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STUDIES IN THE NEUROPHYSIOLOGY OF TWO IDENTIFIED POPULATIONS OF CELLS AND THEIR COMMON SYNAPSE IN *APLYSIA CALIFORNICA*

A Thesis Submitted To The Yale University School Of Medicine In Partial Fulfillment Of The Requirements For The Degree Of Doctor Of Medicine

WILLIAM DAVIS GAILLARD

Anno Domini MCMLXXXV

ABSTRACT

STUDIES IN THE NEUROPHYSIOLOGY

OF TWO IDENTIFIED POPULATIONS OF CELLS

AND THEIR COMMON SYNAPSE IN

APLYSIA CALIFORNICA

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I. Receptors to putative transmitters on A and B cells of Aplysia calicornica were identified and characterized. Each cluster of neurons exhibited receptors to acetylcholine, dopamine, GABA, glutamate, histamine, and serotonin, but not to octopamine or phenylethanolamine. Glutamate, histamine, and serotonin produced depolarizing responses on B cells. The proportion of ionophores associated with each transmitter receptor was characteristic for each cell cluster examined. The A cell receptor profile appeared to be more uniform than that of the B cells.

II. Studies of the A to B synapse did not demonstrate any effect by the sodium and chloride channel blockers curare and strychnine or by the histamine antagonists burimamide and pyrilamine. Bufotenine, a serotonergic antagonist, at high concentrations affected the psp by secondary effects on the membrane. Desipramine, the serotonin re-uptake inhibitor, did not affect the psp, nor did serotonin, glutamate, or aspartate desensitize the synaptic receptor. The study concludes that neither acetylcholine, serotonin, histamine, or glutamate are the transmitters at the A to B synapse.

III. Glycine-induced biphasic hyperpolarizations and a slow depolarization on Aplysia neurons were identified and characterized. The hyperpolarizing responses were mediated by increased permeability to chloride and potassium ions, respectively, which were reversibly abolished by strychnine. These responses, however, could not be elicited during the winter months. The ubiquitous slow depolarization was not associated with a change in membrane conductance. The response was sodium dependent, abolished my metabolic inhibitors, and unaffected by the glycine antagonist strychnine. The depolarization failed to desensitize, but was mimicked by application of the neutral amino acids histidine and glutamine. The glycine-induced depolarization shares many characteristics with sodium coupled cotransport; it does not appear to be mediated by a classical neurotransmitter receptor.

To Goggie,

MARY STAMPS BATESON GAILLARD

My Father's sister who instilled in me from birth
the noble endeavour that is medicine,
her first love.

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This work could not have been made possible without the patience of the kind people who taught me how to use the black boxes of the neuroelectrophysiogist, Drs. Andrew Williamson and N. Traverse Slater. At Yale, Dr. Gordon Shepherd's door was always open for discussion and comment. I owe my greatest thanks to Dr. David Orlo Carpenter -- mentor and friend -- for ideas, thoughts, direction, and much more.

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The faults of this work I claim for my own.

-WDG
New Haven
20 February 1985

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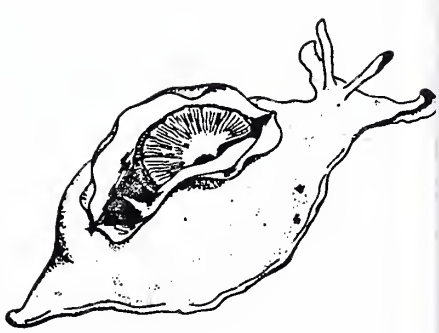


INTRODUCTION

Aplysia californica, a marine mollusc indigenous to the American west coast has played a crucial role for neurophysiologists interested in elucidating the mechanisms underlying neural activity. Aplysia is blessed with a relatively "simple" nervous system; not only are individual neurons identifiable, in a reproducible fashion from animal to animal, but they are accessible to experimental recording (34,55,64 84,96,200). Since the neurons are large, many electrodes can be inserted into any given cell facilitating intracellular recording and making more detailed pharmacological and physiological receptor studies possible. Synaptic pathways can be traced with similar ease. Thus, an understanding of "simple" behaviors of Aplysia has grown as its elementary circuits have been mapped (95).

During the previous twenty years the tools available for studying neurotransmitter pharmacology and synaptic physiology have grown. Single cell biochemical analysis of endogenous levels of transmitter candidates can be carried out, synthetic enzymes can be detected, uptake of precursors can be measured, while the metabolic processes of synthesis and release can be monitered (13). Perfusion or ionophoresis of neurotransmitters, and their agonists and antagonists, has expanded the practicability of receptor pharmacology. Microelectrode techniques have been invaluable for measuring changes in membrane potential, membrane resistance and conductance, and ionic basis of response (207).

The limited spectrum of behavior available to sea slugs has attracted the attention of neurobiologists interested in studying basic elements of behavior. An Aplysia can feed, be it on food to continue self existence, or on another Aplysia to perpetuate the species. Alternatively, an Aplysia can flee to preserve the self while making possible future attempts at procreation (76). With currrent methods of electrophysiological research it is possible to analyze the neural mechanisms and synaptic organization underlying these behaviors. Knowledge has been collected, ranging from reflexes -- such as tentacle, tail, gill and siphon withdrawal (57,166;196,197;18,115), mucous release (164), and inking (22) -- up to the more sophisticated activities of eating (92,93,205) and locomotion (61,76).

The cerebral ganglion neurons of Aplysia are intimately associated with the reception of sensory information, principally from the head and tentacles, and are implicated in the initiation, maintenance, and modulation of the basic behaviors of feeding, locomotion, and defensive withdrawal. The present study focuses on these neurons. It examines how neurotransmitters interact with their receptors on two populations of neurons, the A and B cells of the cerebral ganglion, and attempts to identify the neurotransmitter at their common synapse. Both clusters of cells play a major role in the reception and integration of information essential to Aplysia decision making. By elucidating the physiology of this synapse greater light will be shed on the neural mechanisms that allow for the plasticity, flexibility, and integrative capacity that is crucial to behavior.

To preserve perspective on the ultimate goal of this study, I will begin with a review of the neural organization underlying behaviors associated with the cerebral ganglia. The principal level of this investigation is the neurotransmitter receptor; thus current knowledge of transmitter and receptor identity in Aplysia will be briefly presented as well. A more detailed discussion regarding A and B cluster neurons and the A to B synapse, the subject of this study, is provided to complete the review of the background material.

The experimental results are organized in three sections: first, a survey of receptors found on A and B cells; second, results from studies designed to identify the transmitter at the A to B synapse; and third, characterization of previously undescribed glycine-induced alterations of membrane potential which were encountered during the preliminary receptor survey.

NEURAL BACKGROUND OF BEHAVIOR IN APLYSIA

Organisms exist in and interact with their environment which, in turn, influences being in some manner. They react to the continuous stream of stimuli, for to survive all creatures must constantly strive to affect and alter the environment in which they live. react to the constant stimulus of the environment against which they constantly strive to influence and alter. Aplysia, for example, respond to their surroundings in a number of ways -- generally as variations on common themes. The uniqueness of response, however, is determined by individual experience; this is behavior. Positive action ranges from the simple reflex, generally defensive in nature, to the far more complicated goal directed behaviors, such as feeding and sex, that involve varied levels of arousal, motivation, and satiation (166).

An Aplysia must base its decisions to act on information gleaned from the environment; the interface between animal and world is tenuous, resting on limited means of sensory input. There are sensors for detecting chemical, proprioceptive, coarse visual, tactile, and noxious stimuli (34, 85). Behaviors are centrally mediated; they all have their basis in hard neural circuitry that must, however, be organized with sufficient plasticity to meet an incessant stream of new demands (20, 57, 61, 76, 85, 86, 92, 93).

The importance of chemical stimuli will illustrate the point. The anterior tentacular grooves of Aplysia contain a dense collection of chemoreceptors. These sensors relay information to clusters of neurons in the cerebral ganglion where it is processed to initiate the

appropriate behavior. If proline is presented to the tentacles of an Aplysia it will initiate sex seeking behavior. On the other hand, alanine, cystine, or leucine, dissolved in seawater, will cause the animal to withdrawal. Glutamate (10^{-6} to 10^{-7} M), or aspartate (10^{-5} to 10^{-6} M), will arouse food seeking behavior (81), whereupon the animal will begin to search, will move toward the direction of the stimulus, and then will begin to masticate (81, 93, 92). If the threshold to commence a series of eating related motor programs is lowered, arousal is hightened. Activated metacerebral giant cells alter the excitability of buccal motoneurons and buccal muscle; B cell receptivity to mechanosensory input is heightened (57, 205). Motoneurons in the cerebral and buccal ganglia begin to fire synchronously and in repetitive fashion until the food is consumed (81, 92, 93).

Locomotion is a far more complicated behavior (or a pattern central to many behaviors) which has been studied in great detail. The foot, and locomotion, are utilized for a number of goal directed behaviors. To find food, the animal must search, move toward, and reach out to the object of the mouth's desire. Eating requires action of the foot, too (76, 85, 89). It plays an essential role in sexual orientation and in other endeavors that need only be imagined (76). Suction, the means by which Aplysia attach themselves to various objects, also requires the foot as does withdrawal and flight (76, 85, 86, 89).

Goal oriented movement can be initiated by any of the stimuli listed earlier (76, 90). Nociceptive stimuli, salt or an electric shock, applied to the tail will evoke creeping, if not a gallop (76, 90, 191,

198). On the other hand, a nasty stimulus to the head, or satiation, will inhibit locomotion (76, 191).

Locomotion is stereotyped; it consists of a repetitive and rhythmic series of movements that are invariable regardless of the original stimulus or the final objective (86, 76). Initially the head detaches and extends forward to reattach to the surface. A wave of extension and retraction then moves posteriorly bringing caudal portions forward (76, 87, 89). Associated movements include pulling of the head, parapodial contractions and gill withdrawal (85, 86). This series of muscular contractions can propel an animal at the remarkable maximum speed of 30 cm per minute (76).

The neural organization necessary to achieve the coordination of events is great. Decisions must be made to initiate the pattern, and to set the velocity of movement (61, 89, 90). Once the pattern is generated it must be maintained and concomitantly modified to fit current positional requirements (60, 61, 90). All decisions and commands, which are generated centrally (58, 61, 87, 76), do not depend on peripheral feedback other than for proprioceptive input necessary for making minor adjustments (60, 90). The principal motor innervation arises from the pedal ganglion, although the A and B cells provide some innervation of the foot and parapodia (76, 85, 86, 87).

Three paired pedal nerves carry mixed sensory and motor fibers between the foot and the pedal ganglion. Motor fields are ipsilateral, never cross the midline, but do overlap at their longitudinal borders. The motoneurons either excite or inhibit foot muscles, thus changing the

tone of muscle when fired (76, 87). Motoneurons do not form monosynaptic connections with each other, instead they often share common synaptic input (60, 76). The presynaptic neurons are organized to achieve coordinated common firing and inhibition of the respective agonistic and antagonistic muscle groups (90, also 92, 93).

The pedal wave, generated centrally, passes caudally without fail. Indeed, the wave will propagate beyond a denervated region without losing its proper timing (76, 85). Muscular contractions are bilaterally coordinated by neurons presynaptic to the motoneurns via the pedal commissure (76, 85, 89, 91). It is clear that the pattern requires time locked coordination of phasic movement; reflexes contribute little to the sequence (60, 76, 85).

Only some of the higher level organization necessary for controlling locomotion is known. Ablation studies provide crude insight into the key features of the system (87, 89, 90). Without the cerebral ganglion an Aplysia can not initiate locomotion. Without the pleural connectives, the rate of the pedal wave can not be regulated, nor can escape behavior be initiated. The isolated pedal ganglia, the apparent site of the pedal wave generating system, are able to engender limited and weak pedal waves. The control of movement has been made more clear by the discovery of apparent "command neurons" in the cerebral ganglion by Jahan-Pawar and Fredman (6).

Four neurons, located in the C clusters, are able to initiate generation of the pedal wave. Any one of the neurons when fired will do so, while simultaneously inhibiting its fellow command neurons. Not

only does firing one of these neurons evoke locomotion, but they are active throughout the duration of the behavior. It is not clear, however, if locomotion is not possible without these neurons. Finally, the rate of bursting corresponds to the rate of the pedal wave. An additional set of neurons were found that proved less potent but which have similar effect (6).

In the grand scheme it appears that the cerebral ganlion is essential for the initiation and maintenance of locomotion -- after all that is where the initial sensory information arrives. The numerous connections between the cerebral, pleural, and pedal ganglia in association with analysis of proprioceptive feedback, provide the network responsible for modulating the behavior (6).

NEUROTRANSMITTERS IN APLYSIA CALIFORNICA

The neurons of Aplysia are arranged in multilayered fashion within a ganglionic sheath where clusters of cells are separated by walls of connective tissue. The neurons are large, often one hundred to one hundred fifty micrometers in diameter. Their axons reach into the core of the ganglion whereupon they branch to form axo-axonal synapses hence creating the neuropile. Synaptic terminals, lying deeply embedded in glial enfoldings, are filled by many types of vesicles (64, 96). With the exception of GABA and acetylcholine, neurotransmitter receptors are located on narrow and discreet regions of the axon and axon hillock (8, 29, 118). The following section reviews identified receptors on Aplysia neurons and at known synapses. The discussion begins with the definition of a transmitter, and, more importantly, how a substance may be identified as the messenger at a synapse.

Werman addressed the problem of definition in great detail (207). He reviewed the criteria that need to be met before a substance may be considered to be a neurotransmitter. First, identity. The application of the candidate to the postsynaptic membrane must -- classically -- induce alterations in voltage and conductance that mimic the response evoked by presynaptic stimulation. The pharmacological sensitivity of the induced and evoked responses must match. There are other criteria, too. The candidate must be available at the presynaptic terminal -- this is generally understood to be presence in the neural soma, or in reserve in the neuron, but in fact it may be synthesized only in the terminal on demand. Clearly, the neuron must be able to manufacture the transmitter; this requires the ability to take up precursors and the availability of synthetic enzymes. A mechanism for release must exist, as must a method of inactivation -- be it by enzymatic degredation, diffusion, or re-uptake. Theoretically, the substance should be collectible following presynaptic stimulation if the inactivating system can itself be inactivated (207).

Demonstration of each criterion is not always feasible, yet the guideline remains necessary. The corollary is to establish guidelines for identifying the neurotransmitter at a given synapse. The candidate must be available, on demand, in the presynaptic neuron; the precursors, synthetic enzymes, and an inactivating system must be present as well(109). Physiological investigation must demonstrate receptors specific for the transmitter candidate on the postsynaptic neuron. Once identified, the induced ionic sensitivities, membrane conductance changes, and reversal potentials must match. Similarly, the

pharmacological properties must be identical: the effects of agonists and antagonists, and the secondary effects of inhibiting the inactivation system, must mimic each other. At times, ultrastructural studies may be of use. Collection of the candidate from the bath perfusate following presynaptic stimulation provides supportive evidence.

Receptor Physiology And Pharmacology

The number of neurotransmitters and the multiplicity of responses they elicit in Aplysia is most conveniently addressed where the common and distinctive features of receptor pharmacology and physiology may be considered. Ionophoretically induced shifts in membrane potential, usually associated with an increase in membrane conductance, have been described for many substances: acetylcholine (103), dopamine (5, 6, 26, 30, 179), GABA (211, 214), glutamate (64, 107, 211, 212), histamine (27, 30, 75), serotonin (68), aspartate (64, 107, 211, 212), octopamine (25, 30), and phenylethanolamine (26, 169).

The most common response resulting from the binding of transmitters to their receptors is to increase the permeability of sodium, chloride, or potassium ions across the cell membrane. The membrane potential at which a voltage dependent response will flip, the reversal potential, reflects the equilibrium potential for the ion that mediates the response. For example, the chloride reversal potential is approximately -55 to -60 mv, that of potassium is -75 to -80 mv, and that of sodium is between +10 and +30 mv.

The similarity of the time course, the changes in membrane conductance, and the ionic sensitivities of the commonly found responses that may be generated by all transmitters in Aplysia led Swann and Carpenter to propose the Ionophore Theory (23, 30, 179, 213). Based upon receptor and ion channel antagonists they argue that individual neurotransmitter specific receptors are coupled to prefabricated ionophores -- the ion specific channels that penetrate the membrane. Any one transmitter receptor may be linked with any ionophore. Thus, one transmitter may evoke multiple responses mediated by different ions; and, conversely, any ionic response may be generated by any number of transmitters. Given the prevalence of transmitters with a similar spectrum of response, it makes sense, etiologically, that different receptors should be bound to standard ion gates.

Other, less common, receptors have been identified, as well. Carpenter et al. described two slow depolarizations produced by GABA -- one mediated by a simultaneous sodium and chloride conductance increase, the other by a decrease in potassium ion permeability (211, 214). Atypical slow depolarizing responses have also been described for histamine (129, 138, 200, 206), acetylcholine (101, 103, 104), and for serotonin (68).

Gerschenfeld studied three atypical serotonergic responses in detail which are distinguished by differences in membrane conductance and ionic currents (68). A depolarizing alpha response is mediated by a decrease in potassium ion permeability (reversal potential -75 mv). A hyperpolarization generated by decreased permeability of sodium and

potassium ions, resulting in a reversal potential between -20 and -30 mv, is a beta response. In addition, a second slower depolarization, called an A' response, is associated with increased membrane conductance and is generated by a sodium current.

Pellmar et al. characterized a serotonin-induced voltage dependent calcium conductance increase response that is not cAMP dependent, although other serotonin responses that are voltage dependent have been reported to use cAMP as a second messenger (147, 148, 149 and 146 for a contrary view). Serotonin may be responsible for a form of presynaptic facilitation observed by Kandel et al. where the messenger probably acts by decreasing a voltage dependent resting potassium permeability. This prolongs the action potential which, in turn, allows more transmitter to be released at the terminal (17, 31, 49, 113).

Serotonin also excites cardiac tissue and enhances buccal muscle contraction (38, 100, 109; 204, 205). On the other hand, acetylcholine, which inhibits cardiac tissue, is an important excitatory transmitter at the neuromuscular junction (109, 124; 187, 205). Dopamine may act as a transmitter for some motoneurons, and may also modulate gill muscle contractions -- possibly through cAMP (180, 182, 183, 184, 187; 181). Glutamate excites muscles at discreet locations (187).

Although the ionic mechanisms may be identical among different neurotransmitters, specific pharmacological agents reveal common and unique receptor properties (179, 23). Curare blocks all sodium and chloride channels (30); so too strychnine, save for the gates guarded by GABA receptors where steric hinderance created by GABA receptor and

ionophore interaction prevents strychnine binding (23, 51, 214; Carpenter, personal communication). Penicillin appears to block chloride responses generated by acetylcholine, dopamine, GABA, and serotonin (150). Bicuculine antagonizes acetylcholine and GABA chloride mediated responses (213).

Receptors manifest distinctive characteristics that define their identity. For example, any receptor activated by one neurotransmitter binding to it can not be activated by a different transmitter. Cross sensitivity, on the other hand, is one of the criteria for agonist activity. Hexamethonium specifically antagonizes the acetylcholine generated sodium response, and no other; TEA and PTMA block the potassium response; there is no specific antagonist for the chloride ionophore-receptor complex, however (103).

There are specific antagonists for the many serotonin responses as well: 7-methyltryptamine (7-MT) specifically blocks A receptors (which mediate the common fast depolarizing responses generated by increased sodium ion permeability); 5-methoxygramine (5-MG) closes receptors guarding potassium ionophores; neostigmine blocks receptors linked with chloride channels. Bufotenine eliminates A, A', and chloride responses; curare does not affect either A' or potassium responses (68). Ergots block dopamine receptors which are bound to potassium ionophores(5, 6, 26, 30). Picrotoxin abolishes GABA dependent chloride hyperpolarizations (155, 213; blocks sodium responses only, 214). Cimetidine and burimamide selectively block histaminergic potassium responses. The histamine antagonist pyrilamine eliminates sodium mediated responses (27, 30, 75).

Some transmitters and their receptors do not have specific antagonists -- this makes studies of their receptors more difficult. The example of one transmitter, important for this study, will convey many of the difficulties encountered in characterizing receptors while demonstrating some of the pitfalls associated with generalizing from analogous preparations. Glutamate is a well defined neurotransmitter in the mammalian central nervous systems (137), and also at the neuromuscular junction in crustations and arthropods (137; crayfish, 123; lobster, 62; locust, 4). In the latter systems glutamate increases permeability of sodium and potassium ions; evidence also exists for distinct junctional and extrajunctional receptors.

For molluscs, however, the evidence is less clear. Responses have been reported for Onconidium, but the study is not as rigorous as one would wish (99). More extensive investigation in Helix identified the following reseponses and characterized some of their receptor properties: a depolarization caused by increased permeability to sodium ions that easily desensitizes; a fast hyperpolarization mediated by an increased chloride current that exhibits variable vulnerability to desensitization; and a potassium subserved hyperpolarization associated with increased membrane conductance that desensitizes with difficulty. We have seen the same basic responses before; this is not a surprise (66, 67, 122, 195).

The material for Aplysia is far more scarce. In addition to the the responses mediated by receptors similar to those described in Helix, Kehoe identified a fourth response subserved by sodium that is unmasked

by concanavalin A (64, 107, 211, 212). Receptors have been observed at discreet sites on buccal muscle as well as on gill muscle (181, 187). Unlike their counterparts in Helix, glutamate receptors readily desensitize (64, 211). Even though abundant evidence exists supporting glutamate's candidacy as a neurotransmitter, no glutaminergic synapses have been identified.

Further study of these receptors is hampered by lack of consistently effective agonists and antagonists (134). What work exists is limited to the neuromuscular junction and to Helix -- work that is not applicable to, or reproducible in, the Aplysia central nervous system. In Helix , quisqualic acid and L-aspartate are agonists at receptors which activate sodium ionophores. Other agonists include DL ibotenic acid for chloride responses and DL-amino-adipic acid which has greater agonist activity on chloride than on sodium or potassium responses; DL-ibotenic acid and quisqualic acid also activate receptors linked to potassium ionophores. Curare, as is expected, eliminates sodium and chloride mediated responses. Dinitrophenyl-alpha-aminobutyrate is a more or less effective sodium receptor antagonist. DL-alpha-aminopimetic acid is a mixed agonist and antagonist for all glutamate responses. The efficacy of L-glutamic-gamma-methylester is disputed (30, 137).

The only proven antagonist for Aplysia glutamate receptors is the non-specific curare (211). In contrast to Helix the following compounds appear to be specific agonists for the responses generated by increased permeability of thefollowing ions in Aplysia: sodium, D-glutamate

chloride, DL-ibotenate; and, potassium, quisqualic acid (64, 107, 211, 212).

Aspartate interacts with glutamate receptors, and, in the lobster, is released with glutamate at the neuromuscular junction where it acts to potentiate the glutaminergic response. (62, 137). Aspartate has distinct receptors in Aplysia, some of which cross desensitize with glutamate (29, 133, 134, 212). Glutamate generated responses are potentiated by aspartate at low concentrations (10^{-6} M), which interferes with glutamate re-uptake, and also acts by directly interacting with the receptor (134).

Atypical responses associated with decreased membrane conductance are not sensitive to any known antagonists. As for transmitter and hormone receptors elsewhere, most receptors on Aplysia neurons show desensitization (68, 103, 211). The serotonergic A' receptor, which does not desensitize, is an exception.

From the culmination of data regarding putative neurotransmitter receptors and their agonists and antagonists a reference chart may be constructed (Table 1). The matrix of pharmacological sensitivities allows the investigator to work backwards: the nature of a postsynaptic potential can be ascertained by determining its pharmacological profile which is unique for any given receptor.

TABLE 1: Receptor Antagonists. Chart demontrating the matrix of pharmacological sensitivities of the known receptors in Aplysia. Sodium, chloride, and potassium ionophores are common to all messengers. A' receptors only exist for serotonin; serotonergic A receptors are listed as "Na+". Receptors for less common responses (associated with decreased membrane conductance) are not shown as they do not have any known antagonists. Antagonists specific for transmitter receptors that guard potassium ionophores are not shown: TEA for actylcholine; 5-methoxygramine for serotonin; and ergometrine for dopamine. ACH = acetylcholine, DA = dopamine, GLUT = glutamate, HIST = histamine, 5HT = serotonin, ASP = aspartate, OCT = Octopamine, PEE = phenylethanolamine 7-MT = 7-metyltrptamine. (#) denotes full receptor antagonism, (0) denotes no effect, (#/-) denotes mixed effect. Compiled from references 5,6,23,26,27,51,68,69,74,75,102,103,104,130,155,169,170,211,212,213,214.

RECEPTOR ANTAGONISTS

ANTAGONIST	ACH	DA	GABA	GLUT	HIST	5HT	ASP	OCT	PEE
CURARE									
Na+	#	#	#	#	#	#	#	#	#
Cl-	#	#	#	#	#	#	#	#	#
K+	0	0	0	0	0	0	0	0	0
A'						0			
STRYCHNINE									
Na+	#	#	0	#		#			
Cl-	#	#	0	#	#				
K+	0	0		0	0	0			
A'					0				
BURIMAMIDE									
Na+					#(?)				
Cl-	0				0				
K+	0				#				
PYRILAMINE									
Na+					#				
Cl-	0				0				
K+	0				0				
BUFOTENINE									
Na+	0					#			
Cl-	0					0			
K+	0					#			
A'						#			
7-MT									
Na+						#			
Cl-						0			
K+						0			
A'						0			
NEOSTIGMINE									
Na+	0					0			
Cl-	0					#			
K+	0					0			
A'						0			
HEXAMETHONIUM									
Na+	#	0				0			
Cl-	0				0	0			
K+	0				0	0			
A'						0			
DESENSITIZATION									
Na+	#	#		#		#			
Cl-	#	#?		#/-		#			
K+	#/-	#/-		#/-		#			
A'						0			

TABLE 1

Biochemical And Monosynaptic Studies In Aplysia

Numerous studies have determined the presence of proposed and putative neurotransmitters in nervous tissues as well as in individual neurons. Glutamate (15, 218), aspartate (131), GABA (36), and phenylethanolamine (52, 127) are found unevenly distributed in nervous tissue, but in greater concentrations than in non-nervous tissues. For some substances the levels are uniformly high, as for glutamate, aspartate, and GABA, or are not all that remarkable, as for phenylethanolamine. Octopamine, too, is found throughout the nervous system, but the significance of the quantities found in individual neurons is disputed (52, 127, 168). Far more knowledge exists for acetylcholine, serotonin, histamine and dopamine -- each been found in individual cells and identified at known synapses. A discussion of cellular biochemical analysis and identification of these messengers at known synapses by use of pharmacological and electrophysiological studies follows -- for these investigations provide models for the present study. Evidence regarding glycine as a neurotransmitter is considered in the final section of this thesis.

Acetylcholine. Acetylcholine is the best studied of the putative transmitters. Not only can acetylcholine be isolated from identified neurons, but the principal synthetic enzyme, choline acetyltransferase, can be found as well (70, 128, 132). Even though choline, the precursor to acetylcholine, is ubiquitous, choline acetyltransferase is not (70, 132). Intracellular injection of tritiated choline results in the

production of labeled acetylcholine (48). Identified cholinergic neurons include R2, LPGC, L10, L11, RB(he), LV(vc), numerous buccal cells, and the LD motoneurons to the gill and heart (70, 109, 128, 132). Once liberated from the presynaptic terminal, acetylcholine is inactivated by the ever present acetylcholine esterase (201).

Kandel and co-workers, working in the abdominal ganglion, identified Interneuron I to be the cholinergic cell L10. When activated, L10 increases heart rate while decreasing vasomotor tone (114, 124). This particular neuron monosynaptically innervates at least fourteen cells. It excites R9, R15, and RB(he) [R17] through an increase in sodium permeability. Most follower cells, including L1 through L6, L8, and L11, are inhibited, usually by a chloride mediated psp. Many neurons exhibit biphasic psps generated by increased permeability of chloride and potassium ions. Curiously, L7 is excited when L10 is stimulated at low frequencies; this response desensitizes at higher frequencies to unmask a chloride dependent ipsp. L24 also innervates L7 and acts in a similar fashion (20). Finally, L10 inhibits LD(hi)(1-2) and LB(vc)(1-3) in addition to exhibiting electrotonic synapses. These deductions concerning ionic basis of synaptic activity are generally confirmed by ion exchange studies, and by receptor sensitivity to curare and hexamethonium when appropriate (13, 95A, 192, 193). Similar results are found in the buccal ganglion (63).

Kehoe provided a model for determining pharmacological synaptic identity. In the pleural ganglion one presynaptic neuron forms monosynaptic connections with four cells. Postsynaptic potentials

include biphasic iipsps and triphasic eiipsps mediated by increased permeability to sodium, chloride, and potassium ions. Detailed study of the iipsp demonstrates ionic dependence on chloride and potassium ions; ionophoresed acetylcholine produces responses which mimic that of the synapse. The matching pharmacological sensitivities of the induced and evoked potentials to curare, hexamethonium, and other selective antagonists support the suspicion that acetylcholine is the synaptic messenger (101, 102, 104).

Serotonin. Comprehensive investigation identified serotonin to be the resident neurotransmitter of the metacerebral giant cell (MGC) of Aplysia and its analogue in Helix. Intra-ganglionic concentrations of serotonin vary, but the MGC contains high amounts of the substance (24, 36, 202). Tritiated serotonin has been radioautographically identified in the MGCs and in two other cerebral cells (12, 65), and confirmed by fluorescence studies (64). Uptake of precursors, synthesis of serotonin, transport to the periphery, and collection of labled serotonin following presynaptic stimulation can be demonstrated in Helix (38, 144). Reserpine depletes serotonin stores; tricyclics inhibit the uptake of liberated serotonin which suggests that re-uptake, rather than extracellular enzymatic degradation by ubiquitous monoamine oxidases, is the principal method of inactivation (24, 38, 65, 103; 64).

The MGC makes multiple monosynaptic connections with buccal interneurons, motoneurons, and buccal muscle (39, 69, 145, 209). Of the follower cells, nine respond with epsps, three with ipsps, and one with

an atypical ipsp. Ionic sensitivities, and alteration of membrane resistance, suggest mediation by sodium and potassium currents. Pharmacological investigations indicate that the epsp is generated through A and A' receptor activation since it is abolished by bufotenine, partially blocked by curare, and insensitive to hexamethonium; the ipsp is produced by increased potassium ion permeability since it is eliminated by bufotenine and 5-MG, and is unaffected by curare, 7-MT, and hexamethonium; the atypical ipsp is a beta response which is insensitive to known pharmacological agents. Ionophoretic studies compliment the pharmacological work at each respective postsynaptic membrane. Imipramine and desipramine, serotonin uptake inhibitors, augment the amplitude and duration of postsynaptic potentials as well as those of ionophoretic responses.

Buccal muscle, including the accessory radula closer, is innervated by the MGC. Stimulation of the MGC, however, does not induce muscle contractions; rather, it augments buccal muscle contractions evoked by motoneuron stimulation (204, 205). MGC stimulation also enhances firing of its postsynaptic motoneurons, effects which are also mimicked by serotonin (49, 58).

Histamine. Recent single cell biochemical analysis found high histamine concentrations in LC2 and RC2 of the cerebral E cluster, in addition to neurons in the D and L clusters (138, 203). The synthetic enzyme histidine decarboxylase is also present in these neurons (129). McCamen identified many monosynaptic follower cells of neurons LC2 and

RC2; one neuron has ten follower cells. Ion replacement studies, measurements of ionic conductance changes and reversal potentials suggest the following combinations of response: an epsp mediated by increased sodium ion permeability; an eepsp generated by a similar sodium current and an uncharacterized slow depolarization; an iipsp mediated by chloride and potassium ions associated with increased membrane conductance; and, a number of electrical synapses. Although ionophoretic application of histamine mimics these responses, pharmacological studies have not been performed (129, 138, 200, 206). Similar results are found for RC3 and LC3 (206).

Dopamine. Dopamine is found in high quantities in the ganglia, gill muscle, and walls of the branchial vein, but, unlike acetylcholine, not in the cardiac tissue of Aplysia (24, 181, 182). The synthetic enzyme, aromatic acid decarboxylase, is ubiquitous as is COMT (64, 202). Inactivation is probably achieved by re-uptake, which is antagonized by tricyclic compounds (64, 143). The giant cell of Planorbis corneus contains high levels of dopamine where precursor label studies demonstrate synthesis of dopamine (10, 38, 157). Synaptic studies, however, have only been performed in Planorbis. Epsps, ipsps, and eipsps have been identified in three follower cells of the giant cell; ionophoretically applied dopamine mimics these responses. As the epsp is sensitive to curare, but not hexamethonium, and as the ipsp is decreased by 6-hydroxydopamine and ergometrine, but is unaffected by either curare or hexamethonium, dopamine is thought to be the neurotransmitter at the synapse (10, 38).

Dopamine is also a proposed neuromuscular transmitter. It causes direct contraction of muscle, which is blocked by ergometrine, and also, perhaps independently, increases levels of cAMP in gill tissue. Dopamine acts either directly at the neuromuscular junction or modulates acetylcholine induced contractions (178, 180, 181, 182, 183, 184).

THE CEREBRAL GANGLION: ANATOMY

The highest ordered collection of neurons in Aplysia, the cerebral ganglion, forms a loop about the eosophogus by its connections with the pleural and pedal ganglia. It neighbors the important primary sensory areas of the head: the eyes, statocysts, lips, and the anterior and posterior tentacles (84, 167). In return for what they receive, cerebral neurons send many of their processes to the nearby ganglia as well as to the tentacles and to the eyes. There are also numerous, if not intimate, connections with the buccal ganglion (84). The cerebral ganglion is an important integrative station for the reception of sensory information and for the initiation and modulation of behaviors essential to the well being of Aplysia: feeding, locomotion, and procreation (61, 166). The function of the cerebral ganglion may be compared to the role of the abdominal ganglion and its regulation of gut motility, respiration, circulation, inking, and mucous release (20, 22, 114, 124, 164).

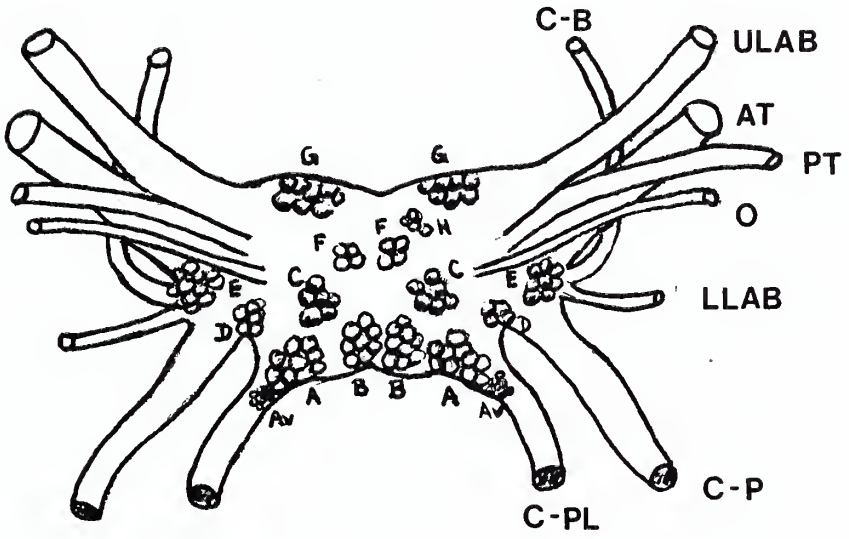
Just as clusters of neurons have been identified in the abdominal ganglion, so, too, have the cells of the cerebral ganglion been described (55, 96; 84). Individual, and populations of, neurons may be identified by morphological and electrophysiological criteria. The

constellation of position, color, appearance, size, and location when considered with firing pattern, evoked synaptic input, response, and axonal output defines a nerve cell. Jahan-Parwar and Fredman described seven pairs of neural clusters, the A through G cells, and one unpaired cluster, the H cells in the cerebral ganglion [Figure A] (84). Initially the cells did not appear to be organized into restricted functional areas. Since Jahan-Parwar's original observations additional groups of cells and their connections have been described that are relevant to the study at hand: the paired J, K, L, and M clusters (138, 166). Identification of sensory afferent and motor efferent connections, as well as the functional purpose of some neurons, has accompanied recent morphological work.

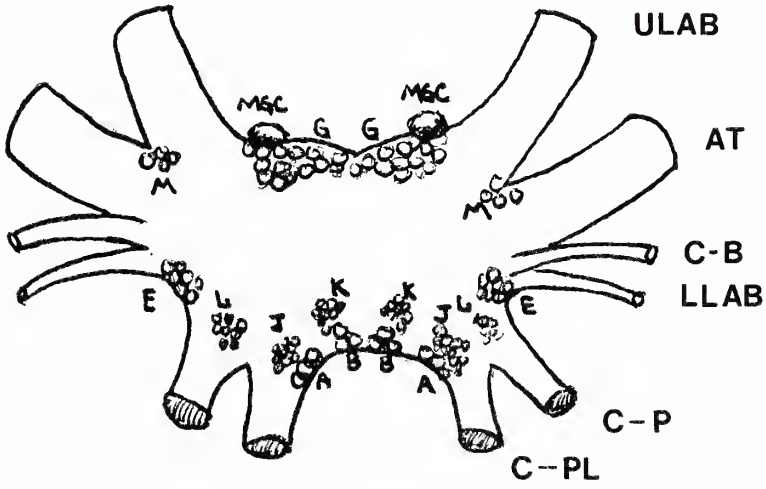
The eyes form numerous monosynaptic connections with cerebral neurons (167, 14), as do the statocysts (89). The anterior tentacles relay chemosensory information to second order sensory cerebral neurons (81, 82). There are also two sets of mechanosensory neurons about the head and tentacles that relay data to the ganglion. Of these cells, the J cluster, located laterally and ventrally to the A cells , have specific, small, and narrowly defined tactile receptive fields which partially overlap along their longitudinal axes. There are twenty small neurons, twenty to sixty micrometers in diameter, in each group. They may well be the Av neurons described by Jahan-Parwar and Fredman, which are of similar size, position, and electrophysiological silence (166, 58). In similar fashion, the K neurons, ventral to the B cells, have touch receptive fields about the lips which are small, specific, and overlap with one another. They are the same in size, number, and in

FIGURE A: Diagram of the cerebral ganglion showing locations of neuron clusters, connectives, and nerves. C-P, cerebral - pleural connective; C-Pl, cerebral - pleural connective; AT, anterior tentacular nerve; PT, posterior tentacular nerve; O, optic nerve; ULAB, upper labial nerve; ULAB, lower labial nerve; MGC, metacerebral giant cell. Dorsal view from Jahan-Parwar and Fredman (84); ventral view from Ono and McCamen (138).

CEREBRAL GANGLION



dorsal



ventral

electrophyiological behavior to the J cells. Both J and K cells form monosynaptic connections with the nearby B cells; however, the relationship is not reciprocal (166). Just rostral to the B neurons lie the B' cells, which, like the B cells, receive second order mechanosensory and chemosensory input. Unlike the B cells, however, they burst irregularly (58).

The cerebral ganglion contains a number of motoneurons. The A and B cells innervate the foot and parapodia (86). Some C cells are presynaptic to foot motoneurons (61). The B cells also encompass large motor fields in the anterior tentacles (57). The extrinsic buccal mass receives the bulk of its motor innervation from E, G and B neurons. E2, described by Jahan-Parwar and Fredman, corresponds to Weinreich's C2 histaminergic neuron (92). In addition to the motoneurons, the metacerebral giant cells of the G cluster modulate the receptive tone and excitability of motoneurons of the buccal ganglion, which innervate the intrinsic buccal mass (205), and, also, enhance the contractility of buccal muscle (205).

The command function of the cerebral ganglion is becoming increasingly evident. Some cerberal neurons are able to initiate the rhythmic stereotyped motor patterns of chewing and locomotion (61, 93, 92). Undoubtedly, there is much integration of information at the synapse and at higher organizational levels of which we are unaware.

MATERIALS AND METHODS

Experimental Design

The purpose of this thesis is to identify the transmitter at the A to B cell synapse. First, receptors on A and B cells for putative neurotransmitters where identified, and then characterized, in order to determine potential synaptic messenger candidates. Then, specific antagonists and agonists of the candidates were selected to assess their affect on the A to B postsynaptic potential (psp). Finally, experiments were performed to characterize glycine-induced alterations of membrane potential found on A and B neurons during the course of the preliminary receptor survey.

The Cerebral Ganglion Preparation

For each preparation a cerebral ganglion of Aplysia californica, supplied by the Pacific Biomarine Supply Co. of Venice, California, was excised and then pinned dorsal surface up, with stainless steel insect pins, to the bottom of a lucite perfusion chamber, volume 3.5 ml, lined with Sylgard 184 silicone elastomer. The preparation was continuously bathed in artificial seawater at a constant rate of 1.5 ml/min; the volume of seawater in the chamber was kept constant by continuous suction. The composition of the artificial seawater (ASW) was as follows:

	NaCl	KCl	$CaCl_2 \cdot 2H_2O$	$MgCl_2 \cdot 6H_2O$	$MgSO_4 \cdot 7H_2O$	$NaHCO_3$	TRIS BASE	MANNITOL
ARTIFICIAL SEA WATER	480	10	10	20	30	2.5	--	--
NA FREE ASW (TRIS)	--	10	10	20	30	--	480	--
NA FREE ASW (MANNITOL)	--	10	10	20	30	--	--	480

TABLE 2 : Composition of artificial seawaters used in these studies. All values are mM. PH of ASW ranged from 7.9 to 8.0 Tris base ASW was titrated to pH 8.0.

Antagonists and agonists for perfusion application were dissolved in ASW in concentrations never greater than 10^{-3} M as needed. A RTE Neslab refridgerated circulation bath maintained the temperature at 15 degrees centigrade for the receptor and perfusion studies.

The connective tissue sheath enveloping the ganglion, visualized through either a Zeiss OPMI or Wild Heerbragg microscope, was surgically removed with a Gibson's breakable razor blade to expose the neural clusters. A and B cells were identified according to the criteria established by Jahan-Parwar and Fredman (56,84).

Fiber filled micropipets were pulled on Kopf 700c or Narishige vertical pipet pullers. Recording and current electrodes (4 to 8 megaohm resistance), filled with 2 M potassium acetate, were inserted into half cells filled with 3 M potassium chloride. The indifferent electrode was filled with Sigma agar impregnated by 3 M potassium chloride. Microelectrodes, directed by Prior manipulators, were inserted into target neurons. Signals recorded by BAK, Livengood, and

Dagan 8500 or 8700 preamplifiers were displayed on a Textronex 5111 storage oscilloscope (Livengood preamp design from Livengood, personal communication). Permanent copies of data were made on a Honeywell 1858 Visirecorder, or on either a Gould Brush 481 or 220 pen recorder.

Receptor Profile And Characterization

The membrane potential of A or B cells was monitered by the first of two microelectrodes inserted into the cell through a Livengood or BAK preamp. A second electrode was used to pass current into the cell, in order to alter the cell's membrane potential, by a Dagan 8700 preamp. Membrane resistance was periodically determined by passing a constant square current pulse through the current electrode from a WPI pulse generating system composed of a Stimulus Isolater 1850A, an Interval Generator 1830, and a Pulse Module 1831.

Selected neurotransmitters were expressed onto the neural surface by a Loess 74 Ionophoretic Stimulator, designed to control the total charge passed, or by pressure ejection. Five-barrelled ionophoretic pipets were pulled on the Narishige pipet puller; single-barrel fiber filled pipets were pulled on the Kopf 700c puller. Neurotransmitter candidates, dissolved in distilled water in concentrations ranging from 0.5 to 1 M and placed in the micropipets, were passed as cations except for glutamate and aspartate which were released as anions. Pulses varied from 100 to 1000 nc; a backing current prevented unwanted leaking of transmitter from the pipet tip.

Fiber filled electrodes used for pressure ejection were pulled on the Kopf 700c puller and filled with a neurotransmitter previously dissolved in distilled or artificial seawater. Fast green was added to the solution to assist visualization of the pipet and efficacy of the pressure ejection. Compressed carbon dioxide was relayed to the pipet by the necessary assortment of regulators, tubing and a half cell. The duration of the pressure pulse was controlled by the WPI pulse generating system.

Transmitter generated responses were measured at different membrane potentials, set by the Dagan 8700 preamp through the current electrode, to determine reversal potentials. A pulse of current was also passed through the cell at the peak of the induced response to assess alterations in membrane conductance. At times, the preparation was perfused with specific antagonists or agonists dissolved in ASW to discern their affect on the induced response. The results of these experiments were then compared with the results of the synaptic perfusion experiments described below. Ideally the effect of the antagonist on ionophoretic response and on the A to B psp could be studied simultaneously.

Criteria For Determining The Ionic Basis Of Response

These studies identified and characterized the receptors, activated by various transmitters, found on A and B cells. After a response was found -- a voltage dependent change in resting membrane potential -- information was gathered to define the receptor and ionophore: the conductance change, reversal potential (reflecting the equilibrium

potential of the ion mediating the response), direction of voltage change from resting membrane potential, and, when necessary, pharmacological sensitivity were determined. There are a limited number of ionic pathways described for Aplysia (see introduction) that hold true for all neurotransmitter induced voltage changes:

ION	CONDUCTANCE	REVERSAL POTENTIAL	VOLTAGE CHANGE
Na+	increase	(+)	depolarization
Cl-	increase	-50 to -60	hyperpolarization
K+	increase	-75 to -80	hyperpolarization
Na+	decrease	(+)	hyperpolarization
K+	decrease	-75 to -80	depolarization

It is clear from viewing the table that determination of voltage change, reversal potential, and alteration of membrane conductance will define the receptor and the ionic basis of response. Actually any two are sufficient; documentation of altered conductance changes argues against the possibility of artifact. Characterization of atypical or previously undescribed responses receives more detailed analysis in the presentation of experimental data below.

Synaptic Circuitry And Perfusion Experiments

Synaptic potentials were studied by placing an electrode in the presynaptic cell which recorded membrane potential through a BAK or Livengood preamp and by placing a pair of electrodes into the postsynaptic cell. Axon spikes were elicited by passing a depolarizing current pulse from the WPI pulse generating system through the recording electrode. Follower cell membrane potential was recorded by a

microelectrode that relayed its signals to a Dagan or Livengood preamp. A current electrode was also inserted into the postsynaptic cell in order to adjust membrane potential and to assess membrane resistance.

The monosynaptic nature of the A to B synapse has been described previously (56, 44, 77). The criteria used here were a one-to-one relationship of presynaptic spike to the psp at a constant latency. By stimulating many neurons while recording from numerous follower cells synaptic connections could be demonstrated among clusters of neurons. Characterization of the A to B synapse was achieved by altering presynaptic stimulation frequency, as well as by bathing the preparation with agonists or antagonists specific for selected neurotransmitter receptors or ionophores while constantly monitering the psp and follower cell membrane resistance. Agonists and antagonists, listed below, were dissolved in ASW ranging in concentrations from 10^{-6} M to 10^{-3} M and then fed into the constant perfusion system of the experimental apparatus. With the exception of bufotenine, membrane resistance was not significantly altered by any of the compounds used in these experiments. Comparison could then be made between known or demonstrated effects of each agonist or antagonist on the ionophoretically induced response and on the concomitantly evoked psp.

Glycine-Induced Response Characterization

Initial studies of a glycine induced response on current clamped A and B cells were made according to the methods outlined above: measurement of reversal potentials, conductance changes, and the effects of perfusion with antagonists and with sodium free ASW. Further studies

were undertaken with voltage clamped neurons. A neuron was impaled with two microelectrodes: one measured membrane voltage while the other passed current to maintain a constant voltage through a Dagan 8500 Intracellular Preamp Clamp. Membrane resistance was determined by passing square wave pulses, generated by the WPI system, through the current electrode. Pressure ejection of glycine and perfusion of antagonists was performed as described above. One series of experiments was performed on Dr. Norman T. Slater's microperfusion apparatus, with an electrical system identical to the system described here, which enabled rapid perfusion of seawater, agonists, and antagonists directly onto neurons in a 1 ml chamber. Glycine 0.001 M dissolved in ASW was rapidly perfused over A cells to study response desensitization.

Chemicals And Compounds

All substances were supplied by Sigma unless noted to the contrary.

NEUROTRANSMITTERS: Acetylcholine Chloride, Adenosine, Gamma-Aminobutyric Acid (GABA), 3-Hydroxytyramine (Dopamine), Glutamine, Glycine, Histamine Hydrochloride, L-Histidine Monochloride, 5-Hydroxytryptamine (Serotonin), Phenylethanolamine, Octopamine Hydrochloride. Sodium L-Glutamate and Sodium L-Aspartate were supplied by K and K Labs.

AGONISTS AND ANTAGONISTS: Bufotenine Hydrogen Oxalate, Burimamide, Desipramine (supplied by USV Laboratories), 2,4-Dinitrophenol, 5-Methoxygramine (5-MG), 7-Methyltryptamine (7-MT), Pyrilamine Maleate, Strychnine Nitrate, Strychnine Sulfate, D-Turbocurarine Chloride, Cobalt II Dichloride ($2H_2O$).

OTHER: Fast Green, Isethonic Acid Sodium Salt, Sodium Chloride, Sodium Bicarbonate, Tris Base, Manntol, Potassium Chloride, Calcium Chloride ($2H_2O$), Magnessium Chloride ($6H_2O$), Magnessium Sulfate ($7H_2O$),

I. CHARACTERIZATION OF NEUROTRANSMITTER RECEPTORS ON A AND B NEURONS

A And B Neurons: Morphology And Electrophysiology

This section provides necessary morphological and electrophysiological background before identification and characterization of the receptors on the A and B cells may be considered. Much of the material here is equally relevant for section II where the question of identifying the transmitter at the A to B synapse is addressed.

The B cells are darkly pigmented neurons approximately one hundred micrometers in diameter. They are formed into paired clusters numbering twenty cells each, and lie in the dorsal, caudal, and medial portion of the cerebral ganglion. Each cell sends forth multiple axonal processes which generally extend ipsilaterally through the cerebral-buccal connective, and out the optic, anterior and posterior tentacular, and the upper and lower labial nerves. Although usually silent electrically, most of the spontaneous input is excitatory (84). For all appearances, the B cells are homologous, a sense reinforced by knowledge of their afferent connections.

The bulk of input to the B cells is sensory. All B cells are second order sensory neurons, for they receive chemosensory and mechanosensory information from the head (59, 166). Chemical stimuli presented to the anterior tentacles causes tonic firing of B cells that is proportional to the stimulus strength (57, 166). Activation of B cells by chemical

stimuli may also make them more sensitive to later sensory input. Mechanosensory stimuli elicit a phasic response that rapidly adapts; increasing the stimulus strength does not lead to an increase in B cell firing (151, 166). This phenomenon is best seen at the J or K to B cell synapses. These two clusters of first order tactile sensory neurons of the head also make monosynaptic connections with B cells (166). Repeated stimulation of either J or K cells decreases the epsp which, however, is restored by rest. The habituation of the synaptic potential is paralleled by the habituation of the anterior tentacular withdrawal reflex. This circuit exhibits heterosynaptic summation, but not heterosynaptic facilitation: the greater the number of J or K cells that are stimulated, the greater is the B cell response (166). In addition, B cells receive secondary sensory information from the eyes (167). Although there have not been any studies of B cell nociceptive response, examination of Aplysia tails indicates that primary nociceptive neurons do not exist (18). The organization of B cell input at this level creates an ability to distinquish among different types of sensory input, subsequently encoded in firing frequency for relay to other neurons. Some forms of input alter B cell resting membrane potential which serves as a memory influencing the integration of forthcoming information.

B cells receive information from other, non-sensory, cerebral neurons. B' cells, also second order sensory cells, send axons to B cells (58). So too the Av cells mentioned above (58). Each B cell makes a synapse with every ipsilateral B cell, and, most likely, forms synapses with their contralateral neighbors (56, 58, 77). Of course,

each A cell forms synaptic connections with most, if not all, B cells. The character of this synapse will be discussed in far greater detail below. All identified cerebral synaptic input is excitatory.

The final series of known afferent connections arises from the pleural ganglion. B cells receive excitatory input from pleural cell 6 (Pl 6), Pl 12, and Pl 14. Pl 6 and Pl 14 also make excitatory connections with the left giant cell (LGC) of the pleural ganglion. And then there is inhibitory input from Pl 3, Pl 5, Pl 7, Pl 9, and Pl 11. It is interesting to note that Pl 5 simultaneously excites the LGC and Pl 9 elicits an iepsp on B cells (58). In general, neurons which excite B cells inhibit presynaptic neurons that are B cell inhibitors (58). It is readily seen that there is much synaptic exchange between the cerebral and pleural ganglia.

Knowledge of B cell output is more limited. B cells do not form synapses with A, Av, J, or K cells (56, 58, 77, 166). There appears to be a neuron that lies between the A and the B cells: B cell excitation of this interneuron, at least at low to medium frequencies, results in inhibition of A cells (56, 58). Two B cell excitatory synapses on pleural cells have also been identified (58).

The remainder of B cell output is motor. Whether B cells are primary motor neurons or not is unclear, as neuromuscular potentials have never been recorded, but if they are not motoneurons then they are presynaptic to peripheral motoneurons (57, 86, 92). B cells have large ipsilateral motor fields in the anterior tentacles. They appear to be the neurons that mediate the anterior tentacular withdrawal reflex (57,

166). Before a contraction can be elicited, however, many B cells must be driven (57). Similarly, stimulation of B cells results in contraction of large foot and parapodial motor fields (86). Finally, one B cell in each cluster innervates the extrinsic ventrolateral protractor of the extrinsic buccal muscle mass (92). Although similar in many regards, B cells may be defined by their efferent connections. The general appearance of B cell output is excitatory -- to fellow neurons as well as to muscle.

The twenty darkly pigmented neurons, one hundred to one hundred twenty micrometers in diameter, of each paired A cluster lie in the dorsal, caudal and lateral portion of the cerebral ganglion. A cells are adjacent to the cerebral-pleural connective and are lateral to the B cells. They, too, have multiple axonal processes that are generally, but not uniformly, ipsilateral projections extending through the cerebral-buccal connective as well as into the optic, anterior tentacular, upper labial and lower labial nerves (84). Electrically they are silent, although the bulk of spontaneous input, unlike the B cells, is inhibitory (84, 58). Their resting membrane potential lies between -50 and -60 mv (88).

Input to the A cells is not as clearly defined as for the B cells. There does not appear to be any primary or secondary sensory input into the A cells, rather, they are fourth order sensory neurons. Sensory information is passed to the A cells via the B cells and the mysterious interneuron described above (56, 58, 59). Given a weak tactile stimulus, the A cells will be inhibited; they will often exhibit a slow

and long lasting hyperpolarization that pulls the resting membrane potential below the chloride equilibrium potential (88). This phenomenon is important because much of the rapid and spontaneous input then becomes excitatory, revealing that chloride currents provide the ionic basis for much of this activity (88). Strong tactile stimuli usually excites A cells. The remaining known input arises from the pleural ganglion: Pl 2 excites both A cells and the LGC, and Pl 3 inhibits both A and B cells (58). In contrast to B cells, A cells do not form synaptic connections with each other.

A cells make many connections with pleural cells; there are no fewer than five identified excitatory monosynaptic connections, including Pl 7. In addition, A cells form ipsilateral and contralateral excitatory connections with the LGC. The low amplitude, rapidly decrementing epsps evoked by A cells on the LGC are very much like the A to B synaptic response (58). In a fashion analogous to the B cells, A cells also appear to be motoneurons. They innervate either the foot or the parapodia and exhibit small, well defined motor fields (86). However, these motoneurons do not fire unless there is a very strong tactile tentacular stimulus. It is interesting to observe that the output here, as with the B cells, is all excitatory.

The precise function of the A and B circuit remains a bit of a puzzle. These neurons are second and fourth order sensory neurons, and are both motoneurons, yet it is unclear if the latter property is their primary function. They mediate tentacle withdrawal, pedal and parapodial movement, and buccal chewing (57, 58, 86, 92, 166). Thus

motor output and sensory input is involved with motor control of the anterior body for headwaving, eating, and moving in response to chemical and tactile stimuli (56, 57). The intimate connections with the buccal and pleural ganglia suggest an important relationship to locomotion and feeding. Yet A and B cells are not command neurons as was once thought (61). Perhaps, then, they play a role in arousal for these important functions (57, 88, 166).

In sum, the B cells receive chemosensory and mechanosensory information from the anterior tentacles which is then encoded and relayed to various regions of the nervous system including the A cells. The B cells innervate muscle in the tentacles, foot, parapodia, and mouth of Aplysia. A cells relay their input to B cells, pleural cells and to some muscles of the foot and parapodia.

A And B Cell Receptors

Reports from two groups of investigators note diverse, but not identical, receptor populations on B cell somata. Hinzen and Davies found acetylcholine generally induced a depolarization of membrane potential, although an occaisonal inhibitory response could be found (77). McCamen, to the contrary, did not find any excitatory responses (130). Ionophoresed dopamine and GABA evoked uncharacterized hyperpolarizing alterations of membrane potential. Octopamine generaterd an excitatory response that failed to appear when repeated in high Mg++ artificial seawater (77). Only glutamate and serotonin -- of 43 unlisted compounds in one account -- gave rise to depolarizing responses which were both caused by an increase in sodium ion

permeability (77, 130). The serotonergic response, however, proved more difficult to elicit as the receptors were located deep in the neuropile (77). Receptors have not been described for histamine or aspartate (77, 130).

The receptor pharmacology of A cells is better studied. All cells respond to acetylcholine; a biphasic inhibition is most commonly elicited, although monophasic inhibition is also seen (7, 75). The faster component is mediated by increased permeability to chloride ions, while potassium subserves the slower phase. Most cells display dopamine receptors the vast majority of which guard potasium channels (7, 75). Closer analysis discerned a fast sodium mediated depolarization either found alone, or as the initial component of a biphasic response coupled with the slow hyperpolarization (7, 33). This discrepancy is a matter of localization of receptors on the neuron soma -- the receptors linked to sodium ionophores are far more restricted. Although Ascher (7) could find only a monophasic potassium based hyperpolarization induced by histamine, Gruol (75) found twenty percent of his responses were generated by chloride currents. Rarely could the fast chloride component (less than 5 seconds) be completely isolated from the slower potassium response (8 to 20 seconds) (7, 75). All identified responses were associated with increased membrane conductance.

RESULTS

A And B Cell Membrane Properties

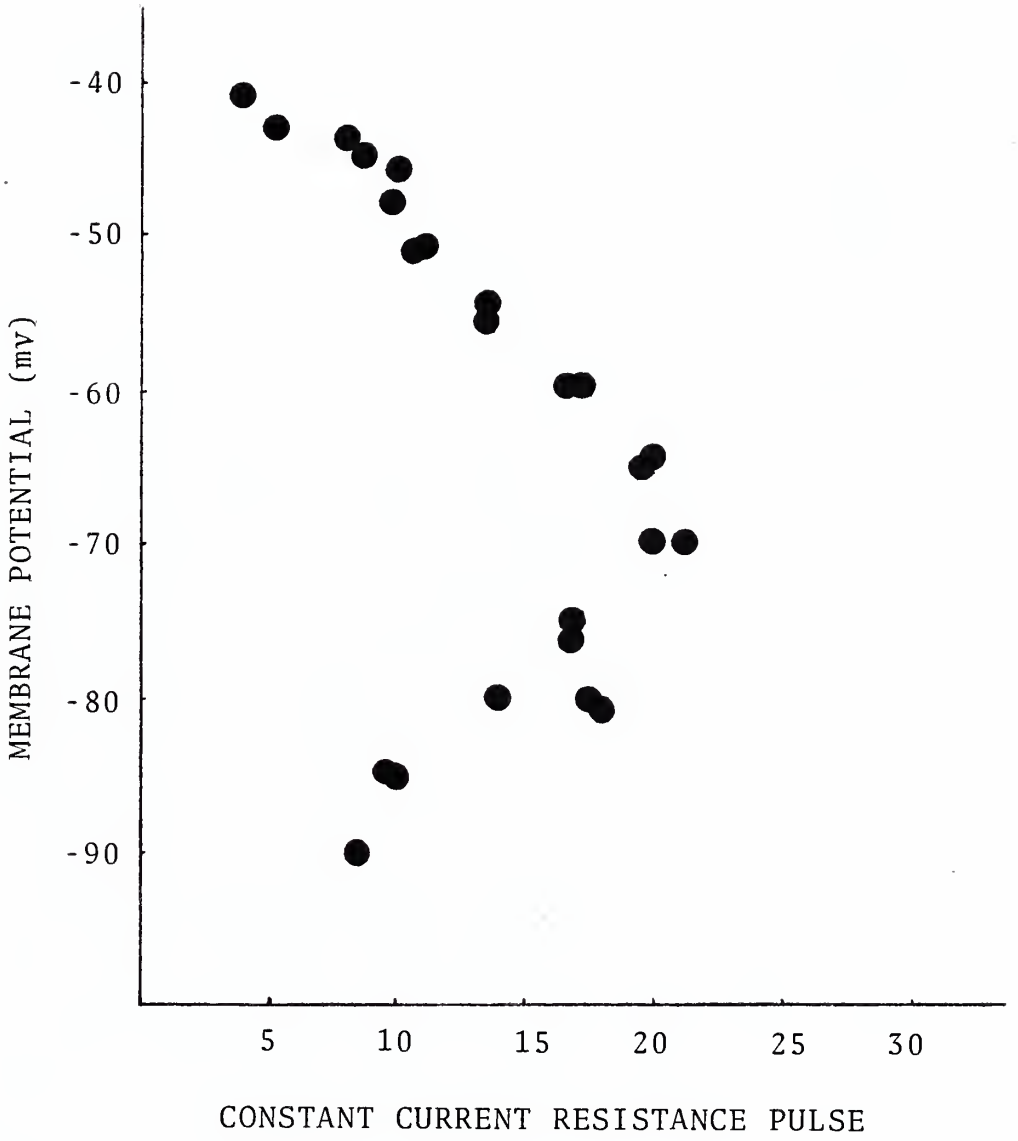
Immediately following penetration of the soma with a microelectrode A cells gave off a series of action potentials after which the membrane stabilized to attain a resting membrane potential of approximately -55 mv. A cells remained silent, although occaisonally spontaneous ipsps appeared. A cell membranes exhibited marked delayed and anomalous rectification with the peak resistance occuring at -65 mv (Graph 1). The membrane rectification, in part accentuated by current clamp recording techniques used here, affects the interpretation of the change in potential induced by a ionophoretically applied transmitter. At membrane potentials above and below the point of peak resistance, responses appeared to be smaller for the same degree of conductance change.

B cells unleashed a series of action potentials, too, when a microelectrode penetrated their soma whereupon the cells assumed a resting membrane potential close to -50 mv. Spontaneous background activity was greater than for the A cells; epsps occured often and more frequently than ipsps -- occaisonally B cells spiked. These cells also exhibited delayed and anomolous rectification (Graph 2), but to a lesser extent than A cells. Peak resistance occured at -60 mv.

B Cell Receptors

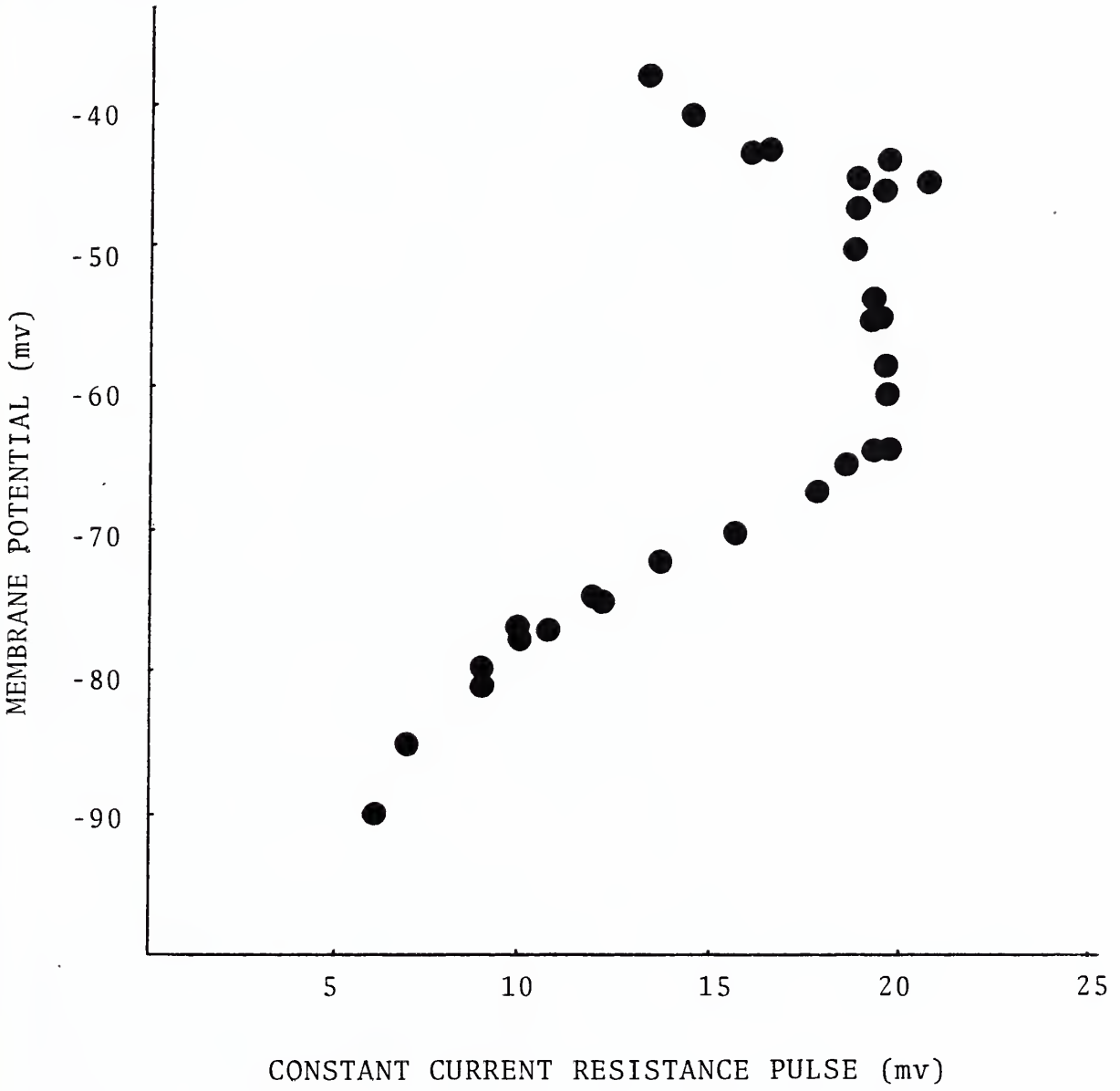
Numerous responses to many transmitters were found for individual cells as well as for the population of B cells. Tables 3 and 4 , at the

GRAPH 1: A representative current clamped A cell which manifested delayed and anomalous rectification. Ordinate: cell membrane potential (mv). Abscissa: pulse amplitude of a constant current pulse (mv) passed through the current electrode. Note the peak resistance near -70 mv. Resting membrane potential (RMP) was -52 mv.


-40
-50
-60
-70
-80
-90
MEMBRANE POTENTIAL (mv)
5
10
15
20
25
30
CONSTANT CURRENT RESISTANCE PULSE

A CELL

GRAPH 2: A representative current clamped B cell which manifested delayed and anomalous rectification. Ordinate: cell membrane potential (mv). Abscissa: pulse amplitude of a constant current pulse (mv) passed through the current electrode. Note the peak resistance near -60 mv. Resting membrane potential (RMP) was -42 mv.


-40
-50
-60
-70
-80
-90
MEMBRANE POTENTIAL (mv)
5
10
15
20
25
CONSTANT CURRENT RESISTANCE PULSE (mv)

B CELL

end of the section, summarize the receptor data results for both the A and the B cells.

Acetylcholine evoked alterations in membrane potential that were universally inhibitory, and were usually mediated by an increased permeability to chloride ions. Occaisonally an inhibitory response mediated by increased permeability of potassium ions could be brought forth. Figure 1 portrays a response that had a reversal potential at -50 mv. Figure 2 shows a similar hyperpolarization, with a similar reversal potential, that demonstrated decreased resistance even though a voltage change induced by acetylcholine was not apparent (by definition, the reversal potential). Together these figures define a response mediated by an increase in chloride permeability. Figure 3 shows a response that hyperpolarized the cell at a resting potential of -70; assessment of membrane resistance revealed increased conductance, thus it was a response mediated by potassium.

Glutamate, when applied to the surface of a B cell, resulted in varied changes in membrane potential. A response mediated distinctly by one ion rarely could be found, rather most responses were multiphasic or were generated by combined ionic currents. In the analysis of ten potential changes associated with an increased conductance, sodium mediated six responses, chloride seven, and potassium five. A pure chloride mediated hyperpolarization, similar to the acetylcholine response, is shown in figure 4. The more common mixed response, seen in figure 5, began with an initial depolarization which was then partially masked by the chloride current that had a later peak response and a

FIGURE 1: Ionophoresis of 100 nc acetylcholine onto a B cell which activated a chloride ionophore. Reversal potential -50 mv, peak response at 5 seconds.

ACH 100 nC

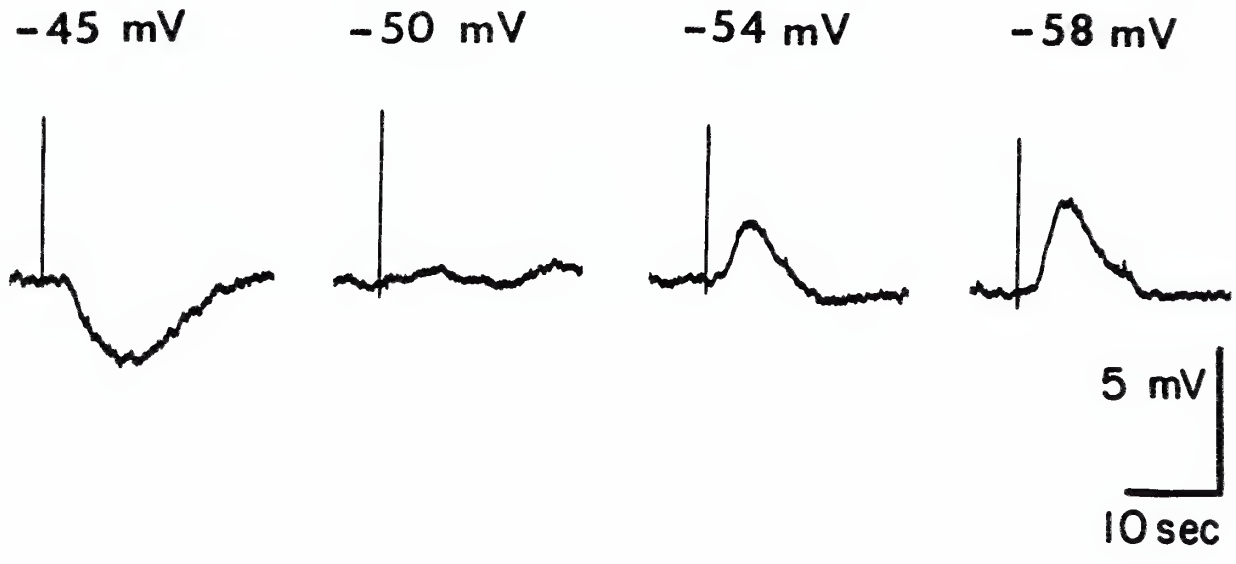


FIGURE 2: Ionophoresis of 1000 nc acetylcholine onto a B cell which activated a chloride ionophore. Decreased membrane resistance, reversal potential at -60 mv, peak response at 4 seconds.

ACH 1000 nC

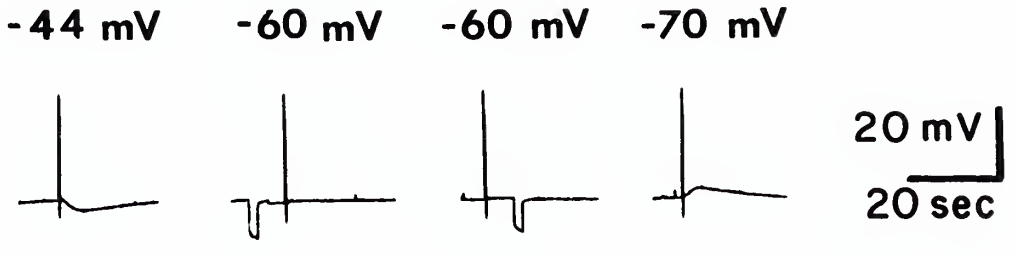


FIGURE 3: Ionophoresis of 1000 nc acetylcholine onto a B cell which activated a potassium ionophore. Decreased membrane resistance, reversal potential less than -70 mv, peak response at 8 seconds.

ACH 1000 nC

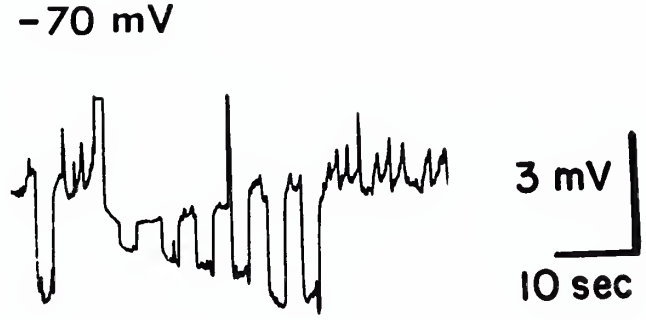


FIGURE 4: Ionophoresis of 1000 nc glutamate onto a B cell which activated a chloride ionophore. Decreased membrane resistance, reversal potential -56 mv, peak response at 2 seconds.

Glutamate 1000 nC

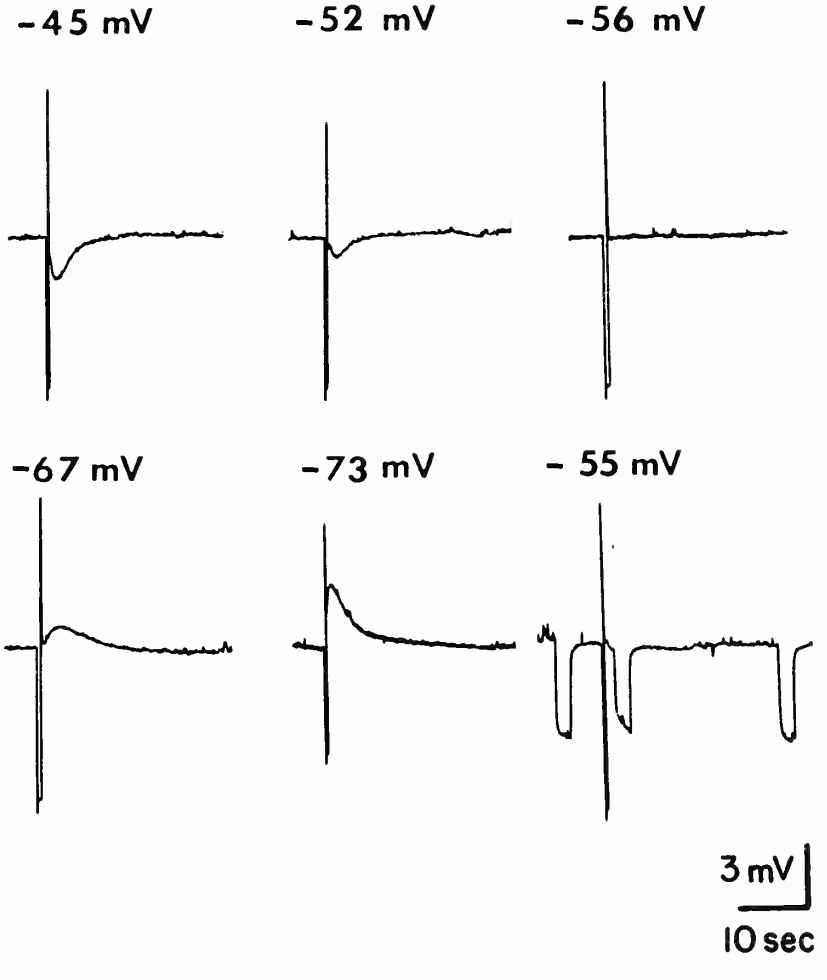
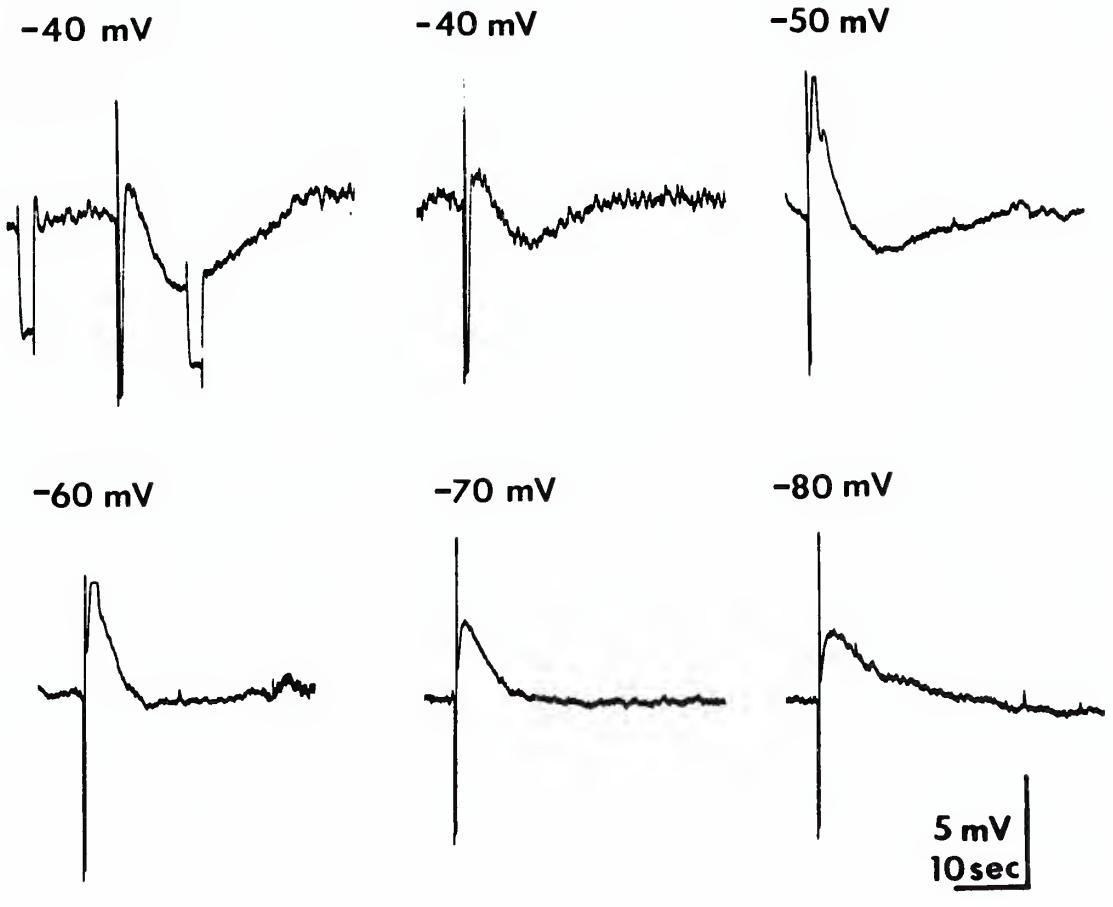


FIGURE 5: Ionophoresis of 1000nc nc glutamate onto a B cell which activated a series of ionophores for sodium, chloride, and potassium ions. sodium: reversal potential greater than -40 mv, peak response at 2 seconds; chloride: reversal potential approximately -50 mv, peak response at 5 seconds; potassium: reversal potential less than -70 mv, peak response at 12 seconds.

Glutamate 1000 nC



reversal potential between -50 and -55 mv. The chloride current and the effects of membrane rectification probably dampened the degree of depolarization brought forth in the -40 mv range. The late, and weak, contribution of a potassium current, which had a reversal between -70 and -80 mv, occured at twelve seconds. Other cells revealed increased membrane resistance associated with similar responses (figure 6).

Aspartate never gave rise to a response on a cell that did not display similarly appearing glutamate-induced potentials. In fact, aspartate-evoked depolarizations were always weaker reflections of their glutaminergic counterparts (compare figure 7 with figure 5, both from the same cell).

Serotonin elicited a wide spectrum of responses many of which, in a fashion akin to glutamate, proved to be multiphasic. A preliminary survey evoked nine uncharacterized depolarizations. Definitive experiments identified A, A', chloride, potassium, and alpha responses. Figure 8 portrays one of the more complex sequence of alterations in membrane potential seen. The initial depolarization, an A response, generated a spike within the first two seconds. The rapid hyperpolarization which followed was mediated by chloride whose reversal potential was -54 mv. At eighteen seconds a depolarization occured which resulted in an action potential -- this was an A' response. Later, the faint suggestion of the end of a hyperpolarization could be discerned which was mediated by potassium. The final figure demonstrates that all the components were associated with increased membrane conductance. A and A' receptor mediated depolarizations may be

FIGURE 6: Ionophoresis of 1000 nc glutamate onto a B cell which elicited a typical depolarization. Note the decreased membrane resistance.

Glutamate 1000 nC

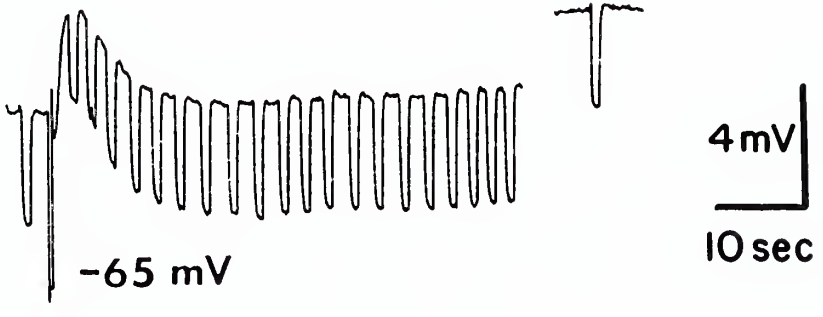


FIGURE 7: Ionophoresis of 1000 nc aspartate onto a B Cell which elicited a depolarizing response mediated by sodium and chloride, compare with the depolarizing responses generated by glutamate. Note the similar configuration but weaker response.

Aspartate 1000 nC

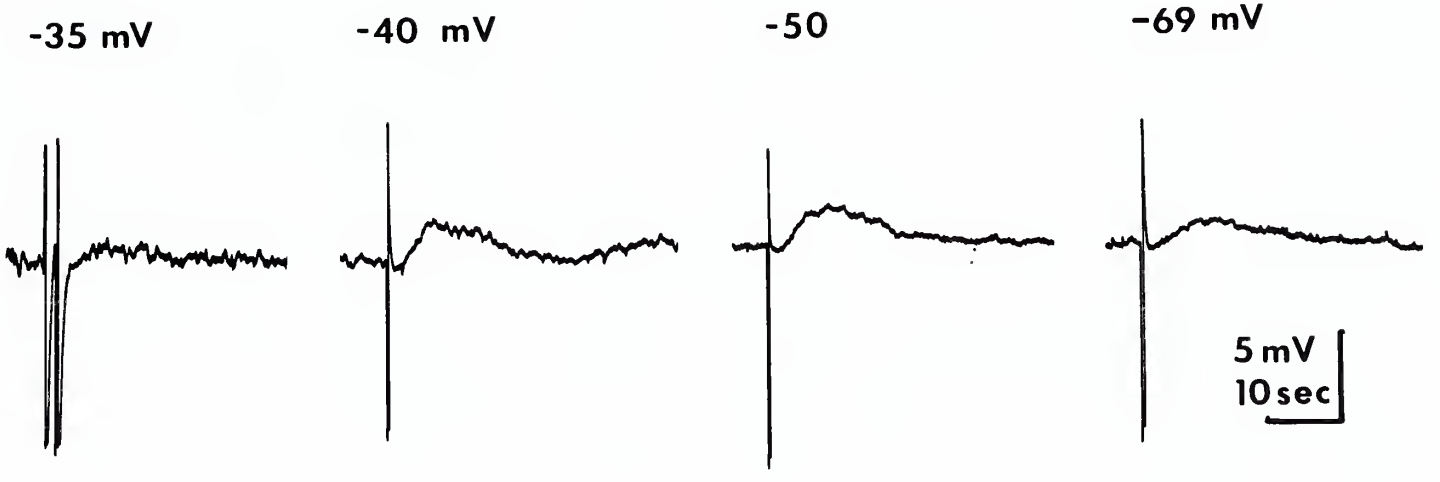
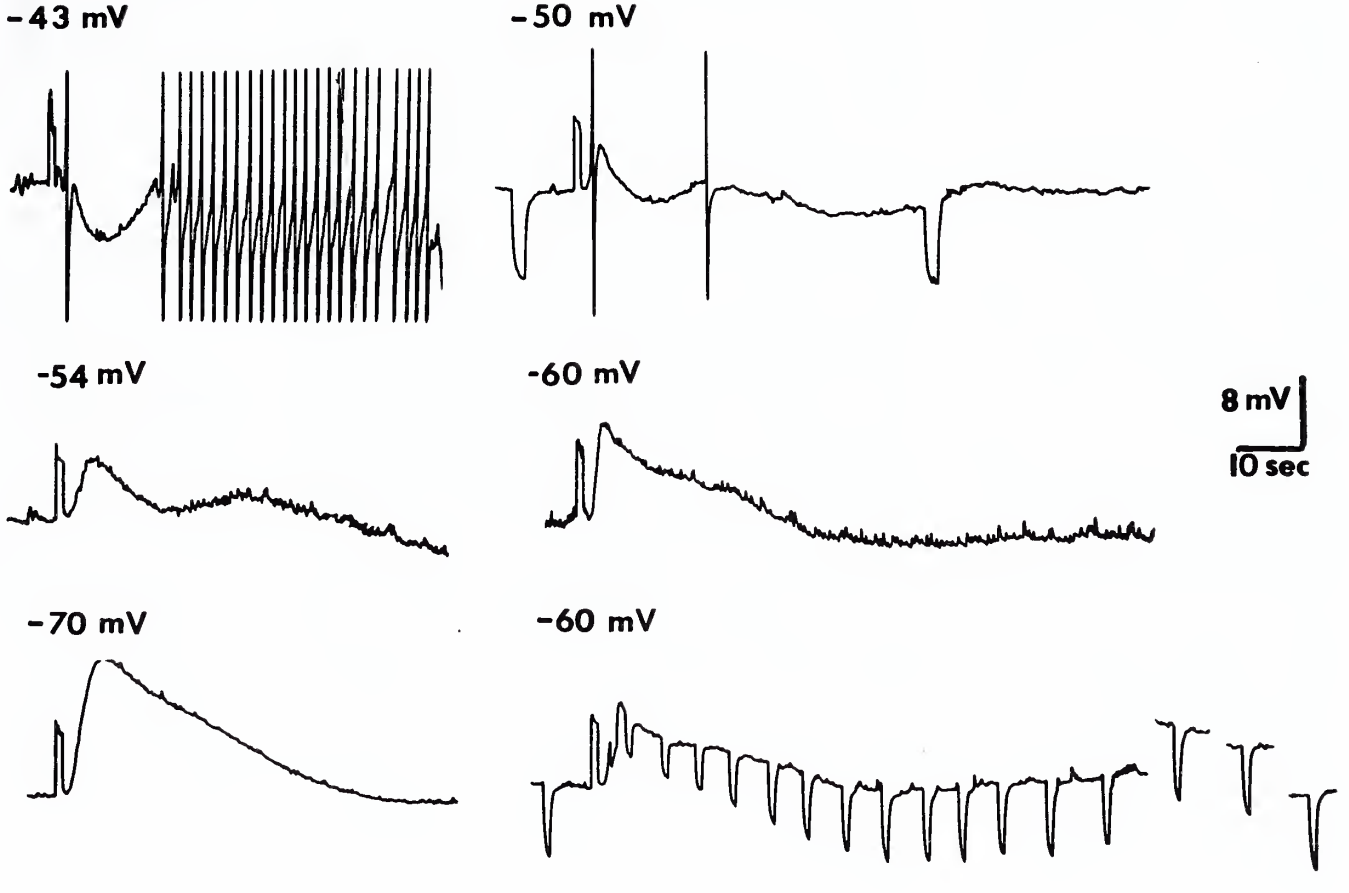


FIGURE 8: Ionophoresis of 1000 nc serotonin onto a B Cell which elicited a multicomponent response mediated by A, chloride ionophore linked, A', and potassium ionophore linked receptors, all associated with decreased membrane resistance. A receptor: reversal potential greater than -45 mv, peak response at 2 seconds; chloride receptor; reversal potential -50 to -54 mv, peak response at 7 seconds; A' receptor; reversal potential less than -45 mv; potassium receptor: reversal potential at -70 mv, peak response at 25 seconds.

5HT 1000 nC



distinguished by the longer latency, duration, and insensitivity to both curare and desensitization characteristic of the A' response; both receptors are blocked by bufotenine.

Pharmacological tools were used to discriminate among different receptors. On one cell 10^{-5} M bufotenine reversibly eliminated A, A' receptor and potassium mediated currents to unmask a chloride component (figure 9). As expected, curare only eliminated the A and chloride responses. Perfusion of the preparation with 3 x 10^{-4} M serotonin desensitized the A, but not the A' receptor.

Histamine brought forth a variety of coupled and mixed responses: chloride ions subserved the commonly elicited fast hyperpolarizations; sodium channel activation occured frequently; and a rare potassium current could be found. A peculiar depolarizing response often elicited by histamine was similar to the glutamate generated depolarization (figure 10). The response may be dampened by either pronounced membrane rectification, or by the simultaneous contribution of a chloride or potassium current to the predominant flow of sodium. A typical biphasic chloride and potassium mediated hyperpolarization is shown in figure 11; occaisonally a lone chloride response was seen.

GABA almost always brought forth an increase in chloride permeability (figure 12): a hyperpolarization mediated by potassium was rarely observed. Dopamine invariably resulted in increasing potassium ion permeability. A representative response is shown in figure 13; note the conductance change as well as the low reversal potential and long latency. Glycine induced a slow depolarization that could induce an

FIGURE 9: B cell exhibiting A, chloride ionophore linked, A', and potassium ionophore linked receptors activated by ionophoresis of 1000 nc serotonin. Perfusion of bufotenine (10^{-5} M) reversibly abolished the A, A', and potassium receptor mediated responses, without affecting the chloride current.

5HT 1000 nC −50 mV

Control

Bufotenine (10^{-5} M)

30 m

Wash

40 m

8 mV

10 sec

FIGURE 10: Ionophoresis of 1000 nc histamine onto a B cell which elicited a depolarizing response mediated by sodium ions, perhaps coupled with a chloride current. Reversal potential was greater than -35 mv with a peak response at 2 seconds. Similar responses on other cells were associated with increased membrane conductance. Note the rapid decrement of response amplitude with higher membrane potentials.

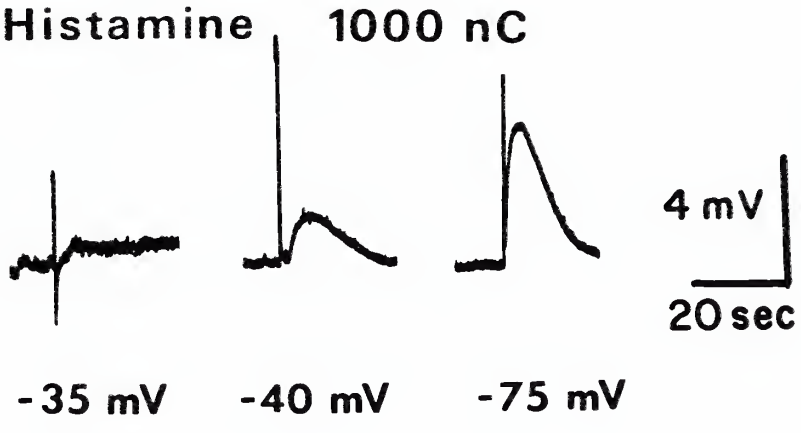

Histamine
1000 nC
4 mV
20 sec
-35 mV
-40 mV
-75 mV

FIGURE 11: Ionophoresis of 1000 nc histamine onto a B cell which elicited a biphasic hyperpolariztion mediated by chloride and potassium ions, respectively. Peak chloride response at 2 seconds with a reversal potential of -55 mv. Peak potassium response between 15 to 18 seconds.

Histamine 1000 nC

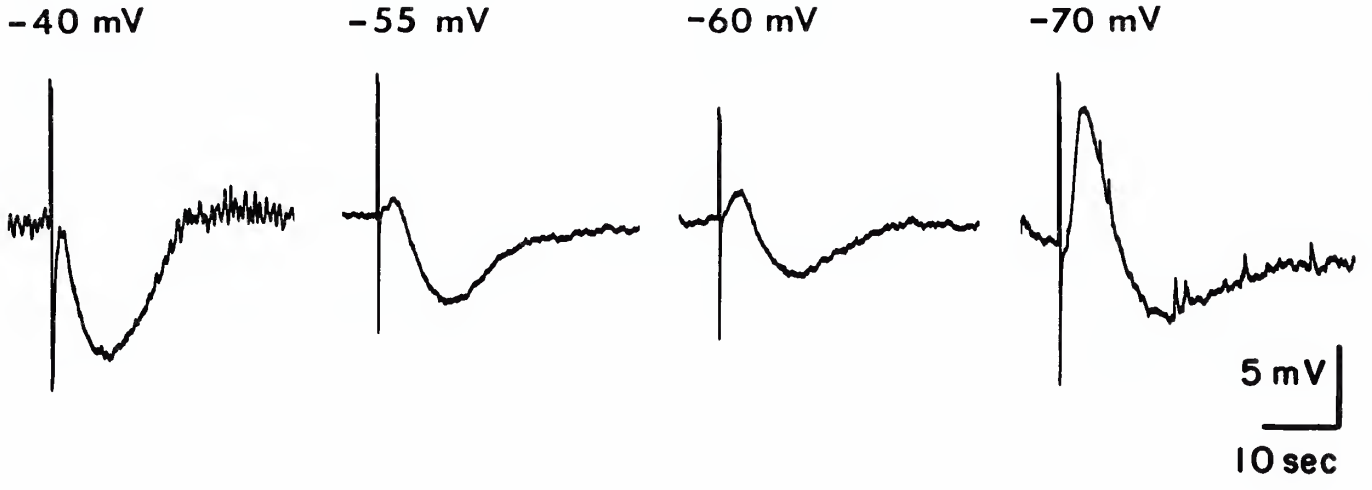


FIGURE 12: Ionophoresis of 100 nc GABA onto a B cell which elicited a hyperpolarization mediated by increased conductance of chloride ions. Reversal potential between -56 and -59 mv with peak response at 5 seconds.

GABA 100 nC

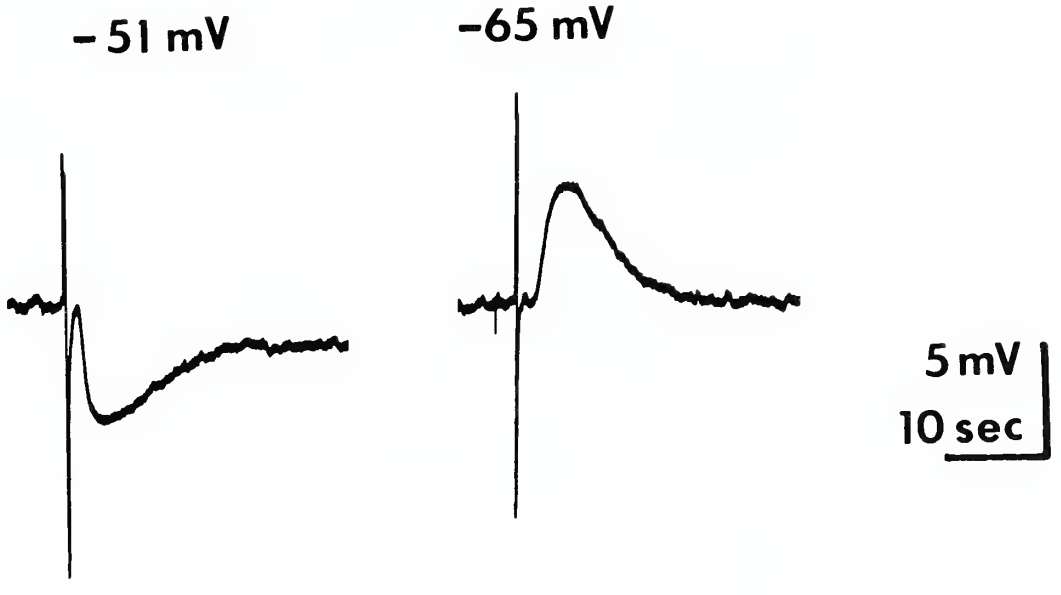
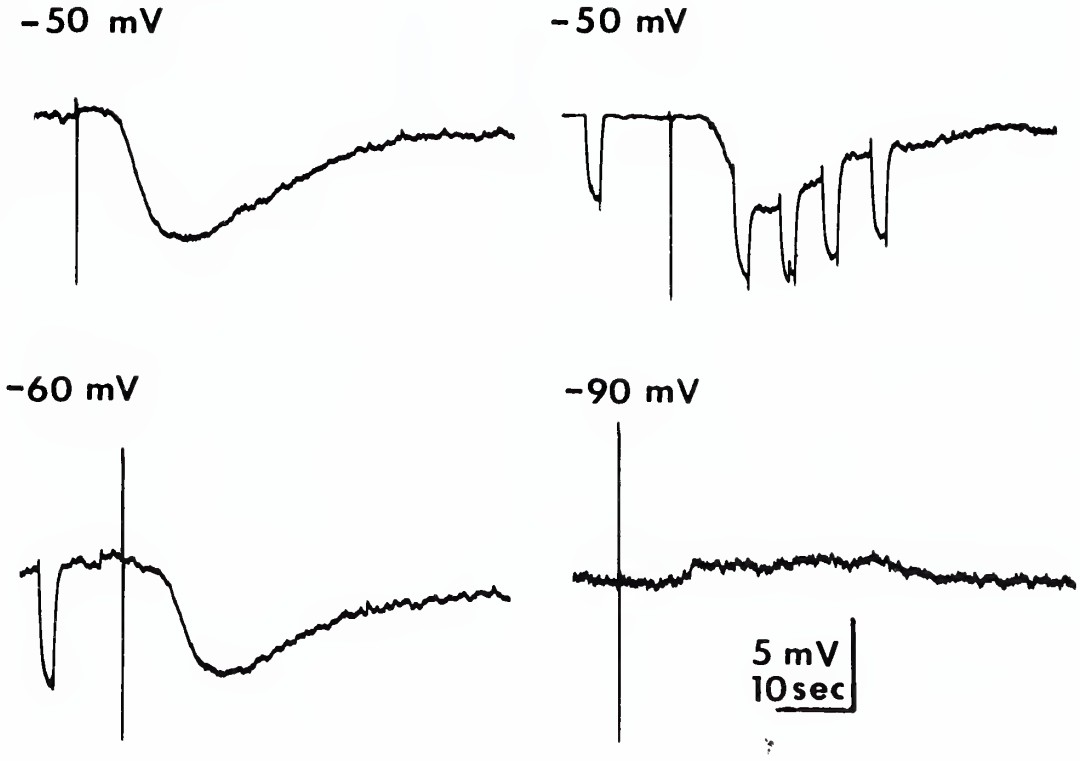


FIGURE 13: Ionophoresis of 500 nc dopamine onto a B cell which elicited a hyperpolarization mediated by increased conductance of potassium ions. Reversal potential less than -67 mv with peak response between 17 and 20 seconds.

Dopamine 500 nC



action potential: the nature of this depolarization is discussed in a later section (figure 14). Evidence for Octopamine and phenylethanolamine receptors was not found.

B cells displayed a number of different receptors on their membranes. Any given cell, then, might respond to a number of different transmitters in various ways; one neuron was found to have receptors for acetylcholine, dopamine, GABA, glutamate, aspartate, histamine, and serotonin. Acetylcholine opened chloride channels; dopamine gave rise to an increase in potassium ion permeability; GABA elicited a hyperpolarization mediated by chloride; glutamate evoked a mixed response subserved by sodium, chloride, and potassium; aspartate-induced changes in voltage were similar to those of glutamate; serotonin gave rise to A , A', and potassium responses; histamine brought forth a biphasic hyperpolarization wrought by chloride and potassium.

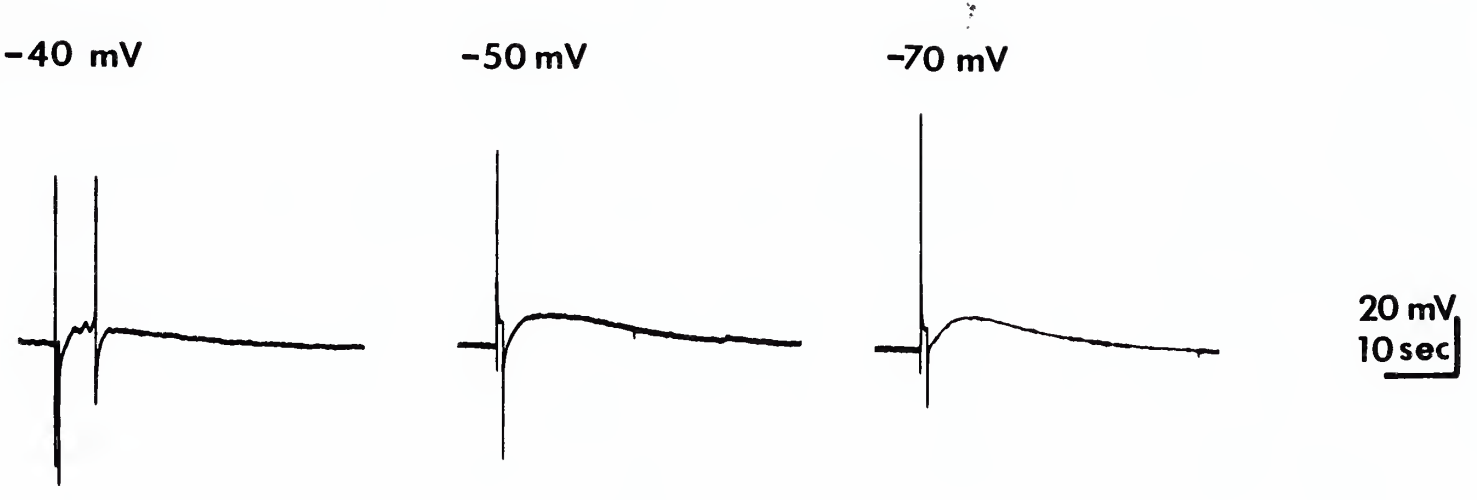
A Cell Receptors

The A cells displayed a different set of receptors on their somata that were also uniformly more consistent than those on their B cell colleagues. Acetylcholine receptors were ubiquitous; responses were usually biphasic hyperpolarizations produced by increased permeability to chloride and potassium ions -- an isolated potassium response could rarely be found (figure 15).

Glutamate generally evoked depolarizing changes of membrane potential which were probably mediated by an increased conductance to sodium ions (figure 16). The unremarkable degree of depolarization at

FIGURE 14: Ionophoresis of 500 and 1000 nc glycine onto a B cell. Reversal potential greater than -40 mv, peak response between 5 to 8 seconds. Note the generation of an action potential following application of glycine.

Glycine



500 nC

1000 nC

1000 nC

FIGURE 15: Ionophoresis of acetylcholine onto an A cell which elicited a biphasic hyperpolarization mediated by increased permeability to chloride and potassium ions respectively. All responses generated by application of 250 nc acetylcholine, except for the response seen at -72 mv when 750 nc was released. Chloride component reversal potential at -60 mv with peak response at 2 seconds. Potassium component reversal potential less than -72 mv with peak response at 10 seconds.

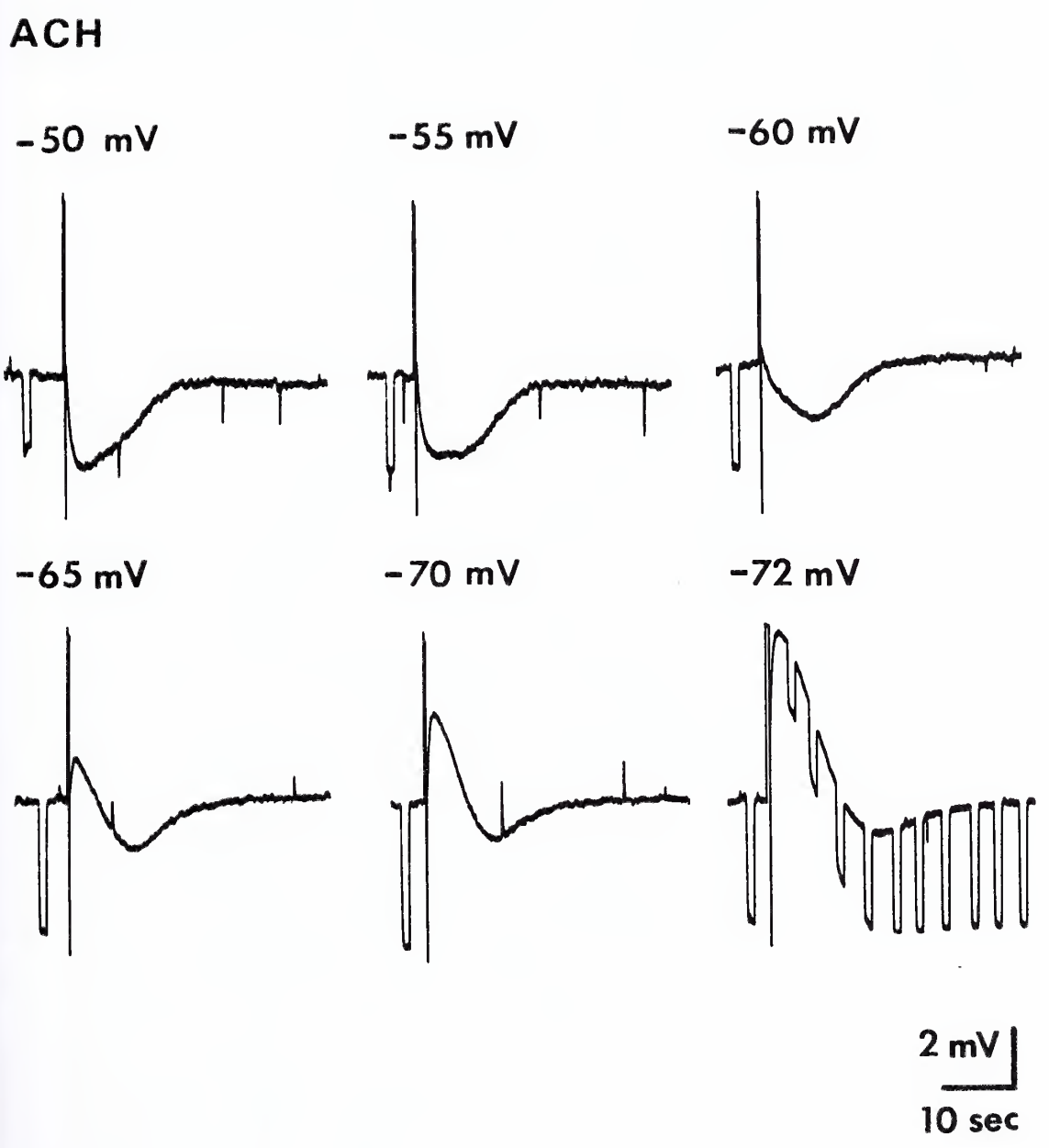
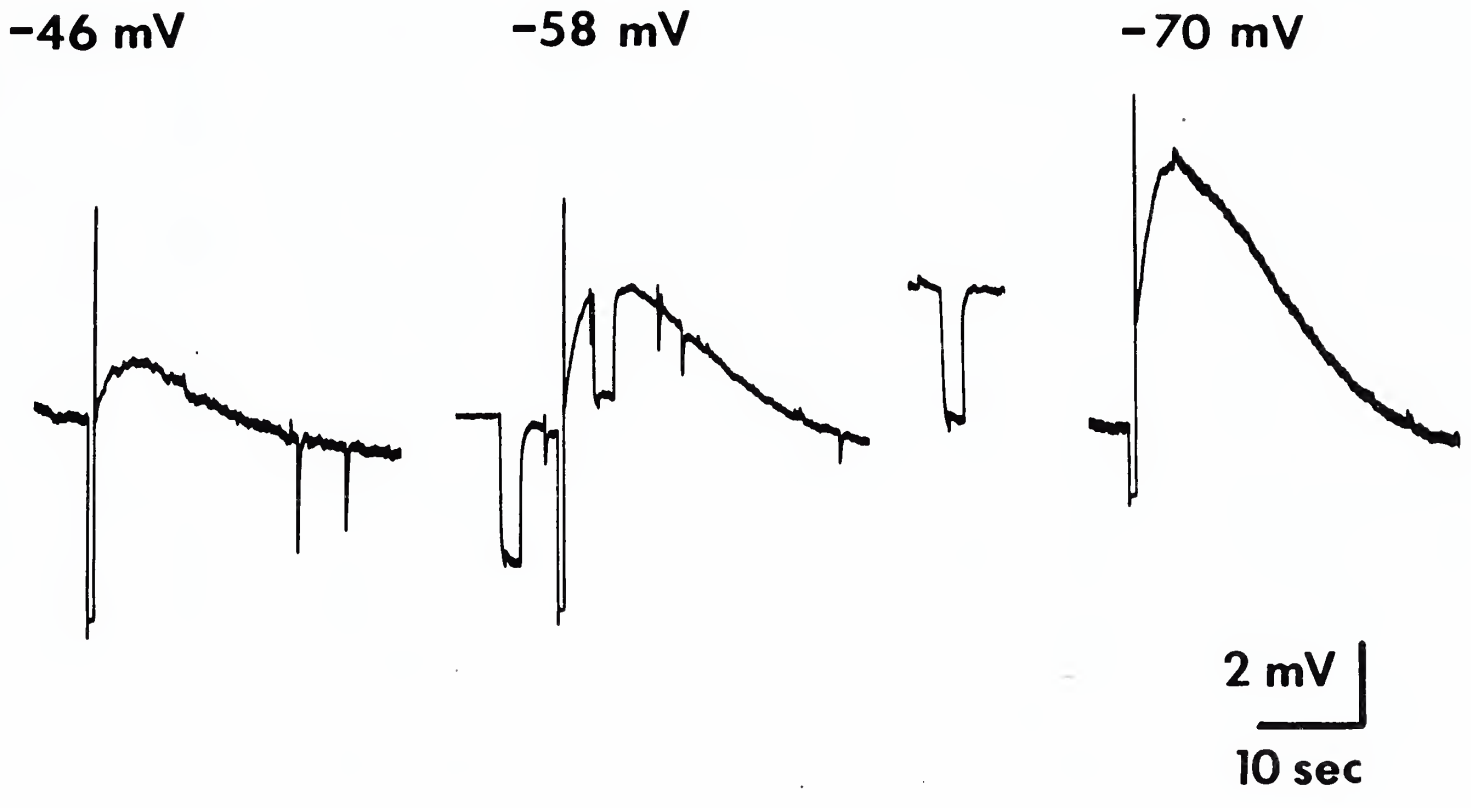

ACH
-50 mV
-55 mV
-60 mV
-65 mV
-70 mV
-72 mV
2 mV
10 sec

FIGURE 16: Ionophoresis of 1000 nc glutamate onto an A cell which elicited a depolarization mediated by sodium ions and perhaps by chloride ions as well. Note the increased membrane conductance and the reversal potential which was greater than -43 mv.


Glutamate 1000 nC
-46 mV
-58 mV
-70 mV
2 mV
10 sec

lower membrane potentials may reflect a mixed, but not separate, chloride component (figure 17). On the other hand, this may also be a property of the marked membrane rectification which A cell membranes exhibit. Aspartate-induced responses, when found, were identical to those produced by glutamate, and invariably occured on cells that manifested glutamate receptors (see figure 18 from the same cell as figure 17).

A mixed collection of depolarizing and hyperpolarizing responses occured when serotonin was applied to A cells. The hyperpolarizing component, frequently accompanied by a depolarization, was mediated by increased permeability to potassium (figure 19). The strong potassium current, associated with increased membrane conductance, partially obscured the earlier depolarizing response. The weak showing of the early component and pronounced rectification of the cell membrane precluded discerning its underlying nature. Diligent searching identified a single alpha response (figure 20).

One example of a biphasic adenosine induced voltage change was observed. In a fashion reminicent of aspartate, all adenosine evoked responses were identical to the serotonin responses and were found only on the same cells (figure 21).

GABA commonly brought forth biphasic chloride and potassium responses. Of nine cells studied, eight revealed biphasic hyperpolarizations, and one, the exception, produced a potassium generated response (figure 22). Dopamine reliably evoked a potassium dependent hyperpolarization (figure 23). One third of the cells

FIGURE 17: Ionophoresis of 1000 nc glutamate onto an A cell which elicited a depolarization mediated by sodium ions and perhaps chloride ions. Reversal potential is greater than -42 mv, but the response exhibited marked decreased amplitude with greater depolarization of membrane potential. Peak response at 3 to 4 seconds. Associated increase in membrane conductance is not shown for this cell.

Glutamate 1000 nC

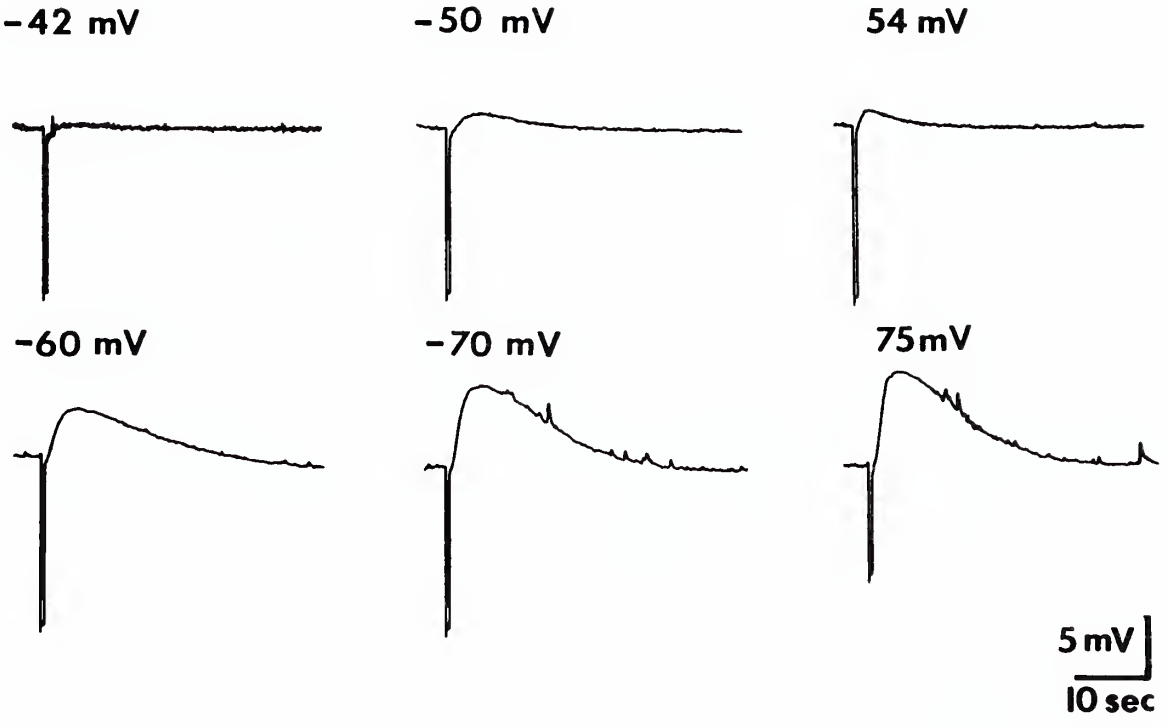


FIGURE 18: Ionophoresis of 1000 nc aspartate onto an A cell which elicited a depolarization. Compare to the glutamate response, figure 17.

Aspartate 1000 nC

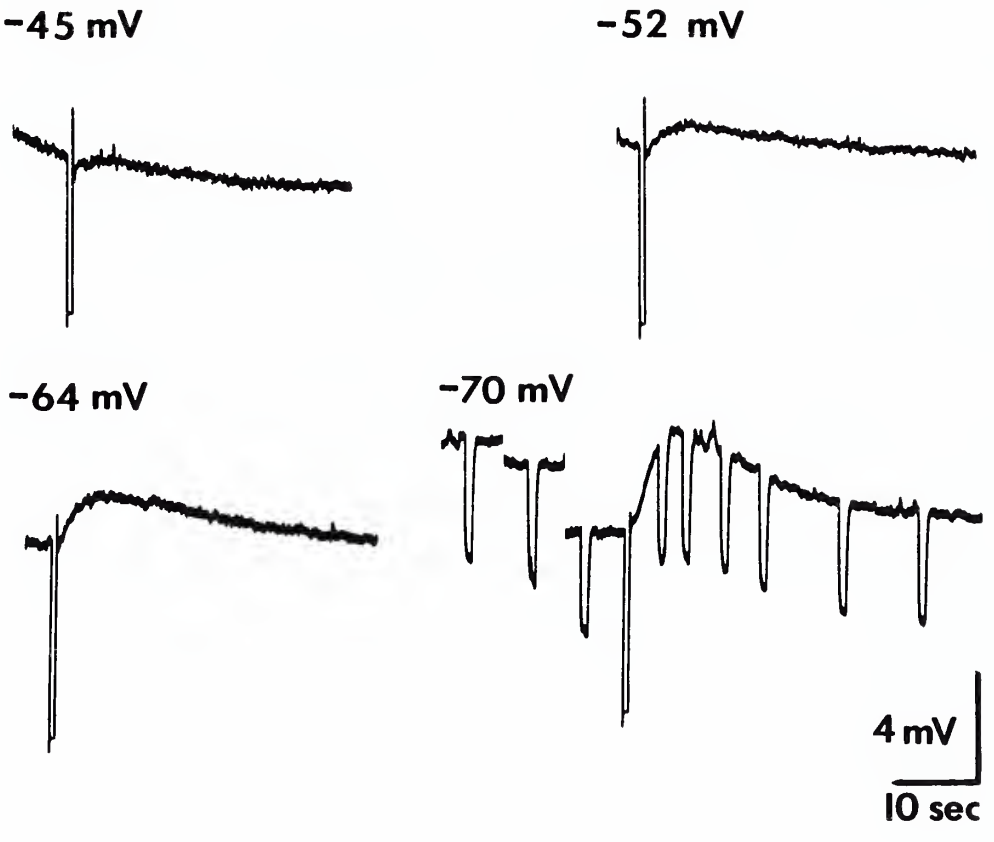


FIGURE 19: Ionophoresis of 1000 nc serotonin onto an A cell which elicited a depolarizing response as well as a more pronounced hyperpolarizing response. The reversal potential of the first component is greater than -52 mv with a peak response at 4 seconds, but there is insufficient information to distinguish between sodium and chloride ion currents. The late component, with peak at 17 seconds, had a reversal potential lower than -70 mv, and was thus mediated by potassium ions. All responses were associated with increased membrane conductance.

5HT 1000 nC

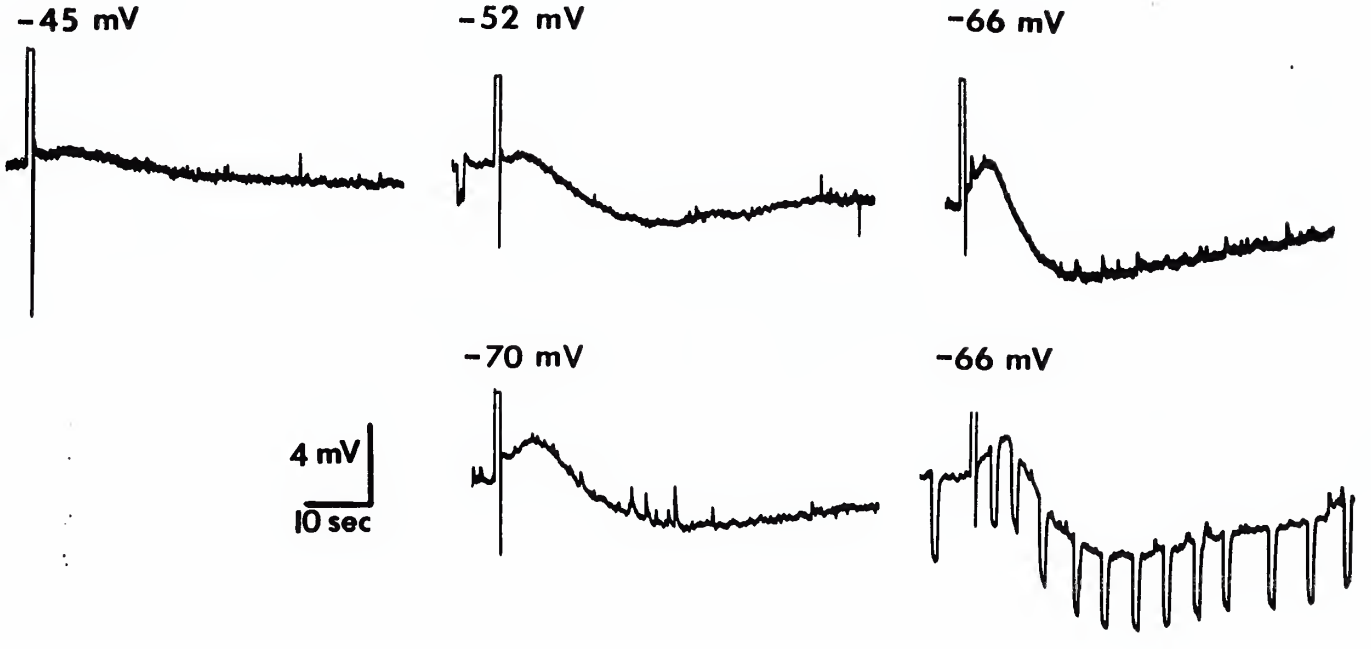


FIGURE 20: Ionophoresis of 1000 nc serotonin onto an A cell which elicited an alpha response. Note the decreased membrane conductance, the late peak response and duration. Reversal potential between -67 and -85 mv.

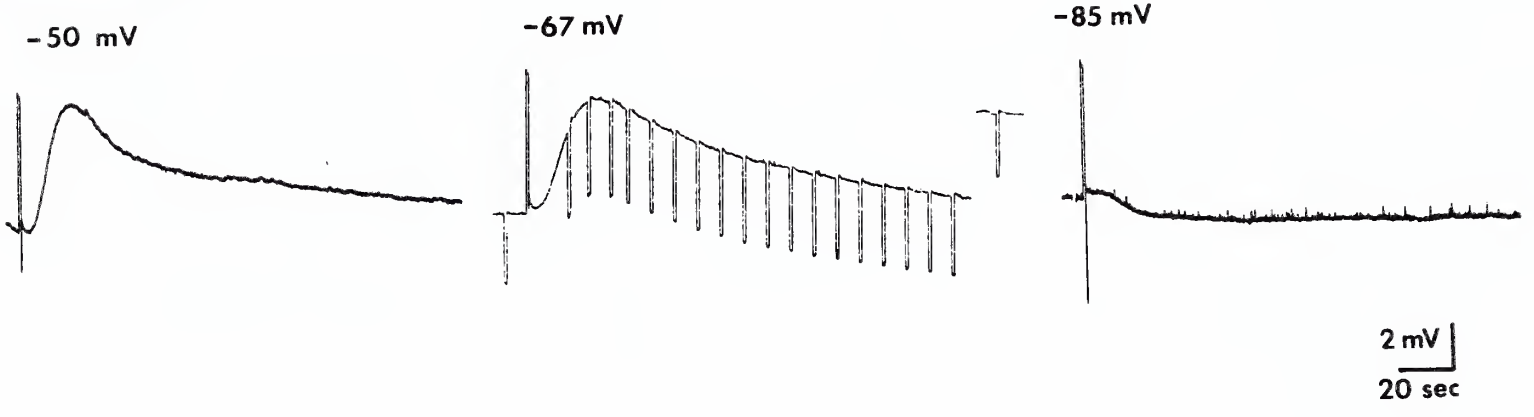

5HT 1000 nC
-50 mV
-67 mV
-85 mV
2 mV
20 sec

FIGURE 21: Ionophoresis of 1000 nc adenosine onto an A cell which elicited a biphasic response. The initial depolarization had a reversal potential greater than -52 mv with a peak response at 1 second. The hyperpolarization had a reversal potential lower than -75 mv with a peak response at 6 seconds. Compare to the serotonergic responses seen on A cells (figure 19).

Adenosine 1000 nC

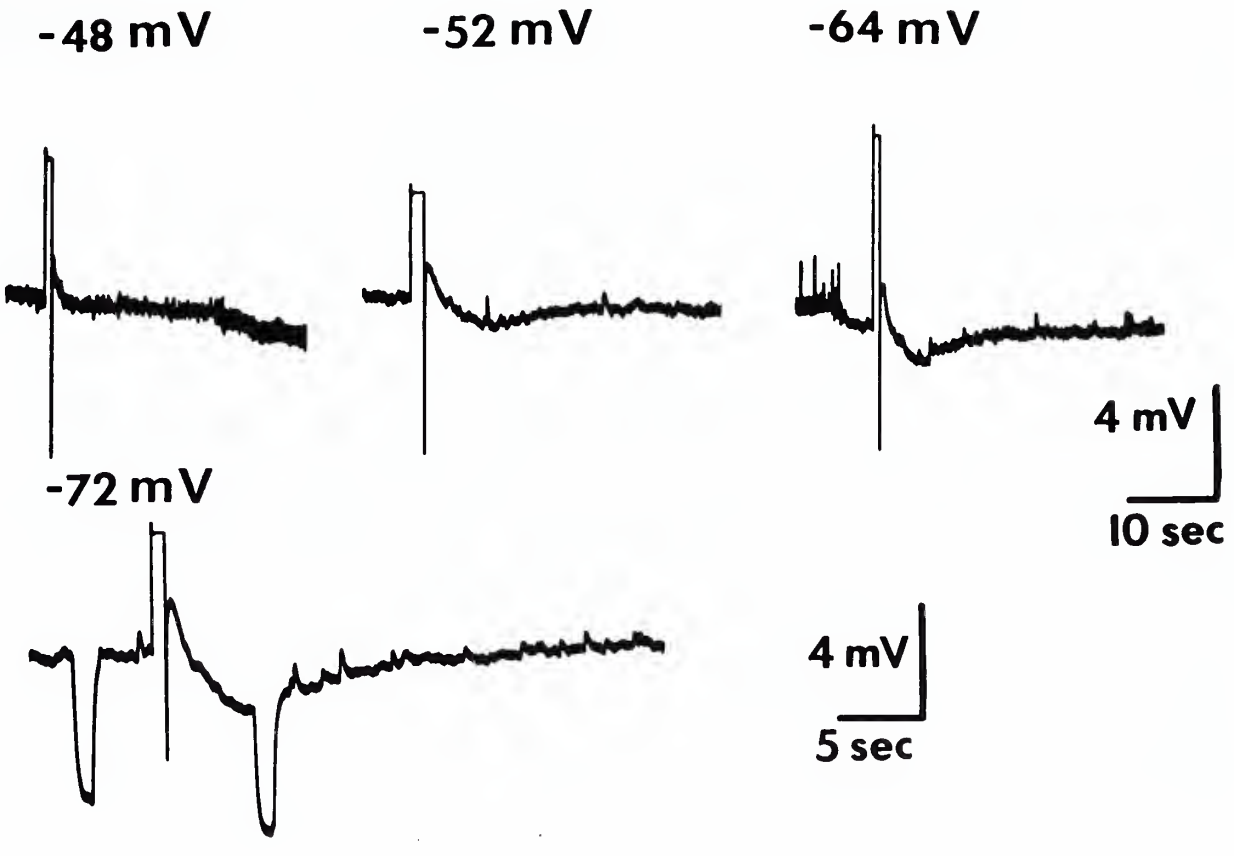


FIGURE 22: Ionophoresis of 100 nc GABA onto an A cell which elicited a biphasic hyperpolarization generated by increased conductance to chloride and potassium ions. The early component reversed between -57 and -65 mv with a peak response at 3 seconds. The latter component, with a peak response at 10 seconds, had a reversal potential below -80 mv.

GABA 100 nC

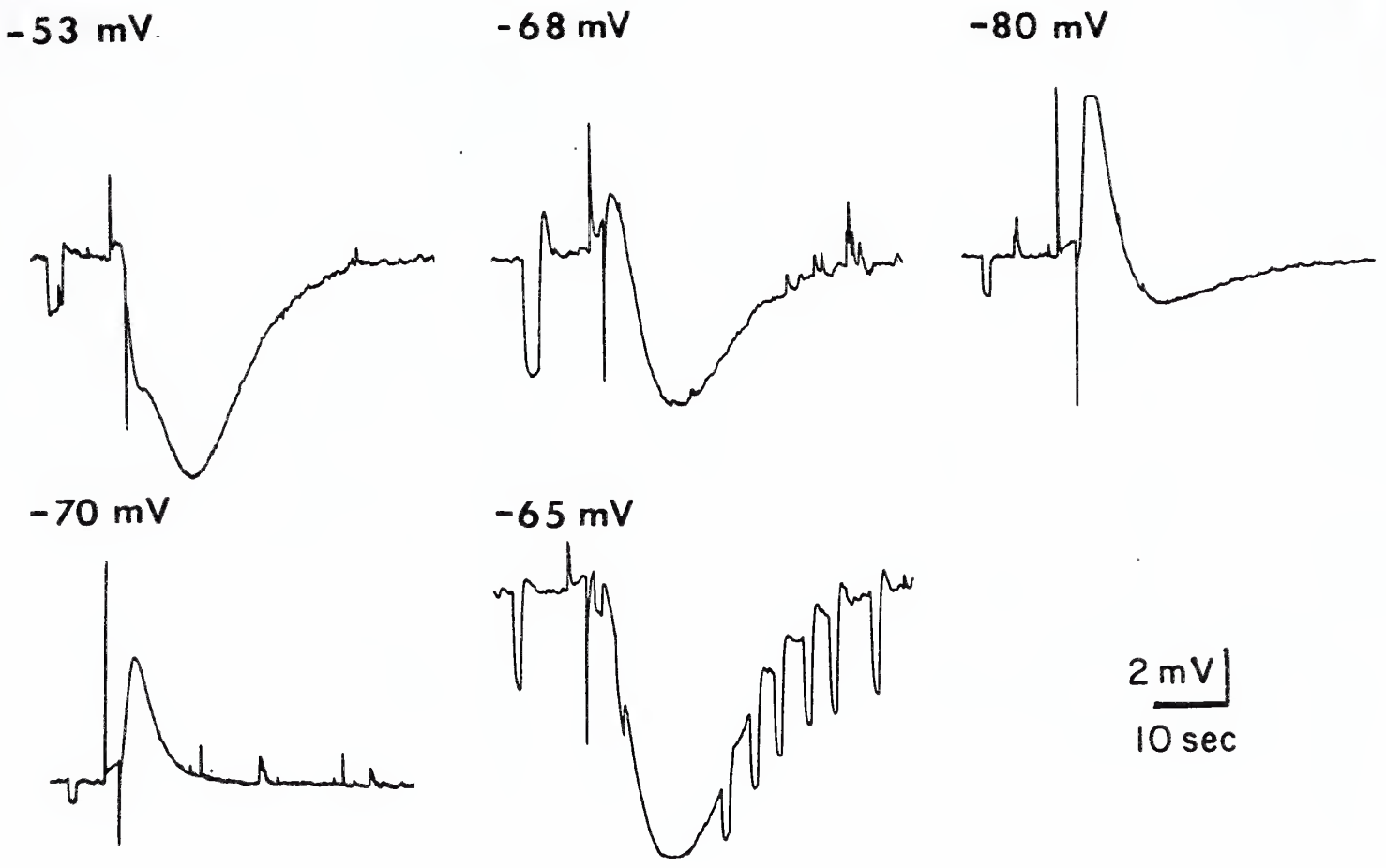
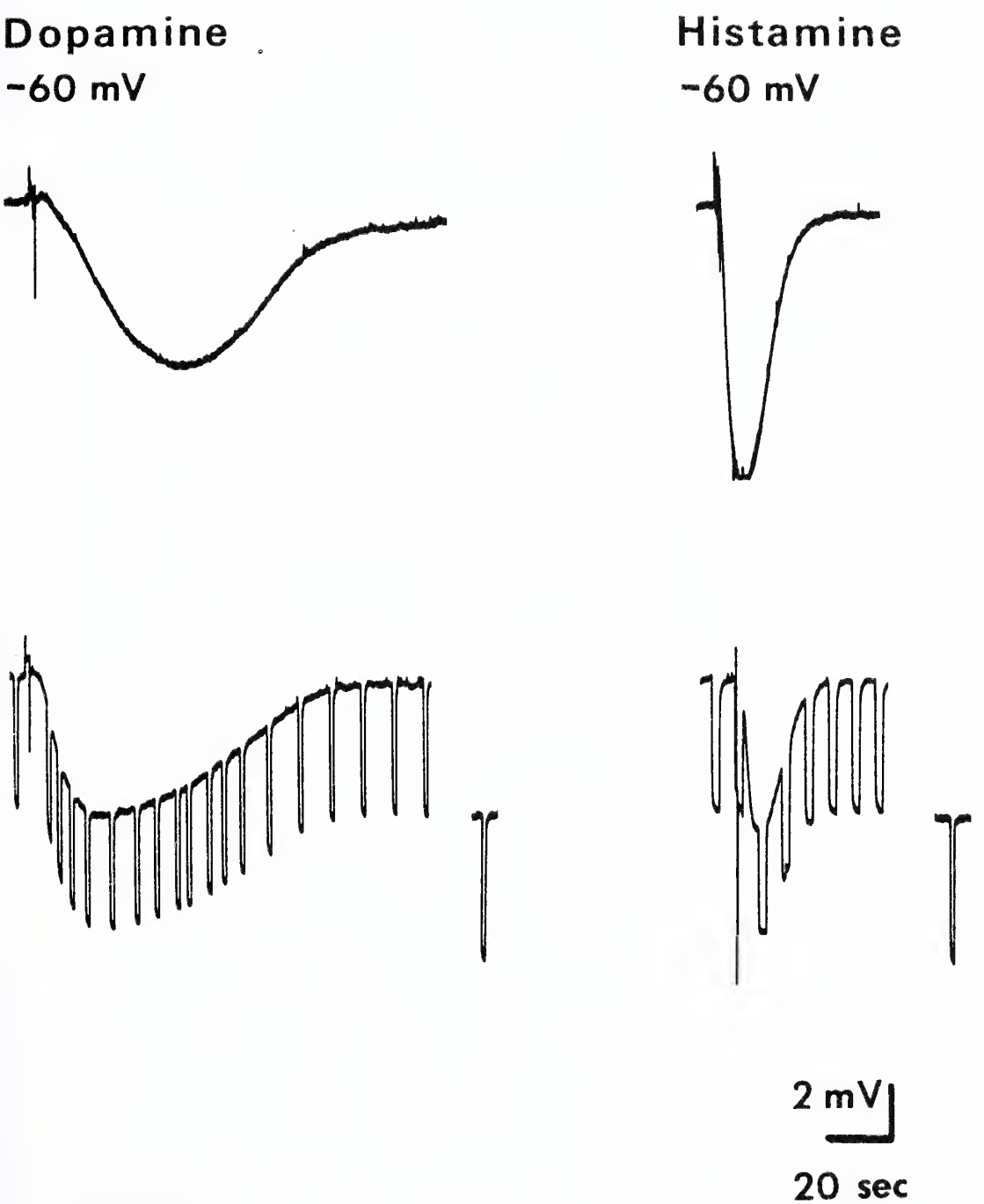


FIGURE 23: Hyperpolarizing responses evoked by dopamine and histamine mediated by increased permeability to potassium ions. Dopamine, 425 and 1000 nc; histamine 425 and 100 nc. Note the marked difference in peak response and decay.


Dopamine
-60 mV
Histamine
-60 mV
2 mV
20 sec

examined, however, exhibited a fast depolarizing response subserved by a sodium conductance increase; this response was rarely seen by itself (figure 24, an A cell upon which dopamine elicited a series of action potentials). Ubiquitous and unvarying, histamine never failed to generate a hyperpolarization of A cell membranes through an increase in potassium ion permeability (figure 23).

Glycine, on the other hand, gave rise to a number of unusual alterations of membrane potential. Briefly presented here, for this matter will be discussed in greater detail below, a slow depolarization could always be found which usually was not associated with an increase in conductance. Occaisonally a biphasic hyperpolarization, subserved by chloride and potassium, was elicited (figure 25, see section III). Evidence for octopamine and phenylethanolamine receptors could not be found.

As with the B cells, individual A cells exhibited a variety of receptors specific for numerous transmitters. For example, one neuron displayed the following distinct collection of responses: acetylcholine and GABA each evoked biphasic hyperpolarizations mediated by chloride and potassium ions; glutamate and aspartate both generated depolarizing responses subserved by sodium, and perhaps chloride; serotonin brought forth a complex series of alterations in membrane potential wrought by increased permeability of sodium, chloride, and potassium ions; adenosine evoked a response similar to that of serotonin; dopamine elicited standard currents mediated by sodium and potassium; histamine gave rise to a potassium generated hyperpolarization; and, finally,

FIGURE 24: Ionophoresis of 500, 500, 50, and 50 nc dopamine onto an A cell which elicted a depolarization mediated by increased permeability to sodium ions. Reversal potential greater than -43 mv, peak response between 3 to 5 seconds. At higher membrane potentials greater quantities of dopamine were needed to generate similar peaked responses or action potentials, an effect of marked membrane rectification which A cells exhibit.

Dopamine

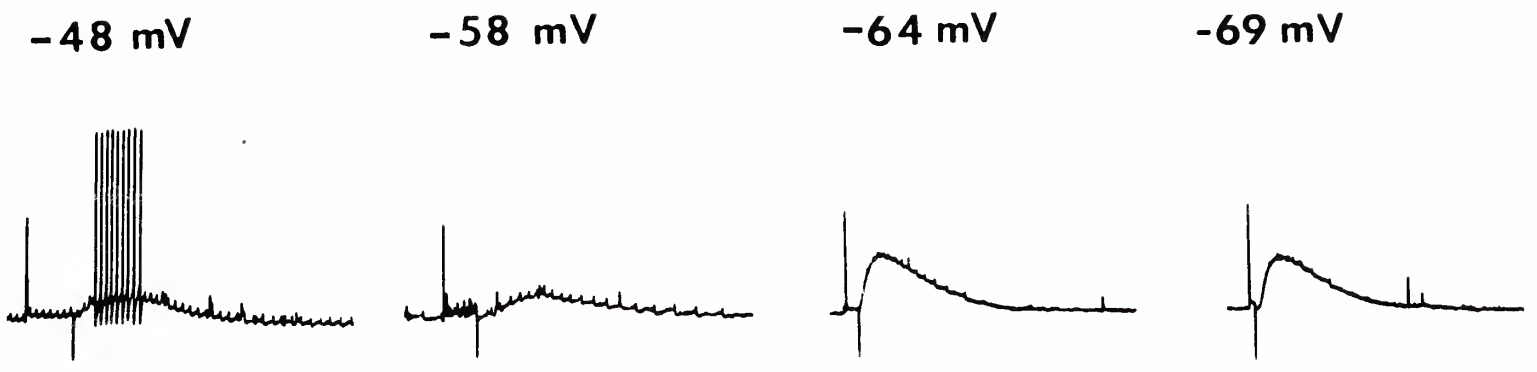
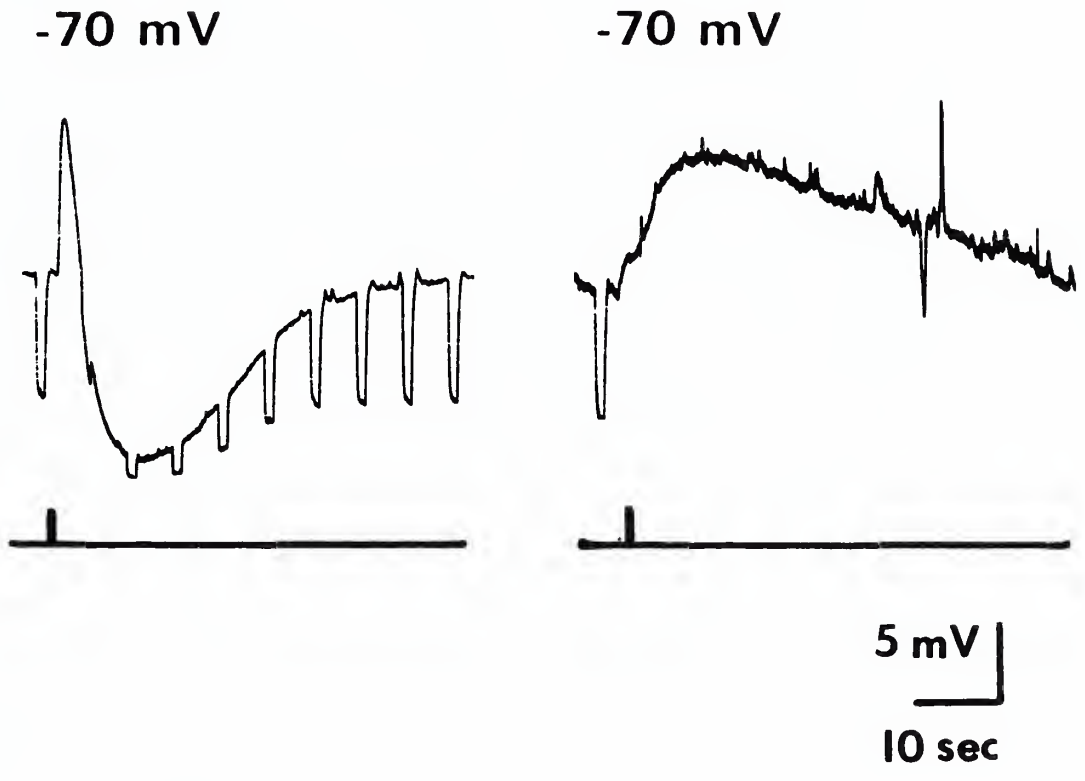


FIGURE 25: Pressure ejection of glycine onto two different locations on an A cell soma which elicited two different responses. At one location a biphasic response, most likely generated by chloride and potassium ions, was produced (note membrane potential was at -70 mv). At a different patch of membrane the slow depolarizing response was seen -- see section III for discussion of the nature of these responses.

Glycine



glycine drew out a slow depolarization and a biphasic chloride and potassium mediated hyperpolarization.

The receptor and ionophore profiles were consistent from cell to cell. The reproducible spectrum of these responses is shown in Table 5 where results from four of the more thoroughly examined cells are presented.

DISCUSSION

A And B Cell Membrane Properties

Both A and B cells manifest delayed and anomalous rectification -- a phenomenon made apparent by using current clamp techniques, and a difficulty that can be avoided by using a voltage clamp. The finding is significant for the analysis of the receptor data since membrane rectification dampens the strength of response at increasingly positive membrane potentials. It is unclear what effect this characteristic of Aplysia membrane has on their ability to receive and process information. Certainly properties of the membrane, and not just the receptor, are crucial to how a given cell will respond to a stimulus (188, 98). It is curious that the peak resistance occurs at a potential midway between the chloride and potassium equilibrium potentials, and that membrane rectification is more pronounced on the more silent and inhibited A cells. This phenomenon may belie the nervous system's penchant for emphasizing the inhibitory message.

B CELL RECEPTORS

TRANSMITTER	NA	CL-	K	OTHER	MULTIPHASIC
ACH		4	1		
DOPAMINE			11		
GABA		8	1		
GLUTAMATE	6	7	5		5
ASPARTATE	1	1			1
HISTAMINE	3	6	1		3
SEROTONIN	A: 5	1	1	A':3;Alpha:1	4

TABLE 3: Table presenting summary of receptor mediated alterations of ionic conductance. For Na+, Cl-, and K+ conductance was increased. GLYCINE results are presented in section III. Table does not include uncharacterized depolarizing or hyperpolarizing responses.

A CELL RECEPTORS

TRANSMITTER	NA	CL-	K	OTHER	MULTIPHASIC
ACH		5	6		5
DOPAMINE	3		9		3
GABA		8	9		8
GLUTAMATE	5	5			5
ASPARTATE	3	3			3
HISTAMINE			12		
SEROTONIN			3	Alpha: 1	

TABLE 4: Table presenting summary of receptor mediated alterations of ionic conductance. For Na+, Cl-, and K+ conductance was increased. GLYCINE results are presented in section III. Table does not include uncharacterized depolarizing or hyperpolarizing responses. There were four depolarizing responses induced by serotonin that were either A responses or mixed Na+ and Cl- responses.

SINGLE A CELL RECEPTOR PROFILES

TRANSMITTER	CELL 1	CELL 2	CELL 3	CELL 4
ACH				
Na+	0	0	0	0
Cl-	#	#	#	#
K+	#	#	#	#
DOPAMINE				
Na+	#	#	#	#
Cl-	0	0	0	0
K+	#	#	#	#
GABA				
Na+	0	0	0	0
Cl-	#	#	#	#
K+	#	#	#	#
GLUTAMATE				
Na+/Cl-	#	#	#	#
K+	0	0	0	0
ASPARTATE				
Na+/Cl-	#	#	0	#
K+	0	0	0	0
HISTAMINE				
Na+	0	0	0	0
Cl-	0	0	0	0
K+	#	#	#	#
SEROTONIN				
A/Cl-	#	#	#	0
A'	0	0	0	0
K+	0	#	#	#

TABLE 5 : Profile of receptors for putative neurotransmitters found on four A cells. {#} denotes presence of receptor mediating the ionic response for the listed transmitter; {0} denotes the response was not elicited. GLYCINE data presented in a later section. There were no receptors found for responses not shown.

A And B Cell Receptors

The spectrum of receptors on B cells sets the limits for the range of action as well as the candidacy of effective presynaptic neurotransmitters -- this knowledge is essential for the identification of the transmitter at the A to B synapse, discussed in the following section, as well as for the messengers at the other B cell synapses. The results of this study also shed light on the nature of the receptors and the responses they mediate.

The interpretation, or identity, of response needs to be addressed. The depolarizations generated by glutamate and histamine on B cells, and serotonin and dopamine on A cells demonstrate that difficulty: the potential changes produced by these transmitters at higher membrane potentials should be more pronounced. Either a property of the membrane or the receptor is responsible. The contribution of membrane rectification minimizes potential change across the membrane the more depolarized the membrane becomes. If this is the explanation then all depolarizing responses should be affected uniformly and to a greater degree on A cells than on B cells. Histamine and glutamate were equally affected on B cells, serotonin less so, but affected nevertheless. On the A cells, the glutamate, dopamine, and serotonin potential changes were markedly affected (the serotonergic response may also be attenuated by a possible biphasic chloride component).

The other possibility is that some responses are not mediated by a single ion or even by two ions utilizing two different channels -- an event which should appear biphasic-- but by multiple ions traversing the

membrane simultaneously. Glutamate and histamine appear to evoke monophasic responses in some cells even though other receptor linked ionophores are known to exist on the same population of cells. The response may be mixed, generated simultaneously by sodium and chloride, or by sodium and potassium. In this instance the reversal potential would rest between the equilibrium potentials of the ions depending upon the mixture of the actors mediating the current. Dual component responses have been described for GABA and serotonin (68, 214). Ion exchange experiments would clarify the matter.

Another question of identity arises when analyzing the adenosine and aspartate responses. Distinct aspartate receptors have been described before yet most aspartate and glutamate receptors cross desensitize (29, 133, 134, 212). Aspartate clearly has the ability to interact with glutamate receptors in other preparations (62, 137, 211). On the cells examined in this study the aspartate responses may not be distinct: they are only found on cells that exhibit glutamate receptors, while they manifest weaker versions of glutamate induced responses. A similar situation has been found for most neurons of the buccal ganglion (134).

One assumption of the ionophore theory is that all ionophores and the currents they mediate should be the same. The different time course, peak latency, and decay of the histamine and dopamine evoked potassium currents in A cells are markedly different (figure 23). If the dopamine receptors are deeper in the neuropile than their histaminergic counterparts, then the rate of diffusion may account for the observed differences. If not, then the A cells present a convenient

preparation for studying the kinetics of different potassium responses (see 7, 33).

Gruol and Weinreich described a histamine induced chloride current on twenty percent of the A cells they examined (75). This study, along with Ascher, did not find the chloride mediated component (7). The differences may be due to the low sample number here, or to the narrow localization of this histamine receptor. Discreet localization is not uncommon, as the dopaminergic sodium receptors described here are only found on small patches of membrane.

The pharamacological sensitivity of serotonin A, A', chloride and potassium responses to curare, bufotenine and desensitization was confirmed (68, 69).

The most relevant finding of this study concerns the association of receptors found on Aplysia neurons. This study reports the existence of two separate populations of neurotransmitter receptors on two distinct populations of cells. In fact, the spectrum of receptors defines the cell, in addition to anatomic and other physiological criteria (55, 96). A and B cells are unique not only in the neurotransmitter receptors they carry, but also in the mixture of ionophores linked to those receptors displayed on postsynaptic membrane. Table 6 summarizes the differences found between the A and B cells.

Receptor mediated responses on A cells tend to be more biphasic than their B cell counterparts. Acetylcholine, dopamine, and GABA activate chloride and potassium channels on A cells, whereas these transmitters

B CELL RECEPTOR PROFILE

TRANSMITTER	PREPONDERANT	FREQUENT	RARE
ACH	Cl		K
DOPAMINE	K		
GABA	Cl		K
GLUTAMATE	Na Cl	K	
ASPARTATE	Na (Cl)		
HISTAMINE	Cl	Na	K
SEROTONIN	A A'		Cl K
OCTOPAMINE	--	--	--
PHENETHAM	--	--	--

A CELL RECEPTOR PROFILE

TRANSMITTER	PREPONDERANT	FREQUENT	RARE
ACH	Cl K		
DOPAMINE	K	Na	
GABA	Cl K		
GLUTAMATE	Na (Cl)		
ASPARTATE		Na (Cl)	
HISTAMINE	K		
SEROTONIN		A/Cl K	K (g dec)
OCTOPAMINE	--	--	--
PHENETHAM	--	--	--

TABLE 6 : Receptor profiles for A and B cells. All responses were associated with increased membrane conductance unless noted to the contrary. () denotes a contributing component to the primary response. "g dec" denotes decreased conductance. Precise identity of the serotonin A/Cl response is not known. PHENETHAM is phenylethanolamine. ACH is acetylcholine.

open chloride, potassium, and chloride gates, respectively, on B cells. Some receptors are held in common, as for glutamate and serotonin excitatory receptors -- although glutaminergic potassium ionophores are

more common on B cells. The other principal difference occurs with histamine: on B cells histamine gives rise to sodium and chloride currents, however on A cells only hyperpolarizing potassium gates are opened.

Uniformity of transmitter profile is greater on A cells than on B cells. This may be because B cell receptors are less accessible, lying more deeply in the neuropile. Or, it may be that B cells are less homogeneous than previously thought. The receptor profiles reflect the function of these cells in Aplysia. A cells are silent, most input is inhibitory (56, 58), and almost all A cell receptors mediate hyperpolarization. There is a preponderance of potassium receptors on A cells not found on B cells. In addition, many receptors are linked to chloride ionophores. Curiously the A cell resting membrane potential is close to the chloride equilibrium potential as is the peak resistance of the cell membrane. Jahan-Parwar and Fredman described input that brought the membrane potential to rest under the chloride equilibrium potential for substantial periods of time; they also noted that much of the spontaneous synaptic input to the A cells activated chloride channels (88). It all fits. Chloride is the means by which a cell may be brought to moderation, a means by which the general state of excitability is dampened, and, hence, receptivity to input is modulated. On the other hand, mixed input is sent to B cells, and B cell receptors are a mixed lot. One would expect this, however, as B cells are generally more excitable. The general preponderance of inhibitory receptors on A and B cells belies one of the essential priniples of neural organization and function. Brains are inhibitory by nature.

One striking feature of receptor organization demonstrated on A and B cell somata is the multiplicity of neurotransmitters that generate the same response by the same changes in permeability to the same ions. Are all these receptors on A and B cells physiological? The evidence suggests they are, for if the same receptors were produced by all cells then all cells should carry the same complement of receptors. The variety of receptors reflect the many different messengers used by the presynaptic neurons which are unique for each type of cell.

Research over the previous twenty years has reshaped the early work of Dale and his successor, Eccles. Dale postulated that neurons release the same transmitter at all terminals. Eccles then proposed a series of postulates that defined cellular function and ionic specificity: first, the presynaptically released transmitter will mediate the same action at all synaptic terminals; second, the transmitter will open one type of ionic gate (63). These postulates did not prove to be true for molluscs, or for mammals either.

One presynaptic neuron, releasing one neurotransmitter, can elicit multiphasic psps; indeed, that same neuron may evoke different combinations of response at different terminals onto different postsynaptic neurons (13, 69, 103, 104, 193, 204, 205). This is possible because distinct receptors for the same transmitter may guard many ionophores, a fact known since Kandel and then Kehoe presented their work on acetylcholine receptors more than ten years ago (95A, 103, 104). The A cells may be added to the other known presynaptic neurons which elicit multiphasic psps: L10, the MGC, LC1, LC2, and numerous pleural cells.

Ionic and, hence, functional specificity resides on the postsynaptic membrane -- the presynaptic neuron simply releases the transmitter. It is the complement of receptors, and the ionophores to which they are bound, that is relevant (13, 38, 69, 95A, 113). Therefore, postsynaptic membrane - receptor studies gain in significance.

Not only may one neuron display multiple responses to one transmitter, but the same neuron may respond to many transmitters in numerous ways. R15 is known to have receptors for seven different substances mediating three different ionic responses (29). The MGC, examined in Aplysia and Helix, carries receptors activated by three transmitters (185). Many neurons respond to two subtances (see 213, for example). Here, two populations of neurons each exhibiting a distinct set of responses evoked by numerous transmitters are described. The responses are consistent: A cells repeatedly respond to eight messengers! Although A and B cells respond to the same transmitters, what IS distinctive is the proportional representation of the different ionophores for each neural population. It follows that the anatomic and temporal spacing of receptors results in unique integration of input (8, 29).

Postsynaptic specificity explains, in part, why there are a multitude of receptors on nerve cells. It explains a striking feature of molluscan and mammalian nervous systems: the number of transmitter candidates and the plethora of responses generated by each messenger. It would seem that variation and integration of neural signals could take place with fewer transmitters given the diversity of receptors.

Yet, the number of messengers and ionophores greatly extends the realms of information processing and the spectrum for modulation. The variety of receptors also allows one neuron, utilizing one transmitter, to supervise more readily regulation of antagonistic follower neurons. Economy of purpose and economy of space is achieved (157). Conversely, a greater number of higher order neurons may utilize a common population of follower neurons for completely different purposes (see also 124, 114). The possibilities and the flexibility inherent in the system is fantastic.

As more and more inputs onto the A and the B cells are discovered, and as the transmitters serving at those synapses are identified, a greater understanding of the events that occur on the membrane will be attained. As events at the membrane are understood, the underlying organizational properties will become more clear, and the essence of behavior will be discerned.

II. IDENTIFICATION OF THE TRANSMITTER AT THE A TO B CELL SYNAPSE

The A to B cell circuit meets the criteria established by Berry and Penreath often used to define a monosynaptic connection (11). There is a one-to-one relationship at constant latency between the presynaptic stimulus spike and the postsynaptic potential (psp). The psp persists in elevated Ca++ sea water, and is enhanced by injection of TEA into the A neuron (43, 56, 77, 130).

The postsynaptic potential is excitatory, although occasionally a biphasic eipsp is found (43, 44). Voltage clamp experiments, difficult because the synapse is a fair way from the soma, suggest that the excitatory response is mediated by sodium (130). The epsp fatigues readily, albeit not completely, with repeated stimulation (43, 44).

Preliminary microchemical analysis of the A neurons failed to detect significant quantities of acetylcholine, serotonin, GABA, histamine, or octopamine. Concentrations of glutamate, aspartate, taurine, and glycine are the same as found in other pigmented neurons (130). The biochemical data does not support the candidacy of any single messenger, nor does it preclude synthesis of the transmitter in the terminals.

Agonist and antagonist perfusion studies present mixed findings. The psp was not sensitive to curare, atropine, hexamethonium, strychnine, eserine, 7-methyltryptamine (7-MT), LSD-25 (all at 10^{-3} M), TEA (2×10^{-4} M), PTMA (5×10^{-4} M), or LSD-25 in prior studies (43, 44, 77). Nor did the psp respond to quisqualic acid, a glutamate agonist in

Helix (30, 137), or to glutamate (10^{-5} to 10^{-3} M), whereas these compounds desensitized the ionophoresed response to glutamate (130). Unfortunately, specific glutamate antagonists do not exist against which the sensitivity of the psp could be tested. Perfusion of serotonin, 7-MT and LSD-25 had neither an effect on the psp, or on ionophoresed serotonin (77). Hinzen and Davies found bufotenine decreased both the psp and the ionophoretically induced serotonin depolarization thus suggesting that the epsp reflects A' serotonin receptor activation. They concluded that serotonin is the neurotransmitter at the synapse; McCamen, however, could not reproduce their results, and thus did not support their conclusions (43, 44, 77, 130).

Hinzen's and Davies' results were achieved at concentrations of 10^{-3} M bufotenine, orders of magnitude greater than those reported by Gerschenfeld and Paupardin-Tritsch (69, 145). They also claim they achieved their results after 10 to 15 minutes perfusion and without any changes in membrane resistance (77). 10^{-4} M bufotenine reportedly augmented the psp and the ionophoretic response evoked by serotonin (44). On the other hand, McCamen observed that the same concentration of bufotenine blocked the ionophoretic response without affecting the psp (130).

In sum, there is little evidence to support acetylcholine as the transmitter. Serotonin, by activating A' receptors, is a disputed candidate. And the evidence against glutamate is not overwhelming. This study attempts to resolve the controversy.

RESULTS

A And B Cell Synaptic Connections

A brief survey of A and B cell monosynaptic connections was made which confirmed previous reports (56, 58, 77). The criteria were similar to those used by Jahan-Parwar and Fredman (56): constant latency and a one to one following of presynaptic spike to postsynaptic potential. Of 147 A to B synapses examined a positive connection was found on 83 occaisons, a synaptic response could not be elicited 56 times, and in 8 preparations responses were ambiguous. With greater experience the vast proportion of cells examined were found to have synaptic connections. A cells innervated many B cells; conversely, B cells received input from many A cells. Contralateral studies were not performed.

Of 54 B to B synapses tested confirmation of a synaptic connection was found on 9 occaisons, no response could be discerned between 40 pairs of cells, and results were unclear 5 times. Of the 6 B to A circuits tested, one synapse was found and the other 5 were negative. Both of two A to A tests were negative; both of two Av to B tests were positive (Table 7).

A To B Synapse Characteristics

The monosynaptic nature of the A to B synapse has been ambly proved by Hinzen and Davies (77) as well as by Jahan-Parwar and Fredman (56); no further discussion is needed. The postsynaptic potential was

SYNAPTIC CONNECTIONS

PRESYNAPTIC NEURON	POSTSYNAPTIC NEURON	
	A	B
A	0/2	83/139
B	1/6	9/49
Av	---	2/2

TABLE 7 : Monosynaptic connections among A, B, and Av cells. Number Positive Results/ Total Number Tested Ambiguous data is not included.

invariably excitatory; initially biphasic eipsps were often encountered, but the inhibitory component rapidly disappeared by the third or fourth stimulus. The excitatory potential also decreased from 40 to 60% of its initial amplitude. After the initial decrement the epsp did not fatigue further (figure 26). The magnitude of the excitatory potential and the appearance of the inhibitory potential were restored by rest. Rapid stimulation resulted in excitatory summation of synaptic potentials (figure 27).

A To B Synapse Perfusion Experiments

The cerebral ganglion was perfused with a series of agonists and antagonists to ascertain the pharmacological identity of the receptors mediating the psp at the A to B cell synapse. All compounds tested, with the exception of bufotenine, did not affect synaptic transmission.

Curare, the "non-specific" fast sodium and chloride channel blocker in concentrations as high as 3×10^{-4} M, did not have any affect on the

FIGURE 26: The presynaptic neuron is an A cell, the postsynaptic neuron is a B cell (A to B synapse). Note the initial eipsp which rapidly fatigued to become an epsp; note, too, the ensuing partial fatigue of the epsp.

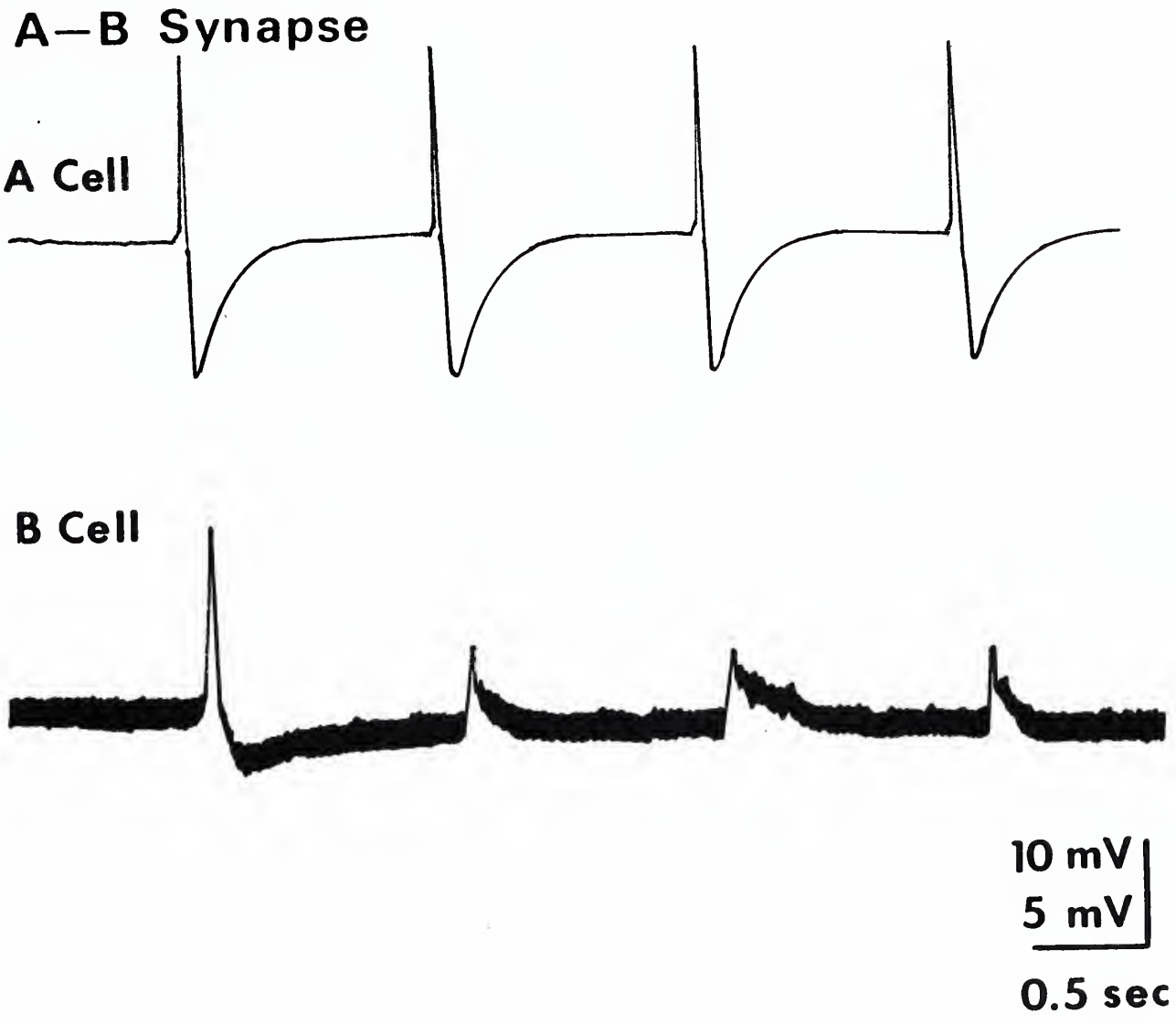
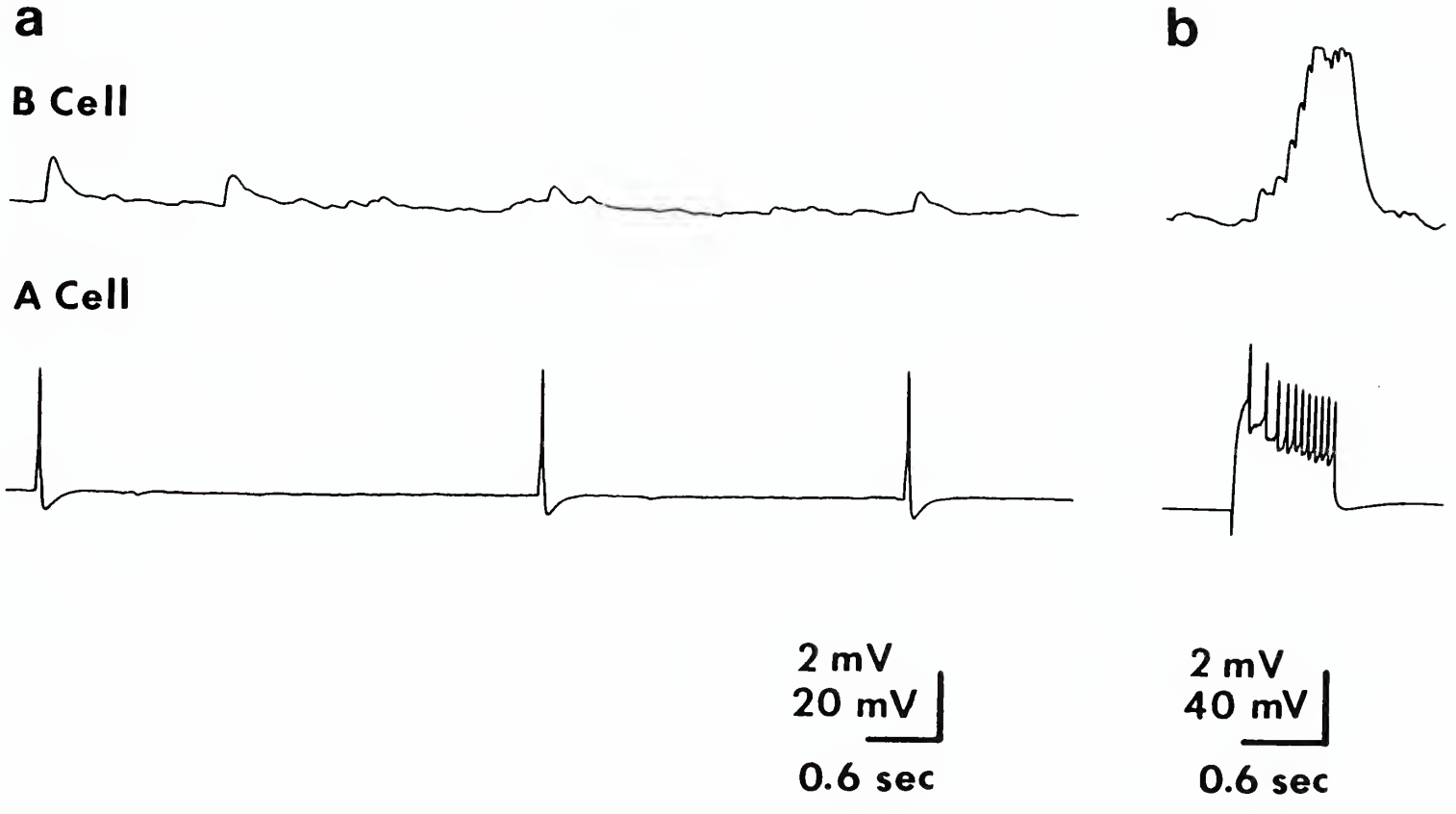

A—B Synapse
A Cell
B Cell
10 mV
5 mV
0.5 sec

FIGURE 27: A to B synapse. Note the fatigue of the epsp as well as the temporal summation of synaptic potentials.

A—B Synapse



psp. Nevertheless, curare effectively eliminated serotonin A and chloride mediated responses and failed to affect the A' depolarization. Strychnine (5×10^{-4} M), another "non-specific" fast sodium and chloride channel blocker, also did not affect the psp. The histamine antagonists burimamide (3×10^{-4} M) and pyrilamine (3×10^{-4} M) never altered the psp.

In similar fashion, the specific serotonin antagonists 7-methyltryptamine (3×10^{-4} M) and 5-methoxygramine (3×10^{-4} M) did not attenuate the synaptic potentials. Desipramine, reported to inhibit the uptake of serotonin and thus potentiate postsynaptic responses (65), also did not alter either the amplitude or duration of the psp when perfused at concentrations ranging from 10^{-6} to 10^{-5} M (figure 28). Bufotenine, on the other hand, influenced the psp in a bizarre fashion.

Bufotenine, 10^{-5} M, abolished ionophoretically induced A, A' and potassium ionophore linked receptor responses without altering membrane resistance, yet did not modify the A to B psp. However, bufotenine, 10^{-4} M, in four of five experiments, caused noisy baseline membrane potential, and concomitantly increased the membrane resistance. At the same time, psp amplitude was augmented, and frequently became a pronounced eipsp (figures 29 and 30). The alteration in magnitude and character of the psp occured after ten to fifteen minutes of perfusion; only once, following one hundred minutes of perfusing bufotenine 10^{-4} M, did the psp become smaller (figure 30). Perfusing the synapse with higher concentrations of bufotenine (10^{-3} M) gave similar results with a more rapid sequence of effect. Alterations in membrane resistance and

FIGURE 28: A to B synapse. Perfusion of desipramine (DMI) 10^{-6} M did not affect the psp or membrane resistance. The tracings which record resistance pulses were run at 2 cm/min.

A—B Synapse

Control

DMI (10^{-6} M)
35 m

B Cell

A Cell

2 mV
20 mV

0.6 sec

FIGURE 29: A to B synapse. Perfusion of bufotenine (10^{-4} M) which, at 30 minutes, markedly increased membrane resistance and concomitantly brought forth the compound nature of the epsp and augmented background activity. Prior resistance pulse tracing at 5 cm/min.

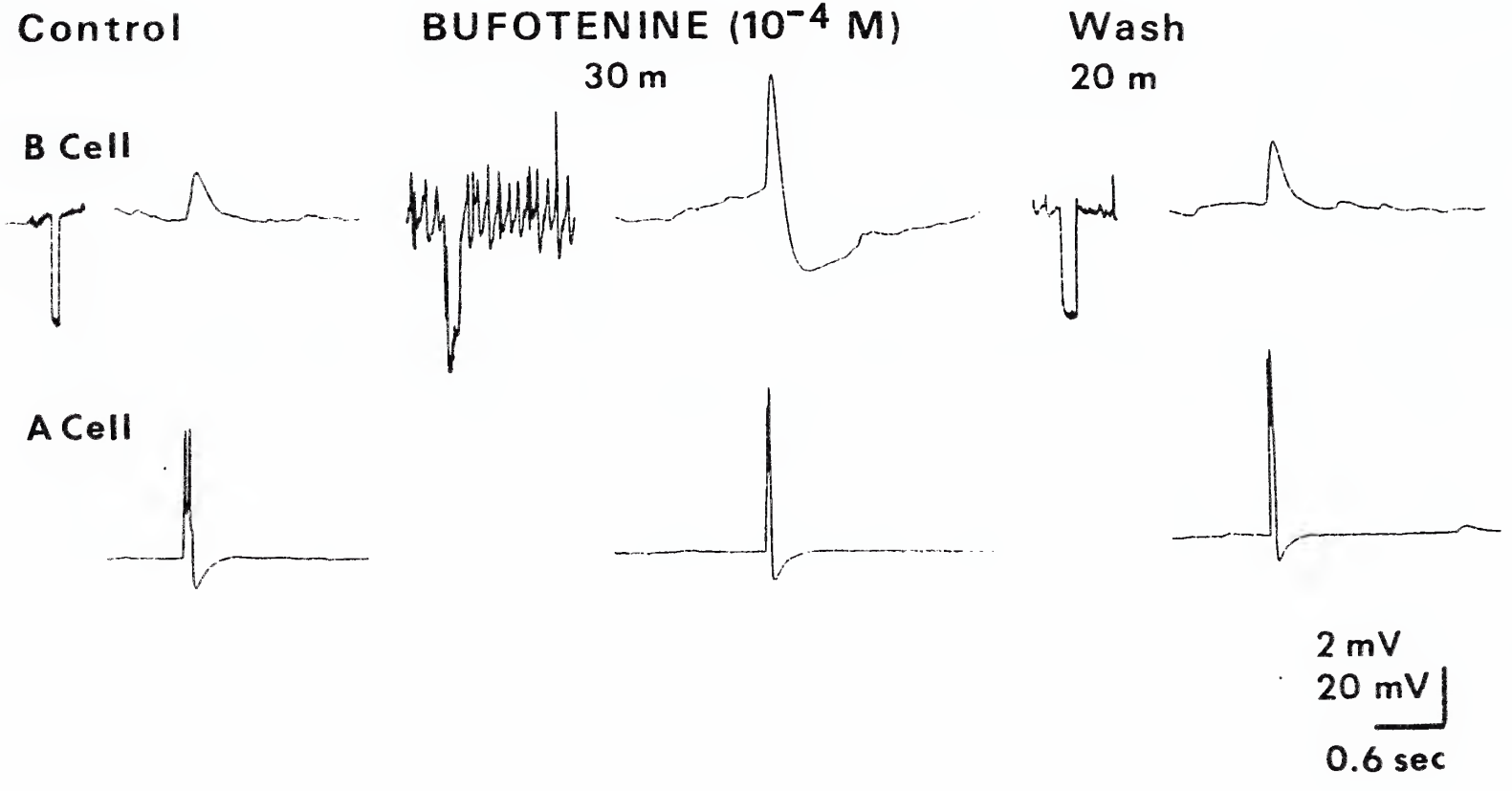
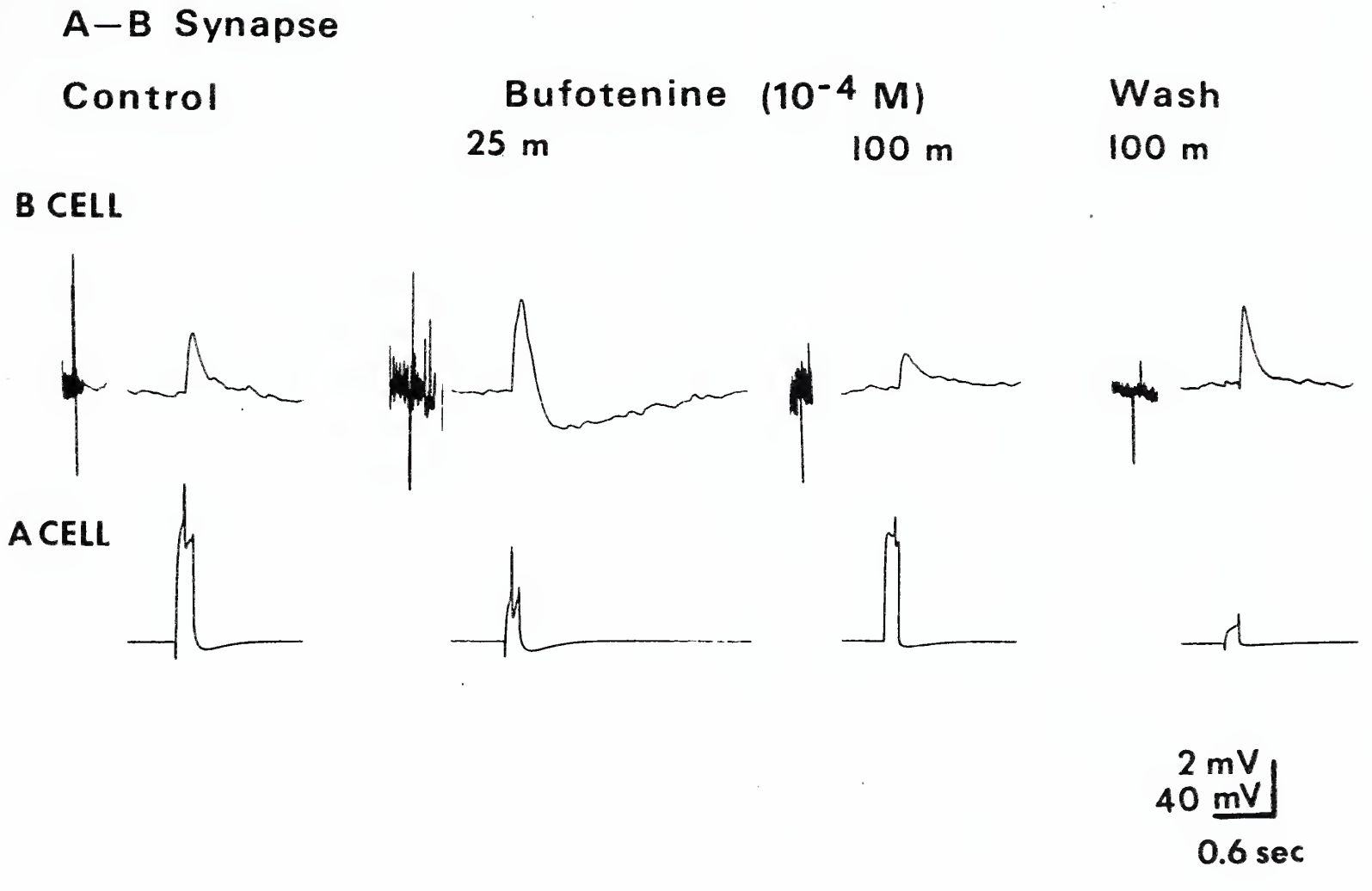

Control
BUFOTENINE (10^{-4} M)
30 m
Wash
20 m
B Cell
A Cell
2 mV
20 mV
0.6 sec

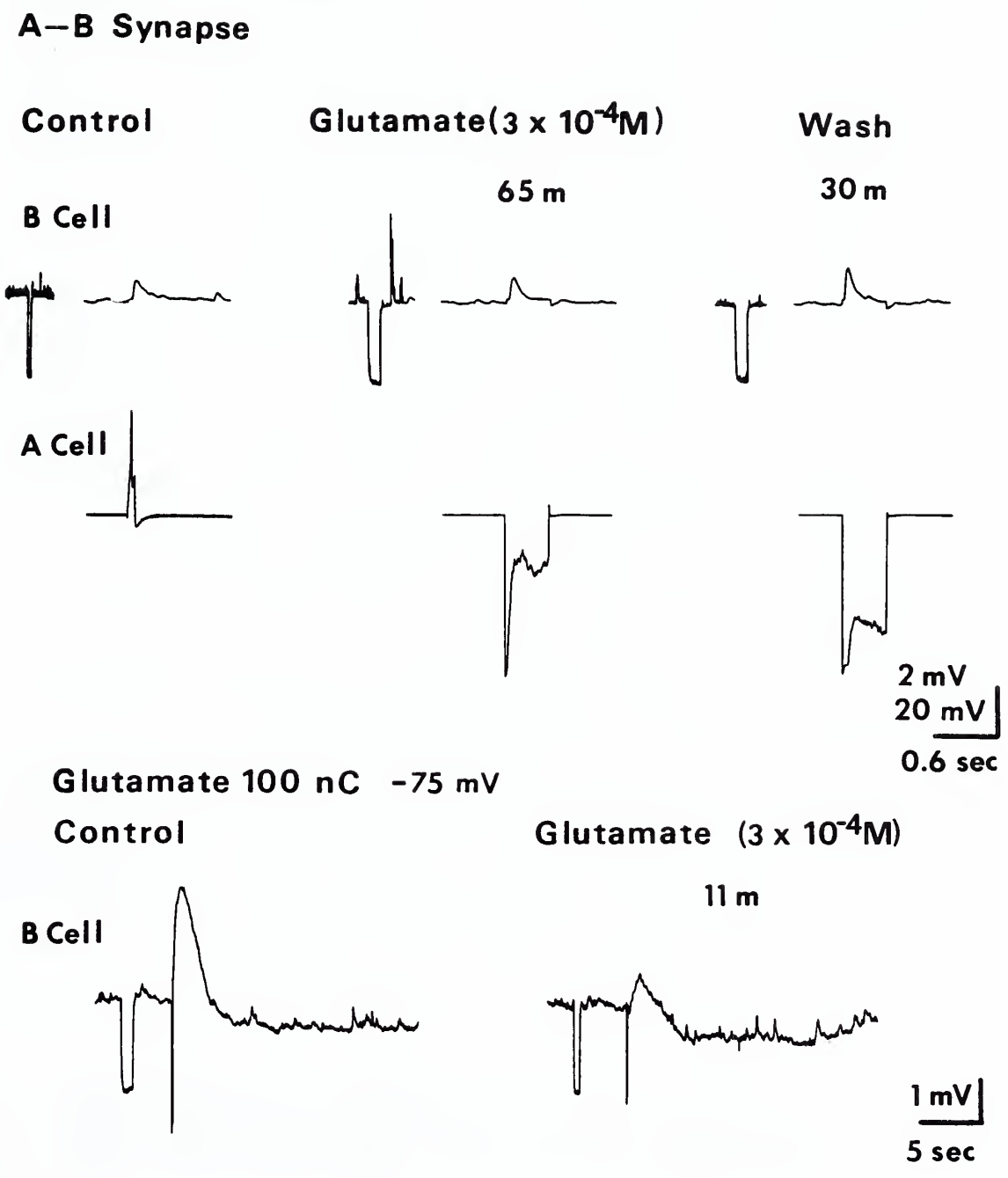
FIGURE 30: A to B synapse. Perfusion of bufotenine (10^{-4} M) which, at 25 minutes, increased membrane resistance, brought forth a biphasic psp, and augmented background activity. After 100 minutes of perfusion (!) the resistance decreased as did the psp. Prior resistance pulse tracing at 2 cm/min.


A—B Synapse
Control
Bufotenine (10-4 M)
25 m
100 m
Wash
100 m
B CELL
A CELL
2 mV
40 mV
0.6 sec

background activity were typically more pronounced. During one experiment, the effect on membrane resistance was a slight increase without any real affect on the psp.

Bathing the ganglion with 3×10^{-4} M serotonin did not have any affect on the psp even though this desensitized A, but not A' responses. The glutamate response desensitized as well, as demonstrated in figure 31. Perfusion of glutamate attenuated a glutamate evoked depolarization, yet did not desensitize the psp (3×10^{-4} M). Aspartate, at low concentrations , augments glutamate mediated responses; at higher concentrations aspartate cross desensitizes glutamate receptors (211). No affect could be found on the psp by perfusing the preparation with aspartate in concentrations ranging from 5×10^{-6} to 3×10^{-4} M. Table 8 summarizes the results of the agonist and antagonist perfusion experiments.

FIGURE 31: Upper tracing: A to B synapse. Perfusion of glutamate (3×10^{-4} M) did not desensitize the psp. Lower tracing: Ionophoresis of 100 nc glutamate onto a B cell elicited a typical depolarizing response that was desensitized by perfusion of glutamate (3×10^{-4} M), or by repeated ionophoresis of glutamate (not shown).


A—B Synapse
Control
Glutamate(3 x 10-4M)
Wash
65 m
30 m
B Cell
A Cell
2 mV
20 mV
0.6 sec
Glutamate 100 nC -75 mV
Control
Glutamate (3 x 10-4M)
11 m
B Cell
1 mV
5 sec

EFFECTS OF AGONIST AND ANTAGONIST PERFUSION ON THE A TO B CELL SYNAPSE

ANTAGONIST/ AGONIST	CONCENTRATION (M)	N	EFFECT ON PSP	EFFECT MEM RESISTANCE
CURARE	2.5 TO 3 x 10^{-4}	6	NONE	NONE
STRYCHNINE	3 TO 5 x 10^{-4}	3	NONE	NONE
5-MG	1.3 TO 3 x 10^{-4}	2	NONE	
	1 x 10^{-5}	1	NONE	
7-MT	3 x 10^{-4}	1	NONE	
BURIMAMIDE	3 TO 3.5 x 10^{-4}	3	NONE	
PYRILAMINE	3 x 10^{-4}	2	NONE	
DESIPRAMINE	1 x 10^{-5}	3	NONE	
	1 x 10^{-6}	3	NONE	
BUFOTENINE	1 x 10^{-3}	2	AUGMENTED (2)	INCREASED
			BIPHASIC (2)	
	1 x 10^{-4}	6	AUGMENTED (5)	INCREASED (5)
			BIPHASIC (5)	DECREASED (1)
			DECREASED (1)	
	1 x 10^{-5}	1	NONE	NONE
SEROTONIN	3 x 10^{-4}	3	NONE	
GLUTAMATE	3 x 10^{-4}	4	NONE	
ASPARTATE	3 x 10^{-4}	1	NONE	
	1 x 10^{-5}	1	NONE	
	1 x 10^{-6}	2	NONE	

TABLE 8 Summary of agonist and antagonist perfussion experiments for the A to B Synapse preparation 5-MG = 5 methoxy gramine; 7-MT = 7 methyltryptamine

DISCUSSION

A And B Cell Connections And Synaptic Characteristics

The results of this study confirm the findings of Jahan-Parwar and Fredman (56, 58) and those of Hinzen and Davies (77) concerning the properties and connections of A and B cells. Both groups of investigators found that all A cells formed synapses with all B cells. Although not found to occur for every set of cells tested, these discrepancies are explained by technique and health of the cells studied. Greater experience with the dissection resulted in greater success in tracing monosynaptic connections. The survey was not undertaken with strict criteria, and thus the results are only supportive. They were designed only as a quick survey of the potential extent of synaptic organization.

This investigator was not as successful as Jahan-Parwar and Fredman in finding B to B synapses -- though a healthy number were identified. These studies did not attempt to determine whether or not contralateral connections exist -- a point of controversy between Jahan-Parwar's and Hinzen's groups. B to A synapses have not been described in the literature: the sole finding here merits further investigation. As previously noted, no researcher has ever found evidence for an A to A cell synapse. The success of demonstrating Av to A cell connections is promising; the small size of the Av cells, however, makes study of this synapse difficult (see also 58).

McCamen is the only investigator who has previously mentioned the existence of an initial eipsp at the A to B cell synapse -- an observation confirmed by this study (130). The A to B synapse joins a growing selection of multiphasic postsynaptic potentials found in Aplysia -- perhaps it is the rule (39, 69, 102, 103, 104, 114, 124, 145, 205, 206). The strength of the initial psp coupled with the biphasic nature of the potential emphasizes the importance placed on the first incoming signal. It also means that the transmitter is acting at two receptor ionophores on the postsynaptic membrane. The partial fatigue of synaptic potentials is similar to the character of the J and K cell to B cell synapses (166). The fatigue of this message is thought to underlie habituation of the tentacle withdrawal reflex (166).

The A To B Cell Synapse Messenger

Of the numerous neurotransmitters found in the Aplysia nervous sytem, many may be dismissed as the messenger of the A cells. Octopamine and phenylethanolamine either do not evoke responses on B cells, or do not exist in suspiciously high concentrations in A cells (130). Dopamine and GABA, are also not found in significant quantities in A cells (130), and, more importantly, do not evoke excitatory responses on B cells.

Acetylcholine, too, can not be the transmitter. This study failed to find any excitatory induced responses by acetylcholine. McCamen could not find either excitatory responses on B cells, or acetylcholine in A cells (130). Perhaps the depolarizing responses described by Hinzen and Davies (77) are mediated by chloride since many of their

experiments were conducted at -70 mv, a membrane potential below the chloride equilibrium potential. Debate aside,

the acetylcholine antagonist studies by Hinzen and Davies together with the negative results of curare and strychnine perfusion presented here eliminate acetylcholine from contention.

Histamine is a potential candidate as it gives rise to varied responses some of which are sodium mediated depolarizations. Yet, the psp is unaffected by the histamine antagonists pyrilamine and burimamide. In addition, the non-specific channel blockers curare and strychnine would abolish a histamine mediated synaptic potential. Histamine, too, is not found in high concentrations in the presynaptic neuron (130).

There is much evidence to support serotonin. Both A and A' excitatory responses can be found. Since strychnine and curare do not have any effect, and because the psp does not desensitize when washed with serotonin, then if the response is due to serotonin the psp must be mediated by an A' receptor. The only evidence favoring serotonin comes from Hinzen and Davies who observed an augmentation of the psp by bufotenine at 10^{-4} M, and the abolition of the psp at 10^{-3} M (44, 77). A repeat of their studies here, however, has not given all the same results.

The results of bufotenine perfusion are disputed. McCamen could not demonstrate an effect with 10^{-4} M bufotenine (130). The results of this study find only a decrement of the psp, not abolition, and then only rarely; more often, no effect can be discerned even at 10^{-3} M. The

most common effect of bufotenine, usually at 10^{-4} M, is enhancement of the psp. In fact, the augmentation is so great that the biphasic nature of the synaptic potential is brought forth. Nevertheless, such effects are accompanied by greater excitability of the postsynaptic neuron, and an increase in membrane resistance. The effects of bufotenine, then, may be explained by secondary effects on the membrane rather than on the receptor. The extrasynaptic events may be a product of direct interaction between bufotenine and the membrane, or, alternatively, they may be a consequence of altered synaptic activity throughout the ganglion caused by the antagonist. These experiments were not performed in high Mg++ seawater which would guard against the latter possibility. This phenomenon demonstrates how important secondary membrane effects may be in modulating synaptic reception of signals.

Additionally, the results of Hinzen and Davies, and some of those here, were achieved at tremendous concentrations of bufotenine and over considerable time. Although the A' receptors are located deep in the neuropile, and such synapses are often snugly enfolded by layers of tenacious glial sheath, Gerschenfeld et al. succeeded in abolishing serotonin mediated synaptic potentials in Aplysia with concentrations of bufotenine orders of magnitude less than 10^{-4} M [10^{-6} to 10^{-5} M (69, 145)]. Those same receptors deep in the neuropile, which are activated by ionophoresed serotonin are inhibited rapidly by bufotenine 10^{-5} M (see also 69, 145).

There are two other pieces of evidence that argue against serotonin. First, desipramine, which augments serotonin generated potentials (38,

69, 144), did not affect the psp. Second, McCamen was unable to isolate serotonin from the A cells in quantities that approach those found in proven serotonergic neurons (87).

There remains to consider glutamate, a candidate supported by the presence of a depolarizing response on B cells. Glutamate exists in fair quantities in A cells (130) -- similar to those found in most Aplysia neurons, which is not sufficiently high to strongly suggest a neurotransmitter function. It would be just that the one component of seaweed most able to fire the chemoreceptors and arouse the B cells to make Aplysia feed should also be the transmitter at the synapse, but such is not to be. The glutamate receptors on B cells, not the receptors at the A to B synapse, desensitize with repeated application, or perfusion, of glutamate. In similar fashion, aspartate did not have any effect. At low concentrations aspartate augments glutaminergic synaptic potentials; and, at higher concentrations aspartate cross desensitizes glutamate mediated responses (211). The failure of these experiments argues agaianst glutamate as the A cell transmitter. Additional support comes from McCamen who found that quisqualic acid blocked glutamate induced responses, but did not have any affect on the psp (130). The argument surrounding glutamate as a neurotransmitter, at this synapse and in the Aplysia nervous system, is constrained by its ubiquitous presence and by the lack of selective antagonists and agonists (64).

A few other messengers need to be briefly considered. Glycine, a neutral amino acid, was found to induce a slow depolarization in B

cells. Further studies, presented below, indicate that the depolarization is generated by sodium coupled cotransport, a mechanism of neutral amino acid uptake common among neurons, and thus is not a viable transmitter candidate. Recently, Carpenter and Hall have described a depolarizing response on A and B cells evoked by enkephalin, a possibility which merits further consideration (28A).

If the neurotransmitter can not be identified to be any one of those currently known to neurophysiologists, then either the methods are inadequate, the synapse is inaccessable, or the transmitter is as yet unknown. Access in the A to B synapse may be a problem since the synapse appears to be deeply embedded in the neuropile where it is difficult to place electrodes, and may prove difficult for antagonists to diffuse toward. The pharmacological methods are inadequate for studying glutamate. Not that much is known of the interaction of most poisons with postsynaptic membrane. It is entirely conceivable that the transmitter is not glutamate, but is a similar compound. Debate raged for years over the identification of the messenger in the rat lateral olfactory tract. Aspartate and glutamate both had their advocates based upon ambiguous experimental data (77A). It now appears that N-acetylaspartylglutamate (NAAG), a glutamate-like substance, is the culprit. NAAG and the lateral olfactory tract epsp are specifically inhibited by aminophosphonobutyric acid but other amino acid responses are unaffected by this antagonist (53, 54).

The final possibility is a previously undescribed substance. This is not surprising: given the complexity of the nervous system and the

simplicity of the human cortex, the natural instinct is to ascribe all mechanism of action to what is known. In this instance, the few of the known transmitters are given credit for all the work done by the myriads of undiscovered messengers and their cousins. It remains to be known.

Whatever it is, it must meet a few conditions. It must be present in the A cells, unless it is produced only in the terminals. It must induce depolarizing responses in B cells that are immune from the action of curare and strychnine. It must evoke an excitatory response that decreases somewhat in amplitude with repeated application, and is able to induce a rapidly disappearing hyperpolarization. Finally, if the A cells are primary motoneurons, then it must also be effective at inducing muscular contractions.

III. CHARACTERIZATION OF GLYCINE INDUCED-ALTERATIONS OF MEMBRANE POTENTIAL

Glycine is an acknowledged transmitter in the mammalian central nervous system (41A, 42, 136, 189, 208, 209). Early studies demonstrated the inhibitory action of glycine in the spinal cord, an effect specifically antagonized by strychnine (41, 41A, 117, 215, 216, 217). There is little direct evidence, however, for glycine receptors in "simpler" systems such as molluscs.

One set of receptors have been described by Oomura et al. on two identified cells in the eosophageal ganglion of Onchidium (112, 139, 140). They elicited a depolarizing response, generated by increased permeability to sodium ions (equilibrium potential +25 to +30), by perfusing the preparation with 1.3 mM glycine -- raising the concentration to 13 mM caused the cells to fire. Oomura discerned two phases: the first, found at lower concentrations of glycine, was mediated by a decreased permeability to potassium ions; the second, predominant at higher concentrations, was generated by a conductance increase to sodium ions. Replacing sodium from the seawater, and not potassium, decreased the magnitude of response. Curiously, strychnine partially antagonized the sodium component, as expected, but augmented the potassium component. Also unusual, the receptors failed to desensitize. Nevertheless, tetrodotoxin, ouabain, and 2, 4 dinitrophenol did not have any affect on the depolarization.

Although receptors for glycine have not been identified on neurons in Aplysia there is much evidence to suggest that glycine may be a messenger in this noble mollusc -- the result of the extensive work of McAdoo, Price, and Sawada et al. (79, 80, 125, 158, 159, 160, 161, 162, 165, 172). Glycine is found in much higher concentrations in the ganglion than in the hemolymph; Cells R3 through R14 of the abdominal ganglion -- the ganglion with the highest concentration of this amino acid -- contain great amounts of glycine (R3-13: 600 picomoles; R14: 1400 picomoles) exceeded only by the bag cells (1500 picomoles) which are known to be involved with protein synthesis (80). This is also greater than their content of aspartate and glutamate (105, 190). Perhaps significant, the same R cells synthesize a 6000 dalton polypeptide (80).

These same neurons utilize a rapid and specific system for glycine uptake which is distinct from the means common to all neurons for neutral amino acid membrane transport (125, 158, 161). Autoradiographically and biochemically determined uptake rates are greater than four times that for other neurons (125, 158). The system is specific for glycine since alanine, leucine, and serine are not affected, nor do glial cells display preferential uptake. The process is sodium, and not energy, dependent; it is abolished in sodium free water but is unaffected by either ouabain or 2, 4 dinitrophenol.

Free glycine is moved about by a rapid axonal transport mechanism that is specific for the amino acid (162). Tritiated glycine is found in vesicles located at the axon terminals of these cells (160); it is

not shunted for protein synthesis (125, 158, 161). Collection of perfusate reveals tonic release of glycine, however when the nerve containing the R3-14 axons is stimulated the release of glycine is greatly increased (165).

The axons of this group of neurons extend down the branchial nerve to the pericardial region to surround the efferent gill, and vein, and also to the heart where the terminals end blindly in sheath bathed by hemolymph. In addition, R14 sends axon processes to the major arterial branches of the aorta to make contact with the arterial wall smooth muscle. The terminals, free of glia, unlike the extensive glial sheath which surrounds the axons, are filled with dense core vesicles (159, see also 79, 171).

Stimulation of R14 does not cause direct contraction of the vessel muscles, nor does local application of glycine. There is no affect on junctional potentials or on membrane permeability. Stimulation of R14 does, however, enhance the force of muscular contraction; bathing the preparation with 0.5 mM glycine similarly enhances serotonin induced muscle contraction. These effects are not produced by alanine, serine, taurine, or histidine (79, 172). Thus, R14 appears to act on arterial muscle in much the same way as the metacerebral giant cell affects buccal muscle contractility (204, 205).

It is not known how glycine enhances muscle contraction. Nor is it clear if glycine is a neurotransmitter rather than a modulator, for distinct receptors have not been identified. Glycine may be a neurohormone, although the low concentrations of glycine found in the

hemolymph argue against this possibility (11). The role of the 7000 dalton peptide also is not clear since Lloyd described a similar polypeptide that enhances cardiac tissue contractility in Helix (121, 119, 120).

In sum, glycine is found in identified neurons which contain mechanisms for the rapid uptake and axonal transport of glycine. The amino acid, found in vesicles at axon terminals, is released following neural stimulation. The neurons, which release glycine, innervate cardiac and arterial muscle. Glycine mimics the modulation of muscular contractility produced by stimulation of glycine containing neurons although glycine receptors have never been identified. The role of an accompanying polypeptide is unclear.

During the course of surveying the identity of receptors on A and B cells in the cerebral ganglion of Aplysia a nearly ubiquitous slow depolarization induced by glycine was found. Less commonly a biphasic hyperpolarization could be elicited. A series of experiments were then performed to characterize the nature of these glycine generated responses.

RESULTS

Fast Hyperpolarizations

Biphasic and triphasic responses were found on many, but not on all, A cells. The early biphasic hyperpolarizations of membrane potential appear to be generated by increased permeability to chloride and

potassium ions. Characterization of the third component, a slow depolarization, is discussed below. Clear evidence of fast sodium subserved depolarizations was not found.

An example of the more typical complex triphasic response found on a current clamped A cell is shown in figure 32. Since the first phase of the response reversed between -55 and -60 mv and occured while membrane conductance was increased, the hyperpolarization must be mediated by chloride. The second phase of the hyperpolarization, also accompanied by increased membrane conductance, reversed between -70 and -80 mv and was thus mediated by potassium. The final, slow depolarization neither reversed between -45 to -80 mv or was associated with an alteration in conductance.

Isolated biphasic or single component responses were occaisonally encountered (figure 33); on a rare cell the slow depolarization could be separated from glycine evoked hyperpolarizations (figure 25). Both the chloride and potassium mediated hyperpolarizations generated by glycine were completely and reversibly abolished by strychnine (10^{-4} M) (figure 34).

All told, seven chloride responses and eight potassium responses were found. Two uncharacterized depolarizations were found at moderately hyperpolarized membrane potentials -- they were probably generated by chloride currents. One response typical of a conductance decrease of potassium was found but could not be reproduced. Glycine induced hyperpolarizations were not ubiquitous -- rather, they were often restricted to localized regions of cell membrane.

FIGURE 32: Pressure ejection of glycine (dissolved in distilled water) onto a current clamped A cell which elicited a triphasic response. The first component with a peak response at 1 second and a reversal potential of -59 mv was generated by increased chloride ion permeability. The second component, peak response at 5 seconds and reversal potential between -75 and -78 mv, was generated by increased permeability to potassium ions. A slow depolarization was seen at 20 seconds that had a reversal potential greater than -50 mv. NOTE: vertical scale is 10 mv. Tracing below each recorded response indicates timing and duration of pressure ejection pulse.

Glycine

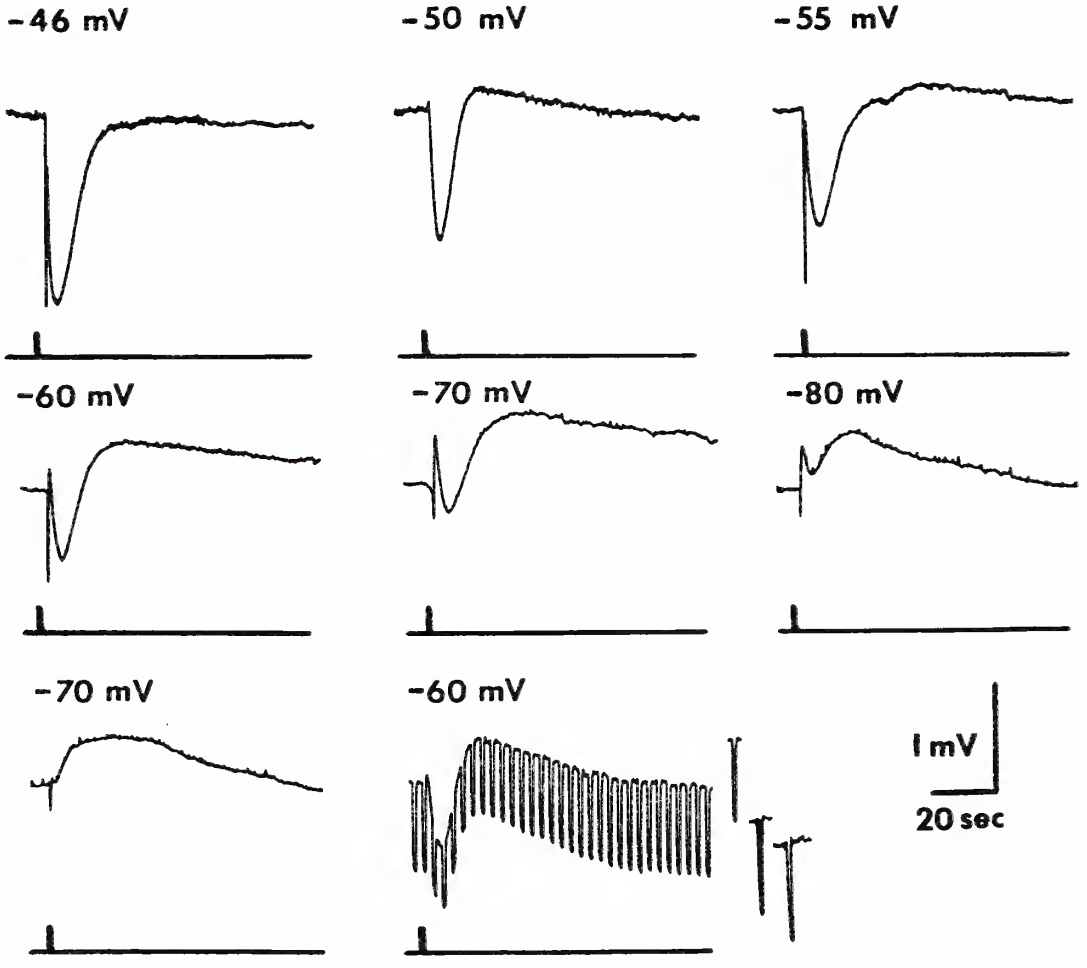


FIGURE 33: Pressure ejection of glycine (distilled water) onto current clamped A cell which elicited a hyperpolarization mediated by potassium ions, and associated with increased membrane resistance. Reversal potential -95 mv, peak response 10 seconds.

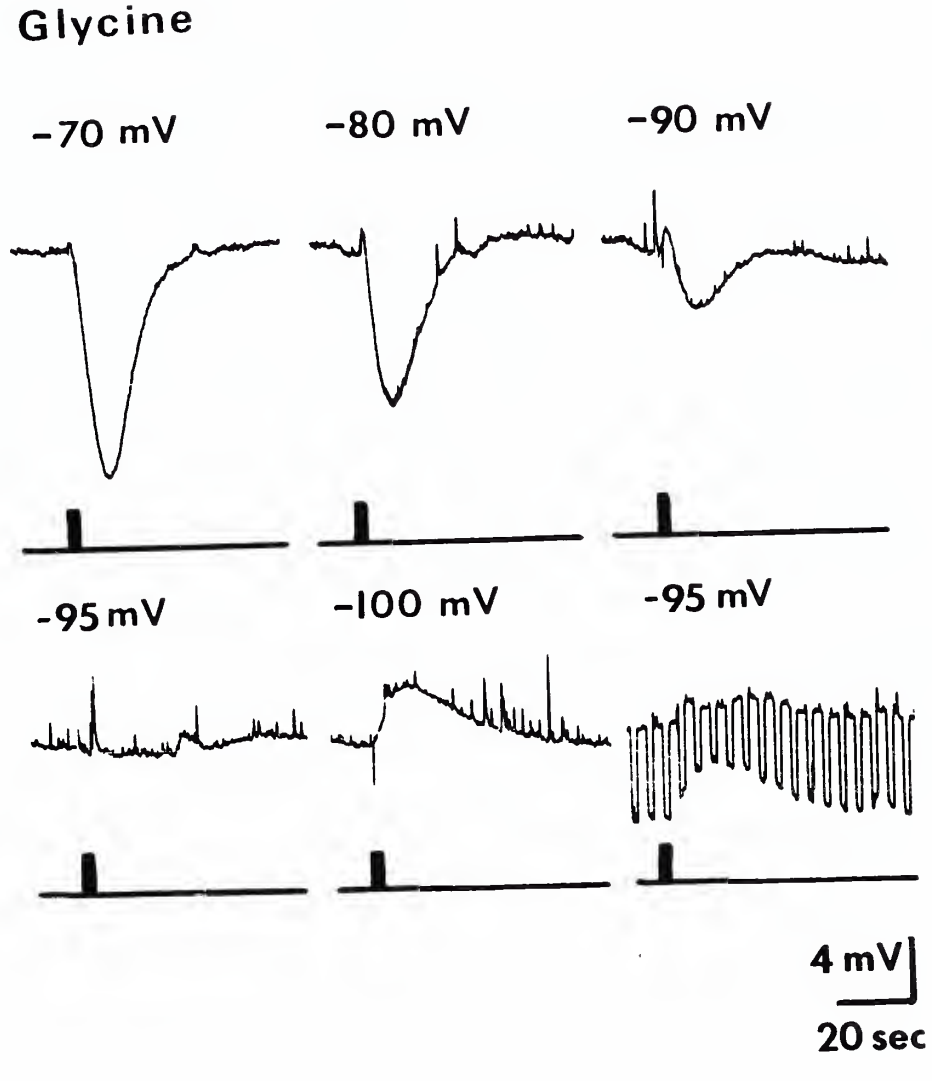
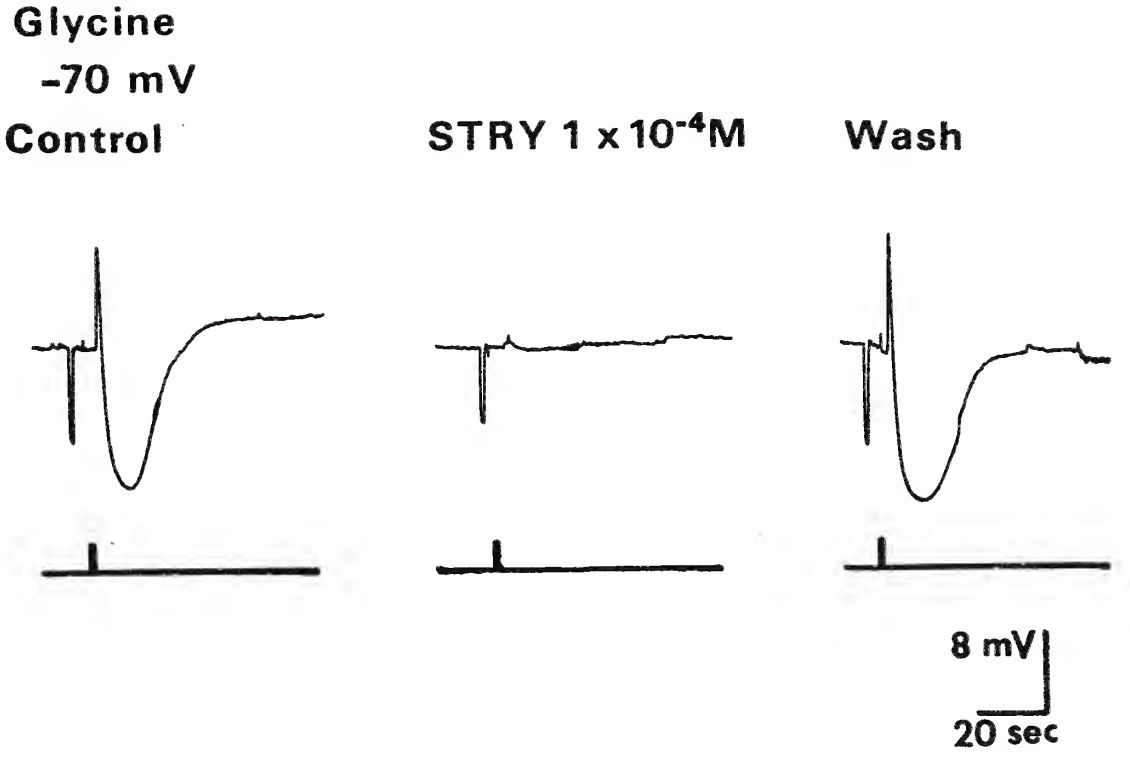

Glycine
-70 mV
-80 mV
-90 mV
-95 mV
-100 mV
-95 mV
4 mV
20 sec

FIGURE 34: Pressure ejection of glycine (distilled water, pH 3.5) onto a current clamped A cell which elicited a biphasic hyperpolarization mediaited by chloride and potassium ions. Perfusion of strychnine (10^{-4} M) rapidly and reversibly abolished both components of response. Note, the tip of the pressure ejection tip was leaking, thus the pipet was brought away from the cell after each pulse.


Glycine
-70 mV
Control
STRY 1 x 10-4M
Wash
8 mV
20 sec

Many of the initial experiments were conducted with glycine dissolved in distilled water or in water with a low pH. Curiously, most of the biphasic responses were found when distilled water was the solvent. However, pressure ejection of distilled water never resulted in biphasic hyperpolarizing responses associated with increased membrane conductance. Similarly sucrose, (1.0 to 0.1 M) did not have any effect on A cell membrane potentials. The experiments which identified the hyperpolarizing responses were undertaken during the summer; the following winter, when the experiments were repeated using 1.0 to 0.01 M glycine dissolved in seawater with a pH between 6.5 and 7.8, only two weak hyperpolarizing responses, one generated by chloride, the other by potassium, were found.

GLYCINE-INDUCED RESPONSES

RESPONSE	A CELL	B CELL	OTHER
Na+	1?		
Cl-	7		
K+	8		2
Slow Depol	32	12	1
K (g dec)	1		

TABLE 9 : Glycine induced changes in membrane potential. Results using glycine in low pH solution are not shown. "g dec" denotes conductance decrease. Responses mediated by Na+, Cl-, and K+ were associated with increased conductance.

Slow Depolarization

A slow depolarizing response generated by glycine of similar latency (peak 8 to 12 seconds), and similar duration (40 to 90 seconds) was

found on nearly every cell examined: 32 A cells and 12 B cells. Occaisonally the response was obscured by a preceding potassium mediated hyperpolarization. Rarely, ionophoresis of glycine was sufficient to evoke the depolarization (figure 14); pressure ejection, however, proved to be universally effective and which generally elicited responses on any portion of neural membrane. At times a neuron could be stimulated sufficiently to generate an action potential [figure 14 (ionophoresis), 35 (pressure ejection), both cells were current clamped].

Figure 35 demonstrates a typical depolarization found on a current clamped B cell -- note the absence of a change in membrane conductance, a characteristic feature of this response. Only one cell of forty-four examined demonstrated an increased membrane conductance. Figure 36 reflects the depolarization seen on a voltage clamped neuron; the reversal potential for this response was -15 mv (Graph 3). Also, note that an alteration in conductance could not be demonstrated in this cell either. Other voltage clamped neurons manifested pronounced depolarization at -20 mv. Perfusing the preparation in sodium free (Tris) seawater markedly decreased the magnitude of response (figure 39).

Microperfusion of 0.001 M glycine for three minutes revealed a constant plateau depolarization that did not desensitize (figure 37); on one occaison no change occured in the degree of depolarization for fifteen minutes. Strychnine, as high as 2.5×10^{-4} M, failed to affect the slow depolarization on either A or B cells (figures 38 and 39). Bathing the preparation in 2, 4 dinitrophenol (5×10^{-4} M) abolished the

FIGURE 35: Pressure ejection of glycine (ASW) onto a current clamped B cell which elicited a slow depolarization (peak response 12 seconds) and generated an action potential. The response was not accompanied with an alteration of membrane conductance.

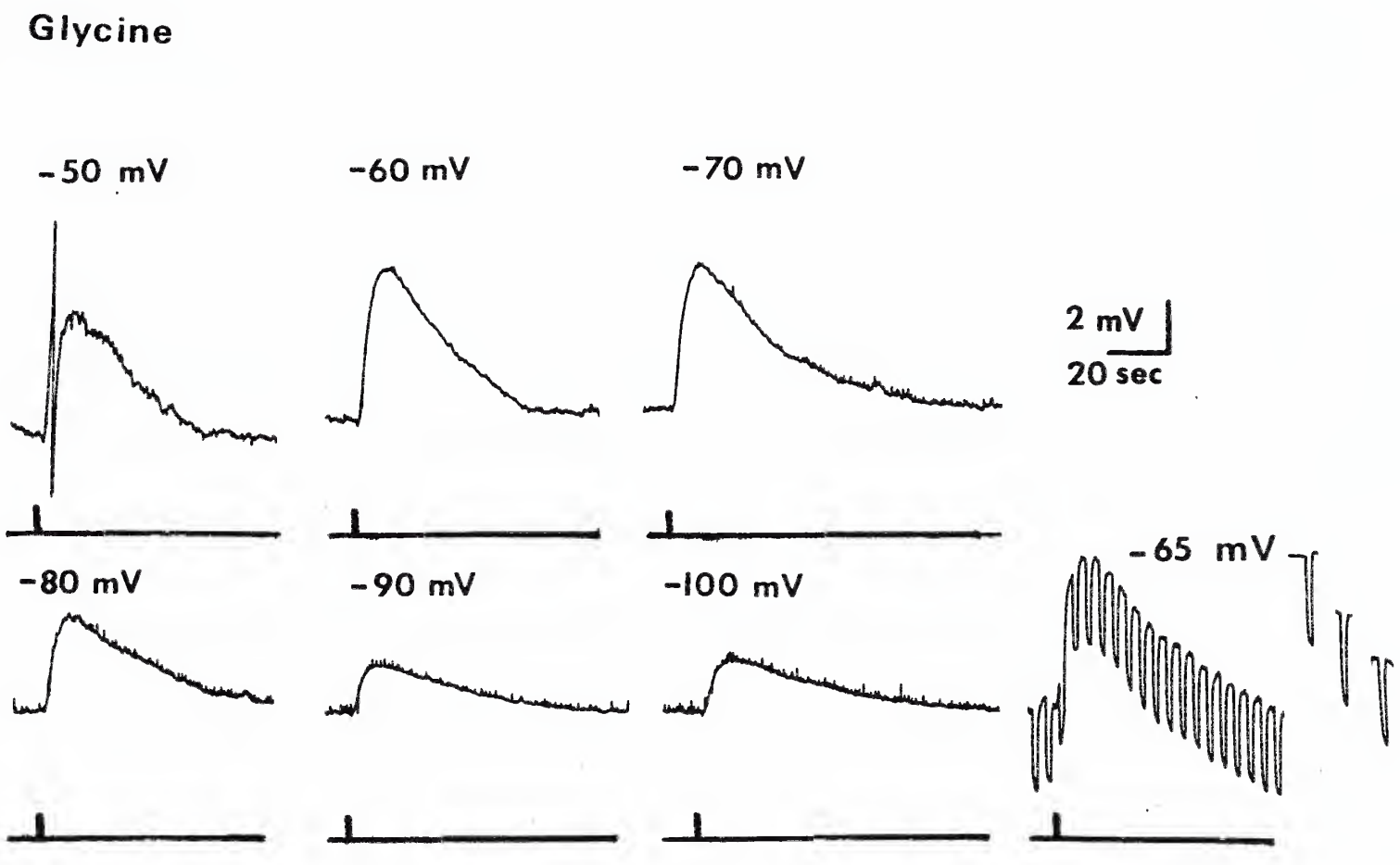
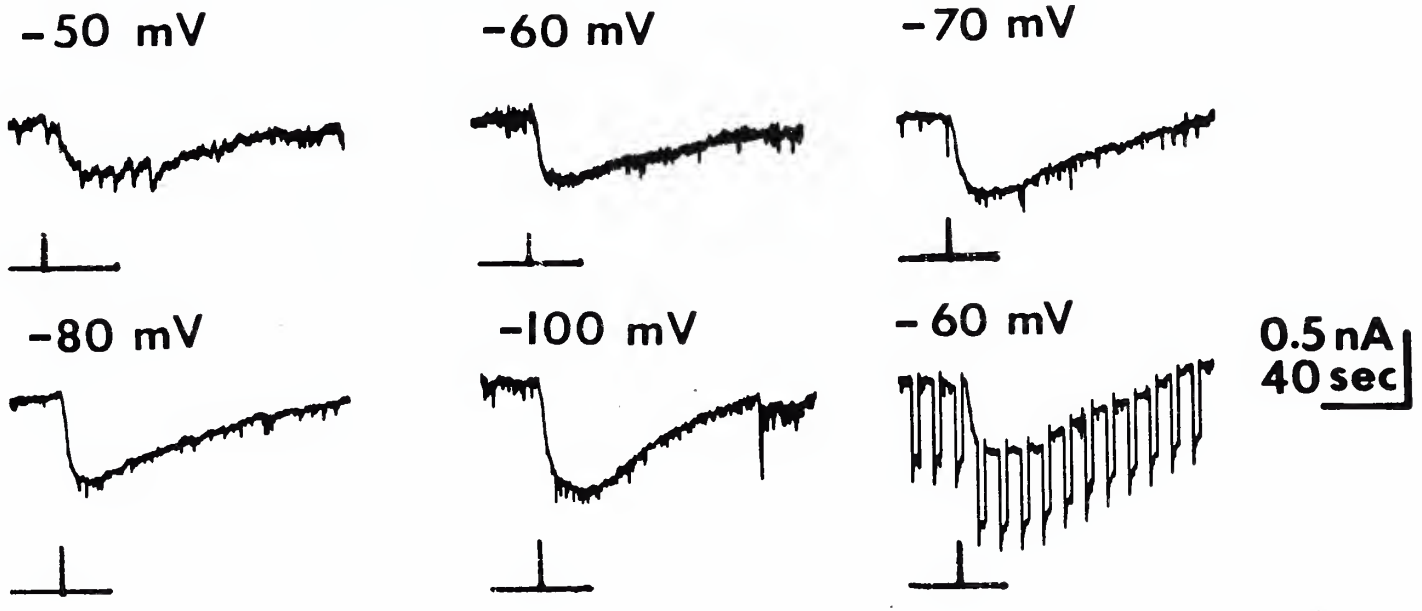
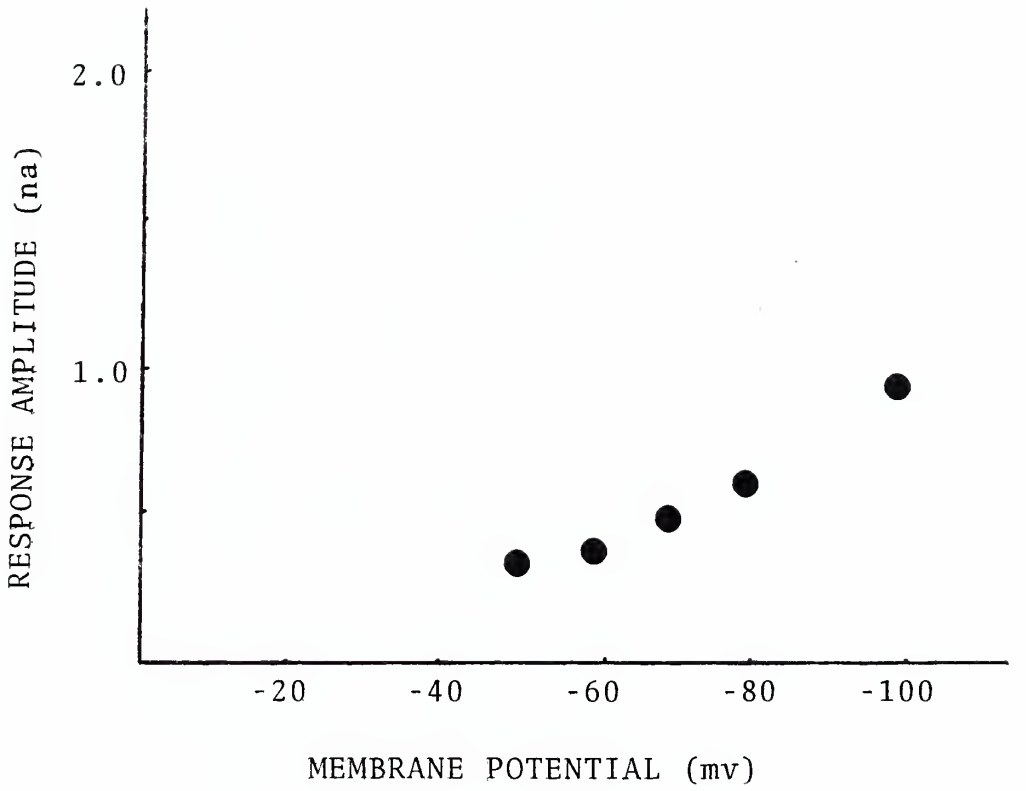

Glycine
-50 mV
-60 mV
-70 mV
2 mV
20 sec
-80 mV
-90 mV
-100 mV
-65 mV

FIGURE 36: Pressure ejection of glycine (ASW pH 7.8) onto a voltage clamped A cell which elicited a typical depolarization with a peak response at 22 seconds. Determination of the reversal potential for this response is presented in graph 3. Note the absence of an alteration of membrane potential.

Glycine



GRAPH 3: Reversal potential of a typical glycine-induced slow depolarization determined from a voltage clamped A cell (see figure 36). Ordinate: amplitude of response (na) evoked by pressure ejection of glycine onto an A cell. Abscissa: membrane potential of the A cell (mv).



GLYCINE RESPONSE

FIGURE 37: Microperfussion of 0.001 M glycine (ASW) onto a voltage clamped A cell. Arrows denote beginning and end of perfusion. Each response failed to desensitize.

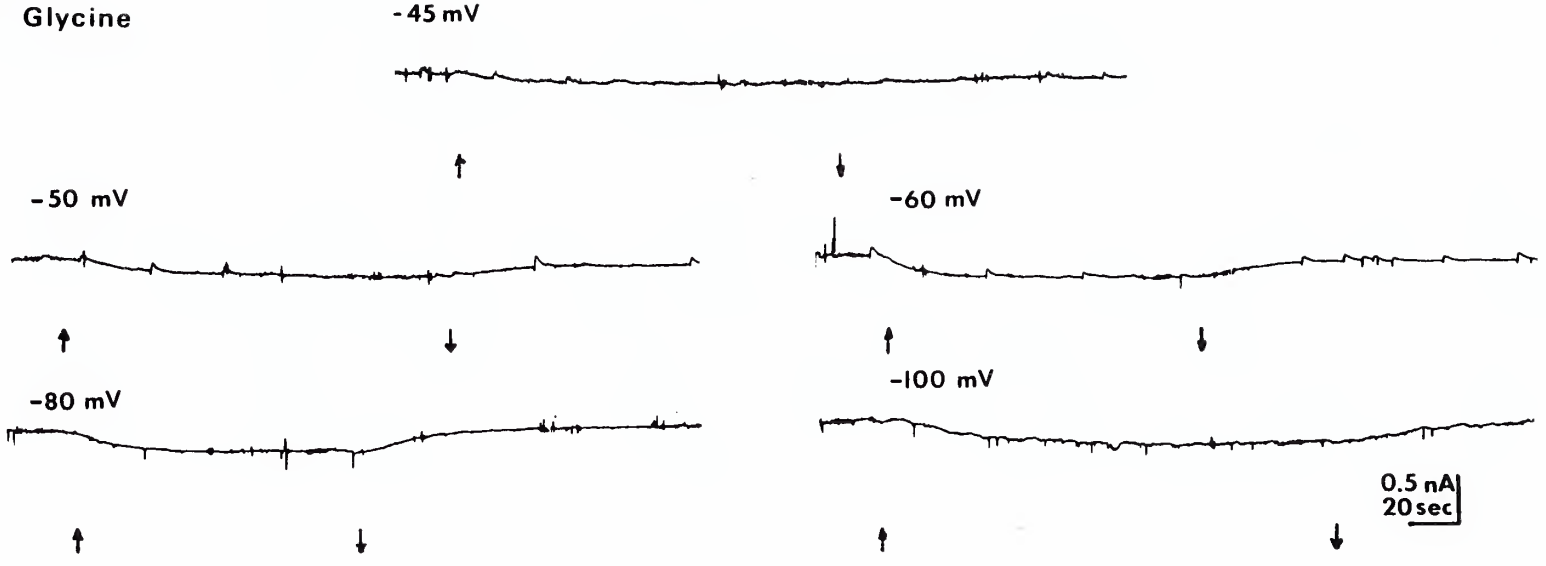

Glycine
-45 mV
-50 mV
-60 mV
-80 mV
-100 mV
0.5 nA
20 sec

FIGURE 38: Pressure ejection of glycine (ASW) onto a current clamped A cell which elicited a typical slow depolarization. Perfusion with strychnine (2.5×10^{-4} M) did not affect the response.

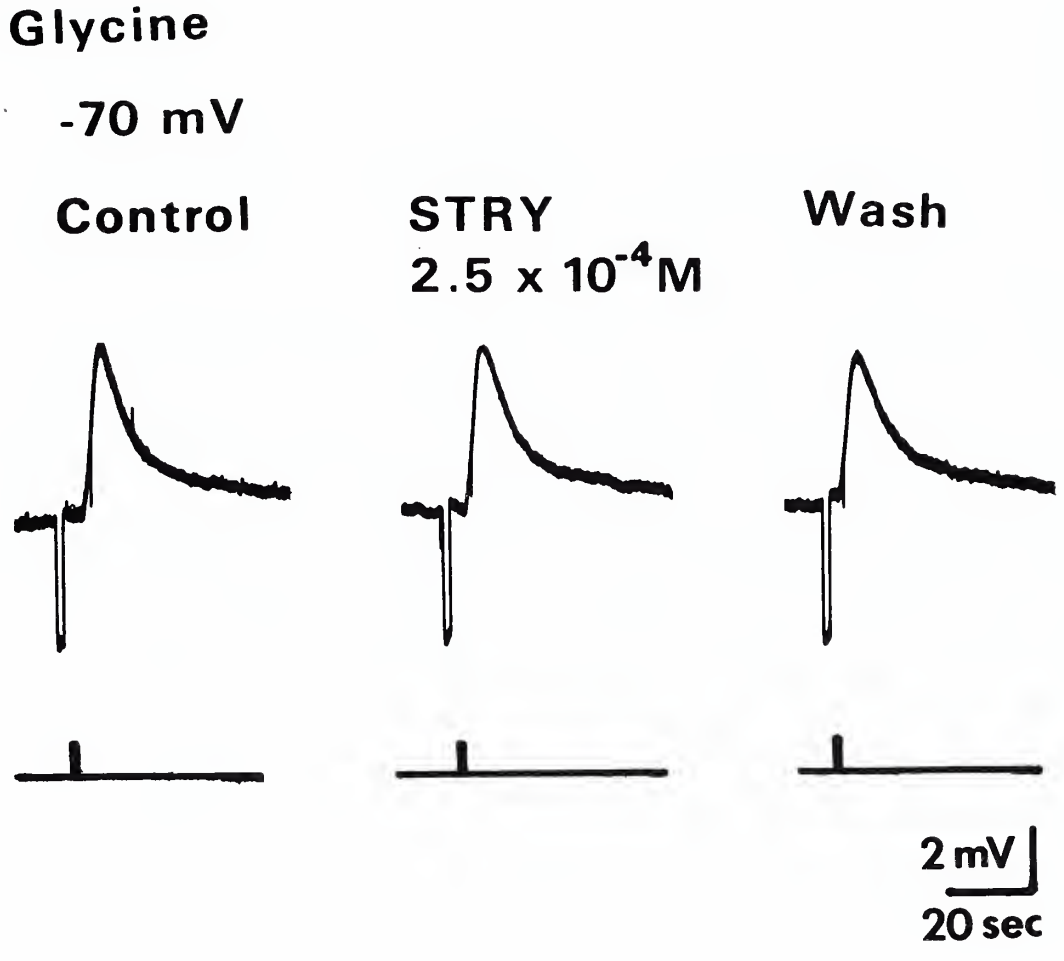
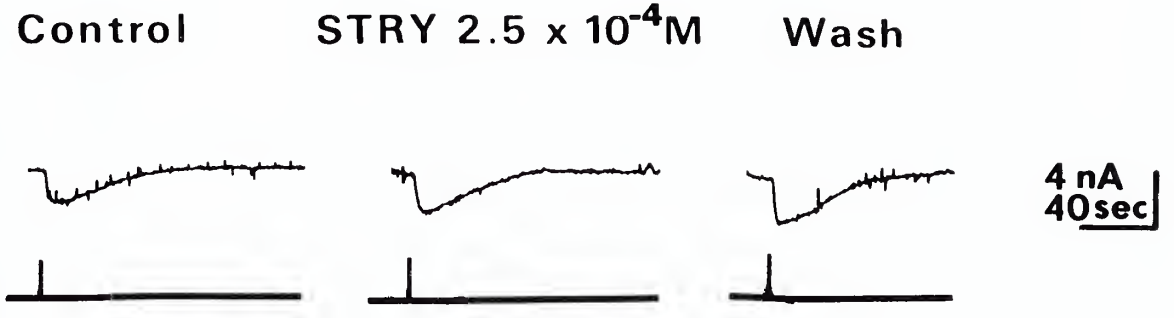
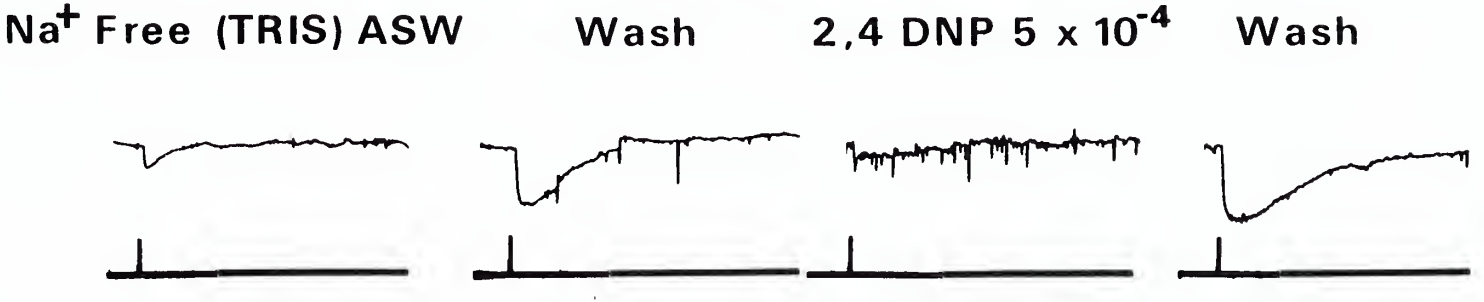

Glycine
-70 mV
Control
STRY
2.5 x 10-4M
Wash
2 mV
20 sec

FIGURE 39: Pressure ejection of glycine (ASW pH 7.8) onto a voltage clamped A cell which elicited a typical slow depolarization sequentially perfused (and then washed) with strychnine, Na+ free (Tris) ASW, and 2,4 dinitrophenol (2,4 DNP). Strychnine (2.5×10^{-4} M) did not affect the response. Na+ free (Tris) ASW markedly decreased the amplitude of depolarization. 2,4 DNP (5×10^{-4} M) reversibly abolished the response.

Glycine


Control
STRY 2.5 x 10-4M
Wash
4 nA
40sec


Na+ Free (TRIS) ASW
Wash
2,4 DNP 5 x 10-4
Wash

glycine induced depolarization. Both the effects of sodium free seawater and 2, 4 dinitrophenol perfusion were reversible (figure 39). Perfusion of Cobalt II, a calcium channel antagonist, did not have any effect on the response (figure 40).

Pressure ejection of the neutral amino acids histidine and glutamine caused depolarizing responses similar to those elicited by glycine and that were also not associated with an alteration of membrane conductance. The configuration, latency, and time course of response were remarkably similar even though the histidine and glutamine responses were weaker than the glycine-induced counterpart (figure 41). Table 10 summarizes the effects of perfusion and ion exchange experiments on the slow depolarization brought forth by glycine.

Pressure ejection of artificial seawater, distilled water, 1 M sucrose (a hypertonic solution), or artificial seawater with low pH did not reproduce the glycine-induced slow depolarization.

DISCUSSION

Hyperpolarizations

That glycine-induced hyperpolarizations are mediated by chloride and potassium ions is suggested by alterations in membrane conductance and reversal potentials; it is an effect seen by all neurotransmitters in Aplysia. Strychnine abolished both components. Although it is unusual for strychnine to block potassium channels (51, 179), strychnine is a

FIGURE 40: Pressure ejection of glycine (ASW) onto a current clamped A cell which elicited a typical slow depolarization. Perfusion of the Ca++ current antagonist cobalt II (5×10^{-3} M) did not affect the response.

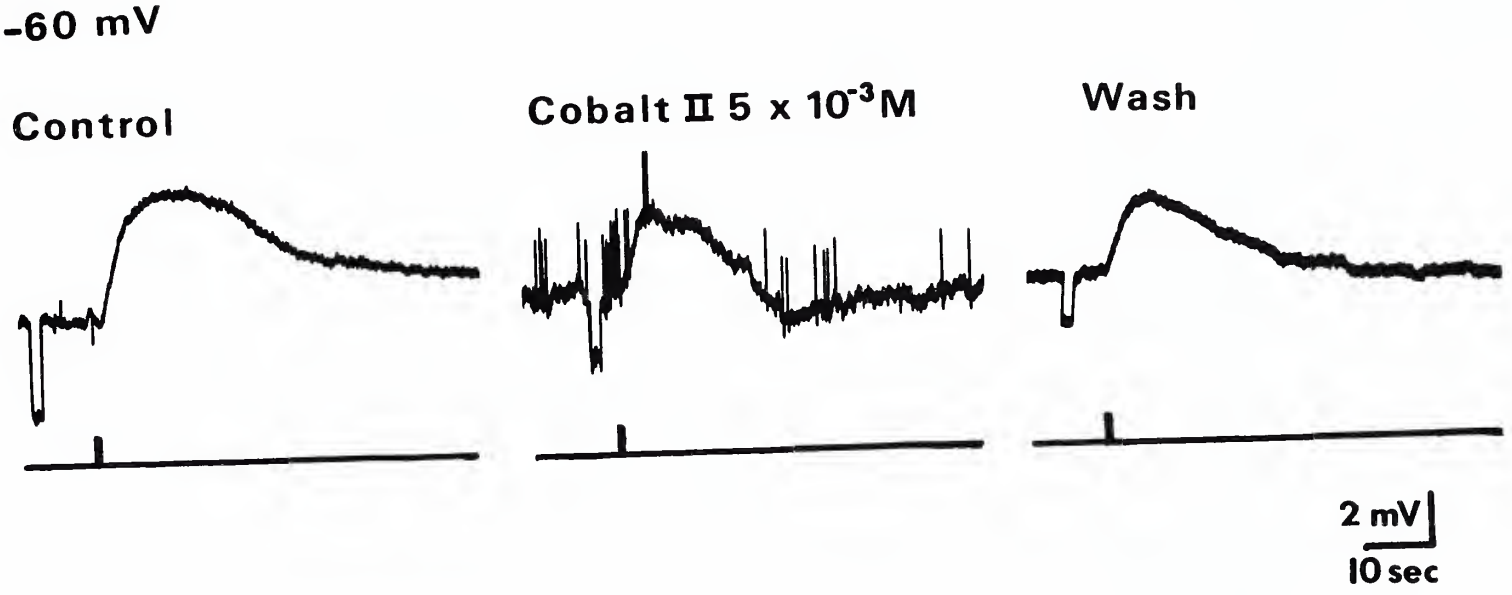
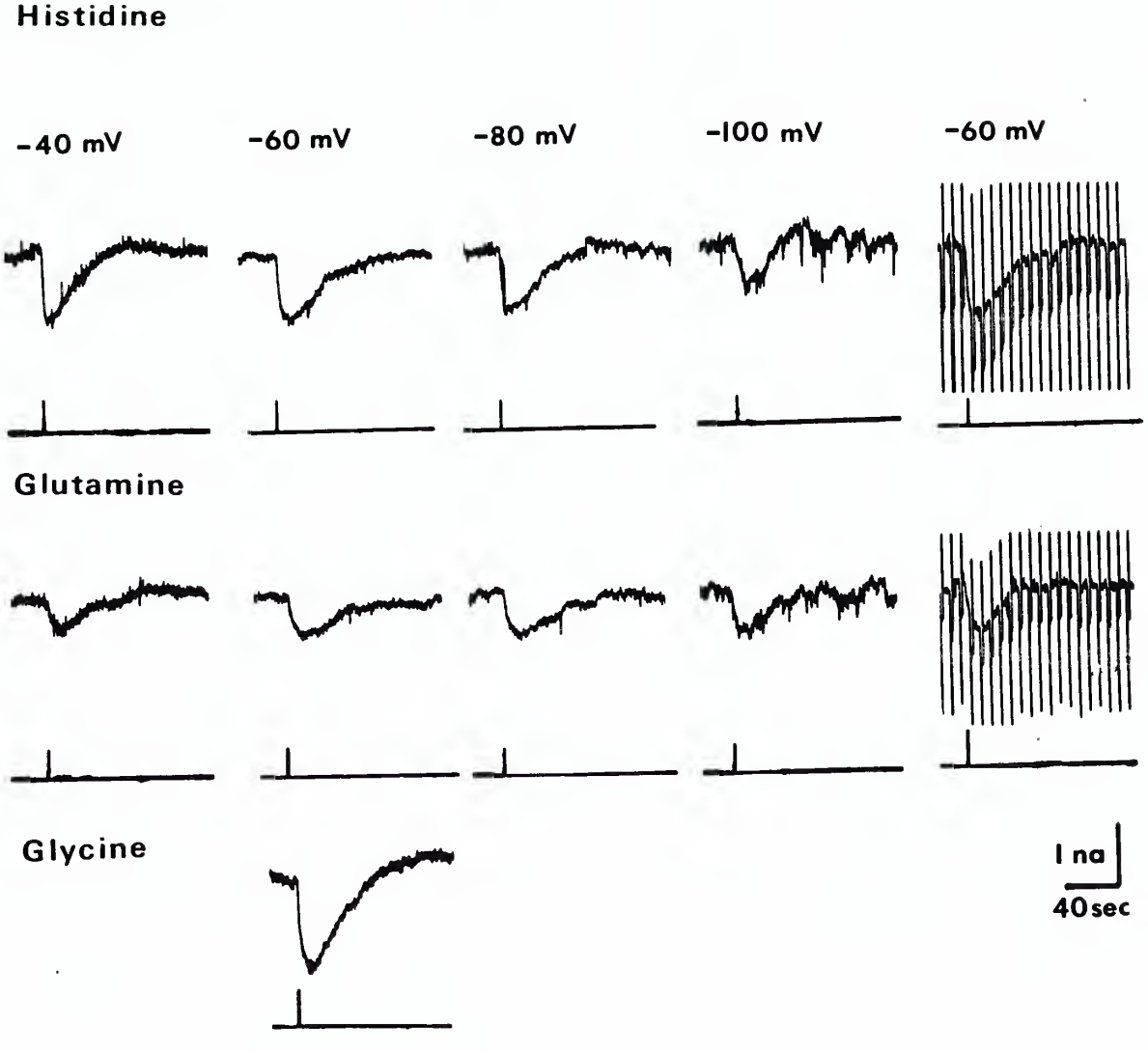

-60 mV
Control
Cobalt II 5 x 10^{-3}M
Wash
2 mV
10 sec

FIGURE 41: Pressure ejection of histidine, glutamine, and glycine (ASW) onto a voltage clamped A cell. All substances generated similar slow depolarizations, with similar latencies, peak responses, and decay, that were not associated with alterations of membrane conductance. Note the histidine and glutamine responses were weaker than those of glycine, although this may have been a function of pipet placement.


Histidine
-40 mV
-60 mV
-80 mV
-100 mV
-60 mV
Glutamine
Glycine
I na
40sec

GLYCINE INDUCED SLOW DEPOLARIZATION: PERFUSION AND ION EXCHANGE EXPERIMENTS

AGONIST/ ANTAGONIST	CONCENTRATION (M) (IF RELEVANT)	EFFECT
GLYCINE	0.001 M Microperfusion	No Desensitization
NA FREE(TRIS) ASW		Decreased Response (R)
STRYCHNINE	2.5×10^{-5} M	No Effect
2, 4 DNP	5×10^{-4} M	Abolished Response (R)
COBALT II	5×10^{-3} M	No Effect
HISTIDINE	Pressure Ejection	Mimicked Response
GLUTAMINE	Pressure Ejection	Mimicked Response

TABLE 10: Effect of agonists and antagonists on the slow depolarization induced by glycine. (R) denotes reversible; 2, 4 DNP is 2, 4 dinitrophenol.

well known glycine receptor antagonist in other preparations (41, 117, 135 139, 140, 215, 217; may act only on chloride channels in mammals 216). The biphasic hyperpolarizing responses evoked by glycine, however, were not reproduced the following winter. The question arises whether or not the elicited responses were artifact.

Four factors are important: use of pressure ejection, high concentrations of glycine (1.0 to 0.01 M), pH , and solvent. It is unusual that glycine induced responses could only be found with pressure ejection, a technique that dumps a large amount of substance onto a cell -- even if the receptors are located deep in the neuropile encased in

glia it is possible to bring forth responses induced by other transmitters with greater ease. One molar glycine was initially used for pressure ejection; if the response can only be evoked by using fantastic amounts of a substance it may not be physiologically relevant. Yet, pressure ejection alone does not create an artifactual response, nor do hypertonic solutions of sucrose (1 M). PH of seawater ranging from 6.5 to 8.0 did not have any independent effect, either. Nevertheless, a low pH may alter the charge of glycine and thus have a secondary effect. Most of the hyperpolarizing responses were found early in the studies when distilled water was used as the solvent, however pressure ejection of distilled water , by itself, did not elicit a similar response. Perhaps, though this is unlikely, the pipets were contaminated with other transmitter substances. More important, the response was not universally found, as would be expected for an artifact, rather biphasic hyperpolarizations were found only on localized and narrow regions of the cell.

Another possibility may be that glycine interacts with other receptors much as aspartate activates glutamate receptors, but this does not account for the paucity of later findings (68, 105, 156). There is one other alternative explanation. The initial findings occured during the summer, when the animals were young; the second series of experiments were performed in the winter, when the Aplysia were in the twilight of their lives. As Aplysia age their neurons undergo changes in their electrophysiological properties; perhaps, with time, the receptor profiles on their neurons are altered, too (152, 153).

The solution will come with a continued search for glycine receptors, during the summer months, and, when found, they can be properly characterized by the necessary ionic exchange and antagonist studies suggested by these results.

Slow Depolarization

The glycine-induced slow depolarization described here shares a few characteristics with conventional putative neurotransmitter receptor evoked responses, yet there are a number of features that are atypical. The depolarization can be generated by the little amount of substance delivered to a cell soma by ionophoresis. The configuration of the response -- latency, peak, and duration -- is typical of classical transmitter generated changes in membrane potential. Also, sufficient depolarization can be generated to initiate an action potential.

The unusual characteristics, however, outnumber superficial similarities. Pressure ejection, which produces very high concentrations of substance outside the cell, is needed to elicit the depolarizations on most neurons. The response does not densensitize unlike most transmitter generated responses -- although there are exceptions, for example, the serotonin A' receptor (68). Most unusual is the failure to discern any change in conductance across the membrane. Either the receptors are a far ways away from the recording electrode or, indeed, there is no change in conductance. Although the depolarization is sodium dependent it is unaffected by strychnine, a fast sodium channel blocker and a specific glycine receptor antagonist for sodium mediated depolarizations in Onchidium and inhibitory

responses in mammals (41, 112, 117, 135, 139, 140, 215, 217). Unlike common transmitter evoked responses subserved by increases in sodium ion permeability, the glycine response is sensitive to the metabolic inhibitor 2, 4 dinitrophenol (106). Nor is the response completely specific: the neutral amino acids histidine and glutamine evoke similar responses -- the only possible analogy here is for activation of glutamate receptors by aspartate (62, 137, 211).

The glycine induced depolarization is best compared to, and contrasted with, the responses generated by glycine that are described by Oomura in Onchidium, the depolarization of membrane potential evoked by neutral amino acids investigated by Kehoe, and the glycine uptake systems analyzed by McAdoo et al. (112, 139, 140; 106; 125, 158, 161). Kehoe described a depolarization induced by neutral amino acids, in particular histidine and glutamine, in Aplysia pleural neurons (106). The depolarization was generated by bathing the preparation with high concentrations of amino acid -- 0.5 to 2.0 mM; the response maintained a constant level of depolarization and did not desensitize, just like that seen in figure 37. There was minimal dose dependent response. Kehoe never mentioned the generation of an action potential, however. Like the responses described here, the depolarization was sodium dependent, had a reversal potential that ranged between -7 and +54 mv, and was unaccompanied by a change in conductance. Other neutral amino acids had the same effect -- similar Km's -- on the same cells. It was sensitive to 2, 4 dinitrophenol and cooling, but not to ouabain. Although one must be careful in analyzing the effects of metabolic inhibitors, the response described by Kehoe suggests a nonspecific energy and sodium dependent system of neutral amino acid transport (106).

Oomura's glycine induced depolarization exhibits some peculiar features, too. He had to use very high concentrations of glycine; the response did not desensitize; nor did he demonstrate a clear change in conductance (139, 140). Neutral amino acids evoked similar but weaker responses, the amplitude of which proved to be a function of side chain length. On the other hand, the response was partially antagonized by strychnine and not affected by 2, 4 dinitrophenol, unlike the responses described here and by Kehoe. And, similar to the depolarization characterized here, an action potential could be generated, albeit with greater amounts of glycine (139, 140).

There are many features characteristic of sodium dependent cotransport, the means by which many neutral amino acids and sugars are carried across membranes. These mechanisms are not specific, they do not alter membrane conductance, nor do they desensitize. A sodium gradient is necessary to rush material into the cell, and thus the system is usually energy dependent. Typically, the cell is minimally depolarized, although there is usually an initial peak depolarization that then partially subsides (3, 47, 110, 116, 174).

McCadoo et al. described a glycine specific uptake system peculiar to cells R3 through R14 of the abdominal ganglion. Their system is sodium dependent, is sensitive to mercury, but is unaffected by 2, 4 dinitrophenol or ouabain (and is partially decreased by cold). Whereas the non-specific glycine uptake system is not sensitive to mercury, 2, 4 dinitrophenol or ouabain (125, 158, 161, 162). Glycine uptake in mammals, however, is dependent on sodium, is sensitive to mercury,

ouabain, cold, AND 2, 4 dinitrophenol -- but not to strychnine (45, 94, 116, 136). Curiously, application of glycine to cardiac tissue in Aplysia enhances contraction without altering membrane conductance (79).

The glycine induced slow depolarization shares many features of sodium coupled cotransport -- certainly many characteristics of classically evoked changes in membrane potential are lacking. Yet the phenomenon described here does not fit the uptake systems described by McAdoo et al. Rather it compares most favorably, but not perfectly, with the response described by Kehoe. Some of the features of the Onchidium glycine response do not match the classical neurotransmitter model either, and, in fact, share some common characteristics with the responses described here, by Kehoe, and by McAdoo et al. If such great amounts of glycine are needed to generate an action potential then the response measured by the recording electrodes may not by electrophysiologically significant. Application and recording techniques may have made more evident the means by which necessary nutrients are picked up. There is much room, and need, for further clarification. The search for the elusive glycine receptor in Aplysia continues.

CONCLUSION

This study examined the physiological properties of the A and B cells of the cerebral ganglion, mapped their synaptic connections, determined their receptor profiles, and performed experiments designed to identify the transmitter at the A to B synapse.

A brief survey of A and B cell synaptic connections support the previous reports of Jahan-Parwar and Fredman and of Hinzen and Davies (56, 58, 77). A cells form synapses with nearly all B cells; A cells do not form synapses with each other. The A to B synaptic potential is initially a biphasic eipsp. The inhibitory component rapidly disappears while the remaining exitatory component partially fatigues with continued stimulation. The potential also exhibits temporal summation.

The neurotransmitter receptor profiles of two populations of cells in Aplysia californica are described here for the first time. A and B cells each exhibit a different spectrum of receptors that is characteristic; indeed, they define the cell. A cells appear to be more homogeneous in regards to their receptor population than do the B cells. Inhibitory responses are predominant, more so for A cells than for B cells; this profile correlates with the dominant activity of, and input onto, each cell cluster.

Serotonin, glutamate, and histamine produced excitatory responses on the B cells, thus limiting the possibilities of the A cell transmitter -- the messenger at the A to B cell synapse. Perfusion

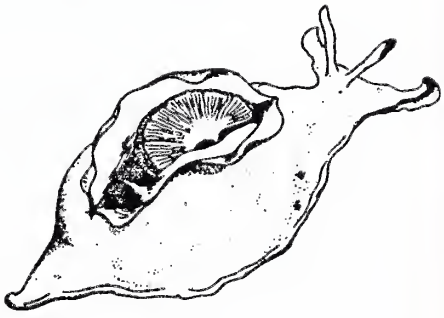
studies of the A to B synapse preparation indicate that neither serotonin, histamine, or glutamate, however, is the transmitter at the synapse. The "non-specific" ionophore antagonists curare and strychnine are without effect on the synaptic response, as are the histamine antagonists pyrilamine and burimamide. Bufotenine, the serotonin A and A' receptor blocker, does not directly affect the psp. Apparent augmentation of the synaptic potential, and the appearance of a biphasic eipsp, at 10^{-4} M bufotenine probably results from secondary effects on the membrane. Further, the psp can not be desensitized by serotonin or glutamate, nor does aspartate have any effect on the synaptic potential. In part, investigations of this nature are restricted by the paucity of adequate antagonists. Clearly, the identity of the messenger remains unknown. The transmitter will most likely prove to be an as yet unidentified neurotransmitter, for surely there are many synaptic messengers and modulators that await discovery.

A series of alterations of membrane potential are found with pressure ejection and ionophoresis of glycine. A biphasic response, mediated by increased conductance of chloride and potassium, respectively, was identified. Both components were reversibly eliminated by the classical glycine antagonist strychnine. However, a repeated search for these responses during the winter months failed to reproduce the initial findings made during the previous summer.

A nearly ubiquitous slow depolarizing response, that is not accompanied by an alteration of membrane conductance, is also found. The response proved to be energy and sodium dependent, but is not

sensitive to strychnine. Nor does the depolarization ever desensitize. Application of the neutral amino acids histidine and glutamine evoke weaker responses that share many characteristics of the glycine-induced response. This response shares many features with energy dependent sodium coupled cotransport of neutral amino acids that is common to many neurons; it does not appear to be generated by activating a classical neurotransmitter receptor.

The spectrum of receptors on Aplysia neurons is vast and the complexity of synaptic integration is great. Moluscan behavior may be "simple" and limited in scope, yet it must remain plastic, and at the same time maintain economy of function and purpose. That is, after all, one of the features that makes the study of the nervous system fascinating. In time, greater information will be gathered concerning the myriad transmitter receptors on a given collection of cells, such as the B cells, that underlie a particular behavior; the inputs to the cells will be completely mapped and the transmitters at each synapse will be identified. Neural organization and integrated activity at the synaptic level will provide the basis on which behavior at higher levels will be understood. With time, as more becomes known, the elegance of the nervous system will become increasingly evident.



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