FINAL TECHNICAL REPORT ON CONTRACT N00014-85-K-0242 / UNIVERSTY OF CALIF., DAVIS

PRINCIPAL INVESTIGATOR: D.W. Deamer

CONTRACTOR: Office of Naval Research

CONTRACT TITLE: Role of water in proton-hydroxide conductance across model and biological membranes.

RESEARCH OBJECTIVES:

Our goals over the contract period can be summarized as follows:

1. To understand the role of proton flux in the action of general anesthetics.
2. To characterize the effects of transmembrane peptides on proton permeation of bilayers, and to relate this to bioenergetic functions of coupling membranes.
3. To establish a model system for measuring proton flux along hydrogen bonded chains of water in hydrophobic phases.

TRAINING ACTIVITIES:

Three doctoral and two post-doctoral students have been or are being supported by the ONR contract.

Dr. Gail Barchfeld, currently at Chiron Inc., Emoryville CA, was supported by the contract during the last three years of her doctoral research.

The pre-doctoral studies of Ann Oliver and Joseph Curtis (a minority student) are presently being supported by an extension of the fifth year of the contract.

Dr. Mark Akeson and Dr. Roscoe Stribling were supported by the contract. Dr. Akeson accepted a position at NIH, starting September 1, 1990, and Dr. Stribling is now a Research Associate at the Cancer Research Institute, UC San Francisco Medical Center.

Summary: Women or minorities - 3
Post-doctoral students - 2

AWARDS:

The PI, D.W. Deamer, was a Guggenheim Fellow in 1986-87

PUBLICATIONS AND REPORTS RELATED TO ONR CONTRACT NOOO14-85-K:
(Year 1 to date)

1. Barchfeld, G.L. and Deamer, D.W. (1985) Effect of general anesthetics on proton and potassium permeability of liposomes. Biochim. Biophys. Acta 189:161-169.

2. Deamer, D.W. and Gutknecht, J. (1986) Methods Enzymol. 127:471-480.

3. Deamer, D.W. and Bramhall, J. (1986) Permeability of lipid bilayers to water and ionic solutes. Chem. Phys. Lipids 40:167-181.

4. Deamer, D.W. (1987) Proton permeability of lipid bilayers. J. Bioenerg. Biomembr. 19:457-479.

5. Barchfeld, G.L. and Deamer, D.W. (1988) Effect of alcohols on proton permeability: relation to general anesthetics. Biochim. Biophys. Acta 944:40-48.

6. Deamer, D.W. and Nichols, J.W. (1989) Proton flux in model and biological membranes. J. Membr. Biol. 107:91-103.

7. Akeson, M. and Deamer, D.W. (1989) A test of the pump-leak hypothesis for general anesthesia. Biochemistry 28:5120-27.

8. Deamer, D.W. and Akeson, M. (1991) General anesthetics and membranes: a critical review. Advances in Membrane Fluidity, vol 5. pp. 71-89.

9. Deamer, D.W. Annual reports to the Office of Naval Research in 1986, 1987 and 1988, and distribution to other Principal Investigators.

10. Akeson, M. and Deamer, D.W. (1991) Proton conductance by the gramicidin water wire: Model for proton conductance in the F1Fo ATPases? Biophys. J. 60: 101 - 109.

SUMMARY OF RESEARCH PROGRESS TO DATE

Our research effort focused on proton flux mechanisms, building on our original observation that protons diffuse across lipid bilayer membranes by a process quite different from that of other cations (Nichols and Deamer, 1980; Deamer and Nichols, 1983). As a working hypothesis, we proposed that proton equivalents move along hydrogen bonded chains of water molecules which occur in transient defects in the bilayer. We have extended this concept to the action of certain membrane perturbants on the bilayer. We are also working with a known hydrogen bonded chain of water - the gramicidin channel - and have proposed that proton conductance along similar water structures may play a role in biological membranes as well (Deamer and Nichols, 1989).

The results provide insight into the nature of the lipid bilayer, and the manner in which hydrated defects contribute to ion permeation across the bilayer barrier. It has also permitted us to better understand the effects of anesthetic molecules on the ability of synaptic vesicles to maintain proton gradients necessary for neurotransmitter uptake. Finally, recent results suggest that hydrogen bonded chains of water may be involved in conducting proton equivalents through the Fo subunit of coupling membranes, and we are

now in a position to test this exciting possibility in reconstituted planar membranes.

General anesthetics and proton permeability.

A variety of compounds perturb lipid bilayers in such a way that the barrier to ion flux is reduced. This can lead to inhibition of certain membrane functions that depend on ionic gradients. The classic example is the effect of uncoupling agents on mitochondrial and thylakoid membranes. Uncouplers introduce "leaks" that allow gradients of protons or other ions to decay, with the result that chemiosmotic phosphorylation is uncoupled from electron transport.

Physical perturbation of membranes has also been used to account for the action of general anesthetics (for review, see Akeson and Deamer, 1989). The perturbing effect on physical properties of lipid bilayers - fluidity, volume, phase transitions - does not appear to be sufficient to explain anesthetic effects (Franks and Lieb, 1978; 1982; 1984; 1986). However, Bangham and co-workers have suggested that increased permeability to ions may in fact offer a unitary explanation for the action of general anesthetics. These considerations led to the pump-leak hypothesis (Bangham and Mason, 1980; Bangham and Hill, 1986) which integrates several aspects of anesthetic effects on the bilayer phase of nerve cell membranes. First, it focuses on synaptic transmission, which is the most plausible cellular site of anesthetic action. Second, it incorporates the effect of general anesthetics on lipid bilayer ionic permeability in a way that accounts for other anesthetic effects, particularly the manner in which cold, heat and hypoxia might produce anesthesia. It therefore offers a general and broadly based explanation of anesthetic action on any organism sufficiently complex to have a nervous system.

The pump-leak hypothesis notes that synaptic vesicles have an ATP-dependent proton transport enzyme (the pump) which works against a continuing proton leak across the vesicle membrane. The pump is required to maintain an electrochemical proton gradient equivalent to perhaps 2 pH units, which in turn is used as an energy source to concentrate neurotransmitters such as catecholamines and indolamines. The hypothesis proposes that anesthetics increase membrane permeability to protons, leading to a collapse of the gradient. As a result, neurotransmitters are lost from the vesicles, synaptic transmission is inhibited, and if this occurs in sensitive portions of the CNS, anesthesia ensues.

The pump leak hypothesis is valuable because it makes clear predictions that can be tested experimentally. Results of such tests have provided information about quantitative aspects of anesthetic effects on lipid bilayers. For instance, one would expect anesthetics to cause marked increments in bilayer permeability to protons in model systems. Second, anesthetics should release neurotransmitters such as catecholamines from vesicles in which they have accumulated in response to a proton gradient. Finally, these effects must be consistent with the principles stated in the introduction. That is, they will be measureable at ED_{50} concentrations of anesthetics, and the kinetics of the process should match the known time factors with which anesthetics produce the anesthetic state.

Bangham and Mason (1980) measured effects of benzyl alcohol and other anesthetics (halothane, chloroform, butanol) on permeability of synaptic vesicles isolated from rat brain. The vesicles were shown to

accumulate labeled dopamine in an ATP-dependent process corresponding to their function in the synapse. It was demonstrated that benzyl alcohol did in fact increase proton permeability in a dose-dependent manner, with the result that the dopamine was released. It was also shown that liposomes with pH gradients accumulate dopamine, which was released at a more rapid rate in the presence of benzyl alcohol, butanol or halothane. Barchfeld and Deamer (1985, 1988) in research supported by the Office of Naval Research, confirmed these results in liposome systems, and extended the observations to comparative proton and potassium permeabilities. Several general anesthetics produced similar increments in both proton and potassium permeabilities, demonstrating that the leak was due to a general defect in the bilayer permeability barrier, and not specific for protons.

Despite the early positive results, significant questions remained. At ED_{50} concentrations, anesthetic effects on permeability were minimal. For instance, if we assume that a significant permeability increment would be a doubling of the proton permeability coefficient, concentrations of anesthetics several times ED_{50} are required. A second concern is that anesthetic-induced decay of proton gradients had half-times in the range of 15 - 30 minutes, much longer than required for the onset of anesthesia in organisms.

To define the pump-leak mechanism more precisely, Akeson and Deamer (1989) initiated direct measurements of catecholamine loss from chromaffin granules. This system was chosen because it has a robust proton ATPase activity which produces large proton gradients. Furthermore, the electrochemical proton gradient and membrane potential are responsible for the accumulation and maintenence of internal catecholamines to concentrations approaching 0.4 M (Johnson, 1988). These factors permitted direct measurements of ATPase activity, proton permeability and catecholamine flux in the presence and absence of anesthetics.

Our results show that there is a measureable loss of catecholamine from the chromaffin granules at ED_{50} concentrations of anesthetics. This correlates closely with an increased proton permeability which was also measured, and a corresponding decrease in the magnitude of the pH gradient. Moreover, the kinetics of catecholamine efflux are similar in the chromaffin granules and synaptic vesicles.

These positive results qualitatively agree with the pump-leak hypothesis. However, analysis at a more quantitative level is less favorable. First, the leak is measureable only because of the sensitivity of the method. In fact, the pH gradient in chromaffin granules decays just 0.05 pH units in 20 minutes, and perhaps 5% of the catecholamine is lost. Clearly, the release is slow. Using the principle that an anesthetic effect must correlate with onset times in organisms, the amount of catecholamine lost in the first minute following anesthetic addition is miniscule. Even when the pH gradient was completely released by addition of uncouplers or ammonium chloride, loss of catecholamine had half times of 30 minutes.

In summary, we have concluded that anesthetic effects on ionic permeability of membranes, while measureable, is not sufficient to account for the effects of general ansethetics on the nervous system. Our publication in Biochemistry summarizes these results, and we will not continue further investigative effort on this anesthetic mechanism. Instead, there is good evidence that anesthetics such as the benzadiazepines, barbiturates, steroids and alcohols all act on the GABA receptor, and we have initiated collaborative research with Dr. Mark McNamee (Biochemistry, UC Davis) which will focus on this receptor, particularly the effects of steroids.

The nature of the proton-conducting bilayer defect.

Plots of proton flux and driving force

Nagle (1987) proposed three mathematical treatments of proton flux through transient hydrogen-bonded defects. These depend on the lifetime of the defect and the mechanism of proton transfer. Each model produces a characteristic curve when driving force (ΔpH or voltage) is plotted against flux. Results from earlier investigations show a surprising lack of concensus for such curves. In an attempt to clarify such results for lipid bilayers, we have made a series of careful measurements for liposome systems in which ΔpH was the driving force. These results, together with similar results from other laboratories, show that proton flux is linear with ΔpH.

The conclusion that the relationship between proton flux and ΔpH is linear suggests a transport mechanism in which the effect of the rate limiting step increases exponentially with driving force. Our next goal is to determine the nature of the rate limitation by direct comparisons of voltage-driven proton currenta in liposomes and PLM systems. This will also test the generality of the result, assuring that it is not related to arbitrary choices of lipid, driving force and buffers.

Chain length effects lipid bilayer permeability to protons

We measured proton permeability of homologous phospholipids containing hydrocarbon chains ranging from 14 to 22 carbons. The remarkable observation is that permeability increases approximately an order of magnitude between C14 and C18, even though this represents only a fractional decrease in the thickness of the bilayer.

Our interpretation of these results is that the hydrophobic effect maintaining bilayer stability becomes increasingly weaker with shorter chain lengths, so that thermal fluctuations become more numerous or longer lived. Finally, at ten carbons, thermal fluctuations overwhelm stabilizing forces and the phospholipid becomes micellar.

Proton flux in the gramicidin channel

We have completed our investigation of proton flux in the gramicidin channel, and reported our results at the annual meeting of the Biophysical Society in February, 1990, and in the Biophysical Journal (see refs. below). Our results can be summarized as follows:

1. The proton conductance of the gramicidin channel has at least three kinds of rate-limiting steps, depending on proton concentration. In the neutral pH range, the rate limiting step appears to be proton production by hydrolysis of water at the channel mouth. At intermediate acidity (pH 2 - 5) conductance is limited by proton diffusion to the channel. And at high acidity (pH 0 to 2) conductance is limited by the hopping defect along hydrogen bonded water chains in the channel.

2. At high proton concentrations, the conductance of the channel is at least as high as the equivalent amount of water hydrogen-bonded in the form of ice..

3. D_2O has clearly defined isotope effects on proton conductance that are related to the above conductive mechanisms.

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