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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The research program was and continues to be directed to the functional interactions between nerve fibers and their satellite glial cell investment. The long term aim is to determine the role of the glial cell in maintenance of the ionic homeostasis of the perineural environment during resting and active neuron states, the mechanisms which glial cells and neurons use to modulate each others metabolic state and the chemical, electrical and neurohumoral communication links that exist between the axon (neuron) and its associated glial (Schwann) cells.

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The nervous system of the freshwater crayfish, *Procambarus clarkii* was the preparation of choice for most of the studies the axon has been well characterized electrically and metabolically by a number of investigators including the P.I. The glial cell investment of the crayfish medial giant axon has been partially characterized by this investigator. At the time this research program was started the crayfish was only one of two such axon-Schwann cell preparations characterized for this work. The other being a tropical squid axon Schwann cell preparation not available in this country.

In latter phases of this research program the emphasis, as originally planned, was shifted to the development of a mammalian cell culture model for axon-Schwann cell interaction studies for the purpose of determining the generality of our findings in the invertebrate systems.

These studies were expected to provide us with a greater understanding of the mechanisms by which axons/neurons and their glial cell investments communicate to actively regulate the ionic microenvironment of the nervous system and protect the signaling properties of nerve tissue. Possible clinical correlates in which glia are especially suited to be actively involved include nerve nutrition, nerve regeneration and the action of physiological as well as toxic or therapeutic psychoactive agents.

NEURON-GLIA INTERACTIONS AND NERVOUS SYSTEM HOMEOSTASIS

FINAL REPORT

U.S. Army Research Office
Proposal No. P-23114-LS, Modification P00003
Contract No. DAAL03-86-K-0023

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LIST OF APPENDIXES

1. Copies of manuscripts and abstracts published, in press, accepted for publication or submitted.

REPORT

Neuron-glia Interactions and Nervous System Homeostasis.

Statement of the problem

The research program was and continues to be directed to the functional interactions between nerve fibers and their satellite glial cell investment. The long term aim is to determine the role of the glial cell in maintenance of the ionic homeostasis of the perineural environment during resting and active neuron states, the mechanisms which glial cells and neurons use to modulate each others metabolic state and the chemical, electrical and neurohumoral communication links that exist between the axon (neuron) and its associated glial (Schwann) cells.

The nervous system of the freshwater crayfish, *Procambarus clarkii* was the preparation of choice for most of the studies the axon has been well characterized electrically and metabolically by a number of investigators including the P.I. The glial cell investment of the crayfish medial giant axon has been partially characterized by this investigator. At the time this research program was started the crayfish was only one of two such axon-Schwann cell preparations characterized for this work. The other being a tropical squid axon Schwann cell preparation not available in this country.

In latter phases of this research program the emphasis, as originally planned, was shifted to the development of a mammalian cell culture model for axon-Schwann cell interaction studies for the purpose of determining the generality of our findings in the invertebrate systems.

These studies were expected to provide us with a greater understanding of the mechanisms by which axons/neurons and their glial cell investments communicate to actively regulate the ionic microenvironment of the nervous system and protect the signaling properties of nerve tissue. Possible clinical correlates in which glia are especially suited to be actively involved include nerve nutrition, nerve regeneration and the action of physiological as well as toxic or therapeutic psychoactive agents.

Summary of Results

The following projects have been pursued during the period covered by the research agreement:

1. Nerve energy metabolism.

2. Ionic basis for the membrane potential of invertebrate Schwann cells, its response to cholinergic agents and the role of these responses in Schwann cell transport and ionic regulation of the neural microenvironment.

3. Neuron-glia (axon-Schwann cell) interactions.

4. Role of the perineurium (Blood-Brain Barrier) in ionic homeostasis of the central nervous system in the crayfish.

1. Nerve Energy Metabolism

As a corollary to our previous experiments on the high energy phosphate metabolism of single isolated giant axons and their associated glial cells of the crayfish ventral nerve cord we have extended our experiments to the oxygen metabolism of the same preparation. Our experiments suggest that the energy metabolism of the nerve fiber is extra-odinarily sensitive to the presence of the glial cell. The inverse is also true. These results presented the problem of the development of a method for the measurement of minute amounts of oxygen consumed by single isolated giant axons. Differentiation between the oxygen consumed by the axon and that consumed by the glial cells without changing their metabolic and physiological interactions compounds the problem.

We have developed a method for oxygen consumption based on oxygen quenching of fluorescence. A 200 um dialysis tube containing the fluorescent chemical dissolved in paraffin oil is placed in a capillary tube chamber in a fluorescence microscope for measurements. See the submitted reprint for a detailed description of the method Hargittai, et al (1988). In initial experiments we compared the oxygen consumption of the intact axon-Schwann cell preparation to the preparation in which the axon had been internally perfused with extracellular fluid. We found that the glial layer which comprises approximately 5% of the total volume of the axon accounted for approximately 50% of the ouabain sensitive oxygen consumption. Although this is an extra-ordinary amount of oxygen consumption when the axon/glial cell volume ratio is considered, the high energy phosphate consumption to oxygen consumption ratio was approximately .3 rather than 3:1. This data suggests 2 possibilities: (1) that our previous measurements of phosphate consumption are incorrect (low) by a factor of 10 which seems unlikely in light of the extensive controls performed to insure measurement of total phosphate content of the axoplasm or (2) the axon and Schwann cell have such a close relationship that in the absence of a viable axon the glial layer down-regulates its energy consuming functions. Structural relationships, for example the amount of glial plasma membrane of the sheath is approximately 10X that of the axon, support the concept that the axon-glial cell relationship was disturbed.

To study this possibility we have completed initial experiments with a non-permeant mitochondrial poison (carboxy-atractylocide), testing the effectiveness of the agent on mitochondrial oxygen uptake in pure cell free

systems. In experiments to test its toxicity on the intact axon we have also found that we can inject 1000 to 10,000 times the quantity necessary to block oxygen uptake without effecting the electrophysiological function of the axon for up to 1 1/2 hr. The last experiments to be performed are now under way. These will measure oxygen consumption of the glial (Schwann) layer in the presence of electrophysiological normal but metabolically deficient axons. It is expected that these experiments will be more representative of the normal *in vivo* situation. It is expected that the oxygen consumption of the glial layer will represent 90% of the total in the intact nerve-Schwann cell preparation. The importance of such experiments, as based on earlier findings with phosphate consumption, is that the intact resting axon conserves energy by decreasing its membrane permeability by a factor of 10-20 times to ions and other substances which require high energy phosphate for transport. That is, we propose that the resting nerve has a membrane resistance in the range of 20,000 rather than the 1 - 2,000 usually estimated by methods that shift the membrane out of its true zero current steady-state. The data that is beginning to accumulate from our studies also suggests that the glial layer is a major contributor to maintenance of nervous system steady-state conditions by doing much of the transport work previously attributed to the nerve fiber.

2. Ionic Basis for the Low Membrane Potential of Glial Cells and Its Relationship to Periaxonal Ionic Homeostasis.

The experiments related to the investigation of the ionic basis for the low membrane potential of glial cells and its relationship to transport of ions out of the periaxonal space were completed in the early phase of the research program period covered by this agreement.

We discovered that the intracellular K concentration of the glial cell is low, about 65mM compared to the axon which is about 275 mM, giving an equilibrium potential for the glial cell of -60 to -65 mV. The membrane potential is about -40 mV due to a high permeability and outward electrochemical gradient for Cl ions. Cholinergic agents, applied externally or acetylcholine released physiologically by axon stimulation cause the glial cell to hyperpolarize toward the K equilibrium potential as a result of a cholinergically-induced decrease in Cl ion permeability. The increased glial cell membrane potential decreases the K outward gradient reducing its efflux into the periaxonal space. This mechanism allows for an apparent larger dilution volume for the K released by the axon during stimulation and with an equal or greater K concentration in the periaxonal space the glial cell is able to take up K in excess of its normal loss its electrochemical gradient.

In a large series of additional studies on the intact axon-glial cell preparation two separate K uptake systems were shown to participate in the control of the periaxonal space K concentration. We discovered that the resistance in series with the axon membrane was a reliable, although qualitative, measure of glial cell volume and indicator of the manner in which K is taken up by the glial cell. In situations where small amounts of K enter the periaxonal space the main route for K uptake into the glial cell is the ouabain-sensitive Na-pump. Under these conditions the cell volume decreases. In the case where large amounts of K enter the periaxonal space both the ouabain-sensitive Na-pump and furosemide-sensitive Na-K-2CL co-transport system act to take up K. In this case cell volume increases with increasing participation of the co-transporter.

The results of these experiments are in press at this writing. They will be published as a a series of three articles (Brunder and Lieberman, Hassan and Lieberman and Lieberman and Hassan) in Neuroscience Vol.25 No.3, 1988 (see appendix).

3. Neuron-Glia Interactions

The fact that axon stimulation causes the release of acetylcholine suggests that an agent is released during stimulation that acts on the glial cell initiating a cascade of events that ends, at the least, in a significant hyperpolarization of the glial cell. Since K alone does not cause the hyperpolarization one must assume that another agent is involved. A series of experiments using the giant axon of the squid mantle nerve were performed in an attempt to identify the agent released from the stimulated axon. We were able to provide convincing evidence that glutamate was released in direct proportion to axon stimulation and that quisqualte/kainate type glutamate receptors were present on the glial cell membrane. On activation of these receptors with glutamate or the appropriate glutamate receptor agonist, the glial cell membrane depolarized leading to the release of acetylcholine from the glial cell which acts back upon itself to lead to glial cell hyperpolarization. A published abstract describing these results is found in the appendix along with a manuscript which is expected to be submitted to Glia within the month. Experiments have been performed on crayfish axon-glial cell preparation with results similar to those seen with the squid preparation.

An additional study has been completed in collaboration with Dr. Robert Grossfeld of the Department of Zoology at N.C. State University. In these studies we examined the transfer of macromolecules between the axon and glia concentrating on the axon to glia direction. We found that both a fluorescent protein and dextran transport marker was effectively transferred between the two cells. Since most of the markers were found in the cytoplasmic regions of the sheath, not the extracellular space it is proposed that the transfer is cell to cell. Further experiments are being planned for the future to test this hypothesis. A manuscript describing these results are IN PRESS in Glia.

4. Role of the perineurium in nervous system ionic homeostasis

All vertebrates and higher invertebrates are known to have a blood brain barrier separating the circulating blood from the nervous system with a primary role to maintain a stable neural environment compatible with integrative nervous activity. The crayfish is a freshwater crustacean with a partially open circulatory system requiring an efficient barrier to protect the nervous system from disturbances in body fluid ionic content. The purpose of this work was to study the mechanisms present in the *perineurial* glia for nervous system ionic homeostasis for comparison with the mechanisms reported for the periaxonal glia. Our findings in this area are fascinating in that we have discovered that the rather loose tissue that makes up the perineural sheath has a significant electrical resistance (300-400 ohms.cm^2) and has the capability to restrict Na movement through the sheath almost completely. It is most selective for K ion and a selectivity for Cl of about 1/10th that of K. The sheath appears to be symmetric with respect to permeability restriction although permeability is sensitive to internal and external K concentration and the voltage across the sheath membrane. Some of the restrictiveness for ions appears to be attributable to the mucopolysaccharide matrix that makes up the basement membrane and the intercellular matrix

between cells. Sheath permeability and selectivity are sensitive to hyaluronidase treatment. The voltage generating capacity of the membrane is related to the fact that K and Cl are not of equal permeability. It has been shown that even in preparations that have no voltage generating capacity it is generally a function of the decrease in K permeability rather than the increase in Cl permeability, thus maintaining a high degree of restrictiveness of the barrier. The selectivity of the barrier is quite sensitive to a number of treatments such as pH, Ca depletion, La and enzyme treatment. It is of interest that we were unable to find significant numbers of tight junctions between cells that can account for the permeability restriction and thus must tentatively conclude that a combination of perineurial glial cell transport and membrane K-conductance channels are responsible for the K selectivity over a base selectivity of both selectivity and restrictiveness provided by the basement membrane and intercellular matrix. We further propose that the mucopolysaccharide matrix of the perineurium may be a model for the role of extracellular matrix substance in ionic regulation of the intercellular space.

A manuscript has been submitted to the J. Physiol. (Lond.) fully describing our results in this topic area and several others are in preparation concern the structure of the blood-brain barrier, metabolic properties of the perineurial glia and current-voltage properties of the perineurial lamella.

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STUDIES OF THE CARDIAC-LIKE ACTION POTENTIAL IN CRAYFISH GIANT AXONS INDUCED BY PLATINIZED TUNGSTEN METAL ELECTRODES

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Accepted 22 October 1986

SUMMARY

A lightly platinized tungsten (Pt-W) wire electrode, axially inserted into a crayfish giant axon, causes the development of cardiac-like action potentials with durations of up to 4 s. The plateau in membrane potential typically occurs within 10 min of the start of action potential elongation. The effect occurs without passing current through the Pt-W electrode and is temporally related to a dramatic decrease in intracellular pH (pH_i). Such an effect cannot be induced by a decrease in pH_i produced by equilibrating the axon with HCO_3^--CO_2 solution (pH 6), an NH_4Cl rebound or direct intracellular injection of PO_4^{3-} buffer (pH 4·5). Action potential elongation is accompanied by a block of delayed rectification and the possibility that inward rectification also develops cannot be ruled out. Plateau generation requires Na^+ and Ca^{2+} inward currents as demonstrated by abolition of the plateau by $[Na^+]_o$ or $[Ca^{2+}]_o$ depletion or treatment with tetrodotoxin (TTX) or verapamil. The block of outward rectification by Pt-W requires external Na^+ or Ca^{2+}. Action potential elongation produced by 3,4-diaminopyridine is not sensitive to verapamil and the waveform is different from that produced by Pt-W. The data support the possibility that different classes of excitable membranes have similar channel populations and that the functional differences between them reside in the inhibitory or masking influences that are present in the microenvironments of the various membrane channels.

INTRODUCTION

This study arose from a chance observation made while using tungsten (W) wire as a substitute for platinum (Pt) as an intracellular current-passing electrode. Tungsten was expected to be a good alternative to Pt because of its superior mechanical properties and equivalent electrical properties.

When crayfish giant axons were cannulated with a platinized tungsten (Pt-W) wire, prepared similarly to platinized platinum electrodes, modification of the action potential (AP) occurred within 15 min. Whereas the normal action potential has a duration of approximately 1 ms, action potentials with durations of up to 4 s were

Key words: axons, crayfish, cardiac-like action potentials, tungsten electrodes, K^+, Ca^{2+}, channels.

ANALYTICAL BIOCHEMISTRY 163, 418–426 (1987)

A Pyrene Fluorescence Technique and Microchamber for Measurement of Oxygen Consumption of Single Isolated Axons

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Received October 17, 1986

Pyrene fluorescence is quenched by oxygen in an inverse and linear manner related to the partial pressure of O_2 in solution. We have developed a microchamber for measuring $\dot{Q}_{O_2}$ of a single isolated axon, monitoring the change in fluorescence of a pyrene probe. The probe consists of a Spectra/Por dialysis hollow fiber filled with 2.5 mM pyrene in paraffin oil. The probe is inserted into a 1-mm-i.d. 2-cm-long quartz capillary tube with a freshly isolated crayfish medial giant axon. The capillary is mounted in an apparatus that forms an air- and water-tight seal except for a 0.2-mm-i.d. stainless steel tube at both ends permitting the exchange of solutions. An Olympus inverted microscope, equipped with epifluorescence optics and a 150-W xenon lamp, is used to view the preparation, generate the excitation light, and monitor the emitted fluorescence with a photomultiplier tube placed in the microscope TV port. A dichroic filter unit is utilized to select an excitation wavelength of 350 nm and collect emitted light above 420 nm. The signal is amplified with a Keithley 480 picoammeter and recorded on a strip chart. $\dot{Q}_{O_2}$ of isolated axons was $552 \pm 70 \times 10^{-6}$ nmol O_2/liter tissue × min. Following sequential treatment with 2 mM ouabain and 2 mM NaCN, $\dot{Q}_{O_2}$ decreased by 22 and 82%, respectively. These data are consistent with $\dot{Q}_{O_2}$ measurements of whole nerve cord made with a Clark electrode O_2 monitor. With minimal modification this system could be used for metabolic measurements on small quantities of cells in culture, microgram quantities of biopsy material, and simultaneous measurements of $\dot{Q}_{O_2}$ and contraction of single muscle fibers.

KEY WORDS: pyrene; fluorescence; oxygen probe; ultramicrotechniques; neurochemistry.

The ratio of high energy phosphate (~P) production to oxygen consumption ($\dot{Q}_{O_2}$) is indicative of the metabolic pathways and major substrates used by cells in maintaining their physiological and structural integrity. As such, it is an important parameter when studying cellular metabolism. The P/O ratio may also serve as a useful reference in experiments in which ~P requirements of a specific cell function are studied. That is, confidence is increased that a measured value of P/O is representative of the actual energy requirement of the process if this ratio is within the normal range for metabolism of aerobic cells.

A search of the literature for a convenient, reliable, and cost-effective method to measure the $\dot{Q}_{O_2}$ of single isolated axons and to differentiate between the axon and its glial sheath proved fruitless. Therefore, we designed a simple air- and water-tight chamber to measure the $\dot{Q}_{O_2}$ of single isolated axons and their glial sheaths using a fluorescent probe and a standard inverted fluorescence microscope. The oxygen probe used for continuous oxygen monitoring is a modification of the fluorescent pyrene technique originally developed by Longmuir and colleagues for intracellular O_2 measurements (1,2). Pyrene, a highly fluorescent, hydrophobic, and nontoxic compound, is dissolved in paraffin oil and encapsulated within a transparent microdialysis tube. The principle of the method is based on fluorescence quenching by oxygen, which is proportional to $[O_2]$ in the solution.

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BRIEF COMMUNICATION

PERIAXONAL K^+ REGULATION IN THE SMALL SQUID *ALLOTEUTHIS*

Studies on Isolated and In Situ Axons

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ABSTRACT A novel giant axon preparation from the squid *Alloteuthis* is described. Properties of in situ and isolated axons are similar. Periaxonal K^+ accumulation is a function of the physiological state of the animal and of the axon and its sheathing layers. Carefully dissected isolated axons, and axons in situ in a healthy mantle, show much less K^+ accumulation than previously reported in squid. It is suggested that the Schwann cells are involved in the observed K^+ regulation.

INTRODUCTION

In classic experiments on the isolated giant axon of the squid *Loligo forbesi*, Frankenhaeuser and Hodgkin (1956) showed that stimulation at high frequency caused accumulation of K^+ in the periaxonal space. The $[K^+]$ was estimated from the undershoot (hyperpolarization) of the action potential during a train of impulses. The results were compatible with K^+ clearance via a permeable barrier that they identified with the Schwann cell layer.

Since that study, it has generally been assumed that an increase in extracellular $[K^+]$ is a necessary consequence of neural activity, and indeed a rise in $[K^+]$ around active neurones has been demonstrated in numerous vertebrate and invertebrate preparations (Orkand et al., 1966; Baylor and Nicholls, 1969; Lux and Neher, 1973; Heinemann and Lux, 1977; Syková and Orkand, 1980; Orkand, 1980). However, it is now known that the nervous system has several mechanisms for minimizing K^+ accumulation, including specializations of glial cell membranes (see, e.g., Coles, 1985). Glial mechanisms have generally been investigated in isolated and cultured glia (Kettenman et al., 1983; Hertz, 1986; Kimelberg et al., 1986), and it has been difficult to extrapolate from these to the in vivo condition.

We chose to reexamine the role of glial cells in extracellular K^+ regulation, using a novel squid axon preparation which has several advantages, and in which normal geometrical relations between axon and glia are preserved. Squid giant axon physiology has been thoroughly investigated, and knowledge of the kinetics of K^+ clearance from the axon surface is important for interpretation of the membrane ion currents (Adelman and Fitzhugh, 1975; Clay, 1986). A great deal is known about the pharmacology of the periaxonal glial (Schwann) cells, and the way they respond to axonal stimulation (Villegas, 1984; Evans et al., 1985, 1986).

The small squid *Alloteuthis subulata*, mantle length 6–10 cm, can be caught with minimal damage and survives well in the laboratory. The thinness and transparency of the mantle makes it a promising preparation for recording from giant axons in situ. The vasculature is accessible for cannulation and perfusion. In this paper we describe the preparation, and compare the properties of isolated and in situ axons. We present evidence that K^+ accumulation is much less marked than previously shown if the axons/Schwann cell sheaths are in good physiological condition. In the subsequent paper, we present a quantitative treatment that explores the mechanisms responsible (Astion et al., 1988).

Preliminary accounts have been published (Abbott et al., 1985; Pichon et al., 1987, 1988).

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STUDIES OF AXON–GLIAL INTERACTIONS AND PERIAXONAL K^+ HOMEOSTASIS—I. THE INFLUENCE OF NA^+, K^+, Cl^- AND CHOLINERGIC AGENTS ON THE MEMBRANE POTENTIAL OF THE ADAXONAL GLIA OF THE CRAYFISH MEDIAL GIANT AXON

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Abstract—The ionic basis for the low (−40 mV) resting membrane potential of glial cells surrounding the giant axons of the crayfish and their hyperpolarization by cholinergic agents (to −55 mV) was studied using standard electrophysiological techniques, ionic substitutions and pharmacological agents. The resting membrane potential of the glial cell was depolarized by increasing $[K^+]_o$, but the response was not Nernstian. Na^+ depletion caused a small depolarization of the glial resting membrane potential, whereas Cl^- depletion resulted in a hyperpolarization comparable to that seen with carbachol at various $[K^+]_o$. Both furosemide (1 mM) and bumetanide (0.1 mM) produced an 8–10 mV hyperpolarization as compared to 15–17 mV seen with Cl^- depletion or carbachol. Carbachol has no further effect on the potential following furosemide treatment of Cl^- depletion. After carbachol administration or Cl^- depletion the resting membrane potential of the glial cell responded to $[K^+]_o$ in a more Nernstian manner.

The data indicate that the low resting membrane potential of glial cells is due to a combination of a low $[K^+]$ and an outwardly-directed (depolarizing) Cl^- electrochemical gradient. Carbachol acts to decrease Cl^- conductance, resulting in the hyperpolarization of the glial cell membrane and a decrease in the outwardly-directed K^+ electrochemical gradient by approximately two-thirds. We hypothesize that this mechanism for modulation of the glial cell membrane potential and the K^+ electrochemical gradient serves to enhance the uptake of K^+ by the glial cell transport system.

The role of the glial cell layer in restricting the free movement of substances between the adaxonal or perineural space and the bulk extracellular space has been the subject of extensive studies[2,5,6,14,28] because of its importance to regulation of $[K^+]_o$ and maintenance of integrative nervous activity. Accumulation of $[K^+]_o$ in the immediate vicinity of axons and neurons during their excitation can drastically affect a number of properties including resting membrane potential (E_m), action potential amplitude, synaptic transmission, excitability threshold and action potential propagation velocity of the nerve fiber. Frankenhaeuser and Hodgkin[14] proposed that the Schwann cell layer surrounding the squid giant axon corresponds to a barrier restricting the passive diffusion of K^+ into the bulk extracellular fluid with a time constant of 50–100 ms. This idea was later modified by Orkand *et al.*,[28] who proposed that the glial cells of the central nervous system acted as passive "spatial buffers" in moving K^+ from areas of high concentration to areas of low concentration by a mechanism involving the asymmetric depolarization of glial cells.

In contrast to the findings of Frankenhaeuser and Hodgkin[14] the more recent investigation by Shrager *et al.*[34] showed that K_o^+ can be cleared from the perineural space of crayfish giant axons with a time constant of 5 ms and Abbott *et al.*[1] have demonstrated that K^+ does not accumulate in the perineural space of the giant axon of the small squid, *Alloteuthis*, during 100 Hz stimulation which is equivalent to a K^+-clearance time of less than 10 ms.

After the first few milliseconds of high frequency stimulation, the clearance of $[K^+]_o$ in the giant axons of both *Alloteuthis* and the crayfish cannot be accounted for by passive diffusion alone. Similarly, spatial buffering,[28] which depends on high sensitivity of the glial cell membrane potential to a small increase in $[K^+]_o$ (a Nernstian response to K^+), is unlikely to be the sole mechanism for rapid K^+ clearance from the adaxonal space of giant axon systems since the low membrane potential of adaxonal glia and their hyperpolarization during stimulation of the axon[11,12,31,41] would limit clearance forces. Therefore, active ion transport mechanisms of glia, in addition to diffusion and spatial buffering, would appear to be necessary for long-term $[K^+]_o$ homeostasis. Rapid clearance of perinaxonal K^+ can be enhanced by glial cell uptake of K^+ maintaining an apparent large extracellular volume for dilution of the entering K^+.

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Abbreviations: E_m, resting membrane potential; P_{Na}, P_K or P_{Cl}, permeability of Na or K or Cl; R_s, series resistance; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; d-TC, d-tubocurare.

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STUDIES OF AXON–GLIAL CELL INTERACTIONS AND PERIAXONAL K^+ HOMEOSTASIS—II. THE EFFECT OF AXONAL STIMULATION, CHOLINERGIC AGENTS AND TRANSPORT INHIBITORS ON THE RESISTANCE IN SERIES WITH THE AXON MEMBRANE

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Abstract—The small electrical resistance in series with the axon membrane ~~[illegible]~~ is generally modeled as the intercellular pathway for current flow through the periaxonal glial (Schwann cell) sheath. The series resistance of the medial giant axon of the crayfish, *Procambarus clarkii*, was found to vary with conditions known to affect the electrical properties of the periaxonal glia.

Series resistance was estimated from computer analysed voltage waveforms generated by axial wire-constant current and space clamp techniques. The average series resistance for all axons was $6.2 \pm 0.5\ \Omega cm^2$ ($n = 128$). Values ranged between 1 and $30\ \Omega cm^2$. The series resistance of axons with low resting membrane resistance ($< 1500\ \Omega cm^2$) increased an average of 30% when stimulated for 45 s to 7 min (50 Hz) whereas the series resistance of high membrane resistance ($> 1500\ \Omega cm^2$) axons decreased an average of 10%. Carbachol (10^{-3} M) caused the series resistance of low membrane resistance axons to decrease during stimulation but had no effect on high membrane resistance axons. d-Tubocurare (10^{-4} M) caused the series resistance of high membrane resistance axons to increase during stimulation but had no effect on low membrane resistance axons. Bumetanide, a Na–K–Cl cotransport inhibitor and low $[K^+]_o$, prevented the stimulation-induced increase in series resistance of low membrane resistance axons but had no effect on the high membrane resistance axons.

The results suggest that the series resistance of axons varies in response to the activity of the glial K^+ uptake mechanisms stimulated by the appearance of K^+ in the periaxonal space during action potential generation. An increase or decrease in the series resistance is a function of which K^+ uptake system (bumetanide-sensitive or ouabain-sensitive) dominates. Which system dominates depends on the quantity of periaxonal K^+ and the action of the glial cell cholinergic system on the electrochemical gradient for K^+.

The electrophysiological properties of glial cells change as a result of action potential (AP) generation in their associated axon or application of cholinergic agents, transport inhibitors or transport activators.[6,24] These changes should be reflected in the physiological interactions between axons and their associated glial cells. To study these interactions we examined the resistance in series with the axon membrane (R_s) under conditions known to affect glial cell properties. The R_s, in theory,[3,9] represents the electrical resistance of the transglial pathway to the flow of current and is presumed to be primarily the transglial intercellular space because of its low electrical resistance (1–30 Ωcm^2).

The rationale for this approach was based on the effects of stimulation or inhibition of glial ion transport on glial intercellular space. Glial uptake of K^+ in exchange for Na^+, by the ouabain-sensitive exchange mechanism should cause glial volume to decrease[14,17] and result in a decrease in R_s by increasing the size of the intercellular space. The bumetanide-sensitive Na–K–Cl cotransporter tends to cause cell volume expansion[22] which, in turn, should result in an increase in R_s by restricting the transglial intercellular spaces.

EXPERIMENTAL PROCEDURES

The medial giant axon of the ventral nerve cord of the fresh water crayfish *Procambarus clarkii* was dissected and isolated from the animal according to the method described by Wallin.[26] During cleaning of the nerve cord and the partial isolation of the giant axon, the preparation was continually superfused with crayfish physiological solution, modified from an original recipe for fresh water crustaceans.[20] It contained (in mM) 190 NaCl, 5.4 KCl, 13.5 $CaCl_2$, 2.6 $MgCl_2$ and 20 Tris buffer; the osmolarity was 430 mOsm. The pH of all solutions was adjusted to 7.4. Physiological solutions with low $[K^+]$ were made by equimolar substitution of Tris-HCl for KCl. The chamber design allowed for a rapid (10 s) exchange of bathing solution. All experiments were performed at ambient temperature (20–23 C). Pharmacological agents were added directly to the appropriate solution.

Measurement of Resting Membrane and Action Potentials

The apparatus employed for membrane potential recording, space and current clamping was as previously

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Abbreviations: AP, action potential; C_m, membrane capacitance; E_m, resting membrane potential; I, transmembrane current step; R_m, membrane resistance; R_s, series resistance; d-TC, d-tubocurare.

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STUDIES OF AXON–GLIAL CELL INTERACTIONS AND PERIAXONAL K^+ HOMEOSTASIS—III. THE EFFECT OF ANISOSMOTIC MEDIA AND POTASSIUM ON THE RELATIONSHIP BETWEEN THE RESISTANCE IN SERIES WITH THE AXON MEMBRANE AND GLIAL CELL VOLUME

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Abstract—The effect of anisosmotic physiological solutions and $[K^+]_o$ on the resistance in series with the axon membrane were studied in medial giant axons of the crayfish, *Procambarus clarkii* to determine if changes in series resistance are correlated with changes in glial cell volume and volume regulatory responses.

Series resistance was estimated from computer analysed voltage waveforms generated by constant current and space clamp techniques using piggy-back axial wire current passing and glass pipette recording electrodes. Axons subjected to anisosmotic physiological solution in the range of 23 to 175% of isosmolar solution demonstrated that the series resistance of axons changes in a manner similar to that expected for a volume change in isolated cells. In hyperosmotic solution the series resistance changes biphasically, initially decreasing followed by a recovery of the series resistance, similar to the regulatory volume increase described for glial cells in culture. The increase in series resistance following the initial decrease is inhibited by bumetanide (0.1 mM). Ouabain (1 mM), an inhibitor of the volume decreasing Na–K pump, causes the series resistance to increase significantly above that seen for the no drug control. Bumetanide, an inhibitor of the volume increasing Na–K–Cl cotransporter, inhibits the volume regulatory response to anisosmotic media. Treating the axon with three times normal external $[K^+]$ causes the series resistance to decrease approximately 15% while five times normal $[K^+]$ leads to a 15% increase in series resistance. *Both ouabain and d-tubocurare (10^{-3} M) prevent the three-fold* $[K^+]$*-induced* decrease in series resistance while carbachol (10^{-3} M) and bumetanide have little effect. On the other hand, ouabain enhances the five-fold $[K^+]$-induced increase in series resistance while carbachol and bumetanide cause the five-fold $[K^+]$ response to be in a decreasing direction. D-tubocurare has little effect on the five-fold $[K^+]$-induced increase in series resistance.

The study demonstrates that under the conditions of these experiments changes in series resistance are a reflection of changes in cell volume modulated by ouabain- and bumetanide-sensitive K^+ uptake mechanisms. The effects of carbachol and d-tubocurare on the series resistance suggest that their effects are modulated through their action on the glial cell membrane potential and the electrochemical gradient for K^+, which in turn controls the amount of K^+ that appears in the periaxonal space. A comprehensive model for K^+ homeostasis of the periaxonal space is presented and integrates the influences of glial transport processes and cholinergically controlled membrane potential of the glial cell at rest and during action potential generation.

The first two papers in this series[5,14] provide evidence that the glial cell investment of the medial giant axon of the crayfish actively modulates the periaxonal $[K^+]$ during action potential (AP) generation. Modulation occurs through a combined action of K^+ entering the periaxonal space and cholinergic signals[3,4,11,22,25,27,28,29] that activate glial cell ouabain- and bumetanide-sensitive ion transport mechanisms.[5,14] The systems controlling periaxonal ion homeostasis are so effective that 95% of K^+ that flows from a crayfish giant axon during a voltage clamp pulse is cleared from the space during the pulse and that the time constant for clearance of the remainder is approximately 5 ms.[24]

As pointed out in the previous paper[14] changes in the resistance in series with the axon membrane (R_s) that occur during stimulation and that are modified by $[K^+]_o$, cholinergic agents and transport inhibitors, are probably secondary to changes in volume of the glial cell. Recent studies with mammalian astrocyte cultures have demonstrated that both the ouabain- and furosemide bumetanide-sensitive transport systems are involved in glial cell volume regulation where the ouabain-sensitive K^+ uptake is a volume contracting process[20] and the bumetanide-sensitive K^+ uptake is volume expanding.[16,35] Since it has been shown that glial cells from invertebrates also accumulate K^+ from the periaxonal[1] and perineural spaces[8,9] as a result of excitation, similar volume regulatory

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Abbreviations: AP, action potential; E_m, resting membrane potential; R_m, membrane resistance; R_s, series resistance; d-TC, d-tubocurare.

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POTASSIUM HOMEOSTASIS IN THE NERVOUS SYSTEM OF CEPHALOPODS AND CRUSTACEA

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Running Title: K^+ homeostasis in invertebrates

Key words: K^+ homeostasis, invertebrate, crustacea, cephalopods, crayfish, squid, glia.

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SUMMARY

1. Previous work has shown that nerve activity is associated with a significant release of potassium in the vicinity of the axonal membrane. Several mechanisms are normally present which reduce K^+ accumulation in the extra-axonal space.

2. In intact connectives of the crayfish, Procambarus clarkii, repetitive stimulation of the giant axons was associated with an apparent hyperpolarization measured by an interstitial microelectrode, which most probably corresponds to depolarization of the inner face of the perineurial cells by K^+ ions leaving the axons.

3. In desheathed connectives of the crayfish, potassium accumulated during long depolarizing voltage-clamp pulses but cleared away very quickly at the end of the pulse.

4. In the small squid .Alloteuthis subulata, repetitive stimulation of giant axons in situ in fresh and well-perfused animals did not result in a large decrease in the positive after potential (undershoot), reflecting the absence of potassium accumulation. A similar absence of accumulation was observed in vitro for carefully and freshly dissected isolated axons from live squids.

5. In both cases, deterioration of the physiological state of the axon was accompanied by a significant potassium accumulation.

Potassium accumulation could also be reversibly enhanced by decreasing the osmotic pressure of the bathing medium, whereas hyperosmotic solutions had the opposite effect. These results are compatible with the idea that Schwann cells around the axon play a key role in K^+ homeostasis.

6. Experiments on giant axons of the large squid species, Loligo forbesi confirmed the observations made on Alloteuthis in that fresh preparations exhibited little potassium accumulation. Under voltage-clamp conditions, 10 ms depolarizing pulses to various potential levels did not induce any accumulation in these preparations as reflected by the outward tail current. Large accumulation was observed in older axons under similar experimental conditions.

7. A large peri-axonal space associated with healthy glial cells appears to be a prerequisite for efficient K^+ homeostasis in both crayfish and squid. Other mechanisms involving specific transport mechanisms across axonal and glial membranes are also likely to be involved.

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AN ELECTROPHYSIOLOGICAL STUDY OF THE IONIC PERMEABILITY AND SELECTIVITY OF THE CRAYFISH PERINEURIUM

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RUNNING TITLE: POTASSIUM ION SELECTIVITY

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SUBJECT INDEX: GLIAL CELL, POTASSIUM, BLOOD-BRAIN BARRIER

POTASSIUM ION SELECTIVITY

SUMMARY

1. Potassium permeability and selectivity of the crayfish perineurium were studied using conventional electrophysiological techniques and application of the Goldman-Hodgkin-Katz equation.

2. Potentials were generated across the perineurial sheath in elevated $[K^+]_o$ in a Nernst-like manner. In 100 mM $[K^+]_o$, the sheath potential was characterized by a transient 40 mV potential, which peaked within 10-20 seconds before falling over 60-90 seconds to a potential plateauing around 20 mV. The response to a $[K^+]$ gradient across the sheath was not symmetrical, and below normal $[K^+]_o$ small potentials of 2 mV were recorded, deviating sharply from the Nernst equation. The response to 100 mM $[K^+]_o$ was not consistent, but varied between animals; three classes of sheaths could be distinguished, with decreasing K-selectivity and decreasing peak potential amplitude. Most of the results were taken from class 1 or 2 sheaths, which showed the highest degree of K^+-selectivity.

3. The peak potential was a result of high K^+-selectivity of the perineurium. The sheath was effectively impermeable to Na^+, and was ten times less permeable to Cl^- than K^+. The selectivity sequence to monovalent cations was K = Rb >> NH_4 > Cs > Li $\geq$ Tris > Na; only Rb^+ was capable of

generating a large peak potential. During the plateau the permeability ratio (P_{Cl}/P_K) increased from 0.10 to 0.29 due to a decrease in K^+-permeability, and was unaffected by Cl^-_o

4. The peak potential generated by Rb^+ was strongly concentration dependent, and was greatly reduced in less selective sheaths. In 100 mM $[Rb^+]_o$ the peak potential was of equal amplitude to that in K^+, but was of briefer duration (10-20 seconds) and fell to a significantly lower plateau potential of 8-10 mV. At this potential, in highly selective sheaths, spontaneous spike potentials equivalent to the first peak potential were generated without any further experimental manipulation.

5. The axon membrane potential was calibrated for $[K^+]_o$ in the desheathed preparation (in which the perineurium was removed surgically) under a number of experimental conditions, and it was used as a biological "K^+-electrode" to monitor $[K^+]_{ad}$ in the intact nerve cord in order that K^+ transport rates across the perineurium could be estimated. Potassium movement between the bulk solution and adaxonal space was rapid in the desheathed preparation, with a transport rate of 3-4 x 10^{-3} $.s^{-1}$. In the intact nerve cord, flux rates fell with increasing electrochemical gradient, from an extrapolated value of 2 x 10^{-3} $.s^{-1}$ in normal, 5.4 mM $[K^+]_o$, down to 0.4 x10^{-3} $.s^{-1}$ in 100 mM $[K^+]_o$. In the absence of an electrochemical gradient, i.e.

in the normal case, K^+ movement does not appear to be particularly restricted, but when the system is disturbed, K^+ is considerably decreased. Efflux and influx of K^+ across the perineurium was symmetrical. Removing Cl^-_o had no significant effect on K^+ transport rates. Transport of Rb^+ was slightly slower than for K^+.

6. It is suggested that K^+-selective channels, acting like inward rectifying channels, are activated in high $[K^+]_o$ when a voltage of 8 to 10 mV (inside positive) is generated across the perineurium, giving rise to the peak potential. The presence of shoulders in the rising phase implies that K^+-selective channels may be activated in voltage steps. At a potential of 40 to 45 mV, the channels inactivate in a manner which appears to be $[K^+]_i$ dependent, and the potential falls to a lower level. At least two populations of K^+-selective channels were implicated; one with a very high selectivity which also allows the passage of Rb^+ and results in the Rb^+ peak potential and spontaneous spikes, and the spike potential in class 1 sheaths; and a second channel which restricts Rb^+ and gives rise to the broad peak potential in 100 mM $[K^+]_o$. The major route for K^+ transport is through these channels, and K^+ "leak" across the perineurial barrier does not appear to be significant.

7. It is concluded that the crayfish perineurium is an effective permeability barrier, compatible with a role in

central nervous system homeostasis, and illustrates a K^+ selectivity not observed in the vertebrate blood-brain barrier, which is characterized by extremely low ionic permeability and active ion transport. The nature of the perm-selective barrier is unclear, but the perineurial glia as the site of modulated K-selectivity is consistent with their role in K homeostasis and spatial buffering in the central nervous system. The limitations in explaining all of the observations by such a cellular barrier coupled with a tight epithelium are discussed. It is proposed that the thick outer neural lamella and extracellular matrix, made up of glycosaminoglycans (most likely predominantly hyaluronic acid) embedded within a collagen matrix, influences the K^+ perm-selective properties of the crayfish perineurium, and may explain some of the discrepancies between this tissue and others with similar functions.

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Evidence for glutamate as the mediator of axon–Schwann cell interactions in the isolated giant axon of the squid

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Schwann cells surrounding the giant axons of the squid hyperpolarize upon high-frequency stimulation of the axon, and application of cholinergic agonists (Villegas, 1984), octopamine, agents that raise intracellular cyclic AMP, and some peptides (Evans *et al.* 1986). The endogenous signal from axon to Schwann cell is unknown, although glutamate has been suggested (Villegas, 1984).

In isolated axons of *Alloteuthis subulata* bathed in artificial sea water (Abbott *et al.* 1985), 100 Hz stimulation of the axon caused E_m, the Schwann cell resting potential (−40 mV), to hyperpolarize in direct proportion to train duration by up to a maximum of 11 mV at 15–30 s stimulation. The hyperpolarization produced by 100 Hz for 15 s was a linear function of action potential amplitude ($r = 0.86$).

The E_m of the Schwann cell hyperpolarized (by up to 17 mV) with the application of L-glutamate (10^{-9} to 10^{-6} M) in a dose-dependent manner. The response to glutamate and to axon stimulation was blocked by 10^{-5} M 2-amino-4-phosphonobutyrate (2APB or AP4) as well as by 10^{-7} M D-tubocurarine. In the presence of 2APB the Schwann cell was still normally responsive to 10^{-7} M carbachol. L-aspartate (10^{-7} M) was nearly as effective as glutamate in causing a hyperpolarization (10·7 ± 0·7 and 12·8 ± 1·1 mV respectively, mean ± S.E.), but this was not blocked by 2APB. Quisqualate (10^{-5} M) produced a hyperpolarization (8·2 ± 0·9 mV, $n = 5$) blocked by 10^{-4} M L-glutamic acid diethylester (GDEE), which also blocked the response to axonal stimulation. Kainate (10^{-4} M) also caused a hyperpolarization (7·2 mV) but NMDA (10^{-4} M), ibotenate (10^{-5} M), α-amino-3 hydroxy-5-methyl-isoxazole propionate (AMPA, 10^{-4} M) and isethionate (10^{-5} M) were ineffective.

The results suggest that glutamate mediates the communication between the active axon and its surrounding Schwann cells by acting on quisqualate/kainate receptors to activate the cholinergic mechanism of the Schwann cell. This may in turn activate further Schwann-cell processes, including K^+ homeostasis of the periaxonal environment, as suggested for the equivalent system in the crayfish (Brunder & Lieberman, 1986).

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3427 ION SELECTIVITY OF THE CRAYFISH BLOOD BRAIN BARRIER: AN ELECTROPHYSIOLOGICAL STUDY. A.M.Butt*, P. Hargittai* and E.M. Lieberman. (SPON: W. R. Woolen) Dept. Physiology, Sch. Med., East Carolina U., Greenville, NC 27834.

Previous studies from this laboratory (Lieberman et al., Biophys J., 46,146a, 1985) presented evidence that the perineurium surrounding the ventral nerve cord (blood brain barrier) is highly selective to the major ions, being 10 times more permeable to K^+ than Cl^- and practically impermeant to Na^+.

The ventral nerve cord is removed intact from the animal with special regard to avoiding physical stretch and exposure to air, thus maintaining the integrity of the permeability properties of the sheath. Using standard electrophysiological techniques the perineurium and the medial giant axon are impaled. In this way the potential across the sheath and the axon membrane can be monitored by differential recording.

In control solution (190mM Na, 5.4mM K crayfish physiological solution) there is no potential across the sheath. Exchange of the control solution with one containing 100mM [K] (Na substituted) causes a rapid change in potential to +42±2mV, inside positive, peaking within 10 seconds. This is followed by a fall in potential with a plateau value of approximately +25mV. The peak potential is used to evaluate ion selectivity of the barrier.

The permeability selectivity sequence for several monovalent cations is $K^+ = Rb^+ >> NH_4^+ > Cs^+ = Li^+ > Na^+ > Tris^+$. Based on the effect of Na depletion, and Cs^+, Li^+, and $Tris^+$ substitution on the action potential these ions are effectively impermeant within a time frame of 30 minutes exposure.

The potential response of the perineurial sheath to 100mM Rb^+ is of particular interest. Although the peak response is the same (40±1mV) as in 100mM K, the plateau is much lower (11±1mV). In several experiments spontaneous spikes occurred at this potential while the preparation was continuously bathed in Rb solution. Current-Voltage relations of the sheath showed a negative resistance region in the range of 10-15mV. The additional spikes had the same approximate amplitude and time course of the initial spike. The removal of Ca^{2+} from 100mM K solution greatly depressed the spike behavior of the barrier and slowed the potential response.

The data suggest that the permeability across the perineurial sheath of the crayfish nerve cord is modulated by K-selective channels. These channels appeared to be voltage and ion sensitive, yet were not affected by 30 minutes exposure to Ba^{2+} (2-5mM), TEA (1mM) and 3,4-DAP (0.5mM).

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SITE OF ION SELECTIVITY OF THE CRAYFISH BLOOD-BRAIN BARRIER. Butt, A. M.*, Hargittai, P.* & Lieberman, E. M., East Carolina Univ., Sch. Med., Greenville, N.C. 27834.

The perineurial sheath surrounding the ventral nerve cord of the crayfish is highly selective to K^+, being almost impermeable to other monovalent cations. However, the site of selectivity was unclear: the mucopolysaccharide layer at the sheath surface, membranes of the glial cells which make up the sheath, or ion selective tight junctions between the glial cells. Superfusion with 100mM K^+ causes a rapid change in the potential difference across the sheath to +42mV, used to evaluate ion selectivity, followed by a fall in potential to a plateau around +25mV, a measure of sheath permeability. The axon membrane potential depolarizes slowly 5-10mV over a 2 minute 100mM K^+ pulse and is a measure of the $[K^+]$ in the adaxonal space. Superfusion with 1% NaCN + 1% iodoacetic acid (30 mins) had no effect on sheath selectivity or permeability. Opening of the intercellular junctions by osmotic shock (HCS+100mM mannitol) had no effect on the selectivity of the sheath, but increased the permeability to K^+ 3-4 times. Bathing the cord in 1% hyaluronidase or ruthenium red for 30 mins almost completely abolished the selectivity of the sheath but did not increase its overall permeability. The results suggest the site of ion selectivity is the mucopolysaccharide layer, acting as a highly efficient ion exchanger, which is quite separate from the reduced permeability of the sheath due to the intercellular junctions. Supported in part by ARO DAAL03-86-0023.

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ROLE OF MUCOPOLYSACCHARIDES IN THE ION PERM-SELECTIVITY OF THE CRAYFISH NERVE CORD PERINEURIUM. A. M. Butt, P. Hargittai and E. M. Lieberman. Dept. Physiol., East Carolina Uni. Sch. Med., Greenville, NC 27858.

Ion perm-selectivity and I-V relations of the perineurial sheath were studied by conventional microelectrode techniques. The sheath is 10 times more permeable to K than Cl and effectively impermeable to Na. Selectivity is asymmetric, is both ion and voltage sensitive and has a selectivity sequence typical for K-channels, but is unaffected by K-channal blockers or metabolic inhibitors. Hyaluronidase completely abolishes the selectivity, and osmotic disruption of the intercellular tight junctions increases permeability 2-3 fold, while not reducing selectivity. Selectivity appears to reside at the mucopolysaccharide matrix of the sheath neural lamella in series with limited permeability across intercellular junctions between perineurial glia. It is sensitive to Ca, La, and Ruthenium red, suggesting strong negatively charged sites of proteoglycan heparin sulfate and/or sialic acid side chains are responsible. It is proposed that the ion selective properties of proteoglycans may prove to be important in ion homeostasis of the extracellular space in general, as suggested in studies on the frog node of Ranvier, vertebrate sciatic nerve perineurium, and myocardial cells. The perineurial sheath of the crayfish may prove to be a useful model which allows the components of the extracellular matrix, cell membranes and intercellular junctions to be functionally separated. Supported by ARO DAAG-29-82-K-0182.

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