# Substance P Receptor Activation and Desensitization as Monitored By M Current Inhibition

**by**

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**Substance P Receptor Activation and Desensitization as Monitored**

**By M Current Inhibition**

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## ABSTRACT

Substance P (SP) is a neuropeptide that plays a variety of roles in the human body, including inflammation, nociception, and smooth muscle regulation. In the central and peripheral nervous systems, SP indirectly acts as an excitatory neurotransmitter by inhibiting the M-current, ($I_M$), a time- and voltage-dependent outward potassium current. Activation of $I_M$ produces an outward (hyperpolarizing) current which acts to resist neuronal depolarization and action potential production. By inhibiting $I_M$, SP has the ability to modulate neuronal excitability. Since activation of the SP receptor results in $I_M$ inhibition, $I_M$ amplitude was utilized in the present study as an indirect measure for SP receptor activation and desensitization. Whole-cell voltage-clamp recordings were used to monitor $I_M$ in acutely isolated bullfrog sympathetic ganglia neurons. The cell was voltage-clamped at a holding potential of -30 mV, where $I_M$ is largely activated. Steps to -60 mV deactivated $I_M$, producing a slow, exponential decrease in current amplitude, followed by an exponential increase in current (reactivation) upon stepping back to -30 mV. Single exponential curve fits were utilized to measure $I_M$ amplitude during the reactivation phase. SP application (1 μM) initially produced a rapid and significant inhibition in $I_M$ amplitude. However, with continuous SP application, $I_M$ amplitude significantly recovered, returning to the control amplitude. This desensitization, defined as a decrease in responsiveness following prolonged or repeated exposure to an agonist, suggests a similar desensitizing loss of functioning by the SP receptor during SP application. The findings presented here support previous studies showing that SP application initially produces inhibition of $I_M$, but continued drug application results in desensitization and loss of $I_M$ inhibition.

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# CHAPTER 1

## I. Introduction

Substance P (SP) is a neuropeptide that serves as a neurotransmitter in both the central nervous system (CNS) and peripheral nervous system (PNS). It is a member of a group of closely related peptide neurotransmitter molecules known as tachykinins (Harrison and Geppetti, 2001).

Gaddum and Schild first discovered SP in the early 1930s in extracts of intestinal and brain tissue from horses (Leeman and Ferguson, 2000; Snijdelaar et al., 2000). The newly found substance received its name from their experimental results of obtaining a dry powder. Thus, the 'p' in powder was used to create the name substance P (Leeman and Ferguson, 2000; Harrison and Geppetti, 2001). During their experimentation, they proposed that SP was a neuronal sensory neurotransmitter and was coupled to pain transmission, a conclusion which was drawn from its location in the dorsal root (Snijdelaar et al., 2000; Harrison and Geppetti, 2001).

SP is mostly expressed in the CNS and PNS, but has been localized to non-neural sites. It has been found to have many roles in inflammation, nociception, and smooth muscle regulation (Leeman and Ferguson, 2000; Snijdelaar et al., 2000; Harrison and Geppetti, 2001; Perrine et al., 2003). Immunoreactivity experiments have shown SP to be abundantly expressed in the CNS, including the cerebrum, hippocampus, basal nuclei, amygdala, septal area, thalamus, hypothalamus, pons, medulla, cerebellum, and spinal cord (Shults et al., 1984). Other techniques, such as polyclonal antisense, *in situ* hybridization, and Northern blot analysis, have also found SP to be expressed peripherally in the cell bodies of primary afferent neurons in nodose, trigeminal, and

dorsal root ganglia (DRG) (Kiyama, et al., 1988; Hamid et al., 1991; Sternini, 1991). Furthermore, SP has been found in the stomach and small and large intestines, where it functions to increase intestinal peristalsis (Gartner and Hiatt, 2007).

Despite the advancements made in localizing SP and determining its function, its amino acid structure was not discovered until 1971. Chang et al. (1971) found that SP extracted from bovine hypothalamus had the undecapeptide sequence of H-Arg$^{1}$-Pro$^{2}$-Lys$^{3}$-Pro$^{4}$-Gln$^{5}$-Gln$^{6}$-Phe$^{7}$-Phe$^{8}$-Gly$^{9}$-Leu$^{10}$-Met$^{11}$-$NH_2$. Subsequently, SP was officially included in the tachykinin family (Erspamer, 1983). Tachykinin peptides share a common amino acid sequence at their carboxy terminal of Phe-X-Gly-Leu-Met-$NH_2$, where X represents Phe/Tyr or Val/Ile (Otsuka and Yoshioka, 1993). Their total amino acid sequence varies in length, but ranges from 9 to 42 residues (Liu and Burcher, 2005). In addition to SP, tachykinins include neurokinin A (NKA), neurokinin B (NKB), neuropeptide γ (NPγ), and neuropeptide K (NPK) (Otsuka and Yoshioka, 1993; Liu and Burcher, 2005). Hemokinin 1 and endokinins A-D have recently also been added to this family (Page et al., 2003).

**A. SP Synthesis**

Synthesis of SP begins with the precursor preprotachykinin-A (PPT-A) gene (Carter and Krause, 1990). This gene has four PPTs, (α-, β-, γ-, δ-PPT-A) that encode for SP, NKA, NPγ, NPK, NKB, hemokinin 1, and endokinins A-D (Page et al., 2003; Liu and Burcher, 2005). Synthesis of PPT-A takes place on the membrane-bound ribosomes of the rough endoplasmic reticulum within the neuron cell body and the resulting prepropeptide is packaged into storage vesicles by the Golgi apparatus (Harmar et al.,

1980; Harmar and Keen, 1982). During axonal transport of the prepropeptide filled vesicles from the neuron cell body to the axon terminal, differential processing takes place (Snijdelaar et al., 2000). First, convertases cleave doublets of cationic residues from the PPT protein. At this point, peptidyl-Gly-α-amidating monoxygenase can use glycine as an amide donor to produce amidation of the methionine at the carboxy terminal (COOH-) sequence (Regoli et al., 1994; Snijdelaar et al., 2000). Finally, the physiologically active peptide, SP, is produced (Snijdelaar et al., 2000).

Two enzymes have been discovered that take part in the cleavage and inactivation of SP following its synaptic release. Neutral endopeptidase (NEP) has been found to play a role in SP metabolism at synapses in the brain, spinal cord, and peripheral tissue while angiotensin-converting enzyme (ACE) acts to degrade SP in the plasma (Sakurada et al., 1990; Wang et al., 1991). Both enzymes are active in the cleavage of SP by catalyzing the hydrolysis of either the $Gln^6$ –$Phe^7$, $Phe^8$-$Gly^9$, or $Gly^9$-$Leu^{10}$ bonds within SP and preventing the COOH- region of SP from binding to its receptor (Skidgel and Erdos, 1987; Lantz et al., 1991).

**B. Neurokinin Receptors**

The biological function of SP and other tachykinin peptides results when they bind to a tachykinin receptor, which are also referred to as neurokinin (NK) receptors. The structure of the NK receptor consists of seven hydrophobic transmembrane domains that are connected via intra- and extracellular loops. Receptor actions are mediated through the activation of a guanine nucleotide-binding protein (G-protein) (Simmons and Mather, 1991; Harrison and Geppetti, 2001; Datar et al., 2004). Three subtypes of NK

receptors have been discovered and each show preference for certain tachykinin peptides. SP preferentially binds to the $NK_1$ receptor, while neurokinin A has the highest affinity for the $NK_2$ receptor, and the third receptor, $NK_3$, shows preference for neurokinin B. Although each of these receptors has its highest binding affinity for a particular tachykinin peptide, it should be noted that any of the tachykinins can bind to all three subtypes of NK receptors (Snijdelaar et al., 2000; Harrison and Geppetti, 2001; Datar et al., 2004). The $NK_1$ and $NK_2$ receptors have been found in both peripheral tissues and the CNS, whereas the $NK_3$ receptor has only been localized to the CNS (Snijdelaar et al., 2000).

The preferential receptor for SP is the $NK_1$ receptor, which has a length of 407 amino acids and a molecular weight of 46 kDa. Agonist and antagonist binding sites are found on the second and third membrane-spanning regions of the receptor, while the third cytoplasmic loop mediates binding with a G-protein (O'Connor et al., 2004). Studies have shown that the $NK_1$ receptor is a single copy gene and is located on human chromosome 2 (Gerard et al., 1991; Harrison and Geppetti, 2001). Substance P binding is associated with the Asn-85, Asn-89, Tyr-92, and Asn-96 residues located in the second transmembrane domain and the Tyr-287 residue in the seventh transmembrane domain of the $NK_1$ receptor. Moreover, the Glu-78 in the second transmembrane domain, along with Tyr-205 in the fifth transmembrane domain are both involved with activation of the $NK_1$ receptor (Huang et al., 1994).

Activation of the $NK_1$ receptor results in subsequent G-protein and second-messenger system activation. In the phosphatidylinositol pathway ligand binding to the $NK_1$ receptor leads to activation of the phospholipase C (PLC) enzyme. This results in

the breakdown of phosphatidylinositol bisphosphate ($PIP_2$) into two components: inositol-1,4,5-triphosphate ($IP_3$) and diacylcerol (DAG). At this point, $IP_3$ binds to and opens calcium ($Ca^{2+}$) channels located on the endoplasmic reticulum to stimulate the release of intracellular $Ca^{2+}$ stores. $Ca^{2+}$ binds to protein kinase C (PKC) and DAG recruits PKC from the cytoplasm to the membrane. PKC binds to DAG in the membrane and subsequently becomes active. Activation of PKC is also assisted by the protein calmodulin. When $Ca^{2+}$ binds to calmodulin, activation of the $Ca^{2+}$-calmodulin-dependent protein kinase (CAMK) occurs allowing it to phosphorylate different proteins and produce cellular effects. Kinase activity is thought to mediate $NK_1$ receptor activity via this mechanism (Siegelbaum et al., 2000; Snijdelaar et al., 2000; Datar et al., 2004; O'Connor et al., 2004; Meyer and Quenzer, 2005).

### C. SP Release and Function

SP release from afferent neurons has been found to play a variety of roles in the body that are clinically important, such as in nociception, inflammatory responses, and control of neuronal excitability. The neuron cell bodies of primary afferent neurons are located in both dorsal root and trigeminal ganglia. These neurons have two axonal processes that extend into either the periphery or CNS. For dorsal root ganglia neurons, the longer peripheral process terminates in the periphery while the shorter central process extends into the spinal cord, where it terminates in the dorsal horn (Siegelbaum et al., 2000). SP is released from these sensory neurons at both its central endings within the dorsal horn of the spinal cord and from its peripheral endings into the surrounding tissue (Augustine et al., 2004).

SP neurons which terminate in the dorsal horn of the spinal cord are associated with nociception. These neurons are nonmyelinated and are known as C-fibers. Nociceptors at their peripheral endings initiate the sensation of pain in response to noxious temperature, mechanical, or chemical stimuli. These neurons project into the dorsal horn of the spinal cord and form the first neuron in a three neuron pathway for transmission of nociceptive information to the brain (Siegelbaum et al., 2000; Augustine et al., 2004).

Several pathways are used for pain transmission, but the primary system is the spinothalamic tract, which is the major ascending pathway for pain and temperature stimuli. Axons of the first-order DRG neurons enter the spinal cord via the dorsal root where they synapse onto a second order projection neuron located in lamina V of the dorsal horn (Siegelbaum et al., 2000; Augustine et al., 2004). The axon of the second-order neuron crosses the midline and ascends within the anterolateral region of the contralateral spinal cord. It continues through the brainstem to the thalamus, where the second synapse occurs. The third-order thalamic neuron then ascends to the cerebral cortex, where it terminates in the primary somatosensory cortex (Augustine et al., 2004).

A noxious stimulus causes SP to be released centrally from C-fibers at their synapse with the second-order dorsal horn neurons (Kidd and Urban, 2001). SP is co-released with the excitatory neurotransmitter glutamate and is thought to enhance and prolong the actions of glutamate (Kandel et al., 2004). Glutamate acts on α-amino-3-hydroxy-5-methylisoxazole (AMPA) receptors creating an excitatory postsynaptic potential (EPSP) resulting in depolarization of the dorsal horn neuron. With increased stimuli and depolarization *N*-methyl-D-aspartate (NMDA) receptors become activated

and increased excitability in dorsal horn neurons occurs. When SP is released it binds to its $NK_1$ receptor located in the dorsal horn and activates the previously mentioned phosphatidylinositol pathway. After $IP_3$ activation and subsequent PKC activation, PKC, which may be co-activated, phosphorylates the NMDA receptor allowing the receptor to become more easily responsive to glutamate. By activating NMDA receptors, central sensitization, which is an increase in receptor responsiveness to prolonged exposure to a noxious stimulus, can occur (Kidd and Urban, 2001).

A second important functional role of primary nociceptive neurons is the release of SP from their peripheral axon endings, which mediates a variety of inflammatory effects. These responses may involve arterioles, the respiratory tract, gastrointestinal tract, or body joints (Harrison and Geppetti, 2001). Moreover, four times as much SP immunoreactivity is located in peripheral tissue than in the dorsal horn, indicating an important role for SP in the periphery (Harmar et al., 1980). The peripheral locations of SP have lead to the proposal that blocking SP actions at these locations could serve a therapeutic role in the treatment of pain and inflammation (Harrison and Geppetti, 2001).

The inflammatory response that results from SP release by nociceptive neurons is known as neurogenic inflammation. One part of this response is plasma protein extravasation (Harrison and Geppetti, 2001). When SP is released from primary sensory neurons, it binds to its $NK_1$ receptor at post-capillary venules and produces mast-cell degranulation, increased plasma leakage through endothelial gaps, and vasodilation with subsequent increased blood flow to that particular location (Baluk, 1997; Maggi, 1997). When mast cells degranulate, they release a variety of mediators, such as histamine, serotonin, and prostanoids (Maggi, 1997). Histamine and other mediators disrupt the

endothelial layer and induce the formation of endothelial gaps to allow the passage of proteins out of the venules causing inflammation (Mehta and Malik, 2005).

Inflammation can be prevented by inhibiting stimulation of sensory neurons, thereby inhibiting peripheral release of SP, or by blocking the $NK_1$ receptor which mediates the actions of SP. Beta-adrenergic agonists and steroids can also be used to inhibit the formation of the endothelial gaps, which prevents the edema and swelling associated with plasma leakage (Baluk, 1997).

Another site of SP action is the respiratory tract, where SP has been shown to produce inflammatory responses in airways and could thus be of importance in mediating the symptoms of asthma and chronic bronchitis. A similar vasodilation-induced inflammatory response occurs in the walls of airways due to activation of the $NK_1$ receptors. This in turn stimulates seromucous glands to increase secretion. Simultaneously inflammatory cells, including macrophages, eosinophils, lymphocytes, and dendritic cells, are recruited and activated causing an inflammatory response. This results in airway hyperresponsiveness, which is an enhanced bronchoconstriction response that may be stimulated by a variety of stimuli and is an important feature of asthma (Advenier et al., 1997).

SP is also thought to play a role in the gastrointestinal tract and perhaps produce inflammation that contributes to diseases like *Clostridium difficile* induced pseudomembranous colitis, Crohn's disease, and ulcerative colitis. SP nerve fibers have also been localized in the ureter and bladder where they are thought to participate in cystitis and increased contraction of bladder smooth muscle (Harrison and Geppetti, 2001; O'Connor et al., 2004).

Further support for the role of SP in inflammation comes from studies which have shown that SP stimulates the recruitment of inflammatory cells. As a result, leukocytes accumulate at the site of SP release and the inflammatory response is amplified. These results have led to the proposal that SP is involved in arthritis (Harrison and Geppetti, 2001; O'Connor et al., 2004). Levine et al. (1984) found a considerable number of SP-containing neuronal endings in the joints of rats that suffered severe arthritis in comparison to rats that suffered only mild arthritis. To test whether SP had a primary role in the arthritic inflammatory response, SP was injected directly into the joints of these rats, which caused the inflammatory response to amplify. It was concluded that although SP had a secondary role to the immunological mechanisms that occur in the joint, it did act to enhance the inflammatory response. Therefore, diminishing SP levels in the joint could provide a therapeutic option in reducing the inflammation associated with arthritis.

A third role SP has is in neuronal excitability. When SP is released from primary afferent neurons it acts as an excitatory neurotransmitter causing a depolarizing response (Yamada and Akasu, 1996; Snijdelaar et al., 2000; Harrison and Geppetti, 2001). This depolarizing response is primarily caused by inhibition of the time- and voltage-dependent potassium current known as the M current ($I_M$) which will be discussed later (Akasu et al., 1996; Brown and Yu, 2000; Perrine et al., 2003).

### D. SP Agonists and Antagonists

Peptide agonists have been discovered that have similar efficacy as SP to activate the $NK_1$ receptor. These agonists have been categorized into two groups based on their

pharmacological action: classical agonists and 'septide-like' agonists (Harrison and Geppetti, 2001). Classical agonists, such as [$Sar^9$]-substance P sulfone, substance P methyl ester, and [$Sar^9$Met(02)11]substance P, bind readily to $NK_1$ receptors at the SP binding site. "Septide-like" agonists bind to a site on the $NK_1$ receptor that is different from the SP binding site (Beaujouan et al., 2000). Examples of 'septide-like' agonists include septide itself ([$pGlu^6$,$Pro^9$]substance P(6-11)), and aminovalery1[$Pro^9$,$NmeLeu^{10}$] substance P(7-11). "Septide-like" agonists have similar actions as SP at the $NK_1$ receptor (Meini, et al., 1995). Despite the current findings, more research needs to be conducted to find clinical relevance for both categories of $NK_1$ receptor agonists (Harrison and Geppetti, 2001).

SP antagonists have an important clinical relevance due to their production of an analgesic effect (Snijdelaar et al., 2000). From early studies, it was recognized that a double-D-amino substitution in the COOH-terminal portion of SP resulted in a SP antagonist (Regoli et al., 1994). The first antagonist developed was [D-$Pro^2$, D-$Trp^{7,9}$]substance P, which inhibited SP-induced smooth muscle contractions in the three model systems of the guinea-pig tenia coli, rat colon, and rabbit eye (Holmdahl et al., 1981; Leander et al., 1981; Bjorkroth et al., 1982). Despite its SP antagonistic features, it also produced neurotoxicity and thus is no longer an approved antagonist (Devillier et al., 1985). Other SP antagonists have been discovered, but these exhibited signs of neurotoxicity so another approach that produced conformational changes in the SP molecule was tried (Harrison and Geppetti, 2001).

$NK_1$ antagonists were developed by making various conformational changes to SP. One such antagonist, GR 71251, had a high affinity for the $NK_1$ receptor. In

addition, FR 113680 and FK 888, were also developed and possessed similar qualities. Despite these compounds having a high affinity and selectivity for the $NK_1$ receptor, deficiencies in oral bioavailability and blood-brain barrier penetration were a problem and prevented these antagonistic compounds from being utilized in clinical studies (Hagiwara et al., 1992, 1994; Harrison and Geppetti, 2001).

The hurdle of keeping a high $NK_1$ affinity and selectivity, but allowing CNS penetration was finally overcome with the development of non-peptide $NK_1$ receptor antagonists, such as CP 96 345 and CP 99 994. Both of these new antagonists were able to cross the blood-brain barrier and therefore could readily enter the CNS (Snider et al., 1991; McLean et al., 1993). Other non-peptide $NK_1$ antagonists have also been developed that showed similarities to CP 96345, but affinity to the $NK_1$ receptor strongly varied between species and these variations may cause single mutations in the receptors amino-acid sequence (Harrison and Geppetti, 2001)

Despite these problems, some success has been achieved in the search for beneficial $NK_1$ antagonists. Several drugs, including CP 99 994, have made it to clinical trials. At low doses, CP 99 994 does not produce side effects and it has been used as an analgesic after dental extraction (Dionne et al., 1998). Other successful drugs include MK-869, which is being utilized in depressive disorders, and an anti-emetic drug, L-754, that is being used after chemotherapy treatment with cisplatin (Kramer et al., 1998; Navari et al., 1999).

More recently, another method of pain reduction has been considered that utilizes capsaicin, a drug derived from the red-hot chili pepper (Harrison and Geppetti, 2001). Capsaicin is currently sold in the United States and Canada as a topical cream and serves

as a treatment for the pain associated with diseases such as osteoarthritis, rheumatoid arthritis, and postherpetic or diabetic neuropathy (Park et al., 1995). When capsaicin is applied to the skin, it serves as an agonist for vanilloid receptors located on the peripheral afferent nerve endings. Binding to these receptors activates ligand-gated cation channels and causes an excitatory response on most C-fibers, with action potential production and SP release from the central terminals of the afferent neuron (Maggi and Meli, 1988). Although capsaicin initially causes a burning sensation due to the stimulated release of SP, this is followed by a long-lasting sense of pain relief as SP stores are depleted. With multiple short-term applications of capsaicin, a period of prolonged reduced afferent neuron functioning occurs which gives capsaicin its antinociceptive effect. A major setback to this drug is its neurotoxic feature of causing degeneration of sensory nerves in the epidermis with topical application (Simone et al., 1998). Capsaicin analogs are being tested to reduce the burning effect, which is another major drawback of this drug (Snijdelaar et al., 2000).

**E. Bullfrog Tachykinins**

Over forty different tachykinin peptides have been isolated in a variety of species. Four tachykinin peptides have been isolated from the brain and intestine of the bullfrog (*Rana catesbeiana*). These are ranatachykinin A (RTKA), ranatachykinin B (RTKB), ranatachykinin C (RTKC), and ranatachykinin D (RTKD) (Kozawa et al., 1991). Another tachykinin, SP, has also been localized in the brain of the bullfrog and can be found in the hypothalamic periventricular zone, amygdaloid complex, septal area, dorsal and ventral thalamus, olfactory bulb, and central gray matter, among a variety of other

areas. The locations of these SP immunoreactive cell bodies in the *R. catesbeiana* brain implies tachykinin peptides play a role in numerous areas, including the visual, auditory, and olfactory systems (Inagaki et al., 1981).

Using a Sodium Dodecyl Sulfate (SDS) micelle system, researchers have been able to develop ideas about the structure and activity of tachykinins (Keire and Fletcher, 1996; Perrine et al., 2000). With this system, ranatachykinins A-D, in addition to SP, were discovered to have helical prebinding ligand conformations from the midregion of the peptide to the C-terminus, and a high degree of flexibility at the N-terminus. Numerous mammalian studies have indicated the C-terminus is important for biological activity and receptor binding. A study completed in CHO cells which were transfected with the bullfrog $NK_1$ receptor, supports this hypothesis for amphibian tachykinins. However, more studies need to be conducted to determine if biological activity occurs at other locations besides the C-terminus (Perrine et al., 2000).

Similarities can be seen between the bullfrog and mammalian tachykinin receptors. A 69% identity exists between the cloned mammalian $NK_1$ receptor and the amino acid sequence of the bullfrog tachykinin receptor, which is composed of 408 amino acids. Another CHO study showed similar SP binding potency between the bullfrog tachykinin receptor and the mammalian $NK_1$ receptor. In both models, the binding affinity was SP>NKA>>NKB (Simmons et al., 1997).

### F. Desensitization of the $NK_1$ Receptor

Activation of nociceptic neurons causes the release of SP at their synapses with second-order neurons within the dorsal horn of the spinal cord. With repetitive

application of a stimulus and prolonged release of SP, the physiological response of receptor desensitization may occur (Kidd and Urban, 2001). Desensitization is defined as a decrease in receptor responsiveness following prolonged or repeated exposure to an agonist and results in decreased activation of the postsynaptic neuron. Resensitization is the opposite phenomenon where the return of responsiveness occurs following recovery of desensitization (Bennett et al., 2005).

Numerous G-protein coupled receptors show desensitization, which results from receptor phosphorylation and subsequent uncoupling of the receptor from its G-protein (Hausdorff et al., 1990). The mechanisms underlying desensitization have been extensively studied using the $\beta_2$-adrenergic receptor (Hausdorff et al., 1990; Krupnick and Benovic, 1998; Lefkowitz, 1998). Activation of the $\beta_2$-adrenergic receptor following ligand binding stimulates phosphorylation of the receptor by β-adrenergic receptor kinases 1 and 2. This modification then allows the binding of β-arrestins 1 and 2, which inhibit the interaction between the receptor and its G-protein, thus preventing signal transduction. At this point, a state of desensitization occurs (Hausdorff et al., 1990).

The $NK_1$ receptor is another G-protein coupled receptor whose desensitization is thought to be mediated by G-protein receptor kinases. This preliminary conclusion comes from models expressing SP receptors. In Kristen murine sarcoma virus-transformed rat kidney cells, the SP receptor may be phosphorylated and result in desensitization by G-protein receptor kinases 2 and 3 (Vigna, 1999). Similarly, phosphorylation of the human SP receptor has also been established (Nishimura et al., 1998), but information regarding direct changes in receptor function and their relation to

desensitization is unknown. Moreover, little information is known of G-protein coupled receptors and their role during desensitization in intact neurons (Simmons, 2001).

## G. M Current

The M channel ($I_M$) was first discovered in bullfrog sympathetic ganglion neurons by Brown and Adams (1980), but has since been shown to be present in a wide variety of mammalian peripheral and central neurons (Constanti and Brown, 1981; Womble and Moises, 1992; Coggan et al., 1994). $I_M$ is a voltage- and time- dependant $K^+$ channel that is activated by depolarization beginning at voltage levels (-40 to -55 mV in bullfrogs) near the resting membrane potential. It is an outward potassium current that is non-inactivating and has a slow time-course of activation and deactivation. $I_M$ acts as a primary contributor to membrane conductance within the -30 mV to -60 mV range, where it can thus be observed in relative isolation. Activation of $I_M$ produces an outward (hyperpolarizing) current which acts to resist neuronal depolarization and action potential production (Brown and Adams, 1980; Adams et al., 1982; Brown, 1988; Simmons and Mather, 1991). One example of this is cell accommodation, which is defined as the slowing and/or stopping of action potentials despite the continued application of an excitatory stimulus. The accommodation response is produced by activation of $I_M$ and is thus an important feature of how M-current plays a role in modulating neuronal excitability.

Several neurotransmitters act on neurons to produce inhibition of $I_M$, including agonists at muscarinic cholinergic receptors, SP receptors, and luteinizing hormone-releasing hormone receptors (Kuba and Koketsu, 1976; Jan and Jan, 1982; Adams et al.,

1983; Pfaffinger, 1988; Simmons et al., 1994). When $I_M$ is suppressed, membrane resistance is increased and the resting membrane potential becomes more depolarized. This greatly increases neuronal excitability by enabling excitatory synaptic inputs to more easily move toward the threshold for action potential production, thereby increasing the probability that excitatory synaptic inputs that normally fall below threshold will produce action potential firing (Brown and Adams, 1980; Adams et al., 1982; Brown, 1988; Simmons and Mather, 1991).

When SP is released from primary afferent neurons it activates the $NK_1$ receptor and produces a depolarizing response through inhibition of $I_M$ (Adams et al., 1983; Akasu et al., 1996; Simmons et al., 1997). With continued SP application, SP receptor desensitization occurs and inhibition of $I_M$ subsides, which allows M current to recover after a few minutes (Simmons, 2001). By suppressing $I_M$, SP has the ability to control neuronal excitability.

The cellular mechanisms underlying the neurotransmitter-induced suppression of $I_M$ are currently being sought. Numerous studies have demonstrated G-protein involvement in $I_M$ inhibition, more specifically the $G_{q/11}$ subtype (Pfaffinger, 1988; Mullaney et al., 1993; Simmons and Mather, 1991). Since $G_{q/11}$ proteins are linked to the phosphatidylinositol pathway, research has focused downstream on PLC and $PIP_2$ hydrolysis (Ford et al., 2003; Suh and Hille, 2005). Application of U73122, a PLC inhibitor, showed a decrease in the level of agonist induced inhibition of $I_M$ in mammalian sympathetic neurons, thus supporting a role for $PIP_2$ in the control of M channel opening (Bofill-Cardona et al., 2000). More direct evidence from rat sympathetic neurons and Chinese Hamster Ovary Cells (CHO) has indicated $PIP_2$ is

needed in order to have a stable conformation of the M channel. In the absence of $PIP_2$, the M channel becomes unstable preventing it from opening (Suh and Hille, 2002; Zhang et al., 2003; Liu et al., 2006). With this evidence in rat and hamster cell types it can be suggested a similar mechanism of $I_M$ suppression, which may involve activation of PLC and $PIP_2$ hydrolysis, also exists in bullfrog sympathetic neurons (Ford et al., 2003).

## H. Conclusion

SP is an important neurotransmitter in pain pathways and plays a variety of roles in the human body, including inflammation, nociception, and smooth muscle regulation. When SP is released from primary afferent neurons it can indirectly act as an excitatory neurotransmitter causing a depolarizing response. This depolarizing response is primarily caused by inhibition of M current. Inhibition of $I_M$ by SP has been well studied in both mammalian and bullfrog sympathetic neurons. With the continued presence of SP, a desensitizing response in the SP receptor may be shown with inhibition of $I_M$. Thus, this current can be used as a marker to indirectly measure activation and desensitization of the SP receptor.

## I. Hypotheses and Specific Aims

This research project uses the method of whole-cell patch clamp electrophysiology on sympathetic neurons of bullfrogs to examine M current as a marker to indirectly look at SP receptor activation and desensitization. The hypotheses for this experiment were as follows:

1. Does SP inhibit IM and does SP response show desensitization?

2. If so, what is the time course and magnitude of the desensitization?

To determine changes in SP receptor functioning, $I_M$ amplitude will be monitored before, during, and after SP application. Receptor activation will result in $I_M$ inhibition, while subsequent receptor desensitization will be associated with recovery of $I_M$ amplitude. Within this context, the specific aims will be to:

1. Determine control (baseline) level of $I_M$ amplitude.
2. Measure $I_M$ inhibition and subsequent recovery during continuous SP application, which indicates desensitization and loss of receptor functioning.
3. Determine extent of $I_M$ recovery after wash out of SP to reflect state of SP receptor activity.

Finding answers to these specific aims will result in a better understanding of SP receptor functioning and desensitization.

# CHAPTER 2

## II. Methods

### A. Cell Preparation

Single neurons of bullfrog sympathetic ganglia were isolated as previously described (Simmons et al., 1990). Bullfrogs were purchased from a commercial vendor (Rana Ranch, Twin Falls, Idaho, U.S.A.) and kept in an enriched environment on a 12hr/12hr day/night cycle. Animals were anesthetized by hypothermia and euthanized according to approved procedures. Experiments were approved by the Institutional Animal Care and Use Committee, Youngstown State University, Protocol #02-06. During dissection, bullfrogs were rinsed with a Ringer's saline solution. The sympathetic chain ganglia from both sides of the vertebral trunk were removed. Isolated ganglia were placed in a bath of Dissociation Medium and cleaned of excess tissue around the ganglia through the use of tweezers and dissection scissors. The ganglia were cut into small pieces before being placed in the enzyme. Next, the ganglia were placed in two enzyme solutions for the duration of one hour and thirty minutes each in a bath heated to 31˚ C. Enzymes were dissolved in Dissociation Medium. Enzyme solution 1 consisted of collagenase 1 (19.4mg/10ml) and trypsin (8.7 mg/10ml) and enzyme solution 2 contained collagenase 2 (13.6mg/10ml). At fifteen minute increments, the enzyme mixture was inverted five or six times to keep ganglia suspended for maximum enzyme activity. After enzyme 2 treatment, ganglia were used immediately or placed in Growth Medium and refrigerated from one to three days before use. Cell dishes and trituration pipettes were prepared with the protein BSA to prevent the cells from sticking to the sides of the dishes and pipettes. Ganglia were placed in a bath of Dissociation Medium 3 and triturated to

liberate individual neurons. Four pipettes of narrowing sizes were used for trituration: pipette one was used for the first cell dish, pipette two and three for dish two, and the third dish had ganglia that were triturated with pipette four. Cell dishes were then placed on a lab rotator at a continuous speed to reposition cells to center of dish until needed.

**B. Electrophysiology**

An Axoclamp 2B amplifier was used for whole-cell recordings of isolated neurons at room temperature. Recordings utilized patch electrodes with resistances of 4-7 MΩ, filled with Intracellular Solution. Drug application (1 μM SP) occurred through a capillary tube directed at the cell and extracellular perfusion with Extracellular Solution of the recording dish occurred separately before, during, and after drug application. Both intracellular and drug solutions were placed on ice until needed.

Action potentials were generated in Bridge mode by the application of a depolarizing stimulus, consisting of a 500 ms current pulse (50-115 pA), to determine the quality of each neuron. In addition, only cells with resting membrane potentials more negative than -45 mV were considered of good quality. $I_M$ was observed in Voltage-clamp mode during 500 ms voltage steps from a holding potential of -30 mV to -60 mV, allowing $I_M$ to become suppressed during the step. Responses were monitored every 8 sec. Duration of voltage step was chosen based on the slow time-course and marked delay of M current. P-clamp software was used for data acquisition, digitization, and subsequent analysis.

**C. Salines Used**

All solutions are in mM unless otherwise stated.

Ringer's Solution: NaCl 115, KCl 2.5, $CaCl_2$ 1.8, $MgCl_2$ 1.8, HEPES 2.5, pH 7.4 (with 1N NaOH).

Dissociation Medium: NaCl 98, KCl 2.4, $NaH_2PO_4$ 0.6, $MgCl_2$ 1.8, Na Pyruvate 5, Creatine 5.7, Glucose 5, HEPES 20, M199 medium 10 ml/L, Penicillin 100 U/ml, Streptomycin 100 μg/ml, pH 7.4 (with 1N NaOH).

Growth Medium: NaCl 118, KCl 2.4, Na Pyruvate 5, Creatine 5.7, Glucose 5, HEPES 20, 100x MEM Vitamins 10 ml/L, 50x MEM Essential amino acids 20 ml/L, 100x MEM Non-essential amino acids 10 ml/L, Penicillin 100 U/ml, Streptomycin 100 μg/ml, Bovine Serum Albumin 1 g/L, pH 7.4 (with 1N NaOH).

Dissociation Medium 3: NaCl 118, KCl 2.4, $NaH_2PO_4$ 0.6, $MgCl_2$ 1.8, Na Pyruvate 5, Creatine 5.7, Glucose 5, HEPES 20, M199 medium 10 ml/L, Penicillin 100 U/ml, Streptomycin 100 μg/ml, pH 7.4 (with 1N NaOH).

Extracellular Solution: NaCl 118, KCl 2.4, $CaCl_2$ 1.8, $MgCl_2$ 1.8, Na Pyruvate 5, Glucose 5, HEPES 20, pH 7.4 (with 1N NaOH).

Intracellular Solution: KCl 120, $MgCl_2$ 2, HEPES 10, ATP 1.5, $K_4$ BAPTA 1, GTP 0.4, pH 6.8 (with 1N KOH).

**D. Analysis**

P-clamp software was used for the analysis of $I_M$. Changes in $I_M$ amplitude were measured and plotted against time. In addition, percent inhibition of $I_M$, maximum percent inhibition, percent desensitization, and percent wash were calculated. Rate of

desensitization was measured during the linear phase of desensitization. During this portion of the phase, two points were used, $I_M(-30)_{t1}$ and $I_M(-30)_{t2}$. Rate of desensitization in percentage per second was calculated using the following formula: $\{[(I_M(-30)_{t2}-I_M(-30)_{t1})/(a-b)]/(t2-t1)\} \times 100$, a and b correspond to Fig. 3 (Simmons et al., 1994). Data are represented as the mean ± Standard Error of Mean (S.E.M.). T-tests were conducted to determine significance. Significance was determined to be $p < 0.05$.

## CHAPTER 3

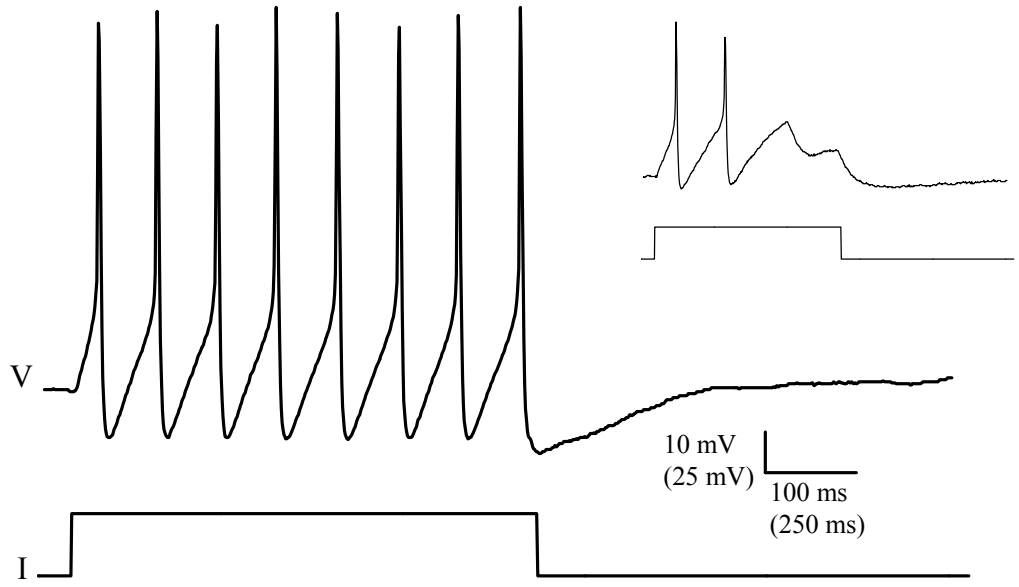
### III. Results

Patch electrodes filled with intracellular solution were utilized for whole-cell recordings from isolated bullfrog sympathetic neurons. The quality of each neuron was initially determined in Bridge (current-clamp) mode by measuring the resting membrane potential of the cell. Resting potentials averaged -62.2 ± 6.0 mV (n = 5), which is similar but slightly more negative than the previously reported range of -40 to -55 mV for bullfrog sympathetic neurons (Brown and Adams, 1980). Discrepancies in resting potentials could be attributed to differences in recording techniques, since Brown and Adams used a two electrode voltage-clamp instead of the patch-clamp method. This may have caused damage to the cell membrane, thus contributing to the resting potential differences.

Another method used to determine the quality of each neuron was its ability to produce overshooting action potentials, which were also examined in Bridge mode. A depolarizing stimulus, consisting of a 500 ms current pulse (50-115 pA), was given to the cell through the recording electrode to generate action potentials. For the cell shown in Fig. 1, the depolarizing stimulus generated eight overshooting action potentials during the 500 ms current pulse. The insert is of another cell, which was given a minimum depolarizing stimulus and produced two action potentials. Note that this cell demonstrated accommodation, which is defined as the slowing and/or stopping of action potentials despite the continued application of an excitatory stimulus. The accommodation response is due to activation of $I_M$. By the end of the second action potential, $I_M$ was activated and by resisting neuronal depolarization, $I_M$ prevented

**Figure 1. Action potential production.** Action potential production in response to a 500 ms depolarizing current pulse (50 pA). Resting membrane potential of cell was -53 mV.

**Insert:** In a different cell, minimal stimulation (20 pA) did not generate action potentials throughout the 500 ms current pulse. Resting potential was -58 mV (unpublished data, Womble 2007). Scale bar values in parentheses apply to insert figure.


V
I
10 mV
(25 mV)
100 ms
(250 ms)

subsequent action potential generation even though the current pulse was still being applied. Accommodation is an important feature of how M-current plays a role in modulating neuronal excitability.

The M-current was observed directly in Voltage-clamp mode. Fig. 2A shows a chart recording of the current responses from a single cell. The cell was voltage-clamped at a holding potential of -30 mV, a voltage level where $I_M$ is largely activated. The holding current at -30 mV is mostly due to $I_M$ although leak current also contributes to the observed current. Every 8 sec, a 500 ms step to -60 mV was applied (downward deflections) to monitor $I_M$ amplitude. Once the baseline holding current at -30 mV had stabilized (1 in Fig. 2A), SP (1 μM) was applied directly to the cell (indicated by bar) via an extracellular drug perfusion tube. With drug application, there was a large and rapid decrease in the holding current (2). However, with continued drug application, there was a gradual recovery in holding current to a new plateau (3). The recovery in holding current during drug application shows that a desensitizing response to SP application has occurred.

Individual $I_M$ responses are shown in Fig. 2B at a faster time speed, corresponding to the numbers in Fig. 2A. Fig. 2B-1 shows a control response, prior to SP application. Stepping the voltage to -60 mV from the holding level of -30 mV causes the M-channels to close, producing a slow, exponential decrease in current amplitude, which represents deactivation of $I_M$. Stepping back to the -30 mV holding potential allows M-channels to slowly reopen, producing an exponential increase in current amplitude, which represents $I_M$ reactivation.

Several methods were initially utilized to determine $I_M$ amplitude. First, the holding current at -30 mV was used as an estimate for $I_M$ amplitude. Since leak channels, which are always open, contribute more to the overall current at this voltage than at more negative voltages, this method was not used. Next, single exponential curve fits were used to measure current amplitude, either during $I_M$ deactivation following the step from -30 mV to -60 mV, or during $I_M$ reactivation, following the step back to -30 mV from -60 mV. Since the M-channel shows a slow time course for deactivation and reactivation, these curves allow for measurements of $I_M$ without leak channels contributing to the measured current (Simmons and Mather, 1991; Simmons et al., 1994). Similar results were seen with all three measurements, but since the $I_M$ reactivation curve produced the largest current amplitude, it was chosen as the standard method to measure $I_M$ amplitude.

As shown in Fig. 2, during the application of SP, there was an initial large decrease in $I_M$ amplitude (Fig. 2B-2). In the continued presence of SP, $I_M$ amplitude gradually recovered to the control level and then to a somewhat larger amplitude (Fig. 2B-3). This recovery in $I_M$ amplitude during continuous SP application indicates that desensitization has occurred.

Fig. 3 shows the changes in $I_M$ amplitude in response to SP application in the same cell shown in Fig. 2. $I_M$ amplitude was determined for each voltage step, which were applied every 8 sec (downward deflections in Fig. 2A), and plotted against time. Measurements of $I_M$ were made before, during, and after drug application. Prior to drug application (Fig. 2B-1; Fig. 3 point a), $I_M$ amplitude averaged 174.4 pA. The SP bar in Fig. 3 represents the time during which the drug was applied. With initial application of SP (1 μM), $I_M$ amplitude rapidly decreased, reaching a minimum or peak inhibition value

(Fig. 2B-2; Fig. 3 point b) of 26 pA. The time from initiation of drug application to peak inhibition (Fig. 3 arrow e) was measured, which for this cell was 64 sec.

With the continued application of SP, $I_M$ inhibition subsided as desensitization occurred. Desensitization was taken to be the largest $I_M$ amplitude during drug application following peak inhibition (Fig. 2B-3; Fig. 3 point c). For this cell, $I_M$ amplitude during desensitization returned to a value of 254 pA. Half desensitization, the $I_M$ amplitude halfway between peak inhibition and full desensitization, was calculated and used to find the time from peak inhibition to half desensitization (Fig. 3 arrow f). Since $I_M$ responses were only measured every 8 sec, intercalation was used to determine the exact time of half desensitization. Time to half desensitization for this cell was 67 sec.

Once the $I_M$ amplitude recovered and a desensitization current plateau was reached, SP application was stopped and an extracellular saline wash begun to remove the drug. $I_M$ responses continued to be monitored to determine any changes in $I_M$ amplitude after drug wash-out (Fig. 3 point d). For this cell, $I_M$ amplitude stabilized at a value of 238 pA.

To compare multiple neuron responses, the average $I_M$ amplitudes prior to drug application was determined for each cell. When all cells were combined, the control $I_M$ amplitude was found to be 145.1 ± 16.4 pA (n = 5), which is lower than previous studies with bullfrog sympathetic neurons (Simmons and Mather, 1991; Perrine et al., 2003). The average control $I_M$ amplitude for each neuron was then set to a standard value of 100% and all subsequent $I_M$ amplitude values, during and after SP application, were normalized to the control level (Fig. 4). Paired Student t-tests or Student t-tests were

**Figure 2. Current responses to SP application.** $I_M$ responses to SP application. A) Slow time scale chart record of neuron voltage clamped at holding potential of -30 mV, with voltage steps to -60 mV given every 8 sec. SP bar indicates timing of SP (1 μM) application. The holding current ($I_{(-30\ mV)}$) was initially greatly reduced by SP application, but subsequently recovered. B) Individual current responses (downward deflections seen in A) shown at a faster time scale produced by 500 ms voltage steps to -60 mV from the -30 mV holding potential. Responses correspond to numbers in A). 1: Control $I_M$ response; 2: $I_M$ response at peak inhibition; 3: $I_M$ response during desensitization.

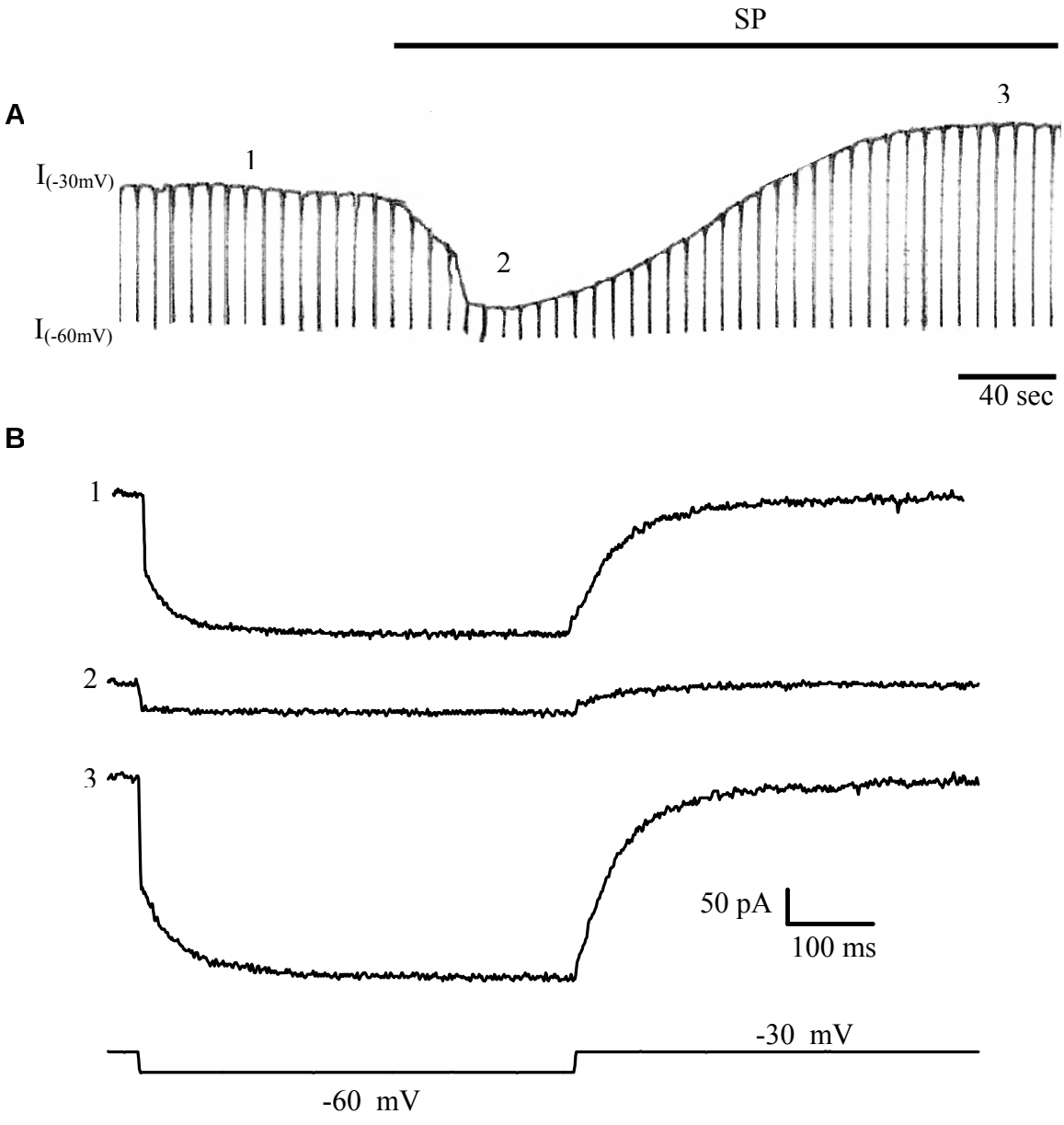
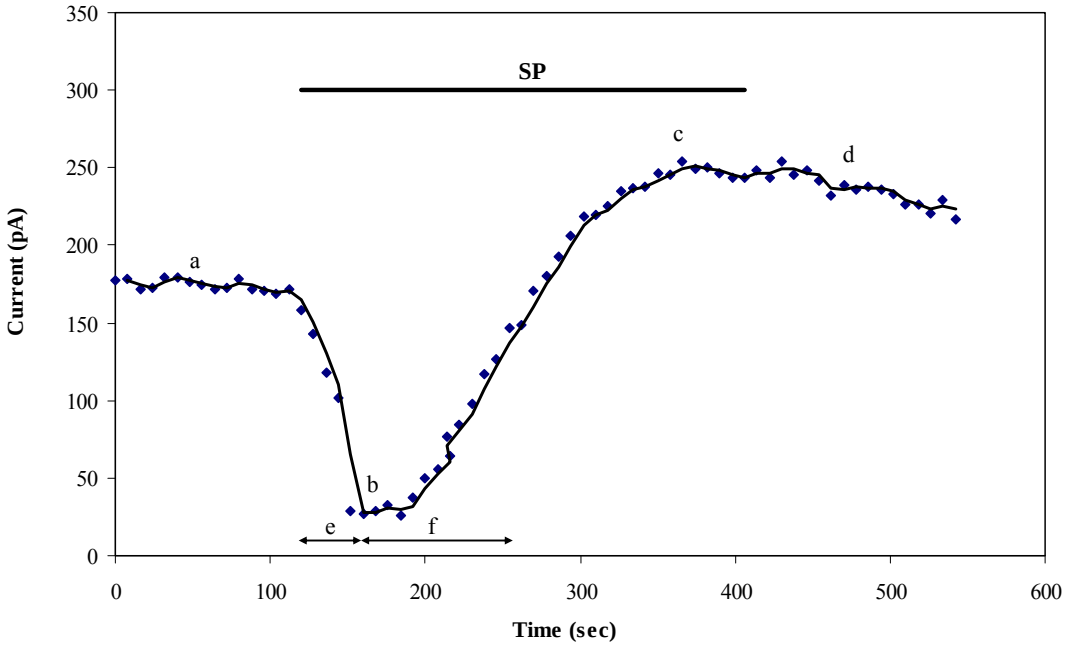

SP
A
3
1
$I_{(-30mV)}$
2
$I_{(-60mV)}$
40 sec
B
1
2
3
50 pA
100 ms
-30 mV
-60 mV

**Figure 3. $I_M$ amplitude responses to SP application.** Individual $I_M$ amplitudes for the cell shown in Fig. 2 (measured every 8 sec) plotted against time. a: Control amplitude, before drug application. b: peak inhibition of $I_M$ during drug application. c: $I_M$ desensitization during drug application. d: $I_M$ recovery following washout of drug. e: Time from start of SP application to peak inhibition. f: Time from peak inhibition to half desensitization. Bar represents time during which SP (1 μM) was applied to the cell.


SP
a
b
c
d
e
f
Current (pA)
Time (sec)
0
50
100
150
200
250
300
350
0
100
200
300
400
500
600

utilized to determine significance between groups. SP application initially produced a significant inhibition in $I_M$ amplitude (peak inhibition), reducing $I_M$ to 22.5 ± 9.6% (n = 5; p = 0.0036) of the control level. During continued SP application, $I_M$ amplitude significantly recovered to a new level (desensitization) of 112.2 ± 16.6% (n = 5; p = 0.0474 vs. peak inhibition). This recovery in current amplitude was not significantly different from the control $I_M$ amplitude, indicating that desensitization occurred even during continuous SP application. The rate of desensitization was found to be 2.0 ± 0.6 % per sec (n = 4). After drug application ceased (wash), $I_M$ amplitude returned to 96.9 ± 20.2% (n = 3), which was not significantly different from either the control or desensitization values. $I_M$ amplitude during wash was significantly larger than the peak inhibition (p = 0.0451).

Fig. 5 shows the average times for Inhibition (time from the beginning of drug application to peak inhibition) and Desensitization (time from peak inhibition to half desensitization). The time to peak inhibition averaged 39.5 ± 12.8 sec (n = 5) and the time to half desensitization averaged 30.6 ± 11.3 sec (n = 4).

**Figure 4. Comparison of $I_M$ amplitudes.** Comparison of $I_M$ amplitudes before, during, and after SP application for all cells tested. $I_M$ amplitudes were normalized to each cell's control level before values were averaged (n = 3-5).

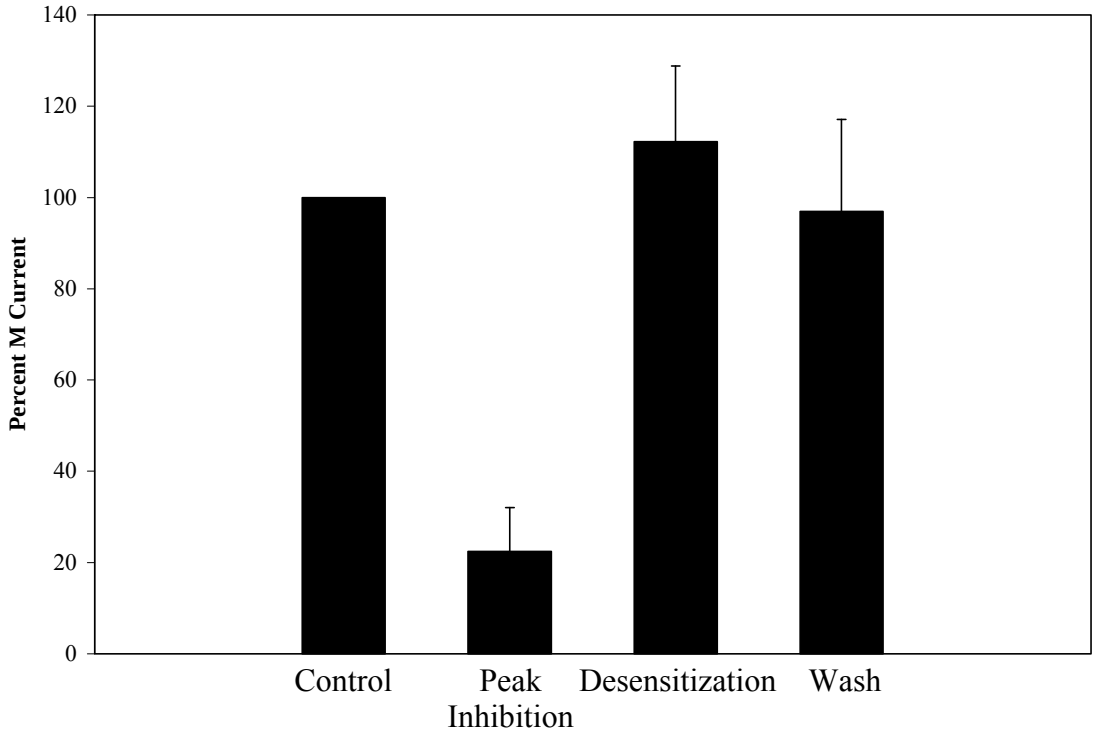
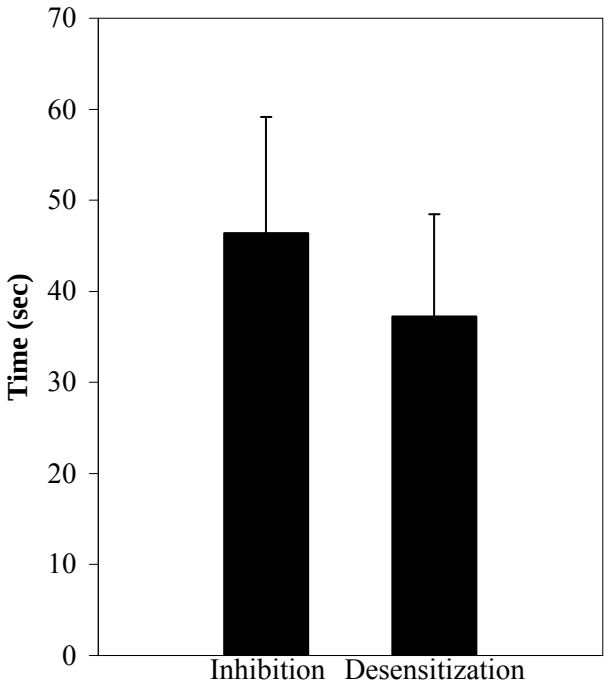

140
120
100
80
60
40
20
0
Percent M Current
Control
Peak Inhibition
Desensitization
Wash

**Figure 5. Peak Inhibition and Half Desensitization times**. Averages from all cells for the time from start of drug application to peak inhibition (Inhibition) and the time from peak inhibition to half desensitization (Desensitization) (n = 4-5).


70
60
50
40
30
20
10
0
Time (sec)
Inhibition
Desensitization

## IV. Discussion

The M-current is a time- and voltage-dependent potassium current which was first described in bullfrog sympathetic neurons (Brown and Adams, 1980). Since then, it has been shown to be present in a wide variety of mammalian peripheral and central neurons (Constanti and Brown, 1981; Womble and Moises, 1992, 1994; Coggan et al., 1994). $I_M$ has slow time-courses of activation and deactivation, and is non-inactivating. This outward current plays an important role in neuronal excitability and thus is an important target for modulation by a variety of neurotransmitters and drugs. Its most important actions are to contribute to the resting potential and to resist neuronal depolarization and action potential production (Brown and Adams, 1980; Adams et al., 1982; Brown, 1988; Simmons and Mather, 1991; Womble and Moises, 1992). When $I_M$ becomes activated by depolarization, the outward current serves to resist additional depolarization. This can cause action potential production to slow or eventually subside. This produces an accommodation response, which is an important feature of $I_M$ in modulating neuronal excitability.

In addition to the response to SP, $I_M$ inhibition has also been observed following agonist-induced activation of muscarinic cholinergic, luteinizing hormone-releasing hormone, opiod, glutamate metabotropic, adrenergic, or serotonergic receptors (Kuba and Koketsu, 1976; Adams et al., 1983; Pfaffinger, 1988; Simmons and Mather, 1991; Simmons et al., 1994; Womble and Moises, 1994; Brown and Yu, 2000; Chen et al., 2001). Several drugs also directly modulate M-channel activity (Wickenden et al., 2000; Tatulian et al., 2001; Passmore et al., 2003). Retigabine, an anticonvulsant drug that is currently in clinical trials, has been found to enhance $I_M$ by producing leftward shift in

the activation curve of $I_M$ to a more negative potential, resulting in greater $I_M$ amplitudes at all depolarized voltage levels. In contrast, linopirdine blocks the M-channel and thus reduces $I_M$ amplitude.

SP produces a depolarizing response through inhibition of $I_M$, which allows SP to modulate neuronal excitability (Adams et al., 1983; Akasu et al., 1996; Simmons et al., 1997). The present findings support previous studies showing that SP inhibits $I_M$ in bullfrog sympathetic neurons at a variety of concentrations (Adams et al., 1983; Simmons and Mather, 1991; Simmons et al., 1994, 1997; Simmons, 2001; Perrine et al., 2003). In the present study, $I_M$ amplitude significantly decreased with SP application by approximately 80% from control amplitude to peak inhibition. Other studies with bullfrog neurons have shown similar reductions in $I_M$ amplitude, with decreases of 70-83% from control amplitude with 1 μM SP (Simmons and Mather, 1991; Simmons et al., 1994; Perrine et al., 2003). The time to peak inhibition with SP application (1 μM) was 39.50 sec in this study, which was somewhat longer than the previously reported range of 8-24 sec (Perrine et al., 2003). In addition, a shorter time from peak inhibition to half desensitization occurred than has previously been seen (Simmons et al., 1994; Perrine et al., 2003). However, these differences may be due to variations in the physical set-up of equipment for SP applications.

Several studies have shown desensitization of the SP receptor occurs during continuous SP application to bullfrog sympathetic neurons (Simmons et al., 1994; Simmons, 2001, 2006; Perrine et al., 2003) or mammalian cells expressing the rat NK-1 receptor (Vigna, 2001). With desensitization of the SP receptor during SP application, studies have shown that $I_M$ amplitude recovers to a desensitization plateau of

approximately 63-79% (Simmons et al., 1994; Perrine et al., 2003). However, a higher final plateau of desensitization (112%) was found in the present study. This difference in percent desensitization may not be due to differences in peptide application, since one study had a similar peptide application time (Perrine et al., 2003). The present study did in fact show a similar percent desensitization to what has been previously found for the bullfrog tachykinin peptides, RTKA, RTKB, and RTKC (Perrine et al., 2003). This study also showed that SP and RTKA had similar binding affinities to cells expressing the rat NK1 receptor, suggesting that these two peptides may also similarly activate the NK1 receptor. This may explain why the percent desensitization seen here is similar to the percent desensitization previously reported for RTKA.

Another aspect that was examined here during SP application was the rate of desensitization. A higher rate of desensitization was seen in the present study, which was 2.0 % per sec, when compared to 0.7 % per sec and 1.2 % per sec in previous literature at the 1μM SP concentration, (Simmons et al., 1994; Perrine et al., 2003). One possible explanation for this would be how far the drug tube was from the cell in the present study compared to these previous studies. If the current study had a shorter distance between the drug tube and cell, the time that it took the drug to reach its maximal concentration of 1 μM would have been shorter, thus causing the rate of desensitization to be faster than previously reported.

In the current study, $I_M$ amplitudes during desensitization or subsequent wash were not statistically different from the control level. A similar full recovery in $I_M$ amplitude during wash has been seen previously (Simmons et al., 1994). However, one cell in the current study showed a large over-recovery during wash of 136% of control

level. Similar over-recovery in $I_M$ amplitude have previously been reported for a minority of bullfrog sympathetic neurons (Simmons et al., 1994). One possible explanation may be the correlation between $I_M$ amplitude and the level of $PIP_2$ in the membrane.

The SP-induced inhibition of $I_M$ results from activation of the SP receptor. This is a G-protein coupled receptor that activates the phosphatidylinositol pathway, resulting in the breakdown of $PIP_2$ from the cell membrane. Several studies have indicated that depletion of membrane $PIP_2$ levels destabilizes the M-channel, causing it to close (Suh and Hille, 2002, 2005; Zhang et al., 2003). Thus, $PIP_2$ hydrolysis is responsible for the muscarinic-induced inhibition of $I_M$ (Suh and Hille, 2002) and a similar mechanism may underlie the actions of SP on $I_M$. One explanation of the over-recovery in $I_M$ amplitude seen in some cells following the wash-out of SP could be an over-production of $PIP_2$ during its resynthesis by the enzymes PtdIns 4-Kinase and PtdIns(4)P 5-Kinase (Suh and Hille, 2002). Since $PIP_2$ levels correlate with the amount of $I_M$, strong activation of these enzymes may result in a higher level of $PIP_2$ in the membrane than was originally present, thus resulting in a higher amount (over-recovery) of $I_M$.

In conclusion, SP was found to significantly inhibit $I_M$ and in the continued presence of SP, $I_M$ significantly recovered. Futhermore, $I_M$ showed a desensitization response in the continued presence of SP application. $I_M$ could therefore be used as a marker to indirectly measure the state of SP receptor activity. Since $I_M$ showed a desensitizing response, this suggests that the SP receptor also shows a desensitization response.

Future improvements could be made to better develop the current research project. By increasing the number of cells, a more accurate depiction of the effects SP has on $I_M$ could be seen. Moreover, by increasing the amount of data, the current findings may better support other relevant studies. In addition, the drug tube distance should be measured. This would help define how fast the drug contacts the cell and could then be compared to a previous study to explain differences in rate of desensitization.

The current research project could also be a foundation for several studies examining the interactions between SP and its receptor in the future. In the big scheme, by developing and utilizing new SP antagonists, a new class of analgesic drugs could be created. Another path this project could take would be to test SP analogs. By testing SP peptide analogs, it could be determined whether the SP peptide could be altered in such a way so that upon peptide binding, the SP receptor would be rapidly forced into either its desensitization or activation mode. If an SP analogue could place the SP receptor in either the desensitization and/or activation state, a better understanding of cell-to-cell communication would occur.

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## Appendix A: Animal Use Approval Form

The letter of approval from the Institutional Animal Care and Use Committee of Youngstown State University for the use of animal subjects throughout the duration of this master thesis research project.

Wednesday, August 29, 2007

Dr. Mark Womble
Department of Biology
University

**RE: IACUC Protocol #02-06**
**Title: Ionic Current Modulation and Control in Sympathetic Neurons**

Dear Dr. Womble:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the aforementioned protocol you submitted for consideration titled **"Ionic Current Modulation and Control in Sympathetic Neurons"** and determined it should be unconditionally approved for the period of **5/17/2006** through its expiration date of **5/17/2009**.

This protocol is approved for a period of three years; however, it must be updated twice via the submission of an Annual Review-Request to Use Animals form ***prior*** to its expiration date of 5/17/2007 and 5/17/2008. You must adhere to the procedures described in your approved request; any modification of your project must first be authorized by the Institutional Animal Care and Use Committee.

Sincerely,

Dr. Peter J. Kasvinsky
Associate Provost for Research
Research Compliance Officer

PJK:dka

C: Dr. Walter Horne, Consulting Veterinarian, NEOUCOM
Dr. Robert Leipheimer, Chair IACUC, Chair Department of Biological Sciences
Dawn Amolsch, Animal Tech., Biological Sciences