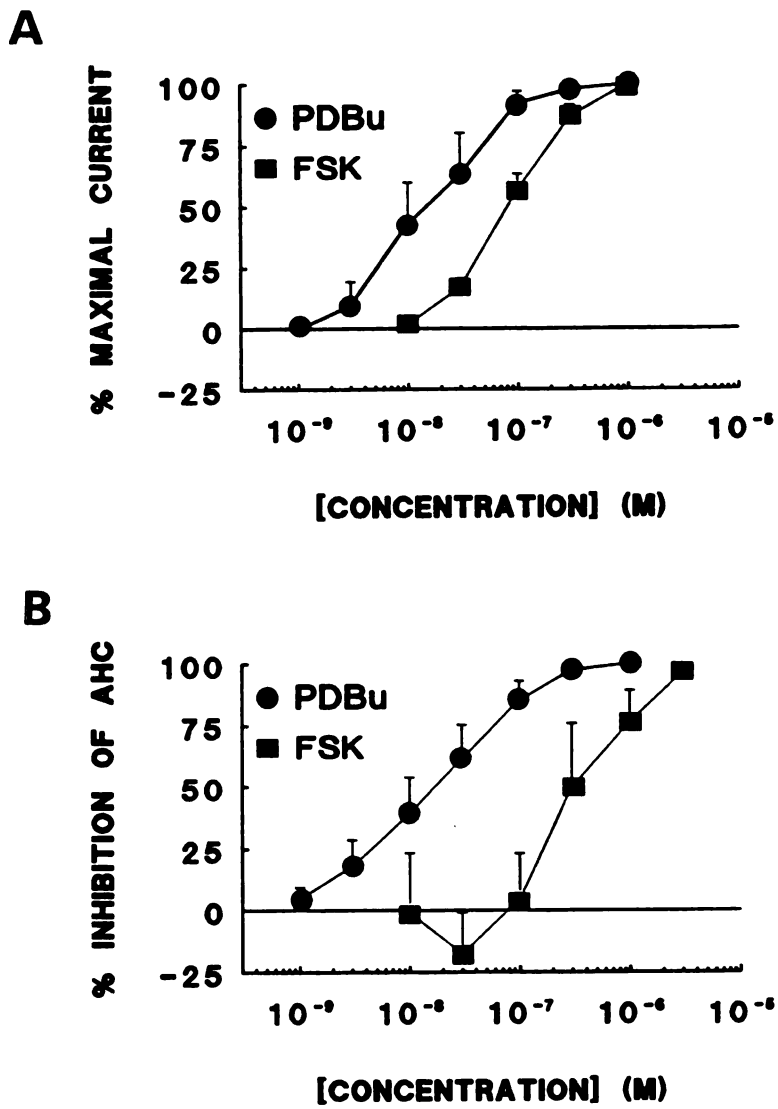

A
PDBu (30 nM)
B
CONTROL
PDBu
(100 nM)
40 s
80 s
120 s
C
PDBu CAUSES AN INWARD CURRENT
CURRENT (pA)
200
0
-200
-400
-600
-800
-100 -90 -80 -70 -60 -50 -40
VOLTAGE (mV)

Figure 15. PDBu and Forskolin inhibit G_K

PDBu (0.001 to 1μM) or forskolin (0.01 to 3 μM) produced a sustained inward current associated with a conductance decrease and an inhibition of the AHC. **A.** EC_{50} values for decreases in resting G_K were FSK = 0.08 μM (n = 6); PDBu = 0.015 μM (n = 5). **B.** EC_{50} values for inhibition of the AHC were FSK = 0.3 μM (n =5); PDBu = 0.05 μM (n = 6).

Figure 15


A
% MAXIMAL CURRENT
100
75
50
25
0
-25
PDBu
FSK
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
[CONCENTRATION] (M)
B
% INHIBITION OF AHC
100
75
50
25
0
-25
PDBu
FSK
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
[CONCENTRATION] (M)

spike. In the presence of forskolin (1 μM), the amplitude of the calcium spike was 94 ± 2 % of control and the duration was 101 ± 4 % of control (n = 6, p > 0.05). 1, 9 dideoxyforskolin (3 μM) did not cause an inward current or reduce the AHC. In the presence of 1, 9 dideoxyforskolin the change in current was + 31 ± 21 pA and the AHC was 94 ± 25 % of control, in the presence of forskolin (1 μM) the change in current was -342 ± 106 pA and the AHC was 13 ± 5 % of control (V_H = -66 ± 4 mV, n = 4, p < 0.05)(Figure 15).

PDBu potentiates the forskolin induced inhibition of the AHC. Forskolin (10 nM) and PDBu (1 to 3 nM) were superfused over 13 AH neurons. In 5 of 13 neurons forskolin or PDBu alone decreased the AHC by more than 30 %. These neurons were not studied further. In 8 of 13 neurons forskolin or PDBu alone caused a small (max <30 % of control, average 7% of control) reduction in the amplitude of the AHC. Forskolin was then added in the presence of superfusing PDBu and in 6 of 8 neurons caused a more than additive (51.7 %) reduction in the AHC (V_H = -62 ± 4 mV; V_{STEP} = -20 mV). The control amplitude of the AHC was +375 ± 20 pA and in the presence of both PDBu and forskolin was +181 ± 43 pA (p < 0.05)(Figure 16). In 2 of 8 neurons PDBu and forskolin caused a slight increase in the AHC, most likely indicating one or both compounds were at a sub-threshold concentration (see Figure 14).

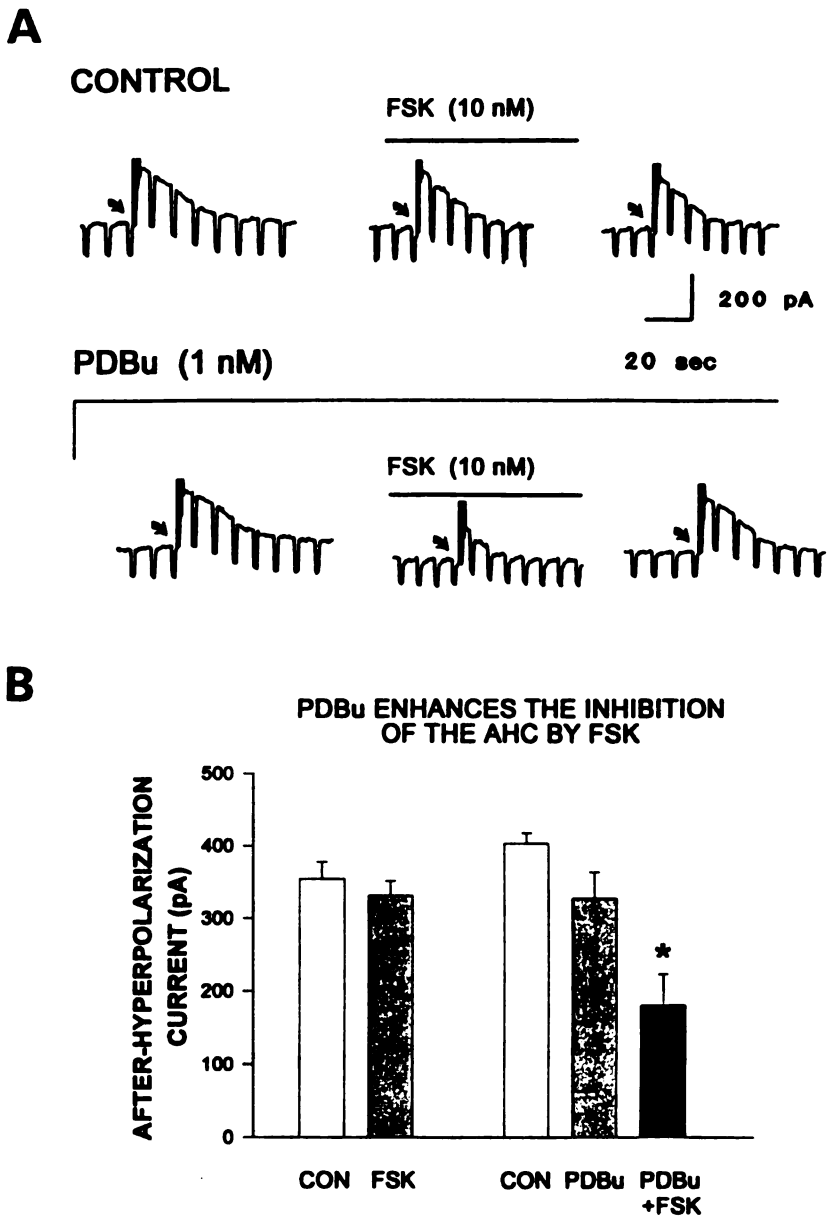
Kinase inhibitors reduce the sEPSC and prevent inhibition of the AHC

Staurosporine (10 - 100 μM) was superfused for .5 to 1.5 hours. It did not produce any effects on resting current (or potential) or on action potential generation. The slow synaptic, senktide, forskolin and PDBu responses were tested alone to obtain a control response and

Figure 16. PDBu enhances the effects of forskolin

Forskolin (10 nM) and PDBu (1 - 3 nM) were superfused over 13 AH-neurons. **A.** In 8 of 13 neurons both forskolin and PDBu alone caused a small ($p > 0.05$), reduction in the amplitude of the AHC. Forskolin was then added in the presence of superfusing PDBu and in 6 of 8 neurons caused a large reduction in the AHC (V_H = -62 ± 4 mV; V_{STEP} = -20 mV) and 2 of 8 neurons caused a slight increase in the AHC. **B.** The control amplitude of the AHC was +375 ± 20 pA and in the presence of both PDBu and forskolin was +181 ± 43 pA ($p < 0.05$). (*) indicates significantly different than other data ($p < 0.05$).

Figure 16


A
CONTROL
FSK (10 nM)
200 pA
PDBu (1 nM)
20 sec
FSK (10 nM)
B
PDBu ENHANCES THE INHIBITION
OF THE AHC BY FSK
AFTER-HYPERPOLARIZATION
CURRENT (pA)
500
400
300
200
100
0
*
CON
FSK
CON
PDBu
PDBu
+FSK

then again in the presence of staurosporine. Most responses showed a time-dependent reduction in amplitude. The sEPSC was inhibited by 38.6 ± 7.6 % of control (n= 15, $p < 0.05$) while the senktide response was inhibited by 59.4 ± 12.8 % of control (n = 6, $p < 0.05$). The forskolin response was inhibited by 64.5 ± 15.1 % of control (n = 8, $p < 0.05$) while the PDBu response was inhibited by 94.4 ± 5.9 % of control (n = 5, $p < 0.05$) (Figure 17). In general, the effects of PDBu were inhibited sooner than those of forskolin while the senktide response or the sEPSC were inhibited last. Fast synaptic transmission was not affected by similar treatments. In control, the fEPSP was 22 ± 8 mV (V_H = -96 ± 22 mV, n = 100, from Galligan & Bertrand, 1994) and in staurosporine (10 μM) treated preparations the fEPSP was 17 ± 7 mV (V_H = -72 ± 15 mV, n = 8, values were within 95 % confidence limits of control).

K-252a and staurosporine prevent the inhibition of the AHC which is normally associated with the slow synaptic, senktide, forskolin or PDBu response (see above). In control, the sEPSC was associated with a 82.1 ± 2.1% inhibition of the AHC (n = 12). During superfusion with K-252a or staurosporine this value was reduced to 6.3 ± 14.3 or 2.9 ± 14.2 % inhibition respectively (n = 16 and 12, $p < 0.05$). Senktide caused a 83.3 ± 4.5 % inhibition of AHC in control and in the presence of inhibitor caused a 78.7 ± 11.4 or 38.4 ± 16.7 % inhibition respectively (n = 7 and 9, $p < 0.05$). Forskolin caused a 80.3 ± 2.8 % inhibition of the AHC in control and in the presence of inhibitor caused a 26.6 ± 9.9 or 21.2 ± 12.4 % inhibition respectively (n = 15 and 9, $p < 0.05$). PDBu caused a 88.4 ± 6.4 % inhibition of the AHC in control and in the presence of inhibitor caused a 39.0 ± 14.6 and 27.0 ± 14.9 % of inhibition respectively (n = 3 and 6, $p < 0.05$)(Figure 18).

Figure 17. Staurosporine inhibits the decrease in G_K

Staurosporine (10 - 100 μM) was superfused over 25 neurons in 9 preparations. **A.** Staurosporine caused a time-dependent reduction of the sEPSC, but did not eliminate it. Staurosporine had only weak effects on the resting membrane potential neurons (V_H = -55 mV; V_{STEP} =). **B.** Histogram illustrating the effects of staurosporine. Ordinate is % inhibition of the sEPSC, senktide (SK), forskolin (FSK) or PDBu response. The sESPC was inhibited the least, while the PDBu response was inhibited the most. Data represents combined current and voltage measurements ($n \geq 5$, * indicates $p < 0.05$).

Figure 17

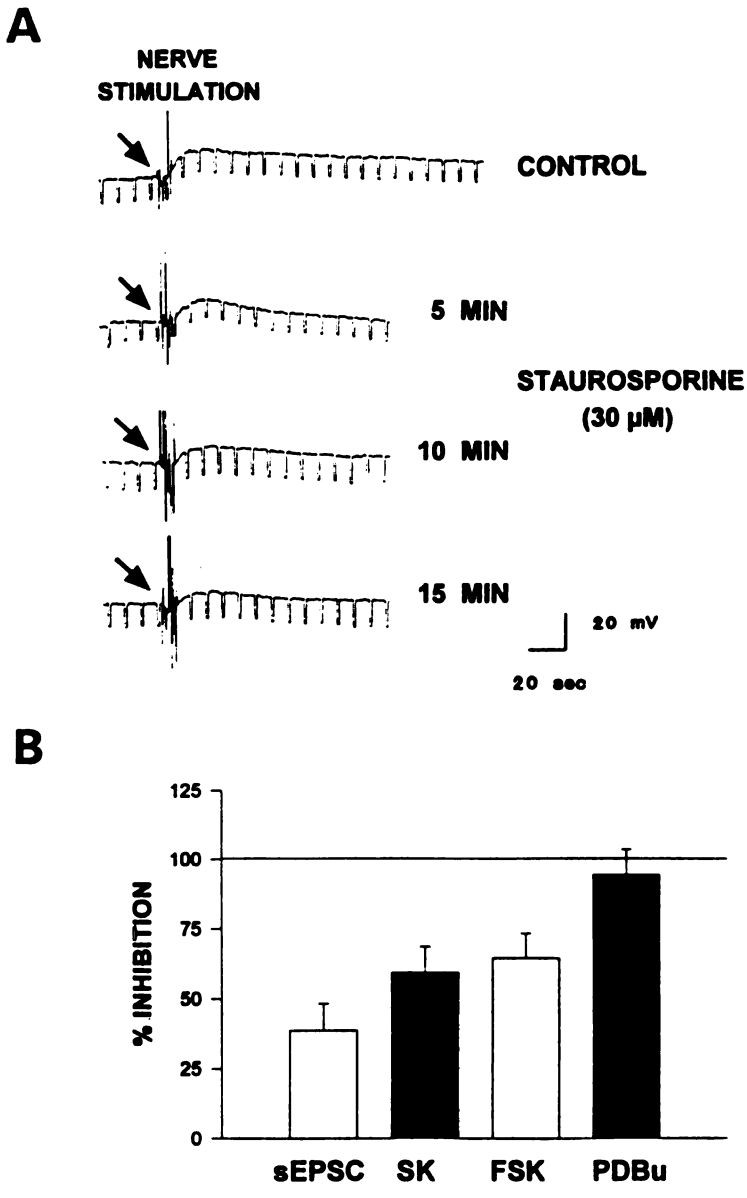
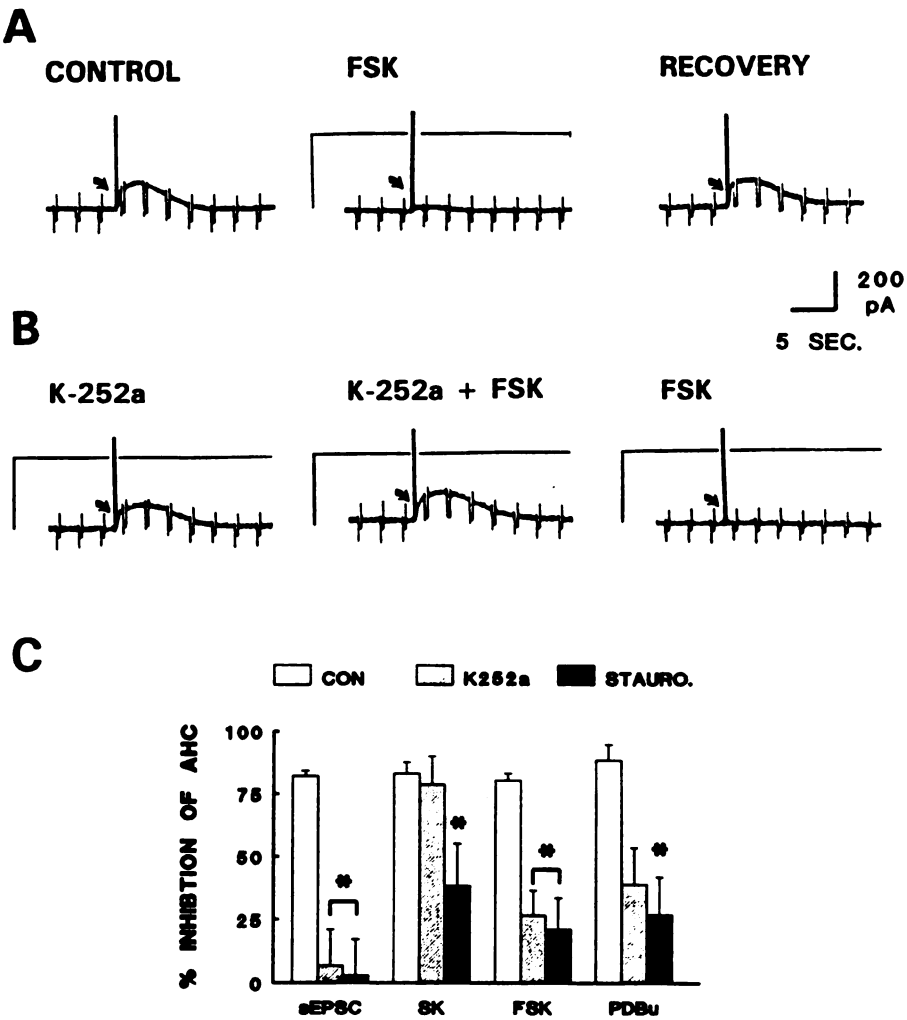

A
NERVE
STIMULATION
CONTROL
5 MIN
STAUROSPORINE
(30 µM)
10 MIN
15 MIN
20 mV
20 sec
B
% INHIBITION
125
100
75
50
25
0
sEPSC
SK
FSK
PDBu

Figure 18. Protein kinase inhibitors prevents inhibition of the AHC

K-252a and staurosporine effectively block the inhibition of the AHC normally seen during the sEPSC or other agonist responses. **A.** Forskolin (1 μM) was superfused during the bar. The AHC (curved arrow ➦) was inhibited by forskolin and recovered upon washout of the drug (V_H = -60 mV; V_{STEP} =). **B.** K-252a (30 μM) was superfused during the first and second traces, while forskolin was present in the second and third traces. The effects of forskolin are blocked by concurrent application of K-252a. **C.** Histogram illustrating the effects of >10 min. superfusion of K-252a or staurosporine versus the sEPSC, senktide (SK), forskolin (FSK) and PDBu. Ordinate is % inhibition of the AHC during ($n \geq 3$, * indicates $p < 0.05$).

Figure 18


A
CONTROL
FSK
RECOVERY
200 pA
5 SEC.
B
K-252a
K-252a + FSK
FSK
C
CON
K252a
STAURO.
% INHIBITION OF AHC
100
75
50
25
0
*
*
*
*
sEPSC
SK
FSK
PDBu

Inhibition of phospholipase C (PLC)

The specific inhibitor of PLC, O-tricylco {5.2.1.0 ^{2,6}] dec-9-yl dithiocarbonate (D609) was effective in reducing the slow synaptic response and the senktide response and causing a shift in the resting membrane potential. Addition of D609 to the superfusion Krebs caused a variable shift in membrane potential which was corrected for with direct current. In 3 of 15 neurons, D609 (300 μM) caused a 6.2 ± 1.6 mV depolarization associated with an increase in resistance. In 5 of 13 neurons, D609 caused a 7.9 ± 3.7 mV hyperpolarization associated with an unclear change in resistance (V_H = -70 ± 2 mV). In control, the sEPSP was +11.7 ± 1.7 mV and in the presence of D609 was +5.6 ± 1.5 mV (52 % inhibition) (n = 10, $p < 0.05$). In control, the senktide response was +20.1 ± 2.7 mV and in the presence of D609 was +7.1 ± 3.6 mV (74 % inhibition) (n = 7, $p < 0.05$). (Figure 19). The amplitude of the fEPSP was not affected by D609 (100 μM). In control, the fEPSP was 16.9 ± 1.1 mV, and in the presence of D609 was 16.7 ± 1 mV (V_H = -100 ± 6 mV, n = 3, $p > 0.05$).

Phosphatase inhibitors modulate G_K

Calyculin A (100 nM, superfusion) caused an average inward current of -254 ± 68 pA (V_H of -65 ± 2 mV) and was associated with a -11.8 ± 2.8 nS change in conductance (n = 9). I/V relationships were estimated to reverse at -90 ± 2 mV and were tested for the presence of a significant increase in G_{Cl}. In 6 of 7 neurons, a one parameter (G_K) equation fit the data as well as a two parameter equation (G_K and G_{Cl}) indicating that calyculin A did not cause a significant increase in conductance. In 1 of 7 neurons, a two parameter equation fit better, indicating a significant contribution of a conductance increase. Calyculin A (100 nM) caused an inhibition of the AHC. In control, the AHC was +428 ± 60 pA and in the presence of

Figure 19. Inhibition of phospholipase C reduces the sEPSP

D609 (300 μM) was superfused over 13 AH-neurons in 8 preparations. sEPSPs were evoked at the (↓) and at the indicated times after start of D609 superfusion. D609 caused a time-dependent decrease in the amplitude of the sEPSP, but only washed out in some neurons. Recovery in this cell is at 10 min. after stopping D609 superfusion (RMP = -70 mV; V_{STEP} = -250 pA). **B.** On average, the control sEPSP was +12.5 ± 2.3 mV and in the presence of D609 was +5.3 ± 2.1 mV (52 ± 13 % inhibition, $p < 0.05$, $n = 10$). The control senktide response was +20.1 ± 2.7 mV and in the presence of D609 was +7.1 ± 3.6 mV (64 ± 18 % inhibition, $p < 0.05$, $n = 7$).

Figure 19

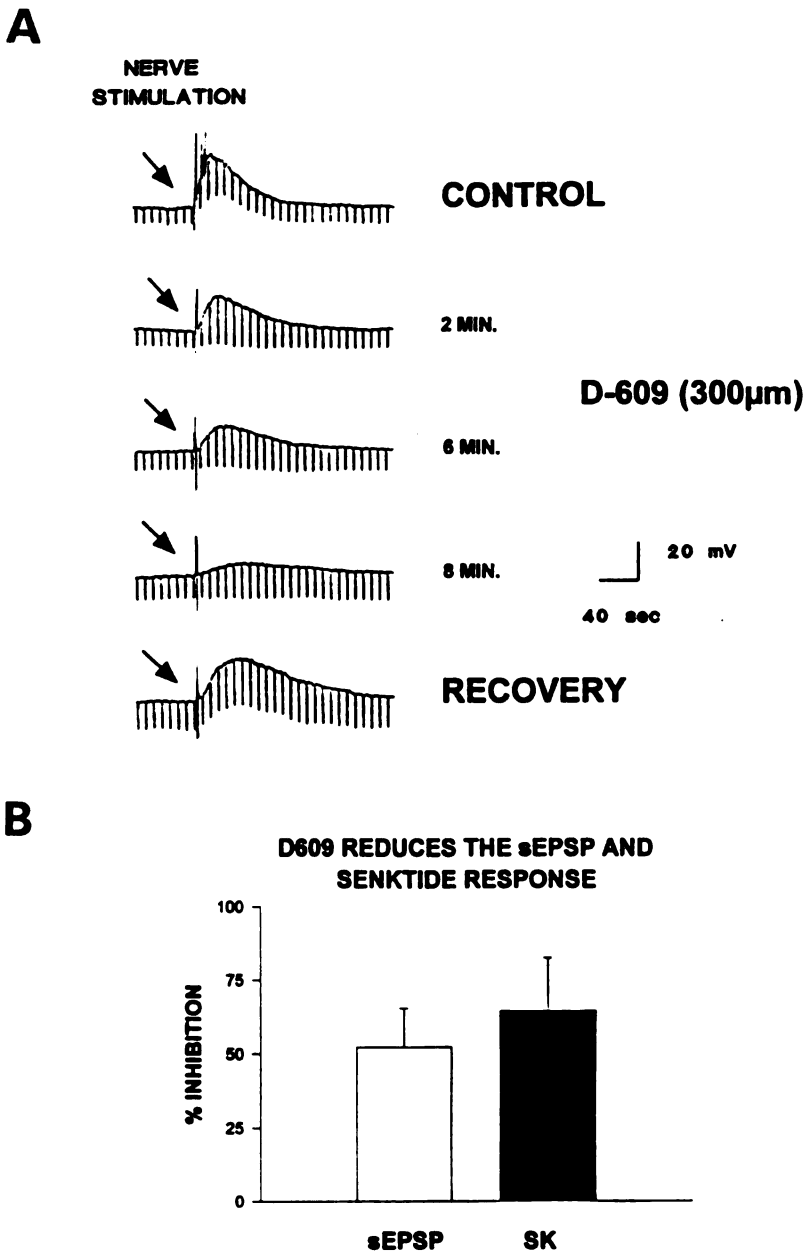
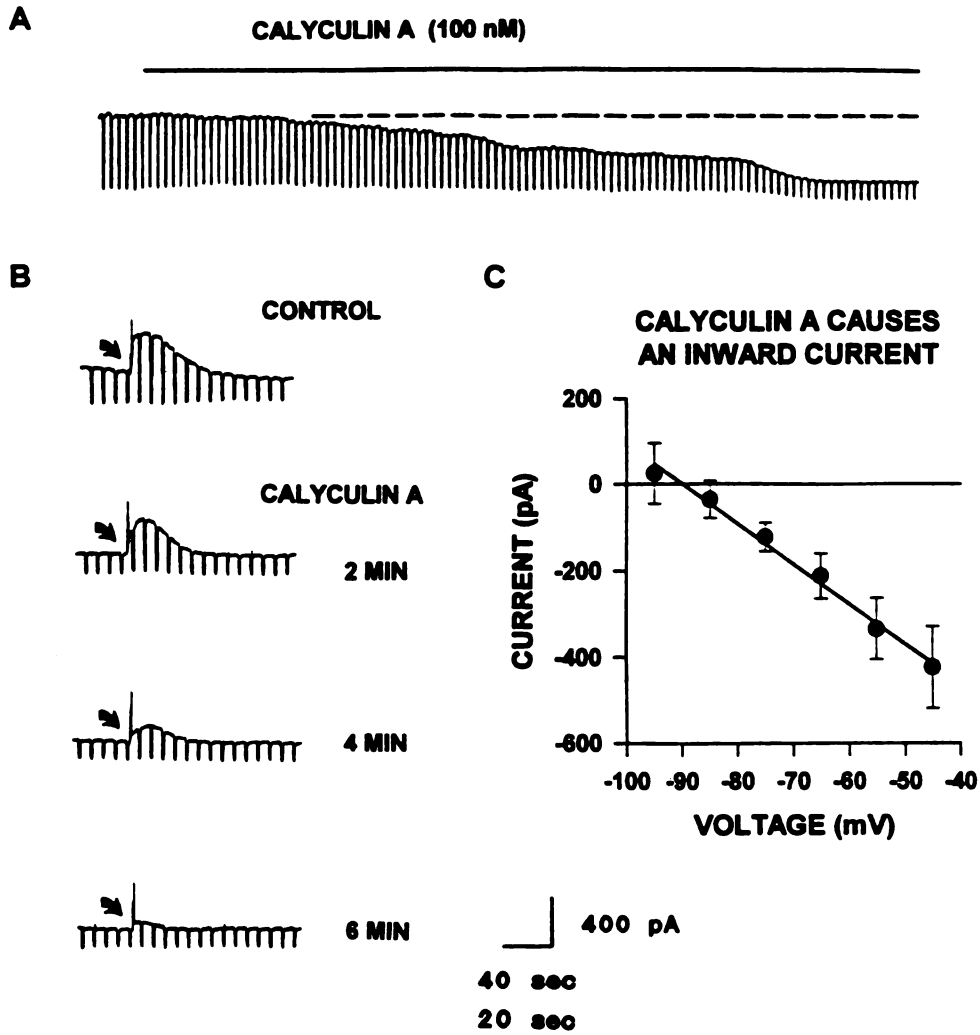

A
NERVE STIMULATION
CONTROL
2 MIN.
D-609 (300µm)
6 MIN.
8 MIN.
20 mV
40 sec
RECOVERY
B
D609 REDUCES THE sEPSP AND SENKTIDE RESPONSE
% INHIBITION
100
75
50
25
0
sEPSP
SK

Figure 20. Inhibition of protein phosphotases mimics the sEPSC

Calyculin A (100 nM) was superfused over 10 AH neurons in 5 preparations. **A.** Calyculin A caused a -650 pA sustained inward current associated with a conductance decrease (V_H = -65 mV; V_{STEP} = -20 mV). **B.** The AHC (curved arrow ➦) was inhibited by calyculin A (Control was +428 ± 60 pA and in the presence of calyculin A was +170 ± 33 pA (n = 10, $p < 0.05$). Successive AHC's were evoked at the indicated times after start of calyculin A superfusion (V_H = -70 mV; V_{STEP} = -20 mV). **C.** The average reversal potential was calculated from the individual linear regressions of I/V relationships and equaled -90 ± 2 mV. These I/V's were averaged and plotted.

Figure 20


A
CALYCULIN A (100 nM)
B
CONTROL
CALYCULIN A
2 MIN
4 MIN
6 MIN
C
CALYCULIN A CAUSES
AN INWARD CURRENT
200
0
-200
-400
-600
CURRENT (pA)
-100 -90 -80 -70 -60 -50 -40
VOLTAGE (mV)
400 pA
40 sec
20 sec

calyculin A was +170 ± 33 pA (40% of the control AHC)($n = 10$, $p < 0.05$). Forskolin (1 μM), when superfused immediately following calyculin A, did not have any significant effects on membrane current ($n = 5$, $p > 0.05$) but did cause a further decrease in the AHC to +45 ± 18 pA (11% of the control AHC)($n = 5$, $p < 0.05$)(Figure 20).

DISCUSSION

IONIC MECHANISMS OF THE SLOW SYNAPTIC RESPONSE

The sEPSC is a multi-conductance event

It is known that slow synaptic responses in enteric neurons frequently do not reverse polarity at E_K and there is often no observed resistance change during the response. While these data have been attributed to actions of neurotransmitters or drugs at electrically distant sites on neurons, Shen and Surprenant (1993) have recently shown that in submucosal neurons of guinea pig ileum, agonist-induced currents and sEPSCs are due to simultaneous inhibition of G_K and activation of a non-specific cation conductance. This conclusion was based on insensitivity of currents to anthracene-9-carboxylic acid and chloride substitution, and current inhibition by extracellular sodium substitution. Simultaneous activation of a cation conductance and inhibition of G_K would account for the inability to reverse the sEPSC or agonist-induced current in these neurons. However, in the guinea pig ileum it has been shown that in a subset of myenteric neurons, the sEPSC is due to inhibition of G_K and activation of a *chloride conductance* (G_{Cl}).

The conclusion that a G_{Cl} is involved in the sEPSC of myenteric neurons is based on the findings that some sEPSCs did not reverse at E_K and had no observable conductance

decrease. Additionally, some sEPSCs clearly contained two phases of conductance change with an early decrease in G_K followed by a more slowly developing increase in G_{Cl}. Senktide was used to mimic the sEPSC. Most senktide currents were associated with a G_K decrease, however as with the sEPSC, in some neurons senktide currents could not be attributed to a G_K decrease alone. Senktide responses often did not reverse polarity at E_K and senktide currents were frequently biphasic with an early G_K decrease followed by a later increase in G_{Cl}. In addition, some senktide currents were associated with only an increase in G_{Cl} and these currents reversed at -17 mV.

The conductance increase is G_{Cl}

Forskolin mimics the sEPSC-induced decrease in G_K in myenteric neurons (Nemeth, *et al.*, 1986). Forskolin was used to occlude the decrease in G_K caused by mediators of the sEPSC and senktide, thus any remaining currents induced by these stimuli would be due to another ionic mechanism. Cesium chloride was also used to block the hyperpolarization activated cation current (I_H) which is present in some myenteric neurons (Galligan *et al.*, 1990). In addition, recordings were obtained more than 30 min after impalement during which time there was the gradual decrease in membrane conductance that commonly occurs in myenteric neurons (Surprenant *et al.*, 1987; Wood, 1989). Under these conditions any senktide or sEPSC induced changes in G_K would be minimized.

A reversal potential for a transmitter- or agonist-induced current between -25 and -10 mV in myenteric neurons is consistent with an increase in a non-specific cation conductance or a chloride conductance (Wood, 1989; Galligan *et al.*, 1990; Galligan *et al.*, 1989; Cherubini & North, 1979). It is unlikely that the conductance increase observed here is due

to a cation conductance as substitution of external sodium by choline, or addition of cesium chloride or cobalt chloride to the extracellular solution did not affect the senktide-induced currents.

Lowering extracellular chloride reduced currents associated with G_{Cl}. In peripheral neurons at rest, the intracellular concentration of chloride is such that a lowered extracellular chloride concentration should augment the efflux of chloride from neurons. Akasu, Nishimura and Tokimasa (1990) demonstrated this effect in rabbit pelvic parasympathetic ganglia where there is a calcium-activated chloride conductance. I studied $GABA_A$-mediated chloride currents in myenteric AH neurons in order to clarify the effects of reduced extracellular chloride solutions on chloride conductances. Prolonged (> 5 min) treatment of preparations with reduced chloride solutions decreased GABA-induced currents and caused a positive shift in the GABA reversal potential. This effect on chloride efflux has been described in sympathetic neurons where it was found that reduced extracellular chloride depleted intracellular chloride over time, or after repeated applications of GABA (Adams and Brown, 1975). The intracellular chloride concentration in AH neurons was calculated based on the reversal potential of GABA-induced currents and the known extracellular chloride concentration. Intracellular chloride is reduced from 65 mM at rest (recorded with a 2M KCl electrode) to approximately 16 mM during superfusion with reduced chloride solutions. The decrease in driving force for chloride would cause an approximately 50% reduction in chloride-mediated currents at a holding potential of -80 mV. These data can explain the reduction of senktide currents and sEPSCs by reduced chloride solutions at similar holding potentials.

Senktide and $GABA_A$ currents were blocked by the fenamates, niflumic and mefenamic acid. These drugs block anion transport (Cousin & Motais, 1979) and anion channels (White & Aylwin, 1990). Fenamates can also block cation conductances (Gogelein, Dahlem, Englert & Lang, 1990), but not cation transport (Cousin & Motais, 1979). It is unlikely that the fenamates were blocking cation channels in the present study as fEPSPs in S neurons were unaffected by these drugs. Cyclo-oxygenase is the enzyme responsible for initiation of the prostaglandin/thromboxane signalling pathway and has been shown to be block by the fenamates (Stutts, Henke & Boucher, 1990). Indomethacin is a potent non-steroidal anti-inflammatory drug which was used in concentrations exceeding those needed to cause maximal inhibition of cyclo-oxygenase *in vitro* (Stutts, Henke & Boucher, 1990). Indomethacin did not affect either senktide- or GABA-induced currents, thus it is unlikely that cyclo-oxygenase inhibition is responsible for the fenamate effects on myenteric neurons reported here.

At high concentrations (100 - 300 μM) the fenamates caused an increase in G_K which is consistent with the observations of others. Toro, Ottolia, Olcese & Stefani (1993) have shown that low concentrations of niflumic acid and flufenamic acid cause an increase in the open probability of calcium-activated potassium channels situated in a lipid bilayer. Also, Farrugia, Rae & Szurszewski (1993) have found that flufenamic acid and mefenamic acid caused a dose-dependent opening of a delayed rectifier-like potassium channel in isolated smooth muscle cells from canine jejunum.

Contribution of G_{Cl} increase to sEPSC

Slow EPSCs, recorded more than 30 min after impalement and in which an I/V relationship had been measured, were categorized based upon a significant contribution of G_{Cl}. These results indicated that up to 29% of these sEPSCs contained an increase in G_{Cl}. In most neurons, an I/V relationship was not measured, but similar analysis could be used to suggest a contribution of G_{Cl}. Responses in which there was an observed conductance decrease were examined using forskolin to occlude changes in G_K, and unmask G_{Cl}. These data indicate that 43% of sEPSCs initially associated with an observed conductance decrease may also contain a significant increase in G_{Cl}. When measured without forskolin these sEPSCs contained approximately a 9 to 1 ratio of G_K to G_{Cl}.

Senktide currents in the presence of TTX, were used to study I_{Cl} and I_K in detail. On average, the rise phase of I_K (τ = 7 s) was faster than I_{Cl} (τ = 17 s). Thus, when the contributions of the two conductances are equal, a clear biphasic conductance change is observed. When the difference in time course of these conductances is less prominent (due to a slower rate of rise for I_K), the observed current is associated with no observed conductance change. The decaying phase for I_K was slower than I_{Cl}. The apparently slower rate may be due in part to the irreversible activation of I_K (ie. decrease in G_K) that is common during impalement of myenteric neurons (Surprenant *et al.*, 1987).

The senktide response and slow synaptic response were both associated with a I_{Cl} which had a slower time to peak than the I_K. The time to peak ΔI in both cases was more closely related to changes in G_K than G_{Cl}. These data indicate that changes in G_{Cl} are involved in the maintenance of the sEPSC, while the peak ΔI is due mainly to changes in G_K. The

measurement of peak ΔI, which is commonly used to construct I/V relationships, may significantly under-represent the contribution of a change in G_{Cl}.

Forskolin was used to mimic the decrease in G_K seen during senktide currents and sEPSCs. The effectiveness of forskolin in isolating G_{Cl} also indicates that forskolin sensitive pathways are not responsible for direct activation of G_{Cl} (see below).

Physiological significance of an increase in G_{Cl}

The resting input resistance of intact myenteric neurons, either *in vivo* or *in vitro*, is expected to be higher than that recorded from neurons impaled using intracellular electrodes. The quality of electrode impalement directly affects the measured input resistance of a neuron. Original estimates of myenteric neuron input resistances were between 20 and 50 MΩs (Nishi & North, 1973). Estimates have varied considerably between studies, but on average have been moved to higher values as intracellular recording techniques have improved (see Wood, 1989 for review). Whole cell patch clamp recordings from cultured myenteric neurons have yielded measured input resistances for AH neurons and S neurons of 234 ± 12 MΩs and 345 ± 23 MΩs respectively (author's unpublished data). These data are similar to others (Tatsumi, Costa, Schimerlik & North, 1990; Baiden, Zholos, Shuba & Wood, 1992). This implies that *in vivo*, many potassium channels which set the membrane potential may be closed and unavailable to mediate sEPSPs. Under these conditions an increase in G_{Cl} could account for a larger proportion of the inward current during the sEPSP. A decrease in G_K and an increase in G_{Cl} is an ideal combination for exciting neurons. During such a response, sodium and calcium gradients remain intact, thus preserving the neurons ability to generate

fEPSPs and action potentials. Conversely, large increases in cation conductance could deplete the transmembrane sodium gradient leading to inhibition of synaptic transmission.

SIGNAL TRANSDUCTION

The slow synaptic response is due to activation of a PTX-insensitive G-protein

GTP-γ-S binds to the free α-subunit of the stimulated G-protein but is not hydrolyzed by the intrinsic GTPase activity of the α-subunit. In this study, all three major components of the sEPSC and senktide current became irreversibly activated in the presence of GTP-γ-S. In cells left were unstimulated, GTP-γ-S caused a slowly developing decrease in conductance. This effect was blocked in some neurons by TTX, suggesting its dependence on axonal action potentials and transmitter release. This implies that release of transmitter and/or G-protein turnover occur in apparently quiescent neurons. The effects of GTP-γ-S were specific to G-protein interaction as ATP-γ-S did not cause a sustained inward current (Esguerra, Wang, Foster, Adelman, North & Levitan, 1994). GDP-β-S can displace GDP at its binding site on the α-subunit of the intact, inactive G-protein and thus prevent its subsequent activation. GDP-β-S did not produce significant inhibition of the slow synaptic or senktide response in this study. One explanation for this lack of effect may be that concentrations of intracellular GDP-β-S obtained were too low. Presumably the concentrations of GDP-β-S were comparable to the GTP-γ-S used previously, however, partial inhibition of G-protein function may not be sufficient to prevent the production of a slow synaptic response provided there is an excess of available G-proteins. Another explanation may be that a portion of the

intracellular transduction machinery is not coupled through a G-protein. G-protein independent second messenger systems include the contraction of guinea pig stomach muscle by angiotensin II and several growth factors *via* tyrosine kinase activity (Hollenberg, 1994) and the changes in intracellular calcium ($[Ca]_i$) created by calcium-dependent action potentials.

PTX is a bacterial toxin which ADP-ribosylates a subset of G-proteins. In the enteric nervous system PTX inactivates the inhibitory G-proteins (G_i) associated with the actions of noradrenalin and somatostatin in submucous neurons (Surprenant & North, 1988). These authors also note that PTX treatment does not affect the generation of submucosal sEPSPs. In the myenteric plexus, this study has shown that PTX treatment does not cause attenuation of slow synaptic, senktide or 5-HT responses. The probable G-proteins which are activated are G_s, which couples the adenylate cyclase and G_q, which couples to PLC (Sternweis & Pang, 1990; Exton, 1994). The coupling of SP through PTX insensitive G-proteins is not unusual in systems such as rat brain (Nakajima, Nakajima & Inoue, 1988). Recent studies by Pan & Gershon (1994) have shown that the slow depolarization produced by 5-HT within the myenteric plexus is PTX-sensitive. However, the lack of control data and the incubation parameters in these experiments (1.5 μg/mL of PTX for 1.5 hr) makes is unlikely that the same pool of G-proteins was being studied.

Diffusible second messenger coupled pathways

The major protein kinases (PKA, PKC and calmodulin kinase II) have been biochemically localized to myenteric ganglia and have been shown to be active versus a range of protein targets (Jeitner, Jarvie, Costa, Rostas & Dunkley, 1991). Previous studies have shown that

application of phorbol esters or forskolin may mimic some aspects of the slow synaptic response (Nemeth, *et al.*, 1986; Bertrand & Galligan, 1993b). In this study, I investigated the specific effects of these drugs on the inhibition of resting and spike-activated G_K and the activation of G_{Cl}. D609 is a specific PLC inhibitor which may be specific for the phosphatidylcholine preferring subtype (PC-PLC) (Schutze, *et al.* 1992). D609 was effective in reducing both the slow synaptic and the senktide response but not the fEPSP. This indicates that a PLC type reaction is activated by the mediator of the slow synaptic response and senktide and is liberating DAG. The actions of PDBu were shown to be sensitive to protein kinase inhibitors. Thus PDBu, by mimicking the actions of endogenously released DAG, is most likely activating PKC. The effects of forskolin at the concentrations used here have been shown to be mediated via activation of adenylate cyclase leading to accumulation of intracellular cAMP (Seamon & Daly, 1986; McHugh & McGee, 1986; Surprenant, 1984; Nemeth, *et al.*, 1986; Akasu & Tokimasa, 1989) as cAMP analogs and inhibition of cAMP degradation mimic forskolin's actions. In this study I show that the current and inhibition of the AH by forskolin are sensitive to protein kinase inhibition. This suggest that the production of cAMP is primarily activating PKA. I further characterized PDBu and forskolin currents under voltage clamp conditions. The inhibition of the AHC by both compounds was dose-dependent and was not based upon direct modulation of the calcium spike. The I/V relationships show these compounds induce currents which reverse near E_K. An analysis of the conductance shows that PDBu does not cause a significant increase in conductance, but that forskolin does in a small sub-population of neurons. I have previously characterized the synaptically-activated conductance increase as a G_{Cl}, however these same studies showed that forskolin was not effective in activating this current (Bertrand & Galligan, 1994). It is

possible this current represents the increase in cation conductance recently described in submucous neurons by Shen & Surprenant (1993). In their study, the major conductance increase activated by agonists such as 5-HT, the mediator of the slow synaptic response and forskolin was sensitive to reduction of external sodium ions.

My efforts to characterize the PDBu and forskolin responses lead to the observation that their actions were very similar. In addition, neurons which responded to one compound more often than not responded to the other. The interaction or cross-talk between second messenger pathways has been reported for many different systems, specifically PKC has been shown to positively regulate adenylate cyclase in S49 cells, olfactory receptor cells and others (Jacobowitz, Chen, Premont & Iyengar, 1993; Frings, 1993). I wished to determine if a significant proportion of the observed effects in the myenteric plexus were due to a positive interaction between the cAMP/PKA pathway and the PKC pathway. By examining the effects of threshold concentrations of forskolin and PDBu on the AHC, I have shown that these pathways do interact in a positive manner. In neurons in which threshold concentrations were obtained most showed a significant PDBu-induced increase in the forskolin response. This increase was greater than would be expected had the effects of PDBu and forskolin been simply additive.

The slow synaptic response is associated with protein phosphorylation

The sEPSC and senktide induced changes in G_K are mimicked by known activators of protein kinases (see above). Calcium activated potassium channels cloned from *Drosophila* (*Slo* channels) have been shown to be regulated by phosphorylation (Esguerra, *et al.*, 1994). In their study, the PKA activity as well as the phosphorylation site were shown to be part of the

channel. In this study I provide evidence that the synaptic response is reduced by inhibitors of protein kinases and mimicked by inhibitors of protein phosphotases. The compounds K-252a (from *Nocardiopsis sp.*) and staurosporine (from *Streptomyces sp.*) are structurally similar non-specific protein kinase inhibitors (Ruegg & Burgess, 1989). Their primary mechanism of action is to suppress protein kinase activity by binding competitively at the ATP binding site (Lo & Breitwieser, 1994). The K_is for staurosporine are between 1 and 10 nM while the K_is for K-252a are between 10 and 50 nM for most kinases including PKC and PKA (Ruegg & Burgess, 1989). Staurosporine was effective in reducing both the inward currents produced by nerve stimulation and by senktide. It is significant that it was not effective in completely inhibiting these currents. The inhibition of the AHC was prevented by staurosporine and K-252a at similar doses. These data do not indicate which if either protein kinase is responsible for the actions of senktide or the mediator of the slow synaptic response. It may be inferred that PKC is involved as D609 was effective in reducing these responses. The D609 insensitive response may represent the actions of the cAMP/PKA system. Staurosporine was effective in completely blocking the PDBu current while only reducing the forskolin induced current. It is likely that activation of PKA is responsible for the residual current.

Recently, the signal transduction of adenosine was studied in submucosal neurons (Barajas-Lopez, 1993). One of the actions of adenosine (acting through adenosine type 2 receptors) is to cause a slow depolarization very similar to the slow synaptic response. The study concluded that, although PDBu and forskolin mimicked the adenosine response, only activation of PKA was involved in the transduction of the response. This was based on the insensitivity of the both the adenosine and forskolin response to brief applications of

staurosporine while the PDBu response was inhibited immediately. The 10-fold selectivity of staurosporine for PKC over PKA was cited as the basis of this result. In this study, staurosporine was most effective in reducing the current produced by PDBu and least effective in reducing the slow synaptic response. This implies that the slow synaptic response is only weakly dependent on the actions of PKC. Interestingly, the inhibition of the AHC by the slow synaptic response was prevented most effectively by staurosporine while PDBu and forskolin were reduced by approximately the same amount. This implies that the inhibition of resting versus spike activated G_K is differentially regulated by either PKA or PKC.

Calyculin A is non-specific inhibitor of protein phosphatases having a K_i of $\leq$ 2 nM for types I and IIA which has been shown to be active in biological systems (Cicirelli, 1991; Ishihara, Ozaki, Sata, Hori, Karaki & Hartshorne, 1989). The effects of calyculin A on myenteric neurons was found to be very similar to those of PDBu and forskolin, Calyculin A was effective in closing resting $G_{K,Ca}$ and in reducing the AHC. These actions occluded those of forskolin. The time to onset of the calyculin A induced current was usually twice as long for forskolin or PDBu. This may represent the slow penetration of calyculin A into the neuron, or may be representative of a gradual build up of phosphorylation due to unchecked activity of protein kinases. Overall, these data indicate that endogenous phosphatase activity in the unstimulated myenteric neuron is negatively coupled to decreases in $G_{K,Ca}$. Further analysis of the conductance change during calyculin A induced currents show that calyculin A does not cause a significant increase in G_{Cl}.

Activation of G_{Cl}

The activation of G_{Cl} is not due to direct activation of ligand-gated ion channels as this study has clearly shown the involvement a G-protein. In addition, the time course of activation of this current (*tau* = 12 to 20s) is several orders of magnitude greater than that of a ligand-gated ion channel (*tau* = 10 ms) or of a G-protein coupled ion channel (Bertrand & Galligan, 1994; Wood, 1989; Clapham, 1994). Thus a diffusible second messenger system is likely responsible for activation of G_{Cl}. Previous studies have shown that indomethacin is not effective, ruling out the role of cyclo-oxygenase products (Bertrand & Galligan, 1994). Similarly, preliminary studies utilizing genistein, a specific tyrosine kinase inhibitor and mepacrine, a PLA_2 inhibitor, were ineffective in blocking activation of G_{Cl} (author's unpublished observations). In this study, the effects of PKC and cAMP, acting at PKA or at other sites, were ineffective in activating G_{Cl}. G_{Cl} is unlikely to be directly regulated by protein phosphatases as calyculin A failed to activate this current. The sensitivity of the slow synaptic response to inhibition by D609 has established that the activation of PLC is important for generation of the slow synaptic response. A messenger candidate for activation of G_{Cl} may be a PLC product other than DAG. IP_3 liberated from a phosphatidylinositol-PLC (PI-PLC) type reaction could directly or indirectly (through changes in intracellular Ca^{++}) cause activation of G_{Cl} (Exton, 1994). However this is unlikely as D609 has been shown to be specific for a PC-PLC and not PI-PLC (Schutze, 1992). Conjecture at this time as to the activator of G_{Cl} does not seem fruitful. Further research is needed in a system in which access to the intracellular space is not a limiting factor as it is with conventional electrophysiology approaches. The use of the patch clamp technique is suggested.

The basis of the slow synaptic response

Attempts to characterize the transduction mechanisms of the slow response in the myenteric plexus focused on the ability of adenosine to inhibit adenylyl cyclase (Palmer, *et al.*, 1987). The mechanism by which adenosine did this is not clear but the marked differences in its inhibitory effects against different substances made the important point that not all agonist in the myenteric plexus use the same signal transduction systems. Palmer concluded that because the actions of SP were not blocked by adenosine, SP did not increase cAMP. Biochemical data from the myenteric plexus dispute this and show that SP can cause PI turnover and cAMP accumulation (Guard *et al.*, 1988; Baiden *et al.*, 1992). Other membrane phopholipids and diffusible second messengers have not been studied in this way. This study has shown that several pathways converge on the same target (ie. $G_{K,Ca}$). Thus it seems likely that SP activates more than one second messenger system. This may explain why no manipulation in this study ever reduced the senktide response or the slow synaptic response by more than 60 %.

A major question left unanswered by this study is the disposition of intracellular calcium $[Ca]_i$ during the slow response. Original observations, which are ascribed here to protein phosphorylation, could be explained by a decrease in $[Ca]_i$ (Grafe, *et al.*, 1980; Morita & North, 1985). The effects of changing $[Ca]_i$ on the inhibition of the AHC and the reduction of resting G_K should be considered separately. First, the inhibition of the AHC takes place without changes in the duration, amplitude or presumably amount of calcium entry during the calcium spike. Resting calcium in myenteric neurons has been measure at 100 nM while a single calcium spike was measured at 10 to 20 nM and a train of 5 at 50 nM (Tatsumi, Hirai

& Katayama, 1988). The large calcium transient evoked by a train of calcium spikes is unlikely to be damped by modest changes in resting $[Ca]_i$. Thus, increased phosphorylation due to kinase activation or phosphatase inhibition must be primarily responsible for reduction of the AHC.

Second, it is known that changes in $[Ca]_i$ are able to regulate resting $G_{K,Ca}$. Morita & Katayama (1992) have shown that A23187 (a calcium ionophore) does open resting $G_{K,Ca}$ while application of calcium channel blockers (such as Mg^{++}) closes $G_{K,Ca}$. These same blockers also have been shown to lower $[Ca]_i$ by 20 nM (Tatsumi, *et al.*, 1988). However, there is no evidence that active modification of $[Ca]_i$ is a mechanism of slow synaptic transmission. In cultured myenteric neurons from the rat, calcium levels have been found to go up during stimulation with SP. It is not clear whether this is due to external of internal sources of calcium, however this strongly suggest that a simple lowering of $[Ca]_i$ is not responsible for the slow synaptic response (Trouslard, et al., 1993). In the simplest case, the mechanism for inhibition of the AHC would also be the mechanism for closing $G_{K,Ca}$ at resting calcium levels. This would imply that decreases in protein phosphorylation would lead to opening of $G_{K,Ca}$. This is not the case. Kinase inhibitors do not cause an increase in $G_{K,Ca}$ or even prevent the normal increase in excitability of neurons seen over time (Barajas-Lopez, 1993). Thus, phosphorylation may primarily regulate the channel's sensitivity to calcium transients such as the AH. The mechanism for regulation of resting $G_{K,Ca}$ may be due to changes in $[Ca]_i$, phosphorylation or a combination of both.

SUMMARY

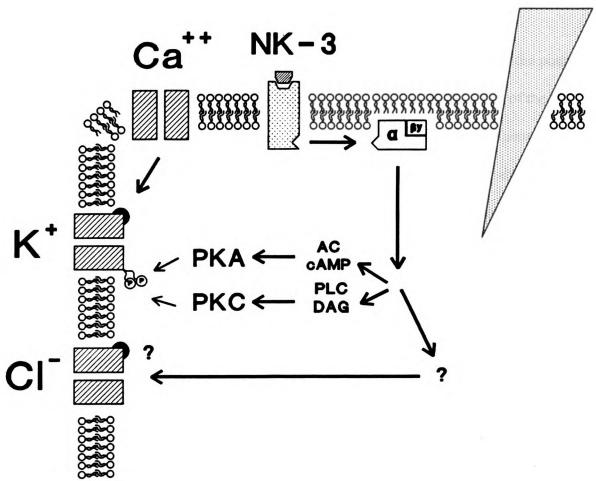
In summary, I have investigated the ionic mechanisms by which slow synaptic responses are generated and have looked at the coupling of these conductance to intracellular messenger pathways. I have characterized the calcium-activated potassium conductance which is the most abundant conductance as well as a novel chloride conductance. I have demonstrated the partial dependence of the slow synaptic potential on PTX-insensitive G-proteins, phospholipase C and the activity of protein kinases and protein phosphatases. It is worthwhile to note that the slow synaptic response is not a single event, but the average of several unknown transmitters acting at many synapses (Furness & Costa, 1987). Thus it may seem an insurmountable task to characterize what in reality are many separate events occurring simultaneously. However, many of the slow synaptic transmitter candidates (with the exception of 5-HT) display a similar profile of activity in their actions on enteric neurons (Tokimasa & North, 1984; Galligan, 1993). This common result may be brought about because these substance share common intracellular transduction pathways. With this caveat, I have in Figure 21 attempted to summarize my findings with those of others whose data formed the basis of this study. In this scheme, the mediator of the slow synaptic response, in this case presumed to be substance P, initiates a biochemical cascade upon binding to the neurokinin-3 receptor (NK-3). The activated receptor binds to and activates one or more PTX insensitive G-proteins present in the membrane. All subsequent intracellular events

appear to mediated *via* G_s and/or G_q type G-proteins. These G-proteins bind to and activate a number of different enzymes. Two which are clearly implicated are adenylate cyclase (AC) and phospholipase C (PLC). It is possible that a third component is activated at this point which eventually leads to activation of a chloride conductance. The identity of this component is unknown. The activation of AC and PLC catalyze the formation of cyclic adenosine monophosphate (cAMP) and diacylglycerol (DAG). cAMP and DAG go on to activate their respective kinases which are most likely the primary effectors of intracellular change (ie. closure of the potassium channels). Up to this point these two pathways have

Figure 21. A proposed model of signal transduction for the sEPSC

An illustration of the intra-cellular changes responsible for the generation of a myenteric sEPSC. The mediator of the sEPSC, possible acting at neurokinin-3 (NK-3) receptors, initiates activation of PTX-insensitive G-proteins (α, β/γ) leading to activation of phospholipase C and adenylate cyclase (PLC, AC) and an unknown signal transduction element (?). The resultant increases in cAMP and DAG leads to activation of PKA and PKC and probable phosphorylation of channel proteins. In the case of $G_{K,Ca}$, this causes an apparent reduction in calcium affinity to resting and action potential generated calcium transients and closure of the channel. The G_{Cl} does not appear to be activated by kinases.

Figure 21



been considered separately. However it is likely that the actions of the protein kinases serve to sensitize or even activate certain steps in one or the others pathway. This would tend to reinforce the actions of either pathway. This suggest there is a great deal of redundancy and reinforcement in the generation of a slow synaptic responses. The reason for this redundancy is not clear, but it underscores the importance of the slow synaptic response as a fundamental mechanism of synaptic communication and its role in enteric function.

The study of the ENS has always been considered important in terms of the potential benefit to gastrointestinal disorders and diseases. More and more, the study of the ENS is being undertaken simply to better understand neurobiology in general. The neurons of the gut, and the complex networks they form, are a rich source of knowledge about neuronal action and interaction. The slow synaptic response is clearly an important component of this interaction. Although the physiological role of the slow synaptic response is unknown, the most likely function is that of sensitization and co-ordination. The gut is fundamentally dependent upon the ability to co-ordinate large groups of neurons in order to carry out its functions. The appropriate transmission of slow synaptic responses is then fundamental to understanding how the complex networks of the ENS do their job.

This study has endeavored to explore the neurobiology of enteric neurons at the cellular level while still maintaining a grasp of the function of the gut. This was accomplished by combining the techniques of electrophysiology, which allows the measurement of the functional output of the neuron, with biochemically basis pharmacology, which allows direct manipulation of cellular processes. This combination has some drawbacks and some benefits. One drawback is the incomplete or inadequate biochemical control of a living, intact cell as opposed to cell fragments commonly used to assay biochemical events. The benefit of

working in an intact system is that the response one is studying is more closely associated with the what is presumed to be occurring in the intact animal. Studies in which one or more techniques are combined provide worthwhile data, but they also serve as a bridge between the two or more disciplines from which they were taken.

BIBLIOGRAPHY

BIBLIOGRAPHY

Adams, P. R. & Brown, D. A. (1975). Actions of gamma-aminobutyric acid on sympathetic ganglion cells. *Journal of Physiology* **250**, 85-120.

Akasu, T. & Tokimasa, T. (1989). Potassium currents in submucous neurones of guinea-pig caecum and their synaptic modification. *Journal of Physiology* **416**, 571-588.

Akasu, T., Nishimura, T. & Tokimasa, T. (1990). Calcium-dependent chloride current in neurones of the rabbit pelvic parasympathetic ganglia. *Journal of Physiology* **422**, 303-320.

Auerbach, L. (1862). Ueber einen Plexus myentericus, einen bisher unbekannten ganglio-nervosen Apparat im Darmkanal der Wirbelthiere. E Morgenstern, Breslau, pp 13.

Auerbach, L. (1864). Fernere vorlaufige Mitteilung uber den Nervenapparat des Darmes. *Arch. Pathol. Anat. Physiol.* **30**, 457-460.

Baidan, L. V., Fertel, R. H. & Wood, J. D. (1992). Effects of brain-gut related peptides on cAMP levels in myenteric ganglia of guinea-pig small intestine. *European Journal of Pharmacology* **225**, 21-27.

Baidan, L. V., Zholos, A. V., Shuba, M. F. & Wood, J. D. (1992). Patch-clamp recording in myenteric neurons of guinea pig small intestine. *American Journal of Physiology* **262**, G1074-G1078.

Barajas-Lopez, C. (1993). Adenosine reduces the potassium conductance of guinea pig submucosal plexus neurons by activating protein kinase A. *Pflugers Archives* **424**, 410-415.

Bartho, L., Holzer, P. & Lembeck, F. (1987). Is ganglionic transmission through nicotinic receptors essential for the peristaltic reflex in the guinea-pig ileum. *Neuropharmacology* **26**, 1663-1666.

Bayliss, W. M. & Starling, E. H. (1899). The movements and innervation of the small intestine. *Journal of Physiology* **24**, 100-143.

Bertrand, P. P. & Galligan, J. J. (1992a). Alfaxalone, pentobarbital and diazepam potentiate γ-aminobutyric acid-induced depolarizations in single myenteric neurons of guinea pig intestine. *Journal of Pharmacology and Experimental Theraputics* **262**, 677-682.

Bertrand, P. P. & Galligan, J. J. (1992b). Substance P and ATP activate novel inward currents in myenteric neurons. *Pharmacologist* **34**, 158.

Bertrand, P. P. & Galligan, J. J. (1993a). Neurokinin-3 receptors couple to a chloride conductance in guinea pig myenteric neurons. *Society for Neuroscience Abstacts* **19**, 1131.

Bertrand, P. P. & Galligan, J. J. (1993b). Protein kinase-mediated inhibition of calcium-activated potassium channel in myenteric neurons. *Gastroenterology* **104**, A1029.

Bertrand, P. P. & Galligan, J. J. (1994). Contribution of chloride conductance increase to slow EPSC and tachykinin current in guinea-pig myenteric neurones. *Journal of Physiology*, in press.

Bertrand, P. P. & Galligan, J. J. (1994). Tachykinin- and slow synaptically-coupled second messenger systems in guinea-pig myenteric neurones. *Journal of Physiology*, in preparation.

Birnbaumer, L. (1990). Transduction of receptor signal into modulation of effector activity by G proteins: the first 20 years or so... *Journal of the Federation of American Scientist and Experimental Biologist* **4**, 3068-3078.

Bodian, M., Stephens, F. D. & Ward B. C. H. (1949). Hirschsprung's disease and idiopathic megacolon. *Lancet* **1**, 6-11.

Bolande, R. P. (1975). Animal model of human disease. Aganglionic or hypoganglionic megacolon; animal model: Aganglionic megacolon in piebold and spotted mutant mouse strains. *American Journal of Pathology* **79**, 189-192.

Bornstein, J. C., North, R. A., Costa, M. & Furness, J. B. (1984). Excitatory synaptic potentials due to activation of neurons with short projections in the myenteric plexus. *Neuroscience* **11**, 723-731.

Boyd, N. D., MacDonald, S. G., Kage, R., Luber-Narod, J. & Leeman, S. E. (1991). Substance P receptor, Biochemical characterization and interactions with G proteins. *Annals of New York Academy of Sciences* **632**, 78-93.

Brookes, S. J. H., Steele, P. A. & Costa, M. (1991). Calretinin immunoreactivity in cholinergic motor neuons, interneurons and vasomotor neurons in the guinea-pig small intestine. *Cell and Tissue Research* **263**, 471-481.

Camardo, J. S., Shuster, M. J., Siegelbaum, S. A. & Kandel, E. R. (1984). Modulation of a specific potassium channel in sensory neurons of *Aplysia* by serotonin and cAMP-dependent protein phophorylation. *Nature*, 213-220.

Cherubini, E. & North, R. A. (1979). The actions of gamma aminobutyric acid on neurones of the myenteric plexus. *British Journal of Pharmacology* **82**, 93-100.

Cherubini, E. & North, R. A. (1984). Inhibition of calcium spikes and transmitter release by gamma-aminobutyric acid in the guinea-pig myenteric plexus. *British Journal of Pharmacology* **82**, 101-105.

Cicirelli, M. F. (1991). Inhibitors of protein serine/threonine phosphatases. *Focus* **14**, 16-20.

Clapham, D. E. (1994). Direct G-protein activation of ion channels?. *Annual Reviews of Neuroscience* **17**, 441-464.

Cook, R. D. & Burnstock, G. (1976). The ultrastucture of Auerbach's plexus in the guinea-pig. *Journal of Neurocytology* **5**, 171-194.

Cousin, J. L. & Motais, R. (1979). Inhibition of anion permeability by amphiphilic compounds in human red cell: Evidence for an interaction of niflumic acid with the band 3 protein. *Journal of Membrane Biology* **46**, 125-153.

DiFrancesco, D. & Tortora, P. (1991). Direct activation of cardiac pacemaker channels by intracellular cyclic AMP. *Nature* **351**, 145-147.

Dogiel, A. S. (1899). Uber den bau der ganglien in den geflechen del darmes und der gallblase des menschen und der saugetiere. *Archives of Anatomy and Physiology* 130-158.

Drapeau, G., Rouissi, N., Nantel, F., Rhaleb, N. E., Tousignant, C. & Regoli, D. (1990). Antagonist for the neurokinin NK-3 receptor evaluated in selective receptor systems. *Regulatory Peptides* **31**, 125-135.

Esguerra, M., Wang, J., Foster, C. D., Adelman, J. P., North, R. A. & Levitan, I. B. (1994). Cloned Ca^{2+}-dependent K^+ channel modulated by a functionally associated protein kinase. *Nature* **369**, 563-565.

Exton, J. H. (1994). Phosphoinostide phospholipases and G proteins in hormone action. *Annual Reviews in Physiology* **56**, 349-369.

Farrugia, G., Rae, J. L. & Szurszewski, J. H. (1993). Characterization of an outward current in canine jejunal circular smooth muscle and its activation by fenamates. *Journal of Physiology* **468**, 297-310.

Frings, S. (1993). Protein kinase C sensitizes olfactory adenylate cyclase. *Journal of General Physiology* **101**, 183-205.

Furness, J. B. & Costa, M. (1980). Types of nerves in the enteric nervous system. *Neuroscience* **5**, 1-20.

Furness, J. B. & Costa, M. (1987). The enteric nervous system., pp 1-290, New York: Churchill Livingstone.

Furness, J. B., Trussell, D. C., Pompolo, S., Bornstein, J. C. & Smith, T. K. (1990). Calbindin neurons of the guinea-pig small intestine: quantitative analysis of their numbers and projection. *Cell and Tissue Research* **260**, 261-272.

Furness, J. B., Bornstein, J. C., Pompolo, S., Young, H. M., Kunze, W. A. A. & Kelly, H. (1994). The circuitry of the enteric nervous system. *Neurogastroenterology and Motility*, in press.

Galligan, J. J. & Bertrand, P. P. (1994). ATP mediates fast synaptic trasmission in myenteric neurons of guinea-pig ileum. *Journal of Neuroscience*, in press.

Galligan, J. J., Tokimasa, T. & North, R. A. (1987). Effects of three mammalian tachykinins on single enteric neurons. *Neuroscience Letters* **82**, 167-171.

Galligan, J. J. & North, R. A. (1988). Drug receptors on single enteric neurons. *Life Sciences* **43**, 2183-2192.

Galligan, J. J., North, R. A. & Tokimasa, T. (1989). Muscarinic agonist and potassium currents in guinea-pig myenteric neurones. *British Journal of Pharmacology* **96**, 193-203.

Galligan, J. J., Tatsumi, H., Shen, K. Z., Surprenant, A. & North, R. A. (1990). Cation current activated by hyperpolarization (I_H) in guinea pig enteric neurons. *American Journal of Physiology* **259**, 966-972.

Galligan, J. J. (1993). Gastointestinal regulatory peptides. In Handbook of Experimental Pharmacology, ed. Brown, D. R., pp. 253-276. Berlin Heidelberg: Springer-Verlag.

Gershon, M. (1981). The enteric nervous system. *Annual Reviews of Neuroscience* **4**, 227-272.

Gogelein, H., Dahlem, D., Englert, H. C. & Lang, H. J. (1990). Flufenamic acid, mefenamic acid and niflumic acid inhibit single nonselective cation channels in the rat exocrine pancreas. *FEBS Letters* **268**, 79-82.

Grafe, P., Mayer, C. J. & Wood, J. D. (1980). Synaptic modulation of calcium-dependent potassium conductance in myenteric neurones in the guinea-pig. *Journal of Physiology* **305**, 235-248.

Gerard, N. P., Bao, L., Xiao-Ping, H. & Gerard, C. (1993). Molecular aspects of the tachykinin receptors. *Regulatory Peptides* **43**, 21-35.

Grundy, D. & Scratcherd, T. (1989). Sensory afferents from the gastrointestinal tract. In Handbook of Physiology - The Gastrointestinal System, eds. Schultz, S. G., Wood, J. D. & Rauner, B. B., pp. 593-620. New York: Oxford University Press.

Guard, S., Watling, K. J. & Watson, S. P. (1988). Neurokinin-3 receptors are linked to inositol phospholipid hydrolysis in the guinea-pig ileum longitudinal muscle-myenteric plexus preparation. *British Journal of Pharmacology* **94**, 148-154.

Hanani, M. & Burnstock, G. (1985). The actions of substance P and serotonin on myenteric neurons in tissue culture. *Brain Research* **358**, 276-281.

Hanani, M., Chorev, M., Chaim, G. & Selinger, Z. (1988). The actions of receptor-selective substace P analogs on myenteric neurons: an electorphysiological investigation. *European Journal of Pharmacology* **153**, 247-253.

Hirst, G. D. S., Holman, M. E. & Spence, I. (1974). Two types of neurones in the myenteric plexus of duodenum in the guinea-pig. *Journal of Physiology* **236**, 303-326.

Hirst, G. D. S., Holman, M. E. & Spence, I. (1975). Two descending nerve pathways activated by distension of guinea-pig small intestine. *Journal of Physiology* **244**, 113-127.

Hirst, G. D. S., Johnson, S. M. & Helden, D. F. (1985a). The calcium current in a myenteric neurone of the guinea pig ileum. *Journal of Physiology* **361**, 297-314.

Hirst, G. D. S., Johnson, S. M. & Helden, D. F. (1985b). The slow calcium-dependent potassium current in a myenteric neurone of the guinea-pig ileum. *Journal of Physiology* **361**, 315-337.

Hodgkiss, J. P. (1981). Slow synaptic potentials in AH-type myenteric plexus neurons. *Pflugers Archives* **391**, 331-333.

Hoffman, H. H. & Schnitzlein, H.N. (1969). The number of vagus nerves in man. *Anatomical Records* **139**, 429-435.

Hollenberg, M. D. (1994). Tyrosine kinase pathways and the regulation of smooth muscle contractility. *Trends in Pharmacological Sciences* **15**, 108-114.

Holzer, P. (1984). Characterization of stimulus-induced release of immunoreactive substance P from the myenteric plexus of the guinea pig ileum. *Brain Research* **287**, 127-136.

Holzer, P. (1989). Ascending enteric reflex: multiple neurotransmitter systems and interactions. *American Journal of Physiology* **256**, G540-G545.

Ishihara, H., Ozaki, H., Sata, K., Hori, M., Karaki, H. & Hartshorne, D. J. (1989). Calcium-dependent activation of contractile apparatus in smooth muscle by calyculin-A. *Journal of Pharmacology and Experimental Theraputics* **250**, 388-396.

Irwin, D. A. (1931). The anatomy of the Auerbach's plexus. *American Journal of Anatomy* **49**, 141-166.

Iyer, V., Bornstein, J. C., Costa, M., Furness, J. B., Takahashi, Y. & Iwanaga, T. (1988). Electrophysiology of guinea-pig myenteic neurons correlated with immunoreactivity for calcium-binding proteins. *Journal of the Autonomic Nervous System* **22**, 141-150.

Jacobowitz, O., Chen, J., Premont, R. T. & Iyengar, R. (1993). Stimulation of specific types of Gs-stimulated adenylyl cyclase by phorbol ester treatment. *Journal of Biological Chemistry* **268**, 3829-3832.

Jeitner, T. M., Jarvie, P. E., Costa, M., Rostas, J. A. P. & Dunkley, P. R. (1991). Protein phosphorylation in guinea-pig myenteric ganglia and brain: presence of calmodulin kinase II, protein kinase C and cyclic AMP kinase and characterization of major phosphoproteins. *Neuroscience* **40**, 555-569.

Johnson, S. M., Katayama, Y. & North, R. A. (1980a). Slow synaptic potentials in neurones of the myenteric plexus. *Journal of Physiology* **301**, 505-516.

Johnson, S. M., Katayama, Y. & North, R. A. (1980b). Multiple action of 5-hydroxytryptamine an myenteic neurones of the guinea-pig ileum. *Journal of Physiology* **304**, 459-470.

Johnson, S. M., Katayama, Y., Morita, K. & North, R. A. (1981). Mediators of slow synaptic potentials in the myenteric plexus of guinea-pig ileum. *Journal of Physiology* **320**, 175-186.

Jones, S. W. (1985). Muscarinic and peptidergic excitation of bull-frog sympathetic neurones. *Journal of Physiology* **366**, 63-87.

Katayama, Y. & North, R. A. (1979a). Does substance P mediate slow synaptic excitation within the myenteric plexus?. *Nature* **274**, 387-388.

Katayama, Y., North, R. A. & Williams, J. T. (1979b). The action of substance P on neurons of the myenteric plexus of the guinea-pig small intestine. *Proceedings of the National Academy of Sciences* **206**, 191-208.

Komuro, T., Baluk, P., Burnstock, G. (1982). An ultrastructural study on neurons and non-neuronal cells in the myenteric plexus of the rabbit colon. *Neuroscience*, 7, 1797-1806.

Kirchgessner, A. L. & Gershon, M. D. (1989). Identification of vagal efferent fibers and putative targets neurons in the enteric nervous system of the rat. *The Journal of Comparitive Neurology* **285**, 38 - 53.

Langley, J. N. (1921). The Autonomic Nervous System, part I., pp 1-80, Cambridge, Heffer.

Levitan, I. B. (1994). Modulation of ion channels by protein phosphorylation and dephosphorylation. *Annual Reviews in Physiology* **56**, 193-212.

Lo, C. F. & Breitwieser, G. E. (1994). Protein kinase-independent inhibition of muscarinic K+ channels by staurosporine. *American Journal of Physiology* **266**, C1128-C1132.

Mawe, G. M. (1992). Intracellular recording from neurones of the guinea-pig gall-bladder. *Journal of Physiology* **429**, 323-338.

McHugh, E. M. & McGee, R. Jr. (1986). Direct anesthetic-like effects of forskolin on the nicotinic acetylcholine receptors of PC12 cells. *Journal of Biological Chemistry* **261**, 3103-3106.

Meissner, G. (1857). Uber die Nerven der Darmwand. *Z Ration Med N F* **8**, 364-366.

Morita, K., North, R. A. & Tokimasa, T. (1982). The calcium-activated potassium conductance in guinea-pig myenteric neurones. *Journal of Physiology* **329**, 341-354.

Murphy, R., Furness, J. B., Beardsley, A. M. & Costa, M. (1982). Characterization of substarnce P-like immunoreactivity in peripheral sensory nerves and enteric nerves by high pressure liquid chromatography and radio-immunoassay. *Regulatory Peptides* **4**, 203-212.

Morita, K. & Katayama, Y. (1992). Substance P inhibits activation of calcium-dependent potassium conductances in guinea-pig myenteric neurones. *Journal of Physiology* **447**, 293-308.

Morita, K. & North, R. A. (1985). Significance of slow synaptic potentials for transmission of excitation in guinea-pig myenteric plexus. *Neuroscience* **14**, 661-672.

Nakajima, Y., Nakajima, S. & Inoue, M. (1988). Pertussis toxin-insensitive G-protein mediates substance P-induced inhibition of potassium channels in brain neurons. *Proceedings of the National Academy of Sciences* **85**, 3643-3647.

Nemeth, P. R., Palmer, J. M., Wood, J. D. & Zafirov, D. H. (1986). Effects of forskolin on electrical behaviour of myenteric neurones in guinea-pig small intestine. *Journal of Physiology* **376**, 439-450.

Nemeth, P. R. & Gullikson, G. W. (1989). Gastrointestinal motility stimulating drugs and 5-HT receptors on myenteric neurons. *European Journal of Pharmacology* **166**, 387-391.

Nishi, S. & North, R. A. (1973). Intracellular recording from the myenteric plexus of the guinea-pig ileum. *Journal of Physiology* **231**, 471-491.

North, R. A. (1993). Mechanisms of Autonomic Integration. In Handbook of Physiology - The Nervous System IV, eds. ???, pp. 115-153, ??????.

North, R. A. & Tokimasa, T. (1983). Depression of calcium-dependent potassium conductance of guinea-pig myenteric neurones by muscarinic agonist. *Journal of Physiology* **342**, 253-266.

North, R. A. & Tokimasa, T. (1984). The time course of muscarinic depolarization of guinea-pig myenteric neurones. *British Journal of Pharmacology* **82**, 85-91.

North, R. A. & Tokimasa, T. (1987). Persistent calcium sensitive potassium current and the resting properties of guinea pig neurones. *Journal of Physiology* **386**, 333-353.

North, R. A., Williams, J. T., Surprenant, A. & Christie, M. J. (1987). Mu and delta receptors belong to a family of receptors that are coupled to potassium channels. *Proceedings of the National Academy of Sciences* **84**, 5487-5491.

Offermanns, S. & Schultz, G. (1994). What are the functions of pertussis toxin-insensitive G proteins G_{12}, G_{13} and G_Z? *Molecular and Cellular Endocrinology*. **100**, 71-74.

Palmer, J. M., Wood, J. D. & Zafirov, D. H. (1987). Transduction of aminergic and peptidergic signals in enteric neurones of the guinea-pig. *Journal of Physiology* **387**, 371-383.

Pan, H., & Gershon, M. (1994). Intracellular transduction pathways in the action of 5-HT_{1P} receptors in the myenteric plexus of the guinea pig ileum. *Gastroenterology Abstracts* **106**, A1044.

Pompolo, S. & Furness, J. B. (1988). Ultrastucture and synaptic relationships of calbindin-reactive, Dogiel type II neurons, in the myenteric plexus of guinea-pig small intestine. *Journal of Neurocytology* **17**, 771-782.

Pompolo, S. & Furness, J. B. (1990). Ultrastructure and synaptology of neurons immunoreactive for gamma-aminobutyric acid in the myenteric plexus of guinea-pig small intestine. *Journal of Neurocytology* **19**, 539-549.

Pompolo, S. & Furness, J. B. (1993). Origins of synaptic inputs to calretinin immunoreactive neurons in the guinea-pig small intestine. *Journal of Neurocytology* **22**, 531-546.

Ruegg, U. T. & Burgess, G. M. (1989). Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases. *Trends in Pharmacological Sciences* **10**, 218-220.

Schutze, S., Potthoff, K., Machleidt, T., Berkovi, D., Wiegmann, K. & Kronke, M. (1992). TNF activates NF-kB by phosphatidylcholine specific phospholipase C-induced "acidic" sphingomyelin breakdown. *Cell* **71**, 765-776.

Seamon, K. B. & Daly, J. W. (1986). Forskolin: Its biological and chemical properties. In Advances in cyclic nucleotide and protein phosphorylation research, eds. Greengard, P. & Robison, G. A., pp 1-150, New York, Raven Press.

Shen, K. Z. & Surprenant, A. (1990). Mechanisms underlying presyaptic inhibition through α_2 adrenoceptors in guinea-pig submucosal neurones. *Journal of Physiology* **431**, 609-628.

Shen, K. Z. & Surprenant, A. (1993). Common ionic mechanisms of excitation by substance P and other transmitters in guinea-pig submucosal neurones. *Journal of Physiology* **462**, 483-501.

Shuster, M. J., Camardo, J. S., Siegelbaum, S. A. & Kandel, E. R. (1985). Cyclic AMP-dependent protein kinase closes the serotonin-sensitive K^+ channels of *Aplysia* sensory neurones in cell-free membrane patches. *Nature* **313**, 392-395.

Siegelbaum, S. A. (1994). Ion channel control by tyrosine phosphorylation. *Current Biology* **4**, 242-245.

Smith, T. K., Bornstein, J. C. & Furness, J. B. (1992). Convergence of reflex pathways excited by distension and mechanical stimulation of the mucosa onto the same myenteric neurons of the guinea pig small intestine. *Journal of Neuroscience* **12(4)**, 1502-1510.

Song, Z. -M., Brookes, S. J. H. & Costa, M. (1991). Identification of myenteric neurons which project to the mucosa of the guinea-pig small intestine. *Neuroscience Letters* **129**, 294-298.

Sternweis, P. C. & Pang, I. (1990). The G protein-channel connection. *Trends in Neuroscience* **13**, 122-126.

Stutts, M. J., Henke, D. C. & Boucher, R. C. (1990). Diphenylamine-2-carboxylate (DPC) inhibits both Cl- conductance and cyclo-oxygenase canine tracheal epithelium. *Pflugers Archives* **415**, 611.

Surprenant, A. (1984). Slow excitatory synaptic potentials recorded form neurones of guinea-pig submucous plexus. *Journal of Physiology* **351**, 343-361.

Surprenant, A., North, R. A. & Katayama, Y. (1987). Observations on the actions of substance P and [D-Arg-1,D-Pro-2,D-Trp-7,9,Leu-11]substance P on single neurons of the guinea pig submucous plexus. *Neuroscience* **20**, 189-199.

Surprenant, A. & North, R. A. (1988). Mechanism of syanptic inhibition by noradrenaline acting at alpha2 adrenoceptors. *Proceedings of the National Academy of Sciences* **234**, 85-114.

Szurszewski, J. H. & King, B. F. (1989). Physiology of prevertebral ganglia in mammals with special reference to inferior mesenteric ganglion. In Handbook of Physiology - The Gastrointestinal System, eds. Schultz, S. G., Wood, J. D. & Rauner, B. B., pp. 519-592. New York: Oxford University Press.

Takaki, M., Branchek, T., Tamir, H. & Gershon, M. (1985). Specific antagonism of enteric neural serotonin receptors by dipeptides of 5-hydroxytryptophan: evidence that serotonin is a mediator of slow synaptic excitation in the myenteric plexus. *Journal of Neuroscience* **5**, 1769-1780.

Takeuchi, T., Okuda, M. & Yagasaki, O. (1991). The differential contribution of endogenous prostaglandins to the release of acetylcholine from the myenteric plexus of the guinea-pig ileum. *British Journal of Pharmacology* **102**, 381-385.

Taylor, S., Bubis, J., Toner-Webb, J., Saraswat, L. D., First, E. A., Buechler, J. A., Knighton, D. R. & Sowadski, J. (1988). *FASEB J.* **2**, 2677-2685.

Tatsumi, H., Hirai, K. & Katayama, Y. (1988). Measurements of the intracellular calcium concentration in guinea-pig myenteric neurons by using fura-2. *Brain Research* 451, 371-375.

Tatsumi, H., Costa, M., Shimerlik, M. & North, R. A. (1990). Potassium conductance increased by noradrenaline, opioids, somatostatin, and G-proteins: Whole-cell recording from guinea pig submucous neurons. *Journal of Neuroscience* **10(5)**, 1675-1682.

Tokimasa T. & Akasu, T. (1993). Biochemical gating for voltage-gated channels: mechanisms for slow synaptic potentials. In Autonomic Ganglia eds. E. M. McLachlan, pp 1-19. Berkshire, Harwood.

Tokimasa, T. & North, R. A. (1984). Many transmitters, few channels. In Coexistence of Neuroactive Substances in Neurons, eds. Chan-Palay, V. & Palay, S. L., pp. 217-296. : John Wiley & Sons, Inc.

Tonini, M. & Costa, M. (1990). A pharmacological analysis of the neuronal circuitry involved in distension-evoked enteric excitatory reflex. *Neuroscience* **38**, 787-795.

Toro, L., Ottolia, M., Olcese, R. & Stefani, E. (1993). Niflumic acid activates large conductance K,Ca channels. *Biophysical Journal* **64**, A2.

Trouslard, J., Mirsky, R., Jessen, K. R., Burnstock, G. & Brown, D. A. (1993). Intracellular calcium changes associatd with cholinergic nicotinic receptor activation in cultured myenteric plexus neurones. *Brain Research* **624**, 103-108.

Weight, F. F. & Votava, J. (1970). Slow synaptic excitation in sympathetic ganglion cells: evidence for synaptic inactivation of potassium conductance. *Science* **170**, 755-758.

White, M. M. & Aylwin, M. (1990). Niflumic and flufenamic acids are potent reversible blockers of Ca2+-activated Cl- channels in Xenopus Oocytes. *Molecular Pharmacology* **37**, 720-724.

Williams, J. T., North, R. A. & Tokimasa, T. (1988). Inward rectification of resting and opiate-activated potassium currents in rat locus coeruleus neurons. *Journal of Neuroscience* **8(11)**, 4299-4306.

Wood, J. D. & Mayer, C. J. (1978). Intracellular study of electrical activity of Auerbach's plexus in guinea pig small intestine. *Pflugers Archives* **374**, 265-275.

Wood, J. D. (1989). Electrical and synaptic behaviour of enteric neurons. In Handbook of Physiology - The Gastrointestinal System, eds. Schultz, S. G., Wood, J. D. & Rauner, B. B., pp. 485-517. New York: Oxford University Press.

Wood, J. D. & Mayer, C. J. (1978). Slow synaptic excitation mediated by serotonin in Auerbach's plexus. *Nature* **276**, 836-837.

Xia, Y., Baidan, L. V., Fertel, R. H. & Wood, J. D. (1991). Determination of levels of cAMP in the myenteric plexus of guinea-pig small intestine. *European Journal of Pharmacology* **206**, 231-236.

Yang, S-G., Saifeddine, M., Laniyonu, A. & Hollenberg, M. D. (1993). Distinct signal transduction pathways for angiotensin-II in guinea pig gastric smooth muscle: Differential blockade by indomethacin and tyrosine kinase inhibitors. *Journal of Pharmacology and Experimental Theraputics* **264**, 958-966.

Yau, W. M., Bowen, D. J. & Youther, M. L. (1991). Evidence for an involvement of eicosanoids in neurokinin-3 receptor mediated acetylcholin release from myenteric neurons. *Neuroscience Letters* **129**, 259-261.

Zafirov, D. H., Palmer, J. M., Nemeth, P. R. & Wood, J. D. (1985). Bombesin, gastrin releasing peptide and vasoactive intestinal peptide excite myenteric neurons. *European Journal of Pharmacology* **115**, 103-107.