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Electrophysiological and Pharmacological Properties of Glial Cells Associated with the Medial Giant Axon of the Crayfish with Implications for Neuron-glial Cell Interactions

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ABSTRACT

Schwann-like glial cells surrounding the medial giant axon of the crayfish (Procambarus clarkii) were impaled with glass microelectrodes to study their responses to cholinomimetics, cholinergic receptor blockers and ouabain. Axon electrical properties were simultaneously monitored. Glial cells have a low membrane potential compared to the axon; -42 mV and -85 mV, respectively. Acetylcholine, carbachol and nicotine hyperpolarized the glial cells but did not affect the axon steady-state or active membrane potentials. The action of the cholinergics was completely blocked by d-tubocurarine and α-bungarotoxin. Ouabain hyperpolarized the glial cell but depolarized the axon. Tubocurarine blocked the ouabain hyperpolarization but not the delayed depolarization of the glial cell or the axon. It is concluded that ouabain causes the release of acetylcholine from the glial cell-axon preparation, inducing the glial hyperpolarization. Studies of the axon-glial cell interaction suggest that a function of the glial cell is to actively modulate the periaxonal potassium concentration on a signal from the axon. Periaxonal potassium can strongly affect axon membrane potential through electrogenic Na transport, modifying axon signalling properties.

INTRODUCTION

Several investigators (2,3,4,6) have recognized that the glial cell layer around axons and neurons restricts free movements of ions in and out of the perineuronal space, especially potassium. Accumulated potassium can modify action potential generation, propagation and electrogenic transport processes and therefore the integrative properties of the nervous system (3,4).

Investigations of the electrical and ionic properties of glial cells have emphasized those from the central nervous system. The passive electrical nature of central glial (3,4,7,14) have lead to the conclusion that they are a passive diffusion barrier that restricts the movements of substances between the extracellular bulk solution and the neuronal membrane. More recent studies

of the Schwann cell investment of the squid giant axon (18,19,20) have identified a neurohumoral interaction between axons and the satellite cells. Aspects of these interactions also occur in crayfish glia (12). The neurohumoral action is mediated by pharmacologically identifiable receptors on the satellite cells. The data suggest that the satellite glial cell layer is an active participant in the regulation of the ionic composition of the perineuronal space.

The following report presents an overview of our earlier attempts, as well as recent new data, to identify and quantitate the electrical, ionic and pharmacological properties of the Schwann-like glial cell surrounding the medial giant axon of the crayfish (12). It is expected that this information will provide a better understanding of the interaction of the glial cell with the axon (or neuron) to modify axonal Na:K electrogenic transport and electrical properties which may be related to integrative activity of the nervous system.

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METHODS

Nerve preparation: The medial giant axon of the ventral nerve cord of the crayfish Procambarus clarkii, was used for all experiments. The entire ventral nerve cord was exposed from a dorsal approach according to the techniques previously described (25). The nerve cord was removed from the animal, placed in a lucite chamber and clamped at both ends with spring clips. Excess tissue was removed, the cord desheathed and most of the surrounding small axons removed between the subesophageal and the fifth thoracic ganglia.

Axon electrophysiological techniques: A hole was cut in the isolated medial giant nerve fiber between the third and fourth thoracic ganglia to permit cannulation of the axon with axial recording and stimulating electrodes. The electrode was placed in the axon so that the recording tip was between the first and second thoracic ganglia. The electrode configuration and electronics have been fully described (9). In addition, external stimulating electrodes were built into the chamber so that propagated action potentials could be initiated at a distance from the recording electrode.

This arrangement made it possible to test the viability of the axon in the region where glial cells were to be explored and to combine external stimulation with local membrane electrical conditioning to test for the effects of the drugs on both passive and active axon membrane electrical properties (Fig. 1).

Glial cell electrophysiological techniques: The medial giant axon is ensheathed by a multilayered investment of glial cells, alternating with dense layers of connective tissue (12). The most successful method found for recording from the satellite cells while avoiding problems associated with penetrat-

ing the connective tissue was to cut several small holes in the dorsal surface of the nerve fiber through which the impaling micropipette could be advanced, crossing the axon to the inner surface of the axolemma at the opposite side of the fiber. Further advance of the electrode allowed for the penetration of the innermost layer of cells, the satellite glial cell layer.

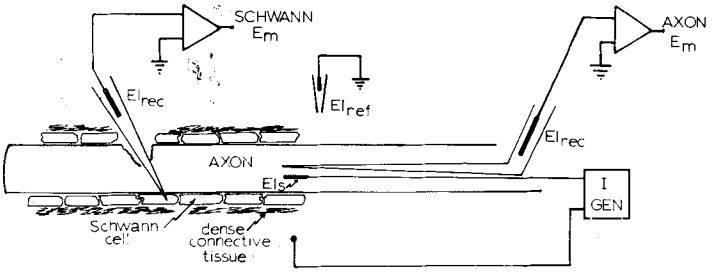

Fig. 1. Diagramatic representation of the axon-glial cell preparation and the electrophysiological set-up for simultaneous recording from axons and glial cells. Abbreviations: E_m, membrane potential; el, electrode; el subscripts rec, ref. and s are abbreviations for recording, reference and stimulating, respectively; I GEN, constant current generator for stimulation of the axon. External stimulating electrodes at cephalic end of nerve not shown in diagram.

During the course of these experiments it became clear that any given control cell, because of its small size, could be impaled and recorded from for only a matter of seconds before slight electrode vibration or bath movement dislodged the electrode from the cell. As a result, the glial cell potential trace was displayed on a storage oscilloscope to facilitate the detection and quantification of such brief impalements (Fig. 2). The preparation and electrophysiological set-up for both the axon and satellite cell are illustrated and described further in Fig. 1.

Solutions: The nerve fiber bathing solution was a modified Van Harreveld (17) crayfish physiological solution containing NaCl, 190 mM; KCl, 5.4 mM; $CaCl_2$, 13.5 mM; $MgCl_2$, 2.6 mM and tris·HCl, 15 mM. Tris base was added in sufficient quantity to bring the solution to pH 7.4. Osmolarity of the solution was 430 mOsm.

Drugs were made up in 1 mM concentrations in normal crayfish solution and stored in the refrigerator until used. For experimental purposes the stock solutions were diluted with crayfish solution to the desired concentration on the day of use. The stocks were kept 3 to 4 days at the most before being replaced with freshly made stock solutions.

The nerve cord preparation in the chamber was constantly superfused with solution during preparation of the giant axon-glial cell system and while recording axon electrical properties. During the period of exploration for satellite glial cells the flow was discontinued to keep movement artifacts to a

minimum. The solution in the chamber could be changed within 15 to 20 seconds. All experiments were performed at room temperature (20-23°C).

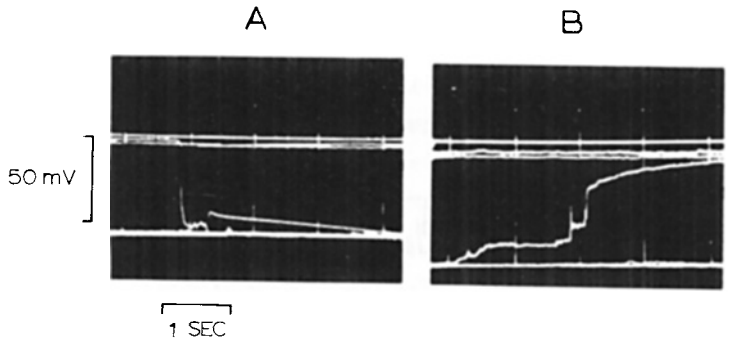

Fig. 2. Oscilloscope tracing of a microelectrode impalement of a single glial cell. Trace A: The rapid downward deflection shows the nature of the potential recorded by a microelectrode as it enters and seals into a Schwann-like glial cell. Trace B: The upward deflection represents the withdrawal of the electrode from the same cell as shown in "A" several seconds later.

RESULTS

The rationale for the study of the Schwann-like glial cells of the crayfish began with the observation (Fig. 3) that the ouabain-sensitive transport current of an axon bathed in a solution depleted of potassium was inhibited to only 80% of its control value (8). Although there is a clear trend of an inhibition of transport with decreasing extracellular potassium, the insensitivity of the transport current to low potassium is in contrast to previous reports in which both steady-state (1) and non-steady-state (stimulated) (16) sodium transport and net transport current could be inhibited to less than 20% of its control value.

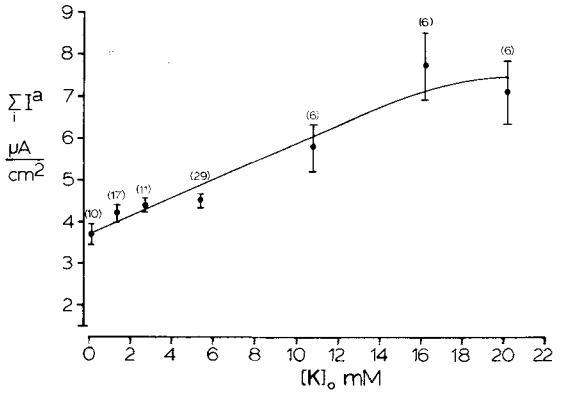

Fig. 3. The effect of extracellular potassium on the ouabain sensitive transport current of axons. Transport current ($\sum_i I_i^a$) is estimated from the ratio between the magnitude of the ouabain sensitive membrane potential and the steady-state specific membrane resistance.

In crayfish axons, even after 3 hours of equilibration in potassium-depleted solutions, both the transport current and intracellular sodium and potassium concentration remained constant (8,26). This observation suggests that the ionic composition of the periaxonal space, was being regulated. One possible source of the periaxonal potassium could be the glial cells, which for a period of time sacrifice their intracellular potassium in order to maintain excitability of the axon.

To understand the nature of the glial cell influence on the periaxonal environment a series of experiments were performed to characterize the electrical and pharmacological properties of the glial cell membrane.

Effect of cholinomimetics and receptor blockers on the membrane potential of glial cells: The resting membrane potential of the glial cell is low compared to the axon; -42 mV and -85 mV, respectively. The membrane potential of the glial cell is also exquisitely sensitive to the application of a variety of cholinomimetic agents, hyperpolarizing the cell about 15 mV with as little as 10^{-8}M drug in the superfusate. Pretreating the axon-glial cell preparation with either d-tubocurarine (10^{-9}M) or α-bungarotoxin prevents the effect of the cholinomimetics or can reverse the cholinomimetic-induced hyperpolarization if applied after pretreatment with these drugs. A histogram presentation of the accumulated data are shown in Figure 4. The following factors need to be noted concerning these data. First, the cholinomimetics used in this study were acetylcholine, nicotine and carbachol. A dose of 10^{-8}M of any one of these agents was sufficient to cause a hyperpolarization of at least 10 mV and 10^{-7}M was a maximal dose resulting in a 15 to 18 mV hyperpolarization. Second, the effect of each drug was similar with respect to its effect on membrane potential and frequency distribution. The center panel of Fig. 4 represents the accumulated data for all compounds. Third, both α-bungarotoxin and d-tubocurarine would block or reverse the cholinergic effect to the same extent so that the lower panel of Fig. 4 combines these data as well. Finally, muscarine (10^{-6}M) or methacholine had no effect on the glial cell potential.

In most experiments, the second of the medial giant axon pair found in the crayfish ventral nerve cord was monitored during treatment of the axon-glial cell preparation with the various drugs used in this study. In no case could we find a discernible effect of these agents, in the concentrations used here, on excitable membrane properties. The question of repetitive, high frequency stimulation will be taken up in a later section.

Effect of Ouabain on the membrane potential of the axon and its associated glial cells: Villegas (22) has proposed that the low membrane potential of the Schwann cell associated with the giant axon of the squid can be accounted for by a depolarizing electrogenic Na:K transport system. This was inferred primarily from the facts that ouabain caused a large hyperpolarization of the

Schwann cell and was appropriately inhibited by low potassium. Similarly, the glial cell of the crayfish exposed to 10^{-3}M ouabain hyperpolarized rapidly. Within 15 to 20 minutes the cell began to depolarize and approached zero membrane potential in approximately 15 minutes. The biphasic nature of this response is illustrated in Fig. 5.

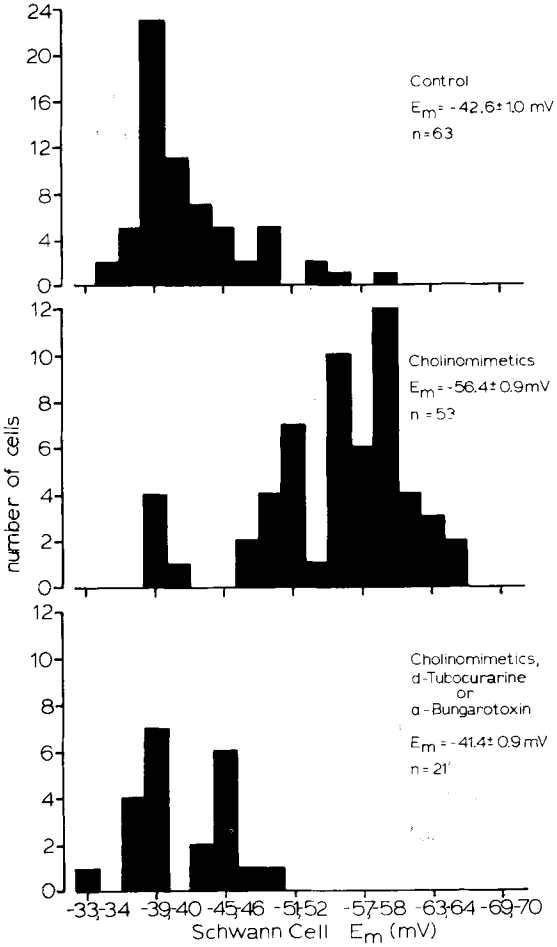

Fig. 4. The effect of cholinomimetics and receptor blockers on the frequency distribution of the resting membrane potentials of individual glial cells. Each division on the abscissa represents 2mV spans. Mean membrane potentials given as the mean ± SEM, n=number of measurements. Top panel, control (untreated) glial cells. Middle panel, axon-glial cell preparation treated with cholinomimetics. Bottom panel, the effect of receptor blockers on the glial cell response to cholinomimetics.

Although the investigation of Villegas (22) was convincing that the low potential could result from a depolarizing electrogenic transport system, recent work has demonstrated that ouabain can release acetylcholine from the neuromuscular junctions (5,23,24). To determine if ouabain was releasing endogenous acetylcholine in the crayfish axon-glial cell preparation, thereby causing the glial cell to hyperpolarize, the axon was pretreated with d-tubocurarine or α-bungarotoxin before exposing it to ouabain. In this case, ouabain did not hyperpolarize the glial cell (Fig. 5). Under these conditions, the glial cell actually depolarized slightly due to ouabain. Steady-state membrane potential data for the experiments presented in Fig. 5 are shown in the inset. Data for all experiments in the series are shown in Table 1.

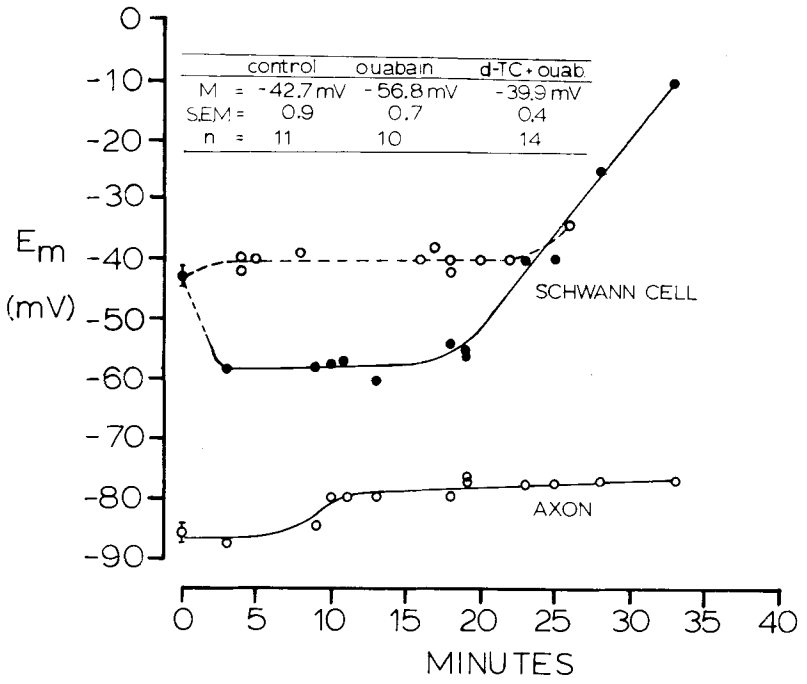

Fig. 5. Effect of 10^{-3}M ouabain on the membrane potential of axons and glial cells. The solid trace identified with closed circles is the time course of the ouabain effect on glial cell potentials. Each point is a single measurement from an individual glial cell. The dashed line trace (open circles) shows the effect of pretreating the glial cell preparation with d-tubocurarine on the response of the glial cell to ouabain. Lower trace (open circles) is the time course of the ouabain effect on the axon membrane potential sampled at the same time that the glial cells were sampled. Inset contains the steady-state membrane potential following each condition. Measurements collected from 8 axons.

Table 1. The effect of ouabain and cholinergic receptor blockers on the resting membrane potential of glial cells.

	Control	Ouabain(10^{-3}M)	d-TC/α-BTX + Ouabain
Em	-42.2 mV	-51.7 mV	-39.5 mV
SEM	1.0 mV	0.8 mV	0.6 mV
n	66	43	21

An additional small number of experiments also demonstrated that ouabain and the cholinomimetics appear to act through the same mechanism. When the axon-glial cell preparation is exposed to ouabain (10^{-3}M) and nicotine (10^{-7}M) simultaneously, the resultant hyperpolarization is no larger than either alone would produce.

Finally, note that the only effect of ouabain on the axon is a depolarization which is unaffected by d-tubocurarine or nicotine.

Effects of cholinomimetics and d-tubocurarine on the post-stimulation hyperpolarization response of the axon: Earlier investigations (18,19) showed that repetitive stimulation of the axon caused the Schwann cell to hyperpolarize. The following preliminary experiments are an attempt to ascertain the nature of the glial cell influence on the axon's electrical properties.

Under normal circumstances repetitive stimulation of the axon produces an axon hyperpolarization during and following the period of stimulation which has been shown to be due to electrogenic Na transport (10,13). The hyperpolarization is accentuated in low potassium (Fig. 6, Top Row). Carbachol or nicotine (10^{-7}M) results in a depressed hyperpolarization during the stimulation period, but an enhanced post-stimulation hyperpolarization. Tentatively, this could be

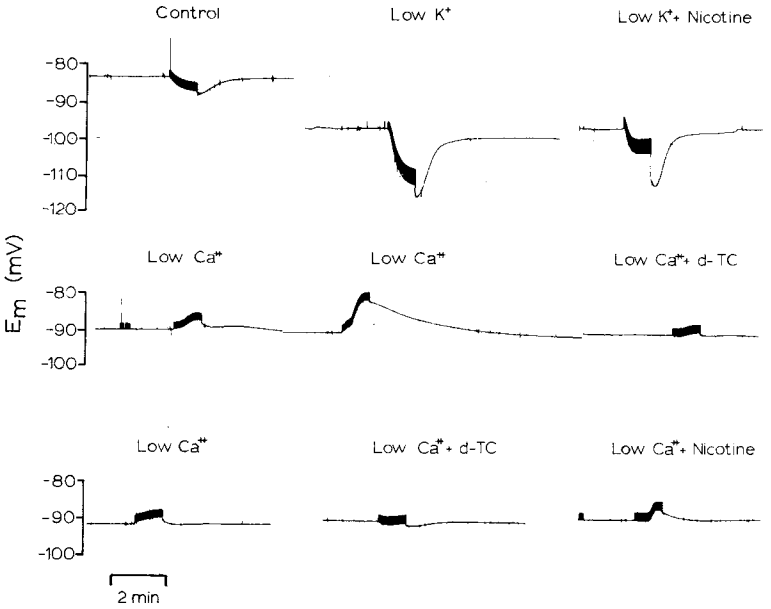

Fig. 6. Effects of cholinomimetics and d-tubocurarine on the post-stimulation hyperpolarization (PSH) of axons. The period of stimulation was 1 minute at 30 stimuli/sec. The wide portion of the trace is the period during which superthreshold stimuli were delivered to the nerve. The top row: the stimulating effect of low potassium on the PSH and the effect of cholinomimetics. Middle row: the depressant action of low Ca^{++} and the restoring effect of d-tubocurarine. Lower row: the restoring effect of d-tubocurarine is opposite the effect of nicotine in low Ca^{++}. Each row of traces represents sequential recordings from one individual axon.

interpreted as an effect of a greater than normal accumulation of potassium in the perineural space due to the cholinomimetics. This would result in a stimulation of electrogenic transport.

In another experiment, the effect of d-tubocurarine appears to be opposite that of the cholinomimetics (middle row). The axon was treated (low Ca^{++}) so

as to depress the post-stimulation hyperpolarization response. The effect of stimulation is a depolarization during the period of stimulation and an exponential return to the baseline following stimulation. Treatment of the axon-glial system with d-tubocurarine tends to restore the normal poststimulation hyperpolarizing response. The enhanced potassium accumulation seen with low Ca^{++} and stimulation appears to be inhibited by the receptor blocker.

The implication of these preliminary findings with respect to the axon-glial cell interaction will be dealt with in more detail in the Discussion to follow.

DISCUSSION

The data presented in this report confirm previous investigations from this laboratory (12) and those of Villegas (18,19,20,21,22) that the membrane potential of Schwann cells associated with the giant axon of the crayfish and squid is normally low (-40 to -42 mV) and that they are exquisitely sensitive to cholinomimetics via the activation of nicotinic receptors on the glial cell membrane.

In studies of the crayfish glial cell (11,12) it was found that ouabain caused the cell to hyperpolarize as does the Schwann cells associated with the giant axon of the squid (22). The latter investigation, although not unequivocal, strongly suggested that the ouabain induced hyperpolarization and low membrane potential of the Schwann cell could be explained by a glial cell depolarizing electrogenic Na:K exchange transport system. That is, a Na:K exchange ratio that was less than 1.

Three factors tend to raise questions concerning the above interpretation. First, the magnitude of the hyperpolarization of the glial cell produced by ouabain is quantitatively similar to that produced by cholinomimetics. Second, the simultaneous treatment of the glial cell preparation with ouabain and cholinomimetics does not produce any more hyperpolarization than either agent alone. Third, recent studies have demonstrated that ouabain can cause the release of acetylcholine from nerve endings (5,23,24).

Our experiments show that d-tubocurarine and α-bungarotoxin prevent the hyperpolarization of the glial cell normally caused by ouabain but not the depolarization that is related to the loss of ionic gradients in both glia and axons.

Recently, Villegas (personal communication) has performed experiments similar to those just described. Interestingly, he found no inhibition of the ouabain-induced hyperpolarization with α-bungarotoxin or d-tubocurarine. He did observe a significant delay in the time of onset of the later depolarization. The delay in depolarization is probably related to the inhibition of the potassium permeability increase normally associated with acetylcholine activation of the glial membrane receptors (20).

It can be concluded that ouabain causes the release of acetylcholine in both the squid and crayfish giant axon-glial cell preparation but the mechanism for the low membrane potential in the crayfish is due primarily to membrane ionic permeability properties where as in the squid it appears to be related to the combined contribution of a depolarizing, ouabain-sensitive electrogenic transport system and ionic permeability properties. Differing mechanisms for the glial cell potential might not be surprising considering the difference in the normal environments of the animals, that is, marine tropical versus freshwater temperate. Although the mechanism for the low membrane potential of the glial cells of the two animals differ, they are both sensitive to transmitter quantities of cholinergics suggesting that their physiological responses to axon-glia interactions may be similar.

Villegas (18,19) demonstrated that a humoral communication link exists from the axon to its glial cell investment, where repetitive stimulation of the axon causes the glial cells to hyperpolarize via activation of nicotinic receptors. In this investigation we attempted to ascertain the nature of the glial cell to axon feedback. It should be emphasized that the experiments described here are preliminary and the approach, in part, was indirect. It is presumed that since the drugs used to effect the glial cell electrical properties have no discernible effect on "single shot" electrical properties of the axon, any effects described on long term axon function is mediated via glial cell-axon interactions. It should be further noted that certain procedures were followed to enhance those properties that are most likely to be affected by a glial cell-axon interaction.

The data presented in this report shows that the low potassium enhanced poststimulation hyperpolarization (PSH) of the crayfish giant axon is modified by carbachol and nicotine. The nature of this response suggests that normal potassium accumulation that occurs with repetitive stimulation is exaggerated in the presence of cholinergic agents. During the stimulation period, as compared to the control, the hyperpolarization is depressed significantly. Following stimulation the hyperpolarization is massively enhanced as though there was a potassium injection into the perineural space with a resultant stimulation of the axon's electrogenic transport system (15).

The depression of the PSH or its conversion to a depolarization of the axon in low Ca^{++} is apparently due to a decreased membrane resistance in both the axon and glial cells and an exceptionally high permeability to potassium. Treatment of the glial cell-axon preparation with d-tubocurarine causes the PSH to be partially restored. Washout of the d-tubocurarine and addition of nicotine enhances the depolarization which occurs in the axon during the stimulation period as compared to the low Ca^{++} control.

This preliminary data suggest that the relationship between the axon and

glial cell is one in which the ionic content of the perineuronal space is modified by accumulation of potassium in the space. The potassium is contributed by both the axon during stimulation and by the glial cell as a result of the activation of its nicotinic receptors (18,19). The result of this accumulation of potassium is a stimulation of electrogenic active transport of the axon leading to a hyperpolarization and a change in such electrical properties of the axon and endings as impulse propagation velocity, stimulation safety factors (threshold), transmitter release, etc. (3,4). The significant difference between the process suggested here and the one apparently operating in the CNS of other animals (3,4) is that the glial cell investment of the crayfish and squid giant axon is an active participant in the process.

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