THE UNIVERSITY OF CALGARY

SMALL CARDIOACTIVE PEPTIDE B MODULATES REFLEX BEHAVIORS OF THE ISOLATED *APLYSIA* GILL *VIA* ACTIVATION OF AN INHIBITORY CHOLINERGIC PATHWAY

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Small Cardioactive Petide B modulates reflex behaviours of the isolated Aplysia gill via activation of an inhibitory cholinergic pathway" submitted by David Cawthorpe in partial fulfillment of of the requirements for the degree of Master of Science.

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Abstract

The purpose of this study was to examine the effects of the endogenous peptide, small cardioactive peptide (SCP_B) on the evoked gill withdrawal reflex (GWR) of the isolated gill of Aplysia californica. In recent years peptides have been found increasingly to be modulators of neural activity throughout the animal kingdom. This is particularly true of Aplysia where peptides have been found to effect behavior both in vivo (Kupferman, 1974) and in vitro (Lukowiak and Colmers, 1987).

In this study it was found that SCP_B when perfused through the isolated gill, suppressed the GWR evoked by tactile stimulation of the gill. In like manner, SCP_B was also found to decrease the amplitude of the siphon evoked GWR and was not found to have an effect upon the rate of habituation of the siphon evoked GWR. SCP_B-like immunofluorescence was found to be present in the ctenidial and siphon nerves which innervate the mantle organs. Fibres, varicosities and spherical somata-like bodies localizing SCP_B-like immunofluorescence were also found to be present in the peripheral plexus of the gill.

Acetylcholine (ACh) is known to have both excitatory and inhibitiory effects when perfused though the isolated gill (Weiss et al. 1985). The inhibitiory effects are known to be mediated by a population of nicotinic-like receptors (Weiss et al. 1985). The effects of ACh perfusion upon the GWR evoked to tactile stimulation had not been examined. In the present study it was found that ACh when perfused through the gill at low concentrations results in suppression of the GWR evoked to tactile stimulation of the gill. The co-perfusion of curare or alpha-bungarotoxin

(α-BTx) through the gill blocked the suppressive effects of ACh upon the GWR. This confirmed the presence of the inhibitory population of cholinergic receptors reported by Weiss et al. (1985) and demonstrated that suppression of the GWR is mediated in part by an inhibitory cholinergic mechanism.

SCP_B could act to bring about suppression of the GWR in a number of ways. Peptides often exert their effects by exerting modulatory effects upon other neural pathways (Kazcmarec and Levitan 1987). Preliminary evidence indicated that the suppressive effects of SCP_B upon the GWR were blocked by co-perfusion with curare (Cawthorpe et al. 1986). These findings led to the hypothesis that SCP_B brought about suppression of the GWR by modulation of an inhibitory cholinergic pathway in the gill periphery. This hypothesis is supported by the findings that both the suppressive effects of SCP_B and ACh are blocked by the same concentrations of either α-BTx or curare. Furthermore, the action of SCP_B does not appear to effect the excitatory pathways which mediate sensitization and dishabituation in the gill periphery.

Taken together, evidence presented indicates that SCP_B modulates the an inhibitory cholinergic pathway known to be present in the peripheral nervous system of the gill. The precise mechanisms of how the effects of SCP_B are brought about in the Aplysia gill remain unknown and an understanding of the mechanisms will require further experimentation. A model of how SCP_B may bring about suppression of the GWR is proposed.

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I dedicate this thesis to my brother

Robert Edward James

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LIST OF ABBREVIATIONS

GWR - Gill Withdrawal Reflex

CNS - Central Nervous System

PNS - Peripheral Nervous System

ISI - Interstimulus Interval

EJP - Excitatory Junctional Potential

IJP - Inhibitory Junctional Potential

EPSP - Excitatory Postsynaptic Potential

IPSP - Inhibitory Postsynaptic Potential

COMPOUNDS

ACh - Acetylcholine

AVT - Arginine-Vasotocin

α-BTx - Alpha Bungarotoxin

ASW - Artificial Seawater

cAMP - cyclic adenosine monophosphate

ELH - Egg Laying Hormone

pCT-cAMP - p-chloro-thio-cyclic AMP

Introduction

Model Systems

One of the primary goals of neuroscience research is to determine the neural, biophysical and biochemical basis of behavior. Several criteria have been developed which an ideal system for the study of behavior should meet. These have been dicussed in detail elsewhere (Byrne, 1987; Rose, 1981; Lukowiak, 1973; Bullock, 1966) and are summarized briefly here.

The system studied should undergo observable behavioral changes. These changes should be consistent from preparation to preparation. The neuronal or cellular correlates of the observed behavioral change should be identifiable from preparation to preparation and exhibit electrophysiological and/or biochemical changes which parallel the onset, duration, and degree of the observed behavioral change.

Meeting these criteria in vertebrate systems is extremely difficult due to the complexity of the neuronal architecture. However, Nature appears to be basically conservative and some behaviors and behavioral adaptions seem to be preserved throughout phyla. Thus, research has been directed toward the use of simpler systems in which to study the neural and biochemical basis of behavior, as simpler systems are well disposed to the use of contemporary experimental techniques. The use of simpler systems, particularly invertebrates, has led to major advances in our understanding of the cellular and subcellular mechanisms underlying behavior (Byrne, 1987).

Use of Aplysia as a Model System

The gill and associated mantle organs of Aplysia californica exhibit a defensive gill withdrawal reflex (GWR) to tactile stimulation of virtually any part of the animal and in particular when external organs of the mantle cavity or the gill itself is stimulated (Kupferman and Kandel, 1969; Peretz, 1970). The GWR evoked by tactile stimulation has been shown to exhibit both associative and non-associative forms of learning which are observed in the intact animal and in reduced preparations (discussed below; see also for review Mpitsos and Lukowiak, 1985; Byrne, 1987). The ability of defensive behaviors to undergo short and long term adaptive changes (Peretz, 1970; Pinsker et al. 1970; Carew et al. 1971; Carew et al. 1972; Pinsker et al. 1973) fulfills the first criterion of a model system used in the study of behavior. Consequently, studies of the neural, ionic and subcellular mechanisms underlying adaptive behavioral changes have focused upon the use of Aplysia as a model system. Aplysia lends itself well to this study for the following reasons:

1. The nervous system of Aplysia has approximately twenty thousand neurons localized in discrete ganglia (Kandel, 1976).

2. The size of individual neurons has allowed the identification of specific neurons or clusters of neurons from preparation to preparation (Koester and Kandel, 1977; Frazier et al. 1967).

3. This has enabled detailed analysis of neurons which participate in mediating the GWR evoked by tactile stimulus of the siphon

(Kupferman et al. 1974; Byrne et al. 1974; Kandel, 1976) and neurons, neurotransmitters and biochemical mechanisms which may be involved in the mediation of behavioral plasticity (Castellucci et al. 1970; Castellucci and Kandel, 1976; Hawkins, 1981; Hawkins et al. 1981 a,b).

The Aplysia Gill

The gill of Aplysia is a delicate fan-shaped structure which lies facing laterally between a pair of wing-like parapodia beneath the mantle. The mantle is a protective flap of tissue in which are localized cells of the purple gland that secrete a dark purple mucus in response to appropriate stimuli. The mantle differentiates caudally to form the siphon, a conical structure which protrudes from between the parapodia. The siphon enhances the circulation of seawater over the gill by allowing the movement of water and waste into and out of the mantle cavity, respectively. A dorsal view of the reduced gill preparation of the gill is shown in Figure 1. For a reveiw of gill anatomy and structure see Carew et al. (1974) and Peretz and Estes (1974).

The gill mediates respiratory exchange of gases between the hemolymph and seawater which passes over the gill. The circulatory system of Aplysia is semi-open. The hemolymph, pumped by the heart through the vasculature of the major internal structures, is collected in the hemocel and enters the gill via the afferent vein which branches into about 13-15 pinnules along the horizontal plane. The afferent vein and associated pinnules branch once in the vertical plane to join on opposite sides of the efferent vein giving

the gill dorsal-ventral symmetry. The hemolymph passes through the pinnules where it is oxygenated and leaves the gill at a right angle via the efferent vein which empties into the heart at the rostral junction with the mantle shelf and body wall. The gill is joined to the mantle shelf at the point of junction of the afferent and efferent veins, as well as along the most rostral pinnule. The pinnules of the gill are longest (about 2 centimeters) near the rostral junction of the efferent vein from where they taper in length to the shortest (a few millimeters) at the most caudal tip of the gill.

The musculature of the gill is complex. The efferent and afferent veins are composed of longitudinal and circular bands of smooth muscle (Carew et al. 1974; Peretz and Estes, 1974). Each pinnule consists of lateral and medial muscle groups which transverse the pinnule longitudinally. As a pinnule approaches the efferent vein the medial external muscles, connective tissue and skin become organized to form venules which empty into the efferent vein (Peretz and Estes, 1974; Carew et al. 1974; Ruben, 1981; Weiss, 1983). Muscle fibres are thought to receive innervation from multiple sources which may arise either centrally or peripherally (Carew et al. 1971; Kandel, 1976). It is also likely that circulating hormones act on gill muscle (Lin et al. 1987).

The Gill Withdrawal Reflex

As mentioned above, the GWR evoked by tactile stimulation of the siphon may undergo both associative and non-associative learning. This means that the GWR changes in a predictable fashion using specific stimulation paradigms.

The GWR of Aplysia has been found to consist of at least four heterogenous action patterns rather than a graded response of singular type (Kupferman and Kandel, 1969; Kupferman et al. 1971, 1974; Kandel, 1976; Leonard et al. 1988). These gill movements may be described further as combinations of ten actions which involve characteristic contractions of veins and pinnules (Leonard et al. 1988). The GWR persists in the absence of the abdominal ganglion which indicates that the peripheral nervous system (PNS) alone is capable of mediating the evoked GWR (Peretz, 1970). The PNS is also capable of mediating certain types of behavioral plasticity that include habituation and sensitization of the GWR (Peretz, 1970; Ruben and Lukowiak, 1982; Leonard et al. 1988). Habituation and sensitization are defined below. In this study the effects of SCP_B on the GWR have been examined in the isolated Aplysia gill which has had the abdominal ganglion surgically removed.

Central Innervation of the Gill

Central innervation of the gill originates entirely within the abdominal ganglion in which are localized the somata of neurons which send axons to the gill and external mantle organs via the branchial, siphon and ctenidial nerves (Kandel, 1976). The spherical neurons of the central ganglia have their somata at the outermost circumference of the ganglia beneath a layer of connective tissue and send out processes which make multiple synapses with the processes of other neurons in the complex neuropil at the centre of the ganglia. The sheath of connective tissue surrounding the abdominal ganglion is vascularized receiving hemolymph from a branch of the aorta and

this may be important in terms of neurohormone release or interaction of central neurons with circulating hormones (Lin et al. 1987; Kandel, 1976).

Several neurons involved in the mediation of the siphon evoked GWR have been identified in the abdominal ganglion (Frazier et al. 1967; Koester and Kandel, 1977; Hawkins, 1981; Hawkins et al. 1981 a,b). Some of these are known to innervate the gill and when depolarized produce characteristic contractions of the gill (Kupferman et al. 1974; Leonard and Lukowiak, 1986; Kurokawa and Kuwasawa, 1985 a,b, 1988). Such motor neurons as L7, LDG1 and LDG2 innervate gill muscle directly (Carew et al. 1974). Similar gill motor neurons in Aplysia kurodai and juliana have been reported to innervate neurons of the branchial or gill ganglion and other components of the peripheral nerve plexus in addition to gill muscle (Kurokawa and Kuwasawa, 1985 a,b, 1988). In Aplysia californica, LDG1 and LDG2 are thought to be cholinergic (Carew et al. 1974); the neurotransmitter of L7 has not yet been identified conclusively. L7 and LDG1 send axons to the gill via both branchial and ctenidial nerves whereas LDG2 sends an axon only via the ctenidial nerve (Kupferman et al. 1974). As well, L7 is reported to have an axon in the siphon nerve (Winlow and Kandel, 1976; Colebrook, 1986). Another neuron, L9 is a modulatory neuron and sends its axon to the gill via a branch of the siphon nerve. Ruben (1981) demonstrated that L9 facilitated the L7 evoked gill contraction via a peripheral dopaminergic pathway.

Siphon mechanoreceptors (LE cluster) are activated by siphon stimulation (Castellucci et al. 1970; Byrne et al. 1974; Byrne, 1980). Activation of sensory neurons produces post synaptic potentials in specific motorneurons and interneurons (Hawkins, 1981; Hawkins et al. 1981 a,b). The synapse between siphon sensory neurons (LE cluster) and identified gill motor neurons (ie. L7) has received considerable attention as this pathway is

involved in the mediation of the siphon evoked GWR. Changes in synaptic efficacy in the mono-synaptic component of the siphon evoked GWR pathway have been correlated with changes in gill withdrawal behavior following specific training paradigms in intact animals and reduced preparations (discussed below). The mono-synaptic component of this reflex pathway will be referred to as the identified sensory-motor synapse.

Interneurons have been identified in the abdominal ganglion which make synapses with both sensory and motor neurons that mediate the siphon evoked GWR (Hawkins et al. 1981a). One such neuron (L29) has been found to influence the efficacy of the synapse between siphon sensory neurons (below) and gill motor neurons. L29 is thought to play a role in the mediation of behavioral sensitization (discussed below), (Hawkins, 1981; Hawkins et al. 1981 a,b).

The accessibility of the central neurons has enabled direct physiological and biochemical experimentation on neurons which participate in the mediation of the GWR. It is a natural consequence of this that some of the neural correlates underlying the behavioral plasticity of the GWR have been identified and studied primarily in the abdominal ganglion. In spite of this, the role played by the peripheral nervous system in the mediation and plasticity of the GWR cannot be overlooked.

The Peripheral Nervous System of the Gill

In the absence of the abdominal ganglion the PNS has been found to be capable of mediating a GWR evoked by either siphon or gill stimulation (this study; Peretz, 1970; Lukowiak and Peretz, 1977; Leonard et al. 1988). This

peripherally mediated GWR undergoes habituation and dishabituation (defined below) which indicates that neural components of the peripheral nervous system are sites of plasticity underlying changes in the reflex behavior of the gill (Peretz, 1970; Peretz and Moller, 1974; Jacket and Rine, 1977; Ruben and Lukowiak, 1982).

The peripheral nerve plexus of the gill is far from simple. There is one discrete bundle of neurons located proximal to the branchial nerve at the point where it enters the gill efferent vein proximal to the heart, termed the gill or branchial ganglion (Peretz and Estes, 1974; Carew et al. 1974). There are sparsely distributed neuronal somata within the nerve trunks innervating the gill though the role played by these neurons in the mediation of gill movements is unknown. Neurons of the genital ganglion have been shown to send processes into the ctentidial nerve though the role played by this ganglion in the mediation of gill behavior is also unknown (Lebeda and Blankenship, 1977). In addition to these there is a diffuse network of neurons throughout the gill which mediate sensory input and motor output in the peripheral nervous system (Peretz and Moller, 1974); some of these neurons may also be interneurons. The gill ganglion has neurons which both innervate gill musculature or have processes which do not leave the ganglion (Colebrook, 1986). The gill ganglion receives input from both central and peripheral neurons and is thought to be important in the control of gill withdrawal amplitude (Peretz and Moller, 1974) and pinnule contractions (Kurokawa and Kuwasawa, 1985b).

As mentioned above muscle fibres of the gill receive multiple inputs which arise both centrally and peripherally. As well, both excitatory junctional potentials (EJP) and inhibitory junctional potentials (IJP) have been recorded intracellularly from muscle fibres (Carew et al. 1974,

Kurokawa and Kuwasawa, 1985 a,b, 1988). EJP's recorded intracellularly from gill efferent vein muscle in response to branchial nerve stimulation have been shown to have mono-synaptic properties (Kurokawa and Kuwasawa, 1988). Increasing the duration of the branchial nerve stimulus recruited IJP's which did not have mono-synaptic latency properties (Kurokawa and Kuwasawa, 1988) and are proposed to arise from peripheral motor neurons (Kurokawa and Kuwasawa, 1988).

Interaction of the Central and Peripheral Nervous Systems

Both the central and peripheral nervous systems modulate gill withdrawal behaviors in reduced preparations (Lukowiak and Peretz, 1977). Removal of the abdominal ganglion results in facilitation of the GWR and a decreased rate of habituation (Peretz and Howieson, 1973). The abdominal ganglion exerts both suppressive and facilitatory influences over the periphery (Peretz et al. 1976). Repeated stimulation of the branchial nerve evokes a decrementing GWR (Peretz et al. 1976). Ctenidial nerve stimulation can reverse this effect and often may result in the facilitation or enhancement of this GWR produced by branchial nerve stimulation (Peretz et al. 1976). On the other hand, repeated ctenidial nerve stimulation also produces a decrementing GWR (Peretz et al. 1976). However, stimulation of the branchial nerve in this case produces further suppression of this GWR produced by ctenidial nerve stimulation (Peretz et al. 1976). These findings indicated that the ctenidial and branchial nerves have facilitatory and suppressive influences over the peripheral nervous system, respectively.

Lukowiak (1977a) also demonstrated that the branchial innervation of the gill mediates the suppressive influences of the abdominal ganglion on the siphon evoked GWR and that facilitatory influences are mediated via the ctenidial nerve. In these experiments the removal of branchial innervation of the gill reduced the latency of a siphon evoked GWR and continued stimulation of the siphon which had previously resulted in habituation, produced facilitation of the GWR. In Aplysia, habituation is the decrement in the amplitude of the GWR with repeated stimulation (Pinsker et al. 1970) whereas dishabituation is the increase in the amplitude of the habituated GWR following presentation of a novel or noxious stimulus (Pinsker et al. 1970). In the experiments of Lukowiak (1977a), the excitatory postsynaptic potential (EPSP) recorded in the gill motor neuron LDG1 decremented as it did during habituation of the siphon evoked GWR when the branchial innervation of the gill was intact (Lukowiak, 1977a). This indicated that habituation of siphon evoked GWR was dependent upon the presence of branchial innervation when the remaining abdominal ganglion innervation of the gill is intact. The fact that EPSP decrement in the motor neuron follower of the identified central synapse persisted and did not parallel gill behavior when the branchial innervation of the gill was removed indicated that plastic changes associated with the siphon evoked GWR habituation must have a branchial component and that EPSP amplitude decrement recorded in the central gill motor neuron does not necessarily parallel gill behavior.

Activity in central motor neuron, L7 (induced by intracellular current injection) can modulate siphon or gill evoked GWR's. Tonic activity in L7 will faciltiate a non-habituated GWR. As well, activity in central gill motor neurons dishabituated GWR's which have been habituated by repeated

tactile stimulation of either the siphon or gill (Lukowiak, 1977b; Lukowiak and Peretz, 1977; Peretz and Lukowiak, 1975). This was a further example of how the CNS can modulate the PNS.

Activity in the peripheral nervous system can modulate the contraction produced by gill motor neuron stimulation. Intracellular stimulation of L7 to produce a fixed number of action potentials produced a gill withdrawal. Repeated activation of L7 resulted in a decrement of this gill withdrawal response. Interposition of a tactile stimulation to the siphon or gill results in a facilitation of the elicited gill withdrawal response produced by motor neuron stimulation (Lukowiak and Peretz, 1977). Furthermore, Jacklet and Rine (1977) observed that the junctional potential evoked by LDG1 stimulation was facilitated following siphon stimulation. If there were no interaction of the CNS and PNS then these data could not be obtained.

In the present study the abdominal ganglion has been removed. Under these circumstances it is assumed that the GWR produced is mediated primarily by the peripheral nervous system (Peretz, 1970). However, participation in the GWR of the terminations of central motor neurons through activation by the peripheral nervous system cannot be ruled out.

Neurotransmitters and Neuropeptides of the Gill

Several types of classical neurotransmitters and neuropeptides have been localized in the peripheral plexus of the gill. Acetylcholinesterase (reflecting the presence of acetlycholine), serotonin, dopamine and FMRF-amide have been demonstrated in the gill using biochemical and immunohistochemical techniques (Peretz and Estes, 1974; Carew et al. 1974;

Weiss et al. 1984; Lehman et al. 1984). These agents have been found to modulate gill contractile behavior.

Serotonin suppresses the GWR at low concentrations (< 1 μM) though serotonin at higher concentrations facilitates the GWR and appears to exert excitatory effects via action at dopamine receptors (Weiss, 1983). Weiss (1983) went on to demonstrate that there were specific receptors mediating the effects of serotonin, dopamine and FMRF-amide.

ACh has both inhibitory and excitatory actions when perfused through the gill (Weiss et al. 1985). The excitatory effects are observed when greater than 10 μM concentrations of ACh are perfused through the gill. These concentrations of ACh bring about a tonic contraction of the efferent vein. This effect was blocked by atropine and enhanced by curare. The fact that curare potentiated the excitatory effects of ACh perfusion of the gill led to the idea that there were excitatory and inhibitory populations of receptors present. The inhibitory effects of ACh were not as obvious. Low concentrations of ACh (< 5 μM) or carbachol (a non-hydrolyzable cholinergic agonist), brought about a decrease in the baseline tension recorded from the gill using a force transducer. As well, carbachol at concentrations as high as 100 μM did not induce a tonic contraction of the gill efferent vein. Weiss et al. (1985) further examined these inhibitory effects using pharmacological techniques. The perfusion of a diffusible cAMP analogue, pCT-cAMP (p-chloro-thio-cyclic adenosine monophosphate) brought about clonic contractions of the gill when perfused through the gill. These contractions could be attenuated by co-perfusion of low concentrations of ACh. This attenuation of pCT-cAMP induced contractions was blocked by co-perfusion of curare. Thus, two populations of ACh-receptors appeared to mediate the effects of acetylcholine in the gill. One nicotinic-like population mediated

the inhibitory effects of ACh when perfused through the gill at concentrations less than about 10 μM. Another muscarinic-like population of receptors mediated the excitatory effects at greater concentrations of ACh (Weiss et al. 1985). In this present study the effects of ACh perfusion on the gill evoked GWR of the isolated gill were examined. ACh was found to suppress the gill evoked GWR. This effect was similar to the suppressive effect of SCP_B upon the gill evoked GWR. Evidence is presented in this study which supports the hypothesis that SCP_B suppresses gill contractile behavior via the activation of an inhibitory cholinergic pathway.

The peptide, FMRF-amide was discovered by Price and Greenberg (1977). Since then FMRF-amide has been found to be present in the Aplysia gill (Weiss et al. 1984; Lehman et al. 1984) and central ganglia (Mackey et al. 1988). When perfused through the gill of Aplysia, FMRF-amide initiates clonic contractions of the gill (Weiss et al. 1984), facilitates the GWR evoked to gill or siphon stimulation and prevents habituation of these GWR's (Cawthorpe et al. 1988, Higgins et al. 1988). Other peptides, such as methionine-enkephalin and arginine-vasotocin appear not to effect the amplitude of the evoked GWR when perfused through gill (Cawthorpe, unpublished data). Evidence presented in this present study shows that SCP_B may be present in the gill where it exerts suppressive effects upon GWR amplitude.

The brief survey presented above indicates that neurotransmitters and neuropeptides are present in the gill of Aplysia. These agents when perfused through the gill can induce gill reflex behaviors or modulate gill reflexes evoked by tactile stimulation of the siphon or gill. These findings suggest that such neurotransmitters and neuro-peptides play a role in the mediation of gill reflex behaviors in vivo.

Non-asssociative Learning: Habituation and Sensitization Habituation

Habituation

Habituation is defined as the the decrement in response amplitude to repeated stimulation. Thompson and Spencer (1966) reported many features of habituation which characterize this process, and distinguish it from receptor adaption or effector fatigue in hind limb reflexes of the spinal cat. Features of habituation have been generalized to many animal systems including Aplysia. These features are:

1. Response decrement to repeated stimulation follows a negative exponential function.
2. Spontaneous recovery occurs when the stimulus is withheld.
3. With successive habituation series the rate of habituation increases with each subsqeuent series.
4. Decreasing the interstimulus interval (ISI) increases the rate and degree of habituation.
5. Weaker stimuli increase the rate and degree of habituation. Strong stimuli may result in little or no habituation.
6. Additional habituating stimuli presented after an asymptotic response level is attained will prolong recovery.
7. Habituation of response to a given stimulus (site) results in generalization or transfer of habituation to other similar stimuli (sites).
8. The presentation of another strong stimulus results in dishabituation of the habituated response.

9. Repeated presentation of a dishabituating stimulus results in habituation of the dishabituated response.

Several mechanisms have been proposed which account for the synaptic plasticity associated with behavioral adaptions of the gill.

Neural Mechanism Underlying Habituation

In Aplysia, habituation has been defined above as the decrement in the response to a repeated tactile stimulus (Thompson and Spencer 1966). Habituation is distinct from receptor adaption or muscle fatigue (Kupferman et al. 1970; Byrne et al. 1974) and may be explained by the mechanisms of homosynaptic and heterosynaptic depression. Homosynaptic depression is modelled as an intrinsic property of a neuron associated with features such as calcium channel inactivation and/or a depletion of the neurotransmitter available for release (Gingrich and Byrne, 1985). Heterosynaptic depression involves the action of a modulatory substance which acts to depress neurotransmitter release. The peptide, FMRF-amide appears to exert its central effects via such a neural mechanism which results in the opening of a potassium channel, the S-channel. This action of FMRF-amide involves the activation of a second messenger cascade involving arachidonic acid metabolism (Piomelli et al. 1987; Mackey et al. 1988). Whether homosynaptic and/or heterosynaptic mechanisms are operating to bring about changes in gill reflex behavior, a decrement in the EPSP recorded in the gill motor neuron is generally observed. The data derived from a quantal analysis was consistent with depletion of available neurotransmitter from sensory terminals at the sensory synapse presynaptic to the gill motor neuron in the

identified monosynaptic pathway (Castellucci and Kandel, 1974). Depletion of neurotransmitter available for release may imply an action upon the mobilization of neurotransmitter or an action upon the release mechanism. These actions underlying homosynaptic depression could be reversed by stimuli which mediate dishabituation. It is possible that both heterosynaptic and homosynaptic mechanisms that have been identified in the abdominal ganglion also operate in the peripheral nervous system.

In the present study the effects of SCP_B on the habituation of the siphon evoked GWR in the isolated gill have been examined.

Sensitization

Sensitization has been defined as the increase in response amplitude to a standard stimulus following the interposition of a novel (strong or noxious) stimulus (Thompson and Spencer, 1966). Habituation and sensitization have been hypothesized to be separate processes. Both processes are thought to operate during habituation. This is known as the dual-process theory of habituation (Groves and Thompson, 1970; but see Marcus et al. 1988).

The gill withdrawal reflex of Aplysia californica has been shown to exhibit all nine parameters of habituation (Kandel, 1976; Goldberg, 1983; Goldberg and Lukowiak, 1982, 1983). Habituation and sensitization in Aplysia conform to the dual-process theory in that sensitization is a separate process and may be superimposed upon the process of habituation (Carew et al. 1971). Also, there may be an initial "hump" in response amplitude prior

to decrement (Lukowiak, 1973) and some preparations fail to exhibit a great degree of habituation (Ruben, 1981).

Dishabituation was for a long time considered a special case of sensitization (Carew et al. 1971). Recently, work on the development of defensive reflexes in Aplysia has shown that habituation, dishabituation and sensitization emerge at distinct developmental stages (Rankin and Carew, 1988, 1987; Rankin et al. 1987). These findings suggest that sensitization and dishabituation are separate processes, though it is unknown whether these two processes employ similar cellular mechanisms.

Neural mechanism underlying Sensitization

In studies using the siphon, mantle, gill and abdominal ganglion preparation, sensitization is the enhancement of a GWR to a siphon stimulus by the interposition of a noxious stimulus (Carew et al. 1971). To obtain a neural correlate of sensitization a preparation was employed that had been reduced even further. This preparation was an abdominal ganglion which contained the reflex arc of the centrally mediated GWR pathways. In some cases the siphon remained connected by the siphon nerve. Stimulation paradigms employed produced a response at the sensory motor synapse which was a correlate of behavioral change. The EPSP recorded in the motor neuron was produced by stimulation of the siphon (afferent input) was mimicked by siphon nerve or intracellular sensory neuron stimulation (Castellucci et al. 1970). The EPSP produced by the stimulation paradigm employed was recorded intracellularly in the motor neuron and the EPSP amplitude served as an index of the behavioral GWR. A sensitizing stimulus was mimicked in such reduced preparations by pleural-abdominal connective stimulation

(Castellucci et al. 1970) or more recently by interneuron L29 stimulation (Hawkins, 1981). Such sensitizing stimuli resulted in facilitation of the EPSP recorded from the motorneuron. The mechanism accounting for EPSP facilitation is heterosynaptic facilitation originally proposed by Kandel and Tauc (1965). Heterosynaptic facilitation involves the action of a modulatory substance at the presynaptic sensory neuron terminal which enhances neurotransmitter release onto the motor neuron.

SCP_B and serotonin mimic the effects of sensitizing stimuli at the identified sensory motor synapse in the abdominal ganglion. Both produce sensory neuron spike broadening and a slow epsp in the sensory neuron (Castellucci et al. 1976, Klein and Kandel, 1980, Abrams et al. 1984). These effects have been associated with the closure of the serotonin sensitive potassium channel, the S-channel (Klein and Kandel, 1980, Klein et al. 1980, 1982, Siegelbaum et al. 1982, Camardo et al. 1983; Occor and Byrne, 1985, 1986). It has been postulated that this leads to an enhancement of the calcium current and neurotransmitter release resulting in a facilitated EPSP recorded in the follower neuron.

Serotonin and SCP_B have been found to mediate their actions via activation of the cAMP second messenger cascade by separate receptors (Abrams et al, 1984; Occor and Byrne, 1985, 1986). The actions of serotonin are mimicked by injection of cAMP into sensory neurons (Brunelli et al. 1976, Kandel and Schwarz, 1982). Serotonin and SCP_B enhance cAMP levels in sensory neurons (Bernier et al. 1982; Occor and Byrne, 1986). The purified catalytic subunit of the cAMP-dependent protein kinase also mimics serotonin (Castellucci et al. 1980) and this effect is blocked by an inhibitor of the kinase (Castellucci et al. 1982). However, neurons localizing either SCP_B or serotonin which produce heterosynaptic facilitation have not yet been

identified. However, the unidentified neurotransmitter of the interneuron L29 is presumed to produce heterosynaptic facilitation since stimulation of this neuron produces heterosynaptic facilitation (Hawkins, 1981).

A homosynaptic mechanism which could account for sensitization is post-tetanic potentiation (Feng, 1941; Fatt and Katz, 1953). In this mechanism enhanced calcium entry into a cell due to tetanic stimulation may result in increased mobilization of transmitter available for release (Gingrich and Byrne, 1985). However this mechanism alone does not account for observations of the effect of post-tetanic potentiation at Aplysia sensory neurons (Walters and Byrne, 1984).

The findings summarized above indicate that endogenous neurotransmitters and neuropeptides such as serotonin and SCP_B may act to modulate synaptic efficacy at the identified central synapse involved in the mediation of the siphon evoked GWR. The changes in synaptic efficacy produced by these agents parallel changes observed at this same synapse following behavioral paradigms which result in behavioral changes (Kupferman et al. 1970) though this does not rule out the involvement of other pathways in the mediation of behavioral changes (Lukowiak and Colmers, 1987). As well, changes in the biochemical pathways through which these agents exert their action parallel biochemical changes following behavioral sensitization (Abrams et al. 1984). Such findings have led to the view that SCP_B and serotonin may play a role in the mediation of behavioral sensitization.

Pathways exist in the peripheral nervous system which are capable of mediating sensitization of the GWR (Peretz, 1970; this study). Although SCP_B is reported in this study to bring about suppression of the GWR, it does not interfere with sensitization or dishabituation. Thus, it appears that the suppressive action of SCP_B does not involve an action upon the neural

pathways which mediate sensitization or dishabituation in the gill periphery.

Behavioral State

Role of Peptides in the Mediation of Behavior

The control of gill contractile behavior is mediated by both central and peripheral components. An understanding of the peripheral components mediating gill behavior is only beginning to emerge. Indeed, how central and peripheral neural networks integrate to bring about such complex behavioral drives as escape, feeding, reproduction and egg laying is only beginning to be understood. In a recent paper by Leonard and Lukowiak (1986), an ethogram of Aplysia has been developed in which the drives are decribed in terms of specific subsets of fixed action patterns or fixed acts which in some cases have been shown to involve central pattern generators (ie. respiratory pumping; Peretz, 1969; Byrne and Koester, 1978). Forty-five such action patterns have been described in Aplysia (Leonard and Lukowiak, 1986). Drives may form particular heirarchies depending upon previous stimulus history and the immediate environmental conditions (Leonard, 1984).

Endogenous peptides appear to play an important role in the mediation of behavioral drives. For example, egg laying hormone extracts of bag cells from a donor animal which are injected into a recipient animal leads to the release of egg laying hormone (ELH) which then produces a complicated sequence of egg laying behavior (Kupferman, 1967, 1974). The gene encoding ELH has now been sequenced and the distribution of cells localizing this

gene examined (Mahon et al. 1985; Scheller, et al. 1984, Scheller and Kirk, 1987). How ELH effects behaviors has been a subject of intense study. This peptide is known to suppress some drives such as feeding and sex and turn others such as egg laying on (Leonard and Lukowiak, 1986).

Studies of some of the ionic mechanisms underlying the firing behavior of bag cells which localize and release ELH, α-bag cell peptide and other peptides are ongoing. In the case of α-bag cell peptide studies indicate that auto-receptors for α-bag cell peptide on bag cells are involved in terminating the bursting behavior of bag cells. The action of α-bag cell peptide at this receptor appears to somehow involve the expression of a potassium current (Kauer et al. 1987).

Three Behavioral States have been Identified in Aplysia

The non-associative learning processes, habituation, dishabituation and sensitization, described above are viewed as being superimposed upon the behavioral state or level of arousal of a given animal. Three behavioral states have been identified in Aplysia (Ruben et al. 1981). These states are the suppressed, normal and facilitated behavioral states (Ruben et al. 1981).

In vitro, such states have been identified on the basis of the GWR amplitude. The first criterion is the comparison of the GWR amplitude evoked by tactile stimulation of the siphon with those occurring spontaneously during respiratory pumping. The suppressed animals have GWR's which are less than thirty-five per cent of the amplitude of the spontaneous contractions. The second criterion is the rate and degree of GWR habituation. In

suppressed animals the rate of habituation is faster and the degree of habituation is greater than in controls (Ruben et al. 1981; Lukowiak, 1980; Lukowiak and Freedman, 1983). Suppressed states have been associated with feeding , sexual activity and aging (Lukowiak, 1980; Lukowiak and Freedman, 1983).

Blood-borne factors act to regulate the level of responsiveness of the animal. This finding comes from experiments in which the blood of satiated animals was found to suppress the GWR of control (ie. non food-satiated) in vitro preparations when superfused over the abdominal ganglion (Lukowiak, 1987). These findings indicate that circulating factors contribute to the suppression associated with satiation. This may also be true of other behavioral states. The identity of blood-borne factors contributing to suppression associated with satiation is not yet known.

Peptides are primary candidates as mediators of behavioral state. Evidence for this comes from the observation that peptides presented both centrally or peripherally can mimic particular behavioral states. For instance arginine-vasotocin (AVT) when superfused over the abdominal ganglion mimics the suppressed state. AVT produces suppression of the GWR evoked by tactile stimulation of the siphon and increases the rate of GWR habituation. AVT also depresses the EPSP at the identified sensory motor synapse. Action at this locus could represent at least part of the neural mechanism through which arginine-vasotocin brings about its effects (Thornhill et al. 1981; Lukowiak and Colmers, 1987). AVT also suppresses the ability of a gill motor neuron to elicit a gill withdrawal reponse (Lukowiak and Colmers, 1987). However the peripheral perfusion of AVT through the gill has no effect upon the GWR evoked by tactile stimulation of the gill

(Cawthorpe, unpublished observation) even though AVT does affect cardiac activity (Wernhem et al. 1982).

A link between the suppressed state produced by superfusion of met-enkephalin over the abdominal ganglion and the suppression observed in animals which have been sexually active has been established. Naloxone has been found to block the suppression of the GWR associated with sexual suppression and the suppression produced by met-enkephalin superfusion. An enkephalin-like opioid has been found to be present in Aplysia (Leung et al. 1984). This suggests that an opiate pathway is involved in the mediation of this behavioral state (Leonard et al. 1984).

The facilitated state has been observed in the GWR of reduced, in vitro preparations. As yet, this facilitated state has not been associated with a specific in vivo behavior (Ruben, 1981) though could be associated with the state of conditioned fear (Carew et al. 1981). Facilitated state preparations have been shown to undergo little or no habituation of the GWR and may be reversibly blocked by dopaminergic antagonists (chlorpromazine and ergotamine), (Ruben, 1981); this state may be mimicked by perfusion of FMRF-amide (Cawthorpe et al. 1988) and dopamine (Ruben, 1981) through the periphery. Ruben (1981) was able to show that the terminations of the indentified abdominal ganglion neuron, L9 were involved in the mediation of the facilitated behavioral state. L9 activity modulated the contraction produced by depolarizing L7. The increase in the contraction produced by L7 with concurrent L9 activity was blocked by dopamine antagonists. This showed that a dopaminergeric pathway was involved in the mediation of the facilitated behavioral state (Ruben, 1981). It may be that several neuropeptides and/or neurotransmitters act in concert to bring about a particular behavioral state.

Though clear identification of the relationship of these peptides with the satiated or facilitated behavioral states has not yet been accomplished, evidence presented in this study represents a necessary step in the development of our understanding of how behavioral state can be mediated in Aplysia. Direct evidence that supports the view that SCP_B does play a role in feeding is that the peptide is localized and released from neurons involved in feeding and potentiates the contractions of muscles involved in biting (Lloyd et al. 1984). The evidence presented in this study shows that SCP_B plays a role in mediating suppression of the evoked GWR.

Small Cardioactive Peptide B

Small cardioactive peptides were discovered by Lloyd (1978) and sequenced by Morris et al. (1982). The structure of the SCP's are compared to AVT and FMRF-amide in Table 1. In this study attention has been focused upon how SCP_B effects the defensive GWR of the isolated Aplysia gill.

Table 1

AVT	Cys-Tyr-Leu-Glu-Asp-Cys-**Pro-Arg-Gly** (C-terminus)
SCP_A	Ala-Arg-Pro-Gly-**Tyr-Leu-Ala-Phe-Pro-Arg-Met**
SCP_B	Met-Asn-**Try-Leu-Ala-Phe-Pro-Arg-Met**
FMRF-amide	**Phe-Met-Arg-Phe**

As indicated by its name, this peptide was found to be a cardiac excitor in Helix (Lloyd, 1978) and in Aplysia (Cawthorpe et al. 1985; Lloyd et al. 1985a). SCP_B is endogenous to Aplysia. SCP_B is found to be localized

primarily in the neurons of the buccal ganglion (B1 and B2) which innervate the gut (Lloyd et al. 1985b). SCP_B is also localized in the cholinergic motor neuron (B15) which innervates the accessory radula muscle of the buccal mass. There are also neurons and processes which localize SCP_B in the cerebral, pleural, pedal and abdominal ganglia including the siphon, ctential and branchial nerves which innervate the gill (Lloyd et al. 1985b). There are considerable amounts of SCP_B localized in the gut and muscle of the buccal mass. In the present study, immuno-histochemical evidence indicates that SCP_B appears to be present in the gill.

Some of the effects of SCP_B appear to be mediated by activation of adenylate cyclase which leads to increased levels of cAMP in heart (Cawthorpe et al. 1985; Lloyd et al. 1985a), accessory radula closer muscle (Lloyd et al. 1984), in central sensory neurons (Occor and Byrne, 1985a) and the gill of Aplysia (Cawthorpe et al. 1985). The action of SCP_B on buccal muscle is similar to to that of serotonin (Weiss et al. 1978). Both potentiate the contractions of the accessory radula closer muscle evoked by stimulation of the neuron B15 or the superfusion of ACh. This potentiation appears to be mediated via activation of cAMP in the muscle. SCP_B and serotonin are not co-localized in neurons of the buccal ganglion and mediate their effects through distinct receptors (Lloyd et al. 1984). Serotonin is thought to play a role in food arousal (Weiss et al. 1978). Because of the similarities of the action of SCP_B and serotonin, it is possible that SCP_B is also involved in the mediation of feeding behavior.

SCP_B has been found to be co-localized with ACh and with the neuropeptide, FMRF-amide in identified neurons of Aplysia (Lloyd et al. 1985b). The co-localization of SCP_B and ACh has also been observed in identified neurons of Tritonia diomedia, where these agents exert opposite

effects upon the postsynaptic neuron which is involved in the modulation of the feeding motor program (Willows, 1985). In this study evidence is presented that shows there is a pharmacological relationship between ACh and SCP_B in the Aplysia gill though it is unknown whether these agents are co-localized in any neurons. Preliminary work has shown that the actions of SCP_B upon the evoked GWR in the isolated Aplysia gill may be mediated via an inhibitory cholinergic mechanism (Cawthorpe et al. 1986).

In order to determine the role of SCP_B in the mediation of a suppressed behavioral state it is first necessary to examine the effects of this peptide on the peripherally mediated GWR. This thesis has been devoted to this end. The primary purpose of this study was to characterize the effects of SCP_B on the gill evoked GWR when perfused through the gill which had been isolated from the abdominal ganglion. As well, the effect of SCP_B on the habituation of the siphon evoked GWR was examined. Immunohistochemical techniques were used to determine whether SCP_B-like immunofluorescence was present in the peripheral nerve plexus or the central innervation of the gill. As well pharmacological evidence points to a possible neural mechanism underlying SCP_B's modulatory action and a model is presented to describe the putative sites of action of SCP_B. Three important findings emerged from the results presented below;

1. SCP_B and ACh exert suppressive effects on the amplitude of the gill evoked GWR. The action of both these agents are blocked by the nicotinic antagonists curare and α-BTx.

2. SCP_B suppresses the amplitude of the siphon evoked GWR during habituation but does not effect the rate of habituation with the stimulus parameters employed. Also, the suppression of the GWR produced by SCP_B does not appear to effect the pathways which mediated dishabituation or sensitization in the periphery.

3. SCP_B appears to be present in neurons of the peripheral plexus of the gill and the nerves which arise centrally and innervate the gill.

Materials and Methods

Aplysia californica weighing between 100 - 250 grams, obtained from Sea Life supply (Sand City, CA) or Pacific Biomarine (Venice, CA.) were used in this study. The animals were maintained in a 1200 litre aquarium containing artificial seawater (ASW, Instant Ocean) at 15 - 17 ^{o}C, pH 7.9 with specific gravity of 1.023-1.025. The animals were fed seaweed once per week. Seaweed was presented to the animals on Fridays and removed on Mondays. Food satiated animals or animals which were observed to be engaged in sexual activity were generally not used for experimentation as these exhibit suppressed gill behavior (Lukowiak, 1980; Lukowiak and Freedman, 1983). Experiments were performed between Tuesdays and Fridays throughout the year.

The Gross dissection

Prior to dissection the animals were weighed and then anesthetized by injection of isotonic (.33 M) $MgCl_2$ (up to 35% of body weight) into the hemocel resulting in the loss of muscle tone and responsiveness within minutes.

The animal was pinned dorsal side up to the dissection board with a pin through the head rostral to the buccal mass and another through the tail. The parapodia were pinned laterally exposing the mantle cavity containing the external mantle organs (the siphon, mantle and gill connected to the mantle shelf and covered by the purple gland). The preparation was rinsed regularly to remove ink and opaline gland secretions. An incision was made

in the dorsal surface of the body wall where the parapodia join the body wall at their most rostral point. The incision was continued rostrally to the head cutting only the body wall and musclulature, avoiding the internal organs and major nerves and connectives. The body wall was pulled laterally and pinned exposing the gut, head ganglia and buccal mass. The salivary ducts and vasculature of the gut was severed. The gut was then pulled outside the body and removed without spilling the contents by cutting anterior to the buccal mass and posterior to the gizzard by the hepatopancreas. At this point the pleural-abdominal connectives were severed. The ctenidia and mantle organs were isolated from the body by an inscision through the body wall encircling the mantle cavity immediately proximal to the parapodia. The ctentidia and mantle organs including the abdominal ganglion were transfered to a dish filled with ASW and pinned dorsal side down. The hepatopancreas, gametolytic and opaline glands were removed by cutting the encasing connective tissue and severing innervation, circulatory vessels and the points of connection to the body wall or ducts. The abdominal ganglion was removed by severing the branchial, ctenidial and siphon nerves in this order to preserve a consistent experimental approach.

Preparation of gills for perfusion experiments

For the isolated gill experiments, the preparations consisted of the siphon, mantle and gill with the abdominal ganglion removed. The preparation was positioned dorsal side down and pinned to the Sylgard base of a Lucite dish, filled with ASW maintained at 15 ^{o}C. The preparation was pinned through

the body wall near the heart, distally opposite the gill and proximal to the siphon by the anus.

Gill perfusion was accomplished using a glass cannula inserted into the afferent branchial vein mimicking the normal flow of hemolymph. Perfusion fluid entered the gill via a cooled tube system which branched through a switching device connected to a gravity flow drip reservoir allowing the delivery of control and experimental perfusion media. The delivery system provided for cooling of the perfusate to 15°C immediately before it entered the gill afferent vein. The control perfusate was ASW; experimental solutions, which consisted of the peptide (SCP_B, Peninsula), neurotransmitter (ACh, Sigma), cholingeric agonists (Carbachol, Sigma) and/or antagonists (Atropine, Curare, alpha-bungarotoxin,Sigma), dissolved in ASW, were prepared immediately prior to use. All agents were stored below 0°C and in the case of SCP_B , 100 µl alloquots of 1.0 mM SCP_B dissolved in distilled water were stored frozen at -70°C. The composition of ASW is shown in the table below. With ASW perfusion the gill can remain viable for up to 72 hours (Cawthorpe, unpublished observations).

Table 2

Compound	Molarity (mM)
NaCl	425
KCl	10
$CaCl_2.6H_2O$	10
$MgCl_2.6H_2O$	22
$MgSO_4.7H_2O$	26
Hepes Buffer	5

After a steady perfusion of the gill was attained, a suture thread was tied to a single gill pinnule; the other end of the suture was then

connected to a Grass type FT03C isotonic force transducer. Again the other end of the wire was attached to the force transducer. The amplitude of gill movements were amplified through a Grass model LP122 low level DC amplifier and displayed on a Grass polygraph and a Hitachi storage oscilloscope. No inhibitors of proteolytic activity were added to any of the perfusion media. Figure 1 shows a diagram of the experimental preparation.

Recording Junctional Potentials

Suction electrodes were used to record extracellular nerve and muscle activity from the gill. The electrode was constructed of a fire-polished glass electrode (tip diameter of 300-400 μm), polyethylene tubing and a syringe filled with ASW. The electrode was connected to the needle and syringe by the tubing. A silver wire which served as a reference electrode was soldered to an insulated wire and wrapped around the tip of the electrode. The internal solution was connected to a wire soldered to the metal needle of the syringe which contacted the ASW sucked into the syringe. When both these electrodes were contacted by the ASW bath and were plugged into the postive and negative inputs of a cable adapted to a Grass EEG amplifier model 7P511H the circuit was complete. Activity could then be recorded and displayed on a Grass polygraph and Hitachi oscilloscope, as described above. The bath was also grounded through this circuit. The tip of the electrode was advanced to the the efferent vein or a pinnule using a Brinkman Mn33 micromanipulator and suction was applied with the syringe, the fire-polished tip of the electrode formed a high resistance seal with the underlying tissue. As a result neural and muscular activity could be

recorded. In Figure 2 (Results) activity recorded in this manner shows that there is a high degree of correlation with spontaneous gill contractions.

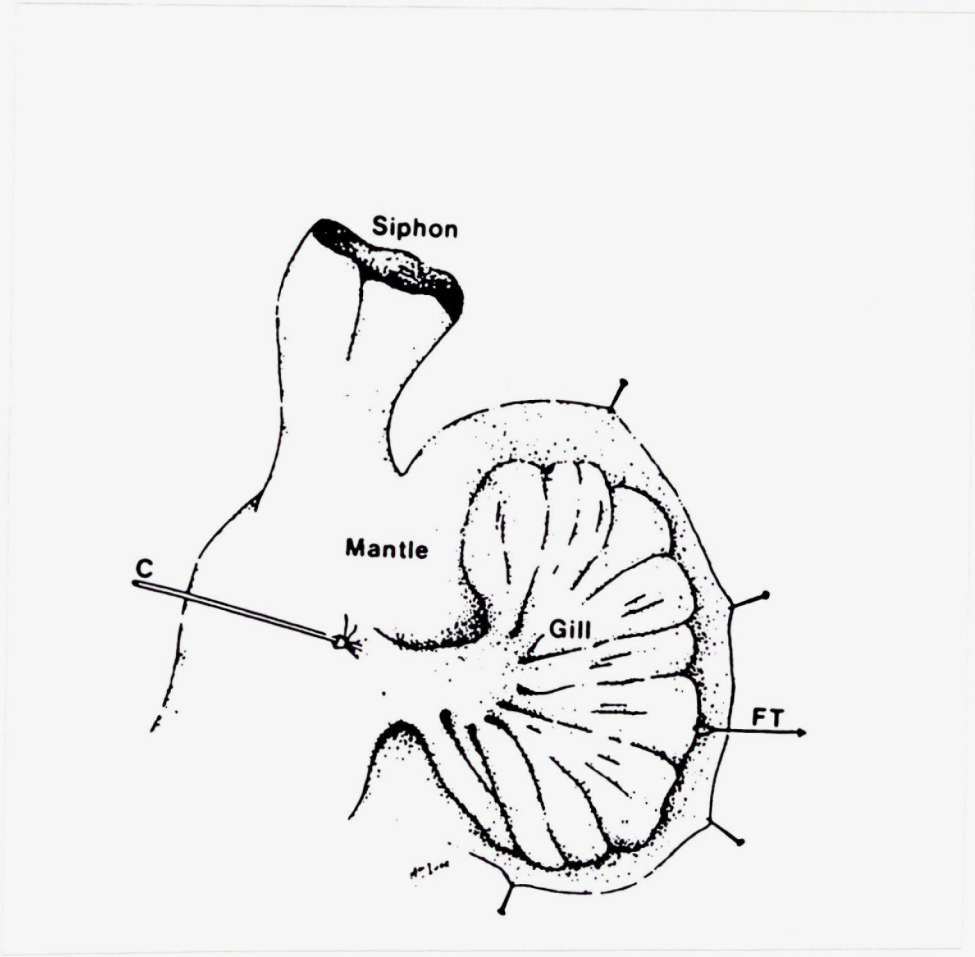


Figure 1.

Diagram of the experimental preparation. The semi-intact Aplysia californica preparation consisted of the siphon, mantle and gill, isolated from the abdominal ganglion. Perfusion was through a glass cannula (C) inserted into the afferent sinus of the branchial vein. Control perfusate (ASW) and experimental solutions were delivered through separate gravity flow drip reservoirs connected through a switching valve to allow continuous perfusion. Temperature was maintained at 15 C by a cooling system. The gill (or siphon) was stimulated using a mechanical tapper. The evoked GWR amplitude was amplified and recorded by connecting a single pinnule of the gill to an isotonic force transducer.

Immunohistochemistry

Monoclonal antibodies to SCP_B were obtained from the department of Zoology, University of Washington. The monoclonal antibody to SCP_B was produced by immunizing mice with SCP_B conjugated to thyroglobulin with glutaraldehyde. Subsequent hybridization and cloning permitted selection of a hybridoma line that produces an IgG1 subclass of antibody. An epitope for this antibody was located on the 6 amino-terminal amino acids. Immunohistochemical studies with this antibody indicate a specific SCP_B-like immunoreactivity in both vertebrate and invertebrate animals (Kempf et al. 1987). The FITC conjugated secondary anti-body, goat anti-mouse (Sigma), was developed in goat using purified mouse immunogen. Affinity isolated antigen specific antibody was isolated by immunospecific purification to remove essentially all goat serum protiens which do not bind to mouse IgG.

The dissection for these experiments was basically the same as described above. The dissection of the entire nervous system out of the animal was accomplished while attempting to preserve ganglionic connectives and samples of tissue where major nerves innervated the gill and mantle organs.

Once dissected the CNS and tissues of the efferent and afferent veins including pinnule muscles were pinned to the sylgard base of a small plastic dish and fixed for approximately twenty-four hours in Zamboni's fixative. The preparations were then washed with phosphate buffered saline (PBS) repeatedly for six hours. Excess PBS was carefully removed and the primary antibody was applied to the preparation for fourty-eight hours. The primary antibody was mouse anti-SCP_B diluted 1:100 with triton X-100 3%, Sodium azide 0.02%, and 30 µl of goat serum to a total volume of 1 ml of PBS. This volume was adequate to cover the preparations. Following the incubation

period the non-specific binding of the antibody was minimized by serial washing with PBS for approxiamtely six hours. The second anitbody was then applied and incubated for twenty-four hours. This was goat anti-mouse gamma globulin-FITC conjugate affinity isolated to be antigen specific. The goat anti-mouse second antibody was similarly diluted 1:100 with sodium azide and goat serum to a final volume of 1 ml in PBS. Following incubation the preparations were washed continually for six hours (ie. once every ten-twenty minutes) to minimize non-specific staining. Each preparation was at this time separated into individual ganglia. The preparations were then mounted in glycerol for examination using Zeiss immunofluorescence optics.

Three series of experiments were conducted. One in which an attempt was made to specifically pre-absorb the primary antibody by including in this solution an excess of SCP_B (100 µl of 1 mM SCP_B). However, pre-absorption controls have had limited sucsess when employed with the SCP_B anti-body. A second control was employed in which the primary anti-body was left out of the recipe in order to determine whether the second antibody exhibited specific staining in the absence of anti-SCP_B. This was not found to be the case. The staining observed in the experimental preparations consisting of the entire CNS resembled the distribution of SCP_B observed by Lloyd et al. (1985b) thus serving as a positive control for SCP_B fluorescence observed in the gill tissue from the same preparations. The SCP_B-like immunofluorescence observed in gill tissue therefore was asssumed to be specific to SCP_B and is termed SCP_B-like immunofluorescence.

Treatment of Data

To normalize data and thereby compare the effects of experimental agents upon the GWR amplitude of different preparations, the amplitude of the GWR obtained during the experimental run was expressed as a percentage of the control GWR amplitude of a given preparation. The experimental values obtained in this manner were used to calculate the mean percentage of control for a series of experiments.

Each point or histogram in subsequent figures of normalized, cummulative data is the mean of all experiments in a series where a particular concentration of SCP_B or neurotransmitter was perfused alone or with cholinergic antagonists prior to and during tactile stimulation of the gill. The error bars are the standard error of the mean and significant differences calculated using the students t-test are noted and the p value given. In the case where rates of habituation are compared, a one-way analysis of variance was performed on the normalized data points and their variances. Resulting F values allowed determination of significance.

Results

Rythmic Contractions in the Isolated Gill

For each experiment, once a steady perfusion was attained, the isolated gill preparation was allowed to rest for a period of one hour in order to minimize the effects of surgery. In some preparations, during this time, the gill was observed to exhibit spontaneous contractions which generally attenuated over the duration of the experiment. In some cases the spontaneous contractions were similar in appearance and frequency to centrally generated respiratory pumping which is dependent upon the recruitment of the interneuron II network, located in the abdominal ganglion (Byrne and Koester, 1978). Figures 2 and 3 show data from two experiments where the gill exhibited such rythmic activity. In Figure 2 the upper trace (A) is a recording of the gill potential recorded from a single pinnule by means of a suction electrode. It can been seen that the gill potential precedes the contraction in each case and lasts until the peak force of the contraction is attained. In Figure 3, only the spontaneous contractions of the gill are shown. By comparison these contractions are more regular and of longer duration though less frequent than those shown in Figure 2. These rythmic contractions were observed to diminish over the course of an experiment. Of importance is that these rythmic contractions occurred in the absence of the abdominal ganglion. The relationship to respiratory pumping is unknown.

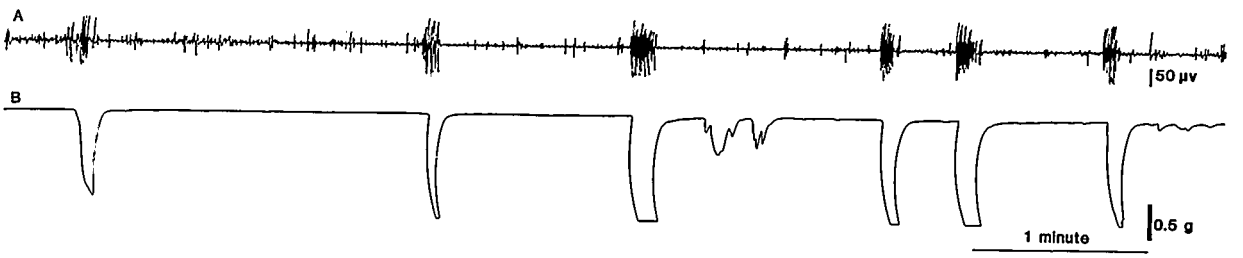


Figure 2.

Spontaneous contractions of the isolated Aplysia gill. The upper trace is an extracellular recording of the gill pinnule potential. The lower trace is a recording of the amplitude of spontaneous gill contractions. Note that the activity in the gill potential record slightly precedes the gill contraction and persists until the gill begins to relax.

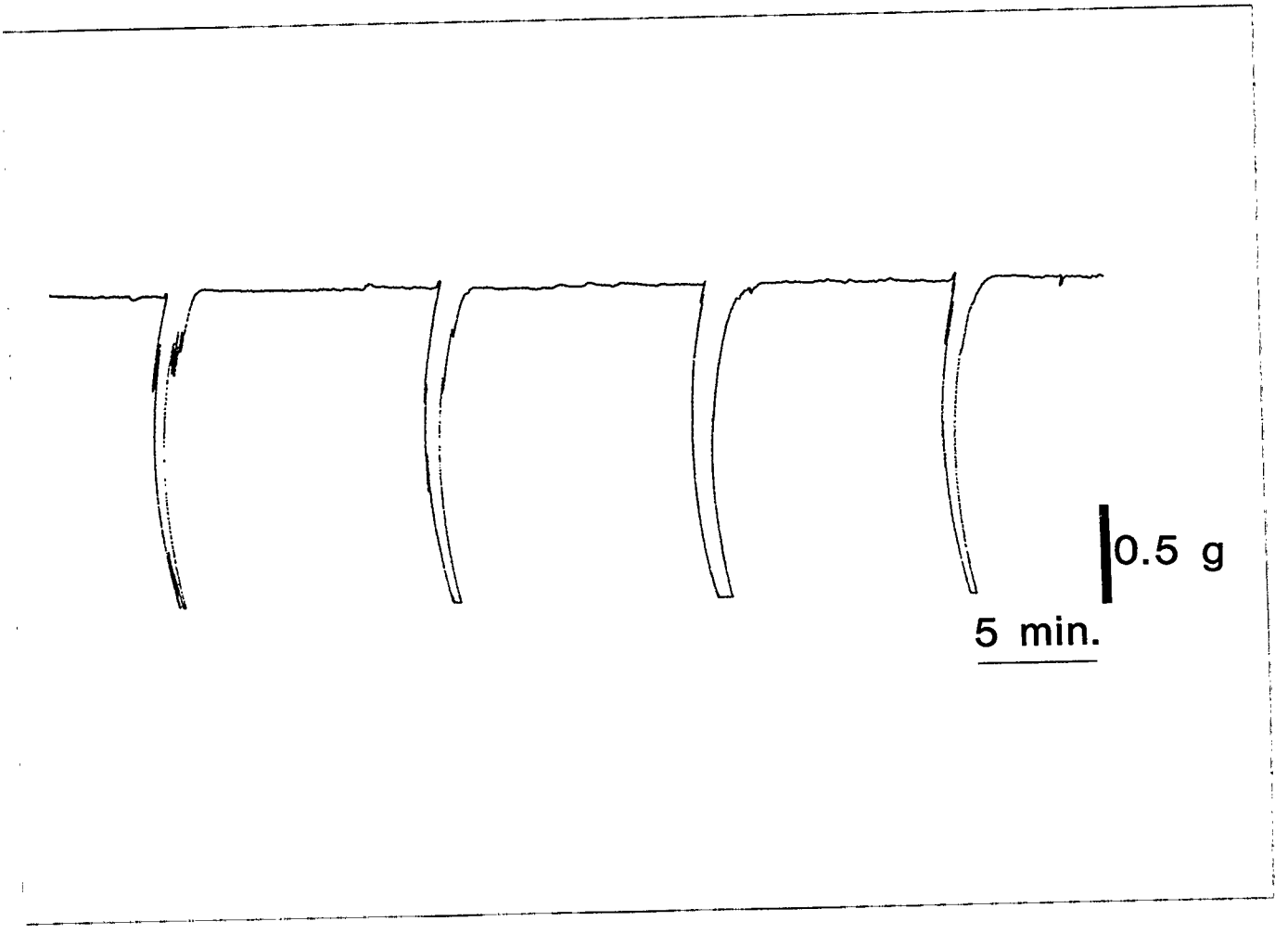


Figure 3.

Spontaneous contractions of the isolated Aplysia gill. Note the time base is compressed by comparison to Figure 2. These contractions are less frequent though more regular.

SCP_B perfusion Suppresses the Gill Evoked Gill Withdrawal Reflex

Following the rest period the gill was stimulated by means of a mechanical tapper. This tapper was calibrated to deliver a stimulus with intensity of about one gram. The gill responded with a brisk contraction involving a contraction of the pinnules with shortening of the efferent and afferent veins. For perfusion experiments the gill was stimulated at an interstimulus interval of no less than twenty minutes in order to prevent habituation (Goldberg and Lukowiak, 1982). Under these circumstances the GWR remains relatively stable with respect to ampltude (of primary interest in this study) and duration. Because of this stability, the GWR was used as an assay to study the effects of SCP_B perfusion. Twenty minutes following the initial stimulus and resultant GWR, a second control GWR was obtained. If the amplitude of this reflex was within ten per cent of the previous reflex then the experiment proceded. Characteristic changes in GWR amplitude following SCP_B perfusion may be seen in Figure 4. The two control GWR's are shown (ASW) as well as the GWR with SCP_B perfusion and following washout (ASW). The perfusion of 10 fM SCP_B through the gill for approximately five minutes resulted in suppression of the GWR. This concentration of SCP_B was the lowest concentration which when tested produced consistent suppression of the GWR. The suppression produced by SCP_B perfusion was reversible; when the perfusion media was switched back to ASW and following a twenty minute washout the GWR has recovered to control levels.

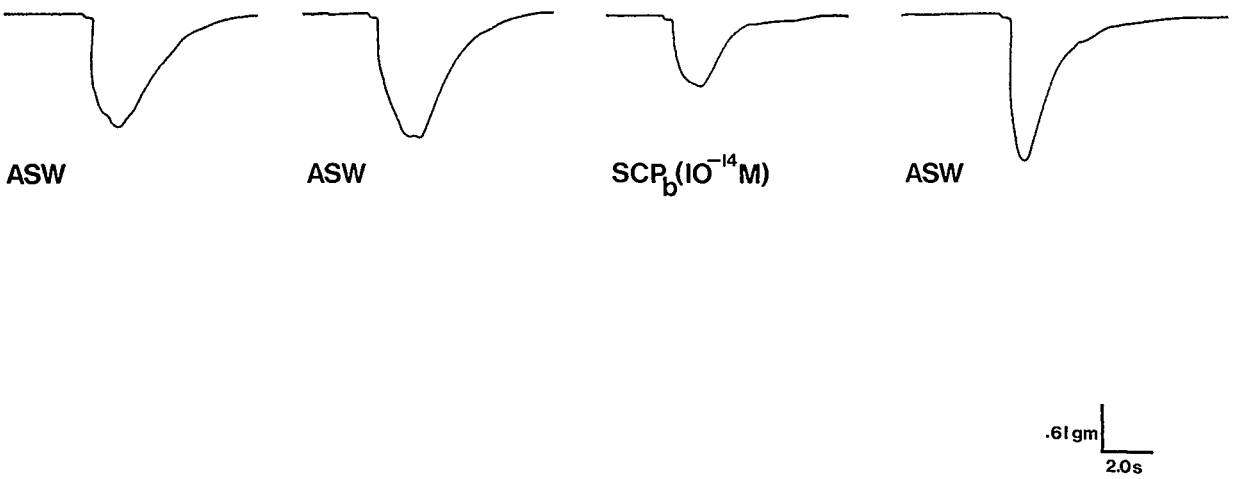


Figure 4.

Individual experiment showing the suppressive effects of SCP_B (10 fM) on the gill evoked GWR when perfused through the isolated Aplysia gill. The two left traces are control the GWR. Five minutes prior to the third GWR, SCP_B (10 fM) was perfused through the gill which resulted in a decrease in the GWR amplitude. Washout with ASW was immediately commenced. The GWR obtained after twenty minutes washout with ASW was within the amplitude range of control.

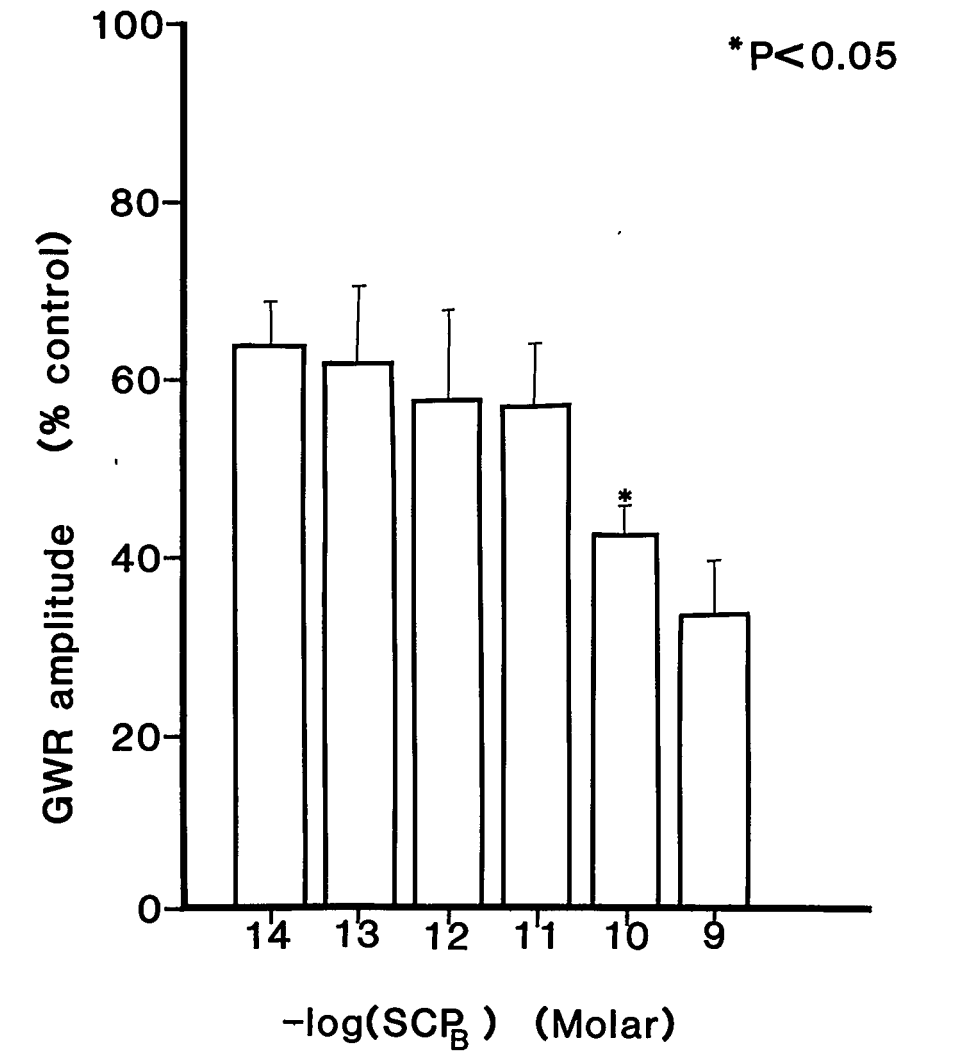


Figure 5.

Dose-response histogram showing the effects of perfusing increasing concentrations of SCP_B through the gill. Graph represents cummulative, normalized data ($n=^B$ 42). All concentrations of SCP_B perfused through the gill brought about suppression of the gill evoked GWR which were significantly different from control ($p<.001$). Increasing concentrations of SCP_B brought about increasing suppression of the GWR with 100 pM being significantly different from lower concentrations ($p<.05$) shown by asterisk.

The normalized, cummulative data from forty two experiments is shown in Figure 5. Every concentration of SCP_B perfused through the gill that is shown in figure 5 brought about suppression of the gill evoked GWR which was significantly different from control ($p<.001$). Increasing concentrations of SCP_B produced greater suppression with 100 pM being significantly different than the suppression of the GWR produced by lower concentrations of SCP_B ($P<.05$).

A Sensitizing Stimulus May Over-ride the Suppression Produced by SCP_B

Data from a single experiment ars shown in Figure 6, it can be seen that the perfusion of 10 pM SCP_B suppressed the GWR; however in this experiment the perfusion of SCP_B was not stopped following the GWR but was continued a further 10 minutes. During this period the interposition of a noxious stimulus (a gill pinch) prior to presentation of the gill stimulus results in a facilitation of the GWR. However, the suppressive effects of SCP_B persisted as indicated by the amplitude of the last trace after the effects of the sensitizing stimulus have diminished. These results show a sensitizing stimulus can activiate the facilitatory pathways in the gill periphery and over-ride the suppressive effects of SCP_B upon the GWR.

SCP_B Suppresses the Amplitude of the Siphon evoked GWR

Figure 7 shows data from a single experiment. Following two control GWR's evoked by siphon stimulation the perfusion of SCP_B (1 nM) through the

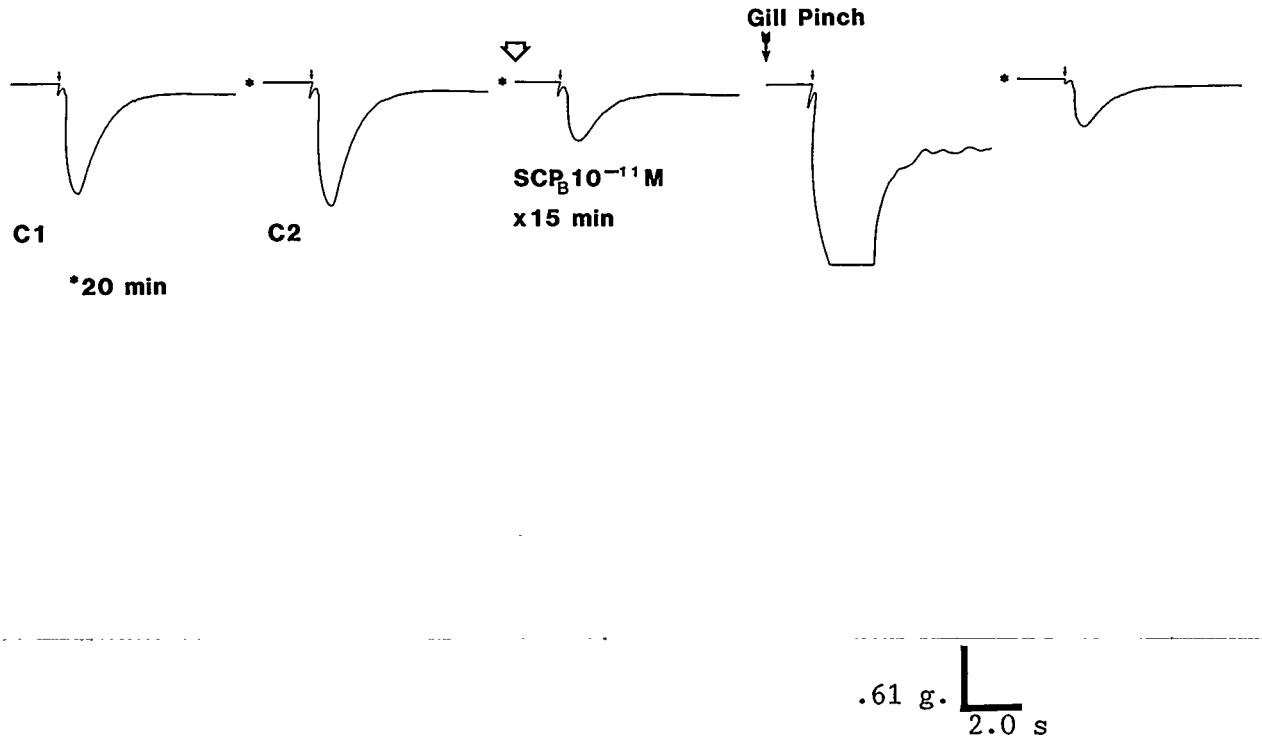


Figure 6.

Sensitization was not blocked by the perfusion of SCP_B. The two left traces are control GWR's, C1 and C2. SCP_B (10 pM) was perfused for five minutes prior to and ten minutes after the GWR shown in the third trace which is suppressed with respect to control. Following this GWR a sensitizing stimulus (gill pinch, not shown) was presented to the gill. When the baseline tension returned to baseline, a tactile stimulus was presented to the gill and the resultant GWR was facilitated (fourth trace) even though SCP_B was continuously perfused through the gill. The fifth trace shows that the suppressive effects of SCP_B are persistent after the effects of the sensitizing stimulus have diminished.

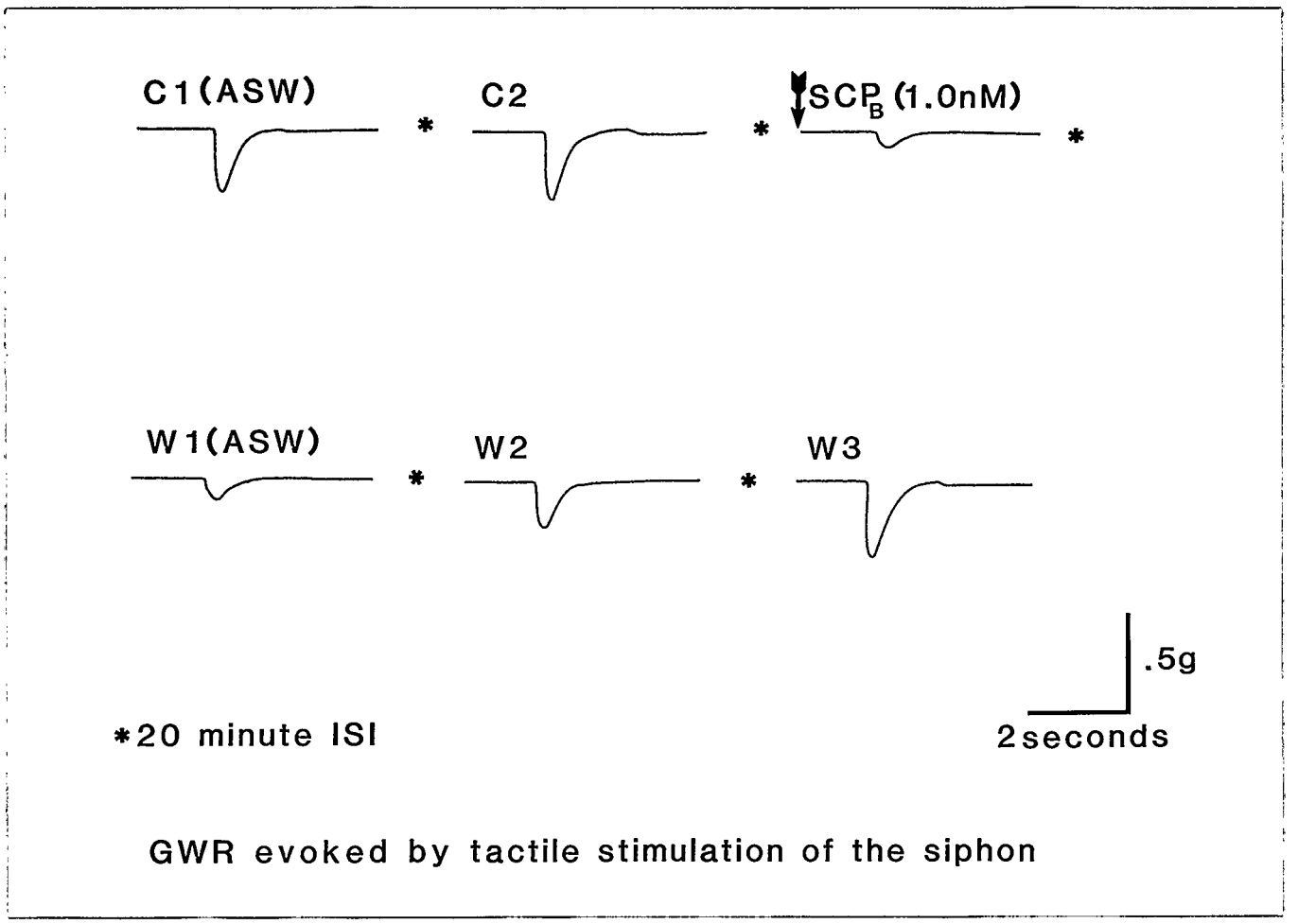


Figure 7.

SCP$_B$ perfusion suppressed the siphon evoked GWR. Two control GWR's are shown (C1 and C2). The perfusion of SCP$_B$ (1 nM) for five minutes results in suppression of the GWR. Following washout for one hour (W3) the amplitude of the GWR has recovered to control levels. ISI- interstimulus interval.

gill produces suppression of the siphon evoked GWR. Normalized cummulative data are shown in Figure 9 (Compare trial 1 from control and experimental habituation runs). The suppression produced by SCP_B is significantly different from control GWR's.

SCP_B Suppresses the Amplitude of the Siphon evoked GWR during Habituation

Habituation has been described as the decrement of the evoked GWR to repeated tactile stimulation. In Figure 8 the effects of perfusing SCP_B through the gill during habituation may be seen. Data from a single experiment are shown. Repeated tacile stimulation of the siphon using an interstimulus interval (ISI) of one minute, with ASW perfusion results in habituation of the GWR (upper traces show trial one, five and ten). Following a three hour rest and recovery period the perfusion of SCP_B (1 nM) suppressed the GWR which still habituated (middle traces). A further three hour rest rest was interposed with ASW perfusion and the amplitude of the GWR was similar to the first control as was the habituation (lower traces). Dishabituation is a parameter of habituation defined as the increase in the amplitude of the habituated GWR following the presentation of a novel (strong) or noxious stimulus. Following trial ten of both the control habituation runs and experimental habituation run where SCP_B was perfused, a dishabituating stimulus (gill pinch) was presented and the subsquent siphon evoked GWR can be seen to be dishabituated (DH) in each case.

Cummulative normalized data (n=9) is shown in Figure 9. These experiments were performed as described above. Repeated tactile stimulation results in habituation of the siphon evoked GWR (open squares). Following a three hour rest period the perfusion of SCP_B (1 nM) resulted in suppression

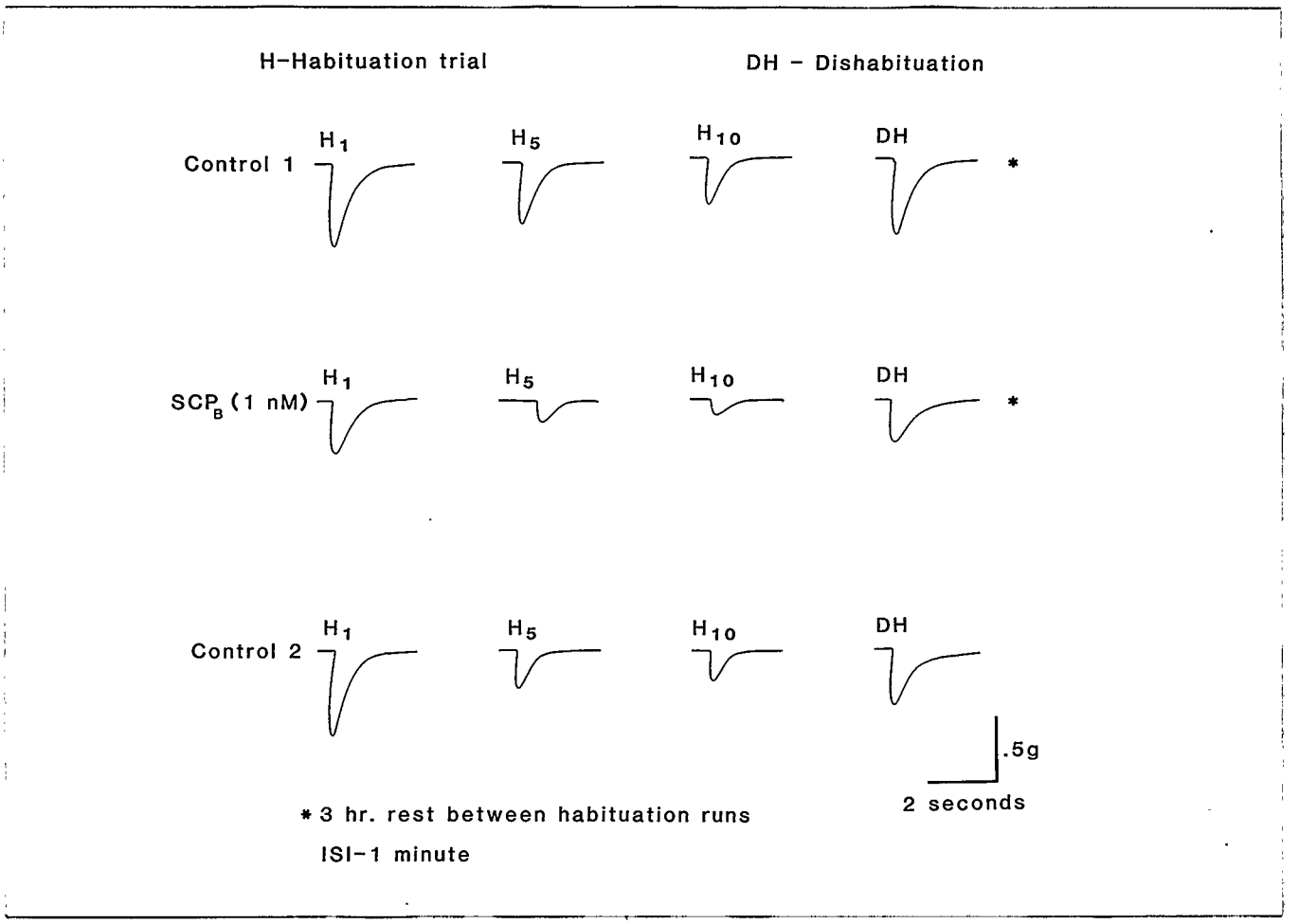


Figure 8.

SCP_B suppressed the amplitude of the GWR during habituation. Data from a single experiment are shown. The initial control habituation run shown in the upper traces (Control 1) was obtained with ASW perfusion. Following a three hour rest a second habituation run was obtained with SCP_B (1 nM) perfusion which resulted in a decrease in the amplitude of the GWR. After a second 3 hr rest period with ASW perfusion, the amplitude of the GWR had recovered to the control level. A second control habituation was obtained with ASW perfusion (Control 2) which was similar to Control 1. After each habituation run a dishabituating stimulus (gill pinch, not shown) was presented and the subsequent dishabituated response is shown for each run (DH). The relative increase in the amplitude of the dishabituated GWR obtained with SCP_B perfusion with respect to trial ten is as great as the dishabituated GWR's observed in each of the control habituation runs.

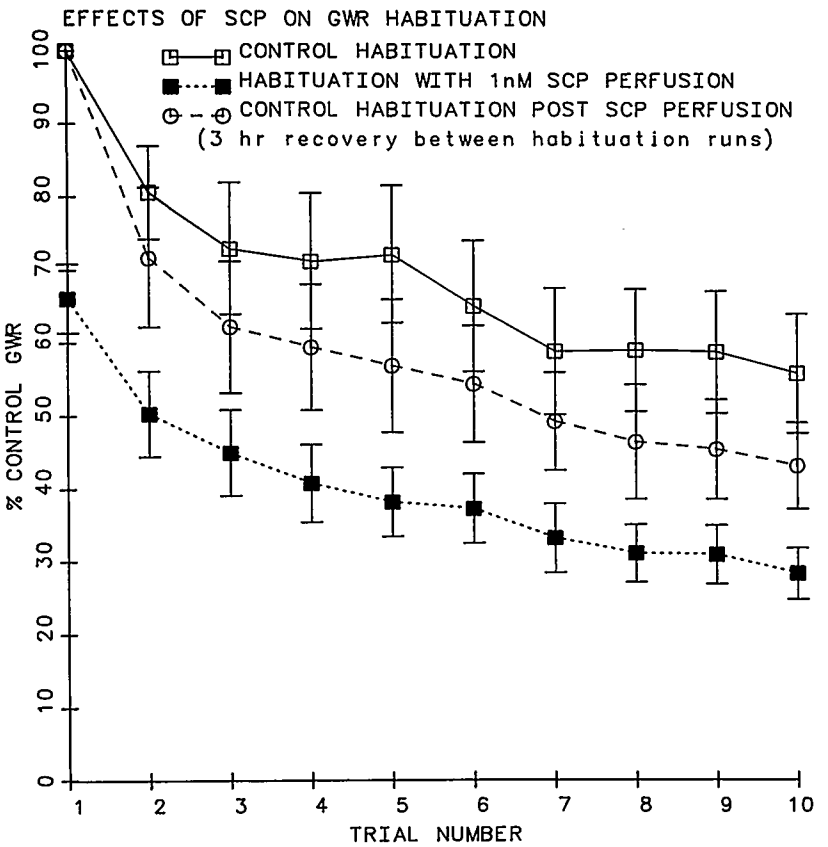


Figure 9.

Effects of SCP_B perfusion of the isolated gill on GWR amplitude during habituation of the siphon evoked GWR. Cummulative, normalized data (n=9) in which SCP_B was continiously perfused through the gill during the experimental habituation run. The ISI for each habituation trial was one minute. The mean data points for each trial in the experimental run are significantly different from those of the initial control habituation run ($p<.01$) and the post SCP_B perfusion habituation run ($p<.05$). Trial 1 shows that the GWR evoked to siphon stimulation is suppressed with respect to control when SCP_B (1 nM) is perfused through the gill.

of the GWR which still habituated and was significantly different from control GWR amplitude at each trial ($p < .01$), (filled squares). Following the interposition of a second three hour rest period with ASW perfusion, the amplitude of the GWR was similar to control as was the habituation (open circles).

SCP_B does not Suppress Dishabituation

Dishabituation is the increase in the amplitude the GWR following the presentation of a strong or novel stimulus to the gill. The cummulative, normalized data in Figure 10 shows the effects of SCP_B perfusion upon the amplitude of the dishabituated siphon evoked GWR. The two left bar graphs allow comparison of the siphon evoked GWR amplitude of the last habituation trial (trial ten) from the control habituation run (open square) and habituation run where SCP_B was perfused through the gill (closed square). SCP_B perfusion resulted in a decrease in the amplitude of the habituated siphon evoked GWR which was siginificantly different from control ($p < .01$). Comparison of the amplitude of the siphon evoked GWR following the presentation of a dishabituating stimulus (gill pinch) shows that the amplitude of the dishabituated responses are similar following control habituation (open circle) and habituation with SCP_B perfusion (closed circle). The amplitudes of both dishabituated GWR's were not significantly different from control or one another. This shows that the perfusion of SCP_B through the gill does not appear to act on the pathways which mediated dishabituation in the gill.

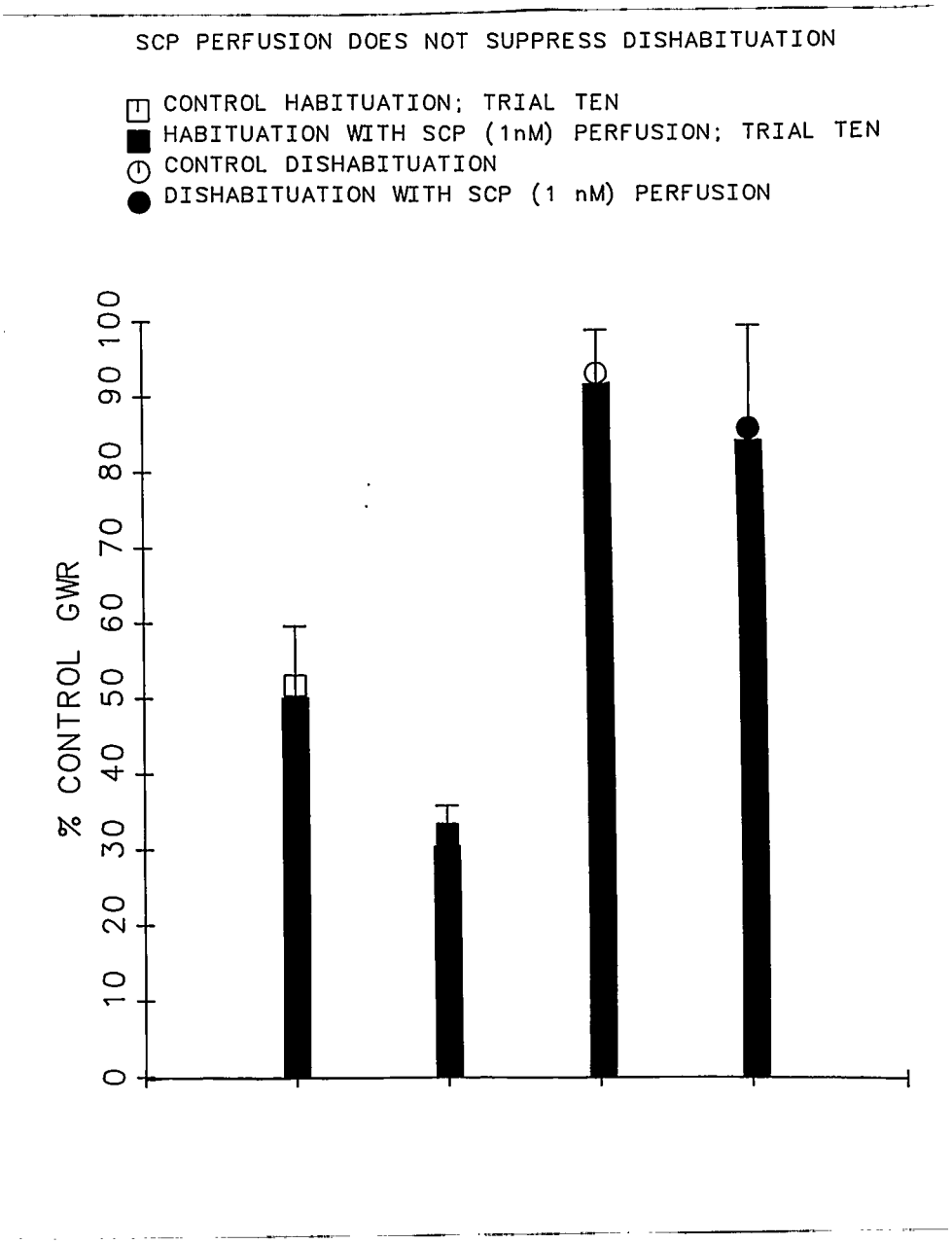


Figure 10.

SCP_B does not suppress dishabituation of the GWR. The two left bar graphs show the the amplitude of the habituated GWR (trial number ten) from the control habituation run and from the habituation run where SCP_B (1 nM) was perfused through the gill. The two right bar graphs show the amplitude of the siphon evoked GWR following the presentation on a dishabituating stimulus. Comparison of the amplitude of the dishabituated GWR's following control and experimental (SCP_B) habituation runs shows that SCP_B perfusion does not suppress the dishabituation of the siphon evoked GWR.

SCP_B Does Not Effect the rate of Habituation

The data shown in Figure 9 were plotted on a log-log graph and presented in Figure 11. Linear regression analysis of the data points for both control and experimental habituation runs gave a best fit curve through the mean GWR amplitude points from each trial. This allowed determination of whether the perfusion of SCP_B through the gill effected the rate of habituation. The rate of habituation is given by the slope of the regression curves representing control and experimental habituation runs. Both the curves are linear with a negative slope as expected since habituation curves follows a negative exponential function (Thompson and Spencer, 1966). In the case where SCP_B was perfused during the experimental run the slope of the line is slightly more negative (-.33) than the slope of the control run (-.25). However, the difference of the slope between the control and experimental curves was not found to be significantly different (F-test) indicating that the perfusion of SCP_B through the gill during habituation does not effect the rate of habituation when an interstimulus interval (ISI) of one minute is used.

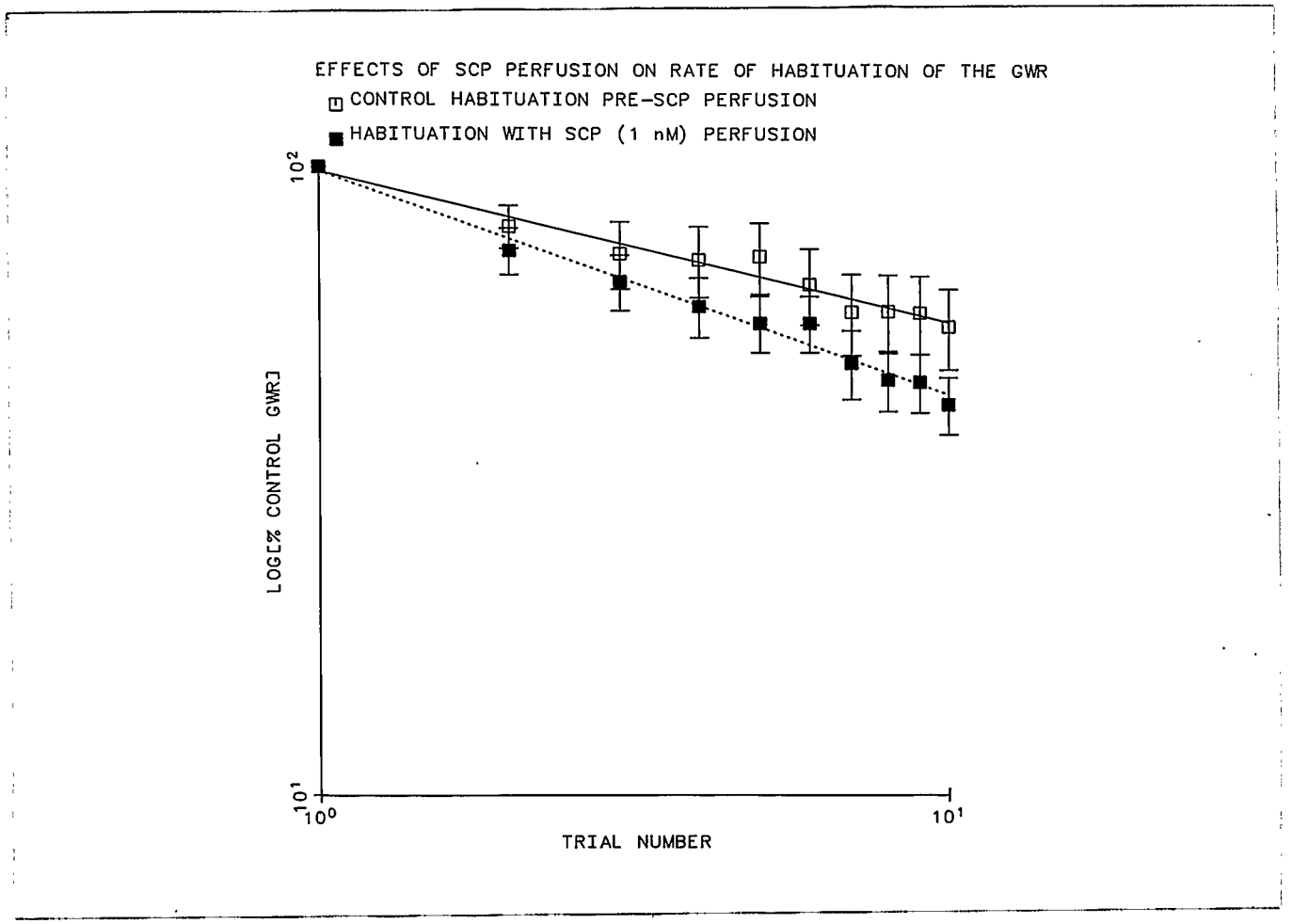


Figure 11.

SCP_B does not effect the rate of habituation of the siphon evoked GWR. Mean data for each trial in the control (open squares) and experimental (closed squares) habituations runs (from figure 9) were plotted on a log-log axis. Linear regression analysis was used to determine the best fit curve through each set of points. The slope of each line gives the rate of habituation for control (-.25) and experimental (-.33) habituation runs. An F-test indicated that there was no significant difference between the two curves. This shows that SCP_B does not effect the rate of habituation when an ISI of one minute is used.

ACh Suppression of the GWR is Blocked by Nicotiniic Antagonists

The data presented above show that SCP_B suppresses the GWR evoked by tactile stimulation of the siphon or gill. SCP_B does not suppress the peripheral pathways in the gill which mediate sensitization and dishabituation, suggesting that SCP_B may bring about suppression of the GWR by acting via an inhibitory pathway in the periphery. To examine the possibility that SCP_B was acting via the inhibitory cholinergic pathway in the gill decribed by Weiss et al. (1985), it was first necessary to examine the effects of ACh perfusion of the gill upon the gill evoked GWR.

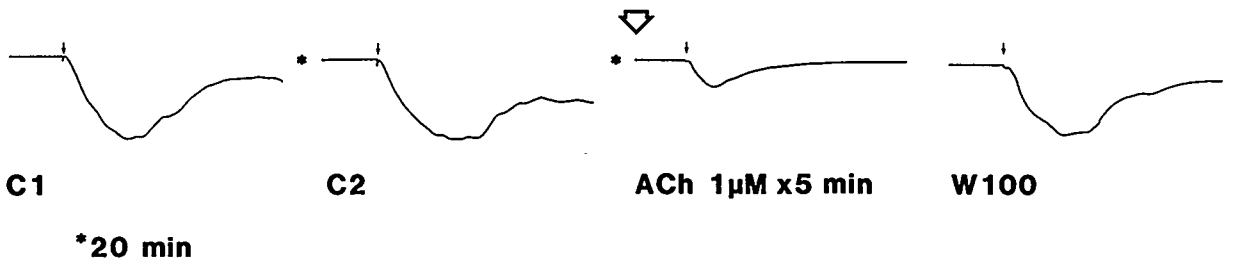
Weiss et al. (1985) demonstrated the presence of both excitatory (muscarinic) and inhibitory (nicotinic) cholinergic receptors in the gill of Aplysia. The inhibitory receptors were revealed when the gill was caused to contract by the perfusion of pCT-cAMP, a diffusable cAMP analogue. Co-perfusion of carbachol, a non-hydrolizable, nicotinic agonist (10 μM) attenuated these contractions and this attenuation was blocked by co-perfusion of curare (10 μM). Low doses of ACh (< 5 μM) do not produce the single tonic, atropine sensitive, contraction of the efferent vein brought about by activation of the excitatory muscarinic-like receptors. Based upon these findings it was possible that the suppressive influences of ACh could be directly revealed by amplitude decrement of the evoked GWR.

In Figure 12 it can be seen that the perfusion of ACh (1 μM) through the gill produces suppression of the GWR. The suppression is reversed after washout for 100 minutes with ASW. As will be seen below the washout time observed in data shown from individual experimnets can vary. This is thought to be a result of differences in the individual animals.

Curare (1 nM and 1 μM) was tested to determine the ability of this nicotinic antagonist to block the suppressive effects of ACh (1 μM) perfusion on the GWR evoked by tactile stimulation of the gill. Figure 13 shows the results of one experiment. Curare reversibly blocked the suppression of ACh perfusion (upper trace, right). Following washout with ASW for two hours, ACh perfusion produced suppression of the GWR.

Figure 14 shows that co-perfusion of atropine, a muscarinic blocker does not block the suppressive effects of ACh perfusion upon the GWR (upper trace, right of centre). In the lower trace (right of centre) co-perfusion of curare (10 μM) again antagonizes the suppressive effects of ACh.

In Aplysia, The action of alpha-Bungaro toxin (α-BTx) has been shown to block the chloride conductance produced by ACh in central neurons (Carpenter et al. 1976). The effects of α-BTx in the Aplysia gill have not previously been tested with respect to the ability of this nicotinic antagonist to block the nicotinic-like or muscarinic-like receptors reported by Weiss et al. (1985). The effects of co-perfusing α-BTx are similar to those of curare. The concentration of α-BTx suggested to be used in these experiments was 1 μg/ml (M.J. Anderson, personal communication). Figure 15 shows that perfusion of 5 μM ACh activates both the excitatory and inhibitory ACh receptors. The slow tonic contraction is apparent in the upper right trace immediately to the left of the evoked GWR which is suppressed. Following recovery, the co-perfusion of α-BTx (1 μg/ml) with 10 μM ACh results in no blockade of the tonic ACh induced contraction which is excitatory and atropine sensitive, whereas the evoked GWR is not suppressed even though a higher concentration of ACh was perfused during this run.



.61 g.
2.0 s.

Figure 12.

ACh suppresses the gill evoked GWR. Small arrows indicate tactile stimuation of the gill. The two left traces are control the GWR's while ASW was perfused through the gill. ACh (1 µM) was perfused through the gill for the last five minutes of the third ISI (large arrow) resulting in suppression of the GWR. Following a twenty minute washout with ASW the amplitude of the GWR recovered to control levels.

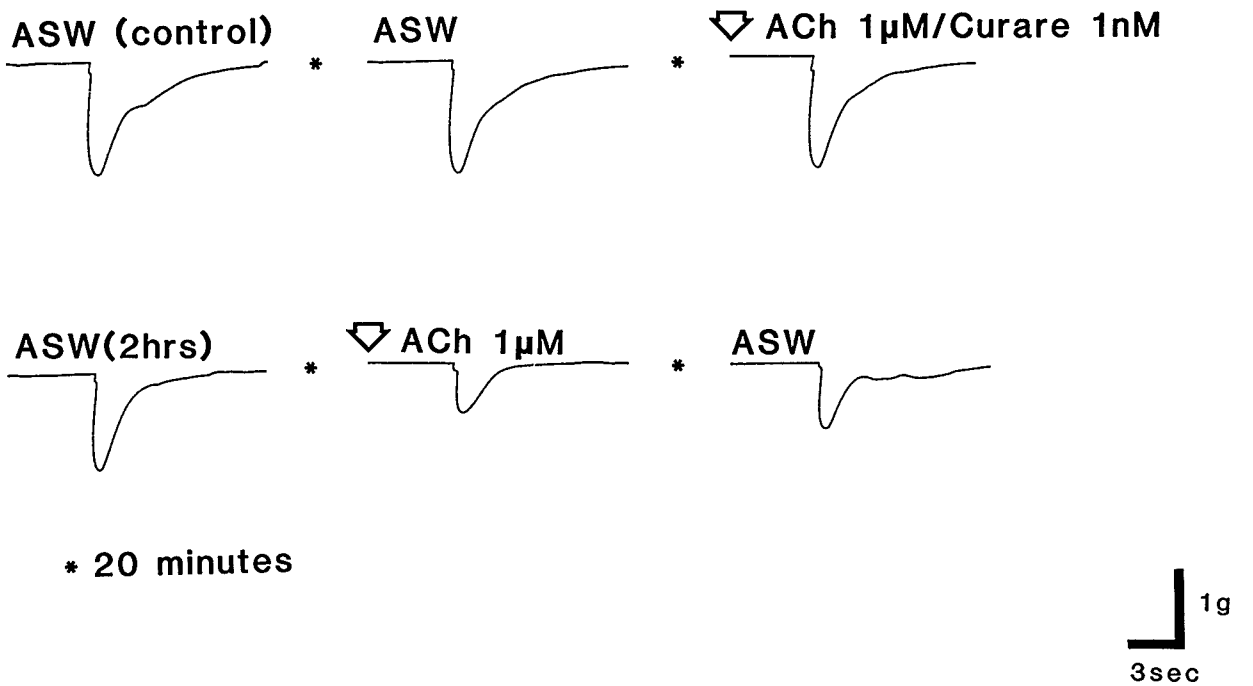


Figure 13.

Curare blocks ACh suppression of the gill evoked GWR. Following control GWR's (upper left two traces) co-perfusion of curare (1 nM) with ACh (1 µM) prevented the suppression of the GWR (upper right hand trace). Following a two hour washout period the amplitude of the GWR is within the control range (lower left trace). Perfusion of ACh (1 µM) during the last five minutes of the next twenty minute ISI results in suppression of the subsequent GWR (lower middle trace). Following a twenty minute washout period a partially recovered GWR is observed (lower left trace).

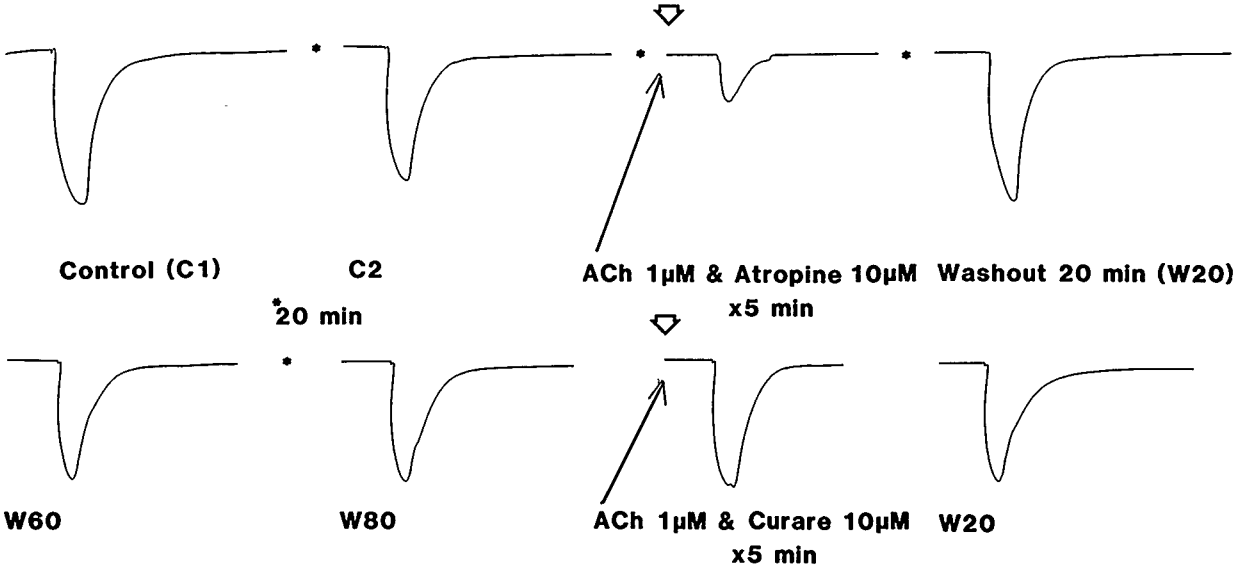


Figure 14.

Atropine does not block the suppressive effects of ACh on the gill evoked GWR. Following two control GWR (upper left two traces), Atropine (10 µM) was co-perfused with ACh (1 µM) though the gill during the last five minutes of the twenty minutes ISI (large arrow). Atropine did not result in a blockade of the suppression produced by ACh perfusion (upper, third trace). Following a twenty minute washout with ASW the amplitude of the GWR has recovered (upper right trace). Two more control GWR's were obtained at sixty and eighty minutes (lower left two traces). When curare (10 µM) was co-perfused with ACh, the suppressive effects of ACh were blocked. Following a twenty minute washout (W20) the amplitude of the GWR returned to control level. The stimulus artifact can be seen at the begining of each GWR (GWR latency 250 ms.).

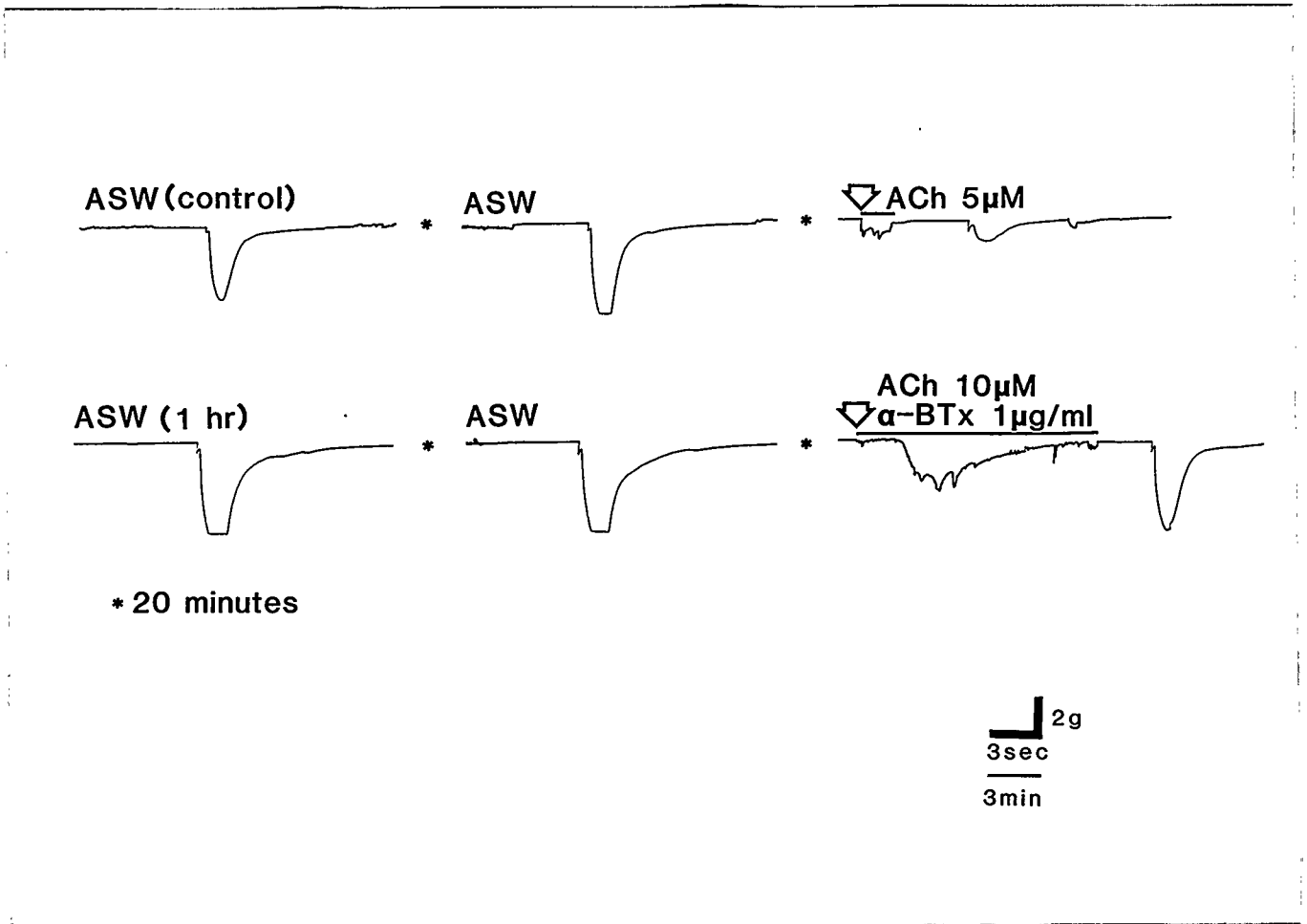


Figure 15.

α-BTx blocks the suppressive effects of ACh on the gill evoked GWR. Following two control GWR's (upper left two traces) the perfusion of ACh (5 µM) during the last five minutes of the subsequent ISI brings about two effects. ACh produces a tonic contraction of the gill (reported by Weiss et al. 1985) which can be seen in the upper right trace. When this contraction ended the evoked GWR amplitude was suppressed (upper right trace). Following a one hour washout the GWR amplitude had recovered from the suppression produced by ACh perfusion. This and the next GWR amplitudes are in the control range (lower two traces). α-BTx (1 µg/ml) was co-perfused with ACh (1 µM) and resulted in a blockade of the suppressive effects of ACh. However this nicotinic antagonist did not attenuate the tonic ACh induced contraction which is mediated by muscarinic-like receptors (lower right trace).

Figure 16 shows that both curare and α-BTx block ACh suppression in the same preparation. The blockade produced by co-perfusion of these agents with ACh can be seen in the upper trace (middle and right, respectively) when these are compared to the suppression produced by perfusion of ACh alone (lower, middle trace). It should be noted that the action of α-BTx in the Aplysia gill is reversible, unlike the blockade produced by this agent at the vertebrate neuro-muscular junction.

The normalized data from twenty experiments shown in Figure 17 indicate that co-perfusion of atropine (10 μM) does not block the suppressive effects of ACh whereas the co-perfusion of curare (10 μM) does block the suppressive effect of ACh upon the GWR.

The normalized data from seventeen experiments depicted in Figure 18 show that both curare (1nM) and α-BTx (1 μg/ml) block the suppressive effects of ACh (1μM) when these agents are co-perfused with ACh through the gill. The perfusion of ACh alone through the gill suppresses the response at concentrations up to 5 μM. At higher concentrations, even though the overall GWR is still suppressed with respect to control, it appears that there is a slight increase in the GWR amplitude. This may possibly be due to the activation of excitatory muscarinic receptors by ACh.

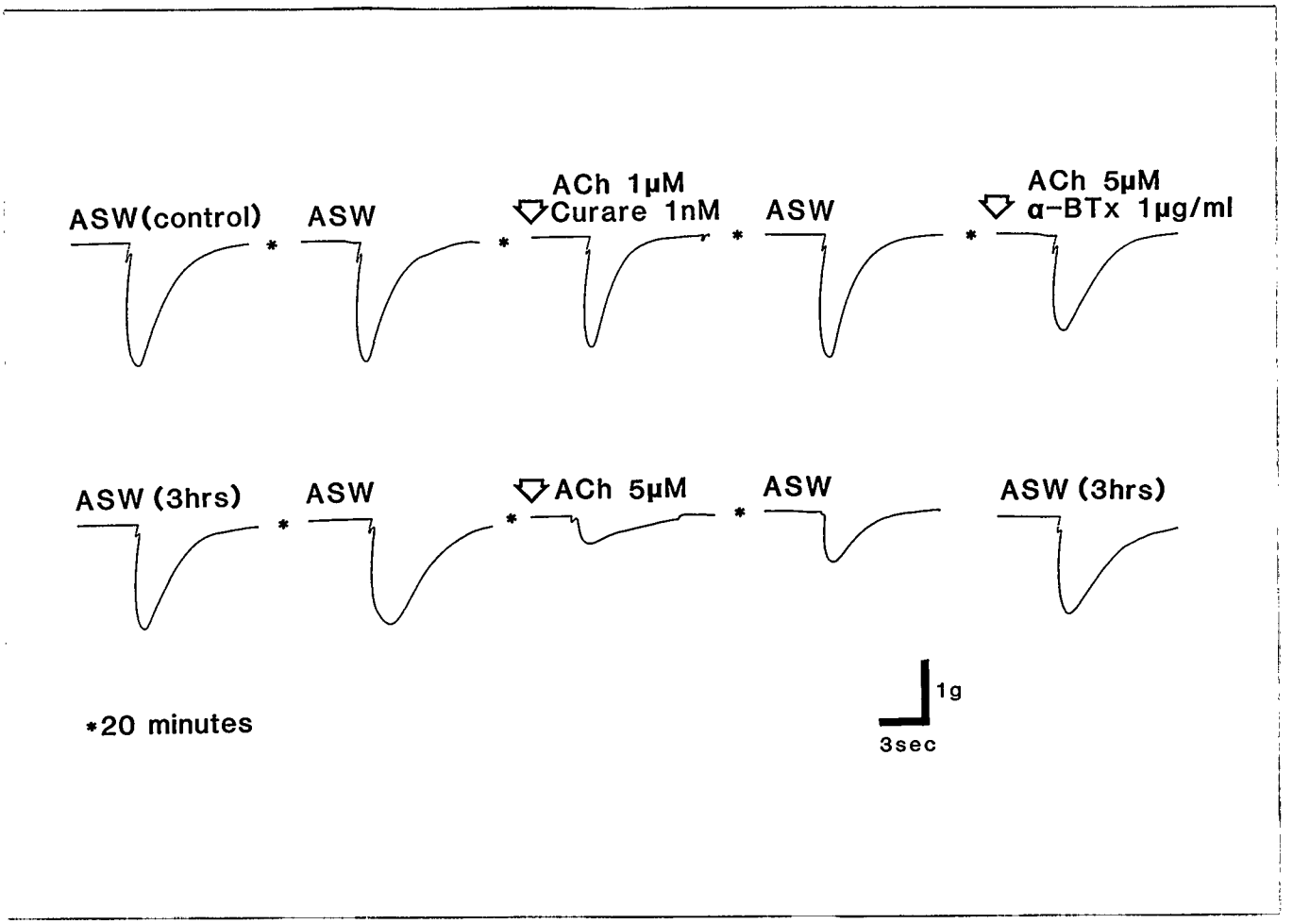


Figure 16.

Curare and α-BTx block ACh suppression of the gill evoked GWR. Following two control GWR's (upper left two traces) curare (1 nM) was co-perfused with ACh (1 µM) for five minutes prior to the GWR (large arrow) which blocked the suppressive effects of ACh (upper middle trace). Following washout the GWR was observed to the control level (upper, fourth trace). α-BTx (1 µg/ml) was perfused with ACh (5 µM) for five minutes prior to the GWR which blocked the suppressive effects of ACh (upper right). The preparation was allowed to rest for three hours while ASW was perfused. Two control GWR's obtained following the rest period were similar in amplitude to the initial controls (lower left two traces). Perfusion of ACh (5 µM) for five minutes prior to the GWR (large arrow) resulted in suppression of the GWR (lower middle trace) which persisted for at least twenty minutes. Following a three hour washout with ASW perfusion the GWR had recovered to within the range of control (lower right trace).

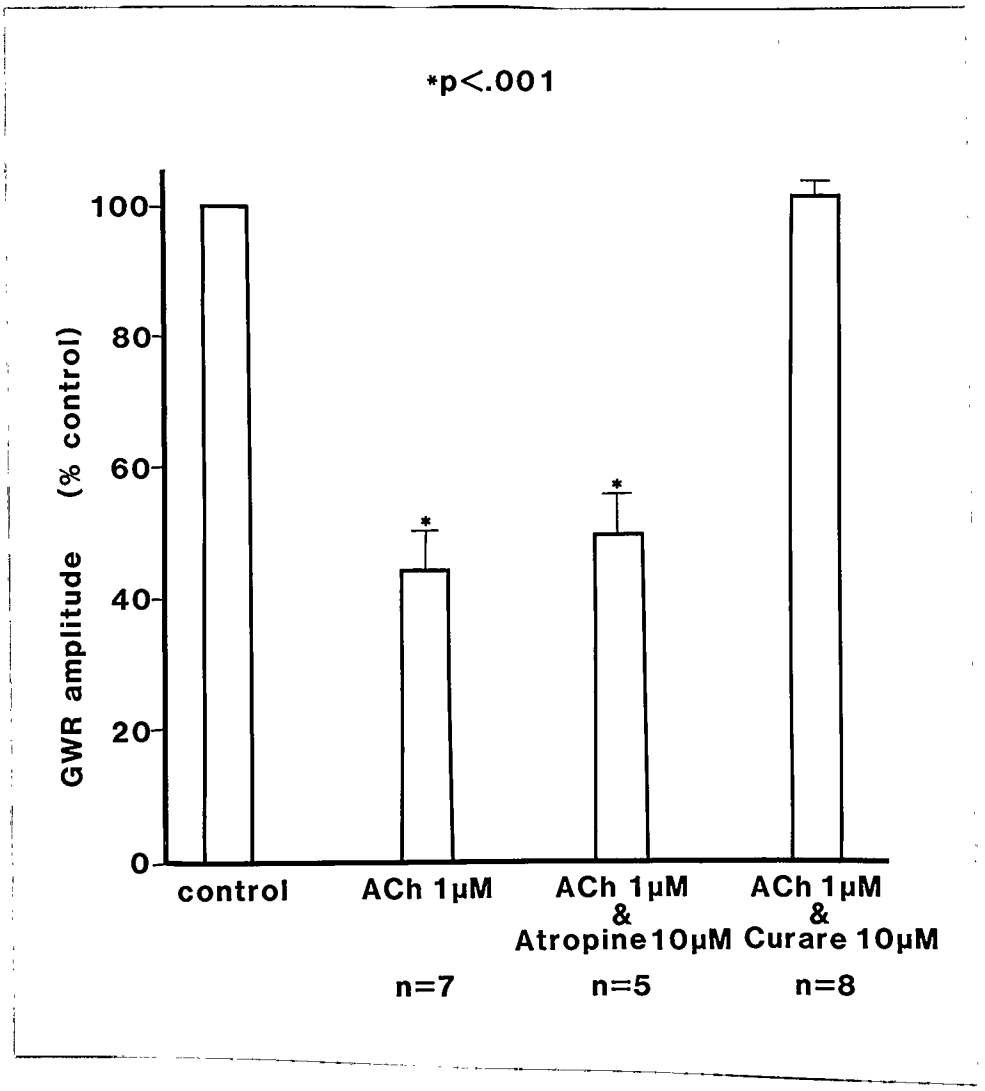


Figure 17.

Histogram showing that curare does block and atropine does not block the suppressive effects of ACh upon the gill evoked GWR. Twenty animals were used in this series of experiments. Perfusion of ACh (1 µM) results in suppression of the GWR with respect to control (100 per cent). When atropine (10 µM) is co-perfused with ACh a similar suppression was observed. The asterisk shows that the suppression produced by perfusion of ACh alone or with atropine is significantly different from control ($p<.001$). This indicates that atropine does not block the suppressive effects of ACh. When curare (10 µM) is co-perfused with ACh (1 µM) the suppressive effects of ACh are blocked completely. There was no significant difference between the effect of ACh when co-perfused with curare and control.

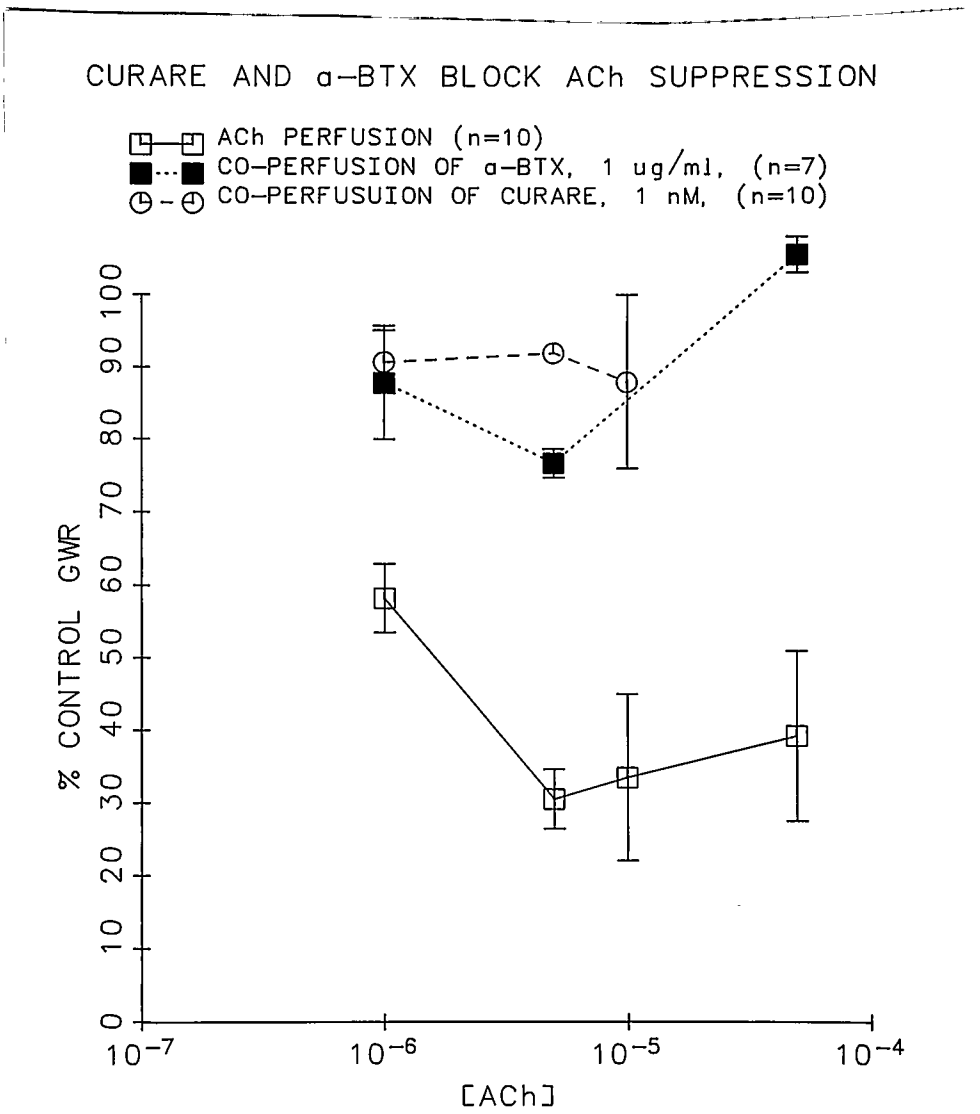


Figure 18.

Cummulative normalized data depicted in this graph show that curare (1 nM) and α-BTx (1 µg/ml) block the suppressive effects of ACh on the gill evoked GWR over a range of ACh concentrations. ACh perfusion of the gill results in a suppression of the GWR at every concentration tested (open squares). This suppression is significantly different from control (100 per cent), ($p<.001$). At higher ACh concentrations (>5 µM) there appeared to be a decrease in the suppression produced by ACh. This is assumed to be due to the activation of excitatory ACh receptors in the gill (Weiss et al. 1985). When α-BTx (filled squares) or curare (open circles) are co-perfused with ACh a blockade of the suppressive effects of ACh were observed. When 50 µM ACh was co-perfused with α-BTx facilitation of the GWR was observed. When curare and α-BTx were co-perfused with ACh the GWR amplitude was not significantly different from control.

Curare Blocks the Suppressive Effects of SCP_B

The above data show the suppressive effects of ACh perfusion upon the gill evoked GWR and indicate that a nicotinic-like ACh receptor mediates this suppression. Peptides often exert their effects by modulation of other neural pathways as is the case in the acessory radula muscle of Aplysia, where SCP_B postsynaptically enhances the action of ACh (Lloyd et al. 1984). Both SCP_B and ACh have suppressive effects upon the gill evoked GWR; because of this similarity, it was postulated that SCP_B may suppress the GWR by modulation of an inhibitory cholinergic pathway.

Thus experiments in which the nicotinic receptor blocker, curare was co-perfused with SCP_B were performed. This tested the hypothesis that SCP_B exerted its action by modulation of the inhibitory, cholinergic pathway characterized above and by Weiss et al. (1985). SCP_B has been shown in this study not to bring aboout its suppressive effects upon the GWR by inhibition of excitatory pathways which mediated sensitization (Figure 6). The data in Figure 19 are from a single experiment showing the suppressive effects of perfusing SCP_B (1 pM) through the gill . Following a 100 minute wash the suppressive effect of SCP_B was not observed (middle trace). Subsequent co-perfusion of curare (1 nM) with a higher concentration of SCP_B resulted in a complete blockade of SCP_B's suppressive effect (lower trace). The normalized data from nine experiments are presented in Figure 20. SCP_B perfused through the gill resulted in a suppressed GWR which was dose-dependent. Co-perfusion of curare (1 nM) blocked the suppressive effects of SCP_B at each concentration of SCP_B tested.

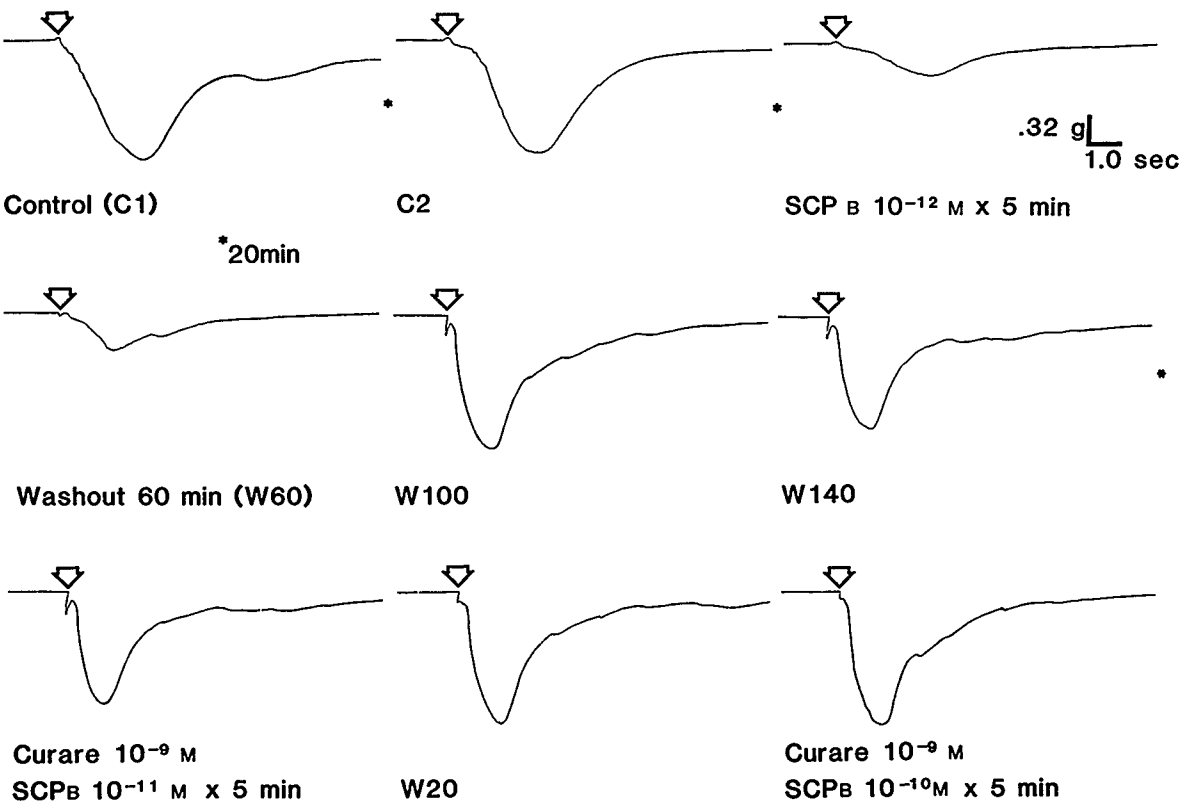


Figure 19.

Curare blocks the suppressive effects of SCP_B upon the gill evoked GWR. Following two control GWR's (upper left two traces), SCP_B (1 pM) was perfused through the gill which resulted in suppression of the GWR (upper right trace). This effect washed out after 100 minutes and the subsequent GWR amplitude was also within the range of control (middle right two traces). The co-perfusion of curare (1 nM) with higher concentrations of SCP_B (10 and 100 pM, respectively) blocked the suppressive effects of the peptide (lower left and right traces). Large arrow indicates stimulus.

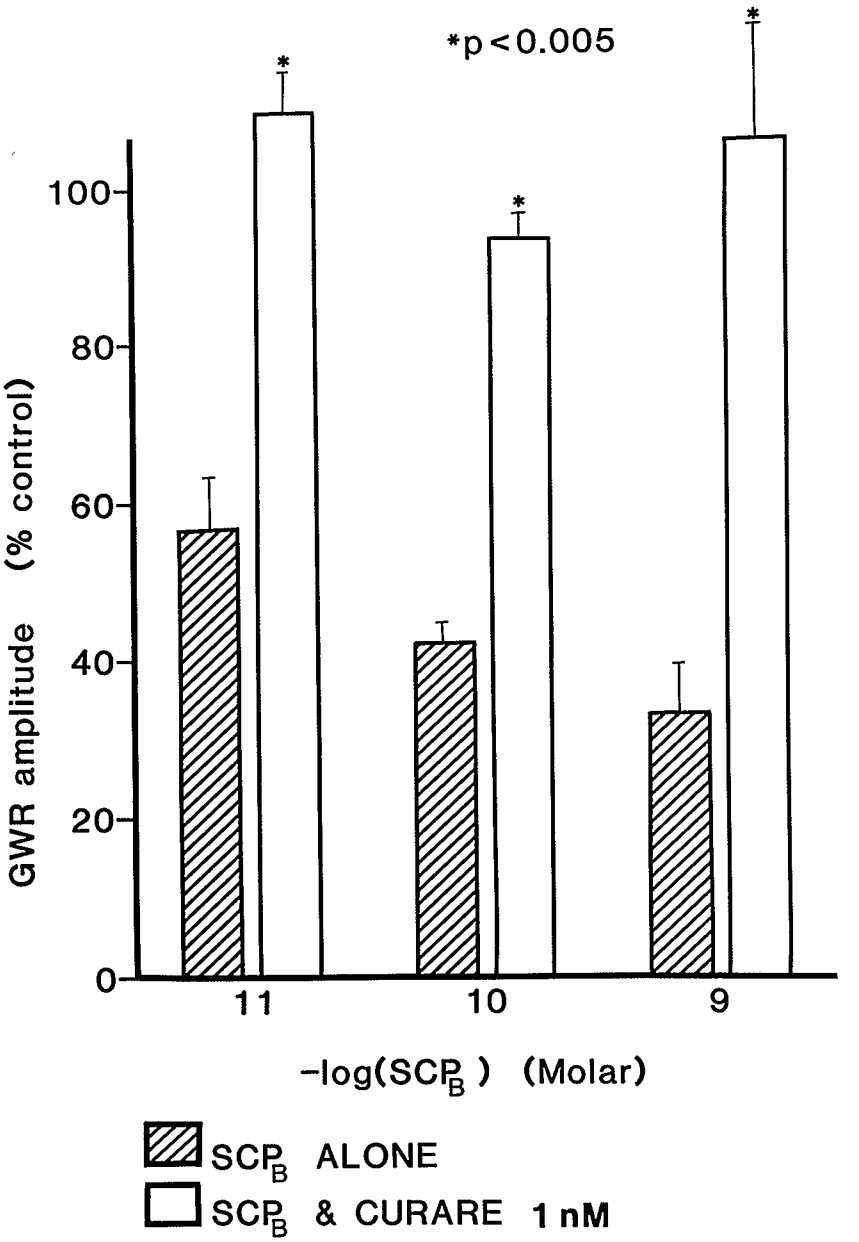


Figure 20.

Cummulative normalized data (n=9) showing that co-perfusion of curare with SCP_B blocks the suppressive effects of SCP_B. When SCP_B is perfused at increasing concentrations a greater suppression was observed. When curare (1 nM) was co-perfused with SCP_B, at each concentration of SCP_B tested a blockade of suppression of the GWR was observed. In some cases a facilitation of the GWR occurred. The asterisks show that the blockade produced by co-perfusion of curare was significantly different from the suppression produced by perfusion of SCP_B alone ($p<.005$). The effect of SCP_B when co-perfused with curare were not significantly different from control.

Alpha-Bungarotoxin Blocks the Suppressive Effects of SCP_B

To further characterize the nature of the nature of the inhibitory, cholinergic pathway that SCP_B was acting on, a second nicotinic antagonist was tested. The alpha snake venom, α-BTx has been shown to be a highly specific blocker of nicotinic receptors in vertebrates. Figure 21 shows the results of one experiment in which α-BTx was perfused alone (upper trace right of centre) and then co-perfused with SCP_B (10 nM) (upper trace far right). When perfused alone, α-BTx had no effect upon the GWR. When co-perfused with SCP_B, the suppressive effects of SCP_B were blocked. Following washout the control GWR's became somewhat larger (lower left two traces). When SCP_B was perfused alone this resulted in suppression of the GWR. The last record shows a partial recovery after 40 minutes. Consistent with the blockade of ACh by α-BTx is that the blockade of the suppressive effects of SCP_B is also reversible.

The normalized data from six experiments are presented in Figure 22 showing that α-BTx blocks the suppressive effect of SCP_B. These data indicate that both curare and α-BTx may block the suppressive action of SCP_B in the Aplysia gill.

The data presented above (figures 20 and 22) show that both curare and α-BTx block the suppressive effect of SCP_B. However it has not been shown that a nicotinic antagonist blocks the suppressive action of both SCP_B and ACh in the same preparation. Data in Figure 23 shows that SCP_B and ACh both produce suppression in the same preparation (upper and lower traces, respectively) and that the suppression is blocked by co-perfusion of α-BTx.

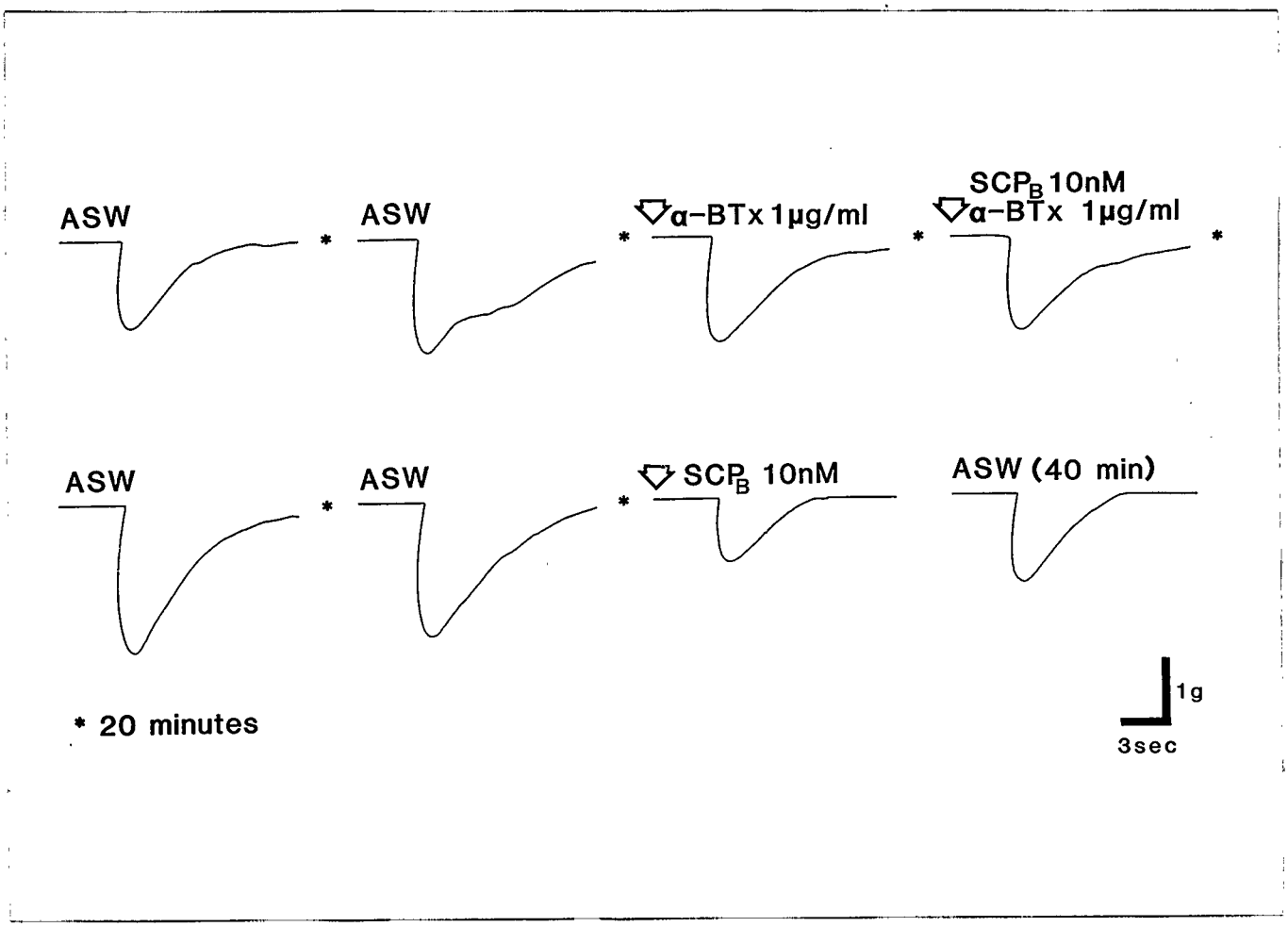


Figure 21.

α-BTx blocks supppressive effects of SCP$_B$ on the gill evoked GWR. Following two control GWR's (upper right two traces), α-BTx (1 µg/ml) was perfused through the gill (large arrow) for five minutes prior to gill stimulation. α-BTx had no effect on the GWR (upper right of centre trace). SCP$_B$ (10 nM) was then co-perfused with α-BTx for five mintues prior to the next gill stimulation (upper right, large arrow). This blocked the suppressive effect of SCP$_B$ (upper right trace). Two more control GWR's were then obtained (lower left two traces). Perfusion of SCP$_B$ (10 nM) for five minutes prior to the next GWR resulted in the suppression of the GWR (lower trace, right of centre). After forty minutes of washout the amplitude of the GWR had partially recovered (lower right trace).

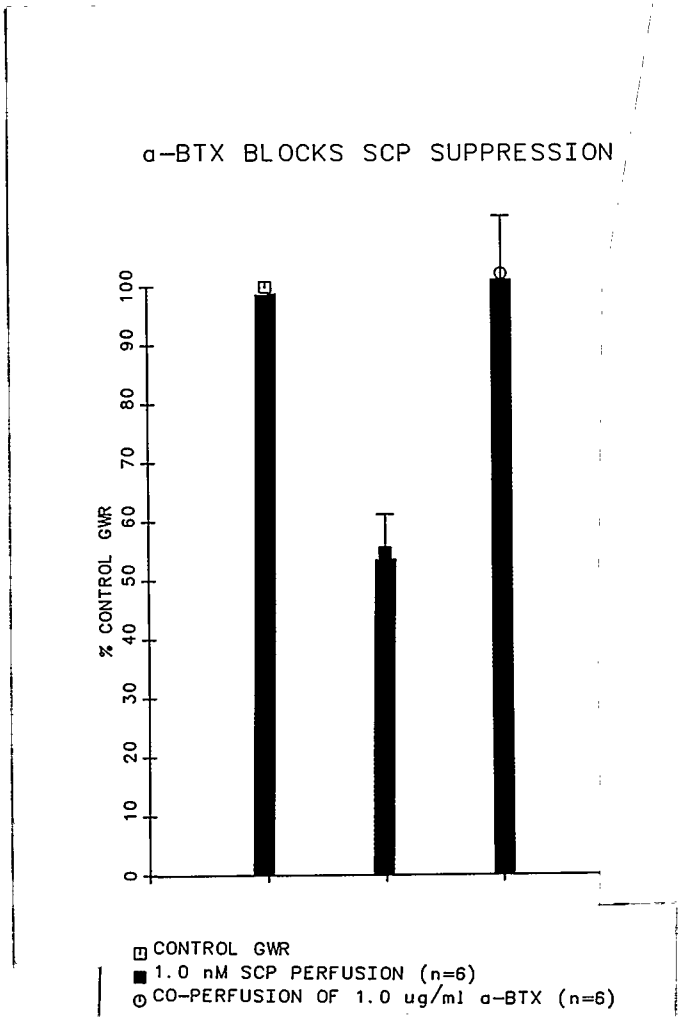


Figure 22.

Cummulative, normalized data (n=6) showing that co-perfusion of α-BTx with SCP_B blocks the suppressive effects of SCP_B on the gill evoked GWR. The perfusion of SCP_B resulted in a significant ($p < .005$) suppression of the GWR. Co-perfusion of α-BTx (1 µg/ml) resulted in a blockade of the suppressive effects of SCP_B. The GWR amplitude when SCP_B and α-BTx were co-perfused was not significantly different from control.

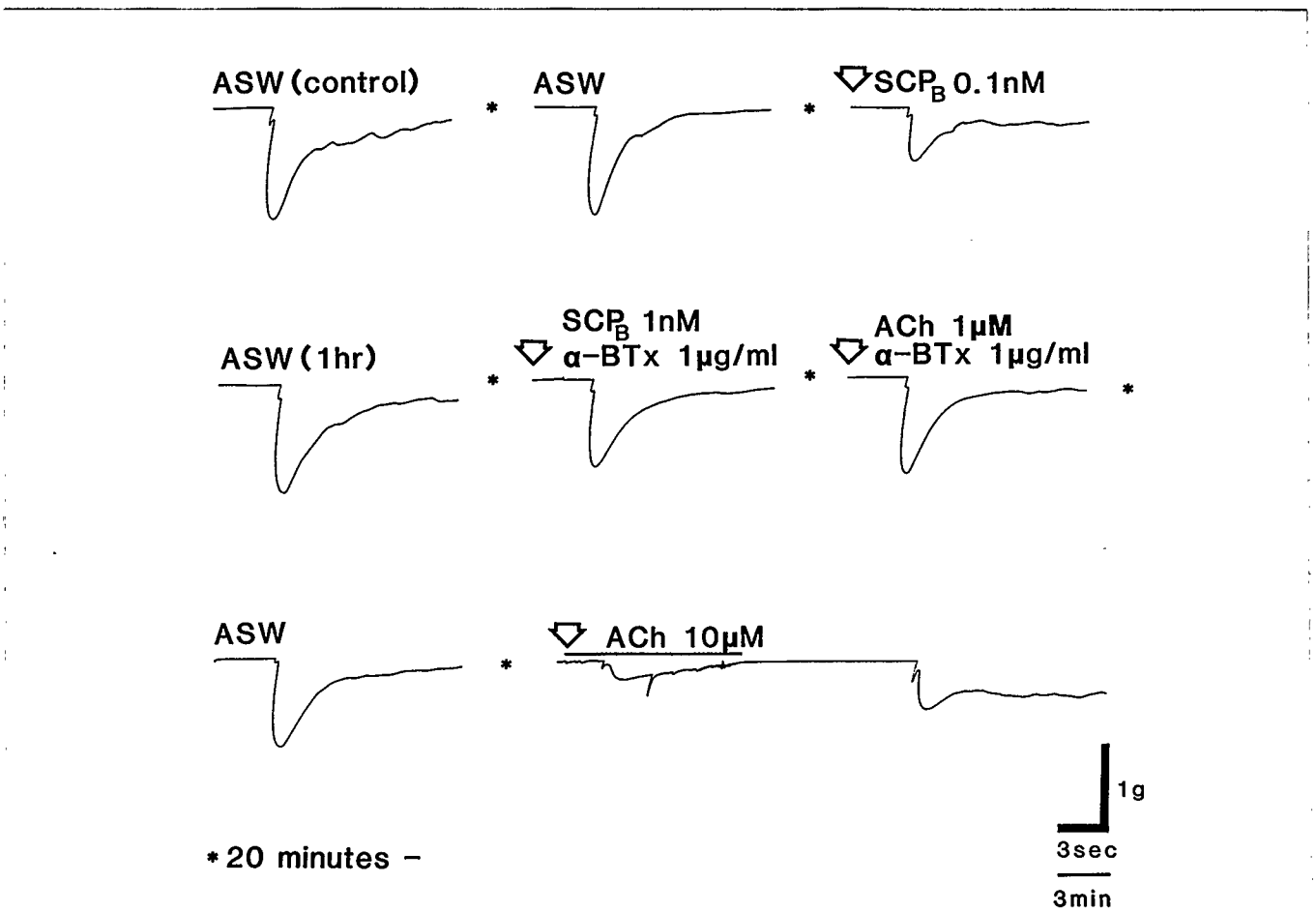


Figure 23.

SCP$_B$ and ACh suppressd the gill evoked GWR and the suppressive effects of both are blocked by α-BTx. Data from a single experiment are shown. Following two controls (upper left two traces), the perfusion of SCP$_B$ (0.1 nM) for five minutes (large arrow, upper right) results in a suppression of the GWR (upper right trace). This suppression washed out after an hour (middle left trace). SCP$_B$ (1 nM) and ACh (1 μM) were subsquently co-perfused with α-BTx (1 μg/ml) which resulted in a blockade of the suppressive effects of both these agents (middle right two traces). ACh (1 μM) was then perfused through the gill resulting in a tonic contraction of the gill which has been shown to be atropine sensitive (Weiss et al. 1985). Following this contraction the evoked GWR was found to be suppressed.

Immunohistochemistry: SCP_B appears to be Present in the Gill of Aplysia

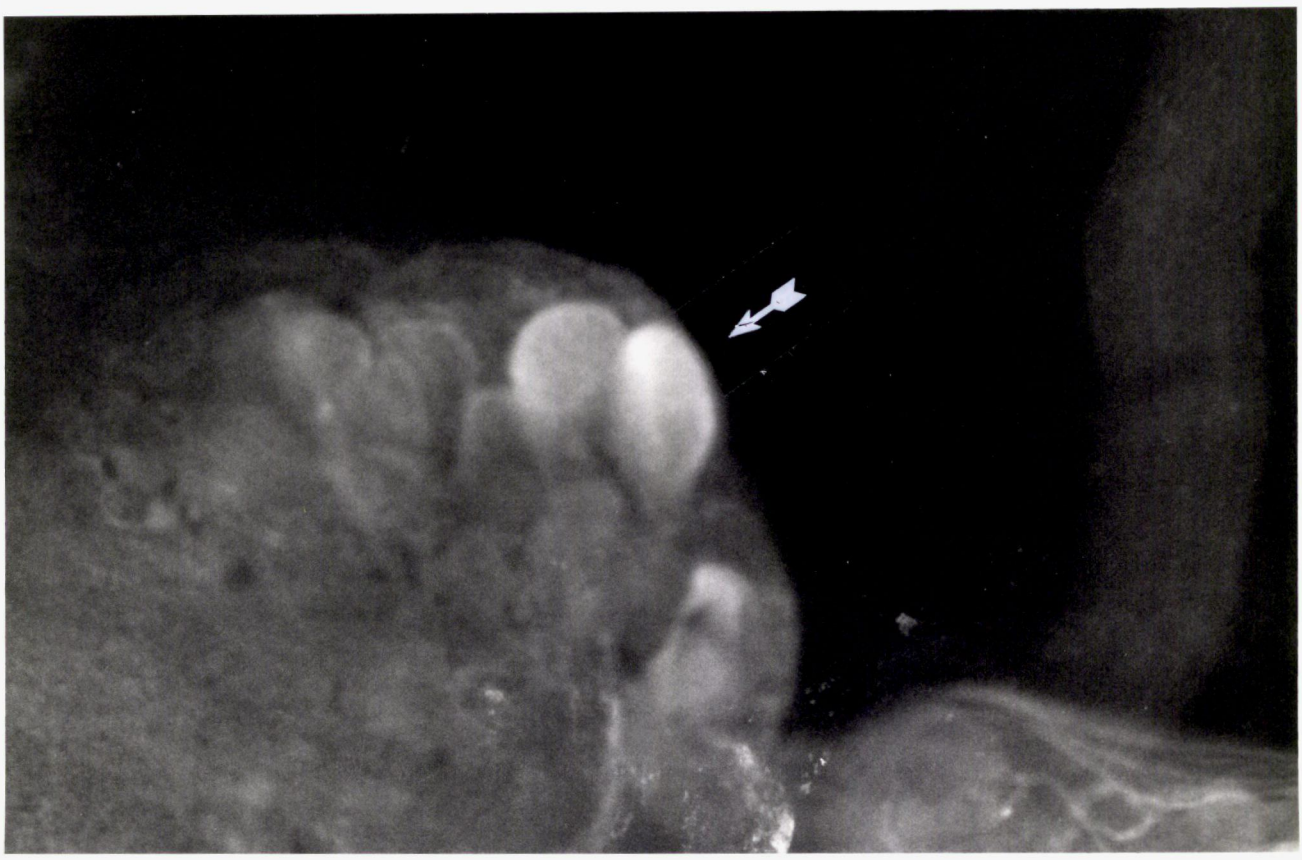
SCP_B-like immunofluoresence was found to be present in the gill plexus and in the nerves innervating the gill from the abdominal ganglion.

Neurons in the buccal ganglion SCP_B-like immunofluorescence (Figure 24). These cells are in the location of B1 and B2, shown by Lloyd et al. (1985b) to synthesized and release SCP_B. As such, these cells represents a positive control for the SCP_B-like fluorescence of the gill. The SCP_B negative control (where the only the second antibody has been used) showed no staining indicating that this antibody predominantly binds the primary antibody.

SCP_B-like immunofluorescence was present in the siphon and ctenidial nerves up to the points where these innervate the mantle shelf and in the surrounding mantle shelf (Figure 25). The immunofluorescence observed in the siphon nerve and the ctenidial nerve up to the respective points of innervation of the mantle shelf by these nerves were in agreement with the findings of Lloyd et al. (1985b). There was some staining in the branchial nerve which was less intense than than found in the siphon and ctenidial nerves. The reason for this may be due to the fact that the branchial nerve has a greater diameter which would result in less penetration by the antibodies. That the pattern of staining of neural tissue by anti-SCP_B was similar to that found by Lloyd et al. (1985b). For this reason the staining found in the peripheral plexus of the gill likely represents immunoreactivity which is specific to SCP_B.

SCP_B-like immunofluorescence was found to be present in the gill. Figure 26 and 27 show photos of pinnule muscles two magnifications, repectively. In each case muscle fibres are covered with a network of

brightly immunofluorescent fibres and varicosities which may be compared with controls where no immunofluorescence is observed. Some spherical somata are present and appear in certain circumstances to be closely associated with the fibrous processes (Fig 25). This type of staining pattern was similar to that found for FMRF-amide in the gill (Weiss et al. 1984; Lehman et al. 1984). It is suspected that this fluorescence is specific for SCP_B and reflects the distribution of modulatory neurons which localize SCP_B. This conclusion was reached primarily because the distribution of SCP_B-like fluorescence in the ganglia and connectives was similar to that reported by Lloyd et al. (1985b).



100 µm

Figure 24.

Two buccal neurons of similar size and location to B1 and B2 are shown to demonstrate SCP_B-like immunofluorescence. B1 and B2 are known to synthesize and release SCP_B (Lloyd et al. 1985b).

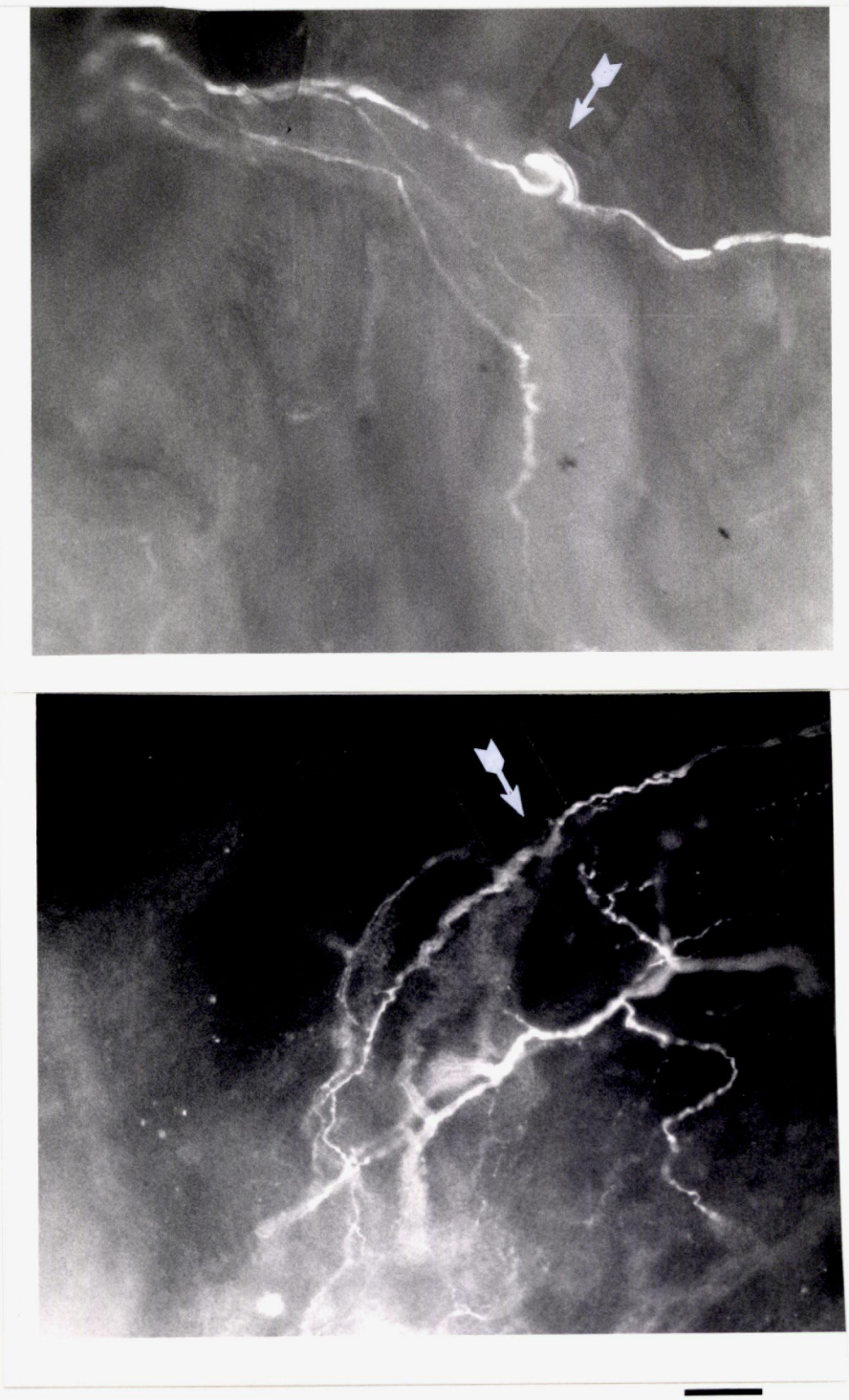


Figure 25.

SCP_B-like immunofluorescence is shown to be present in the siphon nerve at the point of innervation of the mantle shelf (upper photo). As well, axon like processes are apparent in the tissue of the mantle shelf (lower photo).

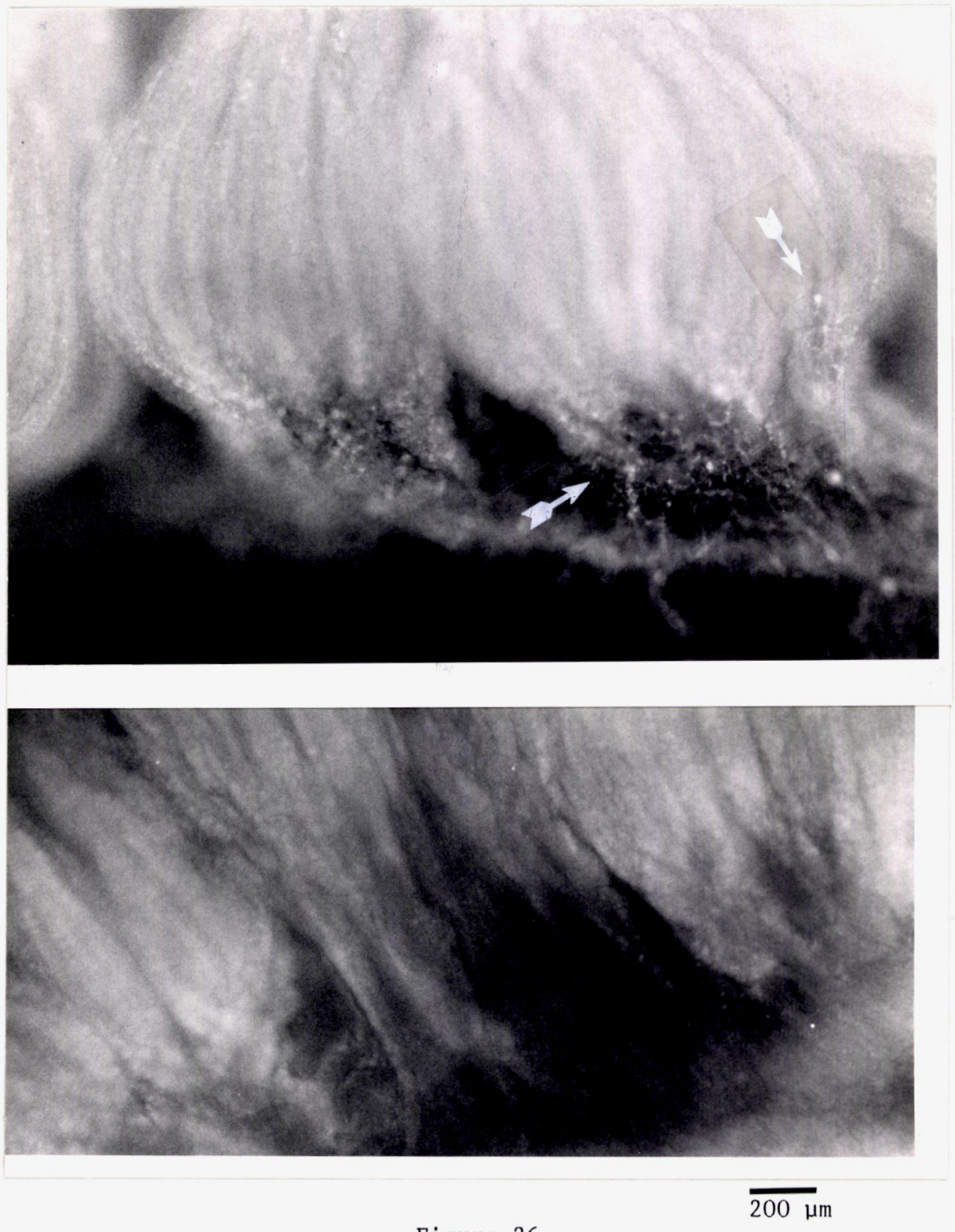


Figure 26.

SCP_B-like immunofluorescence (arrows) is shown in the above photos is closely associated with gill muscle fibres (upper photo). Control where primary antibody was deleted from incubation procedure (lower photo) showing that second antibody is specifically labelling the primary antibody.

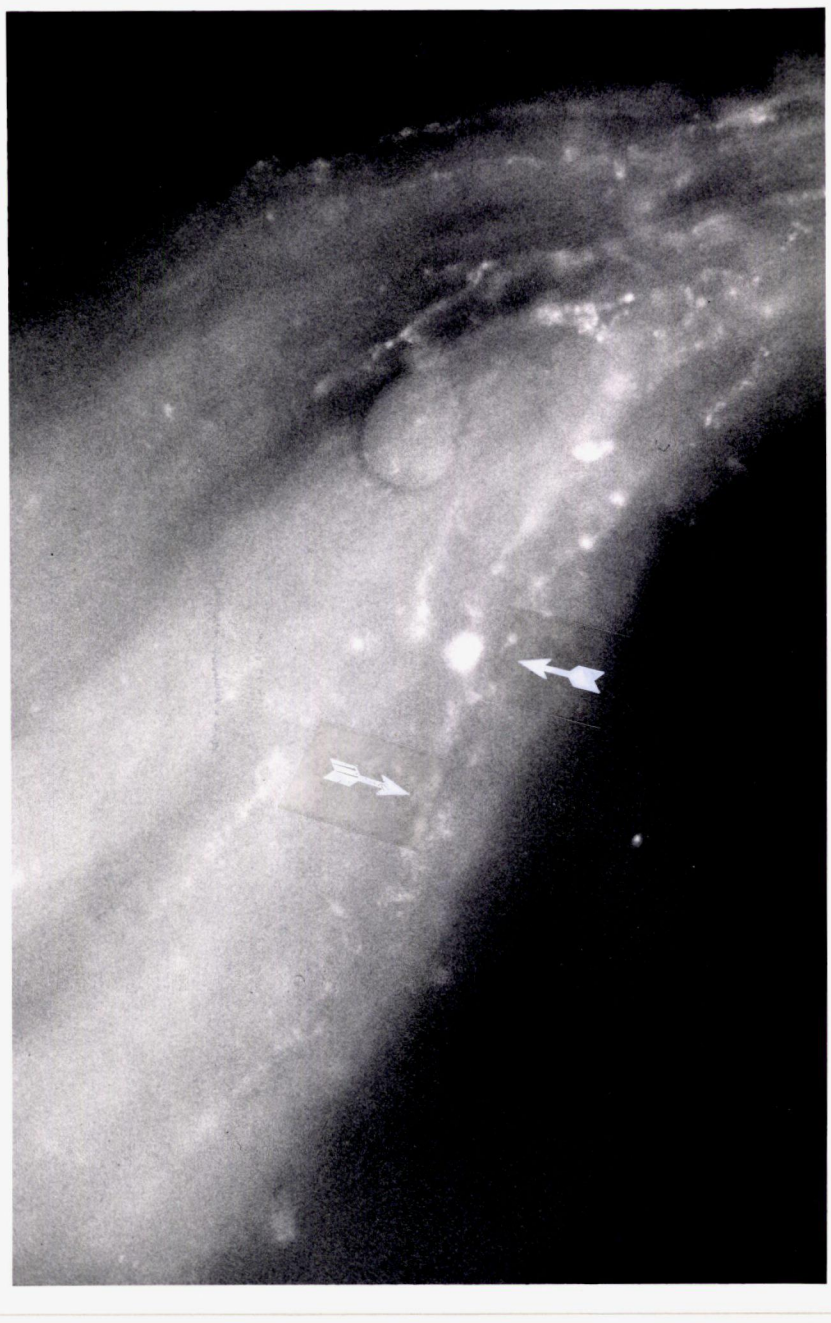


Figure 27.

SCP_B-like immunofluorescence is shown at higher magnification in the above photos in close apposition to muscle fibres. This immunofluorescence may be localized in neurons of the peripheral nerve plexus. Neuron-like processes associated with spherical somata-like bodies (arrows) localize SCP_B staining.

Discussion

The gill of Aplysia is a complex organ involved in respiration. As well as subserving this important homeostatic function, the gill exhibits defensive withdrawal behaviors which may undergo both associative and non-associative learning (Mpitsos and Lukowiak, 1985; Carew and Sahley 1986; Byrne, 1987). The ability of the GWR to exhibit adaptive behavioral changes and the relative simplicity of the neural architecture in the central pathways which participate in the mediation of the GWR makes Aplysia a useful model system in which to study the neural and biochemical basis of behavior.

Understanding the role neuropeptides play in the modulation of GWR behaviors in Aplysia is important because endogenous neuropeptides are known to have important effects on the integrated central and peripheral nervous systems which mediate the GWR (Lukowiak and Colmers, 1987; Cawthorpe et al. 1985; Cawthorpe et al. 1988; Higgins et al. 1988). However, little is known about the organization of the PNS although advances have been made in this regard (Kurokawa and Kawasawa, 1988). An understanding of how the PNS functions in the mediation of GWR behaviors is essential for us to gain an understanding of the neural mechanisms underlying GWR behaviors (Lukowiak and Peretz, 1977). An example of the complexity of the PNS is provided in the discussion below where the PNS is shown to mediate gill contractions which are similar to respiratory pumping (Peretz, 1969) which are thought to be dependent upon a central pattern generator (Byrne and Koester, 1977; Byrne et al. 1983).

The primary goal of this research was to study the effects of SCP_B upon the GWR evoked by tactile stimulation of the siphon or gill using the

isolated gill preparation. SCP_B was found to suppress the GWR evoked by tactile stimulation of the siphon or gill. Also SCP_B suppressed the GWR evoked by stimulation of the siphon. In addition, SCP_B was shown to have no effect on the rate of habituation nor did SCP_B effect dishabituation or sensitization. Finally, SCP_B-like immunofluoresence was found to be present in the gill of Aplysia. Taken together these data indicate that SCP_B may play an important role in the mediation of GWR behaviors in the intact animal.

The second part of this study involved the examination of the effects of ACh on the gill evoked GWR. ACh, like SCP_B brought about suppression of GWR behaviors. Furthermore, the pharmacology of ACh action in the gill has been characterized (Weiss et al. 1985). The suppressive actions of ACh were blocked by the nicotinic antagonists, curare and α-BTx. That both ACh and SCP_B exerted suppressive actions on the GWR led to the hypothesis that SCP_B brought about suppression of the GWR by modulation of an inhibitory cholinergic pathway. This hypothesis was tested by examining the ability of nicotinic antagonists to block the suppressive effects of SCP_B. The data from these experiments demonstrated that the suppressive action of SCP_B was blocked by nicotinic antagonists. This supported the hypothesis that SCP_B acts via modulation of an inhibtiory cholinergic pathway. The possible mechanisms through which SCP_B may act are discussed.

Spontaneous Periodic Contractions of the Isolated Gill are Similar to Those Associated with Centrally Generated Respiratory Pumping

In the present study, some preparations were observed to exhibit spontaneous periodic gill contractions (Figure 2 and 3). The origin of this activity is within the PNS, since the abdominal ganglion had been removed. The spontaneous gill contractions are similar to those observed during respiratory pumping (Peretz, 1969, Byrne and Koester, 1978) which is dependent upon a central pattern generator. This central pattern generator is known as the interneuron II network and involves relatively complex activity and coordination between identified interneurons of the abdominal ganglion (Byrne and Koester, 1978; Byrne, 1983). Neural components of the network in the PNS giving rise to the electrical activity recorded extracellularly from a single pinnule (gill potential; Jacklet and Rine, 1977) and the periodic gill contractions observed in some preparations from this study have not been identified.

The extracellular record of the gill potential (Figure 2) has been shown to reflect the junctional potential recorded intracellularly from muscle cells (Jacklet and Rine, 1977). The gill potential is a recording of the net neural and muscle activity in the pinnule. As such, the gill potential composition may include summated inhibitory and excitatory junctional potentials, in addition to neural activity. However, during the perfusion of an experimental agent through the gill, extracellular recordings of the gill potential are limited with respect to the data these provide. The gill potential does not allow a distinction to be made between increased inhibitory output (ie. increase in IJP's) and modulatory effects leading to decreased excitatory output to muscle (ie. decreased EJP's). Both a decrease

in the EJP or an increase in the IJP would lead to an apparent suppression of the GWR amplitude and the gill potential. Direct demonstration of inhibitory pathways in the PNS whether inhibition arises centrally or peripherally has depended upon recording IJP's intracellularly from gill muscle cells (Carew et al. 1974; Kurokawa and Kuwasawa, 1988).

SCP_B Suppresses the GWR evoked by tactile stimulation of the Gill or Siphon

In the isolated gill preparation the abdominal ganglion has been removed. Thus, the GWR is mediated by sensory and motor pathways within the PNS (Peretz, 1970; Peretz et al. 1976; Lukowiak and Peretz, 1977; Leonard et al. 1988;; Kurokawa and Kuwasawa, 1988). Both excitatory and inhibitory motor pathways have been shown to be present in the PNS (Kurokawa and Kuwasawa, 1988; Carew et al. 1974). In this study it has been shown that SCP_B when perfused through the gill brings about a reversible suppression of the GWR evoked by tactile stimulation of the gill (Figure 4) or siphon (Figure 7). Inititially, the effects of SCP_B perfusion were tested upon the non-habituated GWR. This meant stimulating the gill or siphon at an interstimulus interval (greater than twenty minutes) which did not result in habituation of the GWR and a constant GWR was obtained (Goldberg and Lukowiak, 1982). The perfusion of SCP_B at concentrations as low as 10 fM suppressed the gill evoked GWR (Figure 4). The only concentration tested on the siphon evoked GWR was 1 nM. The suppression of the siphon evoked GWR produced by this concentration was similar to the suppression of the gill evoked GWR by the same concentration of SCP_B. The effects of SCP_B perfusion on the siphon withdrawal reflex have not yet been examined.

SCP_B does not Suppress Sensitization or Dishabituation

One approach taken in this study to test whether SCP_B was acting on excitatory pathways involved in mediating the GWR was to examine the effects of SCP_B on sensitization and dishabituation. Sensitization is the enhancement of the GWR following the interposition of a novel or strong stimulus (Pinsker et al. 1973). The perfusion of SCP_B through the gill did not effect sensitization (Figure 6).

Since dishabituation is now considered to be a separate process from sensitization, potential effects of SCP_B upon dishabituation were also tested. SCP_B did not effect dishabituation (Figure 10). Following habituation of the siphon evoked GWR, the interposition of a strong stimulus resulted in the dishabituation of the siphon evoked GWR. There was no difference between the amplitude of the dishabituated GWR in the control group when compared to the GWR amplitude of the experimental group with SCP_B perfused through the gill (Figure 10). Pathways which mediate sensitization and dishabituation in the gill are excitatory pathways since activity in either results in an enhancement of the GWR.

Decreased activity in an excitatory pathway is one possible mode of SCP_B action. Such an action would be similar to the action of FMRF-amide at the identified sensory-motor synapse in the abdominal ganglion (Mackey et al. 1988). At this site it is thought that FMRF-amide depresses the release of neurotransmitter from sensory neurons through the mechanism of heterosynaptic depression (Piomelli et al. 1987; Mackey et al. 1988). Were SCP_B to act primarily to decrease the activity of an excitatory pathway or the amount of neurotransmitter released at an excitatory NMJ, then it could be expected that SCP_B would decrease the amplitude of the dishabituated GWR

when compared to controls. This could be due to decreased efficacy of an excitatory pathway as a result of the modulatory action of SCP_B. Here it was shown that this was not the case. Thus it seems improbable that SCP_B works via a decrease in the efficacy of an excitatory pathway. Peptides have been previously shown to both enhance and depress the efficacy of central motor neurons (Lukowiak and Colmers, 1987). While the putative action of SCP_B to depress activity in excitatory pathways or the amount of neurotransmitter released at an excitatory NMJ cannot be ruled out completely by these experiments, it seems more plausible to believe that SCP_B works via an inhibitory pathway. Direct examination of SCP_B's effects upon excitatory NMJ efficacy would require intracellular recording from muscle cells directly innervated by excitatory central motor neurons.

SCP_B does not Effect the Rate of Habituation of the Siphon Evoked GWR

The perfusion of SCP_B through the isolated gill suppressed the amplitude of the siphon evoked GWR (Figure 7 and 9 (trial 1)) but did not effect the rate of habituation (Figure 11). Habituation is the decrement in GWR amplitude produced by repeated tactile stimulation of the siphon (Pinsker et al. 1970). During habituation the amplitude of the GWR at each trial with SCP_B perfused through the gill was less than the amplitude of the GWR at each trial during the control habituation run (Figure 8 and 9). However, the rate of habituation with SCP_B perfused through the gill was not significantly different from control (Figure 11). Since the CNS was removed only the PNS could be involved. The fact that SCP_B does not effect the rate is another indication that SCP_B works via an inhibitory cholinergic pathway.

Presumably, were SCP_B to effect an excitatory pathway (ie. heterosynaptic depression) this should have an affect on the habituation rate (see below).

These results may be compared and contrasted with the effects of AVT superfusion of the abdominal ganglion on GWR amplitude and rate of habituation (Thornhill et al. 1981; Lukowiak and Colmers, 1987). AVT decreases the GWR evoked by tactile stimulation of the siphon and increases the rate of habituation (Thornhill et al. 1981). The central effects of AVT include narrowing the sensory neuron action potential, decreasing the EPSP measured in a gill motor neuron, and as well as decreasing the ability of the motor neuron to produce a gill withdrawal response (Lukowiak and Colmers, 1987) possibly by activation of a modulatory pathway in the CNS which affects the peripheral terminations of central gill motor neurons (Thornhill et al. 1981; Lukowiak and Colmers, 1987). The decrease in the amplitude of the GWR appears to be the only similarity of the peripheral actions of SCP_B and the central actions of AVT. AVT does not effect the GWR amplitude of the GWR evoked by tactile stimulation of the gill when perfused through the periphery at concentrations as high as 10 nM (Cawthorpe et al. unpublished data).

However, there were differences in the experimental techniques used in the SCP_B and AVT experiments. To begin with an interstimulus interval of thirty seconds was used in the AVT habituation experiments, whereas an interstimulus interval of one minute was used in the SCP_B habituation experiments. Decreasing the interstimulus interval increases the rate of habituation (Thompson and Spencer, 1966). Further experimentation using a shorter interstimulus interval may result in the observation of an effect of SCP_B upon the rate of habituation. As well, the abdominal ganglion was left intact for the AVT experiments while it was not present for the SCP_B

experiments. When the abdominal ganglion is intact there are differences in the rate and degree of habituation of the siphon evoked GWR (Peretz and Howieson, 1973; Peretz et al. 1976; Lukowiak and Peretz, 1977; Higgins et al. in preparation). In order to directly compare the effects of SCP_B perfused peripherally and AVT superfused centrally, more experimentation is necessary to closely approximate the approach used in the AVT experiments.

That SCP_B only effects the amplitude of the siphon evoked GWR and not the rate of habituation may reveal differences in the mechanism of action of SCP_B and the mechanism underlying habituation. The most likely mechanism underlying habituation is low frequency homosynaptic depression (Castellucci and Kandel, 1974) which has been studied in the abdominal ganglion at sites where changes in neuronal plasticity parallel behavioral changes (Gingrich and Byrne, 1984). This mechanism may also operate in the PNS (Jacklet and Rine, 1977). Homosynaptic depression would have to act within an excitatory pathway in the PNS to bring about habituation. Were SCP_B to act independently of homosynaptic depression and simply reset the amplitude from which habituation of the reflex commenced, for instance by increasing tonic activity in an inhibitory pathway, then a change in the rate of habituation would not be expected. Habituation would commence from and end at a lower GWR amplitude as is observed in this present study (Figure 9). Again, these data lend support to the hypothesis that SCP_B works <u>via</u> modulation of an inhibitory pathway and not <u>via</u> a decrease in the efficacy of an excitatory pathway. In this sense habituation would be superimposed upon the suppression produced by SCP_B.

SCP_B Brings About Suppression of the GWR Through an Inhibitory Cholinergic Pathway

The hypothesis that SCP_B brought about its effects on GWR behavior by modulation of an inhibitory pathway was further tested. SCP_B could bring about its effect by either increasing the activity of an inhibitory pathway or facilitating the amount of inhibitory neurotransmitter released onto gill muscle by a mechanism such as heterosynaptic facilitation. This possibility would be in keeping with the known mechanisms underlying the action of this peptide at other loci in Aplyisa (Abrams et al. 1984; Lukowiak and Colmers, 1987).

To bring about suppression of the GWR by acting on an inhibitory pathway means that SCP_B would have to modulate the release of neurotransmitter from an inhibitory motor neuron. Inhibitory motor neurons have been postulated to be present in the PNS of the gill (Kurakawa and Kuwasawa, 1988). Evidence for this comes from experiments in which stimulation of the branchial nerve with impulses of varying intensity and duration resulted in the production of IJP's recorded intracelluluarly from muscle cells of the gill efferent vein. The latency properties of the IJP's were not mono-synaptic being distinct from constant short latency EJP's (Kurokawa and Kuwasawa, 1988). This indicated that the neurons producing the IJP's that were activated by branchial nerve stimulation resided in the peripheral nerve plexus (Kurokawa and Kuwasawa, 1988). IJP's were previously recorded intracellularly from muscle cells of the efferent vein by Carew et al. (1974). Activation of such inhibitory motor neurons or facilitation of neurotransmitter release from these inhibitiory motor neurons by SCP_B could

explain the suppression of the GWR produced by the action of SCP_B in the gill.

Of the neurotransmitters known to be present in the gill of Aplysia (Peretz and Estes, 1974; Carew et al. 1974; Weiss, 1983), ACh resulted in suppression of GWR behavior when perfused through the gill at concentrations less than 10 μM. ACh had been shown to activate an inhibitory population of cholinergic receptors (Weiss, 1983; Weiss et al. 1985). It was possible then that SCP_B acted through this inhibitory cholinergic pathway to bring about suppression of the GWR. The suppressive effects of ACh and the nature of the receptor mediating these effects were revealed by perfusing the diffusable cAMP analogue, p-chloro-thio-cAMP though the isolated gill which produced contractions of the gill musculature (Weiss et al. 1985). These cAMP induced contractions were attenuated by the co-perfusion of ACh (1 μM) through the gill. Addition of curare (10 μM) a nicotinic antagonist, to the perfusate blocked the ACh attenuation of the cAMP induced gill contractions. Thus, the suppressive effects of ACh appeared to be mediated through activation of a population of nicotinic-like receptors present in the Aplysia gill (Weiss et al. 1985).

In the present study an examination of the suppressive effects of ACh upon the GWR evoked by tactile stimulation of the gill was undertaken. It was thought that this approach would directly reveal the suppressive effects produced by perfusion of ACh though the gill. Indeed, this was found to be the case. The perfusion of ACh through the gill resulted in a suppression of the GWR evoked by tactile stimulation of the gill (Figure 12). This suppression is brought about by the action of ACh upon nicotinic-like ACh receptors in the gill (Weiss et al 1985). It is unkown at present whether

ACh suppresses the GWR evoked by tactile stimulation of the siphon, however ACh is supected to suppress this withdrawal reflex as well.

The perfusion of ACh at contentrations greater than about 5 μM produced a single tonic contraction of the gill efferent vein. These excitatory effects of ACh had previously been observed (Carew et al. 1974; Peretz and Estes, 1974) and the nature of the receptor mediating these effects characterized (Weiss, 1983; Weiss etal. 1985). The excitatory effects of ACh are mediated via activation of a muscarinic-like population of cholinergic receptors since co-perfusion of muscarinic antagonists, such as atropine result in a blockade of the excitatory effects (Weiss et al. 1985). In this study, co-perfusion of atropine with ACh did not block the suppressive effects of ACh on the GWR evoked by tactile stimulation of the gill (Figure 14). Furthermore, even when doses of ACh great enough to produce contractions of the efferent vein were used the suppressive effects of ACh on the GWR were still observed (Figure 15).

The effects of ACh upon sensitization and dishabituation were not examined in the present study. These experiments are necessary in order to determine whether the effects of ACh upon these gill behaviors are similar to those of SCP_B. Problems which may arise in these experiments are that there are two ACh receptors mediating opposite effects upon the gill. The inhibitory ACh receptors have a higher affinity for ACh since these have a lower threshold concentration required for activation of suppressive effects on the GWR. Even so binding of ACh to lower affinity excitatory ACh receptors cannot be ruled out when low doses of ACh are perfused through the gill. This may make a comparison of the effects of ACh on habituation with those of SCP_B difficult.

The suppressive effects of ACh on the GWR were blocked by the nicotinic antagonists, curare and α-BTx (Figure 18). The mechanisms of action of both curare and α-BTx are similar (Katz and Meledi, 1973). Both are competitive antagonists of Ach at its binding site on the α-subunit, comprising two of five subunits making up the pentameric, non-specific ion channel activated by ACh at the NMJ of vertebrates (Noda et al. 1983). The blockade of the suppressive effects of ACh on the GWR produced by both these highly specific nicotinic antagonists suggests that there are similarities between the nicotinic-like receptor in the gill muscle of Aplysia and the nicotinic receptor of vertebrate NMJ. There must be differences as well. α-BTx is known to irreversibly bind to the vertebrate nicotinic ACh receptor. The blockade of α-BTx to the nicotinic-like ACh receptor in the Aplysia gill is reversible (Figure 23). Curare and α-BTx block the suppressive effects of ACh on the GWR at concentrations of 1 nM and 1 μg/ml, respectively.

The results from the present study show that the suppressive effects of ACh may be observed directly as a decrease in the amplitude of the GWR. The suppressive effects of ACh were similar to those of SCP_B. That two specific blockers of the suppressive effects of ACh were known provided a experimental framework in which to test the hypothesis that SCP_B exerted its suppressive action on the GWR through modulation of an inhibitory cholinergic pathway. To support this hypothesis it was necessary to show that the same concentrations of curare and α-BTx could block the suppression of the GWR produced by either SCP_B or ACh.

The results of experiments where the nicotinic antagonists, curare or α-BTx were co-perfused with SCP_B through the gill demonstrate that these agents can block the suppressive effects of SCP_B on the GWR evoked by tactile stimulation of the gill. The concentrations used to block the

suppressive effects of SCP_B are the same as those used to block the suppressive effects of ACh. It is highly unlikely that both α-BTx and curare act at a SCP_B receptor which is distinct from the nicotinic-like ACh receptor in the gill. That the same concentrations of curare and α-BTx block the suppressive effects of SCP_B and ACh indicates that SCP_B acts to modulate the release of ACh from inhibitory neurons in the PNS.

SCP_B could act to bring about suppression by increasing the amount of neurotransmitter released from an inhibitory motor neuron by the mechanism of heterosynaptic facilitation (Kandel and Tauc, 1965; Abrams et al. 1984). SCP_B could also act by modulating excitatory inputs converging on the inhibitory pathway (Lukowiak and Colmers, 1987) by the same mechanism. SCP_B could also be released from neurons which synapse directly upon the inhibitory motor neurons producing EPSPs in these neurons. Such an action would bring neurons of the inhibitory pathway closer to firing threshold.

The absence of an effect of SCP_B upon the rate of habituation while effecting GWR amplitude fits well with the finding that SCP_B modulates an inhibitory pathway in the gill. The effects of ACh upon the rate of habituation is currently unknown however this work is in preparation. The suppression produced by SCP_B was explained as being superimposed upon habituation. For SCP_B to activate an inhibitory cholinergic pathway in the gill, would increase the inhibitory input onto gill muscle. This would decrease the amplitude of the GWR. Habituation, acting through the mechanism of homosynaptic depression (Castellucci and Kandel, 1974; Gingrich and Byrne, 1984) would result in the decrease of neurotransmitter released from neurons at excitatory NMJ's. Were the same degree of habituation to occur, then the amplitude of the GWR would start and end at a lower level in the

presence of SCP_B, due to increased inhibitory input to gill muscle. This would not necessarily effect the rate of habituation.

SCP_B Could Act to Decrease the Efficacy of Excitatory Pathways

SCP_B could also act to decrease the activity in an excitatory pathway. The evidence presented in this study which shows that SCP_B effects neither sensitization nor dishabituation suggests that SCP_B does not act to modulate activity or neurotransmitter release from excitatory pathways in the gill. This is however dependent upon the assumption that the sensitizing or dishabituating stimuli do not simply over-ride and reverse the effects of SCP_B at these loci. The putative action of SCP_B on an excitatory pathway which originates centrally could be examined in the following manner. Identified gill motor neurons in the abdominal ganglion are known to innervate gill muscle directly as EJP's have been recorded intracellularly from gill muscle (Carew et al. 1974, Kurokawa and Kuwasawa, 1988). The central gill motor neurons LDG1 and LDG2 are cholinergic (Carew et al. 1974). Stimulating such gill motor neurons such as LDG1, LDG2 or L7, produces a gill withdrawal response (Lukowiak and Peretz, 1977) and an EJP in gill muscle cells (Carew et al. 1974). The effect of perfusing SCP_B through the gill on the gill withdrawal response and monosynaptic EJP amplitude produced by central motor neuron stimulation could be examined. A decrease in the ampltiude of the EJP in the absence of changes in the input resistance of the muscle could indicate that SCP_B was acting to modulate activity in an excitatory pathway or the release of neurotransmitter from excitatory motor gill motor neurons.

SCP_B Could Act Directly on Gill Muscle

Peptides, such as proctolin have been shown to increase excitation-contraction coupling without changing the conductance of the muscle fibre (Bishop et al. 1985). Similar mechanisms could underly the action of peptides in Aplysia. It was unlikely that SCP_B acted directly on muscle to bring about suppression of the GWR by effecting excitation-contraction coupling in this fashion. This is supported by the finding that the action of SCP_B was blocked by the nicotinic antagonists, curare and α-BTx, indicating that the action of SCP_B was not independent of the action of ACh at the inhibitory ACh receptors. As well, were SCP_B to act to decrease excitation-contraction coupling by a mechanism that was independent of the inhibitory nicotinic-like ACh receptor it would be expected that such an action of SCP_B would also attenuate the contractile response of gill muscle to excitatory inputs. The findings that SCP_B did not attenuate sensitization (Figure 6) or dishabituation (Figure 10) together with the finding that nicotinic antagonists may block the action of SCP_B strongly suggests that the primary action of SCP_B is pre-synaptic to the target muscle. Further experiments are necessary to completely rule out the putative effects of SCP_B on gill muscle.

One possible direct action of SCP_B on muscle that is resolvable with the findings in this study could feasibly take place at the level of the membrane. This could involve the expression or unmasking of nicotinic-like ACh receptors in muscle. This type of mechanism has been proposed to underlie the action of α-bag cell peptide on bag cells in Aplysia which results in the unmasking of potassium channels that terminate the bursting activity of these cells (Strong et al. 1987). SCP_B could also have a direct

effect upon the ACh receptor, resulting in alterations of the channel kinetics. Such mechanisms could account for the suppressive effects of ACh and SCP_B on the GWR and the blockade of this suppression produced by co-perfusion of nicotinic antagonists. As discussed above, such actions of SCP_B may not be revealed when the effects on sensitization or dishabituation are examined as sensitizing or dishabituating stimuli may simply over-ride the suppressive effects of SCP_B. To determine whether such a postsynaptic mechanism is operating to mediate the suppressive effects of SCP_B could be tested employing a culture system of dissociated gill muscle cells. Were this mechanism to operate then a change in membrane potential normally observed upon application of exogenous ACh could be expected when SCP_B was present. The cell attached configuration of the patch clamp technique could be used as well to test for the direct effects of SCP_B upon ACh receptor linked channel number or kinetics in cultured muscle cells. The addition of SCP_B to the extracellular solution inside the pipette could result an increase the frequency of events and/or the conductance of individual events under the patch when ACh was present. This could be interpreted as an increase in the apparent number of channels present in the patch or as an increase in the single channel conductance. In any case such changes measured under these circumstances would indicate the presence of a direct action of SCP_B on muscle.

Figure 28 summarizes the putative sites and possible modes of action of SCP_B in the Aplysia gill. SCP_B could bring about suppression of the GWR in a number of ways. SCP_B could decrease activity in an excitatory pathway or the amount of neurotransmitter released at an excitatory neuro-muscular junction (NMJ) by the mechanism of heterosynaptic depression (Piomeli et al. 1987). Another possiblity is that SCP_B could increase the activity of an inhibitory

pathway or increase the amount of neurotransmitter released at an inhibitory NMJ by the mechanism of heterosynaptic facilitation (Kandel and Tauc, 1965; Castellucci and Kandel, 1976). Finally, SCP_B could also act directly on muscle in a manner similar to the action of α-bagcell peptide on bag cells (Strong et al. 1987).

It must be remembered that many possible configurations of neural circuitry can be devised to explain the effects of SCP_B. This figure represents an explanation of SCP_B action in the gill using the simplest model. It is clear that the determination of the exact mechansims though which SCP_B exerts its action in the periphery requires further experimentation. It has been shown here that at least one mode of SCP_B action is via the activation of an inhibitory cholinergic pathway. This action may be mediated by the mechanism of presynaptic (heterosynaptic) facilitation resulting in an increase in the release of ACh from an inhibitory motor neuron. A presynaptic modulation of neurotransmitter release has been shown to operate between identified pre- and postsynaptic neurons of the buccal ganglion (Baux and Tauc (1987) where pharmacologically distinct autoreceptors were shown to mediate facilitation and depression of ACh release from the presynaptic neuron (Baux and Tauc, 1987). That such mechanisms can operate in Aplysia indicates that the putative presynaptic actions of SCP_B at the sites in Figure 26 is a reasonable assumption. As well the presence of pathways which can modulate the efficacy of gill motor neurons (Lukowiak and Colmers, 1987) also supports the presence of the putative neurons which activate the inhibitory motor pathway in this model.

SCP_B-like immunofluorescence has been shown to be present in the Aplysia gill where it is localized in cells which resemble neurons (Figures 26 and 27). This finding supports the model presented in Figure 28 since this model

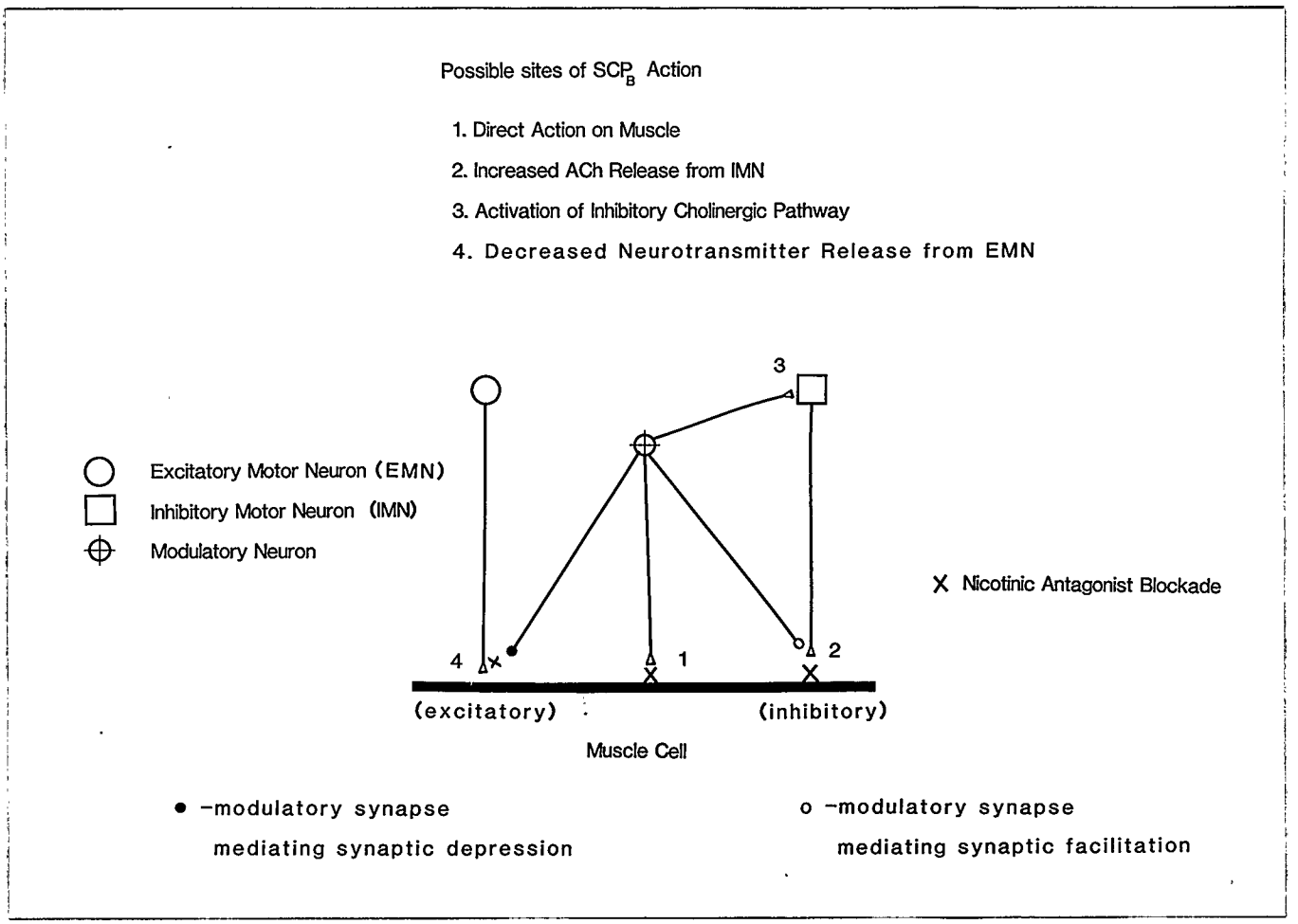


Figure 28.

Model of the possible sites of SCP$_B$ action (see text for details).

depends upon the presence of modulatory interneurons. This does not rule out the possibility that SCP_B acts as a neurohormone in the gill of Aplysia. SCP_B has not been shown to be present in the heart of Aplysia yet has profound effects upon cardiac activity (Cawthorpe et al. 1985; Lloyd et al. 1985a).

Role of SCP_B and the Suppressed Behavioral State

Suppressed GWR behaviors are associated with specific behavioral states have in some cases been related to the modulatory action of a peptide, such as the suppression produced by met-enkephalin which has been related to sexual activity (Leonard et al. 1984). This relationship was demonstrated because a pharmacological agent, naloxone could block the suppression produced by superfusion of met-enkephalin over the abdominal ganglion and the suppression of the GWR associated with sexual activity (Leonard et al. 1984, 1988). Another peptide, AVT, also acts centrally to produce suppression of GWR behavior, though has not yet been directly related to an identified behavioral state (Lukowiak and Colmers, 1987).

SCP_B may play a role in the mediation of the suppression of the GWR associated with satiation. This hypothesis comes from experiments which are currently in progress. In these experiments the perfusion of curare through the gill has been shown to increase the amplitude of the GWR evoked by tactile stimulation of the siphon in animals which are satiated. The approach taken in these experiments was similar to those in which centrally superfused naloxone was shown to reverse the suppression of the GWR associated with sexual activity (Leonard et al. 1984). Furthermore, it could

be expected that SCP_B would produce less suppression in animals which are satiated. More experiments are necessary to define a relationship between SCP_B and the suppression associated with satiation.

It is probable that the action of several peptides and neural transmitters integrate to bring about suppression associated with behavioral state. Suppression of the GWR by the integrated action of several peptides at central and peripheral loci could be brought about by the convergent activation of a final common pathway.

Final Common Pathway Hypothesis

There is convergence of neuronal inputs to the gill which can act to modulate the GWR. This finding has come from studies where activity in the PNS has been shown to modulate gill motor neuron activity. For instance, repeated activation of L_7 produces a decrementing GWR (Lukowiak and Peretz, 1977). Interposed tactile stimulation of the siphon or gill will reverse and facilitate the contraction produced by L_7 stimulation. In these experiments the siphon nerve had been severed leaving the PNS as the only pathway for this facilitatory modulation to be communicated between the siphon and gill (Lukowiak and Peretz, 1977). As well, activity in central motor neurons is known to modulate activity in the PNS. Activity in L_7 or LDG_1 will dishabituate the GWR where habituation has been brought about by repeated tactile stimulation of the siphon or gill (Lukowiak 1977b; Lukowiak and Peretz, 1977; Peretz and Lukowiak, 1975). The central motor neuron L_9 is known to modulate the terminations of L_7 where tonic activity in L_9 enhances the contraction produced by L_7 stimulation (Lukowiak 1977 a,b; Ruben 1981).

These findings demonstated convergence between central motor neurons in the periphery. Thus, convergence occurs between components of the CNS in the periphery, between the CNS and PNS, and finally convergence occurs between the PNS and CNS. Activity in this network integrates to modulate GWR behaviors.

How neural networks converge and interact to modulate behavior or bring about a change in behavioral state remains an ongoing problem facing neuroscience research. In this study Aplysia californica has been used as a model system in which to examine the effects of the peptide, SCP_B on the GWR. It has been found that SCP_B exerts a suppressive influence on the evoked GWR when perfused through the gill. The suppressive actions of SCP_B are possibly brought about via an inhibitory cholinergic mechanism.

It may be that the inhibitory cholinergic pathway acted upon by SCP_B is a final common pathway acted upon by other suppressive peptides. Curare blocks the suppressive influences of both ACh and SCP_B and it is suspected that SCP_B acts to modulate the release of ACh. The blocking action of curare provides a framework in which to test the final common pathway hypothesis. Were other peptides to exert their suppressive influences via this inhibitory cholinergic pathway, then it could be expected that curare would also block the effects of these peptides. For instance AVT could be superfused over the abdominal ganglion where it is known to activate suppressive control neurons which in turn modulate motor neuron efficacy at a peripheral site (Lukowiak and Colmers, 1987). Perfusion of curare through the gill periphery should block this effect were AVT to act via a final common pathway which mediates suppression GWR behaviors.

Summary

In this study the effects of the endogenous peptide, SCP_B on the gill and siphon evoked GWR reflex of the isolated Aplysia gill were examined. SCP_B suppressed the GWR amplitude and was found to have no effect upon the rate of habituation of the siphon evoked GWR. In addition, SCP_B had no effect upon dishabituation or sensitization. Like SCP_B, ACh was found to suppress the gill evoked GWR amplitude. Nicotinic antagonists, curare and α-BTx, blocked the suppressive effects of both ACh and SCP_B. These data suggest that SCP_B brings about its suppressive effects by modulation of an inhibitory cholinergic pathway. The putative sites of SCP_B action where suppressive effects may be mediated are discussed and a model is presented.

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